

**THE EFFECT OF SILVER NANOPARTICLES ON
Trichoderma harzianum, *Rhizoctonia* spp., AND FUNGAL
SOIL COMMUNITIES**

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Declaration

I declare that this thesis is the result of my own investigations and that no part of it has been submitted for any degree other than Doctor of Philosophy at the Newcastle University. Where other sources of information have been used, they have been dully acknowledged.

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Abstract

Silver nanoparticles (AgNPs) have known anti-microbial properties and are applied in many industrial applications ranging from medicine to fabric preservation. Recently, researchers have proposed the use of AgNPs in agriculture to control plant pathogenic fungi. The use of AgNPs to control plant pathogen fungi does raise concerns as they may potentially affect the activity of beneficial soil microorganisms. In this work the effects of AgNPs on the plant pathogenic fungus, *Rhizoctonia* spp., and their biocontrol agent, *Trichoderma harzianum*, and fungal soil communities were investigated. The results showed that *T. harzianum* mycelium growth was very tolerant to high levels of AgNPs (up to 600 mg L⁻¹) while *Rhizoctonia* spp. were more sensitive (mycelial growth was affected at 20 mg L⁻¹). Nevertheless, AgNPs effect on reproductive stage of *T. harzianum*, e.g. spore production, was not clear as it only showed on one concentration. Despite the decrease in spore production of *T. harzianum* after AgNPs exposure, the spores successfully germinated when cultivated on fresh growth medium (more than 60%). Following up these findings, *T. harzianum* and AgNPs were combined to examine the synergistic potential of these chemical and biological controls on growth of *Rhizoctonia* spp. Interestingly, the combination of AgNPs and *Trichoderma* did not appear to act synergistically to reduce *Rhizoctonia* growth in vitro. In subsequent work the effect of AgNPs contamination on soil fungal communities was assessed by Illumina MiSeq Next Generation Sequencing (NGS) and processed using the UPARSE pipeline run with USEARCH. The soil contamination experiments were carried out over a period of 2 years as previous studies have only examined effects of AgNPs contamination over a few months. Before analysing the metabarcoding data from the Illumina sequencer, a method was developed to find a suitable technique to process the data. It was found that single forward read sequences produced more operational taxonomic unit (OTU) than single reverse and paired end sequences. Therefore, single forward read sequences were used to investigate the effect of AgNPs on soil fungal communities in this study. Soil contamination by AgNPs reduced fungal species richness, evenness, and changed the community structure. For example, species such as *Cryptococcus terreus* was the most abundant in controls but these were replaced by other species including *Trichocomaceae* sp. in AgNPs contaminated soil. Tolerant species, such as *T. spirale* were identified in highly contaminated soil (660 mg kg⁻¹ of AgNPs) and this species has been found in previous studies examining metal contamination. Overall the findings from this thesis suggest that more intensive study will be required when considering AgNPs as an alternative to synthetic fungicides to control plant pathogenic fungi as they have a negative impact on the fungal community in soil even at lower levels e.g. 3 mg kg⁻¹ of AgNPs.

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Nomenclature

Abbreviations

AAAS	Association for the Advancement of Science
AgNPs	Silver nanoparticles
AGs	Anastomosis groups
AMF	Arbuscular mycorrhizal fungus
ANOVA	Analysis of Variance
CDA	Czapek dox agar
CEC	Cation exchange capacity
Cfu	Colony forming unit
DI water	Deionised water
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
HIV	<i>Human immunodeficiency virus</i>
HRTEM	<i>High-resolution transmission electron microscopy</i>
ITS	Internal transcribed spacer
LSD	Least significant different
NCBI	National centre for biotechnology information
NGS	Next generation sequencing
NPs	Nanoparticles
OTU	Operational taxonomix units
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
pH	Potential of hydrogen
RBA	Rose bengal agar
ROS	Reactive oxygen species
S.E	Standard error
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscope
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TEM	Transmission electron microscopy
UV	Ultra violet

Symbols

$^{\circ}\text{C}$	Degree celcius
μL	Microliter
μm	Micrometres
cm^2	Centimetre square
g	Gram
Hz	Hertz
kg	Kilogram
m	Metres
mg kg^{-1}	Milligram per kilogram
mg L^{-1}	Milligram per litre
ml	Millilitre
mM	Millimolar
mm	Millimetre
mm^2	Millimetre square
nm	Nanometres
ppm	Part per million
V	Volt
w/v	Weight per volume
$\times \text{g}$	Times gravity

Chapter I. General Introduction

1.1 Introduction

Nanoparticles (NPs) are particles with lengths in at least one dimension of between 1 and 100 nanometres ($1.0 \text{ nm} = 10^{-9} \text{ m}$). Nanoparticles can be made from a variety of compounds and can be metal based e.g. gold (Au), silver (Ag), and silica (Si) or carbon based e.g. carbon nanotubes used for water and oil purification (Zhu *et al.*, 2013). These ultrafine particles are increasingly used in a wide range of applications in science, technology, and medicine.

Silver nanoparticles (AgNPs) are of particular interest as they have known antimicrobial activity and have a wide range of suggested uses e.g. to control postharvest fruit diseases (Martinez-Abad *et al.*, 2012; Derbalah *et al.*, 2012). AgNPs are also applied in many everyday products, such as sunscreen, laundry detergent, kitchen utensils and children's toys. The increased manufacture, marketing and use of silver nanoparticle-containing household and personal care products is prompting concerns about their fate in the environment.

For example, Mitrano (2014) reported that AgNPs contained in textile can be released to the environment through normal laundry washing and therefore can accumulate in sewage sludge. In addition, AgNPs have been suggested as an alternative to conventional fungicides in order to control pathogenic fungi such as *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *S. minor* (Kim *et al.*, 2012). The increasing use of nanoparticles means that these 'new' pollutants will enter the environment and potentially harm beneficial microorganisms in soil when the sludge applied to soil as fertilizer. Moreover, applying AgNPs as a fungicide may not only affect target microorganisms but could affect beneficial soil microorganisms.

Despite the clear potential for AgNPs to enter the environment and cause deleterious effects very little work has been done on the effect of AgNPs on soil microorganisms, particularly fungi. Soil fungi play essential roles in organic matter transformation. They decompose litter on or under soil surface, synthesis vitamin and auxin, produces soil aggregation substrate etc. (Went and Stark, 1968). Furthermore, a number of fungal genera, such as *Trichoderma*, have an ability to control plant pathogens and stimulate plant growth. The overall aim of this thesis is to investigate the impact of AgNPs on soil microorganisms.

1.2 The Applications of Nanoparticles

Nowadays, nanoparticles are subject to intense scientific research, due to a wide variety of potential applications in many fields. Figure 1.1 shows a wide range of nanoparticles application in industries, from textile to electronics industries.

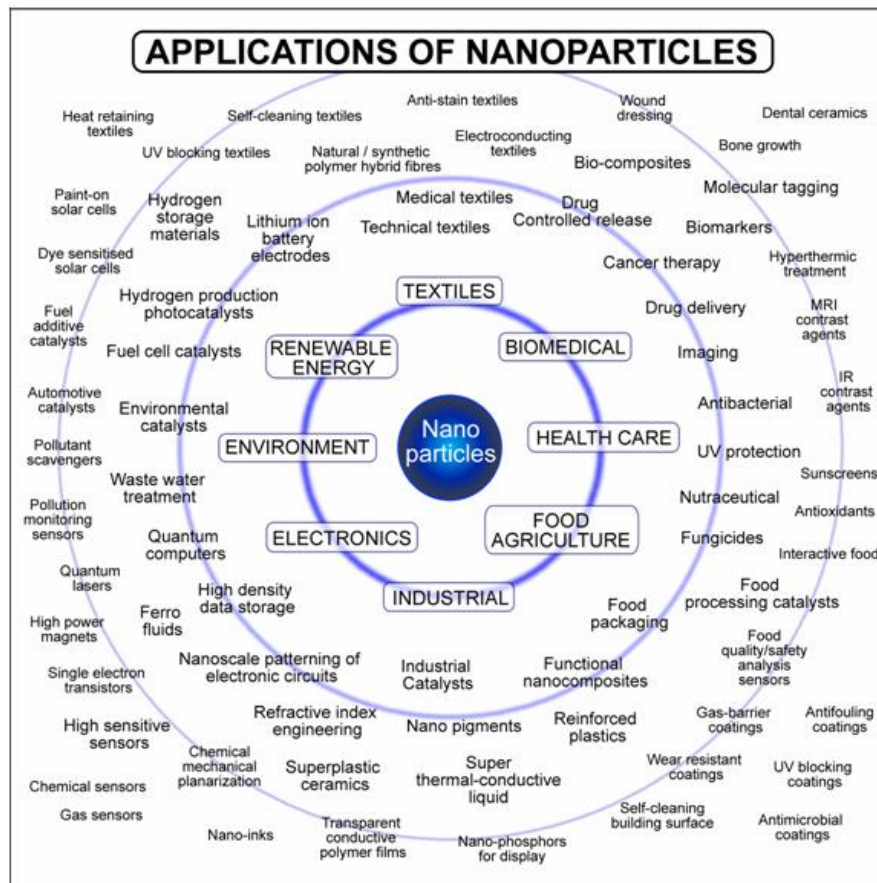


Figure 1.1 The wide range of nanoparticles application in industries. The figure was taken from Tsuzuki (2009)

Different kinds of nanomaterials are employed to fulfil the purposes of each industry. For example, AgNPs is used because of its known anti-microbial properties in medicine, food industry, and electronics e.g. in washing machine produced by Samsung and Daewoo (The Project on Emerging Nanotechnologies, 2013). In medicine nanoparticles have been widely used in a range of biomedical applications (Ge *et al.*, 2014). The use of other types of nanoparticles in industries is presented in Table 1.1.

Table 1.1 The application of nanoparticles in industries

Field of application	Metal	Use as	Reference(s)
Biomedical	gold (Au)	Drug delivery	Alkilany <i>et al.</i> (2012)
Food Agriculture	silver (Ag)	Food packaging	Llorens <i>et al.</i> (2012)
Textile	silver (Ag)	Antibacterial	Xue <i>et al.</i> (2012)
Environment	Iron oxide	Waste water treatment	Xu <i>et al.</i> (2012)

1.3 Classification of Nanoparticles

Scientists have different views on classifying nanoparticles. Chapman *et al.* (2012) divided nanoparticles based on the material such as carbon-based, metal-based, composites, and dendrimers. Carbon-based nanoparticles, may be hollow spheres, ellipsoid, or tube (Figure 1.2). Examples of this type of nanoparticles are spherical fullerenes and carbon nanotubes. Metals like gold (Au), silver (Ag) as well as reactive metal oxides like TiO₂ and ZnO nanoparticles fall into metal-based NPs. Composites are NPs combined with other nano-size atoms or with bulky atoms (e.g. spherical SiO₂ nanoparticles and polycaprolactone) and are used in medical devices as well as for packaging (Llorens *et al.*, 2012). The last classification is dendrimers and these are composed from nano-sized polymers and usually used as catalysts. Dendrimers are compatible with organic structure such as DNA and used particularly in medical and biomedical field e.g. drug delivery (Alkilany *et al.*, 2012).

Nanoparticles can be spherical, tubular, or irregular and found in different forms such as fused, aggregated or agglomerated (Nowack and Bucheli, 2007). The shape of nanoparticles has an important role since it influences their functional behaviour. Other than being naturally formed, nanoparticle shapes can be man-made and carefully controlled. Champion *et al.* (2007) introduced a method to make particles with >20 distinct shapes and characteristic features ranging in size from 60 nm to 30 µm (Figure 1.3).

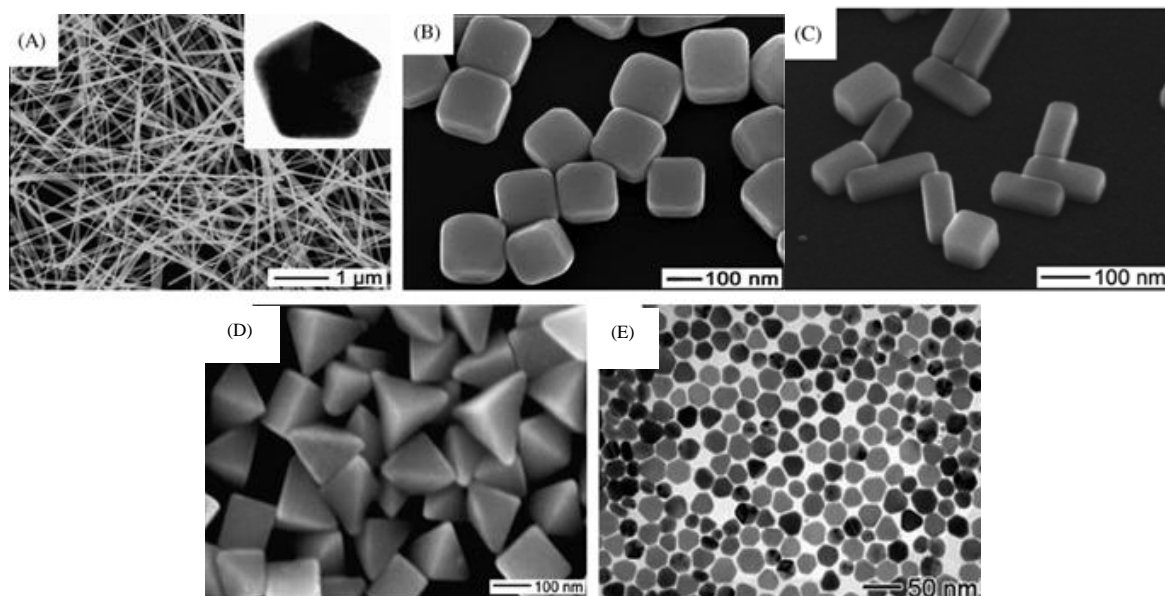


Figure 1.2 Carbon based nanoparticles shape (A) SEM image of Ag nanowires. American Chemical Society, Copyright (2008). (B) SEM image of Ag nanocubes. American Association for the Advancement of Science (AAAS), Copyright (2002). (C) SEM image of the Ag nanobars (D) SEM images of bipyramids approximately 75 and 150 nm in edge length (F) SEM images of silver nanoplates. Royal Society of Chemistry, Copyright (2007).

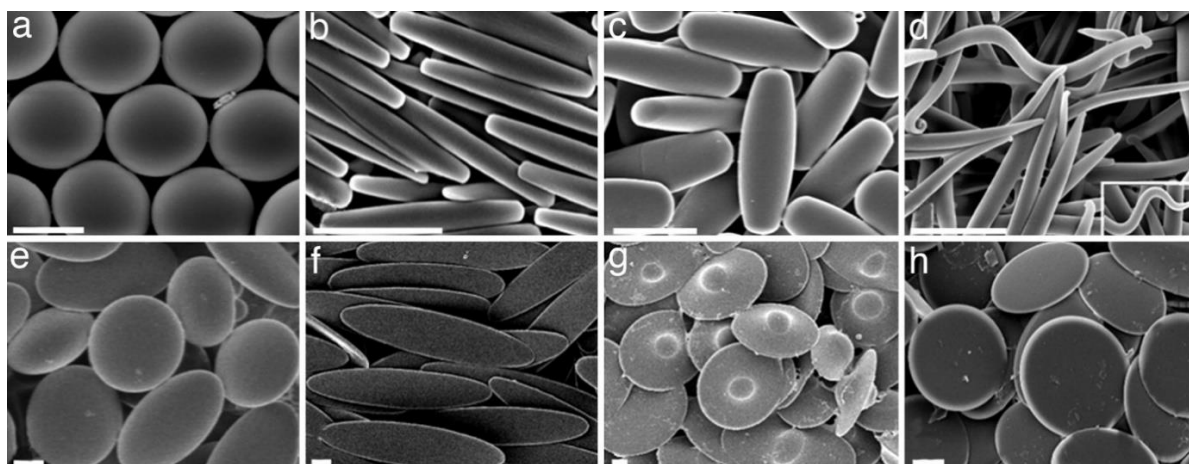


Figure 1.3 Micrographs of shapes made by using scheme A. (a) Spheres. (b) Rectangular disks. (c) Rods. (d) Worms. (e) Oblate ellipses. (f) Elliptical disks. (g) UFOs. (h) Circular disks. (Scale bars: 2 μm .) The figure was taken from Champion *et al.* (2007)

Nanoparticles can be also divided based on how they are formed; natural, incidental, and engineered. Naturally occurring nanoparticles are shaped due to natural process without any human intervention. Examples of naturally occurring nanoparticles include fires, viruses and volcanic ash (Chapman *et al.*, 2012). The second type of nanoparticles are formed as a result of man-made industrial processes e.g. cooking smoke, diesel exhaust, welding fumes, industrial effluents, and sandblasting. Engineered nanoparticles comprise of any manufactured particles with nanoscale dimensions that are produced intentionally for commercial or research application. Examples include controlled shape and size metals, semiconductors, electronics, and optical displays (Nowack and Bucheli, 2007). Furthermore, nanoparticles separated based on their chemical composition into organic and inorganic.

1.4 Synthesis of Nanoparticles

There are two approaches to synthesise nanoparticles; top down and bottom up approaches (Prabhu and Poulose, 2012). The top-down approach is to create smaller devices by using larger ones to direct their assembly. On the other hand, the bottom-up approach is to arrange smaller components into more complex assembly. Several ways are described in the literature to synthesise nanoparticles. These include physical, chemical, and biological methods as represented in Figure 1.4. The primary goal of each method is to control the size, shape, morphology, and crystallinity of nanoparticles to produce a desirable effect. Many physical and chemical methods are expensive and use toxic substance. Therefore, biological methods using microbes and plant extracts are more favourable (Prabhu and Poulose, 2012).

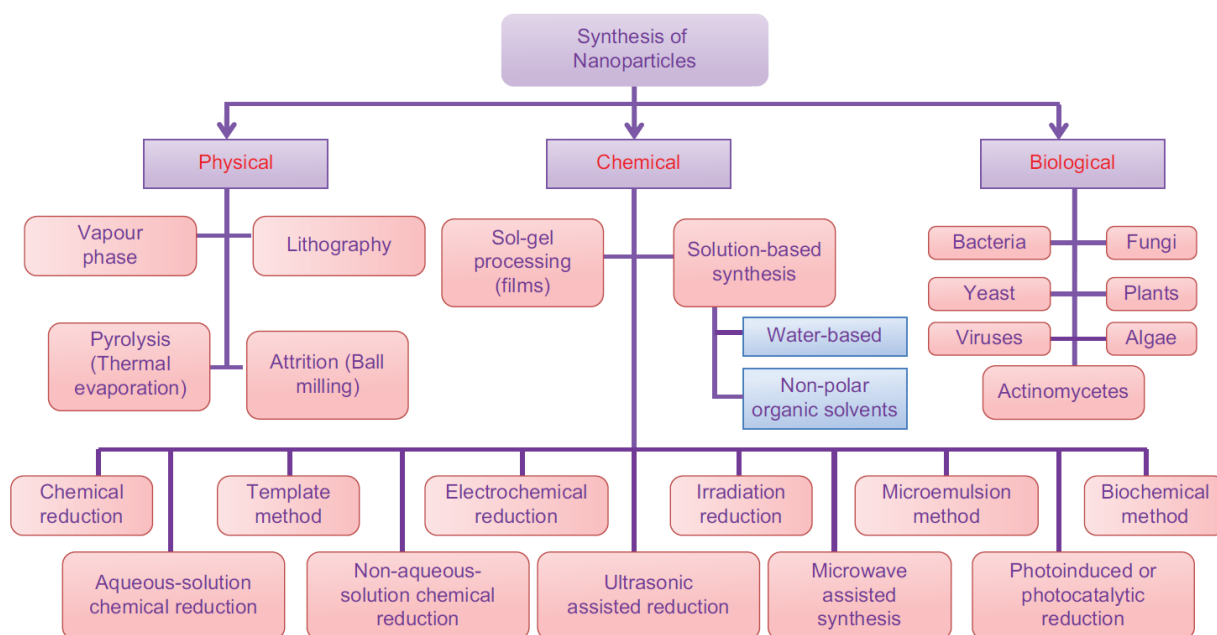


Figure 1.4 Methods employed for nanoparticles synthesis.
The figure was taken from Dhillon *et al.* (2012)

Synthesis of nanoparticles by live organisms is also known as green synthesis. In this method, microorganisms e.g. fungi and bacteria, and plants are employed to synthesis nanoparticles. Sweet *et al.* (2012) claimed that the first report of bacterial based NPs synthesis, by *Pseudomonas stutzeri*, was by Klaus *et al.* (1999). Many reports of nanoparticles synthesis using other bacteria and fungi have appeared since then. Bacteria are more favourable due to several factors including ease of handling, easily genetically manipulated, and the fact that studies on one bacterium can be easily extrapolated to others (Jayaseelan *et al.*, 2012). Electron microscopy analysis confirmed that nanoparticles synthesis by bacteria and fungi occur by an enzymatic process (Iravani *et al.*, 2014). For example, Ag nanoparticles have been synthesized using *Pseudomonas stutzeri* AG259 bacterium via a mechanism involving the NADH-dependent reductase enzyme that donates an electron and oxidises to NAD⁺. The electron transfer results in the biological reduction of Ag ions to Ag nanoparticles (Shah *et al.*, 2015). Fungi are regarded as organisms that produce nanoparticles extracellularly because of their high secretory capacity, which is involved in the reduction and capping of nanoparticles (Sweet *et al.*, 2012).

The biological method is able to create specific characteristic of nanoparticles. For example, *Fusarium oxysporum* produce well-dispersed nanoparticles with size between 5 and 13 nm in spherical form (Husseiny *et al.*, 2015). The numbers of microorganisms and plants recognised to synthesise nanoparticles are presented in Table 1.2.

Table 1.2 Organisms capable of naturally producing NPs

Organism(s)	Type of nanoparticles synthesised	Size (nm)	Shape(s)	Reference(s)
Fungi				
<i>Puccinia graminis</i>	Ag	30-120	spherical	Kirthi <i>et al.</i> (2012)
<i>Humicola</i> sp.	Ag	10	spherical	Syed <i>et al.</i> (2012)
<i>Penicillium citrinum</i>	Ag	109	spherical	Honary <i>et al.</i> (2013)
<i>Cryphonectria</i> sp.	Ag	30-70		Dar <i>et al.</i> (2013)
<i>Fusarium oxysporum</i>	Ag	5-13	spherical	Husseiny <i>et al.</i> (2015)
Bacteria				
<i>Idiomarina</i> sp. PR58-8	Ag	26		Seshadri <i>et al.</i> (2012)

Continued

<i>Proteus mirabilis</i> PTCC 1710	Ag	10-20	spherical	Hebbalalu <i>et al.</i> (2013)
<i>Bacillus flexus</i>	Ag	12-65	spherical, triangular	Priyadarshini <i>et al.</i> (2013)
<i>Bacillus</i> sp.	Ag	42-92		Das <i>et al.</i> (2014)

Plant				
<i>Iresine herbstii</i>	Ag	46-64	cube	Dipankar and Murugan (2012)
<i>Abelmoschus esculentus</i>	Au	45-75		Jayaseelan <i>et al.</i> (2013)
<i>Coleus aromaticus</i>	Ag	44		Vanaja and Annadurai (2013)

1.5 Silver Nanoparticles (AgNPs)

Due to its antimicrobial properties, AgNPs have the most commercial applications as consumer products. There are more than 300 everyday products listed such as bed sheet, socks, toothpaste, and towels (The Project on Emerging Nanotechnologies, 2013). An increasingly common application is the use of silver nanoparticles for antimicrobial coatings, and many textiles, keyboards, wound dressings, and biomedical devices that continuously release a low level of silver ions to provide protection against bacteria.

The size, shape, and surface of the nanoparticles have correlation with their toxicity. Kim *et al.*, (2012) suggested that the AgNPs-induced cytotoxic effects against tissue cells are particle size-dependent. The smaller size the more toxic because when AgNPs is small they release many Ag ions that dominate antibacterial activity. Silva *et al.*, (2014) found size and surface charge of AgNPs explained their toxicity. More recent, researchers demonstrated that surface properties influence both physical and chemical of AgNPs that affect their antimicrobial efficacy (Ouay and Stellacci, 2015).

1.6 Release of AgNPs into the Environment

The increasing and varied use of AgNPs on consumer products increases the risk of AgNPs release into the environment. According to Nowack and Bucheli (2007) the source of AgNPs in the environment may come from point and non-point sources. Release of AgNPs from point source may come from production facilities, landfills, and waste water treatment. The second

source may come from consumer products. For instance, AgNPs contained in textile can be released to the environment through normal laundry washing and therefore can accumulate in sewage sludge (Mitrano, 2014). In many countries e.g. Germany, The UK, Spain, France and Portugal, dry sewage sludge from waste water treatment is produced as agricultural fertiliser and applied on farmland (Schlich *et al.*, 2013). The sewage sludge still contains Ag because water treatment facilities do not always remove them completely (Kaegi *et al.*, 2011).

Other possibility is accidental release during production or transport. In addition to the unintentional release there is also intentional release of AgNPs to the environment. Manufactured AgNPs applied deliberately as fungicide (Kim *et al.*, 2012) and soil remediation (Siripattanakul-Ratpukdi and Fürhacker, 2014) are examples of intentional release of AgNPs to the environment.

The properties of the AgNPs can be modified when they interact with the soil environment. As a result the interaction may change AgNPs stability, availability, and toxicity to organisms (Cornelis *et al.*, 2012; Coutris *et al.*, 2012; Levard *et al.*, 2012; Tourinho *et al.*, 2012; Benoit *et al.*, 2013). Soil pH and organic carbon (OC) have been found to have the greatest effect on AgNPs toxicity (Langdon *et al.*, 2014). In the soil, Ag is known to react strongly with sulphide, chloride, and organic matter (Levard *et al.*, 2012).

1.7 Effect of AgNPs on Soil Microorganisms and Other Soil-associated Organisms

Despite the potential toxicity of AgNPs to soil microbes little work has yet been carried out in this area. However, AgNPs have been shown to disrupt the denitrification process. Kumar *et al.* (2011) reported that plant-associated bacteria, *Bradyrhizobium canariense*, were highly susceptible to AgNPs. It is generally known that *B. canariense* fixes nitrogen to nitrate so that plants can use it to make protein for their growth. It is true that *B. canariense* is not the only nitrogen fixing bacteria and that other bacteria might be able to take their place but AgNPs might also affect those bacteria and other beneficial soil microorganisms and reduce the number of species in the environment. A similar observation was made by Calder *et al.* (2012) during their research on antimicrobial effect of AgNPs towards beneficial soil bacterium, *Pseudomonas chlororaphis*. More recent, a study shows that at environmentally relevant concentrations, AgNPs cause toxic effects on soil microorganisms of the terrestrial ecosystem and changes the diversity (Schlich *et al.*, 2013). Microbes in the soil play important role in soil that any reduction in soil microbial composition results in low soil quality and plant productivity.

A study found that AgNPs affect fertility, lifespan, and body length of non-pathogenic soil nematode, *Caenorhabditis elegans*, (Contreras *et al.*, 2014). AgNPs is also have been reported to affect other soil organisms such as and earthworms, *Eisenia fetida*, (Tsyusko *et al.*, 2012). AgNPs released into soil pore water affect the cocoons and juveniles of earthworms (Schlich *et al.*, 2013b) and affect *Lumbricus rubellus* population in soil (van der Ploeg *et al.*, 2014).

1.8 Effect of AgNPs on Plants

Studies have shown that NPs may accumulate in plants. According to Dietz and Herth (2011), there are two possible pathways for nanoparticles uptake in plants; above ground e.g. shoots and below ground such as plant roots. Uptake of AgNPs has been evidenced in *Arabidopsis thaliana* (Geisler-Lee *et al.*, 2013) and *Oryza sativa*, *Brassica campestris*, *Vigna radiata* seeds (Mazumdar, 2014). From root the AgNPs may distributed to other part of the plant and accumulate in different organs. For example, the nanoparticles were accumulated in *A. thaliana* leaves while the accumulation occurs both in leaves and stem of *Populus deltoids* (Wang *et al.*, 2013).

Several reviews dealing with the effect of AgNPs on plant have been published. Studies showed that AgNPs caused both positive and negative effect on plant growth and germination. AgNPs enhanced the germination rate of *Zea mays*, *Citrullus lanatus*, and *Cucurbita pepo* (Almutairi and Alharbi, 2015). Similar finding reported on the germination of *Trigonella foenum-graecum* (Hojjat and Hojjat, 2015). In contrast, Yin *et al.* (2012) reported that AgNPs reduced germination of *Phytolacca americana*. AgNPs showed a toxic effect on *Zea mays* root elongation, whereas *Citrullus lanatus*, and *Cucurbita pepo* seedling growth were positively affected by certain concentrations of AgNPs (Almutairi and Alharbi, 2015).

1.9 Anti-microbial Mechanism of AgNPs

The exact anti-microbial mechanism of AgNPs is still not fully understood. However, studies suggested nanoparticles exert their toxic effects by a variety of mechanisms. For instance, they may stick to cell surfaces (Derbalah *et al.*, 2012), penetrate the cells (Kim *et al.*, 2009), change cell membrane properties and finally result in DNA damage due to dissolution of Ag ions from the particulate AgNPs (Morones *et al.*, 2005). Conversely, Hwang *et al.* (2008) dispute that damage to DNA may be caused. Other workers propose that silver ions interrupt cellular metabolism and respiration processes (Kim *et al.*, 2009). In addition, AgNPs produces reactive

oxygen species (ROS), particularly superoxide radical and hydroxyl radical, that damage the cell (Hwang *et al.*, 2008).

More recent, Agnihotri *et al.* (2013) demonstrated that direct contact plays a predominant role in disinfection of AgNPs. The Transmission Electron Microscopy (TEM) images showed that AgNPs presence at bacterial cell membrane, just inside the cell membrane, and in the cell interiors. AgNPs may directly damage the cell membranes, disrupt ATP production and DNA replication, alternate gene expressions, release toxic Ag⁺ ion, and produce reactive oxygen species to oxidize biological components of the cell (Sharma *et al.*, 2014).

1.10 *Trichoderma* Species Roles

The genus *Trichoderma* is abundant in soil as they involved in the transformation of soil organic matter. Molla *et al.* (2012) reported that application of *T. harzianum* minimizes the use of NPK fertilizer and enhance s production and nutritional quality of potato (*Lycopersicon esculentum* Mill.). Furthermore, most of *Trichoderma* species are well known as biological control agent against a wide range of plant pathogens. *Trichoderma* species are able to control ascomycetes, basidiomycetes, and oomycetes. The potential of *Trichoderma* species as biological control agent was first demonstrated by Weindling in the early 1930s (Grondona *et al.*, 1997) and used to control many plant pathogenic fungi since then. Researchers reported that *Trichoderma* gave excellent control of nematodes (*Meloidogyne incognita*) on tomato (Radwan *et al.*, 2012), *Fusarium oxysporum* (Perveen and Bokhari, 2012), *F. nygamai* and *Rhizoctonia solani* (Parizi *et al.*, 2012). In the environmenbkt these beneficial fungi are easily isolated from soil, decaying woods, and other forms of organic matter as they play an important role on decomposition process.

There are several methods to apply *Trichoderma* sp. to control plant pathogens including (1) soil application, (2) seed treatment, and (3) aerial application. Soil and seed treatments usually act as preventive treatment while aerial application acts as curative treatment as well. Seed treatment aims to increase seed germination, seed growth and seedling vigour (Jegathambigai *et al.*, 2009). The effectiveness of each method has been shown to be affected by the host and pathogen. Prasad *et al.* (2002) claimed that soil application of *T. harzianum* was found to be more effective than seed treatment for suppression of *Fusarium udum* on pigeonpea. Whereas seed treatment was more effective to control *Rhizoctonia solani* causing root rot on mungbean (Dubey *et al.*, 2011). *Trichoderma* spp. have been also found to stimulate plant defense mechanisms by increasing its basic immunity (Lorito *et al.*, 2010). *Trichoderma* strains are able

to induce strong response in the plant by producing hydrophobins (Djonovic *et al.*, 2006), expansin-like proteins (Brotman *et al.*, 2008), secondary metabolites, and enzyme (Lorito *et al.*, 2010). *Trichoderma* species have also been shown able to induce plant resistant against pathogen. Interaction between *Trichoderma* species with plants rhizosphere leads to enhanced root proliferation, better growth, and protection of the plants against toxic chemicals (Schuster and Schmoll, 2010).

Not limited in agriculture, *Trichoderma* sp. also applied in many fields. They are have also been applied in paper and pulp industry as they produce prebleaching enzyme (Ahmed *et al.* 2012). They are sources of enzymes used in biofuels industry and producers of secondary metabolites used in bioremediation including heavy metal (Mukherjee *et al.*, 2013). Furthermore, secondary metabolites produced by a few *Trichoderma* sp. is potential sources of anti-cancer and anti-microbial drugs (Mukherjee *et al.* 2012). Without a doubt, *Trichoderma* sp. is among the largest microorganism studied. Over 12,000 scientific reports on *Trichoderma* sp. in various field indexed by Scopus since 1930s.

However, apart from the beneficial of *Trichoderma* species, some strains comprise opportunistic human pathogens. *Trichoderma* infections in humans have been related with several risk factors, being associated mostly with peritoneal dialysis, organ transplantation, and hematologic disorders (Hatvani *et al.*, 2013). *T. arundinaceum*, *T. turrialbense*, *T. protrudens* and *Hypocrea rodmanii* are reported to produce trichotoxins (Keswani *et al.*, 2014). They cause severe and persistent disseminated infections, allergic and acute invasive sinusitis, keratitis, otitis externa, skin and subcutaneous infections, peritonitis, deep pulmonary infections, endocarditis, and brain abscess (Sandoval-Denis *et al.*, 2014).

1.11 Biological Control Mechanisms of *Trichoderma*

1.11.1 Competition through rhizosphere competence

Competition over space and nutrition are the most well-known mechanism of *Trichoderma* sp. to control other fungi. Their rapid growth allows these species to directly compete for space and nutrients with phytopathogens by producing metabolic compound that inhibit spore germination (Naher *et al.*, 2014). In addition, species of *Trichoderma* are naturally resistant to pesticide so that they can grow rapidly. The competition between *Trichoderma* sp. and pathogens can be shown easily by plating both fungi on agar medium (Figure 1.6).

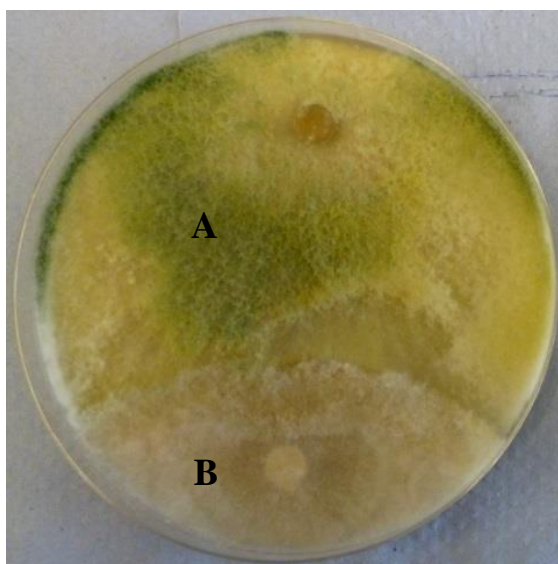


Figure 1.5 Interaction between *Trichoderma harzianum* (A) and *Rhizoctonia solani* (B) on agar medium

1.11.2 Mycoparasitism and antibiotic (toxin) production

Mycoparasitism is generally defined as parasitism of a fungus (host) by another fungus (mycoparasite). Mycoparasitism is divided into two major groups; necrotrophic (destructive) and biotrophic (balanced) parasitism (Manocha, 1991). *Trichoderma* spp. are grouped in the necrotrophic mycoparasites because the species produce antibiotics and/or toxins to cause destruction of their hosts (Howell, 2003). Mycoparasitism mechanisms are well described by Weindling (1941) on interaction between *T. virens* and *R. solani* that including coiling around pathogen hyphae, penetration, and subsequent dissolution of the host cytoplasm by producing a toxin called gliotoxin (Figure 1.7). Coiling of the plant pathogenic fungal hyphae by *Trichoderma* spp. is one parameter used to characterize the mycoparasitism (Rocha-Ramirez *et al.*, 2002; Howell, 2003). The production of pyrone by *T. harzianum* has a strong relation to the antagonistic ability of *T. harzianum* (Scarselletti and Faull, 1994). Later on, a new antibiotic isolated from *T. virens* called gliovirin that strongly inhibited the growth of *Pythium ultimum* and *Phytophthora* species were described (Howell, 2003). *Trichoderma* species have also been reported to produce antimicrobials among their secondary metabolites (Vinale *et al.*, 2008).

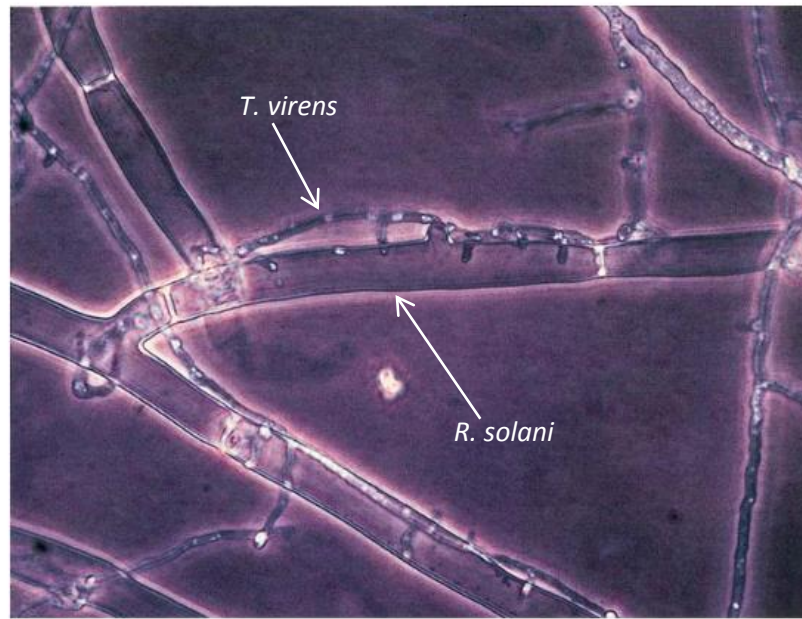


Figure 1.6 Penetration and haustoria formation within the large hyphae of *Rhizoctonia solani* by the smaller hyphae of *Trichoderma virens* (The American Phytopathological Society, 2003)

1.11.3 Enzyme production

More advanced research has led to the discovery of an alternative antagonistic mechanism of *Trichoderma* sp. on pathogenic fungi. Most of the biological control agents are known to produce chitinase and β -1,3-glucanases enzymes produced when there is contact with pathogen to degrade the cell wall of the pathogen leading to the lysis of hyphae (Sangel and Bambawale, 2004). The cell wall degrading enzymes were produced in the presence of phytopathogen cell walls as the carbon source (Gajera *et al.*, 2012). Chitinase produced by *T. harzianum* was reported to destroy the cell wall of *Fusarium graminearum* and *F. culmorum* on rice (Matarese *et al.*, 2012), *Alternaria alternata* on tobacco (Gveroska and Ziberoski, 2012), *Alternaria porri* on onion (Abo-Elyousr *et al.*, 2014), and *F. oxysporum* on tomato (El-Komy *et al.*, 2015).

1.12 Factors Affecting the Success of *Trichoderma* spp. as a Biological Control Agent

When applying *Trichoderma* sp. for the purposes of biological control in the soil, several environmental parameters that affect its efficacy must be considered. Some important parameters are temperature, water activity and pH, and the presence of pesticide and metal ions.

Temperature has an important contribution to *Trichoderma* sp. growth and its ability to control pathogen. It has been reported that growth of *Trichoderma* sp. was highly sensitive to high

temperatures. Optimum growth temperature for *Trichoderma* species, such as *T. harzianum* and *T. viride*, appears to be between 25–30 °C (Singh *et al.*, 2014). At higher temperature (35 °C) growth is extremely slow and stops at 45 °C (Singh *et al.*, 2014; Gupta and Sharma, 2013).

Abiotic factors such as pH and water activity determine physiological quality and quantity of colonies (Daryaei *et al.*, 2016). Acidity has effects on *Trichoderma* sp. growth and enzyme production. Generally, *Trichoderma* sp. is able to grow in a wide range of pH between 5.5 and 7.5 (Singh *et al.*, 2014), while enzymes e.g. cellulose are produced optimally from 4.5-5.5 (Li *et al.*, 2013). Water condition have been shown to strongly affect *Trichoderma* sp. activities, most particularly on spore germination and germ tube growth, mycelia growth, saprophytic ability, interaction with other fungi, and enzyme production (Kredics *et al.*, 2003). Linear correlation is found between water potential and colony growth rate while enzyme production reacts differently. Some enzymes e.g. cellobiohydrolase and NAGase produced optimally at high water potential, while others e.g. β -glucosidase and β -xylosidase occurred at low water potential. Begoude *et al.* (2007) added low water activity prevent the growth of *T. asperellum* under some conditions.

Several pesticides used in agriculture contain metal ions. Application of pesticide particularly fungicide in agriculture land to control pathogens gives a side effect on *Trichoderma* sp. Yan-bing *et al.* (2010) reported pesticide at recommended concentration inhibit the growth of *T. harzianum* up to 20%. Low concentration (10 ppm) of commercial fungicide stops the growth of *T. harzianum* (Sarkar *et al.*, 2010). More recent, insecticide with profenofos active ingredient inhibits 50% growth of *T. harzianum* (Thiruchchelvan *et al.*, 2013). More recently, Mohammadi and Amini (2015) reported that commercial formulation of Ethalfluralin and Amitraz reduced the mycelial growth and spore germination of *T. harzianum*.

Metals may be present as result of contamination in the environment. Even though some heavy metal ions are necessary elements for the growth of fungi, they are toxic at high concentration. For example, 800 ppm manganese ions caused a weakening of the conidial germination of *T. harzianum* and *T. viride* that affect their ability to control pathogens (Jaworska and Dłużniewska, 2007). Inhabitation on *Trichoderma* sp. growth also found with the presence of calcium ions in media (Singh *et al.*, 2014).

1.14 Project Aims

Due to the potential risk caused by the application of AgNPs in soil there is a need to investigate the effect of AgNPs on soil microorganisms particularly fungi. *T. harzianum* was used in this study as a model fungi as it is a beneficial fungi and well known biological control agent against a wide range of plant pathogens. The specific aims of the thesis were to:

- a) investigate the effect of AgNPs on *T. harzianum* growth *in vitro* (Chapter II).
- b) investigate the effect AgNPs on the ability of *T. harzianum* to control plant pathogenic fungi (Chapter III).
- c) develop a method to analyse soil fungal community using Illumina Miseq Next Generation Sequencing (NGS) (Chapter IV).
- d) investigate soil fungal community responses to AgNPs as assessed by Illumina NGS (Chapter V).

**Chapter II. The Effect of Silver Nanoparticles on *Trichoderma harzianum*
Growth *in Vitro***

2.1 Introduction

Silver nanoparticles (AgNPs) are widely used in industry due to their anti-microbial properties. Common applications include the use of AgNPs for antimicrobial coatings on textiles, electronics and biomedical devices (Emerich and Thanos 2007; Rai *et al.* 2009; Lee *et al.* 2010). Recently, researchers have proposed the use of AgNPs in agriculture to control plant pathogenic fungi such as *Rhizoctonia solani*, *Bipolaris sorokiniana*, *Magnaporthe grisea*, and *Colletotrichum* sp. (Min *et al.*, 2009; Jo *et al.*, 2009; Lamsal *et al.*, 2011).

The use of AgNPs to control plant pathogen fungi does raise concerns as they may potentially affect the activity of beneficial soil microorganisms. The nanoparticles applied will end up in the soil and may become toxic to microorganisms. In bacteria, silver ions inhibit cell growth and multiplication by breaking through the cell wall, disrupting respiration and binding (Prabhu and Poulouse, 2012). Antifungal activity of AgNPs damage fungal membrane integrity by interrupting the structure of the cell membrane and inhibiting the normal budding process (Kim *et al.*, 2009).

The genus *Trichoderma* is one of a range of beneficial fungi that live in soil as saprophytes. In particular, *Trichoderma* has the ability to decompose organic matter (Zaidi and Singh, 2013). Furthermore, *Trichoderma* species also have capability to live on other fungi and this property makes them well known as biocontrol agent against a wide range of plant pathogens. The potential of *Trichoderma* species as a biocontrol agent was first demonstrated by Weindling in the early 1930s (Grondona *et al.*, 1997) and has been used to control many plant pathogenic fungi since then. *Trichoderma* suppress plant pathogen by (1) coiling and penetrating the pathogen hypha (Weindling, 1932), (2) produce toxins and enzyme (chitinase and/or glucanases) to destroy pathogen cell wall integrity (Weindling, 1941; Howell, 2003), (3) compete for space and nutrients (Zhang *et al.*, 1996; Harman, 2000; Howell *et al.*, 2000). Today, several species of *Trichoderma* have been produced commercially as biological fungicides.

Due to the ecological importance of *Trichoderma* in soil and as biocontrol, the current work investigates the effect of AgNPs exposure on *Trichoderma* growth *in vitro*. Very little work has been done to study the impact of AgNPs on *Trichoderma* sp. Most studies have only focused on the use of AgNPs to control fungal plant pathogens. Research carried out by Gavanji *et al.* (2012) tested various levels of AgNPs on *T. harzianum* and *T. viridae* growth *in vitro*. They found that 150 mg L⁻¹ of AgNP reduced colony diameter and dry weight of both *Trichoderma* sp. mycelia significantly compared to control. What is not clear yet is the impact of AgNPs on

spore production and its viability. Both parameters are very important because spores are the reproductive cells of fungi. The fungi used in this study were isolated from UK soil and identified morphologically and phylogenetically (ITS1 + ITS4 primers) to ensure the fungi investigated belonging to genus *Trichoderma*.

Overall, the aim of this study was to assess the effect of AgNPs on *Trichoderma* species growth *in vitro*. Individual objectives were to:

- a) identify fungi isolated from soil
- b) characterise the AgNPs
- c) examine colony diameter of *Trichoderma* under AgNPs stress.
- d) examine *Trichoderma* spore production in media containing AgNPs.
- e) examine the viability of *Trichoderma* spores after contact with AgNPs.

2.2 Materials and Methods

2.2.1 Fungal isolation

Five grams of UK soil sample (Newcastle University, 54.9780° N, 1.6150° W) was diluted in 50 ml of sterile water, shaken and left to settle. Soil dilution (50 µl) was spread on to Potato Dextrose Agar (PDA) and incubated at 24 °C until colonies appeared. Morphologically different colonies were transferred to a plate of PDA and incubated at 24 °C. Six colonies were selected for identification and potential further work.

2.2.2 Morphological identification

The cultures were examined based on colony colour, shape and hypha under microscope. To observe the fungal reproductive structures under a microscope, cellophane tape was pressed lightly against the edge of a young colony and then placed in a drop of methylene blue on a microscope slide. The fungal structures were observed under a light microscope and images captured using an Olympus PEN E-PL1 camera.

2.2.3 Molecular identification

A. Deoxyribonucleic acid (DNA) extraction

The DNA was extracted either by using a microwave based method or a PowerSoil kit as described below.

Microwave based methodology

The fungal cultures were grown on PDA for 7 days. mycelium was scraped aseptically from the agar surface and placed into 1.5 ml microtubes containing 100 µl of lysis buffer (50 mM EDTA, 3% w/v sodium dodecyl sulphate) and subjected to heating in a microwave for 30 seconds (sequentially separated into a 15, 10, 5 second segments to prevent boiling). After the addition of 300-350 µl of lysis buffer, tubes were heated at 80°C for 10 minutes. Four hundred µl of a 25:24:1 Phenol:Choloform:Isoamyl Alcohol mixture was then added; samples were mixed and then centrifuged at 10,000 x g for 14 minutes. The aqueous upper layer was removed and placed into a fresh tube. To this 0.54 volumes of isopropanol and 10 µl of 3M sodium acetate pH 5.2 were added. Samples were centrifuged at 10,000 x g for 3 minutes, the supernatant discarded and the pellet washed in 500 µl of 80% ethanol with subsequent centrifugation at 10,000 x g for another 3 minutes. The resulting pellets were air dried for 5-10 minutes to get rid of any remaining ethanol and resuspended in 50-100 µl of TE buffer (10mM Tris-HCL, 0.1mM EDTA pH 8.0) dependent upon pellet size.

PowerSoil kit methodology

DNA from fungal cultures was extracted using the PowerSoil[®] DNA isolation kit as described by the manufacturer (MO BIO laboratories, USA). Fungal hyphae were scraped from plates and added to a PowerBead tube containing a buffer to help break open the fungal cell and protect nucleic acids from degradation. The tube was gently vortexed to mix the components in the buffer and disperse the sample in the solution. C1 solution (60 µl, containing sodium dodecyl sulphate (SDS) and other disruption agents required for complete cell lysis) was added to break down fatty acids and lipids prior to vortexing for 10 minutes. Any debris was removed by centrifugation at 10,000 x g for 30 seconds at room temperature and the supernatant transferred to a clean 2 ml microfuge tube. Solution C2 (250 µl, containing a reagent to precipitate non-DNA organic and inorganic material) was added to the supernatant, mixed by vortexing for 5 seconds and incubated at 4 °C for 5 minutes. The solution then centrifuged at room temperature for 1 minute at 10,000 x g. Approximately 600 µl of supernatant was transferred to a clean 2 ml microfuge tube and 200 µl of solution C3, containing the second reagent to precipitate additional non-DNA organic and inorganic material, was added and the solution vortexed briefly before being incubated at 4 °C for 5 minutes. The aqueous phase containing nucleic acids was separated by centrifugation at room temperature for 1 minute at 10,000 x g. The supernatant (750 µl) was transferred to a clean 2 ml microfuge tube and 1.2 ml of C4 solution containing high concentration of salt solution) was added to the supernatant prior to vortexing for 5 seconds. Approximately 675 µl of the solution was loaded to a spin filter (to

collect the DNA) and centrifuged at 10,000 x g for 1 minute at room temperature. The flow through was discarded. This step was repeated until no solution remained (total three loads). After the third load 500 µl of solution C5 (ethanol) was added to the spin filter and centrifuged (10,000 x g at room temperature) for 30 seconds. The filtrate was discarded and centrifugation repeated for 1 minute to remove any remaining liquid. The spin filter was placed in a new 2 ml microfuge tube and 50 µl of solution C6 (10 mM Tris-EDTA (TE) buffer) was added to the centre of the white filter membrane. The tube was centrifuged (10,000 x g at room temperature) for 30 seconds. The spin filter was discarded and the tube containing nucleic acid was stored at -20 °C for further use.

B. DNA quality check

DNA quality was checked using ND 1000 V3.2.1 spectrophotometer by dropping 1 µl of DNA solution on the chamber. Samples with 260:280 ratios above 1.8 were accepted as “pure” for DNA. If the ratio is appreciably lower, this indicated the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm and the DNA extraction procedure was repeated to obtain a good quality sample.

C. Polymerase Chain Reaction (PCR) amplification

Primers ITS1 (forward) and ITS4 (reverse) were used to amplify both the ITS1 and ITS2 rDNA regions of any fungus in the samples. The two ITS regions are highly variable to discern among very closely related taxa based on the variation found. PCR amplification was performed using a total volume 25 µl reaction mixture consisting of 2.5 µl 10x NH₄ PCR buffer, 2 µl of 50 mM MgCl₂, 0.2 µl of 25 mM dNTP, 0.1 µl of each 100 mM ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers (White *et al*, 1990), 0.25 µl Taq polymerase (Bioline, UK), 18.85 µl of sterile water, and 1 µl DNA template. The PCR reaction was performed on a DNA Engine DYAD™ Peltier thermal cycler (MJ Research, USA) in 0.2 ml tube. A total 35 cycles were performed using the following cycle conditions : 5 minutes at 94 °C, 30 seconds at 94 °C, 1 minute at 55 °C, 3 minutes at 72 °C and a final extension at 72 °C for 3 minutes. Two µl of the PCR product was checked by electrophoresis in 100% agarose gels stained with GelRed™ Nucleid Acid (section 2.2.3.D) prior to being sent to Genevision (INEX Business Centre, Newcastle Upon Tyne, UK) for clean-up and sequencing. At Genevision the templates were cleaned up using either Qiagen QIAquick® PCR purification kit, Qiagen QIAquick® gel extraction kit or Promega Wizard® PCR clean-up kit. The samples were then sequenced using ITS 1 forward primer. Sequence reactions were accomplished using Applied Biosystems BigDye® cycle chemistry. Analyses were performed on ABI 3730xl

capillary sequencers. Raw data from Genevision were matched against the National Centre for Biotechnology Information (NCBI) GenBank database via <http://blast.ncbi.nlm.nih.gov/Blast>. All sequences obtained are in appendix A.

D. DNA agarose gel electrophoresis

Agarose (0.50 g) was dissolved in 50 ml Tris-acetate-EDTA buffer (TAE buffer, pH 8.3) by heating in a microwave until dissolved. Subsequently 2 μ l of GelRedTM Nucleid Acid gel stain (Biotium, USA) was added, the mixture gently swirled and the gel then poured into a gel tank (with gel comb) and allowed to set for 30 minutes.

For electrophoresis, 1 μ l DNA sample from section 2.2.3 was mixed with 5 μ l 6x DNA loading dye (10 mM Tris-HCl pH 7.6, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol 60 mM EDTA) before loading. The gel was run at 120V for 30 minutes in TAE running buffer for electrophoresis. Gels were visualised under UV light using a gel documentation system (Uvitec BXT-20.M).

2.2.4 Characterisation of silver nanoparticle (AgNPs)

The AgNPs used in the experiment were obtained from M K Impex Corp. Mississauga, Canada (MKN-Ag-020). AgNPs were characterised, in terms of structure and particle size distribution. AgNPs structure and size distribution analyses were carried out by L. Siller (School of Chemical Engineering and Advance Material, Newcastle University). The structure of AgNPs was analysed using JEOL 2100F FEG Transmission electron microscopy (TEM) at Durham University. To prepare samples for images a dilute suspension of AgNPs were dropped on a 300 mesh Cu grid with lacey carbon film and then air dried. The particle size distribution of AgNPs was determined by a Dynamic Light Scattering (DLS) technique using a Zetasizer Nano S (Malverin, UK) at Newcastle University. One ml of sample was used for analysis in plastic cuvettes. The suspension was prepared by suspending the AgNPs in DI water (10 ml) with further dilution as needed. The AgNPs were sonicated for 5 minutes immediately prior to making the DLS measurements.

The behaviour e.g. aggregation of AgNPs, in growth media was also observed using light microscopy. AgNPs were added to Potato Dextrose Agar (PDA) media before autoclaving at 121 °C for 15 minutes. Sterile media were shaken thoroughly before being plated into 90 mm petri dishes. Once the media had set, a small amount of AgNPs containing media was placed onto a microscope slide and observed under a light microscope.

2.2.5 The impact of AgNPs on *T. harzianum* colony diameter

Several levels of AgNPs (10, 15, 50, 100, 150, 200, 600, 800 and 1000 mg L⁻¹ of AgNPs) were added to Czapek Dox Agar (CDA) and Potato Dextrose Agar (PDA), before autoclaving at 121 °C for 15 minutes. In a separate experiment, adding AgNPs after autoclaving gave similar growth results on both media employed and so only results obtained using autoclaved AgNPs are presented in this work. Sterile media containing AgNPs were swirled thoroughly before being plated into 90 mm petri dishes. Once the media had set, a 3 mm plug of 7-day-old *Trichoderma* culture was placed in the centre and incubated at 24 °C. The growth of *Trichoderma* was observed by measuring the colony diameter at 24 hours intervals until control plates were fully covered by hyphae (4 days). Control plates were prepared without AgNPs. All experiments were carried out in triplicate.

2.2.6 The impact of AgNPs on *T. harzianum* spore production

The spores number produced by both *Trichoderma* species (from section 2.2.5) was counted at the 28th day after inoculation. The spores were harvested by pouring sterile distilled water on the culture. One ml of spore suspension was transferred to a clean 1.5 ml microfuge tube. Dilutions were made up to 10⁻³, depending on density of spores. Twenty µL of the spores dilution were dropped on the centre of haemocytometer and observed under light microscope. Spores from five random squares (0.04 mm² each square) were counted as the sample. To calculate the number of spores per ml suspension, equation below was used:

$$\frac{\text{number of spores counted in 5 squares}}{5} \times 25 \times 10^4 \times \text{dilution factor}$$

Control plates were prepared without AgNPs. All experiments were carried out in triplicate.

2.2.7 The impact of AgNPs on *T. harzianum* spore viability

Spore viability was studied at the highest level of AgNPs (1000 mg L⁻¹) in PDA. Media were prepared as above (section 2.2.5). The spore viability was observed at the 7th, 14th, 21st, and 28th day after inoculation by pouring sterile distilled water on the culture. One ml of spore suspension was transferred to a clean 1.5 ml microfuge tube. The spore suspension was adjusted to approximately 10 spores in 1 µl by diluting the spore suspension in both control and AgNPs exposed cultures. Ten µL of spore suspension was spread on Rose Bengal Agar (RBA) media and incubated at 24 °C. After incubation, the number of colony forming units (cfu) on agar plates was counted (2 days). Colonies grow on the media were regarded as viable spores. The

number of colonies produced from spores in control (no AgNPs) cultures was compared to the number of colonies produced from equivalent amount of spores in AgNPs exposed cultures.

2.2.8 Statistical analysis

The data on colony diameter, spore production, and spore viability were statistically analysed for Analysis of Variance (ANOVA) on Minitab 17. Significant differences between mean values were determined using LSD (P=0.05).

2.3 Results

2.3.1 Identification of fungal isolates

The six selected colonies purified on fresh PDA were identified morphologically based on colony characters such as pigmentation and reproductive structures under light microscope (Figure 2.1). Based on the colony appearance, two cultures were identified as *Trichoderma* species (Figure 2.1 2A and 4A). *Trichoderma* can be easily recognised due to selected gross morphological characteristics such as mycelial growth and spore colour (green). The conidiophore was branched and aggregated characteristic of *Trichoderma* species (Figure 2.1 2B and 4B) (Gams and Bissett, 1998). The morphological identification was confirmed by molecular identification. The ITS sequences were matched against the National Centre for Biotechnology Information (NCBI) GenBank database via <http://blast.ncbi.nlm.nih.gov/Blast>. Both sequences were identified as strain of *T. harzianum*. The differences between the two sequences are shown on Figure 2.2. The two isolates identified as *T. harzianum* were employed to study the effect of AgNPs on *T. harzianum* in this experiment. Hereafter they are referred to as *T. harzianum* strain 1 and *T. harzianum* strain 2.

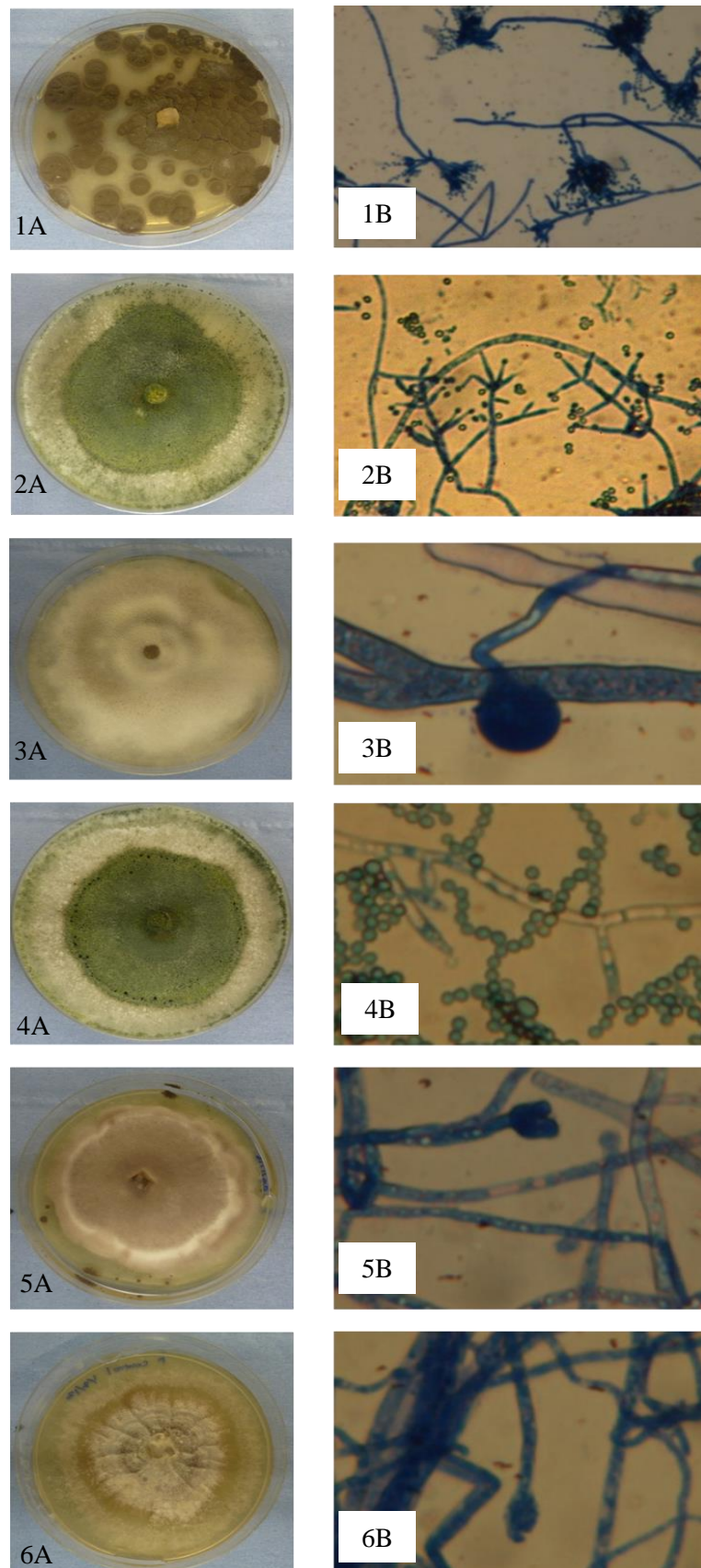


Figure 2.1 Photographs showing the gross morphology of fungi isolated from UK soil on PDA (1A-6A) and microscopic observation of reproductive structure (1B-6B). 1. *Penicillium* sp.; 2 and 4 *T. harzianum*.; 3. *Mucor* sp ; 5 and 6 *Mortierella* sp.

Query	1	AACCCAATGTGAACGTTACCAAACCTGTTGCCTCGGCGGGATCTCTGCCCCGGGTGCGTCCG	60
Sbjct	5	AACCCAATGTGAACGTTACCAAACCTGTTGCCTCGGCGGGATCTCTGCCCCGGGTGCGTCCG	64
Query	61	CAGCCCCGGACCAAGGCGCCCGCCGGAGGACCAACCTAAAACTCTTATTGTATACCCCCT	120
Sbjct	65	CAGCCCCGGACCAAGGCGCCCGCCGGAGGACCAACC-AAAACTCTTATTGTATACCCCCT	123
Query	121	CGCGGGgttttttATAATCTGAGCCTTCTCGGCGCCTCTCGTAGGCGTTTCGAAAATGA	180
Sbjct	124	CGCGGGTTTTTTTATAATCTGAGCC-TTCTCGGCGCCTCTCGTAGGCGTTTCGAAAATGA	182
Query	181	ATCAAAACTTTCAACAACGGATCTCTTGGTCTGGCATCGATGAAGAACGCAGCGAAATG	240
Sbjct	183	ATCAAAACTTTCAACAACGGATCTCTTGGTCTGGCATCGATGAAGAACGCAGCGAAATG	242
Query	241	CGATAAGTAATGTGAATTGCAGAAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG	300
Sbjct	243	CGATAAGTAATGTGAATTGCAGAAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG	302
Query	301	CCC GCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCC	360
Sbjct	303	CCC GCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCC	362
Query	361	GGGGGGTTCGGCGTTGGGGATCGGGCCCTCCCTTAGCGGGTGGCCGCTCCGAAATACAGTG	420
Sbjct	363	GGGGGGTTCGGCGTTGGGGATCGGGCCCTGCCTTGGC-GGTGGCCGCTCCGAAATACAGTG	421
Query	421	GCGGTCTCGCCGCGAGCCTCTCCTGCGCAGTAGTTTCACACTCGCATCGGGAGCGCGGGC	480
Sbjct	422	GCGGTCTCGCCGCGAGCCTCTCCTGCGCAGTAGTTTCACACTCGCATCGGGAGCGCGGGC	481
Query	481	CGTCCACAGCCGTTAAACACCCCAACTTCTGAAATGTTGACCTCGGATCAGGTAGGAATAC	540
Sbjct	482	CGTCCACAGCCGTTAAACACCCCAACTTCTGAAATGTTGACCTCGGATCAGGTAGGAATAC	541
Query	541	CCGCTGAACTTAAGCATATCATAA	564
Sbjct	542	CCGCTGAACTTAAGCATATCATAA	565

Figure 2.2 DNA sequences (ITS) of the 2 *Trichoderma* isolates. Variation in DNA sequences between the strains are shown in the circles.

2.3.2 AgNPs analysis

The average diameter found by DLS to be in the range of 60-120 nm (Figure 2.3) and HRTEM analysis revealed that AgNPs in this study were mainly present as aggregates (Figure 2.4) Microscopic observation of AgNPs in growth media showed agglomeration of AgNPs (Figure 2.5).

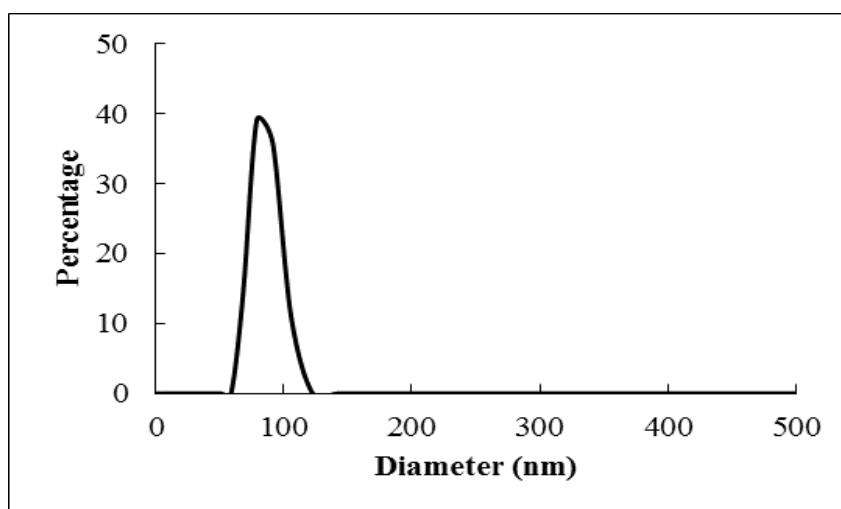


Figure 2.3 DLS showing that the diameter of silver nanoparticles varied from ~60 to ~120 nm.

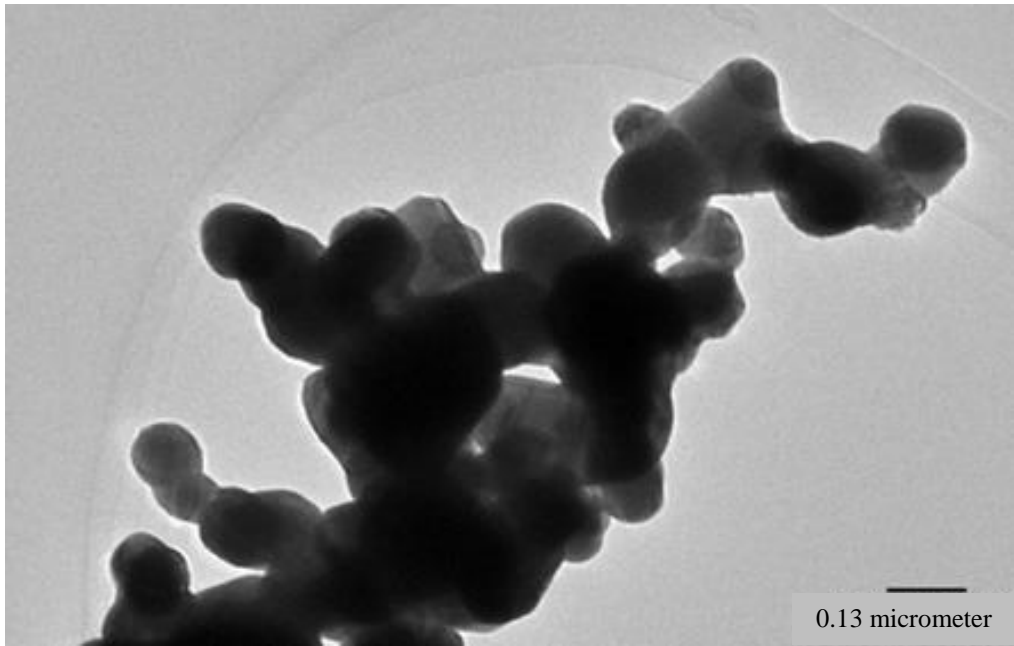


Figure 2.4 HRTEM image showing AgNPs present as aggregates

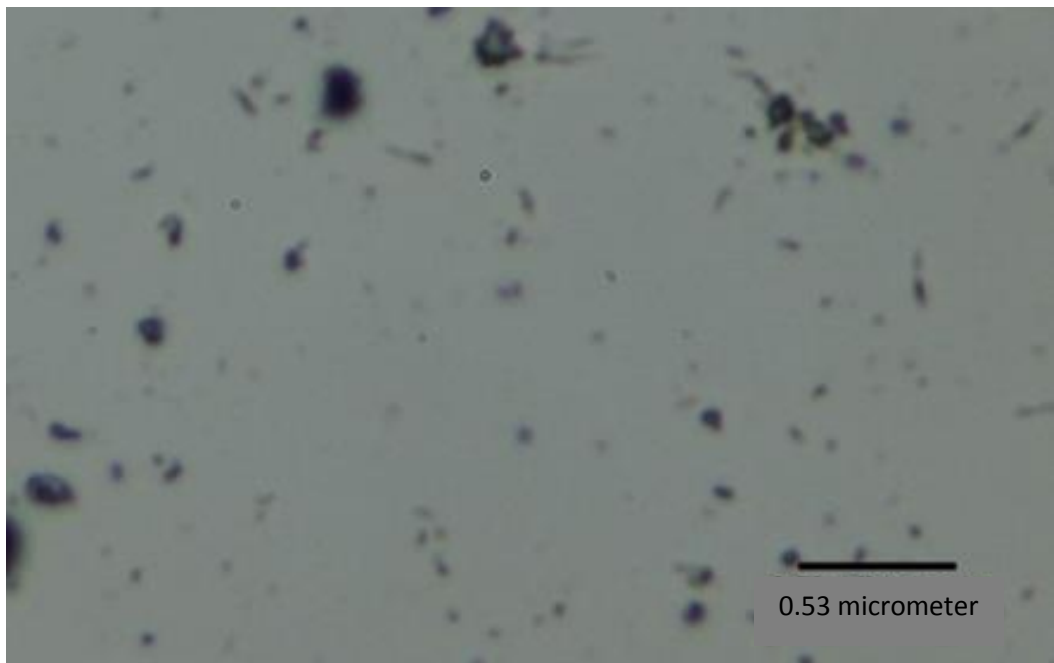


Figure 2.5 Light microscope image of AgNPs in PDA shows agglomeration.

2.3.3 *The impact of AgNPs on T. harzianum colony diameter*

The colony diameter of *T. harzianum* strain 1 and *T. harzianum* strain 2 was measured every 24 hours for four days, when the plate was covered fully by hyphae. Nine levels of AgNPs (10, 15, 50, 100, 150, 200, 600, 800, 1000 mg L⁻¹) were used to determine the level that affected colony diameter of *T. harzianum* strain 1 and *T. harzianum* strain 2. Both strains of *T. harzianum* were grown on two types of media (PDA and CDA) to see if the type of growth media altered the colony response to AgNPs exposure.

On the last day of observation, the control plates were fully covered by both *T. harzianum* strain 1 and *T. harzianum* strain 2 hyphae (8.7 cm colony diameter). Figure 2.6 shows the effect of AgNPs on *T. harzianum* strain 1 and *T. harzianum* strain 2 grown on PDA and CDA. The levels of AgNPs that caused growth reduction varied depending on the growth media used. In all cases the effect of AgNPs on *T. harzianum* growth were more pronounced in CDA than PDA.

In the case of *T. harzianum* strain 1 grown on PDA, colony diameter was only reduces significantly at 800 mg L⁻¹ of AgNPs (Figure 2.6A). However, colony diameter was drop significantly ($P < 0.05$) at 600, 800 and 1000 mg L⁻¹ when the fungi were grown on CDA. The colony diameter of *T. harzianum* strain 2 grown on PDA, was not affected by any level of AgNPs employed (Figure 2.6B). The growth was exactly as the control (without AgNPs). The colony diameter of *T. harzianum* strain 2 grown on CDA reacted similar to *T. harzianum* strain 1 on CDA. Fact that colonies growth of *T. harzianum* strain 1 and *T. harzianum* strain 2 reduced at high level of AgNPs suggest that the colony growth was not affected by media type.

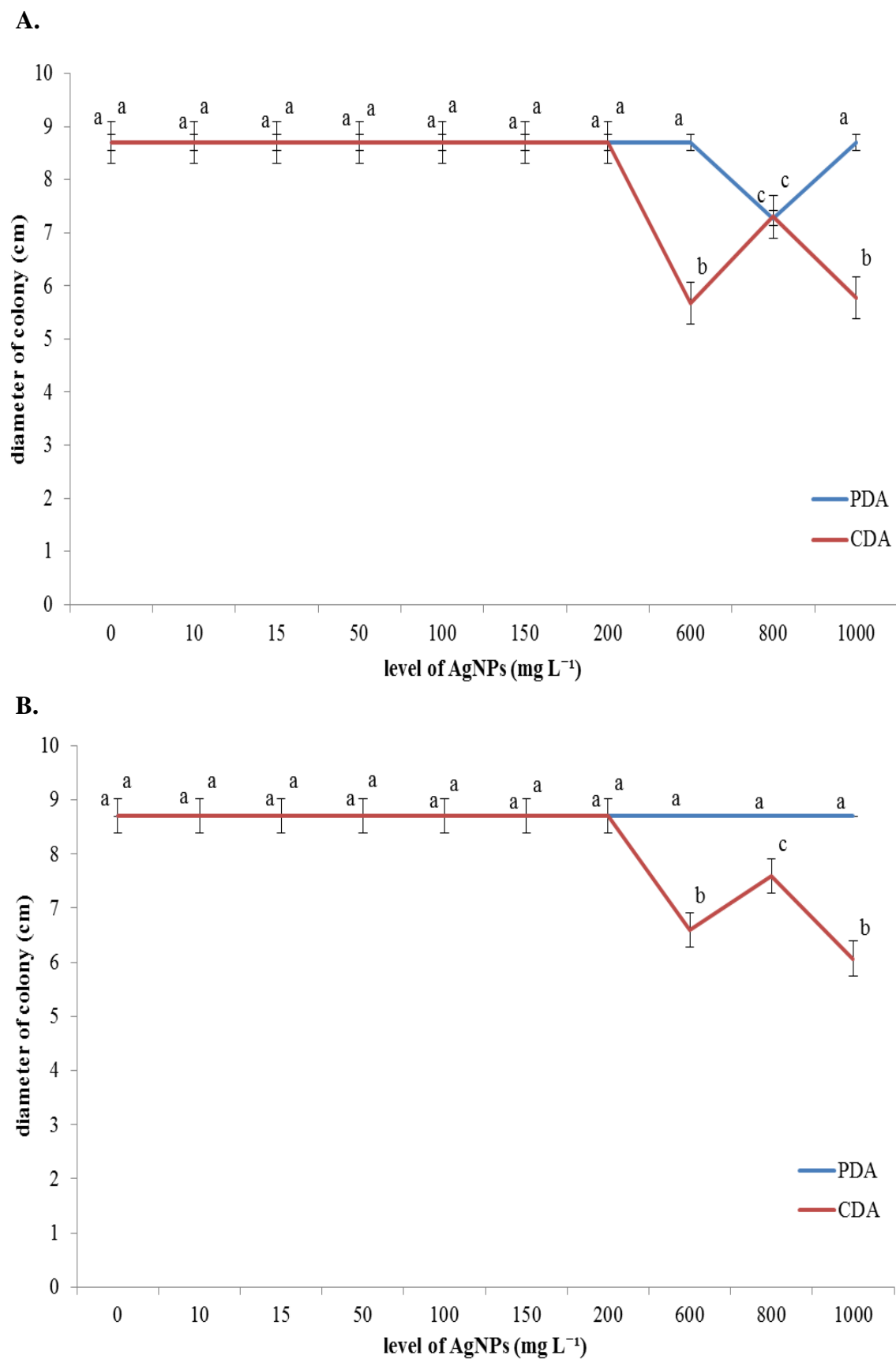


Figure 2.6 Colony diameter of *Trichoderma harzianum* strain 1 (A) and *T. harzianum* strain 2 (B) grown on PDA and CDA at different levels of AgNPs (0, 10, 50, 100, 150, 200, 600, 800, 1000 mg L⁻¹). The colony diameter was measured on the 4th day after inoculation. Data represent means of three replicates with standard error. Different letters above the data points (PDA and CDA) indicate significant difference between treatments at the level of P < 0.05.

2.3.4 The impact of AgNPs on *T. harzianum* spore production

The spore of *T. harzianum* strain 1 and *T. harzianum* strain 2 were harvested and counted on the 28th day after inoculation. Nine levels of AgNPs (10, 15, 50, 100, 150, 200, 600, 800, 1000 mg L⁻¹) were used to see which level that give an affect to spore production of *T. harzianum* strain 1 and *T. harzianum* strain 2. Both strains of *T. harzianum* were grown on two types of media (PDA and CDA) to see if the type of growth media altered the spore production response to AgNPs exposure.

Figure 2.7 and Figure 2.8 show the impact of AgNPs on spore production of *T. harzianum* strain 1 and *T. harzianum* strain 2 grown on PDA (Figure A) and CDA (Figure B). In contrast to the effect of AgNPs on colony growth (section 2.3.3), AgNPs affect spore production at a lower level of AgNPs.

There is no clear pattern on the effect of AgNPs on spore production on both of the strains tested. On *T. harzianum* strain 1 grown on PDA (Figure 2.7A), 50 mg L⁻¹ of AgNPs reduced the spore production significantly. There are no substantial changes on the spore production at 100, 150, and 200 mg L⁻¹ of AgNPs compared to the control. The presence of AgNPs at 600, 800 and 1000 mg L⁻¹ on PDA reduced the spore production by more than 50%. There is also a significant decrease in the number of spore production at 50 mg L⁻¹ of AgNPs. However, the application of AgNPs on CDA growth media did not affect the spore production of *T. harzianum* strain 1 at any level (Figure 2.7B). In the case of *T. harzianum* strain 2 on PDA (Figure 2.8A) a significant decrease on spore production can be seen at 15 and 600 mg L⁻¹ of AgNPs. Interestingly, the presence of AgNPs in CDA only affected the spore production at 15 mg L⁻¹ (Figure 2.8B).

The results also suggested that generally *T. harzianum* strain 1 and *T. harzianum* strain 2 grown on PDA produced more spores than grown on CDA.

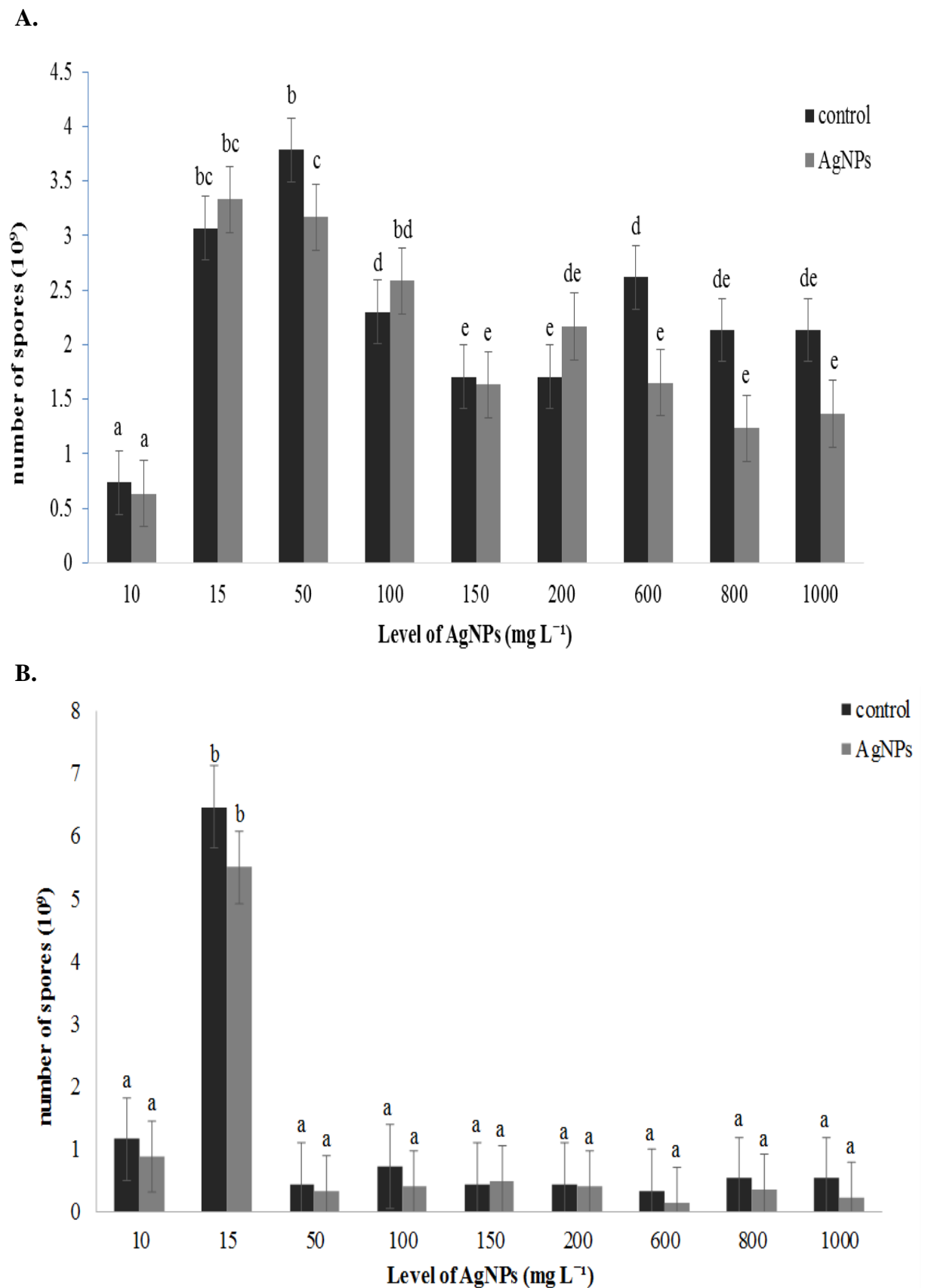


Figure 2.7 Spore production of *Trichoderma harzianum* strain 1 grown on PDA (A) and on CDA (B) at different levels of AgNPs (10, 50, 100, 150, 200, 600, 800, 1000 mg L⁻¹). The number of spores was counted on the 28th day after inoculation. Data represent means of three replicates with standard error. Different letters above the data points (control and AgNPs) indicates significant difference between treatments at the level of P < 0.05.

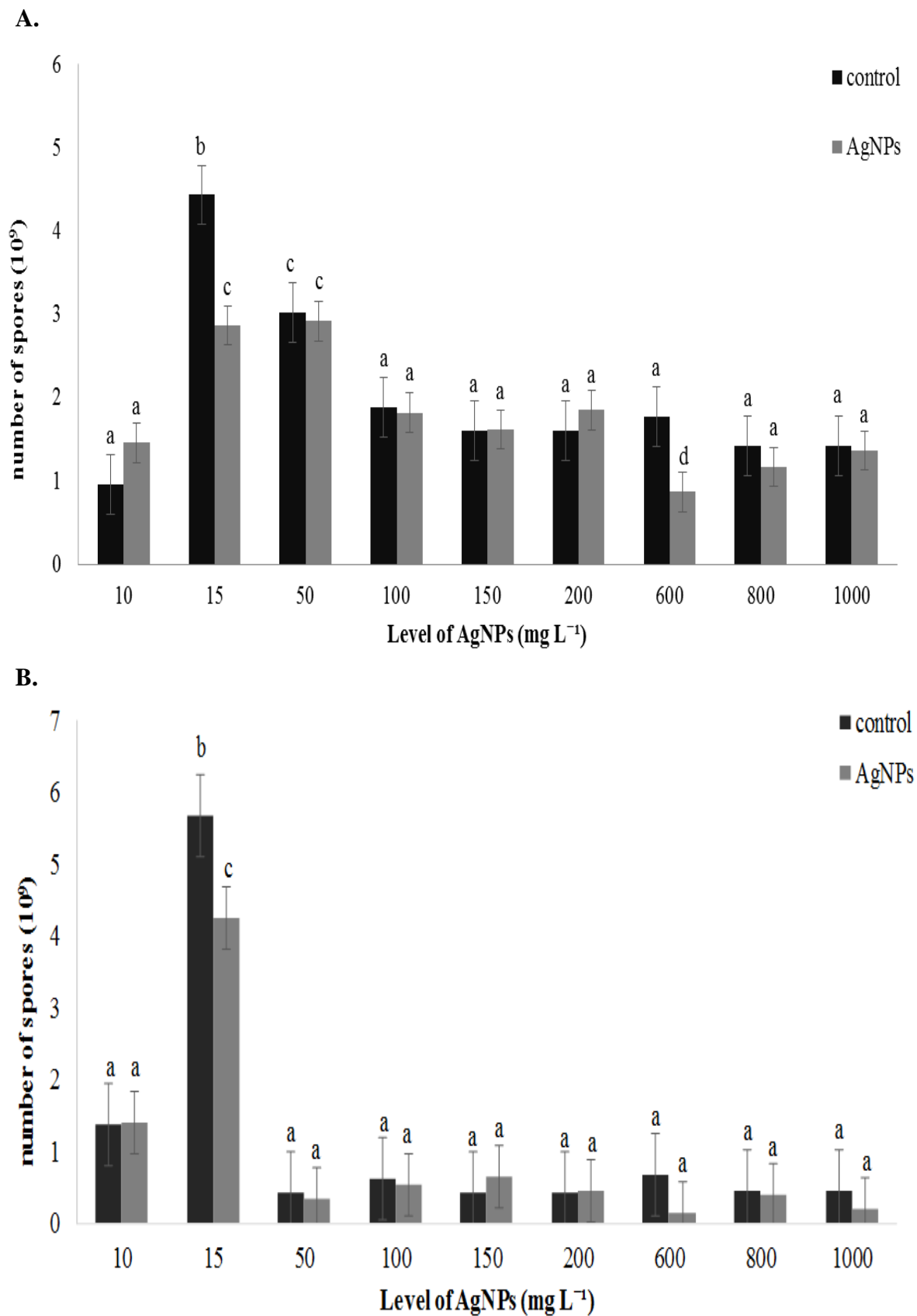


Figure 2.8 Spore production of *Trichoderma harzianum* strain 2 grown on PDA (A) and on CDA (B) at different levels of AgNPs (10, 50, 100, 150, 200, 600, 800, 1000 mg L⁻¹). The number of spores was counted on the 28th day after inoculation. Data represent means of three replicates with standard error. Different letters above the data points (control and AgNPs) indicate a significant difference between treatments at the level of P < 0.05.

2.3.5 The impact of AgNPs on *T. harzianum* spore viability

Based on the previous findings (sections 2.2.3 and 2.2.4) that the colony diameter and spore production of *T. harzianum* strain 1 and *T. harzianum* strain 2 grown on PDA, colony diameter and spore production were not affected by the presence of AgNPs at high level it was decided to investigate the viability of spores produced. The spore viability was tested on the highest level of AgNPs (1000 mg L^{-1}) at different age of culture (7, 14, 21 and 28 days after inoculation). The spores from AgNPs containing media were grown on RBA (without AgNPs) for colony forming unit (cfu) counting.

Figure 2.9 shows that spore viability of the *T. harzianum* strains varied after growth in AgNPs containing media (1000 mg L^{-1}). For *T. harzianum* strain 1, 80% of spores produced by a 7 day old culture were viable. This dropped to 60% viability in 14 day old cultures but returned to initial figures in 28 day old cultures. The percentage of viable spore of *T. harzianum* strain 2 was lower than strain 1 in 7 day old cultures. However, the percentage viability increased significantly in line with the age of culture.

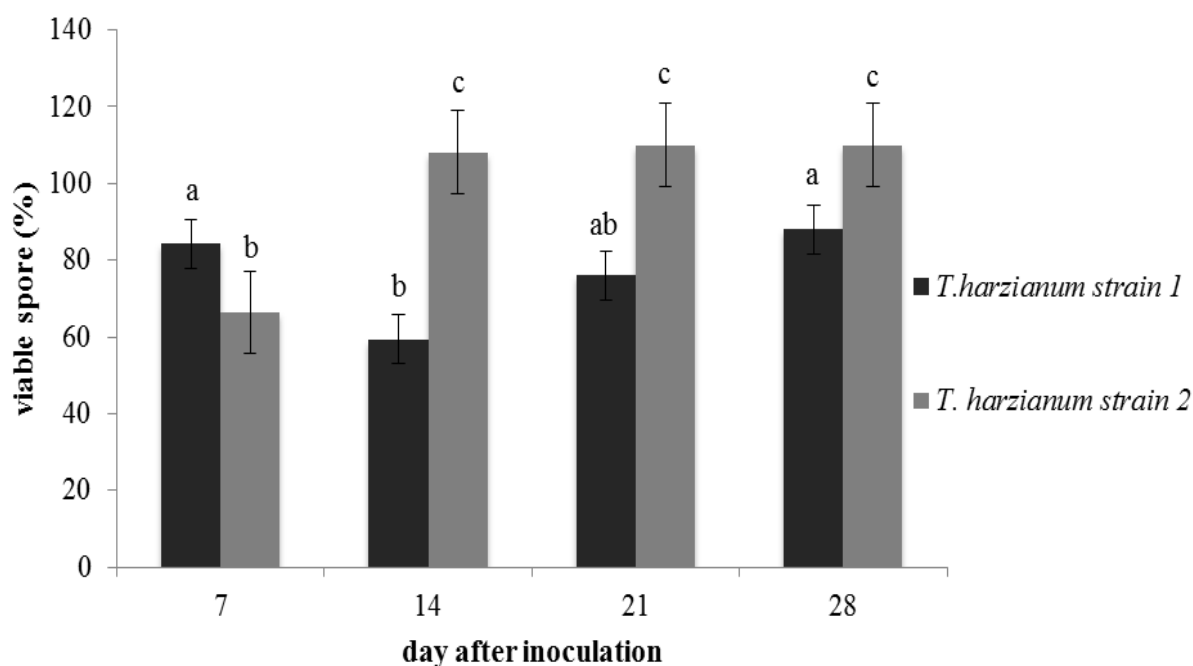


Figure 2.9 Percentage of viable spore produced by *T. harzianum* strain 1 and 2 after growth in AgNPs containing media at 1000 mg L^{-1} . The number of viable spores was counted on the 7th, 14th, 21st, 28th day after inoculation. Data represent means of three replicates with standard error. Different letters above the data points (*T. harzianum* strain 1 and *T. harzianum* strain 2) indicate significant difference between treatments at the level of $P < 0.05$.

2.4 Discussion

2.4.1 *Trichoderma harzianum* identification

The genus *Trichoderma* is easily characterised by rapid growth, mostly bright green conidia and repetitively branched conidiospore structure. However, it is difficult to interpret the species because a descriptive term to describe variation in colour or pattern is insufficient to distinct between species (Gams and Bissett, 1998). Rifai (1969) divided *Trichoderma* into 9 species based on morphological features. The genus was revised and resulted in the establishment of five new sections (Bissett, 1991). Classical microbial identification e.g. morphological and physiological based could only identify at the species level.

Along with advances in technology, DNA-based methods have been routinely used in species identification leading to more accurate *Trichoderma* species discrimination. For example, strains from *Trichoderma* species were identified as *T. harzianum* but molecular identification suggests that they should be identified as *T. inhamatum*, *T. longibrachiatum*, and *T. atroviride*. (Hermosa *et al.*, 2000). Furthermore, molecular identification approaches identified new strains from *Trichoderma* species that are not yet taxonomically established (Oskiera *et al.*, 2015). The complexity in *Trichoderma* species identification comes from the fact that many of these species types are overlapping and therefore, two closely related organisms recognized as a single species (Gherbawy *et al.*, 2014).

The introduction of molecular methods and analysis of DNA sequences in the early 1990's resulted in the dramatic taxonomic changes (Druzhinina *et al.*, 2010). Taxonomy of *Trichoderma* is continuously adjusted and updated (Oskiera *et al.*, 2015). Recently, there are 228 species of *Trichoderma* identified, hence several not yet taxonomically characterized (Jaklitsch and Voglmayr, 2015).

2.4.2 *Mycelia growth under AgNPs-stress*

Overall there is limited literature available regarding the effect of AgNPs on *Trichoderma* species growth. Gavanji *et al.* (2012) reported that 25 mg L⁻¹ of AgNPs inhibited colony growth of *T. harzianum* and *T. viride* by 50%. Similarly, Mahdizadeh *et al.* (2015) reported 90% growth inhibition of *T. harzianum* at 10 mg L⁻¹. This is in contrast to the current finding in this thesis, colony growth was affected at 600 mg L⁻¹ of AgNPs. These findings suggest that sensitivity to silver is likely to be different between fungal strains of the same species but this should be tested further using similar growth conditions. Furthermore, the antimicrobial activity of AgNPs depends on their size. The smaller the size the more toxic because they potentially

release many Ag ions that affect microbial activity (Sotiriou and Pratsinis, 2010). The size of AgNPs applied in this study was much larger than nanoparticles used by Gavanji *et al.* (2012) with their average size of 18-34 nm. The average diameter of AgNPs in this study was found to be in the range of 60-120 nm. In addition, microscopic observation of AgNPs in growth media showed agglomeration of AgNPs which presumably reduces their toxicity by reducing available surface area.

Another possible explanation for the resistance of *T. harzianum* to AgNPs is the ability of *Trichoderma* species to produce nanoparticles when exposed to metal ions. This ability is thought to be a detoxification mechanism e.g. toxic soluble metal ions are reduced to elemental nanoparticles which are less toxic. For example, a previous study showed that *T. asperellum* (Mukherjee *et al.*, 2008) and *T. viride* (Fayas *et al.*, 2010) produced AgNPs as by-product of their metabolism. Vahabi *et al.* (2011) revealed that *T. reesei* produce enzyme and metabolites for their survival when the mycelium was exposed to silver nanoparticles. In this process the toxic metal ions were reduced to the non-toxic metallic AgNPs through the catalytic effect of the extracellular enzyme and metabolites of the fungus. The ability of a variety of fungal species to produce nanoparticles is known as green synthesis and has potential commercial relevance. *Trichoderma* species has been used for synthesis of silver nanoparticles include *T. asperellum* (Mukherjee *et al.*, 2008), *T. viride* (Fayaz *et al.*, 2010), *T. reesei* (Vahabi *et al.*, 2011), *T. virens* (Devi *et al.*, 2013) and *T. harzianum* (Ahluwalia *et al.*, 2014). Biological methods for nanoparticles synthesis are more favourable than chemical means as it relatively simple, cheap, and environmentally friendly (Kulkarni and Muddapur, 2014). Furthermore, species of *Trichoderma* have also been studied for their use in the remediation of environments contaminated with heavy metal. Lima *et al.* (2011) reported the potential of *T. harzianum* as cadmium (Cd) removal. Similarly, Mohsenzadeh and Shahrokhi (2014) suggested that *T. harzianum*, *T. asperellum*, and *T. tomentosum* were able to reduce the amount of Cd in growth media.

The effect of AgNPs has also been studied on other fungal species such as plant pathogenic fungi. Min *et al.* (2009) reported that very low AgNPs concentration (7 mg L^{-1}) inhibited hyphal growth of *R. solani*. However, higher levels of AgNPs, 100 and 180 mg L^{-1} , were needed to significantly reduced the growth of *Colletotrichum* and *Aspergillus paraciticus*, respectively (Lamsal *et al.*, 2011; Mousavi and Pourtalebi, 2015). The reports show that plant pathogenic fungi seem to be more susceptible to AgNPs than *T. harzianum*. It is widely known that *T. harzianum* has ability to control plant pathogenic fungi in soil include *R. solani*. The

resistance of *T. harzianum* and the susceptible of *R. solani* to AgNPs would potentially allow a combination of *T. harzianum* and a lower level of AgNPs to be used in a commercial setting to control *R. solani*. This will be further discussed in the next chapter of this thesis.

Literatures regarding the impact of AgNPs on soil microorganisms is not limited to fungi. Beneficial microorganisms in soil such as *Bradyrhizobium canariense* and *Pseudomonas chlororaphis*. *B. canariense* are also reported to be sensitive to AgNPs. Kumar *et al.* (2011) revealed that the plant-associated bacterium, *B. canariense*, is highly susceptible to AgNPs. It is generally known that *B. canariense* fixes nitrogen to nitrate so that plants can use it to make protein for their growth. It is true that *B. canariense* is not the only nitrogen fixing bacteria and that other bacteria might be able to take their place but AgNPs might also affect those bacteria and other beneficial soil microorganisms and reduce the number of species in the environment. A similar observation was made by Calder *et al.* (2012) during their research on antimicrobial effect of AgNPs towards the beneficial soil bacterium, *P. chlororaphis*. More recent, a study shows that at environmentally relevant concentrations, AgNPs cause toxic effects on soil microorganisms of the terrestrial ecosystem and changes the diversity (Schlich *et al.* 2013). Microbes in the soil play important role in soil that any reduction in soil microbial composition results in low soil quality and plant productivity.

Colony diameter measurement used in the experiment is one of methods of measuring fungal growth rates. The limitation of the technique is there is no necessary correlation between the spread of a mycelia front on a solid surface and the total amount of fungus produced that dry weight measurement is probably the most applicable method to estimate fungal growth (Madan and Thind, 1998).

2.4.3 The impact of AgNPs on *T. harzianum* spore production

This study revealed that spore production was affected by the presence of AgNPs at a lower concentration (15 mg L^{-1}) compared to concentration of AgNPs that affected mycelia growth of *T. harzianum* (600 mg L^{-1}). The finding supports previous study that the production of spores was more sensitive to heavy metal stress than hyphal growth (Cuero, 2003; Miransari, 2016). Raman and Selvaraj (2006) suggesting that distinction should be made between concentrations that inhibit sporulation and those that inhibit fungal growth.

However, at most cases the spore production of *T. harzianum* do not affecting with the presence of AgNPs in the growth media. According to Simonetti *et al.* (1992), Ag has to be in ionised form in order to have anti-microbial properties. They added silver may be used as a metal, but

the active agent appears to be the ions produced. However, the toxicity of AgNPs is still debatable. Navarro *et al.* (2008) presented evidence that AgNPs contribute to toxicity as a source of dissolved Ag ions. On the other hand, some evidence showed a specific nanoparticles effect that could not explained by dissolved Ag⁺ (Fabrega *et al.*, 2009; Yin *et al.*, 2011).

Regardless of the presence of AgNPs in the media used, both *T. harzianum* strains produce more spore on PDA than CDA. This finding in line with Fernando *et al.* (2000) *Colletotrichum acutatum* on PDA produced spore 80% higher than on CDA. Okunowo *et al.* (2010) also observed least sporulation of *Myrothecium roridum* on CDA which may be due to the presence of chloride ion in the test medium.

Work on the effect of AgNPs on spore production of *Trichoderma* species does not appear to have been reported before. There is a related study about the effect of heavy metal on arbuscular mychorrhzal fungi (AMF). Del Val *et al.* (1999) found that the addition of sewage-amended sludge containing heavy metal decrease the number of total AMF spores with the increasing amounts of heavy metal in the soil.

2.4.4 *T. harzianum* spore germination survival after grown on AgNPs contaminated media

Despite the decrease in spore production of *T. harzianum* after AgNPs exposure, the spores successfully germinated when cultivated on fresh growth medium (RBA). Researchers also reported similar finding of the ability *Glomus* sp. spores to germinate after grown on Zn, Mn, Cd, and Pb contaminated media (Hepper and Smith, 1976; Pawlowska and Charvat, 2004). This indicates that fungi from metal environments may be able to survive and germinate when conditions become permissible.

The amount of spores able to produce a separate colony was observed at four different age of culture (7, 14, 21, and 28 days after inoculation). The amount of viable spores increased as the cultures got older. The phenomena is probably due to the changes in cell wall composition. Baldrian (2003) stated that fungal cell wall has the key role in heavy metal sorption capacity. The heavy metal binding capacity is dependent on the mycelia age, young cell held higher adsorption capacities than old ones (Yetis *et al.*, 2000). In line with Ortega-Aguilar *et al.* (2011) that the toxic effect of KHCO₃ on *Trichoderma* sp. growth decreased as the culture get older. Change in cell wall composition or chitin content of the cell wall are suggested as cause of the decrease in metal uptake (Yetis *et al.*, 2000).

2.5 Conclusion

The study revealed that sensitivity to AgNPs is different between two species strains of *Trichoderma*. The *T. harzianum* used in this study was found to be tolerant to high AgNPs concentrations and the tolerance varied depending on the growth medium used. Mycelial growth (as measured by colony diameter) was affected by AgNPs high level of AgNPs. Further study is needed on spore production of *T. harzianum* as the reduction only observed at one low level, not at several higher concentrations. Overall when comparing results from different studies on the effect of AgNPs on fungal growth it is important to take into account the variation in sensitivity due to growth medium used, nanoparticle size and finally note that even fungal strains of the same species can show markedly different responses.

**Chapter III. The Effect of Silver Nanoparticles on the Ability of
Trichoderma harzianum to Control Plant Pathogenic Fungi**

3.1 Introduction

The findings from Chapter 2 demonstrated that *Trichoderma harzianum* is resistance towards silver nanoparticles (AgNPs). On the other hand, plant pathogen such as *Rhizoctonia solani* is reported to be very sensitive to AgNPs (Min *et al.* 2009). This implies the possibility of combining *T. harzianum* and AgNPs at low concentration to control plant pathogens. This could potentially has two outcomes a) the presence of AgNPs could reduce the ability of *T. harzianum* to control pathogens, b) the AgNPs and *T. harzianum* could work synergistically to reduce pathogens growth even further, or c) no interaction between AgNPs and *T. harzianum*.

T. harzianum is well known as biological control agent against a wide range of plant pathogens including soil borne plant pathogenic fungus *Rhizoctonia cerealis* and *R. solani* (Innocenti *et al.*, 2003; Montealegre *et al.*, 2010). *T. harzianum* is the most common antagonist fungi used to control *R. solani* by attacking the mycelium and produced antibiotic (Anees *et al.*, 2010; Hadar *et al.*, 1979). *R. solani* primarily attacks below ground part of plants but it is also capable of infecting above ground plant parts. The pathogen is best known to cause “damping-off”. In the form of sclerotia, *R. solani* can survive in the soil without a host for many years (Georgiou *et al.*, 2000). Sclerotia are compact masses of hardened fungal mycelia with 1-3 mm in diameter containing food reserves formed in response to stress e.g. unfavourable growth condition (Coley-Smith and Cooke, 1971). *R. cerealis* cause sharp eyespot disease on stem base of wheat (*Triticum aestivum* L.) (Lemańczyk and Kwaśna, 2013). The pathogen was not considered to be an important pathogen until the disease become endemic in many countries (Hamada *et al.*, 2011). The antifungal activity of *T. harzianum* on *R. cerealis* has also been studied (Innocenti *et al.*, 2003; Hanson, 2005).

The antagonist mechanisms of *T. harzianum* including competition over space and nutrition, mycoparasitism, antibiotic (toxin) and enzyme production. Competition over space and nutrition are the most well-known mechanism and can be easily studied by plating both fungi on agar medium known as dual culture technique. Therefore, the method was employed to study the effect of AgNPs on the ability of *T. harzianum* to control pathogen in this chapter.

The overall aim of the chapter was to examine the growth of a plant pathogenic fungus, *R. solani* and *R. cerealis*, and theirs biocontrol agent, *T. harzianum*, with the AgNPs presence in the media. Individual objects were to:

- a) examine the colony diameter of *R. solani* and *R. cerealis* with the presence of AgNPs in the media.

- b) examine the ability of *T. harzianum* to control *R. solani* and *R. cerealis* colonial growth with the presence of AgNPs in the media using dual culture technique.
- c) examine sclerotia production of *R. solani* on AgNPs containing media and its germination on fresh media after exposure to AgNPs.

3.2 Materials and Methods

3.2.1 Fungi cultures

Two strains of *T. harzianum* were employed in this study. One was *T. harzianum* strain 1 identified in Chapter 2 and one was obtained from Koppert B.V., The Netherlands, under the name Triatum-P (T22). Using a sterile loop, the powder form of *T. harzianum* (T22) was transferred onto a Potato Dextrose Agar (PDA) plate. Two strains of *Rhizoctonia solani* (AG3 and AG2-1) and *R. cerealis*, kindly provided by J. Woodhal (The Food and Environmental Research, UK), were sub-cultured onto PDA. All *T. harzianum*, *R. solani*, and *R. cerealis* cultures were incubated at 24°C for further use.

3.2.2 Effect of AgNPs on *R. solani* and *R. cerealis* growth

Growth media were prepared by mixing AgNPs powder (20 and 50 mg L⁻¹) with PDA before autoclaving at 121°C for 15 minutes. Sterile media containing AgNPs were swirled thoroughly before being plated into 90 mm petri dishes. Once the media had set, a 3 mm plug of 7-day-old *R. solani* (AG3) culture was placed in the centre and incubated at 24°C. The colony of *R. solani* (AG3) was determined as colony area and measured using graph paper by drawing the colony area on the paper. The number of squares within boundaries was used to calculate the colony area. Same media preparation was applied to *R. solani* (AG2-1) and *R. cerealis*. The growth was observed by measuring the colony every day until control plate was fully covered by hyphae (5 days). Higher concentrations (50 and 150 mg L⁻¹ of AgNPs) were applied to *R. cerealis* cultures. Control plates were prepared without AgNPs. All experiments were carried out in triplicate.

3.2.3 Effect of AgNPs on the ability of *T. harzianum* to control *R. solani* and *R. cerealis*

A dual culture technique was employed to study the effect of AgNPs on the ability of *T. harzianum* to control *R. solani* (AG3 and AG2-1) and *R. cerealis* growth. *T. harzianum* strain 1 (from Chapter 2) were used against *R. solani* (AG3 and AG2-1) while *T. harzianum* (T22) was used against *R. solani* (AG3 and AG2-1) and *R. cerealis*.

Two levels of AgNPs (20 and 50 mg L⁻¹) for *R. solani* (AG3 and AG2-1), and two levels of AgNPs (50 and 150 mg L⁻¹) for *R. cerealis* were prepared as previously described. A 3 mm agar disc of a 7-day-old culture of *T. harzianum* strain 1 was placed at the edge of petri dishes (90 mm diameter). The same size and age of another agar disc of *R. solani* (AG3) was similarly placed on the media but on the opposite end and incubated at 24°C. Antagonistic activity was assessed by measuring diameter of both the pathogen and *T. harzianum* strain 1 colonies daily until the control plate was completely covered by the hypha (5 days). The same technique was applied to *T. harzianum* strain 1 against *R. solani* (AG2-1) and *T. harzianum* (T22) against *R. solani* (AG3 and AG2-1) and *R. cerealis*. Control plates were prepared without AgNPs. All experiments were carried out in triplicate.

3.2.4 Assay for *R. solani* sclerotia production and germination

Observations of sclerotia production and germination were only taken for *R. solani* (AG3). The cultures of *R. solani* (AG3) from section 3.2.2 were incubated at 24°C until sclerotia formed (approximately 6 months). The sclerotia were collected by scraping the surface of the media followed by washing with distilled water. Sclerotia were then rinsed with 70% alcohol (Min *et al.*, 2009) prior to being oven dried at 50°C for 2 hours to determine total dry weights produced.

To test the sclerotia viability, 6 dried sclerotia of approximately similar size ranges were re-grown on a fresh PDA plate (sclerotia was placed approximately 2 cm apart) and incubated at 24°C. A sclerotium was considered to have germinated when any outgrowing hyphae were equal to or greater than the diameter of the sclerotium (Ritchie *et al.*, 2013).

3.2.5 Statistical analysis

All data presented are the mean value of three replicates. Values are expressed as means of three replicates ± standard error (S.E) in each group. All statistical analyses were performed using One-way Analysis of Variance (ANOVA) statistical models on Microsoft Excel 2016. Variance analysis was performed on all experimental data and significant differences (P < 0.05) between individual means (three replicates) was analysed using a post hoc Least Significant Difference test.

3.3 Results

3.3.1 The effect of AgNPs on *R. solani* and *R. cerealis* growth

The current investigation confirmed that AgNPs have a potential to inhibit the plant pathogenic fungus *R. solani*. Figure 3.1 shows that AgNPs at 20 and 50 mg L⁻¹ reduced the colony growth of *R. solani* (AG3) significantly compared to controls (no AgNPs). Interestingly, there is a morphological changes of *R. solani* (AG3) colonies when treated with AgNPs (Figure 3.2). Due to these morphological changes, the colony growth was determined as colony area.

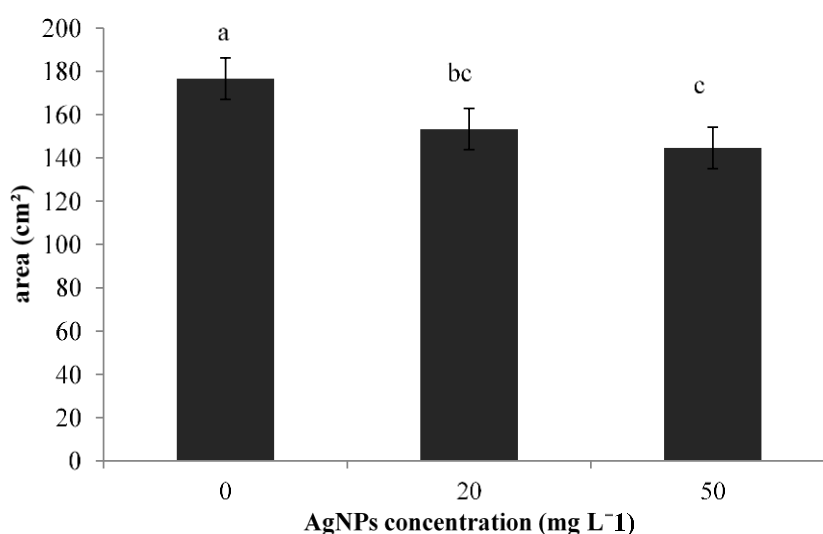


Figure 3.1 Effect of AgNPs at 20 and 50 mg L⁻¹ on *Rhizoctonia solani* (AG3) colony area. Colony areas of *R. solani* (AG3) were decreased significantly by AgNPs at 20 and 50 mg L⁻¹. The data was collected on the 5th day of growth. Data represent means of three replicates with standard error. Different letters above the data points indicate significant difference between treatments at the level of P < 0.05.

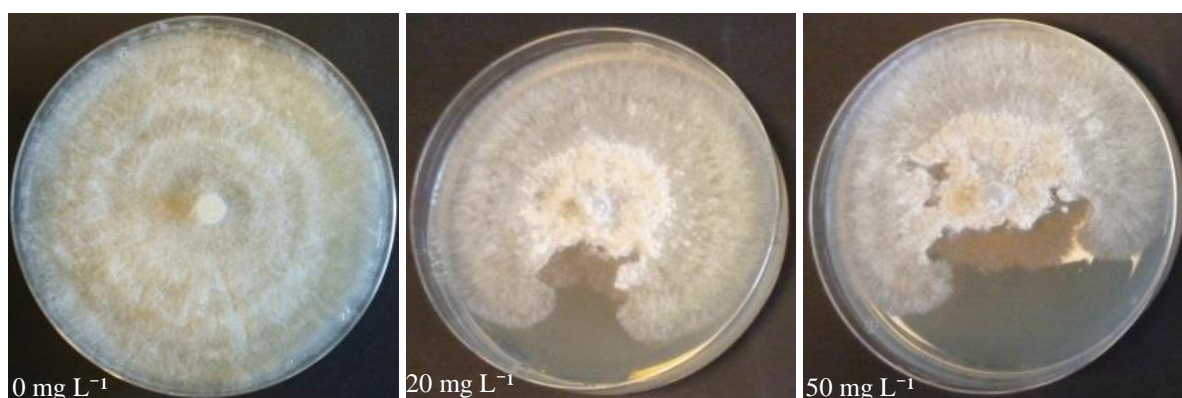


Figure 3.2 Effect of AgNPs at 20 and 50 mg L⁻¹ on *Rhizoctonia solani* (AG3) colony area shows morphological change when treated with AgNPs. The data was collected on the 5th day of growth.

However, the addition of AgNPs in growth media did not affect *R. solani* (AG2-1) and *R. cerealis* growth (Figure 3.3 and 3.4). Even at higher concentration (150 mg L⁻¹ of AgNPs) *R. cerealis* colony grow as much as control (without AgNPs).

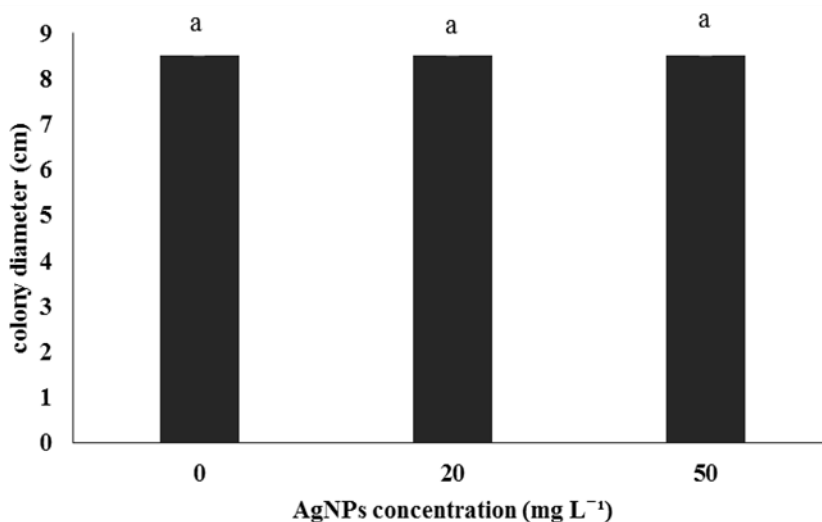


Figure 3.3 Effect of AgNPs at 20 and 50 mg L⁻¹ on *Rhizoctonia solani* (AG2-1) colony diameter. The data was collected on the 5th day of growth. Colony areas of *R. solani* (AG2-1) are not affected by AgNPs presence at 20 and 50 mg L⁻¹. Data represent means of three replicates with standard error. Same letters above the data points indicate insignificant difference between treatments at the level of $P > 0.05$.

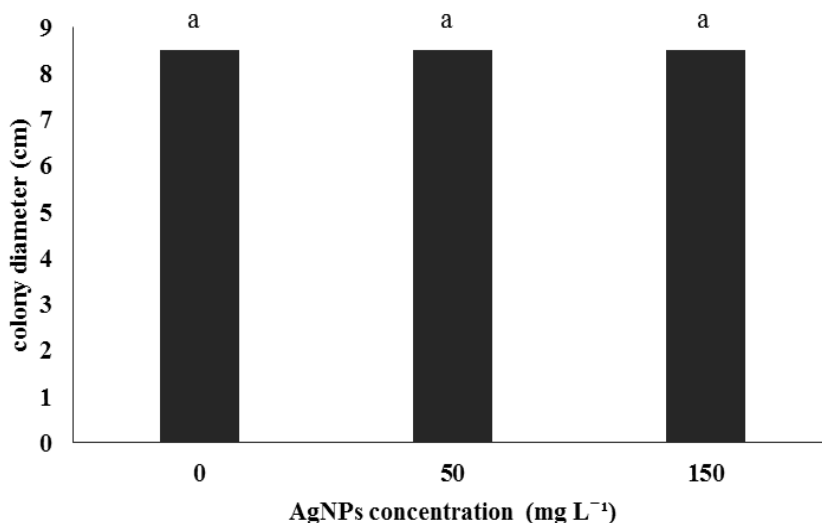


Figure 3.4 Effect of AgNPs at 50 and 150 mg L⁻¹ on *Rhizoctonia cerealis* colony diameter. The data was collected on the 5th day of growth. Colony areas of *R. cerealis* are not affected by AgNPs presence at 50 and 150 mg L⁻¹. Data represent means of three replicates with standard error. Same letters above the data points indicate insignificant difference between treatments at the level of $P > 0.05$.

3.3.2 The effect of AgNPs on ability of *T. harzianum* to control *R. solani* and *R. cerealis* growth

Without *T. harzianum* and AgNPs in the growth media, the colony of *R. solani* (AG3 and AG2-1) and *R. cerealis* grew quickly and reached the edge of the plate within 5 days (4 days for *R. cerealis*). When the pathogens were grown in co-culture with *T. harzianum* the latter fungus significantly restricted their colonies development with and without the presence of AgNPs. *T. harzianum* strain 1 was grown against *R. solani* (AG3 and AG2-1) while *T. harzianum* (T22) grown against the three *Rhizoctonia* spp. Figure 3.5 and 3.6 show the presence of AgNPs in growth media do not give a significant effect on the ability of *T. harzianum* strain 1 to inhibit colony growth of *R. solani* (AG3 and AG2-1).

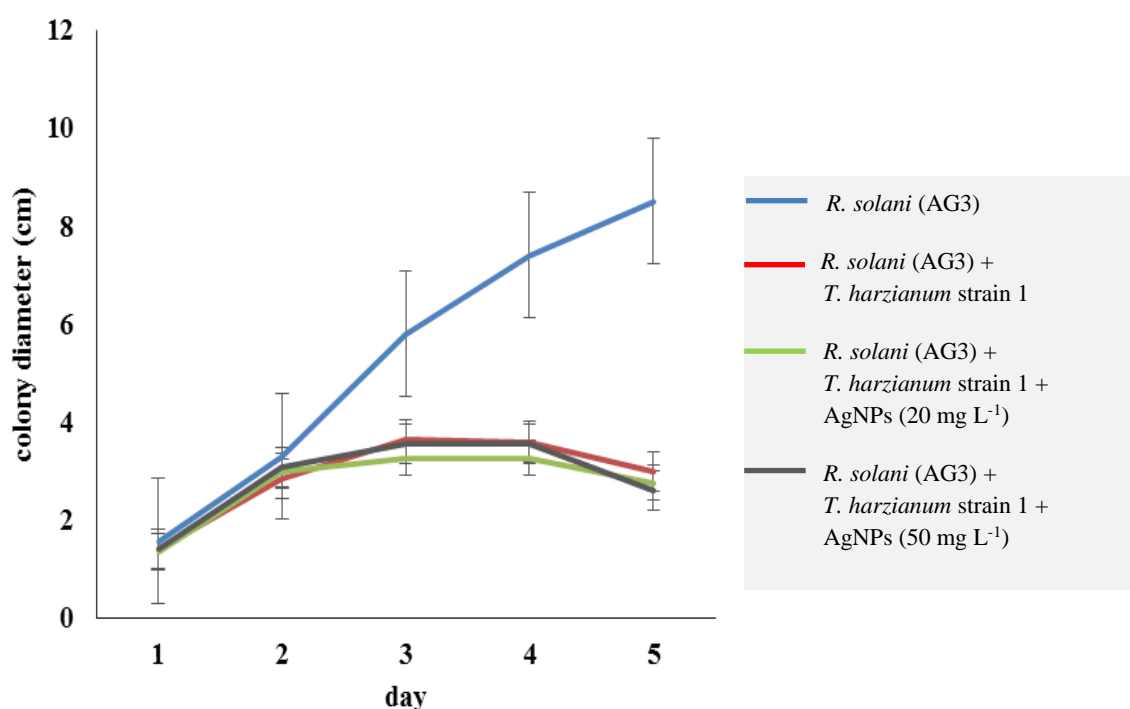


Figure 3.5 Diameter colony of *Rhizoctonia solani* (AG3) with and without AgNPs at 20 and 50 mg L⁻¹ and *Trichoderma harzianum* strain 1 presence in growth media. The colony diameter was measured daily for 5 days. AgNPs do not improve the ability of *T. harzianum* to control *R. solani* growth. Data represent means of three replicates with standard error.

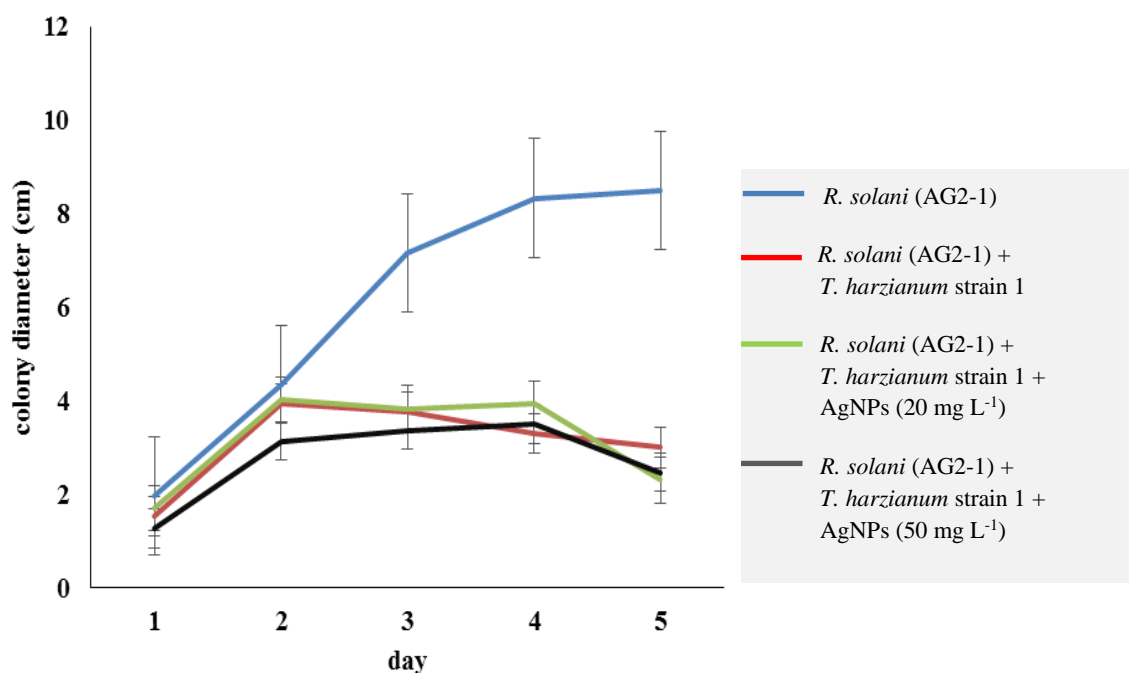


Figure 3.6 Diameter colony of *Rhizoctonia solani* (AG2-1) with and without AgNPs at 20 and 50 mg L⁻¹ and *Trichoderma harzianum* strain 1 presence in growth media. The colony diameter was measured daily for 5 days. AgNPs do not improve the ability of *T. harzianum* to control *R. solani* growth. Data represent means of three replicates with standard error.

A morphological difference was also observed to *T. harzianum* (T22) colony when dual cultured with *R. solani* (AG3) with and without the presence of AgNPs in the growth media. The mycelia of *T. harzianum* (T22) appeared thinner when grown on AgNPs contaminated media. Figure 3.7 shows the change of morphology on *T. harzianum* (T22) appearance.

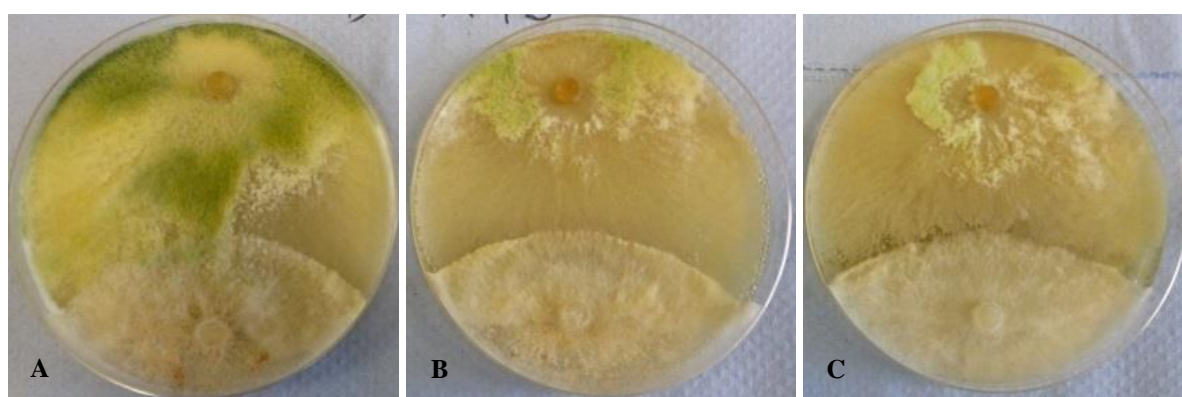


Figure 3.7 *Rhizoctonia solani* (AG3) against *Trichoderma harzianum* (T22) on PDA (A) without AgNPs, control (B&C) with 20 and 50 mg L⁻¹ of AgNPs, respectively.

Figure 3.8 shows that AgNPs at 20 and 50 mg L⁻¹ weaken antagonistic of *T. harzianum* (T22) on fifth day of observation. The colony diameter of *R. solani* (AG3) when dual cultured with *T. harzianum* (T22) at 20 and 50 mg L⁻¹ of AgNPs are significantly increase compared to *T. harzianum* (T22) alone. On the contrary, 50 mg L⁻¹ of AgNPs improve the ability of *T. harzianum* (T22) to control *R. solani* (AG2-1) growth (Figure 3.9). It is shown by a significant decrease of *R. solani* (AG2-1) colony diameter on 4th and 5th day of observation.

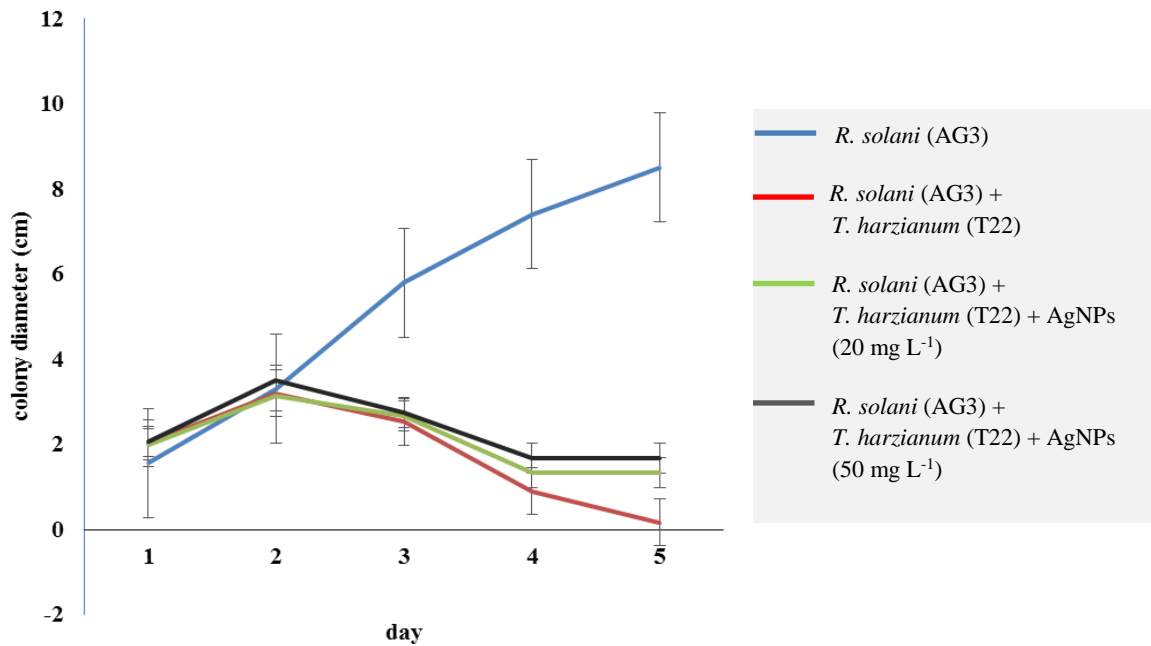


Figure 3.8 Diameter colony of *Rhizoctonia solani* (AG3) with and without AgNPs at 20 and 50 mg L⁻¹ and *Trichoderma harzianum* (T22) presence in growth media. The colony diameter was measured daily for 5 days. AgNPs do not improve the ability of *T. harzianum* to control *R. solani* growth. Data represent means of three replicates with standard error.

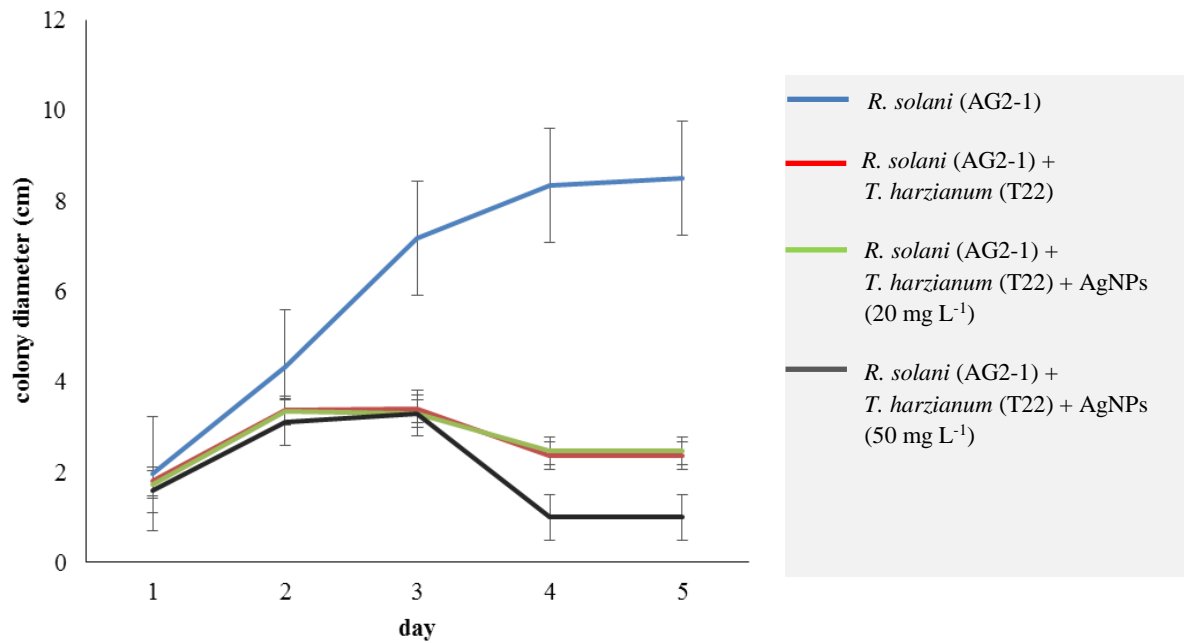


Figure 3.9 Diameter colony of *Rhizoctonia solani* (AG2-1) with and without AgNPs at 20 and 50 mg L⁻¹ and *Trichoderma harzianum* (T22) presence in growth media. The colony diameter was measured daily for 5 days. AgNPs at concentration of 50 mg L⁻¹ improve the ability of *T. harzianum* to control *R. solani* growth significantly ($P < 0.05$) on 4th and 5th day after inoculation. Data represent means of three replicates with standard error.

The combination of *T. harzianum* (T22) and AgNPs do not give a significant effect on antagonistic of *T. harzianum* (T22) on *R. cerealis* at any level employed (Figure 3.10).

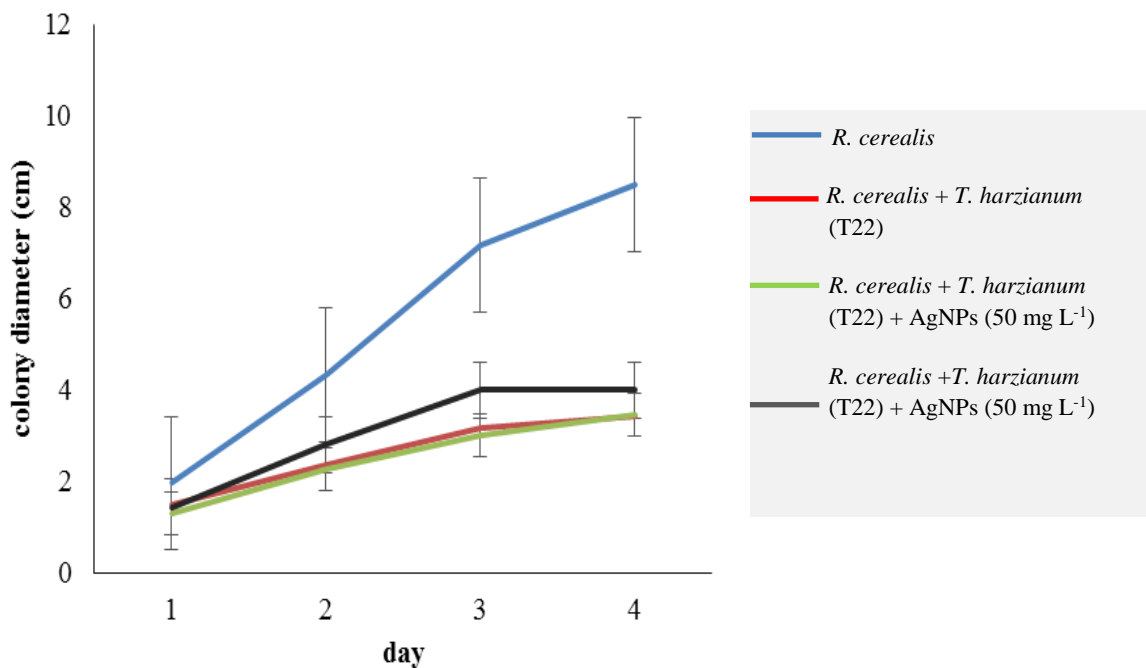


Figure 3.10 Diameter colony of *Rhizoctonia cerealis* with and without AgNPs at 50 and 150 mg L⁻¹ and *Trichoderma harzianum* (T22) presence in growth media. The colony diameter was measured daily for 5 days. AgNPs do not improve the ability of *T. harzianum* to control *R. cerealis* growth. Data represent means of three replicates with standard error.

3.3.3 The effect of AgNPs on *R. solani* (AG3) sclerotia production and germination

Figure 3.11 shows that the lower level of AgNPs (20 mg L^{-1}) used reduced the dry weight of sclerotia by 89% compared to untreated controls. Interestingly, sclerotia production at 50 mg L^{-1} of AgNPs appeared to be unaffected. When the sclerotia were regrown in fresh PDA all sclerotia of *R. solani* produced in the presence of AgNPs germinated fully (Figure 3.12).

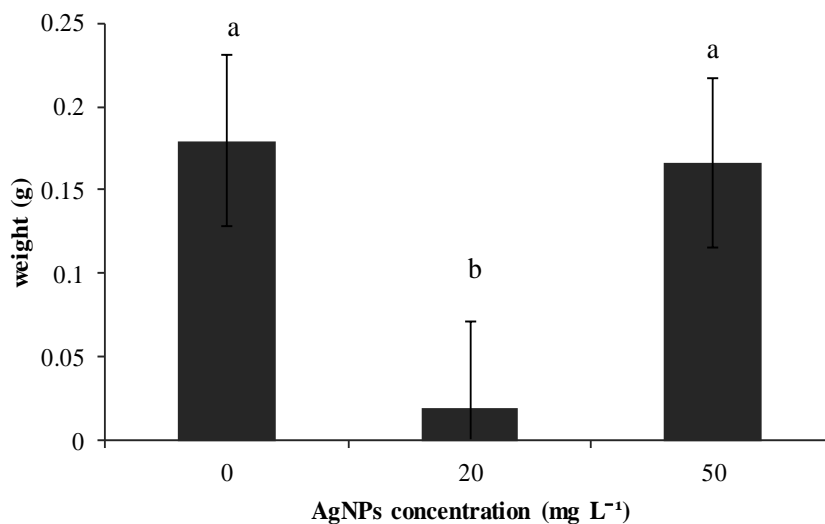


Figure 3.11 AgNPs at 20 mg L^{-1} reduced *Rhizoctonia solani* sclerotia dry weight but no significant difference in sclerotia production was observed at 50 mg L^{-1} AgNPs. The sclerotia produced after 6 months of inoculation were oven dried at 50°C for 2 hours. Data represent means of three replicates with standard error. Different letters indicate significant difference between treatments at the level of $P < 0.05$.

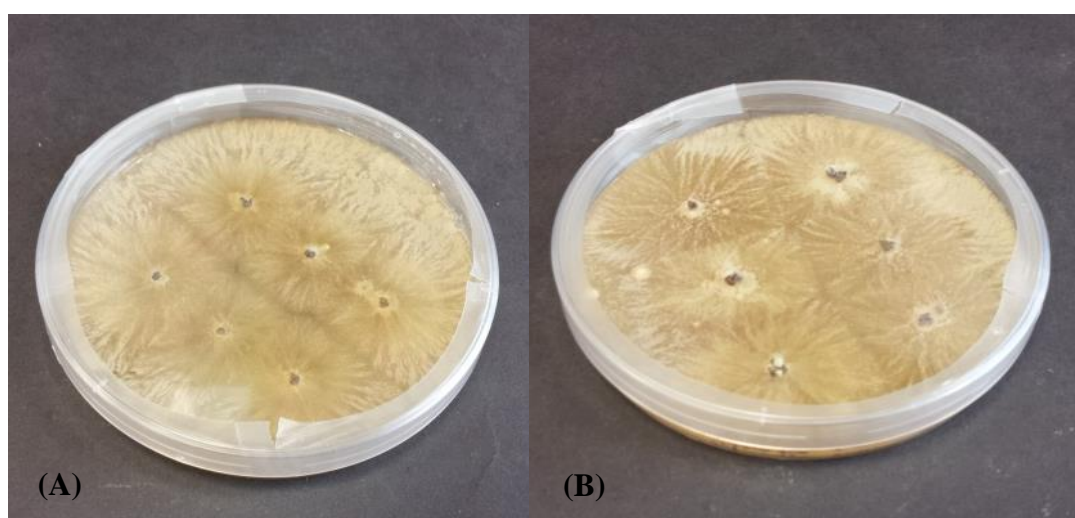


Figure 3.12 Sclerotia of *Rhizoctonia solani* (AG3) after growth in 20 mg L^{-1} of AgNPs (A) and 50 mg L^{-1} of AgNPs (B) shows full germination on fresh PDA. A sclerotium was considered to have germinated when any outgrowing hyphae were equal to or greater than the diameter of the sclerotium.

3.4 Discussion

3.4.1 The effect of AgNPs on *R. solani* and *R. cerealis* growth

The presence of AgNPs in growth media decreased the growth rate of *R. solani* (AG3) but no effect shown by *R. solani* (AG2-1) and *R. cerealis*. These findings suggest that sensitivity to ion silver is likely to be different between fungal strains of the same species but this should be tested further using similar growth conditions. It is known that *Rhizoctonia* species fall into taxonomically distinct groups called anastomosis groups (AGs). *R. solani* is a species complex consisting 13 known AGs which assigned on hyphal interaction base that can be further classified based on the pathogenicity, biochemical and genetic marker (Woodhall *et al.* 2013). Min *et al.* (2009) reported that very low AgNPs concentration (7 mg L⁻¹) inhibited hyphal growth of *R. solani* (AG5) significantly due to the abnormal shape of the hyphal walls which were prone to collapse. Similarly, Elgorban *et al.* (2015) reported that six different strains of *R. solani* (AG-1, AG2-2, AG-5, AG-6, AG-10, and AG-4-HGI) are sensitive to low levels of AgNPs. To the best of author's knowledge, no publication found on the effect of AgNPs on *R. cerealis*. Growth reduction is a typical response of fungi to the toxicity of heavy metals (Baldrian, 2003).

Furthermore, the antimicrobial activity of AgNPs depends on their size. The smaller the size the more toxic nanoparticle are because they potentially release many more Ag⁺ ions that dominate the microbial activity (Sotiriou and Pratsinis, 2010). The size of AgNPs applied in this study was much larger than nanoparticles used by Min *et al.* (2009) and Elgorban *et al.* (2015) with their AgNPs average size of 4-8 nm and 40-60 nm, respectively. AgNPs analysis from chapter 2 shows that diameter of AgNPs used in this study to be in the range of 60-120 nm. In addition, microscopic observation of AgNPs (20 mg L⁻¹) in growth media showed agglomeration of AgNPs which presumably reduces their toxicity by reducing available surface area.

The effect of AgNPs on other plant pathogenic fungi have also been studied. For example, *Sclerotium cepivorum*, *Raffaelea* sp. (Kim *et al.*, 2009), *Bipolaris sorokiniana*, *Magnaporthe grisea* (Jo *et al.* 2009), *Colletotrichum* species (Lamsal *et al.*, 2011), *Alternaria alternate*, *Botrytis cinerea* (Sahar, 2014), *Pythium aphanidermatum*, *Sclerotinia sclerotiorum* and *Macrophomina phaseolina* (Mahdizadeh *et al.*, 2015). Interestingly, some plant pathogenic fungi are used to synthesis nanoparticles (Chapter 1. Tabel 1.2). For example, *Fusarium oxysporum* used to control the size of AgNPs (Ahmad *et al.*, 2003; Korbekandi *et al.*, 2013; Husseiny *et al.*, 2015). Nanoparticles synthesis using microbes is more favourable as many

physical and chemical methods are expensive and use toxic substance (Prabhu and Poulouse, 2012). Nanoparticles biosynthesis would appear to be a common by-product of metal resistance (Sweet *et al.*, 2012). Eukaryotic organisms, such as fungi, produce enzyme and metabolite to defend themselves when exposed to environmental stress e.g. metallic ion, predator and temperature variation (Vigneshwaran *et al.*, 2007; Vahabi *et al.*, 2011).

When contact with heavy metal morphological changes are also commonly observed among fungi. For example, Baldrian (2003) observed morphological changes, including an increasing of areal hyphae formation and irregular appearance of surface hyphae, on several species of white-rot fungi when grown on cadmium containing medium. The phenomenon is potentially a defence mechanism of fungi in the presence of heavy metals. Change on mycelia morphology was also observed in *T. harzianum* when contact with cadmium (Lima *et al.*, 2011). Furthermore, heavy metal affected the morphologies of whole fungal colony of *T. viride* and *Rhizopus arrhizus* (Gadd *et al.*, 2001).

3.4.2 The effect of AgNPs on *T. harzianum* ability to control plant pathogenic fungus

Based on findings that demonstrated that *T. harzianum* is resistance towards AgNPs, an experiment to study the potential synergistic effect of *T. harzianum* and AgNPs to control plant pathogenic fungus was set up. The result shows that a combination of AgNPs and *T. harzianum* has a potential to improve the ability of *T. harzianum* to control plant pathogenic fungus growth. However, it depends on the species of both antagonist and pathogen. Combination of *T. harzianum* (T22) and AgNPs seems to be more promising to control *R. solani* (AG2-1). Again, the toxicity of AgNPs differ between species. This finding support Simonetti *et al.* (1992) who stated that the antimicrobial activity of ion silver depends on microbial species.

The combination of AgNPs and *T. harzianum* did not show synergism effect on *Rhizoctonia* species growth probably due to the ability of *T. harzianum* to synthesis AgNPs. *Trichoderma* species are known to produce enzymes and metabolites for their own survival when in contact with AgNPs (Vahabi *et al.*, 2011; Devi *et al.*, 2013; Hussein, 2016). In this process the toxic metal ions are reduced to the non-toxic metallic AgNPs through the catalytic effect of the extracellular enzyme and metabolites of the fungus (Vahabi *et al.*, 2011). As the result AgNPs may have less toxic to *Rhizoctonia* species.

Trichoderma species has been combined with other fungicide and microorganisms to induce their efficacy. Combinations with other bacteria or fungi often provided more effective disease

control than the application of an individual biocontrol of *Trichoderma* alone (Kumar *et al.*, 2014). Strains combination has been also shown more effective in controlling disease. For example, combination of *T. harzianum* and *T. asperellum* reduced disease severity caused by *Fusarium* (Akrami *et al.*, 2011). Combination of *Pseudomonas fluorescens* and *T. virens* and imazalil (active ingredient of commercial fungicide including Bromazil, Deccoziil, Fungaflor, Freshgard, and Fungazil) and *T. virens* showed synergism effect to control green mould of oranges caused by *Penicillium digitatum* (Zamani *et al.*, 2006).

3.4.3 The effect of AgNPs on *R. solani* sclerotia production and germination

AgNPs shows a potential to control sclerotia of *R. solani* production. However, the inhabitation of AgNPs on sclerotia production did not affect the germination. This study revealed that sclerotia that grown on AgNPs contaminated media germinated successfully on fresh PDA. This finding is contrary to a previous study carried out by Min *et al.* (2009). They found that low levels of AgNPs in growth media inhibit sclerotia germination effectively. These findings suggested that fungi from metal environments may be able to survive and germinate when conditions become permissible.

3.5 Conclusion

In summary, AgNPs have potential to control *R. solani* growth but they do not reduce the viability of sclerotia formed by *R. solani* suggesting that AgNPs may not effectively control *R. solani* survival in soil, unless a high enough level of AgNPs was built up in soil to be able to reduce sclerotial germination (this would likely have detrimental environmental impacts). The combination of AgNPs and *T. harzianum* also have a potential to control pathogen. However, it depends on several factors including the pathogen species. Further work is required on the ability of AgNPs to control *R. solani* growth in a soil environment and on the potential effects of AgNPs contamination on the biocontrol mechanisms of *T. harzianum*.

**Chapter IV. Soil Fungal Community Analysis using Illumina MiSeq Next
Generation Sequencing (NGS) : a Method Development Study**

4.1 Introduction

In vitro studies on the effect of AgNPs on selected soil fungi in pure culture was examined in Chapters 2 and 3. The results showed that sensitivity of soil fungi varied between different species and among strains of the same species. For example, *Trichoderma harzianum* was more tolerant than *Rhizoctonia solani* toward AgNPs. Due to this varying toxicity effect it was decided to examine how AgNPs contamination of soil changed soil fungal communities present using a next generation DNA sequencing (NGS) approach. According to Sagee *et al.* (2012), many soil properties such as grain size distribution, heterogeneity and the presence of soil organic matter influence AgNPs behaviour in soil and subsequently the bioavailability of Ag in soil will be very different to that in agar based media. Further discussion concerning the effects of pollutants (with a focus on nanoparticles and metals) on soil fungal communities are presented in Chapter 5 (Introduction) while this chapter focusses on the development of a NGS method to assess these communities. There are a variety of techniques available to investigate microbial communities in soil ranging from culture-based to culture independent molecular techniques. Culture-based analysis of soil microbial communities can be based on comparisons between direct microscopic counts of microbes in soil samples and recoverable colony forming units (Hilla *et al.*, 2000). However, standard culturing techniques have resulted in a biased evaluation because only small fraction (<0.1%) of the soil microbial community has been accessible with this approach (Rastogi and Sani, 2011) although recent advancements are showing that culture based approaches can in fact isolate a wide range of microbial species. For example, Kaeberlein *et al.* (2002) and Remenár *et al.* (2015) who successfully isolated uncultivable microorganisms using *in situ* incubation techniques.

Along with advances in technology, DNA-based methods are becoming more routinely used and Next Generation Sequencing (NGS) techniques are improving our understanding of the complexity of microbial communities. NGS methods, including 454 pyrosequencing and Illumina Miseq, have been recognised as powerful tools to study fungal communities. However, the Illumina Miseq platform is more suitable to investigate fungal communities compared to 454 pyrosequencing method because it provides greater depth of sequencing and promises a deeper characterisation of fungal communities (Schmidt *et al.*, 2013; Bálint *et al.*, 2014). Accordingly several bioinformatics pipelines to process metabarcoding data including the UPARSE pipeline have been developed (Edgar, 2013). UPARSE is a USEARCH-based pipeline for generating Operational Taxonomic Units (OTU) from next-generation sequencing reads of marker genes such as internal transcribed spacer (ITS) region. The OTU sequences

produced by the UPARSE pipeline are reported to be more accurate than other methods and so were used in this work (Edgar, 2013).

Raw reads received from the Illumina MiSeq sequencer consisted of two reads for each sample (Forward (R1) and Reverse (R2) sequence) provided as FASTQ files. Usually, the two reads are paired to increase sequence quality since two quality scores inform each base (Zhou *et al.*, 2011; Masella *et al.*, 2012; Jeraldo *et al.*, 2014). However, Nguyen *et al.* (2015) reported that paired sequencing might cause some taxa fail to pair largely due to the poor quality and quantity of the reverse direction sequences. Using QIIME analysis pipeline, they recovered more OTU from single highest quality read direction (in their case the forward direction reads) compared to paired and single reverse direction dataset. A study comparing single and paired read was also carried out by Werner *et al.* (2012) who found that choice of paired read and single read made no difference on clustering. However, different sequencing runs and analysis pipelines can produce different results.

Therefore, the aim of this study was to develop a method to analyse Illumina metabarcoding data based on the UPARSE pipeline developed by Edgar (2013) using datasets obtained from fungal communities present in silver contaminated soil. The individual objectives were to:

- a) check the quality of raw reads from Illumina MiSeq sequencer.
- b) process raw reads from Illumina MiSeq sequencer from single forward, single reverse, and paired direction sequences.
- c) count the number of single forward, single reverse, and paired sequences in each sample.
- d) sample the sequences at the same sequence depth.
- e) compare the number of OTUs produced using single and paired direction sequences.

4.2. Material and Methods

4.2.1 Experimental design

To develop the sequence analysis method, samples from an experiment to study the impact of silver contamination on soil fungal communities were used. The exact details of the AgNPs contaminated soil experiment are described in Chapter 5 but an overview is provided here for convenience. A typical arable land soil (see Chapter 5 for soil details), taken from Cockle Park Farm, Northumberland, was experimentally contaminated with AgNPs at several levels and incubated at 25 °C for 24 months. The soil was sampled at the 6th, 12th, and 24th month of the incubation time. A control soil was also prepared in the same manner but without the addition of AgNPs. All experiments were carried out in triplicate.

At the 6th, 12th, and 24th month soil samples were extracted using the PowerSoil[®] DNA isolation kit (MO BIO laboratories, USA) and checked for the quality using ND 1000 V3.2.1 spectrophotometer (Nanodrop). Fungal community analysis was achieved using the fungal ITS gene, utilising primers (ITS1 and ITS2). The details of primers and PCR conditions were described in Smith and Peay (2014). The analysis carried out by NU-OMICS (Northumbria University, United Kingdom) was based on the Schloss wet-lab MiSeq SOP (<https://www.northumbria.ac.uk/about-us/academic-departments/applied-sciences/business/nu-omics/>). For the purposes of this method development study and for clarity) only the control soil and one level of the AgNPs contaminated soil (100 mg kg⁻¹) from the 6th month observation were used as an example but similar results were obtained for all other samples (results not shown).

4.2.2 Bioinformatic processing of Illumina MiSeq output

Raw reads received from the Illumina MiSeq sequencer consisted of forward and reverse sequence provided as FASTQ files. Forward read were marked by R1 (e.g. 41_S23_L001_R1_001.fastq) and reverse reads by R2 (e.g. 41_S23_L001_R2_001.fastq) on the files name. Before being further processed the quality of sequences were checked using FastQC version 0.11.4 (Andrews, 2010). FastQC can be run on single file using commandline *fastqc read_file_name.fastq* or run on all files at once using *fastqc *.fastq* commandline. The FastQC reports were written in the same directory as the fastq files with -fastqc.html appended to the filenames. FastQC performed many tests on the sequences file but produced three summaries of relevance to ITS data including per base sequence quality, overrepresented sequences, and adapter content.

The next step was to process the raw reads sample as single-end (forward and reverse) and paired-end reads following UPARSE pipeline (Edgar, 2013) and run with USEARCH v8.1.1756 (Edgar, 2010). To merge the reads -fastq_merge command was used. According to Edgar and Flyvbjerg (2015) this command is the most appropriate to calculate Q score (the probability that the base call is incorrect) compared to other read mergers such as FLASH, PANDAseq, COPE, PEAR. The command for USEARCH merge pair reads was:

```
usearch -fastq_margepairs forward reads.fastq -reverse.fastq -fastq_maxdiffs 1 -fastq_minovlen 10 -fastqout merged.fastq
```

Explanation: The `-fastq_maxdiffs` sets the maximum number of mismatches allowed in the overlap region while the `-fastq_minovlen` is minimum length of the overlap.

The raw reads paired and unpaired were further analysed using steps as the following:

1. Quality filter, length truncate, convert to FASTA file

The reads were filtered for an average read quality threshold. After that, sequences were truncated to a length of 240 nucleotides. The output of this step was in FASTA file format. The command for quality filtering was:

```
usearch -fastq_filter forward.fastq/reverse.fastq/merged.fastq -fastq_maxee 1.0 -  
fastq_minlen 240 -fastaout filtered.fa
```

The `-fastq_maxee` option used to set an expecting errors threshold, 1.0 was recommended by Edgar and Flyvbjerg (2015).

2. Rarefaction

The rarefaction step was applied to sample each sample at the same depth. The `grep -c ">"` command was used to count the number of sequences in each sample and find the lowest sequence number to use as sample size in rarefaction step using command below:

```
usearch -fastx_subsample filtered.fa -sizein -sizeout -sample_size xxx -fastaout rarified.fa
```

3. Label and pool individual samples

The labelling step purpose was to identify each sample. The `sed` command was used to add a barcode label for each sample, then the reads can be combined into a single input file using `cat` command.

```
sed "-es/^>(.*)/>\1;barcodelabel=samA;/" < samA_rarified.fa > samA_label.fa  
sed "-es/^>(.*)/>\1;barcodelabel=samB;/" < samB_rarified.fa > samB_label.fa  
cat samA_label.fa samB_label.fa > labelled.fa
```

4. Dereplication

The `-derep_prefix` command was used to identify unique sequences in the sample so that only one copy of each sequence is reported.

```
usearch -derep_prefix labelled.fa -fastaout derep.fa -sizeout
```

5. Discard singletons

A singleton is a read with a sequence that is present exactly once. Discarding singletons is recommended for Illumina Miseq outputs because most error are probably singletons (Edgar, 2013). The following command was used to discard singletons:

```
usearch -sortbysize derep.fa -fastaout sorted.fa -minsize 2
```

6. OTU clustering

The `-cluster_otus` command discards reads that have chimeric models built from more abundant reads. The command used was:

```
usearch -cluster_otus sorted.fa -otus otus1.fa -sizein -sizeout -relabel OTU
```

7. Chimera filtering

Chimera filtering is recommended because the `-cluster_otus` command may missed a few chimeras especially if they have parents that are absent from the reads or are present with very low abundance. The `-uchime_ref` command was used to get a chimera-filtered.

```
usearch -uchime_ref otus1.fa -db uchime_sh_refs_dynamic_original_985_11.03.  
2015.fasta -strand plus -nonchimeras otus2.fa
```

8. Mapping

```
usearch -usearch_global labelled.fa -db otus2.fa -strand plus -id 0.97 -uc map.uc
```

9. Creating an OTU table using Edgar's python script (<http://drive5.com/python/>)

Python script convert .uc file to and OTU table in .txt file that can be easily imported into a spreadsheet. The first column indicated the number of OTU assigned. From here the number of OTU from each method used to process Illumina MiSeq metabarcoding data (forward, reverse, and paired reads) can be compared.

```
python python_scripts/uc2otutab.py map.uc > otu_table.txt
```

Generate a Rarefaction curve for each sample

Outputs from the rarefaction step (step no. 2) were used to generate a rarefaction curve. The rarefaction curve is useful to see whether sufficient observations have been made to get a reasonable estimate of a community that has been measured by sampling. The steps were performed using USEARCH v8.1.1756 (Edgar, 2010). The output files were imported into an Excel spreadsheet to generate the curve. The following steps were applied to each sample:

1. Dereplication

```
usearch -derep_prefix rarified.fa -fastaout derep_curve.fa -sizeout
```

2. Discard singletons

```
usearch -sortbysize derep_curve.fa -fastaout sorted_curve.fa -minsize 2
```

3. Compute a rarefaction curve

```
usearch -fasta_rarify sorted_curve.fa -iters 100 -output rare_curve.txt
```

The -iters option specifies the number of iterations to try for each subset size (0, 1%, 2% ... 100% of the unique reads in the input file), default is 32 iterations.

4. Generate rarefaction curve using Excel 2013

The output from -fasta_rarify command was imported to Excel 2013 to generate a rarefaction curve. One sample created one .txt file that combined in one spreadsheet to generate the curve. There were three columns in a file:

- a. Percentage of sequences for subset.
- b. Size of subset (total number of sequences).
- c. Average number of unique sequences.

The average number of unique sequences was used as y axis while the total number of sequences was used as x axis.

4.3 Results

4.3.1 Raw reads sequence quality

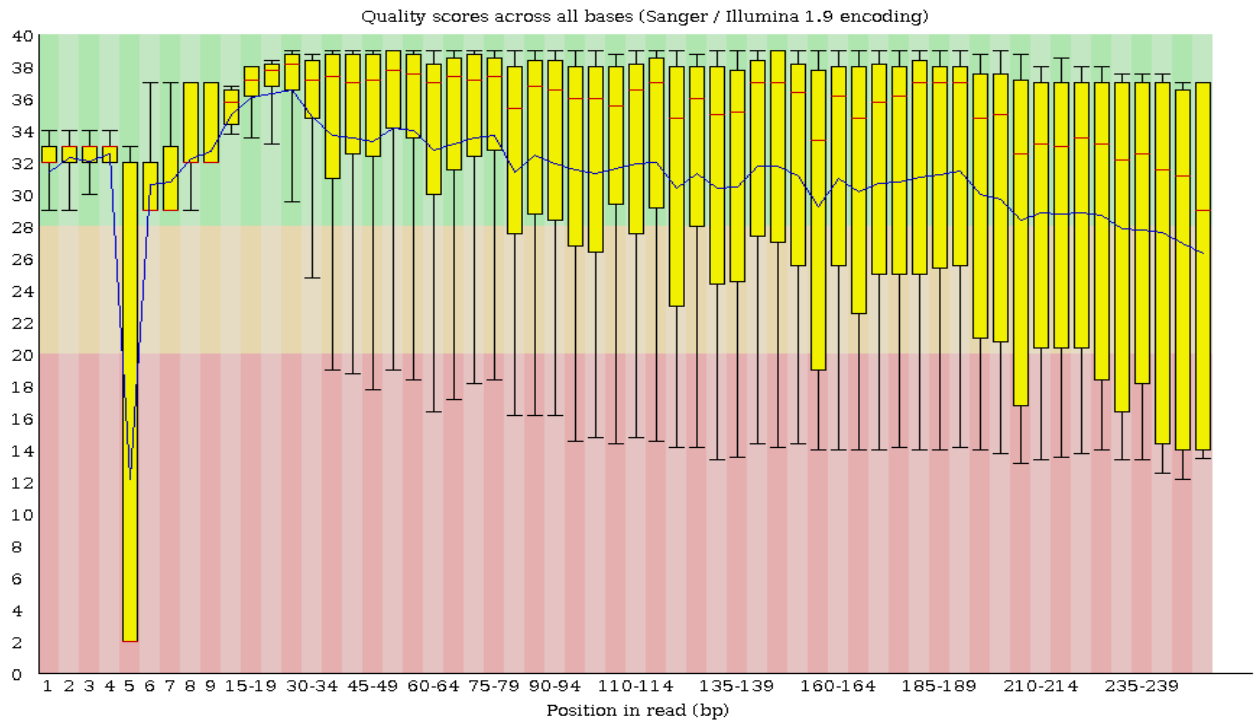
The raw reads of soil and AgNPs contaminated soil samples from Illumina MiSeq sequencer were checked for the sequence quality using FastQC version 0.11.4 (Andrews, 2010). The package produces three summary results that are relevant to look at including per base sequence quality, overrepresented sequences, and adapter content.

Figure 4.1 shows the example of quality output per base sequence quality of soil sample forward and reverse reads. Per base sequence quality showed the base call quality. The y-axis on the graph shows the quality scores. The higher the score the better the base call. The background of the graph divides the y axis into very good quality calls (green), calls of reasonable quality (orange), and calls of poor quality (red). In this particular sample a problem can be seen at the fifth base of the forwards read (Figure 4.1A) as the yellow bar drop to the red zone of the graph. The reverse sequence quality was very poor as seen from the Figure 4.1B.

Overrepresented sequences and adapter content tests looked at any contamination from PCR steps and contamination by sections of DNA that “stick” the amplicons to the sequencing platform. Any contamination from the PCR steps was named as other than ‘No Hit’ in the ‘Possible Source’ column. FastQC report for the overrepresented sequences and adapter content tests for both forward and reverse sequences for both samples (soil control and AgNPs contaminated soil) in this study showed that the samples were not contaminated by any PCR steps or spurious sections of DNA.

For more information on what each piece of FastQC’s output means, the documentation is available online at <http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/Help/>

(A)



(B)

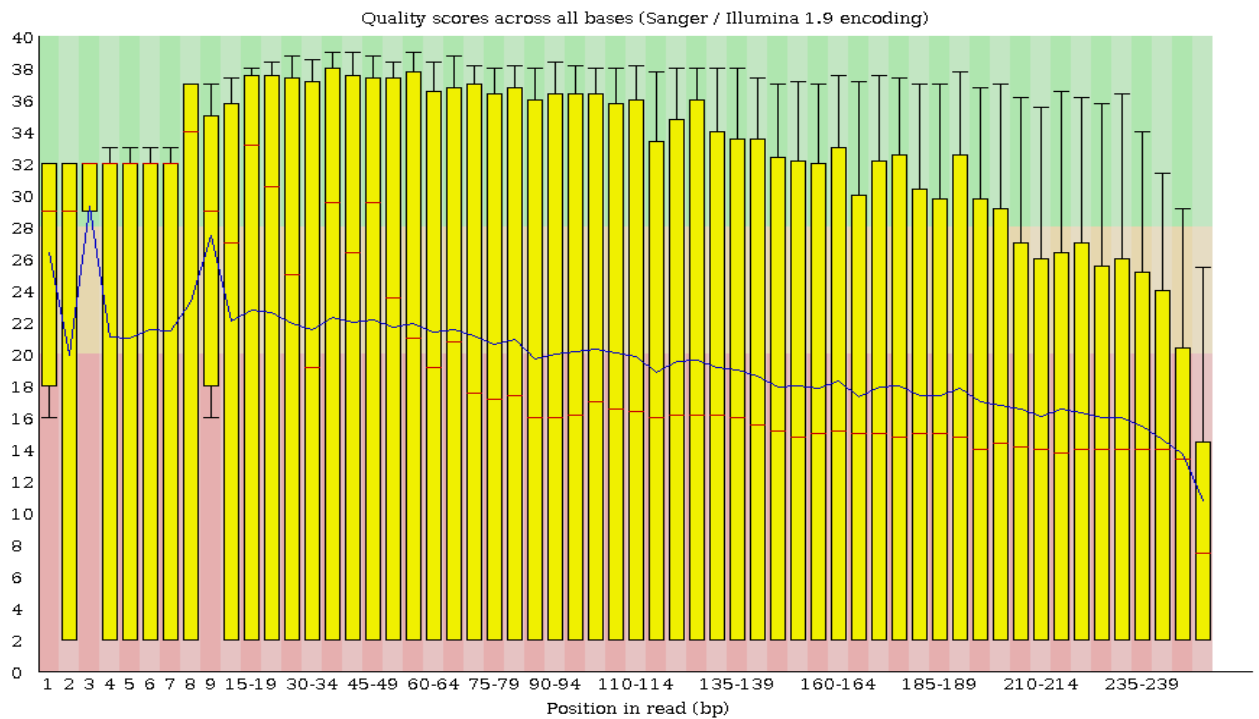


Figure 4.1. Per base quality scores from the FastQC analysis of soil control sample forward (A) and reverse (B) reads. The y-axis on the graph shows the quality scores. The central red line is the median value, the yellow box represents the inter-quartile range (25-75%), the upper and lower whiskers represent the 10% and 90% points, and the blue line represents the mean quality.

4.3.2 Sequences abundance

After being checked for their quality the number of sequence from each sample was counted as single (forward and reverse) and paired reads. It can be seen from Table 4.1 the number of paired reads were much lower than forward and reverse reads. Probably, the poor quality of the reverse reads (Figure 4.1B) affected the number of sequences obtained in the paired reads.

Table 4.1 The number of sequences of single reads (forward and reverse) and paired reads after quality filtering. The data displayed are from the 6th month sampling and sequenced using Illumina MiSeq platform.

Sample (s)	Number of sequences		
	Forward	Reverse	Paired
Soil	8645	2255	438
Soil	16351	2551	200
Soil	11691	3508	269
Soil + AgNPs	10276	2525	846
Soil + AgNPs	21069	3571	1012
Soil + AgNPs	12614	3423	2124
Total sequences	80646	17833	4889

4.3.3 Sequences sampling (generating rarefaction curve)

The lowest number of sequences from each read was used as sample size to make sure that each sample was sampled at the same depth. For example, the lowest sequences number for forward read was 8645 (Table 4.1 forward read column). To show the sample depth, a graph called a rarefaction curve was created. Figure 4.2 shows the rarefaction curve for forward read of six samples (two treatments; control soil and AgNPs contaminated soil and three replicates of each).

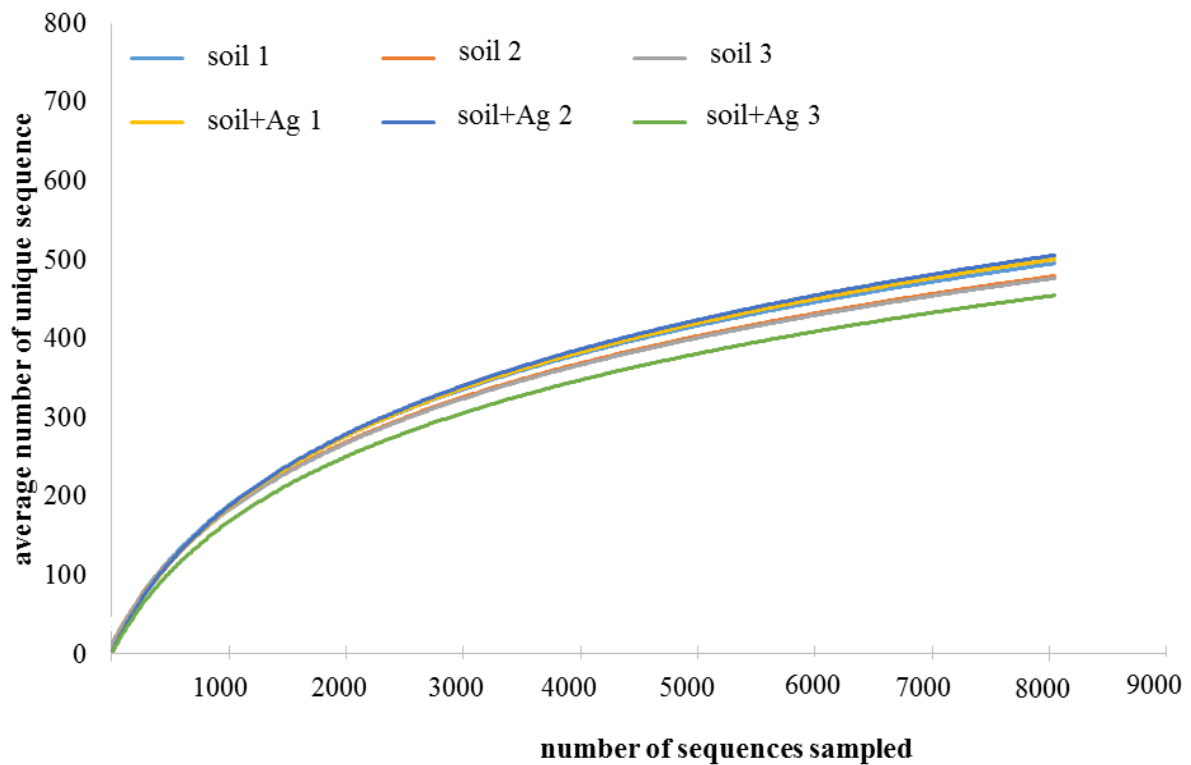


Figure 4.2 Rarefaction curve of soil control and AgNPs contaminated from forward read. The vertical axis shows the number of unique sequence that found after sampling the number of sequences shown on the horizontal axis. Lines of different colours represent soil control and AgNPs contaminated soil with three replications. The sequences were sampled at a depth of 8645 as this the lowest number of sequences number found in the forward reads.

4.3.4 Comparing the OTUs produced by single and paired reads

The single reads (forward and reverse) and paired read were processed separately using the UPARSE pipeline developed by Edgar (2013) to see which reads produced a better result (judging by the number of OTUs produced). The .txt files of OTU tables were imported into spreadsheet to see the number of OTU from each read to be compared. Figure 4.3 shows how the OTU table looks like on a spreadsheet (merged read OTU table). It can be seen from the figure that 10 sequences were successfully clustered into the OTU. This number is considerably low compared to the OTU produced by forward and reverse reads, 110 and 35, respectively (presented in Appendix B).

Table 4.2 OTU table of merged reads on a spreadsheet. The first column indicates the OTU id detected in the samples. The following columns suggest the abundance of each OTU in each sample. The samples were analysed following UPARSE pipeline (Edgar, 2013) and run with USEARCH v8.1.1756 (Edgar, 2010).

OTU Id	Soil 1	Soil 2	Soil 3	Soil+Ag 1	Soil+Ag 2	Soil+Ag 3
OTU 1	67	93	74	85	116	41
OTU 2	34	61	57	90	63	149
OTU 3	97	24	52	17	8	0
OTU 9	2	2	1	0	0	0
OTU 5	0	4	7	0	0	0
OTU 7	0	4	0	0	0	0
OTU 6	0	2	3	1	2	0
OTU 8	0	2	0	0	1	0
OTU 4	0	1	0	3	10	8
OTU 10	0	0	0	0	0	2

4.4 Discussion

Next Generation Sequencing (NGS) has become a more favourable technique as it more cost effective compared to previous Sanger technology for environmental genomic studies. However, the technique has disadvantages such as shorter sequence reads, higher base-call errors, non-uniform coverage and platform-specific artefacts (Chen *et al.*, 2014) that result in lower sequence quality. There are software tools available to check the quality of the sequences. For example, PIQA (Martinez-Alcantara *et al.*, 2009), HTQC (Yang *et al.*, 2013), and FastQC (Andrews, 2010, available at <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). To improve the quality of final data produced many tools have been developed to control the quality of NGS data including CutAdapt (Martin, 2011), Trimmomatic (Bolger *et al.*, 2014) and fastq filtering command (Edgar and Flyvbjerg, 2015). In this study FastQC and fastq filtering were used to check and filter the raw sequence reads. As claimed by Edgar and Flyvbjerg (2015), the quality filter technique reduces error rate effectively by applying the default value of expected error ($E_{\max} = 1$) as filtering threshold (such as that applied in this study). In addition Q score (error probability estimation) of 20 was applied meaning that 99% of the base calls made were correct. Using these parameters the data can be considered to be processed confidently.

After filtering, the sequences from each sample were rarefied at even number to avoid the problem of sequence depth being different from different samples as this will affect diversity estimation. This was confirmed by previous studies proved that a greater sequence depth improves ecological inference from NGS (Smith and Peay, 2014; Song *et al.*, 2015). For

example, if sample A contained 1000 sequences and sample B contained 500 sequences then the species richness of sample A would be much higher than sample B. As this is just an artefact of the Illumina technique (e.g. the samples in this work contained a range of 8000 to over 20000 forward read sequences) then it is important to select an equal sequence number from each sample for downstream data processing e.g. creation of final OTUs. During this process some samples may be eliminated from the sample total as they may contain relatively few sequences compared to others. In this work a sample depth of 8645 from forward read was chosen for the data as this was the lowest sequence in forward read. For comparison Smith and Peay (2014) used a sequence depth of 38,000 as the sample size for data range from 38,423 to 92,189 sequences. Ideally the sample depth chosen should represent a depth that samples the population as effectively as possible e.g. all the fungi in a sample are sampled. To estimate this a rarefaction analysis was carried out (Figure 4.2). Ideally the curves should flatten out demonstrating that any further increases in sampling do not result in an increase in the number of species (or OTU) found. According to He *et al.* (2015) a larger size of sub sample produced a steeper rarefaction curve. In this work the rarefaction curves at a sequence depth of 8645 from forward read has not perfectly reached a horizontal asymptote but it seemed that increasing size of sub sample would not make the lines going up. Schloss *et al.* (2009) produced a rarefaction curve using mother and yet the rarefaction curves continued to climb with increasing sequencing effort. A similar rarefaction curve also found by Sogin *et al.* (2006).

The sequences are generally clustered into OTUs. In this study UPARSE was used to generate clusters (OTUs) from next-generation sequencing reads. Different clustering methods can lead to extensively different biodiversity estimates (Bachy *et al.*, 2013). Flynn *et al.* (2015) compared three different clustering methods (mothur, UCLUST, and UPARSE) and found that UPARSE was more precise and produced more consistent OTU numbers even with relaxed filtering and when including singletons, whereas mothur and UCLUST produced varied and inflated OTU numbers.

The UPARSE pipeline run on USEARCH was used to process metabarcoding data produced by an Illumina MiSeq sequencer. This software and process was used as it provides better quality sequence identification in comparison with other open source software such as QIIME (Edgar, 2013). The data processing can also be quickly carried out on a relatively powerful desktop pc which is accessible to most laboratories worldwide.

Due to the relative paucity of Illumina sequence data for assessing fungal soil communities the first stage of the work involved processing the sequence in different ways to achieve the best (or most suitable) OTU tables. Merging the forward and reverse reads to obtain merged reads should be able to provide a better identification than either forward or reverse sequences alone. However, the work revealed that merging the reads (forward and reverse) dramatically decreased the number of sequences found in each sample. There are potentially several reasons for this but the main cause is probably that merging discards around 40% of the sequences due to uncalled or miscalled bases (Masella *et al.*, 2012). It is clear that the quality of the reverse sequences produced by the Illumina sequence run was low meaning that pairing of forward and reverse reads would be difficult due to the stringency levels used in the UPARSE pipeline. The very low number of sequences obtained after merging also resulted in a very low number of final OTU (10 OTUs) found.

In this study the most stringent parameters (e.g $E_{\max} = 1$, Q score = 20, for filtering step) were applied to each sequence. According to Flynn *et al.* (2015), stringent workflow (USEARCH filtering, singletons removed, UPARSE clustering) recovers less OTU than relaxed filtering. However, stringent filtering is recommended because it reduces redundancy, noise, and problems of generating inflated numbers of OTU (Flynn *et al.*, 2015). At the end of downstream process 110 and 35 sequences were successfully clustered into OTU of forward and reverse direction sequences, respectively. The study revealed that the single forward read produced the most number of OTUs while the lowest number of OTUs was obtained from merged sequences. These findings supports earlier studies that single forward reads generate more OTUs compared to reverse direction sequences (Nguyen *et al.*, 2015). Nguyen *et al.* (2015) recovered 25 and 20 OTUs from single forward and reverse direction sequences, respectively, while 23 OTU was recovered from paired direction sequences. Similarly, Caporaso *et al.* (2011) also found that single reads produce more OTUs than paired reads. For general eukaryotic species, the higher number of OTU (identifications) obtained using forward (ITS1 region) reads can be explained as this section represents a better DNA barcode compared to reverse (ITS2 region) (Wang *et al.*, 2015). Except for *Rhizopus* spp., ITS2 sequences were more variable than that of ITS1 region for identification (Park and Min, 2005).

4.5 Conclusion

The UPARSE pipeline run on the USEARCH programme is a useful method to quickly process data obtained from the Illumina MiSeq sequencer. The program is user friendly but has limitations in graphics. Single forward reads produce the most OTUs compared to single reverse and paired reads and so forward reads with the tested parameters were used to analyse metabarcoding data for soil fungal community studies in the subsequent Chapter (V).

**Chapter V. Soil Fungal Community Responses to the Addition of
T. harzianum (T22) and Silver Nanoparticles Contamination in Soil as
Assessed by Illumina Next Generation Sequencing (NGS)**

5.1 Introduction

In Chapters 2 and 3 three species of soil fungi, *Trichoderma harzianum* (beneficial fungi) and *Rhizoctonia solani* and *R. cerealis* (plant pathogens) were used to test the effect of AgNPs on soil fungi *in vitro*. The results showed that *Rhizoctonia* spp. were more sensitive to AgNPs than *T. harzianum*. *In vitro* tests on the effect of AgNPs on *T. harzianum* (Chapter 2) supports former studies that *Trichoderma* species are highly resistant to heavy metals (Kredics *et al.*, 2001a; Kredics *et al.*, 2001b; Harman *et al.*, 2004). *T. harzianum* is a known biocontrol agent with innate resistance against most chemicals used in agriculture, including metals. AgNPs tolerance showed by *T. harzianum* indicates its potential to use with heavy metal-containing pesticides, as part of an integrated plant protection system. In addition, *T. harzianum* has been explored for removal and recovery of heavy metals such as cadmium and uranium in soil and aqueous streams (Akhtar *et al.*, 2009; Lima *et al.*, 2011). *T. harzianum* has also been employed in phytoremediation to promote root plant growth (Lynch and Moffat, 2005; Adams *et al.*, 2007). Studies revealed that the addition of *T. harzianum* to compost or soil caused an increase in the relative abundance of species of certain chitinolytic bacteria and higher microbial diversity (Blaya *et al.*, 2013; Chen *et al.*, 2012).

Given this variability in fungal sensitivity to AgNPs and the impact of *T. harzianum* addition into soil on microbial diversity it is therefore important to question how AgNPs and *T. harzianum* (T22) affect microorganisms, particularly fungi, in soil. Sagee *et al.* (2012) reported that many soil properties such as grain size distribution, heterogeneity and the presence of soil organic matter influence AgNPs behaviour in soil. AgNPs undergo transformation including physical, chemical, and biological transformations that ultimately affect their persistence, bioavailability/biouptake, reactivity, and toxicity in the environment (Lowry *et al.*, 2012). The degree of toxicity depends mainly on the metallic element and its bioavailability in the soil as affected by abiotic and biotic factors (Bellion *et al.*, 2006). It would also be of interest to see if the purposeful addition of *T. harzianum* to a soil contaminated with AgNPs could help to alleviate any potential toxic effects caused by the contaminant.

Some heavy metals such as nickel, iron, copper, and zinc are essential for fungal metabolism, whereas others such as cadmium, mercury, and silver have no known biological role (Gadd, 1993). Both essential and non-essential heavy metals are toxic for fungi, when present in excess (Baldrian, 2003). The sensitivity of different microbial groups to heavy metals may vary, but usually the total microbial biomass is decreased in heavy metal contaminated sites (Baldrian, 2010). In addition, a reduction in fungal numbers and species diversity will likely be caused by

toxicity from environmental pollutants (Gadd, 2007). Soil microbes play important roles in maintaining soil fertility by cycling nutrients, improving soil structure, supporting healthy plant growth and degrading organic pollutants (Elsgaard *et al.*, 2001; Filip, 2002). It has been suggested that the diversity and activity of the microbial community indicates the quality of soil (Wang *et al.*, 2007). The vast majority of studies on the impact of heavy metal pollutant have been restricted to single-species tests and ecosystem processes or has dealt with abiotic processes such as dissolution, speciation, sorption or transport (Mckee and Filser, 2016). Only more recently have the effects of metal pollution on soil microbial community structure come into focus.

There are few publications on the effect of AgNPs on soil fungi and the work carried out has been of short duration (Kim *et al.*, 2009; Min *et al.*, 2009; Kim *et al.*, 2012; Gavanji *et al.*, 2012; Sillen *et al.*, 2015; Sweet and Singleton, 2015). Accordingly, the present experiment studied the effect of AgNPs on fungal communities in soil for longer duration exposure time (24 months). Since it remains unclear whether AgNPs are a direct cause of enhanced toxicity or the result of Ag ions (Levard *et al.*, 2012), another type of silver, silver nitrate (AgNO₃), was used in the experiment to study which type is more harmful to soil fungal species. Some researchers suggested that the toxicity is mainly the result of Ag⁺ ions (Navarro *et al.*, 2008) while others demonstrated that AgNPs are more toxic than the equivalent dose of Ag ions added as AgNO₃ (Yin *et al.*, 2011). It seems that there are many factors affecting the toxicity of silver metal including the type of microbe studied. For example, bacterial communities are more affected by AgNPs as their composition is significantly modified by nanosilver exposure (Sillen *et al.*, 2015; Carbone *et al.*, 2014). The present work employed three levels of AgNPs. The three AgNPs levels represent amount of Ag release from washing process (Benn and Westerhoff, 2008), concentration applied to control pathogen in agriculture (Min *et al.*, 2009), and polluted type level of AgNPs in environment (Kumar *et al.*, 2011).

Fungal communities were assessed using Illumina MiSeq platform. Data from the sequencer were processed following the method developed in Chapter 4. The data were processed based on the forward reads as they have the greatest number of sequences and give higher operational taxonomic unit (OTU) counts. Each sequence was sampled at an even depth so that the samples can be compared one to another.

There were several aims to be achieved in this chapter including to:

1. investigate the impact of *T. harzianum* (T22) addition into soil on fungal community.
2. compare the effect of different form of Ag (AgNPs and AgNO₃) at one level of concentration (100 mg kg⁻¹) on soil fungal community.
3. assess the impact of *T. harzianum* (T22) addition into Ag (AgNPs and AgNO₃) contaminated soil at one level of concentration (100 mg kg⁻¹) on soil fungal community.
4. Compare three levels of Ag concentration (3, 100, and 660 mg kg⁻¹) added as AgNPs and AgNO₃ with the addition of *T. harzianum* (T22) on soil fungal community.

The soil fungal community was assessed by Illumina Next Generation Sequencing (NGS) and processed using UPARSE pipeline developed by Edgar (2013) and run with USEARCH v8.1.1756 (Edgar, 2013). The objectives of each aim were to examine the species richness, species evenness, and community structure change at the 6th, 12th, and 24th month of observation.

5.2 Material and Methods

5.2.1 Soil sampling and chemical analysis

A typical arable land soil (at a depth of 0-14 cm) from the east headland of Cockle Park Farm, Northumberland, UK, was used in the experiment. The soil was kindly collected by M. Botha (School of Agriculture, Food and Rural Development, Newcastle University, UK). After the removal of stones and larger material, the soil was air dried for 48 h prior to being sieved to 2 mm in the laboratory. The soil sample was analysed by The School of Agriculture, Food and Rural Development, Newcastle University, UK. Soil total C, total N, pH, moisture content and phosphorous were determined and reported in Table 5.1.

Table 5.1 Soil properties

Total C (%)	2.66
Total N (%)	0.25
pH	5.05
Moisture content (%)	1.59
Phosphorous (g kg ⁻¹)	1.147

5.2.2 AgNPs and AgNO₃

AgNPs in powder form with 99.95% purity were obtained from M K Impex Corp. Mississauga, Canada. The AgNPs were characterised in terms of structure and particle size distribution as advised by Dr. L. Siller (School of Chemical Engineering and Advance Material, Newcastle University) and the methods used are as described in section 2.2.4. The AgNPs were mainly present as aggregates with the average diameter to be in the range of 60-120 nm. Three levels of AgNPs (3, 100, and 660 mg kg⁻¹) were used to study its effect on fungal soil community. The three levels of AgNPs were chosen to represent the amount of Ag released from washing processes (Benn and Westerhoff, 2008), concentrations suggested for application to control pathogens in agriculture (Min *et al.*, 2009), and a high level to simulate a pollution event (Kumar *et al.*, 2011).

AgNO₃ in powder form with 99% purity was obtained from Sigma-Aldrich Co. LLC. The dosages of AgNO₃ applied in the soil were adjusted to the equivalent AgNPs concentration using following formula:

$$\left(\frac{100}{\text{Ag purity in AgNO}_3} \times \text{Ag concentration (ppm)} \times \text{soil weight (kg)} \right) \times \left(\frac{100}{\text{AgNO}_3 \text{ purity}} \right)$$

Where:

$$\text{Ag purity in AgNO}_3 = \left(\frac{\text{Ag molecular weight}}{\text{AgNO}_3 \text{ molecular weight}} \right) \times 100$$

5.2.3 *Trichoderma harzianum* (T22)

Commercial *T. harzianum* powder (under name Triatum-P (T22), produced by Koppert B.V., The Netherland) was used as the culture source. A pure culture was prepared by lightly spreading the *T.harzianum* (T22) powder on PDA and incubating at 24 °C for 5 days. The method used to prepare the spore suspension for addition to soil was described in section 2.2.6. The spore suspension was added at 10⁸ into the treated soils (5 g).

5.2.4 Soil treatment

The experiment was carried out with and without *T. harzianum* (T22) addition into the soil to see if *T. harzianum* (T22) addition in soil has an impact on fungal soil communities. Only one level of Ag (100 mg kg⁻¹) was used in the experiment without *T. harzianum* (T22) addition. The Ag was mixed thoroughly with soil (5 g). Soil only also prepared as control. The experiment

with the addition of *T. harzianum* (T22) was prepared by thoroughly mixing each level of AgNPs and AgNO₃ with soil (5 g) followed by *T. harzianum* (T22) suspension. Control soils were treated identically but without Ag addition.

To assess water holding capacity 1 gram of soil was watered and filtered using filter paper and left until no more water drips and weighted (1.7 g). Holding capacity of the soil is 0.7 g (at 100%). *T. harzianum* (T22) needs 70% water in soil so 0.49 g water/g soil (= 490 µl) as spore suspension. All soils were kept in 15 ml conical centrifuge tubes with filter paper as the lids (the paper was pierced using needle to allow airflow) and weighted before being incubated at 25 °C. The tubes weight was kept the same over the duration of the experiment by adding water to maintained water capacity in the soil. DNA from the soil was extracted at 6th, 12th, and 24th month. All experiments were carried out in triplicate. The experiments were arranged as followed:

- A. Soil
- B. Soil + 100 mg kg⁻¹ of Ag as AgNPs
- C. Soil + 100 mg kg⁻¹ of Ag as AgNO₃
- D. Soil + *T. harzianum* (T22)
- E. Soil + *T. harzianum* (T22) + 3 mg kg⁻¹ of Ag as AgNPs
- F. Soil + *T. harzianum* (T22) + 100 mg kg⁻¹ of Ag as AgNPs
- G. Soil + *T. harzianum* (T22) + 660 mg kg⁻¹ of Ag as AgNPs
- H. Soil + *T. harzianum* (T22) + 3 mg kg⁻¹ of Ag as AgNO₃
- I. Soil + *T. harzianum* (T22) + 100 mg kg⁻¹ of Ag as AgNO₃
- J. Soil + *T. harzianum* (T22) + 660 mg kg⁻¹ of Ag as AgNO₃

5.2.5 Deoxyribonucleic acid (DNA) extraction from soil and *T. harzianum* (T22)

DNA was extracted from soil at 6th, 12th, and 24th month after being experimentally contaminated with AgNPs/AgNO₃ using the PowerSoil[®] DNA isolation kit as described by the manufacturer (MO BIO laboratories, USA) with slight modifications. A soil sample (approximately 0.25 g) was added to a 1.5 ml microfuge tube containing a buffer to help break open the fungal cell and protect nucleic acids from degradation. The tube was gently vortexed to mix the components in the buffer and disperse the sample in the solution. C1 solution (60 µl, containing sodium dodecyl sulphate (SDS) and other disruption agents required for complete cell lysis) was added to break down fatty acids and lipids. In order to gain more DNA yield, soil samples in SDS were homogenised using Bead Beater at 30 Hz for 5 minutes to break open

cell walls. Any debris was removed by centrifugation at 10,000 x g for 30 seconds at room temperature and the supernatant transferred to a clean 2 ml microfuge tube. Solution C2 (250 µl, containing a reagent to precipitate non-DNA organic and inorganic material) was added to the supernatant, mixed by vortexing for 5 seconds and incubated at 4 °C for 5 minutes. The solution then centrifuged at room temperature for 1 minute at 10,000 x g. Approximately 600 µl of supernatant was transferred to a clean 2 ml microfuge tube and 200 µl of solution C3, containing the second reagent to precipitate additional non-DNA organic and inorganic material, was added and the solution vortexed briefly before being incubated at 4 °C for 5 minutes. The aqueous phase containing nucleic acids was separated by centrifugation at room temperature for 1 minute at 10,000 x g. The supernatant (750 µl) was transferred to a clean 2 ml microfuge tube and 1.2 ml of C4 solution containing high concentration of salt solution) was added to the supernatant prior to vortexing for 5 seconds. Approximately 675 µl of the solution was loaded to a spin filter (to collect the DNA) and centrifuged at 10,000 x g for 1 minute at room temperature. The flow through was discarded. This step was repeated until no solution remained (total three loads). After the third load 500 µl of solution C5 (ethanol) was added to the spin filter and centrifuged (10,000 x g at room temperature) for 30 seconds. The filtrate was discarded and centrifugation repeated for 1 minute to remove any remaining liquid. The spin filter was placed in a new 2 ml microfuge tube and 50 µl of solution C6 (10 mM Tris-EDTA (TE) buffer) was added to the centre of the white filter membrane. The tube was centrifuged (10,000 x g at room temperature) for 30 seconds. The spin filter was discarded and the tube containing nucleic acid was stored at -20 °C for further use.

T. harzianum (T22) culture was prepared as described in section 5.2.3. DNA of *T. harzianum* (T22) was extracted from the culture using Power Soil kit as described in 2.3.3. DNA extraction of *T. harzianum* (T22) was sent to Genevision (INEX Business Centre, Newcastle Upon Tyne, UK) for clean-up and sequencing. At Genevision the templates were cleaned up using either Qiagen QIAquick® PCR purification kit, Qiagen QIAquick® gel extraction kit or Promega Wizard® PCR clean-up kit. The samples were then sequenced using ITS 1 forward primer. Sequence reactions were accomplished using Applied Biosystems BigDye® cycle chemistry. Analyses were performed on ABI 3730xl capillary sequencers. Raw data from Genevision were matched against the National Centre for Biotechnology Information (NCBI) GenBank database via <http://blast.ncbi.nlm.nih.gov/Blast>.

5.2.6 DNA quality check and sequencing

DNA quality was checked using ND 1000 V3.2.1 spectrophotometer (Nanodrop) by dropping 1 μ l of DNA solution on the chamber. Samples with 260:280 ratios above 1.8 were accepted as “pure”. If the ratio was appreciably lower, this indicated the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm and the DNA extraction procedure was repeated to obtain a good quality sample. Fungal community analysis was achieved using the fungal ITS gene, utilising primers (ITS1 and ITS2) designed by Smith and Peay (2014) and was carried out by NU-OMICS (Northumbria University, United Kingdom) based on the Schloss wet-lab MiSeq SOP (<https://www.northumbria.ac.uk/about-us/academic-departments/applied-sciences/business/nu-omics/>).

5.2.7 Bioinformatic processing of Illumina MiSeq output

Metabarcoding data from Illumina MiSeq sequencer were processed according the method developed in Chapter 4. Based on the results from Chapter 4, only forward reads with the tested parameters were used to analyse metabarcoding data as forward read produced the most OTUs compared to single reverse and paired reads. The steps to analyse the sequences follow the steps on section 4.2.2.

In addition, alpha diversity (α -diversity) was calculated using Shannon diversity index (H). Alpha diversity metrics was calculated from the number of sequences and the cluster size (abundance). After labelled (step 4) the reads were combined into a single input file using *cat* command. The reads were processed per replicates. Therefore, α -diversity was obtained for each treatment. For example, sample 1, 2, and 3 were sample for soil with 3 replicates. The 3 samples were combined into a single input file. The process was followed by dereplication, discard singleton, OTU clustering (step 5-7). The commandline for calculating α -diversity was `usearch -fasta_diversity otus.fa -output diversity.txt -iters 100`

5.2.8 Statistical analysis

All data presented are the mean value of three replicates. Values are expressed as means of three replicates \pm standard error (S.E) in each group. All statistical analyses were performed using One-way Analysis of Variance (ANOVA) statistical models on Microsoft Excel 2016. Variance analysis was performed on all experimental data and significant differences ($P < 0.05$) between individual means (three replicates) was analysed using a post hoc Least Significant Difference test.

5.3 Results

5.3.1 Data processing

As developed in Chapter 4, data from Illumina Miseq sequencer to study fungal communities in soil were processed using single end forward reads following the UPARSE pipeline (Edgar, 2013) using USEARCH version 8.1.1756 sequence analysis tool (<http://www.drive5.com/usearch/>). The sequence quality of each sample was checked using FastQC version 0.11.4 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and filtered using `fastq_filter` command as suggested by Edgar and Flyvbjerg (2015) (http://www.drive5.com/usearch/manual/cmd_fastq_filter.html). The sequences number were counted using `grep -c ">" seqs.fa` commandline to find the lowest number of sequences to use as sample size. As displayed in Table 5.2 there are five samples from the 6th month sampling that have very short sequence (with less than 20 number of sequence after filtered). For example, the number of sequences of sample 'Soil+T22+3 mg kg⁻¹ of AgNPs' was 15 and this was insufficient to allow further analysis of these samples. As the result the five samples from the 6th month observation (3 samples of Soil + T22 + 3 mg kg⁻¹ of AgNPs and 2 samples of Soil + T22 + 100 mg kg⁻¹ of AgNPs) were excluded from the analysis.

Table 5.2 The number of sequences of single forward reads after quality filtering. The data displayed are from the 6th month samples and sequenced using Illumina MiSeq platform.

Sample (s)	Number of sequences
Soil	8645
Soil	16351
Soil	11691
Soil + 100 mg kg ⁻¹ of AgNPs	10276
Soil + 100 mg kg ⁻¹ of AgNPs	21069
Soil + 100 mg kg ⁻¹ of AgNPs	12614
Soil + 100mg kg ⁻¹ of AgNO ₃	16873
Soil + 100mg kg ⁻¹ of AgNO ₃	9291
Soil + 100mg kg ⁻¹ of AgNO ₃	21350
Soil + T22	7195
Soil + T22	6382
Soil + T22	5697
Soil + T22 + 3 mg kg ⁻¹ of AgNPs	15
Soil + T22 + 3 mg kg ⁻¹ of AgNPs	8
Soil + T22 + 3 mg kg ⁻¹ of AgNPs	12
Soil + T22 + 100 mg kg ⁻¹ of AgNPs	23
Soil + T22 + 100 mg kg ⁻¹ of AgNPs	14
Soil + T22 + 100 mg kg ⁻¹ of AgNPs	5984
Soil + T22 + 660 mg kg ⁻¹ of AgNPs	3297
Soil + T22 + 660 mg kg ⁻¹ of AgNPs	2569
Soil + T22 + 660 mg kg ⁻¹ of AgNPs	3037
Soil + T22 + 3mg kg ⁻¹ of AgNO ₃	2195
Soil + T22 + 3 mg kg ⁻¹ of AgNO ₃	4247
Soil + T22 + 3 mg kg ⁻¹ of AgNO ₃	3788
Soil + T22 + 100 mg kg ⁻¹ of AgNO ₃	1520
Soil + T22 + 100 mg kg ⁻¹ of AgNO ₃	17286
Soil + T22 + 100 mg kg ⁻¹ of AgNO ₃	14415
Soil + T22 + 660 mg kg ⁻¹ of AgNO ₃	6895
Soil + T22 + 660 mg kg ⁻¹ of AgNO ₃	9240
Soil + T22 + 660 mg kg ⁻¹ of AgNO ₃	9335

All samples were analysed at the same sequence depth (647 sequences per sample) as this number was the lowest number of the sequences found in samples that didn't exclude a significant number of samples (in 12th month sequences reading). Full numbers of sequences from each sample are displayed in Appendix C. Figure 5.1 shows the rarefaction curve of sample depth. As in Chapter 4, the curve was not reach the horizontal asymptote.

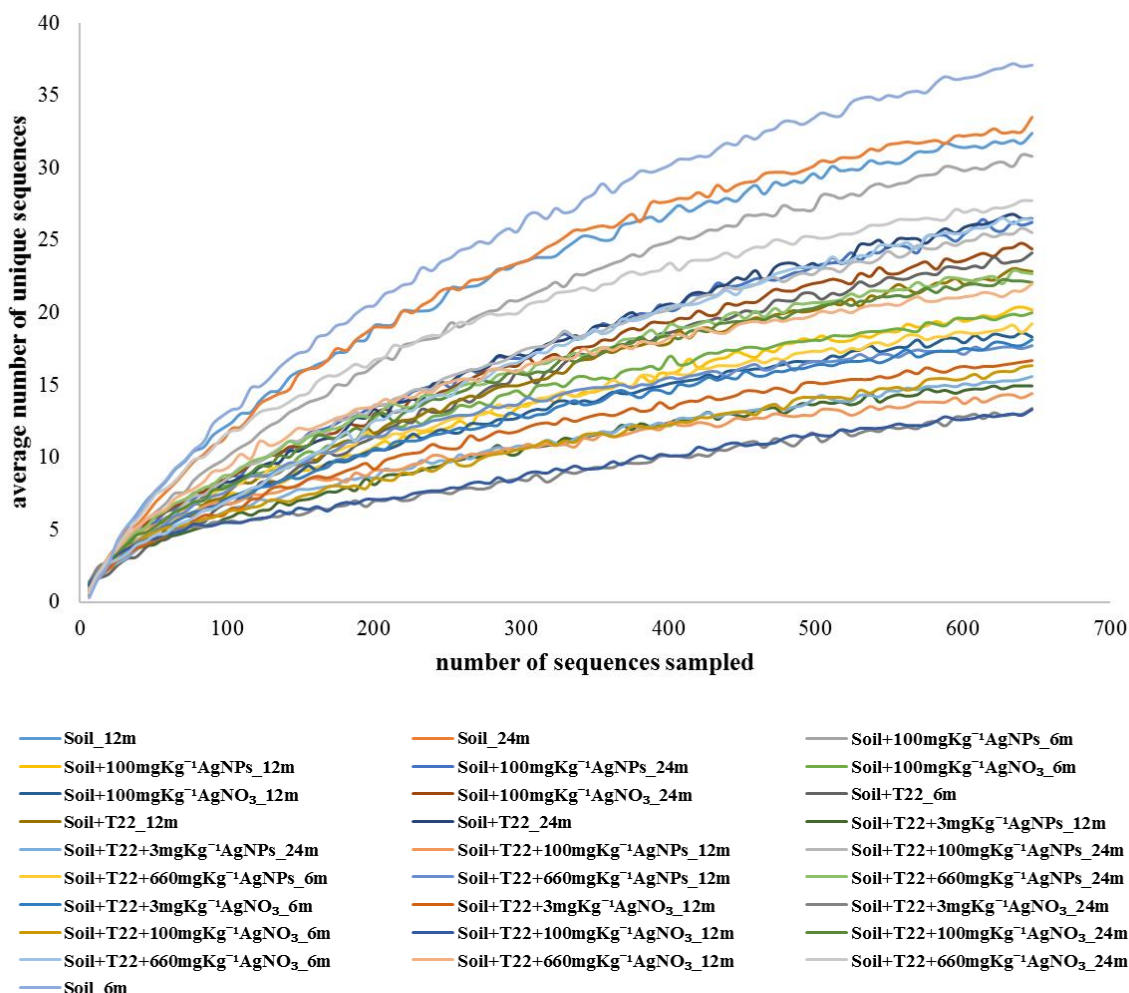


Figure 5.1 Rarefaction curve of soil and Ag contaminated soils with and without *T. harzianum* (T22) addition in soil samples from forward read. The vertical axis shows the number of unique sequences found after sampling the number of sequences shown on the horizontal axis. Lines of different colours represent different soil treatments. The sequences were sampled at 647 as the lowest sequences number found in the forward read.

At the end of the pipeline over 300 sequences of forward reads were successfully clustered into OTU and assigned for taxonomy. The taxonomy annotations were added to OTU sequence labels by using the `utax` command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database. In the case the taxonomy assignment did not give satisfy identification, e.g. Fungi sp., the sequence (the output of OTU clustering file) was matched against NCBI GenBank database (<http://blast.ncbi.nlm.nih.gov/Blast>.). The full taxonomy assignment presents in Appendix D.

Species richness, species evenness, and fungal community structure can be identified manually from the OTU table produced by counting the number of species in each sample for species richness, calculating the species abundance for each OTU for species evenness, and matching the OTU with taxonomy assignment for fungal community structure. In addition, USEARCH also calculating alpha diversity to give an index for the diversity in the community. The `fasta_diversity` command reports several metrics including Shannon index. The Shannon diversity index (H), also known as Shannon entropy, the Shannon-Wiener index and the Shannon-Weaver index, is commonly used to characterize species diversity in a community and so used in this study.

5.3.2 The impact of the addition of *T. harzianum* (T22) into soil on fungal soil community

Knowing the ability of *T. harzianum* to increase relative abundance and microbial diversity (Blaya *et al.*, 2013; Chen *et al.*, 2012), *T. harzianum* (T22) was added into soil to study its impact on fungal soil community. The samples were observed at the 6th, 12th, and 24th month after being experimentally inoculated with *T. harzianum* (T22). The changes in soil fungal communities can be assessed by looking at the fungal species diversity in the soil. Species diversity consists of two components: species richness and species evenness. Species richness is a simple count of species, whereas species evenness quantifies how equal the abundances of each species is. This section looks at the impact of the addition of *T. harzianum* (T22) on both of these diversity components and the community structure in the soil.

The impact of the addition of *T. harzianum* (T22) into soil on fungal species richness

Figure 5.3 displays the number of fungal species in soil with the addition of *T. harzianum* (T22) at three different sampling time. It can be seen that the addition of *T. harzianum* (T22) in the soil did not have a significant change on the fungal species richness. The number of fungal species in control soil increased significantly from approximately 40 at the 6th month observation to around 50 at six months later and back to the initial figure at the 24th month of observation. The same trend occurred to fungal species richness with the addition of *T. harzianum* (T22) in the soil.

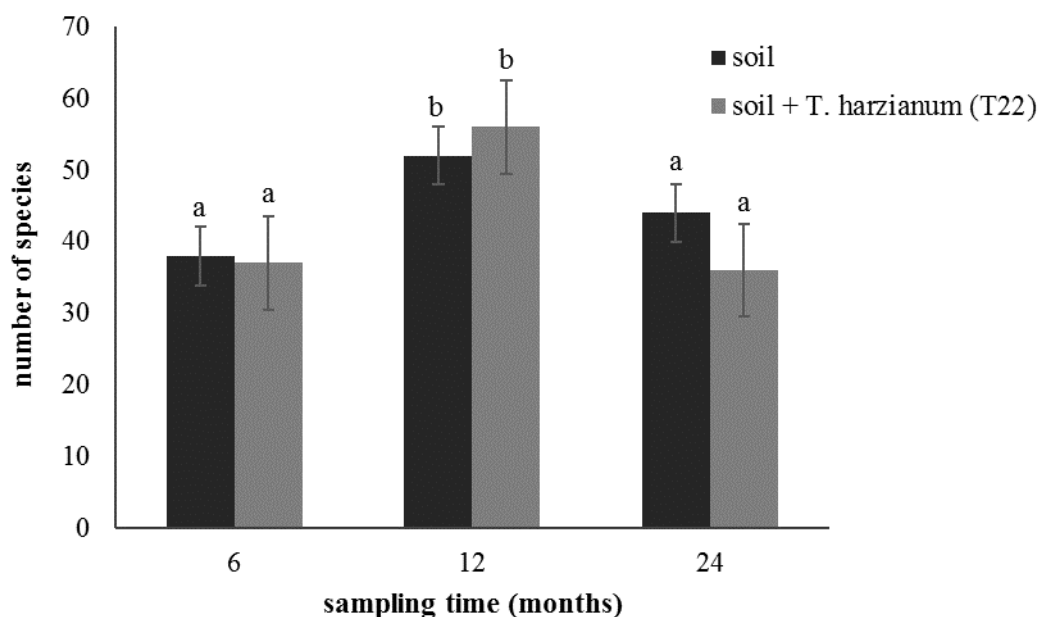


Figure 5.2 The number of fungal species in soil with the addition of *T. harzianum* (T22). The soils were incubated at 25 °C and sampled at 6, 12 and 24 months after being experimentally inoculated with *T. harzianum* (T22). Data represent means of three replicates with standard error. Same letters above the data points indicate insignificant difference between treatments in one sampling time at the level of $P > 0.05$.

The species richness in the soil also measured by Shannon diversity index (H) displayed in Table 5.3. It is shown that the addition of *T. harzianum* (T22) into soil did not have a significant effect on fungal species diversity at the first 12 months of the observation. Interestingly, the presence of *T. harzianum* (T22) in soil at a longer time (24 months) reduced the diversity significantly.

Table 5.3 The fungal species richness in control soil and soil with the addition of *T. harzianum* (T22) measured by Shannon diversity index (H). The species richness was observed at the 6th, 12th, and 24th month after experimentally inoculated with *T. harzianum* (T22) and incubated at 25 °C. The arithmetic mean of three replicates is shown with their arithmetic standard error (Mean±SE). Different letters in the same column indicates significant differences (P < 0.05).

Sample (s)	Shannon diversity index (H) at three different times of observation (month)		
	6 th	12 th	24 th
Soil	2.19±0.11a	2.43±0.11a	2.56±0.11a
Soil+ <i>T. harzianum</i> (T22)	2.40±0.23a	2.37±0.23a	1.70±0.23b

The impact of the addition of *T. harzianum* (T22) into soil on fungal species evenness

Figure 5.4 shows the impact of *T. harzianum* (T22) addition into soil on fungal species evenness. It seems that the addition of *T. harzianum* (T22) into soil improved the fungal species evenness. At the first 6 months after incubation, the community in soil control was dominated by one species (around 40%) while with the presence of *T. harzianum* (T22) in the soil the evenness was equally shared among the species. The addition of *T. harzianum* (T22) maintained the species evenness up to the 12th month of observation. However, 50% of the community was dominated by a species at the last period of observation (the 24th month). In the case of control soil, the species evenness improved at the 12th and the 24th month of observation. Overall, the species evenness in the control soil improved with the time while the converse happened in soil with *T. harzianum* (T22) addition.

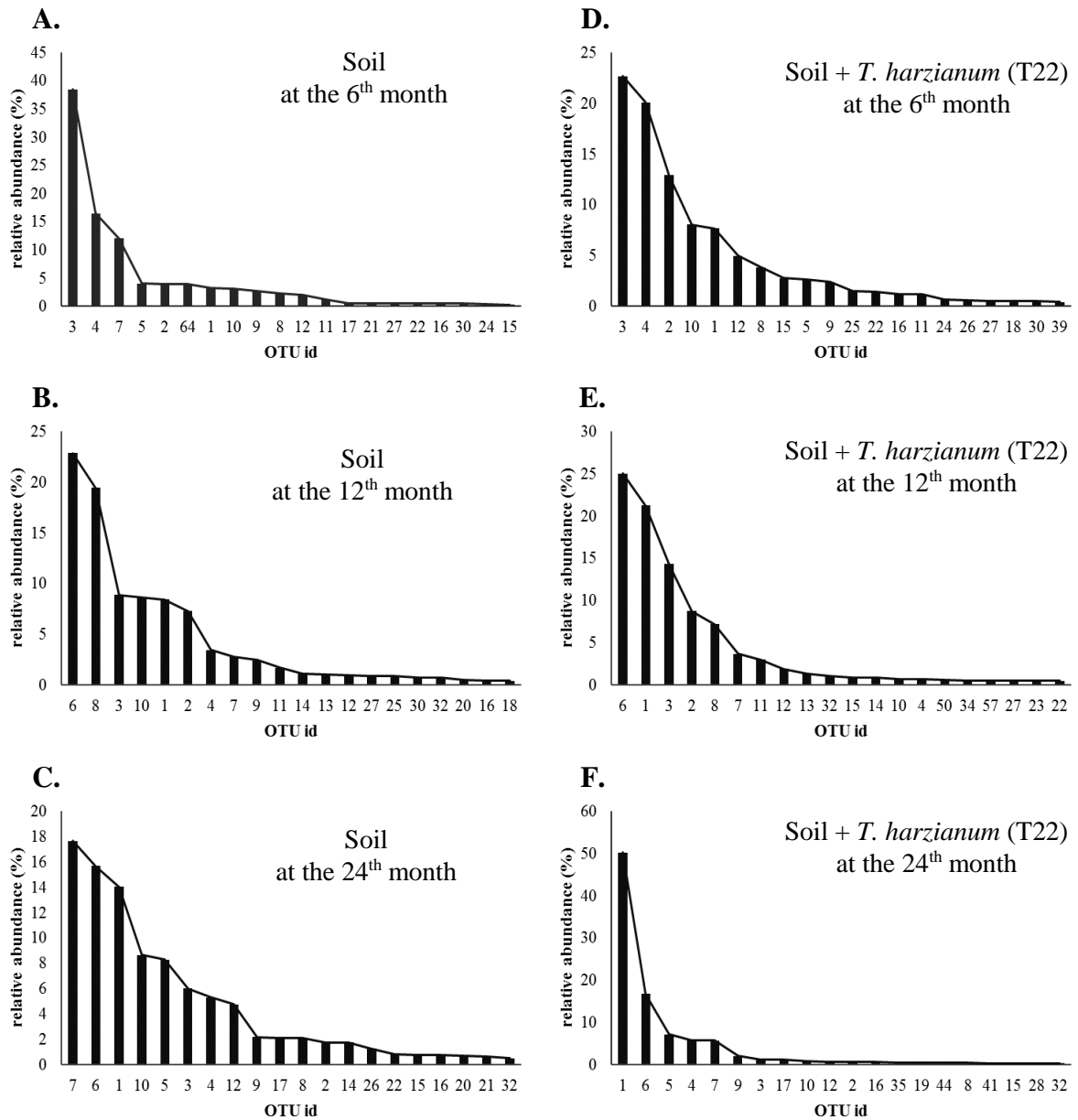


Figure 5.4 The impact of *T. harzianum* (T22) addition into soil on fungal species evenness. A, B, C are control soils sampled at the 6th, 12th, and 24th month, respectively. D, E, F are soil with *T. harzianum* (T22) addition sampled at the 6th, 12th, and 24th month, respectively. All samples were incubated at 25 °C. DNA was extracted from soils and assessed by Illumina MiSeq Next Generation Sequencing.

The impact of the addition of T. harzianum (T22) into soil on fungal community

It also can be seen from Figure 5.4 that the fungal species (indicated by OTU id) relative abundance in the soils were change. For example, Table 5.4 shows the species that structured fungal community in control soil and soil with the presence of *T. harzianum* (T22) at the 6th month observation. *Cryptococcus terreus* and Fungi sp. were the most abundance species in both soil samples. The sequence of Fungi sp. was matched against the National Centre for Biotechnology Information (NCBI) GenBank database (<http://blast.ncbi.nlm.nih.gov/Blast>). The sequence was 96% similar as *Podospora ellisiana* (Sequence ID: AY515360.1, accessed 29th of September 2016). The addition of *T. harzianum* (T22) into soil decreased the relative percentage of *C. terreus*. On the other hand increased the abundance of some species such as *Clitopilus* sp. from 3% in control soil to 12% in soil with *T. harzianum* (T22). The increasing of relative abundance also occurred in *Hypocreaceae* sp. and *Atheliaceae* sp. by 4% and 5%, respectively. Furthermore, there were species in control soil that replaced by other species in soil the addition the of *T. harzianum* (T22) such as *Umbelopsis ramanniana var angulispora*.

At the 12th month, the most abundance species in both soils (Figure 5.4B and 5.4E) was taken over by a “new” species (OTU id 6). The sequence was 99% identified as *Penicillium* sp. (Sequence ID HM036608.1, accessed 29th of September 2016) by matching the sequence against the (NCBI) GenBank database. However, at the 24th month observation the relative abundance of the species decreased by 5% in control soil (Figure 5.4C) and 7% in soil with the addition of *T. harzianum* (T22) (Figure 5.4F). The fungal community in soil with *T. harzianum* (T22) presence at the 24th month was 50% dominated by *Hypocreaceae* sp. (OTU id 1). Full list of the top twenty species in control soil and soil with the addition of *T. harzianum* (T22) at the 12th and the 24th month observation are displayed in Appendix E.

Table 5.4 The top twenty species in soil and soil with the addition of *T. harzianum* (T22). The soils were sampled at the 6th month of incubation time. Red colour indicates different species detected between soil samples. Percentages in brackets show the percentage of relative abundance each species.

OTU id	Soil	OTU id	Soil + <i>T.harzianum</i> (T22)
3	<i>Cryptococcus terreus</i> (38%)	3	<i>Cryptococcus terreus</i> (22%)
4	<i>Fungi</i> sp. (16%)	4	<i>Fungi</i> sp. (20%)
7	<i>Umbelopsis ramanniana</i> var <i>angulispora</i> (12%)	2	<i>Clitopilus</i> sp. (12%)
5	<i>Trichocomaceae</i> sp. (4%)	10	<i>Atheliaceae</i> sp. (8%)
2	<i>Clitopilus</i> sp. (3%)	1	<i>Hypocreaceae</i> sp. (7%)
64	<i>Atheliaceae</i> sp. (3%)	12	<i>Leohumicola minima</i> (4%)
1	<i>Hypocreaceae</i> sp. (3%)	8	<i>Pseudeurotium</i> sp. (3%)
10	<i>Atheliaceae</i> sp. (3%)	15	<i>Scutellinia</i> sp. (2%)
9	<i>Rhytismataceae</i> sp. (2%)	5	<i>Trichocomaceae</i> sp. (2%)
8	<i>Pseudeurotium</i> sp. (1%)	9	<i>Rhytismataceae</i> sp. (2%)
12	<i>Leohumicola minima</i> (0.5%)	25	<i>Pezizomyces</i> sp. (1%)
11	<i>Parmelina</i> sp. (0.5%)	22	<i>Clitocybe</i> sp. (1%)
17	<i>Ascomycota</i> sp. (0.5%)	16	<i>Pezizaceae</i> sp. (1%)
21	<i>Umbelopsis ramanniana</i> (0.5%)	11	<i>Parmelina</i> sp. (1%)
27	<i>Tuckermannopsis platyphylla</i> (0.4%)	24	<i>Physcia magnussonii</i> (0.6%)
22	<i>Clitocybe</i> sp. (0.4%)	26	<i>Humicola</i> sp. (0.5%)
16	<i>Pezizaceae</i> sp. (0.4%)	27	<i>Tuckermannopsis platyphylla</i> (0.5%)
30	<i>Diaporthaceae</i> sp. (0.3%)	18	<i>Ascomycota</i> sp. (0.5%)
24	<i>Physcia magnussonii</i> (0.2%)	30	<i>Diaporthaceae</i> sp. (0.4%)
15	<i>Scutellinia</i> sp. (0.2%)	39	<i>Ascomycota</i> sp. (0.4%)

5.3.3 Comparing the impact of different forms of Ag contamination (100 mg kg^{-1} of Ag as AgNPs or AgNO_3) on fungal diversity in soil

Fungal species richness responses to Ag contamination

It can be seen from Figure 5.5 that the application of Ag in both form AgNPs and AgNO_3 decrease the fungal species richness in soil. The addition of AgNPs in soil reduced the number of fungal species in soil at all sampling times significantly ($P < 0.05$). There was no change in the number of species in AgNO_3 contaminated soil at the first six months of incubation time. However, the number of species in AgNO_3 contaminated soil decreased significantly ($P < 0.05$) when sampled after longer exposure times compared to non-contaminated controls.

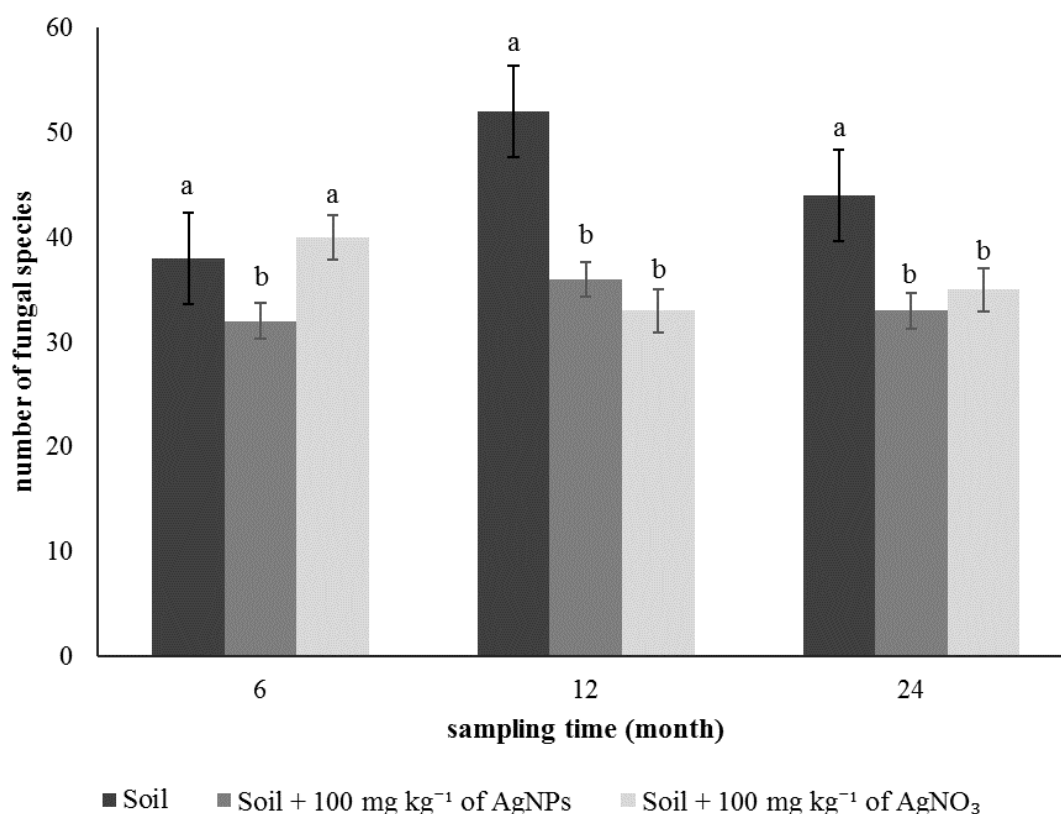


Figure 5.5 The number of fungal species in control soil and with the application of AgNPs and AgNO_3 at 100 mg kg^{-1} . The soils were incubated at $25 \text{ }^\circ\text{C}$ and sampled at 6, 12 and 24 months after being contaminated with the two types of silver. Data represent means of three replicates with standard error. Different letters above the data points indicate significant difference between treatments in one sampling time at the level of $P < 0.05$.

Table 5.5 displays the Shannon diversity index (H) for control soil and Ag contaminated soils at all time points. At all times of sampling the Shannon Diversity values for control soils were significantly higher ($P < 0.05$) than those observed for AgNPs and AgNO₃ contaminated soils. There was an insignificant difference between the diversity index of both AgNPs and AgNO₃ contaminated soil in the first 6 months of observation. However, the fungal diversity in AgNO₃ contaminated soil was higher than AgNPs contaminated soil at the 12th and the 24th month observation.

Table 5.5 The fungal species richness in control soil and Ag contaminated soils at 100 mg kg⁻¹ of Ag added as AgNPs and AgNO₃ measured using Shannon diversity index (H). The species richness was observed at the 6th, 12th, and 24th month after experimentally contaminated with the two types of Ag and incubated at 25°C. The arithmetic mean of three replicates is shown with their arithmetic standard error (Mean±SE). Different letters in the same column indicates significant differences ($P < 0.05$).

Sample (s)	Shannon diversity index at different times of observation (month)		
	6 th	12 th	24 th
Soil	2.19±0.11a	2.43±0.11a	2.56±0.11a
Soil+100 mg Kg ⁻¹ AgNPs	2.00±0.04b	1.94±0.04b	1.85±0.04b
Soil+100 mg Kg ⁻¹ AgNO ₃	2.13±0.03b	2.08±0.03c	2.17±0.03c

Fungal species evenness responses to Ag contamination

Given the effects of Ag contamination on Shannon diversity it was decided to examine the effects of Ag on species evenness more closely. To do this the percentage relative abundance of each species in the community was plotted to visualise the effects of Ag on the soil fungal community evenness. At the first 12 months observation fungal species evenness in control soil and AgNPs contaminated soil were similar (Figure 5.6). At the 6th month observation fungal species abundance in both soils were not equally shared among the species (Figure 5.6A and Figure 5.6D) as around 35% of the communities were dominated by one species. The fungal species evenness was altered in month 12th as the percentage of relative abundance among the species found was more even (Figure 5.6B and Figure 5.6E). For example, the percentage of relative abundance of OTU 6 (the most abundant) in control soil was approximately 23% while the second most abundant OTU has a percentage of relative abundance of approximately 20% (Figure 5.6B). However, at the 24th month of observation control soils showed the ‘best’ species evenness curve as there was less domination by one species in the community (Figure 5.6C). It seems that the longer incubation period improves the species evenness in the soil. On the other

hand, AgNPs contamination appeared to have the opposite effect on species evenness in the soil. After long term exposure the fungal soil community became dominated by a few species. The top 4 fungal species in AgNPs contaminated soil accounted for 80% of the total relative abundance as opposed to approximately 55% in the control soil.

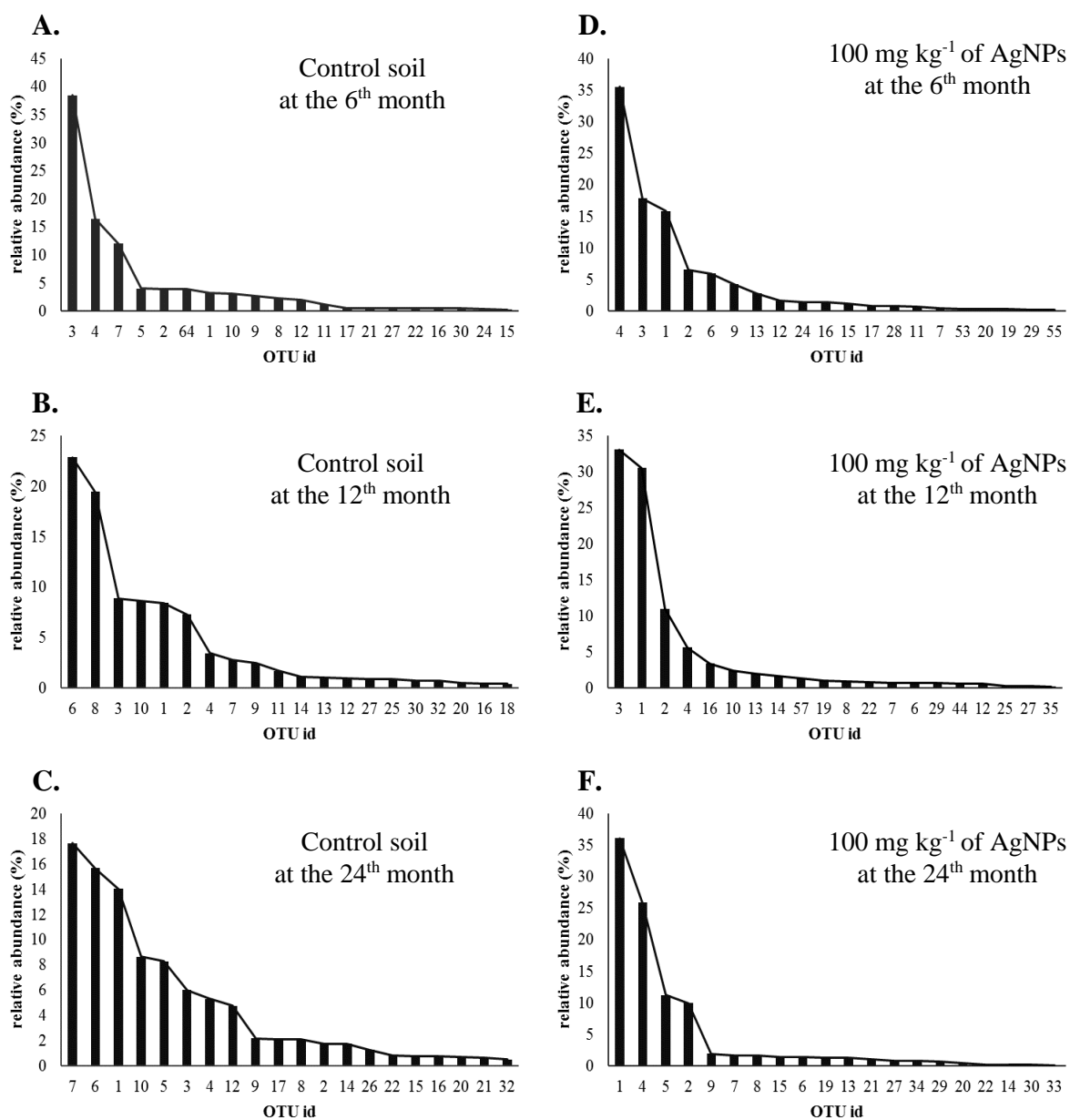


Figure 5.5 The impact of AgNPs application in soil on fungal species evenness. A, B, C are soil controls sampled at the 6th, 12th, and 24th month, respectively. D, E, F are AgNPs contaminated soil sampled at the 6th, 12th, and 24th month, respectively. Both soil control and AgNP contaminated soil were incubated at 25 °C. DNA was extracted from soils and assessed by Illumina MiSeq Next Generation Sequencing.

An equivalent dose of Ag ions (added as AgNO₃) in soil samples was used to compare the toxicity of AgNO₃ and AgNPs on soil fungi. In contrast to AgNPs that decreased fungal species evenness from the 6th month of incubation, the percentage of relative abundance of each species in AgNO₃ contaminated soil was relatively evenly distributed (Figure 5.7D). However, longer term of AgNO₃ exposure decreased fungal species evenness in soil. After 12 months incubation 40% of the community was dominated by one species (Figure 5.7E). The same species (indicated by same OTU id on Figure 5.7E and Figure 5.7F) still dominated the community at the 24th month sample time. From the study it can be seen that species evenness in the soil control is improving with the time while the converse happened in the AgNPs and AgNO₃ contaminated soil.

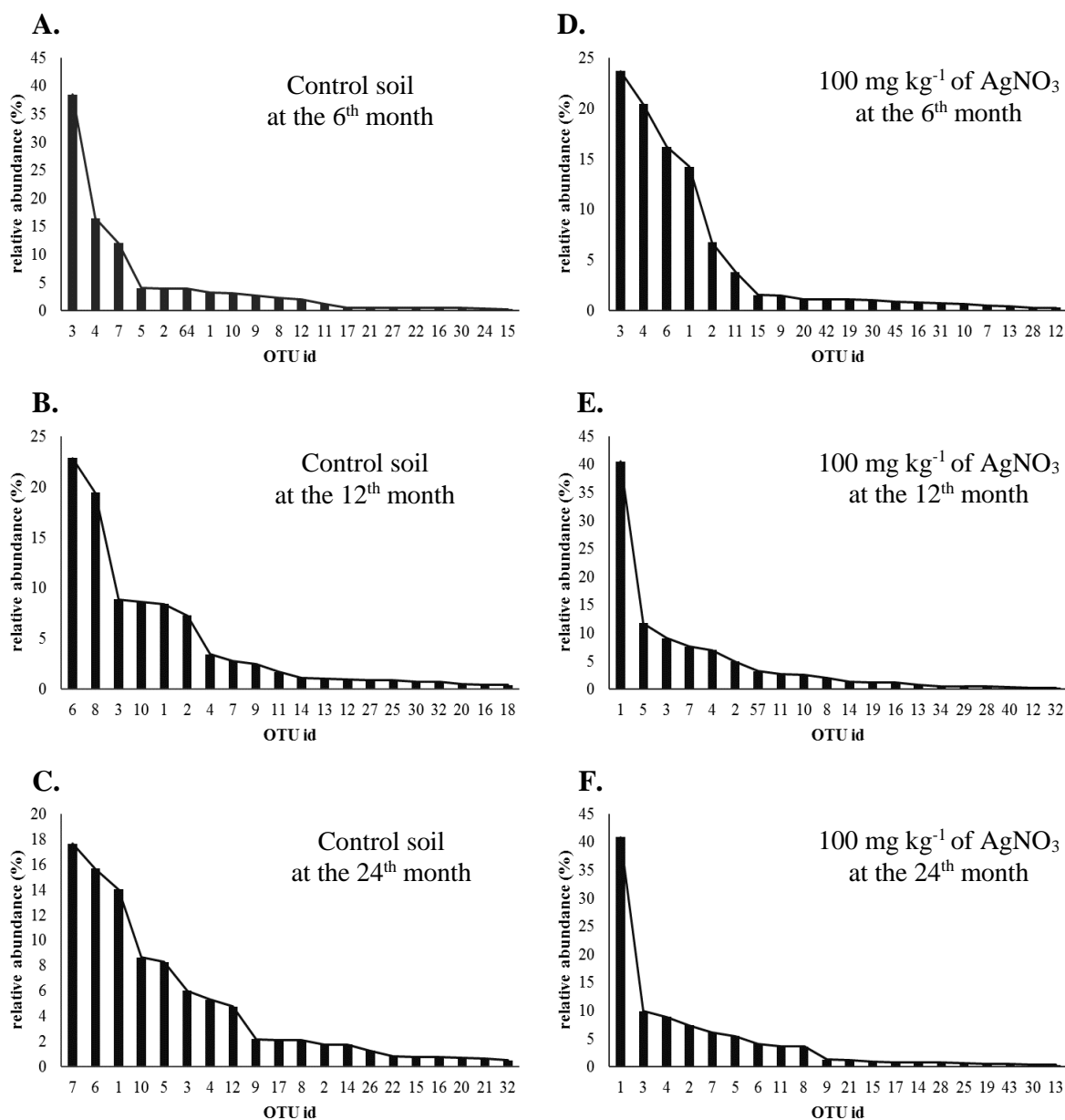


Figure 5.6 The impact of AgNO₃ application in soil on fungal species evenness. A, B, C are soil controls sampled at the 6th, 12th, and 24th month, respectively. D, E, F are AgNO₃ contaminated soil sampled at the 6th, 12th, and 24th month, respectively.

Fungal community structure responses to Ag contamination

The application of AgNPs and AgNO₃ in soil not only changed the fungal species richness and evenness but also the community structure. The results show that the fungal community members in the AgNPs and AgNO₃ contaminated soil were different to the control soil community. There were species that presence in both uncontaminated and contaminated soil and some species were only detected in control soil or Ag contaminated soil. For example, *Cryptococcus tereus* and *Hypocreaceae* sp. were detected in both uncontaminated and contaminated soil over time (Table 5.6, Table 5.7, Table 5.8). Interestingly, *Hypocreaceae* sp. abundance increased in Ag contaminated soil. Longer incubation times of Ag exposure (12 and 24 months) increased the abundance of the species in the soil (30-35% in AgNO₃ contaminated soil and 40% in AgNPs contaminated soil). In the case of *C. tereus*, it was found that the species was less abundance in AgNO₃ contaminated soil.

From Table 5.6, Table 5.7, and Table 5.8 it also can be seen that some species were very sensitive to Ag contamination as they were only detected in control soil e.g. *Rhytismataceae* sp., *Parmelina* sp., and *Leotiomyces* sp. Interestingly some species become more abundant in contaminated soil e.g. *Penicillium* sp., *Oidiodendron truncatum*, and *Trichoderma spirale*. The abundance of *T. spirale* in AgNO₃ contaminated soil tended to increase overtime. It was 1% at the 6th month and this increased at the 12th and the 24th months of incubation to 2% and 3% respectively. It also can be seen that AgNO₃ in soil was more suitable for *Penicillium* sp. as the relative abundance percentage of the species was higher than in AgNPs contaminated soil. For example, the abundance in the 6th month was 5% in AgNPs contaminated soil and 16% in AgNO₃ contaminated soil (Table 5.6). This increase also occurred at the 12th and the 24th month observations (Table 5.7 and Table 5.8) but with smaller percentage different. The abundance of *O. truncatum* in AgNPs contaminated soil decreased overtime (4%, 3%, and 1%). However, the abundance of this same species number increased in AgNO₃ contaminated soil (16% in the 24th month observation).

Table 5.6 The top twenty species in control soils, Ag contaminated soil at 100 mg kg⁻¹ (added as AgNPs and AgNO₃). The soils were sampled at the 6th month of incubation time. Red colour indicates different species detected in each soil sample. Percentages in brackets show the percentage of relative abundance each species.

Soil control	AgNP contaminated soil	AgNO ₃ contaminated soil
<i>Cryptococcus terreus</i> (38%)	<i>Cryptococcus terreus</i> (35%)	<i>Trichocomaceae</i> sp. (23%)
<i>Fungi</i> sp. (16%) (Identified as <i>Podospora ellisiana</i> by NCBI GenBank database)	<i>Trichocomaceae</i> sp. (17%)	<i>Cryptococcus terreus</i> (20%)
<i>Umbelopsis ramanniana</i> var <i>angulispora</i> (12%)	<i>Clitopilus</i> sp. (15%)	<i>Penicillium</i> sp. (16%)
<i>Trichocomaceae</i> sp. (4%)	<i>Hypocreaceae</i> sp. (6%)	<i>Clitopilus</i> sp. (14%)
<i>Clitopilus</i> sp. (3%)	<i>Penicillium</i> sp. (5%)	<i>Hypocreaceae</i> sp. (6%)
<i>Atheliaceae</i> sp. (3%)	<i>Oidiodendron truncatum</i> (4%)	<i>Ascomycota</i> sp. (3%)
<i>Hypocreaceae</i> sp. (3%)	<i>Helotiales</i> sp. (2%)	<i>Pseudeurotium</i> sp. (1%)
<i>Atheliaceae</i> sp. (3%)	<i>Atheliaceae</i> sp. (1%)	<i>Oidiodendron truncatum</i> (1%)
<i>Rhytismataceae</i> sp. (2%)	<i>Umbelopsis ramanniana</i> var <i>angulispora</i> (1%)	<i>Ascomycota</i> sp. (1%)
<i>Pseudeurotium</i> sp. (1%)	<i>Ascomycota</i> sp. (1%)	<i>Atheliaceae</i> sp. (1%)
<i>Leohumicola minima</i> (0.5%)	<i>Pseudeurotium</i> sp. (1%)	<i>Trichoderma spirale</i> (1%)
<i>Parmelina</i> sp. (0.5%)	<i>Pezizaceae</i> sp. (0.8%)	<i>Scytalidium</i> sp. (1%)
<i>Ascomycota</i> sp. (0.5%)	<i>Pezizomycetes</i> sp. (0.7%)	<i>Trichocomaceae</i> sp. (0.8%)
<i>Umbelopsis ramanniana</i> (0.5%)	<i>Ascomycota</i> sp. (0.7%)	<i>Ascomycota</i> sp. (0.8%)
<i>Tuckermannopsis platyphylla</i> (0.4%)	<i>Cryptococcus terricola</i> (0.4%)	<i>Penicillium atrovenerum</i> (0.7%)
<i>Clitocybe</i> sp. (0.4%)	<i>Devriesia</i> sp. (0.3%)	<i>Ceratobasidiaceae</i> sp. (0.6%)
<i>Pezizaceae</i> sp. (0.4%)	<i>Ascomycota</i> sp. (0.3%)	<i>Cryptococcus terricola</i> (0.5%)
<i>Diaporthaceae</i> sp. (0.3%)	<i>Trichoderma spirale</i> (0.3%)	<i>Helotiales</i> sp. (0.4%)
<i>Physcia magnussonii</i> (0.2%)	<i>Leohumicola minima</i> (0.2%)	<i>Pezizomycetes</i> sp. (0.3%)
<i>Scutellinia</i> sp. (0.2%)	<i>Seimatosporium</i> sp. (0.2%)	<i>Atheliaceae</i> sp. (0.3%)

Table 5.7 The top twenty species in control soils, Ag contaminated soil at 100 mg kg⁻¹ (added as AgNPs and AgNO₃). The soils were sampled at the 12th month of incubation time. Red colour indicates different species detected in each soil sample. Percentages in brackets show the percentage of relative abundance each species.

Soil control	AgNPs contaminated soil	AgNO ₃ contaminated soil
<i>Fungi</i> sp. (22%) (Identified as <i>Penicillium</i> sp. by NCBI GenBank database)	<i>Cryptococcus terreus</i> (33%)	<i>Hypocreaceae</i> sp. (40%)
<i>Cryptococcus terreus</i> (19%)	<i>Hypocreaceae</i> sp. (30%)	<i>Sordariomycetes</i> sp. (11%)
<i>Diaporthaceae</i> sp. (8%)	<i>Trichocomaceae</i> sp. (11%)	<i>Cryptococcus terreus</i> (9%)
<i>Umbelopsis ramanniana</i> var <i>angulispora</i> (8%)	Ascomycota sp. (5%)	<i>Clitopilus</i> sp. (7%)
<i>Hypocreaceae</i> sp. (8%)	<i>Oidiodendron truncatum</i> (3%)	Ascomycota sp. (6%)
<i>Rhizoglyphaceae</i> sp. (7%)	<i>Trichoderma spirale</i> (2%)	<i>Trichocomaceae</i> sp. (4%)
<i>Trichocomaceae</i> sp. (3%)	Ascomycota sp. (1%)	<i>Hypocrea virens</i> (3%)
<i>Parmelina</i> sp. (2%)	<i>Seimatosporium</i> sp. (1%)	<i>Simplicillium lamellicola</i> (2%)
<i>Clitopilus</i> sp. (2%)	<i>Hypocrea virens</i> (1%)	<i>Trichoderma spirale</i> (2%)
<i>Tuckermannopsis platyphylla</i> (1%)	<i>Helotiales</i> sp. (1%)	<i>Penicillium</i> sp. (2%)
<i>Leotiomyces</i> sp. (1%)	<i>Penicillium</i> sp. (0.9%)	<i>Seimatosporium</i> sp. (1%)
<i>Physcia magnussonii</i> (1%)	<i>Pezizaceae</i> sp. (0.7%)	<i>Helotiales</i> sp. (1%)
Ascomycota sp. (0.9%)	<i>Clitopilus</i> sp. (0.7%)	<i>Oidiodendron truncatum</i> (1%)
<i>Atheliaceae</i> sp. (0.9%)	<i>Fungi</i> sp. (0.7%) (Identified as <i>Penicillium</i> sp. by NCBI GenBank database)	Ascomycota sp. (0.7%)
<i>Parmelina</i> sp. (0.9%)	<i>Pseudeurotium</i> sp. (0.6%)	<i>Atheliaceae</i> sp. (0.5%)
<i>Umbelopsis ramanniana</i> (0.7%)	<i>Ramalina confirmata</i> (0.5%)	<i>Pseudeurotium</i> sp. (0.5%)
<i>Atheliaceae</i> sp. (0.7%)	<i>Diaporthaceae</i> sp. (0.5%)	<i>Scytalidium lignicola</i> (0.4%)
<i>Pezizaceae</i> sp. (0.5%)	<i>Chaetomium globosum</i> (0.3%)	<i>Umbelopsis ramanniana</i> var <i>angulispora</i> (0.3%)
<i>Humicola</i> sp. (0.4%)	<i>Tuckermannopsis platyphylla</i> (0.2%)	<i>Diaporthaceae</i> sp. (0.2%)
<i>Pseudeurotium</i> sp. (0.4%)	<i>Physcia magnussonii</i> (0.2%)	<i>Talaromyces flavus</i> (0.2%)

Table 5.8 The top twenty species in control soils, Ag contaminated soil at 100 mg kg⁻¹ (added as AgNPs and AgNO₃). The soils were sampled at the 6th month of incubation time. Red colour indicates different species detected in each soil sample. Percentages in brackets show the percentage of relative abundance each species.

Soil control	AgNP contaminated soil	AgNO ₃ contaminated soil
<i>Cryptococcus terreus</i> (17%)	<i>Hypocreaceae</i> sp. (36%)	<i>Hypocreaceae</i> sp. (40%)
Fungi sp. (15%) (Identified as <i>Penicillium</i> sp. by NCBI GenBank database)	<i>Cryptococcus terreus</i> (25%)	<i>Simplicillium lamellicola</i> (9%)
<i>Hypocreaceae</i> sp. (14%)	<i>Ascomycota</i> sp. (11%)	<i>Cryptococcus terreus</i> (8%)
<i>Humicola</i> sp. (8%)	<i>Trichocomaceae</i> sp. (9%)	<i>Trichocomaceae</i> sp. (7%)
<i>Diaporthaceae</i> sp. (8%)	<i>Diaporthaceae</i> sp. (1%)	<i>Oidiodendron truncatum</i> (6%)
<i>Trichocomaceae</i> sp. (6%)	<i>Oidiodendron truncatum</i> (1%)	<i>Ascomycota</i> sp. (5%)
<i>Rhytismataceae</i> sp. (5%)	<i>Trichoderma spirale</i> (1%)	<i>Penicillium</i> sp. (4%)
<i>Umbelopsis ramanniana</i> var <i>angulispora</i> (4%)	<i>Seimatosporium</i> sp. (1%)	<i>Sordariomycetes</i> sp. (3%)
<i>Parmelina</i> sp. (2%)	<i>Penicillium</i> sp. (1%)	<i>Trichoderma spirale</i> (3%)
<i>Tuckermannopsis platyphylla</i> (2%)	<i>Helotiales</i> sp. (1%)	<i>Diaporthaceae</i> sp. (1%)
<i>Ascomycota</i> sp. (2%)	<i>Diaporthaceae</i> sp. (1%)	<i>Ascomycota</i> sp. (1%)
<i>Clitopilus</i> sp. (1%)	<i>Ascomycota</i> sp. (1%)	<i>Seimatosporium</i> sp. (0.8%)
<i>Pseudeurotium</i> sp. (1%)	<i>Devriesia</i> sp. (0.8%)	<i>Pseudochaete rigidula</i> (0.8%)
<i>Atheliaceae</i> sp. (1%)	<i>Atheliaceae</i> sp. (0.8%)	<i>Clitopilus</i> sp. (0.8%)
<i>Pezizomycetes</i> sp. (0.8%)	<i>Umbelopsis ramanniana</i> var <i>angulispora</i> (0.7%)	<i>Ascomycota</i> sp. (0.7%)
<i>Pezizaceae</i> sp. (0.8%)	<i>Pezizaceae</i> sp. (0.4%)	<i>Scytalidium</i> sp. (0.6%)
<i>Leotiomyces</i> sp. (0.7%)	<i>Penicillium atrovenetum</i> (0.2%)	<i>Helotiales</i> sp. (0.5%)
<i>Penicillium atrovenetum</i> (0.7%)	<i>Clitopilus</i> sp. (0.2%)	<i>Devriesia</i> sp. (0.5%)
<i>Physcia magnussonii</i> (0.6%)	<i>Pseudeurotium</i> sp. (0.2%)	<i>Pseudeurotium</i> sp. (0.3%)
<i>Atheliaceae</i> sp. (0.5%)	<i>Cristinia helvetica</i> (0.1%)	<i>Diaporthaceae</i> sp. (0.3%)

5.3.4 Assessing the impact of *T. harzianum* (T22) addition into Ag contaminated soil at 100 mg kg⁻¹ (added as AgNPs and AgNO₃) on soil fungal community

T. harzianum has been explored for the removal and recovery of heavy metals (Lynch and Moffat, 2005; Adams *et al.*, 2007; Akhtar *et al.*, 2009; Lima *et al.*, 2011) and as a soil inoculant to improve plant growth (Molla *et al.*, 2012). This work aimed to determine if the addition of *T. harzianum* (T22) to Ag contaminated soil was able to maintain the number of species and abundance of fungi in the soil and to see if the addition of *T. harzianum* (T22) affected soil fungal communities in any way. Due to poor quality of the sequences of AgNPs contaminated soil at 3 and 100 mg kg⁻¹ obtained from the 6th month samples (Table 5.2), the effect of *T. harzianum* (T22) addition into AgNPs contaminated soil could only be fully observed at the 12th and 24th months sampling at the three levels of concentration. The effect of the addition of *T. harzianum* (T22) into AgNO₃ contaminated soil was observed at the three sampling time.

***The impact of T. harzianum* (T22) addition into Ag contaminated soil on fungal species richness**

Figure 5.8 shows the number of fungal species in control soil, soil with the addition of *T. harzianum* (T22), AgNPs contaminated soil at 100 mg kg⁻¹, and AgNPs contaminated soil with the addition of *T. harzianum* (T22) at the 12th and 24th month observation. It can be seen from the figure that the addition *T. harzianum* (T22) into uncontaminated soil did not give a significant effect on the number of species at any time of observation. However, at the 12th month the addition of *T. harzianum* (T22) into AgNPs contaminated soil increased the number of fungal species significantly from approximately 35 species to over 40 species. At the longer time of incubation there was no significant effect shown on the fungal species richness with the addition of *T. harzianum* (T22) into the soil.

The addition of *T. harzianum* (T22) into AgNO₃ contaminated soil did not improve the fungal species richness at any time of observation. Figure 5.9 shows there was no considerable change in the number of species in AgNO₃ contaminated soil with the addition of *T. harzianum* (T22) at any time of observation.

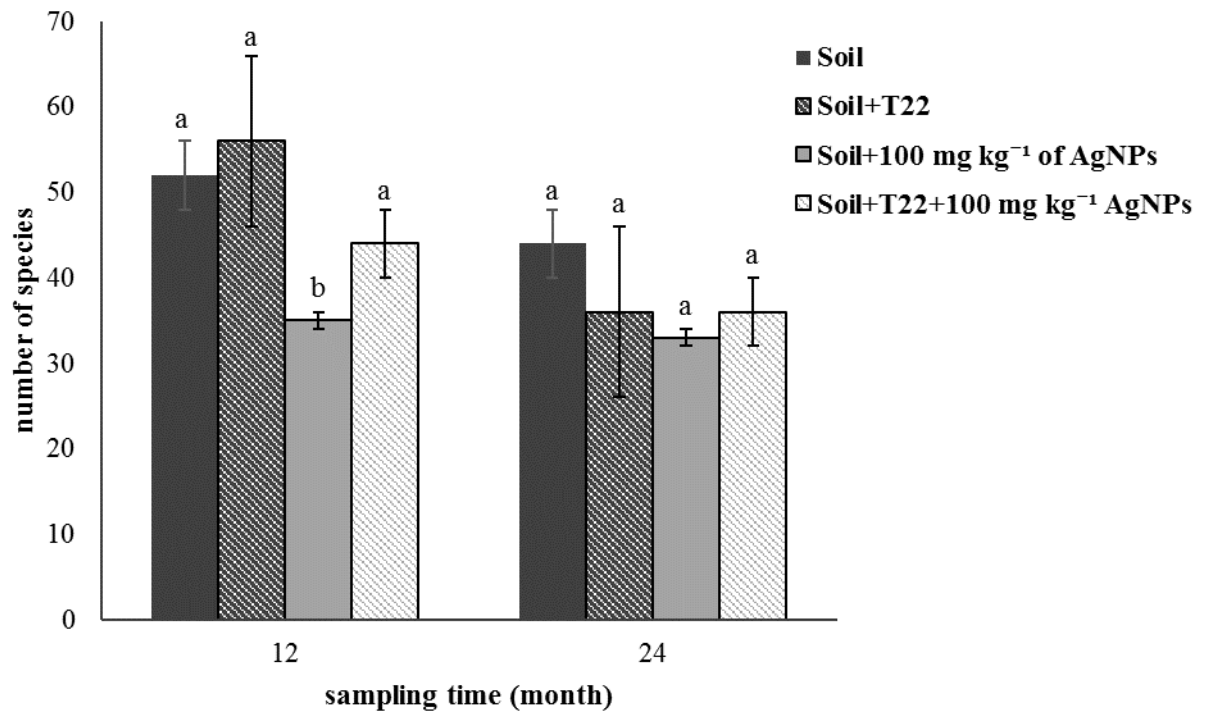


Figure 5.7 The effect of *T. harzianum* (T22) addition in soil and AgNPs contaminated soil at 100 mg kg⁻¹ on the number of fungal species. The soils were sampled at the 12th and 24th month after being contaminated with AgNPs. Data represent means of three replicates with standard error. Different letters above the data points indicate significant difference between treatments at one sampling time at the level of P < 0.05.

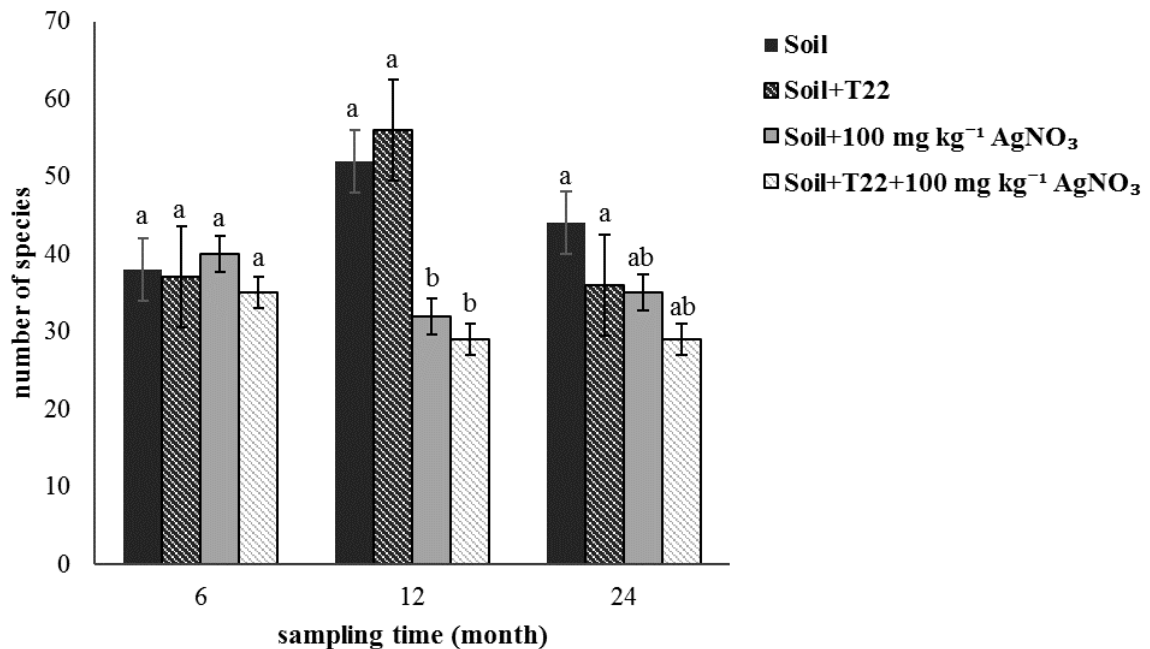


Figure 5.8 The effect of *T. harzianum* (T22) addition in soil and AgNO₃ contaminated soil at 100 mg kg⁻¹ on the number of fungal species. The soils were sampled at the 12th and 24th month after being contaminated with AgNO₃. Data represent means of three replicates with standard error. Different letters above the data points indicate significant difference between treatments at one sampling time at the level of P < 0.05.

The impact of T. harzianum (T22) addition into Ag contaminated soil on fungal species evenness

The addition of *T. harzianum* (T22) into AgNPs contaminated soil at 100 mg kg⁻¹ did not improve fungal species numbers in the soil. In fact, AgNPs contaminated soil without the addition of *T. harzianum* (T22) were more even compared to the one with *T. harzianum* (T22) presence. Figure 5.10C and Figure 5.10D show that community in the soil with *T. harzianum* (T22) addition at the two observation times were dominated by one same species (indicated by same OTU Id). At the 12th month the species dominated 70% of the community in the soil. The relative abundance percentage was decrease to 60% at the 24th month.

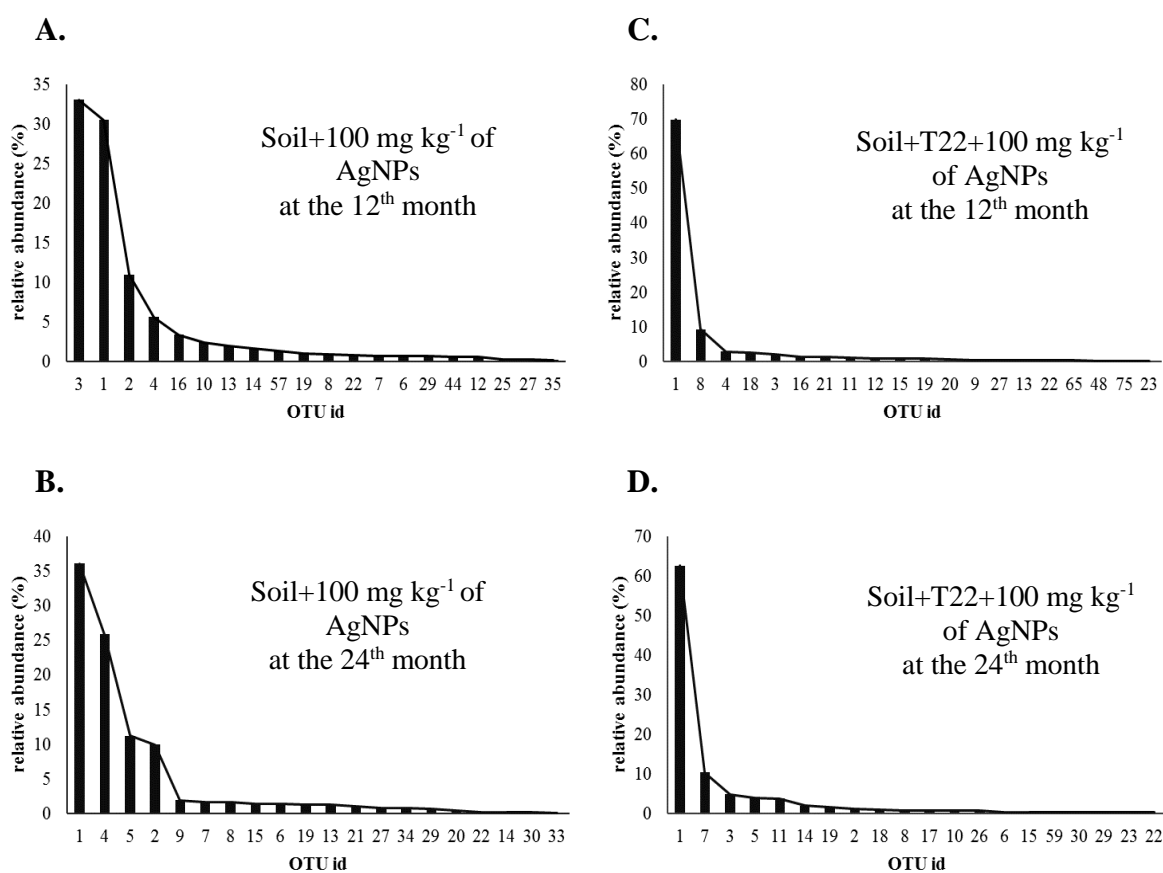


Figure 5.9 The impact of *T. harzianum* (T22) addition in AgNPs contaminated soil on fungal species evenness. A, B are AgNPs contaminated soil at 100 mg kg⁻¹ sampled at the 12th, and 24th month, respectively. C, D are AgNPs contaminated soil with the addition of *T. harzianum* (T22) sampled at 12th, and 24th month, respectively.

Figure 5.11 shows the effect of *T. harzianum* (T22) addition into AgNO₃ contaminated soil. At the 6th month 100 mg kg⁻¹ of Ag (added as AgNO₃) contamination did not give a negative impact on the species evenness in the soil (Figure 5.11A) as so *T. harzianum* (T22) addition into soil only maintained the fungal species evenness for the first 6 months (Figure 5.11D). At the longer time of exposure *T. harzianum* (T22) did not improve the species evenness.

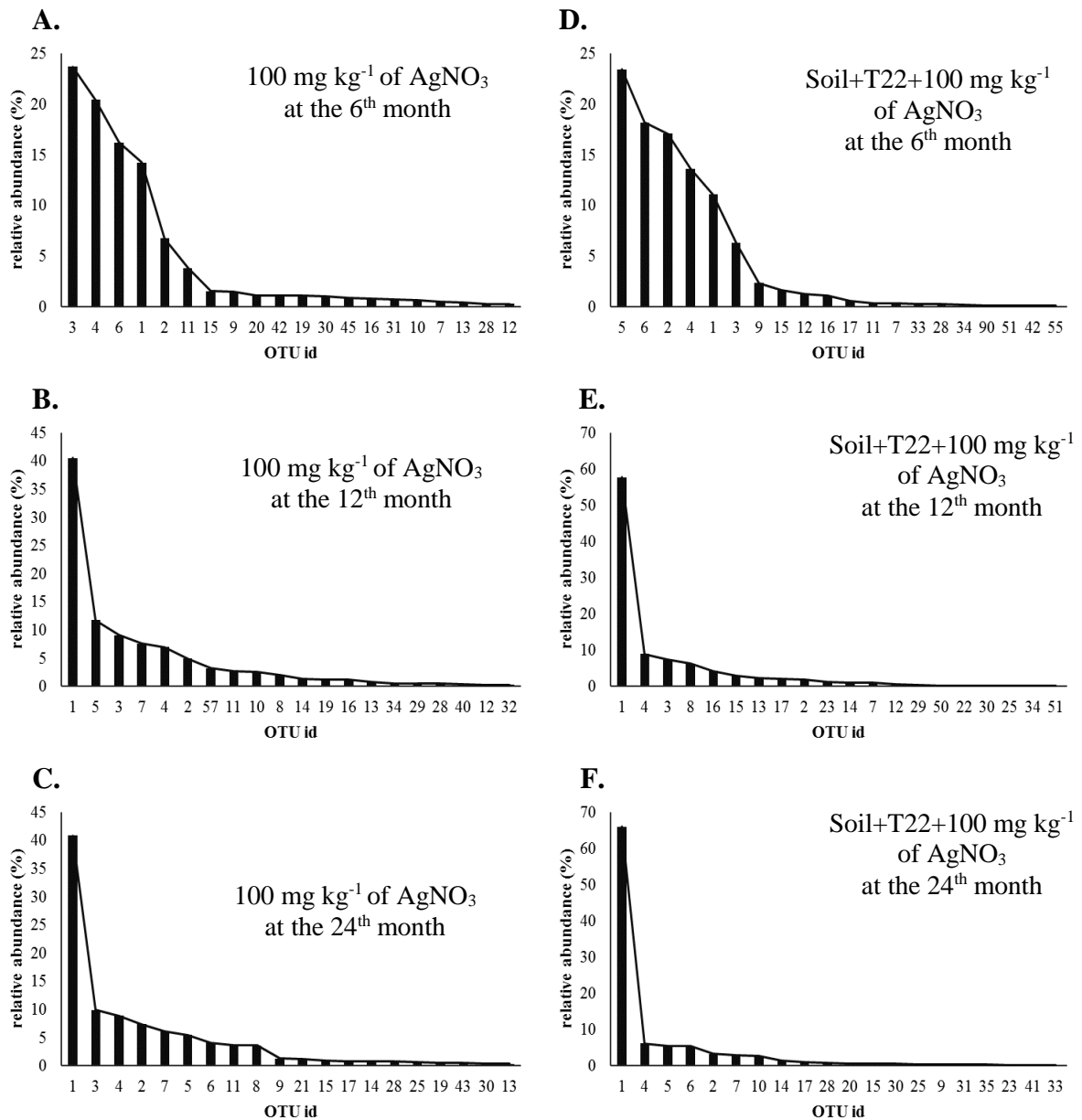


Figure 5.10 The impact of *T. harzianum* (T22) addition in AgNO₃ contaminated soil on fungal species evenness. A, B, C are AgNO₃ contaminated soil at 100 mg kg⁻¹ sampled at the 6th, 12th, and 24th month, respectively. D, E, F are AgNO₃ contaminated soil with the addition of *T. harzianum* (T22) sampled at 6th, 12th, and 24th month, respectively.

The impact of *T. harzianum* (T22) addition into Ag contaminated soil on fungal community structure

As displays in Table 5.6, Table 5.7, and Table 5.8, there were some species become more abundant in Ag contaminated soil e.g. *Penicillium* sp., *O. truncatum*, and *T. spirale*. With the addition of *T. harzianum* (T22) into soil, the abundance of each species were tend to decrease. With the presence of *T. harzianum* (T22) in the Ag contaminated soil, *T. spirale* did not appear in as the top twenty species in the community. On the other hand, *Hypocreaceae* sp. became the most abundance species (approximately 60% of the community) when *T. harzianum* (T22) was applied into the Ag contaminated soil. Full top twenty species in Ag contaminated soil with the addition of *T. harzianum* (T22) presents in Appendix F.

5.3.5 Compareison of three concentrations of Ag concentration (3, 100, and 660 mg kg⁻¹) added as AgNPs and AgNO₃ with the addition of *T. harzianum* (T22) on soil fungal community

Three levels of AgNPs (3, 100, and 660 mg kg⁻¹) and equal dose of Ag ions added as AgNO₃ were applied. *T. harzianum* (T22) was also inoculated into the Ag contaminated soil. The change on fungal species richness, species evenness, and community structure in the soil were can be fully observed at the 12th, and 24th month (due to poor quality of sequences in the 6th month. Shown in Table 5.2).

Impact of Ag concentration and *T. harzianum* (T22) addition into soil on fungal species richness

Figure 5.12 shows the impact of Ag concentration and the addition of *T. harzianum* (T22) into soil on fungal species richness. It can be seen that the increasing in the level of Ag resulted in the reduction in the number of species in the soil. The figure also shows that even with the addition of *T. harzianum* (T22), AgNPs decreased the number of species in the soil. The species richness in two levels of Ag (3 and 100 mg kg⁻¹ added as AgNPs) were similar at both sampling time while the highest level (660 mg kg⁻¹) reduced the number of species significantly. In the form of AgNO₃, each level of Ag reduced the number of species significantly. Shannon diversity index (H) also indicates the increasing of Ag concentration reduced the diversity in the soil (Table 5.9).

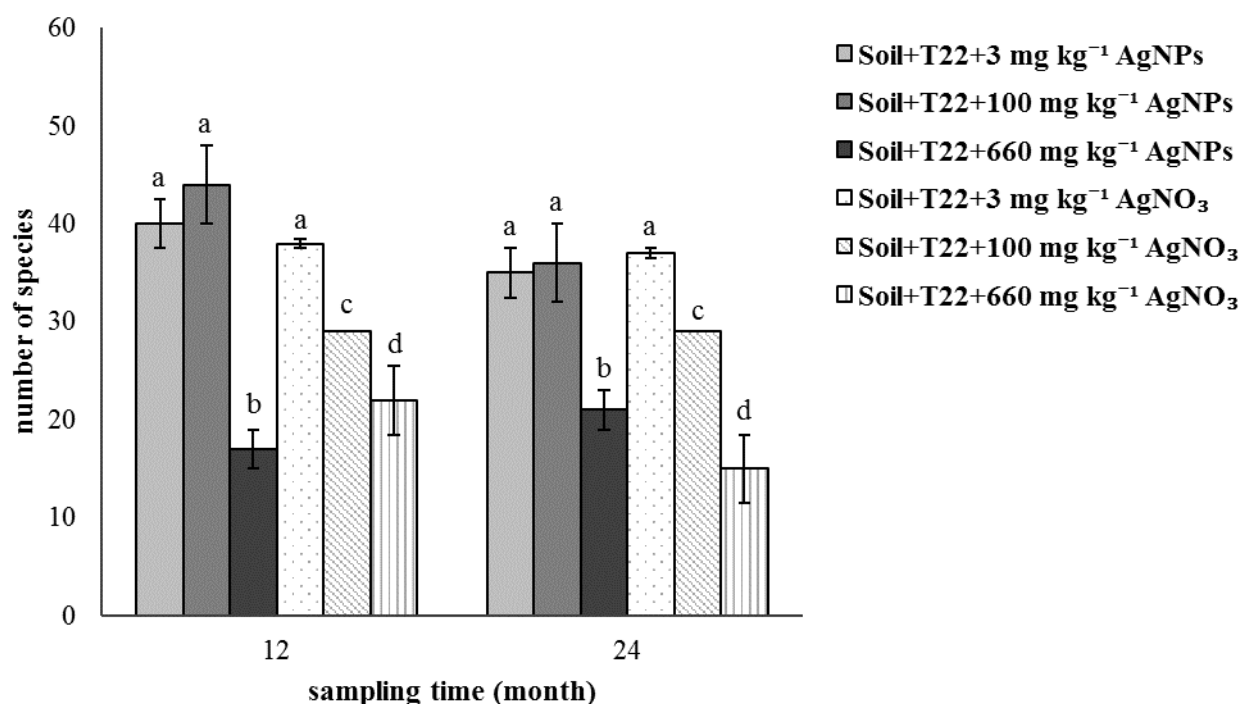


Figure 5.11 The number of fungal species in control Ag contaminated soil (added as AgNPs or AgNO₃) at 3, 100, and 660 mg kg⁻¹ with the addition of *T. harzianum* (T22). The soils were sampled at the 12th and 24th month after being contaminated with both types of Ag. Data represent means of three replicates with standard error. Different letters above the data points indicate significant difference between treatments in one sampling time at the level of $P < 0.05$.

Table 5.9 The fungal species richness in control soil and Ag contamination soils at 3, 100, and 660 mg kg⁻¹ of Ag added as AgNPs and AgNO₃ with *T. harzianum* (T22) addition measured using Shannon diversity index (H). The species richness was observed at the 12th and 24th month after experimentally contaminated with the two types of Ag and incubated at 25°C. The arithmetic mean of three replicates is shown with their arithmetic standard error (Mean±SE). Different letters in the same column indicates significant differences ($P < 0.05$).

Sample (s)	Shannon diversity index at different times of observation (month)	
	12 th	24 th
Soil+T22+3 mg kg ⁻¹ of Ag as AgNPs	1.77±0.14a	1.49±0.14a
Soil+T22+100 mg kg ⁻¹ of Ag as AgNPs	1.31±0.09b	1.48±0.09a
Soil+T22+660 mg kg ⁻¹ of Ag as AgNPs	0.90±0.04c	0.98±0.04b
Soil+T22+3 mg kg ⁻¹ of Ag as AgNO ₃	1.94±0.22a	1.51±0.22a
Soil+T22+100 mg kg ⁻¹ of Ag as AgNO ₃	1.63±0.014a	1.35±0.14a

Impact of Ag concentration and T. harzianum (T22) addition into soil on fungal species evenness

Figure 5.13 shows the community in AgNPs contaminated soil at any concentration were dominated by one species (OTU id 1). The increasing of AgNPs concentration increased the species abundance in the soil. For example, at the 12th month the species abundance in 3 mg kg⁻¹ AgNPs contaminated soil was 40% and increased to 70% in 100 mg kg⁻¹ AgNPs (Figure 5.13A and Figure 5.13C). At the highest level of AgNPs (660 mg kg⁻¹) the abundance was more than 70% (Figure 5.13E). At the 24th month observation the species abundance fluctuated between 55%-70% (Figure 5.13B, Figure 5.13D, Figure 5.13F). It also can be seen that even with the addition of *T. harzianum* (T22) into soil did not maintain the species evenness. Similar trend also occurred to AgNO₃ contaminated soil (results are shown in Appendix G).

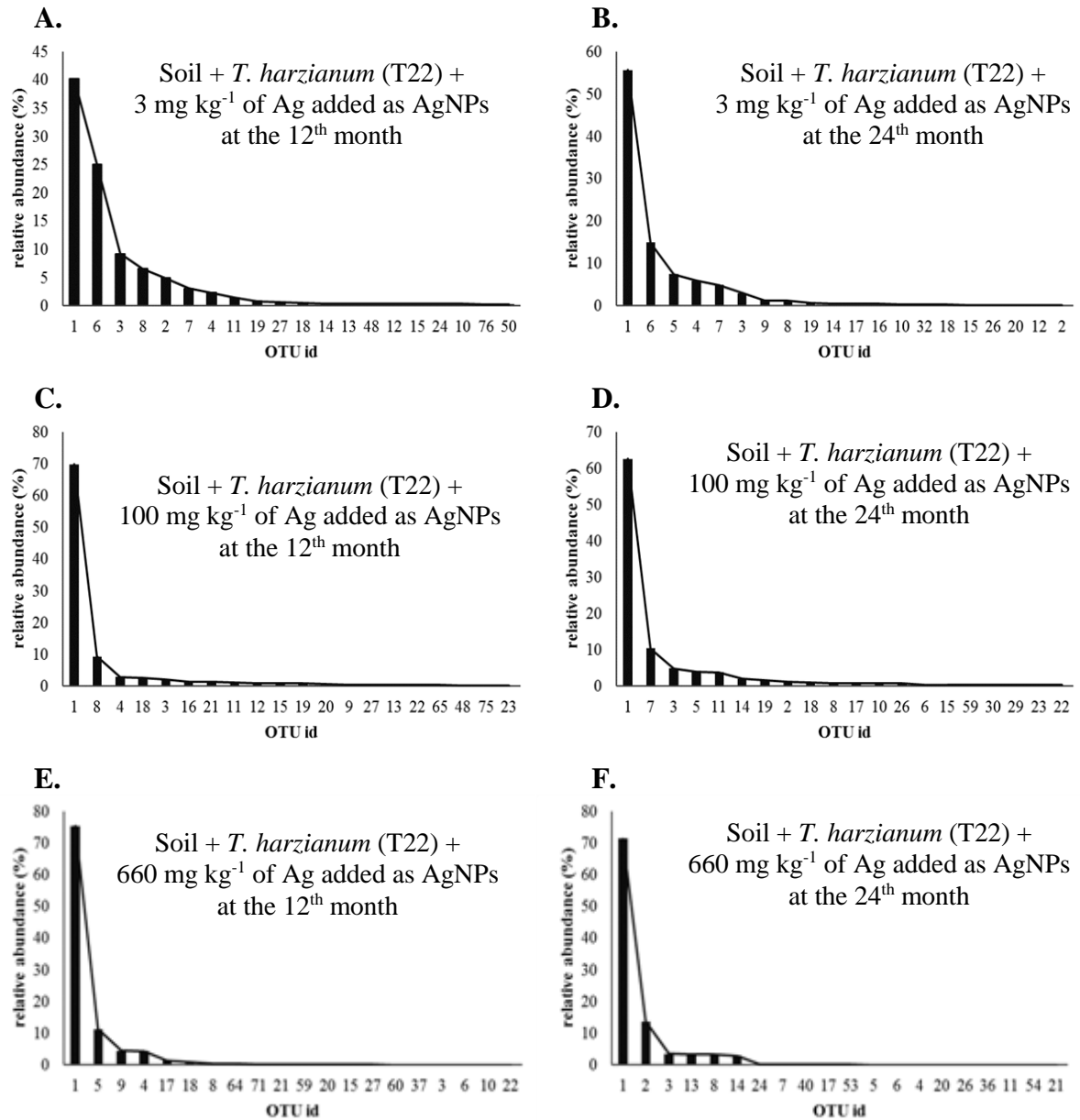


Figure 5.12 The Impact of Ag concentration (added as AgNPs) and *T. harzianum* (T22) addition into soil on fungal species evenness. A, B are Ag contaminated soils at 3 mg kg⁻¹ sampled at the 12th, and 24th month, respectively. C, D are Ag contaminated soils at 100 mg kg⁻¹ sampled at the 12th, and 24th month, respectively. E, F are Ag contaminated soils at 660 mg kg⁻¹ sampled at the 12th, and 24th month, respectively. All samples were incubated at 25°C. DNA was extracted from soils and assessed by Illumina MiSeq Next Generation Sequencing.

Impact of Ag concentration and T. harzianum (T22) addition into soil on fungal community structure

Table 5.10 presents the top twenty species identified in the three levels of AgNPs contaminated soil at the 12th month observation. There were species that detected at any level of AgNPs applied e.g. *Hypocreaceae* sp. and *C. terreus*. The abundance of *Hypocreaceae* sp. increased as the Ag concentration increasing. In the case of *C. terreus* the abundance increased by 3% in 100 mg kg⁻¹ and dropped to 0.4% in 660 mg kg⁻¹ of AgNPs. Similar results also seen at the 24th month observation (Appendix H). It is also noticed that there were only 16 species detected in the highest level of AgNPs contaminated soil. However, more species detected at the 24th month. Ag contamination at the three levels of concentration and at the two times of observation in the form of AgNO₃ showed similar results (results are shown in Appendix I but are not included here to aid the clarity of presentation).

Table 5.10 The top twenty species in Ag contaminated soil at 3, 100, and 660 mg kg⁻¹ (added as AgNPs) with the addition of *T. harzianum* (T22) at the 12th month observation. Percentages in brackets show the percentage of relative abundance each species.

3 mg kg⁻¹ of Ag	100 mg kg⁻¹ of Ag	660 mg kg⁻¹ of Ag
<i>Hypocreaceae</i> sp. (40%)	<i>Hypocreaceae</i> sp. (69%)	<i>Hypocreaceae</i> sp. (75%)
<i>Fungi</i> sp. (25%) (Identified as <i>Penicillium</i> sp. by NCBI GenBank database)	<i>Cryptococcus terreus</i> (9%)	<i>Trichoderma spirale</i> (11%)
<i>Diaporthaceae</i> sp. (9%)	<i>Trichocomaceae</i> sp. (3%)	<i>Clitopilus</i> sp. (4%)
<i>Cryptococcus terreus</i> (6%)	<i>Pseudeurotium</i> sp. (2%)	<i>Trichocomaceae</i> sp. (1%)
<i>Rhytismataceae</i> sp. (4%)	<i>Diaporthaceae</i> sp. (2%)	<i>Simplicillium lamellicola</i> (0.9%)
<i>Parmelina</i> sp. (3%)	<i>Humicola</i> sp. (1%)	<i>Pseudeurotium</i> sp. (0.5%)
<i>Trichocomaceae</i> sp. (2%)	<i>Penicillium</i> sp. (1%)	<i>Cryptococcus terreus</i> (0.4%)
<i>Tuckermannopsis platyphylla</i> (1%)	<i>Tuckermannopsis platyphylla</i> (1%)	<i>Hypocreales</i> sp. (0.1%)
<i>Ascomycota</i> sp. (0.8%)	<i>Ascomycota</i> sp. (0.9%)	<i>Mortierella minutissima</i> (0.1%)
<i>Atheliaceae</i> sp. (0.6%)	<i>Seimatosporium</i> sp. (0.8%)	<i>Penicillium</i> sp. (0.1%)
<i>Pseudeurotium</i> sp. (0.4%)	<i>Ascomycota</i> sp. (0.8%)	<i>Physcia magnussonii</i> (0.1%)
<i>Leotiomyces</i> sp. (0.4%)	<i>Pezizaceae</i> sp. (0.7%)	<i>Pezizaceae</i> sp. (0.1%)

continued

Table 5.10 The top twenty species in Ag contaminated soil at 3, 100, and 660 mg kg⁻¹ (added as AgNPs) with the addition of *T. harzianum* (T22) at the 12th month observation. Percentages in brackets show the percentage of relative abundance each species - continued

<i>Physcia magnussonii</i> (0.4%)	<i>Clitopilus</i> sp. (0.5%)	<i>Seimatosporium</i> sp. (0.1%)
<i>Ramalina confirmata</i> (0.3%)	<i>Atheliaceae</i> sp. (0.3%)	<i>Atheliaceae</i> sp. (0.05%)
<i>Ascomycota</i> sp. (0.3%)	<i>Physcia magnussonii</i> (0.3%)	<i>Mortierella</i> sp. (0.05%)
<i>Seimatosporium</i> sp. (0.3%)	<i>Diaporthaceae</i> sp. (0.3%)	<i>Penicillium atrovenetum</i> (0.05%)
<i>Helotiales</i> sp. (0.3%)	<i>Oidiodendron truncatum</i> (0.3%)	<i>Diaporthaceae</i> sp. (0%)
<i>Umbelopsis ramanniana var angulispora</i> (0.3%)	<i>Ramalina confirmata</i> (0.2%)	<i>Fungi</i> sp. (0%) (Identified as <i>Penicillium</i> sp. by NCBI GenBank database)
<i>Entoloma infula</i> (0.2%)	<i>Ascomycota</i> sp. (0.2%)	<i>Umbelopsis ramanniana var angulispora</i> (0%)
<i>Leohumicola minima</i> (0.2%)	<i>Pezizaceae</i> sp. (0.2%)	<i>Diaporthaceae</i> sp. (0%)

5.4 Discussion

5.4.1 The impact of Ag contamination and the addition of *T. harzianum* (T22) into soil on fungal communities

The results of these first long term experiments indicated that the application of AgNPs and AgNO₃ in soil reduced the species richness, evenness and change the fungal community structure in soil. A similar conclusion has been reached that metal NPs indeed change the composition of soil microbial communities (Shah and Belozeroва, 2008; Hänsch and Emmerling, 2010; He *et al.*, 2011; Ge *et al.*, 2011; Kumar *et al.*, 2011; Kumar *et al.*, 2011; Ge *et al.*, 2012; Nogueira *et al.*, 2012; Shah *et al.*, 2014). Others have reported that soil contamination with AgNPs reduces ectomycorrhizal diversity found in bishop pine root (Sweet and Singleton, 2015). According to Gadd (2007) a reduction in fungal numbers and species diversity are likely caused by toxicity from environmental pollutant. Fungal communities in soil are also affected by other types of heavy metal pollutant. For example, zinc and cadmium were strongly correlated with alteration of the fungal community composition (Beeck *et al.*, 2015).

In this study, the addition of *T. harzianum* (T22) into Ag contaminated soil did not improve microbial diversity in the soil. Today, *T. harzianum* (T22) single strain based products are sold as biopesticides and biofertilizers by many industries e.g. Koppert B.V., The Netherlands (<https://www.koppert.com/products/products-pests-diseases/trianum-p/>). As biopesticides, *T. harzianum* has been used to control wide range of pathogenic fungi (e.g. Fernandez, 1992; Mwangi *et al.*, 2011; Hasan *et al.*, 2012). Their effectiveness against non-fungal organisms such as nematode also has been reported (Dababat *et al.*, 2006; Kyalo *et al.*, 2007; Goswami *et al.*, 2008; Sahebani and Hadavi, 2008). The application of *T. harzianum* (T22) as a biofertilizer is proven to enhance vegetative and reproductive growth, yield and nutritional quality of tomato and save at least 50 % the use of chemical fertilizer (Molla *et al.*, 2012). In addition, *T. harzianum* has been explored for the removal and recovery of heavy metal in soil and aqueous streams (Lynch and Moffat, 2005; Adams *et al.*, 2007; Akhtar *et al.*, 2009; Lima *et al.*, 2011). However, the ability of *T. harzianum* (T22) to decrease heavy metal toxicity as assessed by effects on the soil fungal community in this study is not supported. The addition of T22 alone to soil did not appear to adversely affect the fungal community significantly.

5.4.2 Sensitivity of fungal genera to Ag contamination

The study also revealed that sensitivity to Ag is different among fungal genera. Some are more sensitive to silver pollution than others. There are several factors that influence the ability of fungi to survive in the presence of potentially toxic metals such as physiological and/or genetical adaptation, morphological changes and environmental modification of the metal in relation to speciation, availability and toxicity (Gadd, 2007). For example, Anahid *et al.*, (2011) showed that the survival of fungal species at high metal concentration involved several mechanisms involving (1) extracellular protection, by preventing metal entry into the cell, and (2) intercellular, by reducing the heavy metal burden (Anahid *et al.*, 2011). In this work some fungal species (OTUs) survived at the highest concentration of heavy metal suggesting that they are tolerant to the pollutant. The results seem to be consistent with other research that found that silver shows very different activities against different microbial species (Simonetti *et al.*, 1992). It has been reported that certain fungi such as *Hypocreales* fungi are abundant in soil treated with the high Ag concentration (Kumar *et al.* 2014). Bacterial communities are more affected by AgNPs as their composition is significantly modified by nanosilver exposure (Sillen *et al.*, 2015; Carbone *et al.*, 2014). A study confirmed that plant-associating bacteria, *Bradyrhizobium canariense*, appeared to have a marked sensitivity to AgNPs (Kumar *et al.*, 2011), showing that the variation in sensitivity to metal pollution shown by fungi is consistent with that seen in other kingdoms.

In this work the genus *Trichoderma* was found in silver contaminated soil and this is in agreement with previous authors who found *Trichoderma* in heavy metal polluted soil. For example, *Trichoderma* species were isolated from a forest situated near a chemical factory in Lithuania by Pečiulytė and Dirginčiūtė-Volodkienė (2009). Furthermore, Gadd (2007) reported that *Trichoderma* is one of the most frequent genera isolated from heavy metal polluted habitats in Argentina. The genus *Trichoderma* was referred to as metal tolerant fungus as it was found in agricultural field soil receiving long-term application of municipal and industrial wastewater (Zafar *et al.* 2007). Kubicek *et al.* (2003) isolated *T. spirale* from tropical rain forest, Tangkuban Perahu volcano, Indonesia, while Fomina and Gadd (2014) isolated the same species of *Trichoderma* from depleted uranium particles in soil. These facts suggest that *T. spirale* is metal resistant species and interestingly this species did increase in abundance during the 2 years of this study.

Oidiodendron and *Penicillium* species also show high tolerance to Ag contamination in the soil. Nordgren *et al.* (1985) studied soil microfungi in a heavy metal polluted area in Canada. They found that the growth of some species from the genus *Oidiodendron*, e.g. *O. cf. pilicola* and *O. flavum*, were not affected by metal contamination. This genus was also found to increase in abundance in Ag contaminated soil samples during the current work. These types of fungi perhaps have potential as bioremediation agents to detoxify or remove heavy metal from polluted soil or perhaps could be used as soil inocula to help maintain soil functions (e.g. nutrient recycling) in contaminated environments. Similarly, *Penicillium* species such as *P. funiculosum* and *P. simplicissimum* showed high tolerance toward heavy metal (Valix *et al.*, 2001; Anahid *et al.*, 2011). *Penicillium* sp. also has been isolated from polluted sites in Tangier, Morocco (Ezzouhri *et al.*, 2009).

Interestingly, *Cryptococcus terreus* was found at all Ag soil contamination levels suggesting it is an important member of the soil fungal community. Studies about this species are very limited. Menna (1954) reported the isolation of this yeast from soil samples taken in the province of Otago, New Zealand for the first time. The species was distinguished from other species of the genus by its ability to utilize glucose, maltose, lactose, galactose and potassium nitrate. *C. terreus* closely resembles *C. albidus* on all points but the ability to utilize sucrose. It appears that its distribution may be similar also. Vishniac (1995) suggested that *C. albidus* is an important yeast of arid soils as it has competitive ability and lengthy survival.

Trichocomaceae sp. that identified in both control and Ag contaminated soil is commonly important species to both industry and medicine. For example, *Penicillium* sp. that produce penicillin, a molecule that is used as an antibiotic. However, some species are opportunistic pathogens, e.g. *Aspergillus* sp. Studies showed that both species are used to synthesize silver nanoparticles (Li *et al.*, 2012; Honary *et al.*, 2013).

5.4.3 Which form of Ag more toxic to fungal species?

No apparent differential toxicity of the two forms of silver used in this study was found as a similar impact was shown by both AgNPs and AgNO₃ on the fungal species richness, evenness and community structure. Studies on bacterial communities in sludge shows that AgNPs give a higher impact than Ag⁺ (Yang *et al.*, 2014). Other studies show that AgNO₃ inhibited bacterial activity in natural water more than AgNPs (Das *et al.*, 2012). On the other hand, AgNPs were more toxic to sea urchin than their equivalent Ag⁺ ion dose (Šiller *et al.*, 2013). It seems that the toxicity of AgNPs and AgNO₃ is different to different microorganisms and in different environments. It is difficult to compare results between studies due to the different environments, organisms and levels (and forms) of AgNPs used. The results obtained in this study are of importance as the work was carried out for 2 years. It is possible that the initial toxicity of free Ag⁺ ions has more immediate impact than less readily bioavailable AgNPs but in a fungal soil community over the longer term it appears that the toxic effects of the two silver forms are similar in that they both reduce species richness and evenness and select for fungal species that are resistant to metal pollution.

5.4.4 Significant of the present study

To the best knowledge of the author of this thesis, this work is the first long term study carried out (up to 24 months) on the effect of Ag contamination on fungal communities in soil. Moreover, two types and three levels of Ag applied in this study covered contamination from everyday products, the use of Ag as fungicide in agriculture land, and a high level to simulate a pollution event. This study found significant effects of Ag contamination on fungal communities even at very low level of Ag. The current study has added value as it was performed for a longer time of observation than previous work. Studies of changes in microbial communities are often observed only over short time periods e.g. several months. The two approaches (short term vs long term) may result in different findings. For example, long term studies carried out by Degens *et al.* (2000) showed that the addition of organic matter into soil change microbial community structure. However, the change did not show in a short term study (Crecchio *et al.*, 2001). This work showed that more changes to soil fungal

communities occurred over the longer term highlighting the requirement for the need of studies of sufficient length.

5.5 Conclusion

The application of AgNPs and AgNO₃ even at low levels resulted in a reduction in fungal species richness and evenness and changed the fungal community structure in soil. The addition of *T. harzianum* (T22), as an attempt to improve fungal communities in contaminated soils did not improve fungal diversity in soil. Any change in the community has the potential to affect soil decomposition processes, nutrient cycling and finally soil quality and future studies should examine these soil functions. Intentional silver application, for example to control plant pathogenic fungi in agriculture, should be re-considered as it will boost the level of metal pollutant in soil and lead to potentially deleterious effects.

Chapter VI. General Discussion

6.1 Introduction

Silver nanoparticles (AgNPs) have known antimicrobial activity and have a wide range of suggested uses. Common applications include the use of AgNPs for antimicrobial coatings on textiles, electronics and biomedical devices (Emerich and Thanos 2007; Rai *et al.* 2009; Lee *et al.* 2010). Recently, researchers have proposed the use of AgNPs in agriculture to control plant pathogenic fungi such as *Rhizoctonia solani*, *Bipolaris sorokiniana*, *Magnaporthe grisea*, and *Colletotrichum* sp. (Min *et al.* 2009; Jo *et al.* 2009; Lamsal *et al.* 2011). The increasing and varied use of AgNPs increases the risk of AgNP release into the environment whether unintentional, such as the release from normal laundry washing and subsequent accumulation in sewage sludge (Benn & Westerhoff, 2008) or intentionally, when applied in agriculture (Zhang *et al.*, 2012). Studies have revealed that AgNPs can cause toxic effects on soil microorganisms (Kumar *et al.* 2011; Calder *et al.*, 2012; Sweet and Singleton, 2015). Furthermore, AgNP contamination changes the composition of soil microbial communities (Hänsch and Emmerling, 2010). The toxicity caused by AgNPs is still debatable so that another type of silver, silver nitrate (AgNO₃), was used in this thesis (soil contamination section) to study which type was more harmful to soil fungal species.

In this work, *Trichoderma harzianum* was employed as a model fungus as it has important ecological roles e.g. as an organic matter decomposer and is a well-known biological control agent against a wide range of plant pathogens (Hadar *et al.*, 1979; Innocenti *et al.*, 2003; Montealegre *et al.*, 2010; Anees *et al.*, 2010). In addition, *T. harzianum* has been explored for removal and recovery of heavy metal such as cadmium and uranium in soil and aqueous streams (Akhtar *et al.*, 2009; Lima *et al.*, 2011) and also employed in phytoremediation to promote root plant growth (Lynch and Moffat, 2005; Adams *et al.*, 2007). The effect of AgNPs were tested on *T. harzianum* growth and its ability to control selected soil borne fungal plant pathogens *in vitro*, and also on fungal communities in soil. Soil fungal communities were studied in a long-term experiment (lasting 24 months). The long term observation provides a better understanding of the changes that may occur in the environment due to metal contamination. The fungal communities were assessed using Illumina Next Generation Sequencing (NGS) techniques and analysed following the UPARSE pipeline run with USEARCH v8.1.1756 (Edgar, 2013). Although lacking in downstream graphics applications for data visualisation, the method has been found as a useful method to quickly process data obtained from the Illumina MiSeq sequencer.

In summary, the work found that *T. harzianum* was very tolerant to high level of AgNPs compared to other fungi tested (*Rhizoctonia* species). Interestingly a combination of AgNP and *T. harzianum* did not improve control of fungal pathogen growth likely due to subtle effects of AgNPs on *T. harzianum* that affected its ability to control *R. solani* growth. AgNP contamination of soil changed the fungal soil community and the addition of a commercially available *T. harzianum* strain into Ag contaminated soil as an effort to maintain fungal diversity had no real effect because the inoculum added (T22) appeared not to grow under the experimental soil conditions used.

6.2 The Effect of AgNPs on the growth of Soil Fungi : *In vitro* Experiments

Three strains of *T. harzianum* (two strains were isolated from UK soil and one was a commercial product; T22) and two *Rhizoctonia* species (*R. solani*, AG3 and AG2-1, and *R. cerealis*) were employed in the initial study to examine the effect of AgNPs on the growth of soil fungi. The experiments were carried out using two types of culture media (PDA and CDA) and a wide range of AgNPs concentrations (up to 1000 mg L⁻¹) to understand whether those variations showed any different effects on fungal growth. The study revealed that *T. harzianum* was more tolerant to AgNPs than *Rhizoctonia* spp. AgNPs only affected the growth of *T. harzianum* (as measured by colony diameter) at a very high level (600 mg L⁻¹) while 50 mg L⁻¹ of AgNPs reduced the colony growth of *Rhizoctonia* spp. The reproductive stage of *T. harzianum*, i.e., spore production, was more sensitive to AgNPs than mycelial growth (as measured by colony diameter) as spore production of *T. harzianum* was reduced at 50 mg L⁻¹ of AgNPs while the colony diameter was only affected at 600 mg L⁻¹. Furthermore, different species of the same genus reacted differently to AgNPs. For example, *R. solani* growth was affected by the presence of AgNPs in the culture media at 50 mg L⁻¹ while 150 mg L⁻¹ of AgNPs did not affect the growth of *R. cerealis*. Different types of culture media also had a contribution on the reaction of fungal species toward AgNPs. *T. harzianum* produced more spores when grown on PDA. Less sporulation on CDA may be due to the presence of chloride ion in the test medium (Okunowo *et al.*, 2010). The findings implied that there are several factors affecting metal toxicity on microorganisms including species strain, growth media, metal concentration, and the life stage of the microorganism.

Metal tolerance showed by *T. harzianum* in this study adds to evidence that this species has potential as a bioremediation agent to clean up environmental pollutants or as a species that may be added to contaminated soil to enhance or maintain soil functions e.g. organic matter transformation, when the activity of other fungi have been reduced by the presence of a

contaminant. Previously, *T. harzianum* has been reported to remove cadmium and uranium from the environment (Akhtar *et al.*, 2009; Lima *et al.*, 2011). *T. harzianum* also showed tolerance toward arsenic and aluminium (Arriagada *et al.*, 2007; Arriagada *et al.*, 2009). According to Tripathi *et al.*, (2013) metal bioremediation activities of *Trichoderma* species can be classified into four categories. Firstly, the ability of *Trichoderma* species to accumulate heavy metal (biosorption). Secondly, an active process of metal removal (bioaccumulation). The next strategy is biovolatilization which is generally carried out by intracellular biochemical reactions that convert organic and inorganic compounds of metalloids enzymatically. Finally, *T. harzianum* could be applied as part of a combination of microbes and plants to remediate pollutants (also known as phytobial remediation). Heavy metal removal using microorganisms is studied widely as it is less expensive than conventional physico-chemical remediation methods (Tripathi *et al.* 2013). In situ bioremediation has the added benefit of minimal site disruption (Gabriel, 1991). Furthermore, the natural process of bioremediation is expected to have a minimal impact to the environment when compared to chemical and physical remediation processes.

The tolerance of *T. harzianum* to AgNPs coupled with the sensitivity of *Rhizoctonia* species to AgNPs suggested that *T. harzianum* could be combined with pesticides containing metal ions and chemicals as part of integrated pest management for agricultural or horticultural use. The effects of certain pesticides have been tested on *T. harzianum* in vitro and have demonstrated that combinations of *T. harzianum* and pesticide were compatible (Wedajo, 2015; Bhosale *et al.*, 2015). However, results obtained in this thesis using a combination of *T. harzianum* and AgNPs to control the growth of *Rhizoctonia* in vitro were inconclusive and it appears that *T. harzianum* was affected by the presence of AgNPs in the growth media. For example, AgNPs may have an impact on the biocontrol mechanisms of *T. harzianum* thereby resulting in no additional control of *Rhizoctonia*. This hypothesis would require further research e.g., an assessment of the effect of AgNPs on the production of lytic enzymes by *T. harzianum*.

In addition to potential subtle effects on fungal activity, AgNPs may have indirect detrimental effects on beneficial microorganisms in the soil as reported by previous researchers e.g. Kumar *et al.* (2011); Calder *et al.* (2012); Schlich *et al.* (2013) and AgNP release into the environment is regarded as a dangerous pollutant in other studies (Benn and Westerhoff, 2008; Geranio *et al.*, 2009; Kumar *et al.*, 2011). It is clear that any potential purposeful release of AgNPs for potential agricultural use needs to consider their impact on beneficial soil microbes. This thesis focused on soil fungal communities as they have been understudied in relation to AgNPs

pollution and have an important ecological role in soil. The work also carried out an impact study over a significant time period (2 years) as most previous work only studies impacts of pollutants over relatively short time scales e.g. months.

6.3 The Effect of AgNPs contamination on Soil Fungal Communities

Two types of Ag (AgNPs and AgNO₃) with three levels of concentration were used to investigate the long term effect of silver contamination on fungal communities in soil. The three Ag levels used represented a potential low level potentially caused by chronic accidental environmental release, a medium level to represent potential agricultural use and a high level representative of an accidental industrial release incident. Overall, both forms of Ag contamination in soil reduced the number of species and species evenness and also changed the community structure. Long-term exposure of Ag even at the lowest level applied (3 mg kg⁻¹ of Ag) resulted in the domination of one fungal species in the soil community. Different fungal species reacted differently toward Ag contamination, some were very sensitive and some were very tolerant (being only detected in highly contaminated soil). These tolerant species of fungi (*Penicillium* sp., *O. truncatum*, and *T. spirale*) perhaps have potential to be used in bioremediation to alleviate the toxicity of heavy metals in polluted soil or to maintain soil functions.

Several previous studies have reported that silver contamination affects microbial communities. For example, reductions in the number of ectomycorrhizal fungal species found on pine roots (Sweet and Singleton, 2015), a decrease in the abundance of nitrifying bacteria in activated sludge (Yang *et al.*, 2014) and a change in bacterioplankton communities in natural waters (Das *et al.*, 2012) have been observed. Any change in the community composition might affect ecosystem processes. For instance, a change in the soil community will affect plant decomposition processes and nutrient cycling and finally soil quality. Using Shannon diversity index (H) values, other studies on the effect of heavy metal on microorganisms in soil also showed a higher diversity in uncontaminated soil. For example, Wang *et al.* (2007) and Val *et al.* (1999) reported that heavy metal influenced microbial diversity in soil. Shannon diversity index (H) estimates of the soil samples confirmed that diversity in uncontaminated soil was higher than in Ag contaminated soil (showed by a higher diversity index in uncontaminated soil). The Shannon values estimated in this work are similar to those found in agricultural soils used in previous study giving confidence in the results found (Chen *et al.*, 2014). Interestingly, Shannon values of fungal communities tend to be higher in tropical soils reflecting the differences in diversity between different soils and global climates (Sharma *et al.*, 2015). The

typical values of Shannon index are generally between 1.5 and 3.5 in most ecological studies, and the index is rarely greater than 4 (Magurran, 2004). In this study, the index value for uncontaminated soil was as high as 2.5 and as low as 0.9 in Ag contaminated soil.

T. harzianum has been reported as a potential bioremediation agent to remove heavy metals from polluted soil (Arriagada *et al.*, 2007; Arriagada *et al.*, 2009; Akhtar *et al.*, 2009; Lima *et al.*, 2011). The previous studies have been mostly carried out using short term exposures (months) to heavy metals. The longer period of observation in this study showed that the ability of *T. harzianum* to reduce the toxic effect of AgNPs on soil microorganisms decreased over time and shows the importance of maintaining *T. harzianum* growth conditions at the contaminated site. In order to reduce pollutant levels or detoxify pollutants in contaminated soil using microorganisms, their growth and activity must be stimulated (Naik and Duraphe, 2012). It might be difficult to control growth conditions of bioremediation agents because there are many confounding environmental factors that must be considered such as nutrient levels, pH, temperature, etc. In addition any bioremediation agent added to soil has to be able to grow and compete with the existing soil microbial community present. Results from this work indicated that the T22 strain of *T. harzianum* added to the soil did not grow successfully in competition with the existing fungal community suggesting that the use of microbial inocula is unlikely to be a successful remediation strategy. This is despite the fact that the experimental conditions used (soil pH, moisture and temperature) all should have allowed T22 growth. Indeed the topic of microbial inocula for use in bioremediation, biocontrol of plant pathogens and enhancement of plant growth is one of constant scientific debate with different authors finding contrasting results depending on the experimental parameters used. For example, Mishra *et al.* (2001) reported that inoculum addition was successfully remediate oily-sludge-contaminated soil. In contrast, another study revealed that microbial inoculation into contaminated soil did not increase the bioremediation significantly (Kuhad and Gupta, 2009).

The different forms of Ag (AgNPs and AgNO₃) used in this study showed similar effects on soil fungal communities. Different findings have been reported on which form of Ag is more toxic. Some studies have revealed that AgNPs are more toxic (Yin *et al.*, 2011; Yang *et al.*, 2014; Šiller *et al.*, 2013; Sillen *et al.*, 2015; Carbone *et al.*, 2014) while some showed the opposite results (Das *et al.*, 2012; Boenigk *et al.*, 2014). These studies were carried out over a short term of exposure. The results obtained in this work are of importance as experiments were carried out for 2 years. It is possible that the initial toxicity of free Ag⁺ ions has more immediate impact than less readily bioavailable AgNPs but in a fungal soil community over the longer

term it appears that the toxic effects of the two silver forms are similar. Interestingly, a previous study (Sweet and Singleton, 2015) examining the toxicity of AgNPs towards ectomycorrhizal fungi, showed that a small proportion of Ag appeared to be in an extractable form in soil thus demonstrating the potential of the Ag, in 'insoluble' AgNPs, to become available to living organisms. In addition, it seems that AgNP and AgNO₃ toxicity is different to different microorganisms and in different environment types. Dorobantu *et al.* (2015) confirmed that the toxicity effects observed depend on the species being examined.

6.4 UPARSE Pipeline as a Method to Process Illumina NGS Metabarcoding Data

The long-term effect of AgNPs on soil fungal communities in this study was assessed using the Illumina NGS technique. The Illumina Miseq platform has been claimed as more suitable method to investigate fungal communities as it provides greater depth of sequencing and promises a deeper characterisation of fungal communities (Schmidt *et al.*, 2013; Bálint *et al.*, 2014). Before further processing the quality of sequences from the sequencer was checked using FastQC. Poor quality sequences were also filtered later on using the UPARSE pipeline. Sample depth was decided by finding the lowest number of sequence in the samples to avoid the problem of sequence depth being different from different samples as this would affect diversity estimation. The reads then were processed with the UPARSE pipeline run using USEARCH. Although lacking in graphics capability (compared to QIIME for example) the method was very useful to quickly process data obtained from the Illumina MiSeq sequencer. In previous studies, the UPARSE pipeline has been compared with other different clustering methods and they found that the method was more precise and produced more consistent OTU numbers (Edgar, 2013; Flynn *et al.*, 2015). Flynn *et al.*, 2015 added that UPARSE pipeline produced a precise OTU even with relaxed filtering and when including singletons. Stringent workflow (USEARCH filtering, singletons removed, UPARSE clustering) was applied to obtained high quality sequences because it reduces redundancy, noise, and problems of generating inflated numbers of OTUs (Flynn *et al.*, 2015). Pairing the forward and reverse reads resulted in a reduction in the number of sequence in each sample and also resulted in a very low number of final OTU (10 OTUs) found. It has been suggested that some taxa fail to pair in the process due to the poor quality and quantity of the reverse direction sequences (Nguyen *et al.*, 2015). However, to pair or not to pair sequence reads decision might vary depend on the data quality obtained and primers used. In this study, single forward reads produced the most OTUs compared to single reverse reads so the single forward reads were used to analyse soil fungal communities in Ag contaminated soil. Overall this work similar fungal diversity levels to those found in previous studies and the ability to use USEARCH on an easily available bench top pc

suggests that this method of sequence processing and analysis is of excellent use especially in laboratories that have limited access to high performance computers.

6.5 Conclusion and Future Perspectives

The toxicity of AgNPs depended on the fungal species examined. *T. harzianum* (biocontrol agent) showed a high tolerance toward AgNPs while *Rhizoctonia* spp. (plant pathogenic fungi) were more sensitive to the metal pollutant. These results show the potential of AgNPs to control plant pathogenic fungi. In addition, *T. harzianum* tolerance to AgNPs also indicated that the species could be combined with pesticides containing metal ions and chemicals as part of integrated pest management. However, the application of AgNPs in agricultural land should only be made after considering the impact of the heavy metal on soil community (particularly microbes) as Ag has a strong antimicrobial activity. This study confirmed that silver contamination in soil reduced fungal species richness and changed fungal communities structure over the longer term (2 years) even at low Ag levels and these changes might affect ecosystem processes. To assess fungal communities in soil, UPARSE pipeline run with USEARCH was found as a useful method to quickly process data obtained from the Illumina MiSeq sequencer.

In the future, more intensive study is needed when considering AgNPs to control plant diseases as they clearly have negative impacts on fungal soil communities. Broad antimicrobial activity of AgNPs has advantages because it can be used in many industries. At the same time these also the disadvantages of AgNPs as they affect non-target microorganisms. Future work should consider the effects of AgNPs on soil functional capability in long-term experiments.

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Appendix A: Fungal sequences from to Genevision (INEX Business Centre, Newcastle Upon Tyne, United Kingdom). The fungal were isolated from UK soil (Chapter 2)

Sample 1:

TCTGGGTACCTCCCACCCGTGTAATATTTACCTTGTTGCTTCGGCGAGCCTGCCTTTGGGGCCGGGG
GACGTCAGTCCCCGGGTCCGTGCTCGCCGAGAACCTTAAACTCTGTCTGAAGATTGTAGTCTGAG
ATTAATATAAATTATTTAAACTTTCAACAACGGATCTCTTGTTCCGGCATCGATGAAGAACGCAG
CGAAATGCGATACGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCG
CCCTCTGGTATTCCGGAGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGTT
GGGCCCCGTCTCTCCGGGGGACGGGTCCGAAAGGCAGCGGGCGGCACCCGCTCCGGTCTCAAG
CGTATTGGTCTTTGCACTCGCTTTGTAGGCCTGGCCGGCGCTTGCCGATCAACCAAACCTTTTATCA
GGTTGACCTCGGATCAGGTAGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAGGGGCG
GGGGCGGCGGGCCGGGCGCGGGGCGCGGGCGA

Sample 2:

AACCAATGTGAACGTTACCAAACCTGTTGCCTCGGCGGGATCTCTGCCCCGGGTGCGTCGCAGCCCC
GGACCAAGGCGCCCGGAGGACCAACCTAAACTCTTATTGTATACCCCTCGCGGGTTTTTTTAT
AATCTGAGCCTTTCTCGGCGCCTCTCGTAGGCGTTTCGAAAATGAATCAAACCTTTCAACAACGGATC
TCTTGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGT
GAATCATCGAATCTTTGAACGCACATTGCGCCCGCAGTATTCTGGCGGGCATGCCTGTCCGAGCGT
CATTCAACCCTCGAACCCCTCCGGGGGTGCGCGTTGGGGATCGGCCCTCCCTTAGCGGGTGGCCG
TCTCCGAAATACAGTGGCGGTCTCGCCGAGCCTCTCTGCGCAGTAGTTTGCACACTCGCATCGGG
AGCGCGGCGCGTCCACAGCCGTTAAACACCCAACCTTCTGAAATGTTGACCTCGGATCAGGTAGGAAT
ACCCGCTGAACTTAAGCATATCATAA

Sample 3:

TTAAAAGAATACTCTATGATAAGCGTGAACGAGAAAATTCCTTTTCTGGAGCACTCGGGCGAACA
AGAGGGTCCGCTCTCCGTCCGGCTCGCCCCCTTTTCCCCCCCCCTTGCTGGGGCACCTGGCTG
CGTCCCTACTGCTCCTTCTC

Sample 4:

TCCAACCAATGTGAACGTTACCAAACCTGTTGCCTCGGCGGGATCTCTGCCCCGGGTGCGTCGCAG
CCCCGGACCAAGGCGCCCGGAGGACCAACCAAACCTTATTGTATACCCCTCGCGGGTTTTTT
TATAATCTGAGCCTTCTCGGCGCCTCTCGTAGGCGTTTCGAAAATGAATCAAACCTTTCAACAACGGA
TCTCTTGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
GTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCAGTATTCTGGCGGGCATGCCTGTCCGAGC
GTCATTTCAACCCTCGAACCCCTCCGGGGGTGCGCGTTGGGGATCGGCCCTGCCTTGGCGGTGGCC
GTCTCCGAAATACAGTGGCGGTCTCGCCGAGCCTCTCTGCGCAGTAGTTTGCACACTCGCATCGG
GAGCGGCGCGTCCACAGCCGTTAAACACCCAACCTTCTGAAATGTTGACCTCGGATCAGGTAGGAA
TACCCGCTGAACTTAAGCATATCATAAGG

Sample 5:

TAATATACTACGGAGGTAGGGAGAAGAGAAAATGGGGAGGGTGGGCCGCGGCTGCCTTTGGGCCT
TGGGGGAGGGGGAAAGAGGGGGTGCGGAACGGCCCCCGTGGGGGCCACCCCCCGCCCCCG
GAGGGTTCGGGGTCAATGCGCGGCTCCCATGCCCCCGAAGGCTGGGGGCCAATGCGCGACAA
AGACTCTATGATTCACCGGATTTTGCATTCCCATTACTTCTCCGTTTTCGCTGCTTTCTTCATCCGTTCC
ATCACGTGTTGAGCCGTTTGTGAATGTTTTGTTCTTTTTCGATTGCCCCTTGATGACGGGACCTAG
AATGGGGTCCTCCCCTATTAACACGCATCTCTTAGGGACTGTATGTAACCATGGT

Sample 6:

TACTACGGAGGTAGGGAGAAGAGAAAATGGGGAGGGTGGGCCGCGGCTGCCTTTGGGCCTTGGGG
GAGGGGGAAAGAGGGGGTGCGGAACGGCCCCCGTGGGGGCCACCCCCCGCCCCCGGAGGG
TTCGGGGTCAATGCGCGGCTCCCATGCCCCCGAAGGCTGGGGGCCAATGCGCGACAAAGACTC
TATGATTCACCGGATTTTGCATTCCCATTACTTCTCCGTTTTCGCTGCTTTCTTCATCCGTTCCATCACG
TGTTGAGCCGTTTGTGAATGTTTTGTTCTTTTTCGATTGCCCCTTGATGACGGGACCTAGAATGGG
GTCCTCCCCTATTAACACGCATCTCTTAGGGACTGTATGTAACCAT

Appendix B: OTU tables of forward and reverse reads (Chapter 4)

OTU table of forward reads

OTU Id	soil 1	soil 2	soil 3	soil+Ag 1	soil+Ag 2	soil+Ag 3
OTU14;size=286;	179	48	118	0	0	0
OTU1;size=17239;	2670	3478	3629	2629	3681	2595
OTU3;size=3491;	1225	1644	1077	0	4	0
OTU6;size=2885;	2086	376	532	222	125	1
OTU5;size=2198;	386	297	259	449	577	524
OTU12;size=574;	98	213	129	148	45	15
OTU9;size=1573;	360	642	805	13	24	7
OTU11;size=559;	343	28	306	1	0	1
OTU4;size=4889;	216	392	350	1976	1651	792
OTU16;size=437;	58	165	282	33	5	2
OTU27;size=76;	14	23	31	11	5	6
OTU15;size=250;	86	30	8	123	47	4
OTU23;size=85;	61	10	39	0	1	0
OTU2;size=4444;	265	461	356	703	405	2678
OTU60;size=5;	7	0	0	0	0	0
OTU13;size=473;	11	40	39	83	267	112
OTU34;size=41;	14	19	12	4	0	2
OTU67;size=4;	4	0	3	0	0	0
OTU31;size=106;	66	27	49	0	2	0
OTU19;size=150;	68	76	38	1	0	0
OTU41;size=18;	9	11	8	0	0	0
OTU22;size=64;	7	0	0	10	33	34
OTU33;size=42;	26	0	24	0	0	0
OTU79;size=8;	4	0	0	8	0	0
OTU69;size=3;	2	0	1	1	1	0
OTU28;size=63;	23	13	38	1	6	4
OTU65;size=8;	11	0	0	0	0	0
OTU58;size=8;	14	0	1	0	0	0
OTU36;size=77;	50	9	40	0	3	0

continued

OTU table of forward reads - continued

OTU Id	soil 1	soil 2	soil 3	soil+Ag 1	soil+Ag 2	soil+Ag 3
OTU32;size=35;	18	35	8	0	0	0
OTU17;size=228;	12	107	13	42	58	53
OTU20;size=128;	14	70	83	1	2	0
OTU43;size=25;	6	15	7	5	0	2
OTU7;size=1504;	2	3	5	270	465	988
OTU40;size=33;	9	30	4	2	0	0
OTU78;size=3;	3	0	0	0	0	0
OTU61;size=12;	2	6	2	2	0	0
OTU35;size=36;	6	31	6	0	3	0
OTU55;size=15;	12	4	6	0	0	0
OTU24;size=81;	13	5	7	0	76	0
OTU72;size=8;	6	0	6	0	0	0
OTU52;size=13;	12	1	6	0	0	0
OTU90;size=2;	1	0	0	0	0	1
OTU100;size=2;	2	0	0	0	0	0
OTU106;size=2;	3	0	0	0	0	0
OTU76;size=7;	7	0	9	0	0	1
OTU49;size=7;	3	4	2	2	1	1
OTU89;size=4;	4	0	0	0	0	2
OTU29;size=51;	6	28	28	1	0	1
OTU93;size=2;	2	2	0	0	0	0
OTU88;size=2;	2	0	0	0	0	0
OTU59;size=9;	3	5	5	0	3	0
OTU73;size=3;	3	0	0	0	0	0
OTU83;size=6;	2	4	0	0	0	0
OTU8;size=866;	1	0	0	378	272	361
OTU39;size=40;	2	0	0	1	24	21
OTU21;size=94;	1	1	6	59	30	23
OTU51;size=16;	2	19	0	0	0	0
OTU56;size=8;	2	6	0	0	0	0

continued

OTU table of forward reads - continued

OTU Id	soil 1	soil 2	soil 3	soil+Ag 1	soil+Ag 2	soil+Ag 3
OTU77;size=3;	1	5	0	0	0	0
OTU50;size=20;	4	7	4	0	2	8
OTU101;size=2;	1	0	0	0	1	0
OTU98;size=2;	2	0	0	2	0	1
OTU70;size=8;	2	9	0	0	0	0
OTU38;size=39;	0	2	0	21	10	16
OTU57;size=17;	0	9	11	0	2	0
OTU81;size=5;	0	5	0	0	0	0
OTU92;size=4;	0	5	0	0	0	0
OTU64;size=11;	0	4	3	3	1	5
OTU47;size=14;	0	5	0	7	8	0
OTU62;size=5;	0	6	0	0	0	0
OTU37;size=39;	0	2	3	19	18	10
OTU42;size=14;	0	14	5	0	0	0
OTU71;size=3;	0	7	1	0	0	0
OTU84;size=2;	0	3	0	0	0	0
OTU91;size=2;	0	3	0	0	0	0
OTU68;size=4;	0	4	0	0	0	0
OTU54;size=6;	0	1	0	10	8	4
OTU63;size=9;	0	4	0	3	0	4
OTU111;size=2;	0	1	3	0	0	1
OTU80;size=5;	0	0	12	0	0	0
OTU26;size=75;	0	0	96	0	0	0
OTU87;size=2;	0	0	3	0	0	0
OTU96;size=2;	0	0	1	1	0	0
OTU109;size=2;	0	0	1	0	0	2
OTU66;size=4;	0	0	5	0	0	0
OTU74;size=3;	0	0	1	0	0	2
OTU86;size=4;	0	0	7	0	0	0
OTU10;size=593;	0	0	0	693	0	0

continued

OTU table of forward reads – continued

OTU Id	soil 1	soil 2	soil 3	soil+Ag 1	soil+Ag 2	soil+Ag 3
OTU18;size=289;	0	0	0	94	163	57
OTU75;size=5;	0	0	0	6	0	0
OTU48;size=10;	0	0	0	7	2	3
OTU44;size=13;	0	0	0	18	1	0
OTU45;size=24;	0	0	0	21	1	13
OTU25;size=62;	0	0	0	5	61	10
OTU104;size=2;	0	0	0	3	0	1
OTU53;size=8;	0	0	0	1	4	6
OTU99;size=2;	0	0	0	1	1	0
OTU95;size=2;	0	0	0	0	5	0
OTU30;size=42;	0	0	0	0	16	46
OTU82;size=3;	0	0	0	0	3	0
OTU107;size=2;	0	0	0	0	4	0
OTU94;size=2;	0	0	0	0	6	0
OTU105;size=2;	0	0	0	0	3	0
OTU110;size=2;	0	0	0	0	2	2
OTU102;size=2;	0	0	0	0	3	0
OTU103;size=2;	0	0	0	0	4	0
OTU108;size=2;	0	0	0	0	0	2
OTU85;size=2;	0	0	0	0	0	3
OTU97;size=2;	0	0	0	0	0	4

OTU table of reverse reads

OTU Id	soil 1	soil 2	soil 3	soil+Ag 1	soil+Ag 2	soil+Ag 3
OTU1;size=5366;	800	690	954	1140	1610	979
OTU22;size=16;	13	9	6	0	0	0
OTU2;size=2325;	849	1123	706	2	8	0
OTU4;size=1058;	185	81	110	346	270	318
OTU7;size=208;	36	66	213	12	19	18
OTU6;size=136;	124	3	32	28	5	0
OTU16;size=38;	66	0	0	21	0	0
OTU9;size=64;	3	5	6	18	54	15
OTU8;size=42;	4	0	0	9	17	31
OTU13;size=64;	17	23	17	28	6	5
OTU3;size=1119;	53	26	66	257	122	770
OTU19;size=8;	9	5	2	0	0	0
OTU20;size=6;	8	0	0	0	0	0
OTU26;size=3;	3	2	0	0	0	0
OTU14;size=30;	5	17	25	1	0	0
OTU32;size=2;	4	2	5	0	0	0
OTU34;size=2;	4	0	0	1	0	0
OTU36;size=2;	3	0	1	0	0	0
OTU31;size=2;	2	0	0	0	0	0
OTU29;size=3;	2	1	2	0	3	0
OTU18;size=13;	2	7	5	7	5	4
OTU35;size=2;	1	0	0	1	0	1
OTU11;size=130;	0	119	0	1	36	0
OTU24;size=8;	0	5	3	0	3	0
OTU28;size=3;	0	3	6	0	2	0
OTU25;size=5;	0	0	1	3	6	3
OTU17;size=8;	0	0	5	4	1	2
OTU21;size=8;	0	0	1	7	5	2
OTU5;size=241;	0	0	0	288	0	0
OTU33;size=2;	0	0	0	3	2	0
OTU10;size=36;	0	0	0	18	9	33

continued

OTU table of reverse reads - continued

OTU Id	soil 1	soil 2	soil 3	soil+Ag 1	soil+Ag 2	soil+Ag 3
OTU23;size=7;	0	0	0	2	19	0
OTU12;size=19;	0	0	0	1	7	24
OTU27;size=5;	0	0	0	4	0	3
OTU30;size=2;	0	0	0	2	3	1

Appendix C: The number of sequences of forward reads at the 12th and 24th month observation
(Chapter 4)

The number of sequences of single end forward reads from the 12th month observation.

Sample (s)	Number of sequences
Soil	1972
Soil	1423
Soil	1347
Soil + 100 mg kg ⁻¹ of AgNPs	4223
Soil + 100 mg kg ⁻¹ of AgNPs	2072
Soil + 100 mg kg ⁻¹ of AgNPs	1986
Soil + 100mg kg ⁻¹ of AgNO ₃	2020
Soil + 100mg kg ⁻¹ of AgNO ₃	1648
Soil + 100mg kg ⁻¹ of AgNO ₃	2314
Soil + T22	742
Soil + T22	647
Soil + T22	941
Soil + T22 + 3 mg kg ⁻¹ of AgNPs	1181
Soil + T22 + 3 mg kg ⁻¹ of AgNPs	1982
Soil + T22 + 3 mg kg ⁻¹ of AgNPs	1576
Soil + T22 + 100 mg kg ⁻¹ of AgNPs	1973
Soil + T22 + 100 mg kg ⁻¹ of AgNPs	1639
Soil + T22 + 100 mg kg ⁻¹ of AgNPs	3043
Soil + T22 + 660 mg kg ⁻¹ of AgNPs	2618
Soil + T22 + 660 mg kg ⁻¹ of AgNPs	4103
Soil + T22 + 660 mg kg ⁻¹ of AgNPs	3128
Soil + T22 + 3mg kg ⁻¹ of AgNO ₃	946
Soil + T22 + 3 mg kg ⁻¹ of AgNO ₃	1437
Soil + T22 + 3 mg kg ⁻¹ of AgNO ₃	1972
Soil + T22 + 100 mg kg ⁻¹ of AgNO ₃	2409
Soil + T22 + 100 mg kg ⁻¹ of AgNO ₃	2839
Soil + T22 + 100 mg kg ⁻¹ of AgNO ₃	2472
Soil + T22 + 660 mg kg ⁻¹ of AgNO ₃	3723
Soil + T22 + 660 mg kg ⁻¹ of AgNO ₃	816
Soil + T22 + 660 mg kg ⁻¹ of AgNO ₃	2215

The number of sequences of single end forward reads from the 24th month observation.

Sample (s)	Number of sequences
Soil	43305
Soil	73897
Soil	51821
Soil + 100 mg kg ⁻¹ of AgNPs	65120
Soil + 100 mg kg ⁻¹ of AgNPs	69060
Soil + 100 mg kg ⁻¹ of AgNPs	66040
Soil + 100mg kg ⁻¹ of AgNO ₃	50814
Soil + 100mg kg ⁻¹ of AgNO ₃	26845
Soil + 100mg kg ⁻¹ of AgNO ₃	29120
Soil + T22	72948
Soil + T22	42379
Soil + T22	61124
Soil + T22 + 3 mg kg ⁻¹ of AgNPs	223022
Soil + T22 + 3 mg kg ⁻¹ of AgNPs	45860
Soil + T22 + 3 mg kg ⁻¹ of AgNPs	79248
Soil + T22 + 100 mg kg ⁻¹ of AgNPs	93949
Soil + T22 + 100 mg kg ⁻¹ of AgNPs	43625
Soil + T22 + 100 mg kg ⁻¹ of AgNPs	95130
Soil + T22 + 660 mg kg ⁻¹ of AgNPs	108168
Soil + T22 + 660 mg kg ⁻¹ of AgNPs	78043
Soil + T22 + 660 mg kg ⁻¹ of AgNPs	70800
Soil + T22 + 3mg kg ⁻¹ of AgNO ₃	44100
Soil + T22 + 3 mg kg ⁻¹ of AgNO ₃	48366
Soil + T22 + 3 mg kg ⁻¹ of AgNO ₃	44569
Soil + T22 + 100 mg kg ⁻¹ of AgNO ₃	85784
Soil + T22 + 100 mg kg ⁻¹ of AgNO ₃	98608
Soil + T22 + 100 mg kg ⁻¹ of AgNO ₃	53698
Soil + T22 + 660 mg kg ⁻¹ of AgNO ₃	48219
Soil + T22 + 660 mg kg ⁻¹ of AgNO ₃	19000
Soil + T22 + 660 mg kg ⁻¹ of AgNO ₃	65684

Appendix D. Taxonomy assignment of forward reads (sample 40-54; 6th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database (Chapter 5).

OTU1; size=85 9;	k:Fungi(71.8),p:Ascomycota(37.4),c:Sordariomycetes(37.4),o:Hypocreales(37.4),f:Hypocreaceae(37.4),g:unidentified(35.9),s:Hypocreaceae_sp(35.9)
OTU2; size=69 3;	k:Fungi(85.2),p:Basidiomycota(65.0),c:Agaricomycetes(65.0),o:Agaricales(65.0),f:Entolomataceae(49.2),g:Clitopilus(49.2),s:Clitopilus_sp(49.2)
OTU3; size=11 37;	k:Fungi(63.0),p:Basidiomycota(63.0),c:Tremellomycetes(63.0),o:Filobasidiales(41.0),f:Filobasidiaceae(41.0),g:Cryptococcus(41.0),s:Cryptococcus_terreus(41.0)
OTU4; size=54 9;	k:Fungi(13.4),p:unidentified(6.9),c:unidentified(6.9),o:unidentified(6.9),f:unidentified(6.9),g:unidentified(6.9),s:Fungi_sp(6.9)
OTU5; size=29 7;	k:Fungi(65.5),p:Ascomycota(65.5),c:Eurotiomycetes(36.0),o:Eurotiales(36.0),f:Trichocomaceae(36.0),g:unidentified(36.0),s:Trichocomaceae_sp(36.0)
OTU7; size=17 3;	k:Fungi(40.9),p:Zygomycota(40.9),c:Incertae_sedis(40.9),o:Mucorales(40.9),f:Umbelopsidaceae(40.9),g:Umbelopsis(40.9),s:Umbelopsis_ramanniana_var_angulispora(26.4)
OTU6; size=16 8;	k:Fungi(63.0),p:Ascomycota(63.0),c:Eurotiomycetes(42.6),o:Eurotiales(42.6),f:Trichocomaceae(42.6),g:Penicillium(42.6),s:Penicillium_sp(36.0)
OTU8; size=24 0;	k:Fungi(59.7),p:Ascomycota(59.7),c:Dothideomycetes(38.4),o:Incertae_sedis(38.4),f:Pseudeurotiaceae(38.4),g:Pseudeurotium(38.4),s:Pseudeurotium_sp(31.5)
OTU9; size=74 ;	k:Fungi(9.0),p:Ascomycota(4.9),c:Leotiomycetes(4.9),o:Rhytismatales(4.9),f:Rhytismataceae(4.9),g:unidentified(4.9),s:Rhytismataceae_sp(4.9)
OTU11 ;size=3 7;	k:Fungi(7.9),p:Ascomycota(4.0),c:Lecanoromycetes(4.0),o:Lecanorales(4.0),f:Parmeliaceae(4.0),g:Parmelina(4.0),s:Parmelina_sp_PN_2010(4.0)

continued

Taxonomy assignment of forward reads (sample 40-54; 6th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database – continued

OTU10 ;size=2 34;	k:Fungi(10.1),p:Basidiomycota(5.5),c:Agaricomycetes(5.5),o:Atheliales(5.3),f:Atheliaceae(5.3),g:unidentified(5.3),s:Atheliaceae_sp(5.3)
OTU13 ;size=2 9;	k:Fungi(65.3),p:Basidiomycota(61.3),c:Tremellomycetes(60.0),o:Filobasidiales(58.2),f:Filobasidiaceae(58.2),g:Cryptococcus(58.2),s:Cryptococcus_terricola(36.0)
OTU14 ;size=3 3;	k:Fungi(57.0),p:Ascomycota(57.0),c:Eurotiomycetes(40.5),o:Eurotiales(40.5),f:Trichocomaceae(40.5),g:Penicillium(40.5),s:Penicillium_atrovenetum(30.6)
OTU15 ;size=4 9;	k:Fungi(53.6),p:Ascomycota(53.6),c:Pezizomycetes(53.6),o:Pezizales(39.3),f:Pyronemataceae(39.3),g:Scutellinia(39.3),s:Scutellinia_sp(37.2)
OTU16 ;size=2 5;	k:Fungi(43.5),p:Ascomycota(39.3),c:Pezizomycetes(39.1),o:Pezizales(39.1),f:Pezizaceae(39.1),g:unidentified(39.1),s:Pezizaceae_sp(39.1)
OTU18 ;size=3 2;	k:Fungi(58.0),p:Ascomycota(58.0),c:unidentified(31.7),o:unidentified(31.7),f:unidentified(31.7),g:unidentified(31.7),s:Ascomycota_sp(31.7)
OTU17 ;size=2 7;	k:Fungi(45.8),p:Ascomycota(45.8),c:unidentified(26.1),o:unidentified(26.1),f:unidentified(26.1),g:unidentified(26.1),s:Ascomycota_sp(26.1)
OTU19 ;size=1 7;	k:Fungi(69.7),p:Ascomycota(38.9),c:Sordariomycetes(38.9),o:Hypocreales(38.9),f:Hypocreaceae(38.9),g:Trichoderma(35.3),s:Trichoderma_spirale(35.3)
OTU21 ;size=1 1;	k:Fungi(58.2),p:Zygomycota(58.2),c:Incertae_sedis(58.2),o:Mucorales(58.2),f:Umbelopsidaceae(58.2),g:Umbelopsis(58.2),s:Umbelopsis_ramanniana(29.7)
OTU20 ;size=1 4;	k:Fungi(57.3),p:Ascomycota(48.1),c:Dothideomycetes(46.2),o:Incertae_sedis(46.2),f:Myxotrichaceae(46.2),g:Oidiodendron(45.6),s:Oidiodendron_truncatum(35.1)

continued

Taxonomy assignment of forward reads (sample 40-54; 6th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database – continued

OTU22 ;size=25;	k:Fungi(9.7),p:Basidiomycota(5.5),c:Agaricomycetes(5.5),o:Agaricales(5.0),f:Tricholomataceae(5.0),g:Clitocybe(5.0),s:Clitocybe_sp(5.0)
OTU23 ;size=20;	k:Fungi(53.8),p:Ascomycota(48.7),c:Saccharomycetes(48.7),o:Saccharomycetales(48.7),f:Incertae_sedis(48.7),g:unidentified(48.7),s:Saccharomycetales_sp(48.7)
OTU12 ;size=101;	k:Fungi(41.8),p:Ascomycota(28.0),c:Leotiomyces(26.8),o:Incertae_sedis(21.3),f:Incertae_sedis(21.3),g:Leohumicola(21.3),s:Leohumicola_minima(21.3)
OTU24 ;size=13;	k:Fungi(3.8),p:Ascomycota(1.9),c:Lecanoromycetes(1.9),o:Teloschistales(1.9),f:Physciaceae(1.9),g:Physcia(1.9),s:Physcia_magnussonii(1.9)
OTU25 ;size=37;	k:Fungi(70.6),p:Ascomycota(62.4),c:Pezizomycetes(62.4),o:unidentified(56.3),f:unidentified(56.3),g:unidentified(56.3),s:Pezizomycetes_sp(56.3)
OTU27 ;size=15;	k:Fungi(4.7),p:Ascomycota(2.8),c:Lecanoromycetes(2.8),o:Lecanorales(2.5),f:Parmeliaceae(2.5),g:Tuckermannopsis(2.5),s:Tuckermannopsis_platyphylla(2.5)
OTU26 ;size=11;	k:Fungi(53.4),p:Ascomycota(53.4),c:Sordariomycetes(37.8),o:Sordariales(37.8),f:Chaetomiaceae(37.2),g:Humicola(29.2),s:Humicola_sp_JZ_115(29.2)
OTU29 ;size=6;	k:Fungi(59.6),p:Ascomycota(59.6),c:Sordariomycetes(30.9),o:Sordariales(30.9),f:Chaetomiaceae(30.9),g:Chaetomium(30.9),s:Chaetomium_globosum(30.9)
OTU28 ;size=10;	k:Fungi(47.3),p:Ascomycota(32.8),c:Leotiomyces(32.6),o:Helotiales(25.7),f:unidentified(25.7),g:unidentified(25.7),s:Helotiales_sp(25.7)
OTU33 ;size=5;	k:Fungi(22.3),p:Ascomycota(18.3),c:Pezizomycetes(16.2),o:Pezizales(16.2),f:Pezizaceae(16.2),g:unidentified(16.2),s:Pezizaceae_sp(16.2)
OTU30 ;size=10;	k:Fungi(5.6),p:Ascomycota(3.0),c:Sordariomycetes(3.0),o:Diaporthales(3.0),f:Diaporthaceae(3.0),g:unidentified(3.0),s:Diaporthaceae_sp(3.0)

continued

Taxonomy assignment of forward reads (sample 40-54; 6th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database – continued

OTU36 ;size=7;	k:Fungi(55.6),p:Zygomycota(55.6),c:Incertae_sedis(55.6),o:Mortierellales(55.6),f:unidentified(41.6),g:unidentified(41.6),s:Mortierellales_sp(41.6)
OTU35 ;size=4;	k:Fungi(48.5),p:Ascomycota(48.5),c:Leotiomyces(48.5),o:unidentified(37.6),f:unidentified(37.6),g:unidentified(37.6),s:Leotiomyces_sp(37.6)
OTU34 ;size=5;	k:Fungi(77.1),p:Basidiomycota(70.8),c:Agaricomycetes(70.8),o:Corticiales(68.2),f:unidentified(68.2),g:unidentified(68.2),s:Corticiales_sp(68.2)
OTU37 ;size=6;	k:Fungi(67.1),p:Ascomycota(67.1),c:Eurotiomyces(49.2),o:Eurotiales(49.2),f:Trichocomaceae(49.2),g:Penicillium(49.2),s:Penicillium_sp(46.0)
OTU40 ;size=3;	k:Fungi(46.8),p:Ascomycota(46.8),c:Saccharomycetes(46.8),o:Saccharomycetales(46.8),f:Incertae_sedis(29.3),g:Schwanniomyces(29.3),s:Schwanniomyces_occidentalis(27.2)
OTU39 ;size=6;	k:Fungi(54.1),p:Ascomycota(50.2),c:unidentified(44.4),o:unidentified(44.4),f:unidentified(44.4),g:unidentified(44.4),s:Ascomycota_sp(44.4)
OTU41 ;size=5;	k:Fungi(17.2),p:Ascomycota(17.2),c:Pezizomycetes(12.1),o:Pezizales(12.1),f:Pyronemataceae(11.7),g:unidentified(9.0),s:Pyronemataceae_sp(9.0)
OTU38 ;size=8;	k:Fungi(21.3),p:Basidiomycota(16.5),c:Agaricomycetes(16.5),o:Russulales(12.3),f:Stephanosporaceae(12.3),g:Cristinia(11.7),s:Cristinia_helvetica(11.7)
OTU43 ;size=3;	k:Fungi(86.6),p:Ascomycota(72.1),c:Dothideomycetes(72.1),o:Pleosporales(72.1),f:Pleosporaceae(72.1),g:Drechslera(70.6),s:Drechslera_sp_BAFC_3419(68.5)
OTU42 ;size=5;	k:Fungi(47.9),p:Ascomycota(37.2),c:Eurotiomyces(36.8),o:Eurotiales(36.8),f:Trichocomaceae(35.9),g:unidentified(30.7),s:Trichocomaceae_sp(30.7)
OTU45 ;size=3;	k:Fungi(90.8),p:Basidiomycota(53.8),c:Agaricomycetes(53.8),o:Agaricales(53.8),f:Entolomataceae(53.8),g:Entoloma(53.8),s:Entoloma_conferendum(53.8)
OTU47 ;size=5;	k:Fungi(78.9),p:Basidiomycota(63.5),c:Agaricomycetes(63.5),o:Agaricales(63.5),f:Entolomataceae(63.5),g:Entoloma(63.5),s:Entoloma_sacchariolens(62.0)
OTU46 ;size=3;	k:Fungi(28.7),p:Basidiomycota(22.4),c:Agaricomycetes(22.4),o:Agaricales(20.3),f:Psathyrellaceae(18.6),g:Psathyrella(17.7),s:Psathyrella_friesii(17.7)
OTU44 ;size=3;	k:Fungi(73.8),p:Basidiomycota(61.7),c:Agaricomycetes(61.7),o:Agaricales(61.7),f:Entolomataceae(61.7),g:Entoloma(61.5),s:Entoloma_serrulatum(61.5)
OTU48 ;size=3;	k:Fungi(65.4),p:Basidiomycota(51.7),c:Agaricomycetes(51.7),o:Agaricales(51.7),f:Lyophyllaceae(48.5),g:Tephrocybe(44.3),s:Tephrocybe_gibberosa(44.3)

continued

Taxonomy assignment of forward reads (sample 40-54; 6th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database – continued

OTU49 ;size=5;	k:Fungi(19.7),p:Basidiomycota(11.5),c:Microbotryomycetes(11.5),o:Leucosporidiales(10.7),f:Leucosporidiaceae(10.7),g:Leucosporidium(10.7),s:Leucosporidiu m_escuderoi(10.5)
OTU52 ;size=2;	k:Fungi(46.2),p:Ascomycota(37.4),c:Sordariomycetes(35.9),o:Microascales(35.9),f:Microascaceae(35.9),g:Scedosporium(33.6),s:Scedosporium_prolificans(33.6)
OTU50 ;size=3;	k:Fungi(7.4),p:Ascomycota(3.9),c:Pezizomycetes(3.7),o:unidentified(3.7),f:unidentified(3.7),g:unidentified(3.7),s:Pezizomycetes_sp(3.7)
OTU53 ;size=2;	k:Fungi(51.5),p:Ascomycota(43.9),c:Sordariomycetes(42.1),o:Sordariales(42.1),f:Lasiosphaeriaceae(42.1),g:unidentified(39.1),s:Lasiosphaeriaceae_sp(39.1)
OTU54 ;size=2;	k:Fungi(49.0),p:Ascomycota(49.0),c:Leotiomycetes(41.2),o:Helotiales(38.3),f:unidentified(38.3),g:unidentified(38.3),s:Helotiales_sp(38.3)
OTU56 ;size=2;	k:Fungi(41.8),p:Ascomycota(41.8),c:Pezizomycetes(32.4),o:Pezizales(32.4),f:Pyronemataceae(32.4),g:unidentified(32.4),s:Pyronemataceae_sp(32.4)
OTU57 ;size=2;	k:Fungi(34.5),p:Ascomycota(34.5),c:Sordariomycetes(20.6),o:Coniochaetales(17.9),f:Coniochaetaceae(17.9),g:Lecythophora(17.2),s:Lecythophora_sp(17.2)
OTU59 ;size=2;	k:Fungi(52.7),p:Ascomycota(41.8),c:Eurotiomycetes(30.1),o:Chaetothyriales(30.1),f:Herpotrichiellaceae(30.1),g:Exophiala(30.1),s:Exophiala_equina(28.0)
OTU60 ;size=2;	k:Fungi(48.7),p:Zygomycota(39.5),c:Incertae_sedis(39.5),o:Mortierellales(39.5),f:Mortierellaceae(39.5),g:Mortierella(39.5),s:Mortierella_amoeboidea(26.5)
OTU58 ;size=2;	k:Fungi(63.9),p:Ascomycota(50.8),c:unidentified(49.6),o:unidentified(49.6),f:unidentified(49.6),g:unidentified(49.6),s:Ascomycota_sp(49.6)
OTU61 ;size=2;	k:Fungi(46.6),p:Ascomycota(36.9),c:Dothideomycetes(35.8),o:Capnodiales(34.3),f:Teratosphaeriaceae(34.3),g:Devriesia(34.3),s:Devriesia_sp(34.3)
OTU62 ;size=2;	k:Fungi(85.4),p:Basidiomycota(72.2),c:Agaricomycetes(72.2),o:Agaricales(72.2),f:Entolomataceae(72.2),g:Entoloma(72.2),s:Entoloma_clandestinum(46.2)
OTU63 ;size=2;	k:Fungi(72.4),p:Basidiomycota(56.6),c:Agaricomycetes(56.6),o:Agaricales(56.6),f:Psathyrellaceae(53.7),g:unidentified(43.0),s:Psathyrellaceae_sp(43.0)
OTU51 ;size=2;	k:Fungi(43.0),p:Basidiomycota(43.0),c:Agaricomycetes(43.0),o:Trechisporales(43.0),f:Hydnodontaceae(30.0),g:Trechispora(30.0),s:Trechispora_sp(30.0)
OTU64 ;size=2;	k:Fungi(10.1),p:Basidiomycota(5.5),c:Agaricomycetes(5.5),o:Atheliales(5.3),f:Atheliaceae(5.3),g:unidentified(5.3),s:Atheliaceae_sp(5.3)

Taxonomy assignment of forward reads (sample 55-70; 6th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database.

OTU2;size =1323;	k:Fungi(71.8),p:Ascomycota(37.4),c:Sordariomycetes(37.4),o:Hypocreales(37.4),f:Hypocreaceae(37.4),g:unidentified(35.9),s:Hypocreaceae_sp(35.9)
OTU1;size =1106;	k:Fungi(85.2),p:Basidiomycota(65.0),c:Agaricomycetes(65.0),o:Agaricales(65.0),f:Entolomataceae(49.2),g:Clitopilus(49.2),s:Clitopilus_sp(49.2)
OTU3;size =1121;	k:Fungi(65.5),p:Ascomycota(65.5),c:Eurotiomycetes(36.0),o:Eurotiales(36.0),f:Trichocomaceae(36.0),g:unidentified(36.0),s:Trichocomaceae_sp(36.0)
OTU4;size =1535;	k:Fungi(63.0),p:Basidiomycota(63.0),c:Tremellomycetes(63.0),o:Filobasidiales(41.0),f:Filobasidiaceae(41.0),g:Cryptococcus(41.0),s:Cryptococcus_terreus(41.0)
OTU5;size =655;	k:Fungi(77.6),p:Basidiomycota(77.6),c:Agaricomycetes(77.6),o:Agaricales(69.2),f:Strophariaceae(48.7),g:Gymnopilus(48.7),s:Gymnopilus_junonius(48.7)
OTU7;size =208;	k:Fungi(65.3),p:Basidiomycota(61.3),c:Tremellomycetes(60.0),o:Filobasidiales(58.2),f:Filobasidiaceae(58.2),g:Cryptococcus(58.2),s:Cryptococcus_terricola(36.0)
OTU8;size =122;	k:Fungi(13.4),p:unidentified(6.9),c:unidentified(6.9),o:unidentified(6.9),f:unidentified(6.9),g:unidentified(6.9),s:Fungi_sp(6.9)
OTU10;size =211;	k:Fungi(76.7),p:Basidiomycota(76.7),c:Agaricomycetes(76.7),o:Cantharellales(76.7),f:Ceratobasidiaceae(76.7),g:unidentified(44.7),s:Ceratobasidiaceae_sp(44.7)
OTU9;size =140;	k:Fungi(57.6),p:Ascomycota(48.3),c:Dothideomycetes(46.4),o:Incertae_sedis(46.4),f:Myxotrichaceae(46.4),g:Oidiodendron(45.8),s:Oidiodendron_truncatum(35.3)
OTU11;size =84;	k:Fungi(63.9),p:Ascomycota(50.8),c:unidentified(49.6),o:unidentified(49.6),f:unidentified(49.6),g:unidentified(49.6),s:Ascomycota_sp(49.6)
OTU12;size =162;	k:Fungi(10.1),p:Basidiomycota(5.5),c:Agaricomycetes(5.5),o:Atheliales(5.3),f:Atheliaceae(5.3),g:unidentified(5.3),s:Atheliaceae_sp(5.3)
OTU13;size =47;	k:Fungi(59.0),p:Ascomycota(59.0),c:Leotiomycetes(46.7),o:Helotiales(46.7),f:unidentified(36.8),g:unidentified(36.8),s:Helotiales_sp(36.8)
OTU14;size =69;	k:Fungi(17.2),p:Ascomycota(17.2),c:Pezizomycetes(12.1),o:Pezizales(12.1),f:Pyronemataceae(11.7),g:unidentified(9.0),s:Pyronemataceae_sp(9.0)
OTU15;size =107;	k:Fungi(59.7),p:Ascomycota(59.7),c:Dothideomycetes(38.4),o:Incertae_sedis(38.4),f:Pseudeurotiaceae(38.4),g:Pseudeurotium(38.4),s:Pseudeurotium_sp(31.5)
OTU17;size =29;	k:Fungi(43.5),p:Ascomycota(39.3),c:Pezizomycetes(39.1),o:Pezizales(39.1),f:Pezizaceae(39.1),g:unidentified(39.1),s:Pezizaceae_sp(39.1)

continued

Taxonomy assignment of forward reads (sample 55-70; 6th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database - continued

OTU16;size=56;	k:Fungi(58.0),p:Ascomycota(58.0),c:unidentified(31.7),o:unidentified(31.7),f:unidentified(31.7),g:unidentified(31.7),s:Ascomycota_sp(31.7)
OTU18;size=33;	k:Fungi(9.0),p:Ascomycota(4.9),c:Leotiomyces(4.9),o:Rhytismatales(4.9),f:Rhytismataceae(4.9),g:unidentified(4.9),s:Rhytismataceae_sp(4.9)
OTU19;size=23;	k:Fungi(69.7),p:Ascomycota(38.9),c:Sordariomycetes(38.9),o:Hypocreales(38.9),f:Hypocreaceae(38.9),g:Trichoderma(35.3),s:Trichoderma_spirale(35.3)
OTU20;size=28;	k:Fungi(45.8),p:Ascomycota(45.8),c:unidentified(26.1),o:unidentified(26.1),f:unidentified(26.1),g:unidentified(26.1),s:Ascomycota_sp(26.1)
OTU22;size=29;	k:Fungi(56.9),p:Zygomycota(56.9),c:Incertae_sedis(56.9),o:Mortierellales(56.9),f:Mortierellaceae(30.5),g:Mortierella(30.5),s:Mortierella_minutissima(28.5)
OTU23;size=21;	k:Fungi(7.9),p:Ascomycota(4.0),c:Lecanoromycetes(4.0),o:Lecanorales(4.0),f:Parmeliaceae(4.0),g:Parmelina(4.0),s:Parmelina_sp_PN_2010(4.0)
OTU6;size=757;	k:Fungi(64.3),p:Ascomycota(64.3),c:Eurotiomycetes(43.5),o:Eurotiales(43.5),f:Trichocomaceae(43.5),g:Penicillium(43.5),s:Penicillium_sp(36.8)
OTU24;size=30;	k:Fungi(40.9),p:Zygomycota(40.9),c:Incertae_sedis(40.9),o:Mucorales(40.9),f:Umbelopsidaceae(40.9),g:Umbelopsis(40.9),s:Umbelopsis_ramanniana_var_angulispora(26.4)
OTU25;size=30;	k:Fungi(53.8),p:Ascomycota(48.7),c:Saccharomycetes(48.7),o:Saccharomycetales(48.7),f:Incertae_sedis(48.7),g:unidentified(48.7),s:Saccharomycetales_sp(48.7)
OTU26;size=27;	k:Fungi(20.1),p:Zygomycota(10.0),c:Incertae_sedis(10.0),o:Mortierellales(10.0),f:Mortierellaceae(10.0),g:Mortierella(10.0),s:Mortierella_acrotona(10.0)
OTU28;size=55;	k:Fungi(60.3),p:Ascomycota(52.5),c:Pezizomycetes(52.5),o:unidentified(44.4),f:unidentified(44.4),g:unidentified(44.4),s:Pezizomycetes_sp(44.4)
OTU27;size=28;	k:Fungi(50.2),p:Basidiomycota(50.2),c:Microbotryomycetes(50.2),o:Sporidiobolales(50.2),f:unidentified(31.2),g:unidentified(31.2),s:Sporidiobolales_sp(31.2)
OTU29;size=38;	k:Fungi(42.2),p:Ascomycota(28.3),c:Leotiomyces(27.0),o:Incertae_sedis(21.5),f:Incertae_sedis(21.5),g:Leohumicola(21.5),s:Leohumicola_minima(21.5)
OTU31;size=19;	k:Fungi(57.0),p:Ascomycota(57.0),c:Eurotiomycetes(40.5),o:Eurotiales(40.5),f:Trichocomaceae(40.5),g:Penicillium(40.5),s:Penicillium_atrovenetum(30.6)
OTU30;size=17;	k:Fungi(43.5),p:Ascomycota(43.5),c:Leotiomyces(32.0),o:Helotiales(32.0),f:Incertae_sedis(24.1),g:Scytalidium(24.1),s:Scytalidium_sp(24.1)

continued

Taxonomy assignment of forward reads (sample 55-70; 6th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database - continued

OTU32;size=12;	k:Fungi(39.7),p:Basidiomycota(39.7),c:Tremellomycetes(39.7),o:Tremellales(23.0),f:Incertae_sedis(23.0),g:Cryptococcus(23.0),s:Cryptococcus_aerius(21.5)
OTU21;size=25;	k:Fungi(55.9),p:Zygomycota(55.9),c:Incertae_sedis(55.9),o:Mortierellales(55.9),f:unidentified(41.8),g:unidentified(41.8),s:Mortierellales_sp(41.8)
OTU34;size=20;	k:Fungi(9.7),p:Basidiomycota(5.7),c:Agaricomycetes(5.5),o:Agaricales(5.0),f:Tricholomataceae(5.0),g:Clitocybe(5.0),s:Clitocybe_sp(5.0)
OTU36;size=11;	k:Fungi(48.5),p:Ascomycota(48.5),c:Leotiomyces(48.5),o:unidentified(37.6),f:unidentified(37.6),g:unidentified(37.6),s:Leotiomyces_sp(37.6)
OTU33;size=14;	k:Fungi(4.5),p:Basidiomycota(2.5),c:Agaricomycetes(2.5),o:Hymenochaetales(2.5),f:Hymenochaetaceae(2.5),g:Pseudochaete(2.5),s:Pseudochaete_rigidula(2.5)
OTU37;size=12;	k:Fungi(36.7),p:Basidiomycota(36.7),c:Agaricomycetes(36.7),o:Agaricales(36.7),f:Clavariaceae(36.7),g:Ramariopsis(21.1),s:Ramariopsis_fusififormis(21.1)
OTU35;size=24;	k:Fungi(10.7),p:Ascomycota(6.2),c:Leotiomyces(6.2),o:Rhytismatales(6.0),f:Rhytismataceae(6.0),g:Lophodermium(5.6),s:Lophodermium_australe(5.6)
OTU38;size=9;	k:Fungi(62.3),p:Ascomycota(55.9),c:Eurotiomyces(54.1),o:Eurotiales(51.4),f:Trichocomaceae(51.4),g:Talaromyces(51.2),s:Talaromyces_sp(51.2)
OTU40;size=15;	k:Fungi(21.3),p:Basidiomycota(16.5),c:Agaricomycetes(16.5),o:Russulales(12.3),f:Stephanosporaceae(12.3),g:Cristinia(11.7),s:Cristinia_helvetica(11.7)
OTU41;size=10;	k:Fungi(3.8),p:Ascomycota(1.9),c:Lecanoromycetes(1.9),o:Teloschistales(1.9),f:Physciaceae(1.9),g:Physcia(1.9),s:Physcia_magnussonii(1.9)
OTU43;size=22;	k:Fungi(68.6),p:Basidiomycota(57.2),c:Microbotryomycetes(57.2),o:Sporidiobolales(55.2),f:unidentified(55.2),g:unidentified(55.2),s:Sporidiobolales_sp(55.2)
OTU42;size=31;	k:Fungi(10.1),p:Basidiomycota(5.5),c:Agaricomycetes(5.5),o:Atheliales(5.3),f:Atheliaceae(5.3),g:unidentified(5.3),s:Atheliaceae_sp(5.3)
OTU44;size=17;	k:Fungi(47.3),p:Ascomycota(32.8),c:Leotiomyces(32.6),o:Helotiales(25.7),f:unidentified(25.7),g:unidentified(25.7),s:Helotiales_sp(25.7)
OTU46;size=12;	k:Fungi(5.6),p:Ascomycota(3.0),c:Sordariomyces(3.0),o:Diaporthales(3.0),f:Diaporthaceae(3.0),g:unidentified(3.0),s:Diaporthaceae_sp(3.0)
OTU45;size=11;	k:Fungi(42.6),p:Ascomycota(42.6),c:Eurotiomyces(42.6),o:Eurotiales(26.8),f:Trichocomaceae(26.8),g:unidentified(22.1),s:Trichocomaceae_sp(22.1)

continued

Taxonomy assignment of forward reads (sample 55-70; 6th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database - continued

OTU47;size=5;	k:Fungi(6.4),p:Ascomycota(3.8),c:Leotiomycetes(3.4),o:Helotiales(3.4),f:unidentified(3.4),g:unidentified(3.4),s:Helotiales_sp(3.4)
OTU48;size=13;	k:Fungi(22.8),p:Ascomycota(14.3),c:Leotiomycetes(13.9),o:Helotiales(12.7),f:unidentified(12.7),g:unidentified(12.7),s:Helotiales_sp(12.7)
OTU49;size=5;	k:Fungi(53.8),p:Ascomycota(49.6),c:unidentified(43.7),o:unidentified(43.7),f:unidentified(43.7),g:unidentified(43.7),s:Ascomycota_sp(43.7)
OTU50;size=7;	k:Fungi(48.7),p:Zygomycota(39.5),c:Incertae_sedis(39.5),o:Mortierellales(39.5),f:Mortierellaceae(39.5),g:Mortierella(39.5),s:Mortierella_amoeboidea(26.5)
OTU52;size=18;	k:Fungi(4.7),p:Ascomycota(3.0),c:Lecanoromycetes(2.8),o:Lecanorales(2.5),f:Parmeliaceae(2.5),g:Tuckermannopsis(2.5),s:Tuckermannopsis_platyphylla(2.5)
OTU51;size=4;	k:Fungi(35.9),p:Zygomycota(19.0),c:Incertae_sedis(19.0),o:Mortierellales(19.0),f:Mortierellaceae(19.0),g:Mortierella(19.0),s:Mortierella_exigua(18.6)
OTU53;size=4;	k:Fungi(46.6),p:Ascomycota(36.9),c:Dothideomycetes(35.8),o:Capnodiales(34.3),f:Teratosphaeriaceae(34.3),g:Devriesia(34.3),s:Devriesia_sp(34.3)
OTU55;size=4;	k:Fungi(3.8),p:Ascomycota(2.1),c:Sordariomycetes(1.9),o:Xylariales(1.9),f:Amphisphaeriaceae(1.9),g:Seimatosporium(1.9),s:Seimatosporium_sp(1.9)
OTU54;size=4;	k:Fungi(46.0),p:Ascomycota(37.2),c:Sordariomycetes(35.8),o:Microascales(35.8),f:Microascaceae(35.8),g:Scedosporium(33.5),s:Scedosporium_prolificans(33.5)
OTU56;size=6;	k:Fungi(60.7),p:Basidiomycota(60.7),c:Agaricomycetes(60.7),o:Agaricales(60.7),f:Clavariaceae(60.7),g:Clavaria(46.4),s:Clavaria_sp(46.4)
OTU57;size=4;	k:Fungi(17.2),p:Basidiomycota(12.9),c:Agaricomycetes(12.9),o:Trechisporales(12.9),f:Hydnodontaceae(10.1),g:Trechispora(10.1),s:Trechispora_sp(9.4)
OTU58;size=3;	k:Fungi(90.8),p:Basidiomycota(53.8),c:Agaricomycetes(53.8),o:Agaricales(53.8),f:Entolomataceae(53.8),g:Entoloma(53.8),s:Entoloma_conferendum(53.8)
OTU59;size=3;	k:Fungi(46.9),p:Ascomycota(36.4),c:Eurotiomycetes(36.0),o:Eurotiales(36.0),f:Trichocomaceae(35.2),g:unidentified(30.0),s:Trichocomaceae_sp(30.0)
OTU39;size=7;	k:Fungi(48.1),p:Zygomycota(39.7),c:Incertae_sedis(39.7),o:Mortierellales(39.7),f:Mortierellaceae(27.6),g:Mortierella(27.6),s:Mortierella_humilis(26.2)
OTU61;size=3;	k:Fungi(59.6),p:Ascomycota(59.6),c:Sordariomycetes(30.9),o:Sordariales(30.9),f:Chaetomiaceae(30.9),g:Chaetomium(30.9),s:Chaetomium_globosum(30.9)

continued

Taxonomy assignment of forward reads (sample 55-70; 6th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database – continued

OTU63;size=5;	k:Fungi(46.8),p:Ascomycota(46.8),c:Saccharomycetes(46.8),o:Saccharomycetales(46.8),f:Incertae_sedis(29.3),g:Schwanniomyces(29.3),s:Schwanniomyces_occidentalis(27.2)
OTU60;size=3;	k:Fungi(6.5),p:Ascomycota(3.7),c:Leotiomyces(3.5),o:unidentified(3.5),f:unidentified(3.5),g:unidentified(3.5),s:Leotiomyces_sp(3.5)
OTU65;size=2;	k:Fungi(27.7),p:Ascomycota(27.7),c:Archaeorhizomycetes(27.7),o:Archaeorhizomycetales(18.7),f:Archaeorhizomycetaceae(18.7),g:Archaeorhizomyces(18.7),s:Archaeorhizomyces_sp(18.7)
OTU66;size=12;	k:Fungi(8.6),p:unidentified(4.3),c:unidentified(4.3),o:unidentified(4.3),f:unidentified(4.3),g:unidentified(4.3),s:Fungi_sp(4.3)
OTU68;size=2;	k:Fungi(44.7),p:Ascomycota(35.7),c:Dothideomycetes(34.2),o:Pleosporales(30.5),f:Sporormiaceae(30.5),g:Preussia(30.5),s:Preussia_flanaganii(30.5)
OTU67;size=2;	k:Fungi(35.3),p:Basidiomycota(31.3),c:Tremellomycetes(31.1),o:Tremellales(18.1),f:Incertae_sedis(18.1),g:Cryptococcus(18.1),s:Cryptococcus_podzolicus(18.1)
OTU69;size=2;	k:Fungi(9.8),p:Ascomycota(6.1),c:Leotiomyces(5.9),o:Rhytismatales(5.9),f:Rhytismataceae(5.9),g:Coccomyces(5.7),s:Coccomyces_dentatus(5.7)
OTU70;size=6;	k:Fungi(41.8),p:Ascomycota(41.8),c:Pezizomycetes(32.4),o:Pezizales(32.4),f:Pyronemataceae(32.4),g:unidentified(32.4),s:Pyronemataceae_sp(32.4)
OTU71;size=2;	k:Fungi(27.0),p:unidentified(23.0),c:unidentified(23.0),o:unidentified(23.0),f:unidentified(23.0),g:unidentified(23.0),s:Fungi_sp(23.0)
OTU72;size=6;	k:Fungi(20.7),p:Basidiomycota(16.2),c:Microbotryomycetes(14.1),o:Sporidiobolales(14.1),f:Incertae_sedis(14.1),g:Rhodotorula(14.1),s:Rhodotorula_ferulica(14.1)
OTU73;size=2;	k:Fungi(51.5),p:Ascomycota(43.9),c:Sordariomycetes(42.1),o:Sordariales(42.1),f:Lasiochaeriacae(42.1),g:unidentified(39.1),s:Lasiochaeriacae_sp(39.1)
OTU75;size=2;	k:Fungi(66.5),p:Ascomycota(66.5),c:Eurotiomycetes(66.5),o:Eurotiales(46.8),f:Trichocomaceae(46.8),g:Talaromyces(36.2),s:Talaromyces_flavus(36.2)
OTU76;size=2;	k:Fungi(34.9),p:Basidiomycota(31.1),c:Tremellomycetes(30.7),o:Tremellales(17.9),f:Incertae_sedis(17.9),g:Cryptococcus(17.9),s:Cryptococcus_podzolicus(17.9)
OTU80;size=2;	k:Fungi(14.3),p:Ascomycota(7.8),c:Leotiomyces(7.8),o:Leotiales(7.8),f:Leotiaceae(7.8),g:Alatospora(7.8),s:Alatospora_sp(7.8)
OTU77;size=6;	k:Fungi(28.2),p:Ascomycota(16.4),c:Leotiomyces(16.4),o:Leotiales(15.8),f:Leotiaceae(15.8),g:Alatospora(15.8),s:Alatospora_sp(15.8)

continued

Taxonomy assignment of forward reads (sample 55-70; 6th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database – continued

OTU64;siz e=2;	k:Fungi(21.9),p:Ascomycota(18.1),c:Incertae_sedis(17.5),o:Incertae_sedis(17.5),f:Incertae_sedis(17.5),g:Calcarisporiella(17.5),s:Calcarisporiella_sp(14.8)
OTU81;siz e=2;	k:Fungi(53.1),p:Ascomycota(53.1),c:Sordariomycetes(37.7),o:Sordariales(37.7),f:Chaetomiaceae(37.0),g:Humicola(29.1),s:Humicola_sp_JZ_115(29.1)
OTU82;siz e=2;	k:Fungi(23.2),p:unidentified(20.3),c:unidentified(20.3),o:unidentified(20.3),f:unidentified(20.3),g:unidentified(20.3),s:Fungi_sp(20.3)
OTU83;siz e=2;	k:Fungi(43.5),p:Ascomycota(43.5),c:Sordariomycetes(30.1),o:Sordariales(30.1),f:Lasiochaetaceae(30.1),g:Podospora(30.1),s:Podospora_sp(24.5)
OTU78;siz e=2;	k:Fungi(8.5),p:Basidiomycota(5.1),c:Agaricomycetes(5.1),o:Cantharellales(4.9),f:Ceratobasidiaceae(4.9),g:unidentified(4.9),s:Ceratobasidiaceae_sp(4.9)
OTU85;siz e=2;	k:Fungi(7.7),p:Ascomycota(5.1),c:Lecanoromycetes(4.7),o:Teloschistales(4.1),f:Physciaceae(4.1),g:Rinodina(4.1),s:Rinodina_sp(4.1)
OTU87;siz e=2;	k:Fungi(43.0),p:Basidiomycota(43.0),c:Agaricomycetes(43.0),o:Trechisporales(43.0),f:Hydnodontaceae(30.0),g:Trechispora(30.0),s:Trechispora_sp(30.0)
OTU84;siz e=2;	k:Fungi(10.0),p:Ascomycota(6.0),c:Lecanoromycetes(5.6),o:Teloschistales(5.6),f:Physciaceae(5.6),g:Physcia(5.6),s:Physcia_magnussonii(5.6)
OTU86;siz e=2;	k:Fungi(47.1),p:Ascomycota(47.1),c:Sordariomycetes(33.8),o:Hypocreales(24.2),f:Nectriaceae(24.2),g:unidentified(24.2),s:Nectriaceae_sp(24.2)
OTU88;siz e=2;	k:Fungi(17.6),p:Basidiomycota(12.9),c:Agaricomycetes(12.9),o:Trechisporales(12.9),f:Hydnodontaceae(10.7),g:Trechispora(10.7),s:Trechispora_sp(10.5)
OTU89;siz e=2;	k:Fungi(50.6),p:Basidiomycota(45.8),c:Microbotryomycetes(45.8),o:Sporidiobolales(45.4),f:Incertae_sedis(45.4),g:Rhodotorula(45.4),s:Rhodotorula_cresolica(42.7)
OTU90;siz e=2;	k:Fungi(11.4),p:Ascomycota(7.0),c:Dothideomycetes(6.1),o:Botryosphaeriales(6.1),f:Botryosphaeriaceae(6.1),g:unidentified(6.1),s:Botryosphaeriaceae_sp(6.1)

Taxonomy assignment of forward reads (sample 31-45; 12th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database.

OTU1;size=3532;	k:Fungi(70.4),p:Ascomycota(36.6),c:Sordariomycetes(36.6),o:Hypocreales(36.6),f:Hypocreaceae(36.6),g:unidentified(35.2),s:Hypocreaceae_sp(35.2)
OTU4;size=217;	k:Fungi(64.2),p:Ascomycota(64.2),c:Eurotiomycetes(35.2),o:Eurotiales(35.2),f:Trichomaceae(35.2),g:unidentified(35.2),s:Trichomaceae_sp(35.2)
OTU3;size=525;	k:Fungi(5.5),p:Ascomycota(2.9),c:Sordariomycetes(2.9),o:Diaporthales(2.9),f:Diaporthaceae(2.9),g:unidentified(2.9),s:Diaporthaceae_sp(2.9)
OTU2;size=332;	k:Fungi(8.8),p:Ascomycota(4.8),c:Leotiomyces(4.8),o:Rhytismatales(4.8),f:Rhytismataceae(4.8),g:unidentified(4.8),s:Rhytismataceae_sp(4.8)
OTU6;size=1132;	k:Fungi(13.2),p:unidentified(6.8),c:unidentified(6.8),o:unidentified(6.8),f:unidentified(6.8),g:unidentified(6.8),s:Fungi_sp(6.8)
OTU5;size=174;	k:Fungi(68.3),p:Ascomycota(38.1),c:Sordariomycetes(38.1),o:Hypocreales(38.1),f:Hypocreaceae(38.1),g:Trichoderma(34.6),s:Trichoderma_spirale(34.6)
OTU8;size=594;	k:Fungi(61.7),p:Basidiomycota(61.7),c:Tremellomycetes(61.7),o:Filobasidiales(40.1),f:Filobasidiaceae(40.1),g:Cryptococcus(40.1),s:Cryptococcus_terreus(40.1)
OTU7;size=146;	k:Fungi(7.8),p:Ascomycota(3.9),c:Lecanoromycetes(3.9),o:Lecanorales(3.9),f:Parmeliaceae(3.9),g:Parmelina(3.9),s:Parmelina_sp_PN_2010(3.9)
OTU11;size=108;	k:Fungi(4.6),p:Ascomycota(2.9),c:Lecanoromycetes(2.7),o:Lecanorales(2.5),f:Parmeliaceae(2.5),g:Tuckermannopsis(2.5),s:Tuckermannopsis_platyphylla(2.5)
OTU10;size=133;	k:Fungi(40.1),p:Zygomycota(40.1),c:Incertae_sedis(40.1),o:Mucorales(40.1),f:Umbelopsidaceae(40.1),g:Umbelopsis(40.1),s:Umbelopsis_ramanniana_var_angulisporea(25.8)
OTU12;size=66;	k:Fungi(61.5),p:Ascomycota(61.5),c:unidentified(32.6),o:unidentified(32.6),f:unidentified(32.6),g:unidentified(32.6),s:Ascomycota_sp(32.6)
OTU14;size=35;	k:Fungi(6.4),p:Ascomycota(3.6),c:Leotiomyces(3.4),o:unidentified(3.4),f:unidentified(3.4),g:unidentified(3.4),s:Leotiomyces_sp(3.4)
OTU13;size=46;	k:Fungi(3.7),p:Ascomycota(1.9),c:Lecanoromycetes(1.9),o:Teloschistales(1.9),f:Physciaceae(1.9),g:Physcia(1.9),s:Physcia_magnussonii(1.9)
OTU15;size=25;	k:Fungi(3.7),p:Ascomycota(2.0),c:Sordariomycetes(1.8),o:Xylariales(1.8),f:Amphisphaeriaceae(1.8),g:Seimatosporium(1.8),s:Seimatosporium_sp(1.8)
OTU9;size=122;	k:Fungi(83.5),p:Basidiomycota(63.6),c:Agaricomycetes(63.6),o:Agaricales(63.6),f:Entolomataceae(48.1),g:Clitopilus(48.1),s:Clitopilus_sp(48.1)

continued

Taxonomy assignment of forward reads (sample 31-45; 12th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database.

OTU16;size=33;	k:Fungi(52.0),p:Ascomycota(52.0),c:Sordariomycetes(36.9),o:Sordariales(36.9),f:Chaetomiaceae(36.3),g:Humicola(28.5),s:Humicola_sp_JZ_115(28.5)
OTU19;size=28;	k:Fungi(44.9),p:Ascomycota(44.9),c:unidentified(25.5),o:unidentified(25.5),f:unidentified(25.5),g:unidentified(25.5),s:Ascomycota_sp(25.5)
OTU18;size=77;	k:Fungi(58.4),p:Ascomycota(58.4),c:Dothideomycetes(37.7),o:Incertae_sedis(37.7),f:Pseudeurotiaceae(37.7),g:Pseudeurotium(37.7),s:Pseudeurotium_sp(30.9)
OTU20;size=24;	k:Fungi(42.6),p:Ascomycota(38.5),c:Pezizomycetes(38.3),o:Pezizales(38.3),f:Pezizaceae(38.3),g:unidentified(38.3),s:Pezizaceae_sp(38.3)
OTU17;size=20;	k:Fungi(59.0),p:Ascomycota(43.6),c:Sordariomycetes(43.6),o:Hypocreales(39.3),f:Cordycipitaceae(39.3),g:Simplicillium(39.3),s:Simplicillium_lamellicola(39.3)
OTU21;size=19;	k:Fungi(63.0),p:Ascomycota(63.0),c:Eurotiomycetes(42.6),o:Eurotiales(42.6),f:Trichocomaceae(42.6),g:Penicillium(42.6),s:Penicillium_sp(36.0)
OTU23;size=13;	k:Fungi(21.8),p:Ascomycota(17.9),c:Pezizomycetes(15.8),o:Pezizales(15.8),f:Pezizaceae(15.8),g:unidentified(15.8),s:Pezizaceae_sp(15.8)
OTU22;size=14;	k:Fungi(5.5),p:Ascomycota(3.0),c:Sordariomycetes(3.0),o:Diaporthales(3.0),f:Diaporthaceae(3.0),g:unidentified(3.0),s:Diaporthaceae_sp(3.0)
OTU24;size=14;	k:Fungi(9.2),p:Ascomycota(5.9),c:Leotiomycetes(5.5),o:Helotiales(5.3),f:unidentified(5.3),g:unidentified(5.3),s:Helotiales_sp(5.3)
OTU25;size=18;	k:Fungi(7.8),p:Ascomycota(4.5),c:Lecanoromycetes(3.9),o:Lecanorales(3.9),f:Parmeliaceae(3.9),g:Parmelina(3.9),s:Parmelina_sp_PN_2010(3.9)
OTU26;size=10;	k:Fungi(46.9),p:Ascomycota(36.4),c:Eurotiomycetes(36.0),o:Eurotiales(36.0),f:Trichocomaceae(35.2),g:unidentified(30.0),s:Trichocomaceae_sp(30.0)
OTU27;size=29;	k:Fungi(9.9),p:Basidiomycota(5.4),c:Agaricomycetes(5.4),o:Atheliales(5.2),f:Atheliaceae(5.2),g:unidentified(5.2),s:Atheliaceae_sp(5.2)
OTU30;size=10;	k:Fungi(57.0),p:Zygomycota(57.0),c:Incertae_sedis(57.0),o:Mucorales(57.0),f:Umbelopsidaceae(57.0),g:Umbelopsis(57.0),s:Umbelopsis_ramanniana(29.1)
OTU29;size=7;	k:Fungi(58.3),p:Ascomycota(58.3),c:Sordariomycetes(30.2),o:Sordariales(30.2),f:Chaetomiaceae(30.2),g:Chaetomium(30.2),s:Chaetomium_globosum(30.2)
OTU33;size=6;	k:Fungi(33.7),p:Ascomycota(33.7),c:Sordariomycetes(20.2),o:Coniochaetales(17.5),f:Coniochaetaceae(17.5),g:Lecythophora(16.9),s:Lecythophora_sp(16.9)

continued

Taxonomy assignment of forward reads (sample 31-45; 12th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database – continued

OTU31;size=7;	k:Fungi(6.6),p:Zygomycota(3.3),c:Incertae_sedis(3.3),o:Mucorales(3.3),f:Cunninghamellaceae(3.3),g:Gongronella(3.3),s:Gongronella_butleri(3.3)
OTU32;size=17;	k:Fungi(9.9),p:Basidiomycota(5.4),c:Agaricomycetes(5.4),o:Atheliales(5.2),f:Atheliaceae(5.2),g:unidentified(5.2),s:Atheliaceae_sp(5.2)
OTU28;size=7;	k:Fungi(8.0),p:Ascomycota(4.4),c:Lecanoromycetes(4.4),o:Teloschistales(4.4),f:Teloschistaceae(4.2),g:Caloplaca(4.2),s:Caloplaca_aractina(4.2)
OTU34;size=8;	k:Fungi(38.5),p:unidentified(34.4),c:unidentified(34.4),o:unidentified(34.4),f:unidentified(34.4),g:unidentified(34.4),s:Fungi_sp(34.4)
OTU35;size=8;	k:Fungi(63.1),p:Ascomycota(55.2),c:Pezizomycetes(55.2),o:unidentified(48.5),f:unidentified(48.5),g:unidentified(48.5),s:Pezizomycetes_sp(48.5)
OTU36;size=6;	k:Fungi(8.8),p:Basidiomycota(4.6),c:Agaricomycetes(4.6),o:Agaricales(4.4),f:Marasmiaceae(4.4),g:Marasmiellus(4.4),s:Marasmiellus_paspali(4.4)
OTU37;size=5;	k:Fungi(55.8),p:Ascomycota(55.8),c:Eurotiomycetes(39.7),o:Eurotiales(39.7),f:Trichocomaceae(39.7),g:Penicillium(39.7),s:Penicillium_atrovenetum(30.0)
OTU38;size=5;	k:Fungi(7.3),p:Basidiomycota(3.6),c:Agaricomycetes(3.6),o:Agaricales(3.6),f:unidentified(3.6),g:unidentified(3.6),s:Agaricales_sp(3.6)
OTU39;size=4;	k:Fungi(52.7),p:Ascomycota(48.6),c:unidentified(42.8),o:unidentified(42.8),f:unidentified(42.8),g:unidentified(42.8),s:Ascomycota_sp(42.8)
OTU40;size=4;	k:Fungi(9.0),p:Ascomycota(5.3),c:Lecanoromycetes(4.5),o:Lecanorales(4.5),f:Cladoniaceae(4.5),g:Cladonia(4.5),s:Cladonia_firma(4.5)
OTU41;size=4;	k:Fungi(7.8),p:Ascomycota(4.5),c:Lecanoromycetes(3.9),o:Lecanorales(3.9),f:Ramalinaceae(3.9),g:Ramalina(3.9),s:Ramalina_confirmata(3.9)
OTU42;size=4;	k:Fungi(34.9),p:unidentified(31.1),c:unidentified(31.1),o:unidentified(31.1),f:unidentified(31.1),g:unidentified(31.1),s:Fungi_sp(31.1)
OTU43;size=3;	k:Fungi(7.8),p:Basidiomycota(3.9),c:Agaricomycetes(3.9),o:Russulales(3.9),f:Russulaceae(3.9),g:Lactarius(3.9),s:Lactarius_areolatus(3.9)
OTU47;size=5;	k:Fungi(55.7),p:Ascomycota(55.7),c:Pezizomycetes(55.7),o:Pezizales(40.8),f:Pyronemataceae(40.8),g:Scutellinia(40.8),s:Scutellinia_sp(39.8)
OTU46;size=3;	k:Fungi(58.8),p:Ascomycota(38.7),c:Sordariomycetes(38.7),o:Sordariales(38.7),f:Chaetomiaceae(38.7),g:Humicola(32.9),s:Humicola_grisea_var._grisea(31.1)

continued

Taxonomy assignment of forward reads (sample 31-45; 12th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database – continued

OTU44;siz e=3;	k:Fungi(28.1),p:Basidiomycota(21.9),c:Agaricomycetes(21.9),o:Agaricales(19.8),f:Psathyrellaceae(18.2),g:Psathyrella(17.4),s:Psathyrella_friesii(17.4)
OTU45;siz e=5;	k:Fungi(7.8),p:Ascomycota(4.1),c:Lecanoromycetes(3.9),o:Lecanorales(3.9),f:Ramalinaceae(3.9),g:Ramalina(3.9),s:Ramalina_confirmata(3.9)
OTU48;siz e=7;	k:Fungi(7.8),p:Ascomycota(4.3),c:Lecanoromycetes(3.9),o:Lecanorales(3.9),f:Ramalinaceae(3.9),g:Ramalina(3.9),s:Ramalina_confirmata(3.9)
OTU51;siz e=5;	k:Fungi(8.2),p:Ascomycota(4.7),c:Lecanoromycetes(4.1),o:Lecanorales(4.1),f:Ramalinaceae(4.1),g:Ramalina(4.1),s:Ramalina_confirmata(4.1)
OTU50;siz e=13;	k:Fungi(41.0),p:Ascomycota(27.5),c:Leotiomycetes(26.2),o:Incertae_sedis(20.9),f:Incertae_sedis(20.9),g:Leohumicola(20.9),s:Leohumicola_minima(20.9)
OTU55;siz e=3;	k:Fungi(8.6),p:Ascomycota(4.7),c:Lecanoromycetes(4.3),o:Teloschistales(4.3),f:Teloschistaceae(4.3),g:Caloplaca(4.3),s:Caloplaca_lenae(4.3)
OTU52;siz e=3;	k:Fungi(21.7),p:Basidiomycota(17.0),c:Agaricomycetes(17.0),o:Russulales(12.9),f:Stephanosporaceae(12.9),g:Cristinia(12.3),s:Cristinia_helvetica(12.3)
OTU53;siz e=3;	k:Fungi(10.1),p:Ascomycota(6.4),c:Lecanoromycetes(5.5),o:Teloschistales(5.5),f:Physciaceae(5.5),g:Physcia(5.5),s:Physcia_magnussonii(5.5)
OTU54;siz e=7;	k:Fungi(9.9),p:Basidiomycota(5.4),c:Agaricomycetes(5.4),o:Atheliales(5.2),f:Atheliaceae(5.2),g:unidentified(5.2),s:Atheliaceae_sp(5.2)
OTU56;siz e=3;	k:Fungi(8.2),p:Ascomycota(4.5),c:Sordariomycetes(4.3),o:unidentified(4.3),f:unidentified(4.3),g:unidentified(4.3),s:Sordariomycetes_sp(4.3)
OTU58;siz e=2;	k:Fungi(65.7),p:Ascomycota(65.7),c:Eurotiomycetes(48.1),o:Eurotiales(48.1),f:Trichocomaceae(48.1),g:Penicillium(48.1),s:Penicillium_sp(45.0)
OTU59;siz e=2;	k:Fungi(10.0),p:Ascomycota(6.0),c:Lecanoromycetes(5.6),o:Teloschistales(5.6),f:Physciaceae(5.6),g:Physcia(5.6),s:Physcia_magnussonii(5.6)
OTU60;siz e=2;	k:Fungi(46.9),p:Zygomycota(46.9),c:Incertae_sedis(46.9),o:Mortierellales(46.9),f:Mortierellaceae(46.9),g:Mortierella(46.9),s:Mortierella_sp(27.2)
OTU62;siz e=2;	k:Fungi(84.8),p:Ascomycota(70.6),c:Dothideomycetes(70.6),o:Pleosporales(70.6),f:Pleosporaceae(70.6),g:Drechslera(69.1),s:Drechslera_sp_BAFC_3419(67.1)
OTU61;siz e=4;	k:Fungi(7.4),p:Ascomycota(4.1),c:Lecanoromycetes(3.9),o:Lecanorales(3.9),f:Parmeliaceae(3.9),g:Parmelina(3.9),s:Parmelina_sp_PN_2010(3.9)

continued

Taxonomy assignment of forward reads (sample 31-45; 12th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database – continued

OTU63;size=2;	k:Fungi(8.7),p:Basidiomycota(4.6),c:Agaricomycetes(4.6),o:Agaricales(4.4),f:Marasmiaceae(4.4),g:Marasmiellus(4.4),s:Marasmiellus_paspali(4.4)
OTU64;size=2;	k:Fungi(50.0),p:Ascomycota(44.3),c:Sordariomycetes(42.2),o:Hypocreales(31.1),f:unidentified(31.1),g:unidentified(31.1),s:Hypocreales_sp(31.1)
OTU65;size=4;	k:Fungi(56.4),p:Ascomycota(47.3),c:Dothideomycetes(45.5),o:Incertae_sedis(45.5),f:Myxotrichaceae(45.5),g:Oidiodendron(44.9),s:Oidiodendron_truncatum(34.6)
OTU67;size=2;	k:Fungi(8.4),p:Ascomycota(5.2),c:Lecanoromycetes(4.4),o:unidentified(4.2),f:unidentified(4.2),g:unidentified(4.2),s:Lecanoromycetes_sp(4.2)
OTU57;size=7;	k:Fungi(9.9),p:Basidiomycota(5.4),c:Agaricomycetes(5.4),o:Atheliales(5.2),f:Atheliaceae(5.2),g:unidentified(5.2),s:Atheliaceae_sp(5.2)
OTU68;size=2;	k:Fungi(7.4),p:Ascomycota(4.1),c:Leotiomyces(3.9),o:Helotiales(3.9),f:unidentified(3.9),g:unidentified(3.9),s:Helotiales_sp(3.9)
OTU69;size=2;	k:Fungi(38.6),p:Ascomycota(38.6),c:Eurotiomycetes(29.5),o:Onygenales(29.5),f:Onygenaceae(29.5),g:Auxarthron(29.5),s:Auxarthron_sp_RV26652(27.2)
OTU70;size=2;	k:Fungi(21.7),p:Ascomycota(14.2),c:Sordariomycetes(11.9),o:Sordariales(11.9),f:Lasiochaetaceae(11.3),g:unidentified(11.0),s:Lasiochaetaceae_sp(11.0)
OTU72;size=2;	k:Fungi(19.2),p:Basidiomycota(11.3),c:Microbotryomycetes(11.3),o:Leucosporidiales(10.5),f:Leucosporiaceae(10.5),g:Leucosporidium(10.5),s:Leucosporidium_escuderoi(10.3)
OTU73;size=2;	k:Fungi(55.6),p:Ascomycota(55.6),c:Eurotiomycetes(36.0),o:Onygenales(36.0),f:unidentified(36.0),g:unidentified(36.0),s:Onygenales_sp(36.0)
OTU66;size=2;	k:Fungi(7.8),p:Ascomycota(4.5),c:Leotiomyces(4.3),o:Rhytismatales(4.3),f:Rhytismataceae(4.3),g:unidentified(4.3),s:Rhytismataceae_sp(4.3)
OTU74;size=2;	k:Fungi(34.4),p:Zygomycota(34.4),c:Incertae_sedis(34.4),o:Mucorales(34.4),f:Umbelopsidaceae(34.4),g:Umbelopsis(34.4),s:Umbelopsis_ramanniana_var_angulisporea(21.6)
OTU71;size=2;	k:Fungi(55.7),p:Zygomycota(55.7),c:Incertae_sedis(55.7),o:Mortierellales(55.7),f:Mortierellaceae(29.9),g:Mortierella(29.9),s:Mortierella_minutissima(27.9)
OTU75;size=2;	k:Fungi(62.6),p:Ascomycota(49.8),c:unidentified(48.6),o:unidentified(48.6),f:unidentified(48.6),g:unidentified(48.6),s:Ascomycota_sp(48.6)
OTU76;size=2;	k:Fungi(80.3),p:Basidiomycota(58.0),c:Agaricomycetes(58.0),o:Agaricales(58.0),f:Entolomataceae(58.0),g:Entoloma(58.0),s:Entoloma_infula(52.3)

continued

Taxonomy assignment of forward reads (sample 31-45; 12th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database – continued

OTU77;size=2;	k:Fungi(24.0),p:Ascomycota(15.7),c:Leotiomyces(14.7),o:Helotiales(14.0),f:unidentified(14.0),g:unidentified(14.0),s:Helotiales_sp(14.0)
OTU79;size=2;	k:Fungi(6.3),p:Basidiomycota(3.3),c:Agaricomycetes(3.3),o:Hymenochaetales(3.3),f:Schizoporaceae(3.3),g:Hyphodontia(3.3),s:Hyphodontia_subalutacea(3.3)
OTU78;size=2;	k:Fungi(6.6),p:Basidiomycota(3.9),c:Agaricomycetes(3.7),o:Russulales(3.7),f:Bondarzewiaceae(3.7),g:Amyloporus(3.7),s:Amyloporus_campbellii(3.7)
OTU80;size=2;	k:Fungi(8.4),p:Ascomycota(5.0),c:Leotiomyces(4.4),o:Rhytismatales(4.4),f:Rhytismataceae(4.4),g:Coccomyces(4.2),s:Coccomyces_dentatus(4.2)
OTU81;size=2;	k:Fungi(7.1),p:Basidiomycota(3.5),c:Agaricomycetes(3.5),o:Agaricales(3.5),f:unidentified(3.5),g:unidentified(3.5),s:Agaricales_sp(3.5)

Taxonomy assignment of forward reads (sample 46-60; 12th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database.

OTU2;size=417;	k:Fungi(64.2),p:Ascomycota(64.2),c:Eurotiomycetes(35.2),o:Eurotiales(35.2),f:Trichocomaceae(35.2),g:unidentified(35.2),s:Trichocomaceae_sp(35.2)
OTU3;size=819;	k:Fungi(61.7),p:Basidiomycota(61.7),c:Tremellomycetes(61.7),o:Filobasidiales(40.1),f:Filobasidiaceae(40.1),g:Cryptococcus(40.1),s:Cryptococcus_terreus(40.1)
OTU4;size=328;	k:Fungi(44.9),p:Ascomycota(44.9),c:unidentified(25.5),o:unidentified(25.5),f:unidentified(25.5),g:unidentified(25.5),s:Ascomycota_sp(25.5)
OTU1;size=4257;	k:Fungi(70.4),p:Ascomycota(36.6),c:Sordariomycetes(36.6),o:Hypocreales(36.6),f:Hypocreaceae(36.6),g:unidentified(35.2),s:Hypocreaceae_sp(35.2)
OTU5;size=195;	k:Fungi(53.7),p:Ascomycota(41.0),c:Sordariomycetes(41.0),o:unidentified(30.9),f:unidentified(30.9),g:unidentified(30.9),s:Sordariomycetes_sp(30.9)
OTU6;size=322;	k:Fungi(13.2),p:unidentified(6.8),c:unidentified(6.8),o:unidentified(6.8),f:unidentified(6.8),g:unidentified(6.8),s:Fungi_sp(6.8)
OTU8;size=149;	k:Fungi(63.0),p:Ascomycota(63.0),c:Eurotiomycetes(42.6),o:Eurotiales(42.6),f:Trichocomaceae(42.6),g:Penicillium(42.6),s:Penicillium_sp(36.0)
OTU7;size=147;	k:Fungi(83.5),p:Basidiomycota(63.6),c:Agaricomycetes(63.6),o:Agaricales(63.6),f:Entolomataceae(48.1),g:Clitopilus(48.1),s:Clitopilus_sp(48.1)
OTU10;size=76;	k:Fungi(68.3),p:Ascomycota(38.1),c:Sordariomycetes(38.1),o:Hypocreales(38.1),f:Hypocreaceae(38.1),g:Trichoderma(34.6),s:Trichoderma_spirale(34.6)
OTU9;size=91;	k:Fungi(55.8),p:Ascomycota(55.8),c:Eurotiomycetes(39.7),o:Eurotiales(39.7),f:Trichocomaceae(39.7),g:Penicillium(39.7),s:Penicillium_atrovenetum(30.0)
OTU11;size=69;	k:Fungi(59.0),p:Ascomycota(43.6),c:Sordariomycetes(43.6),o:Hypocreales(39.3),f:Cordycipitaceae(39.3),g:Simplicillium(39.3),s:Simplicillium_lamellicola(39.3)
OTU14;size=56;	k:Fungi(3.7),p:Ascomycota(2.0),c:Sordariomycetes(1.8),o:Xylariales(1.8),f:Amphisphaeriaceae(1.8),g:Seimatosporium(1.8),s:Seimatosporium_sp(1.8)
OTU13;size=82;	k:Fungi(56.8),p:Ascomycota(56.8),c:unidentified(31.1),o:unidentified(31.1),f:unidentified(31.1),g:unidentified(31.1),s:Ascomycota_sp(31.1)
OTU12;size=122;	k:Fungi(5.5),p:Ascomycota(2.9),c:Sordariomycetes(2.9),o:Diaporthales(2.9),f:Diaporthaceae(2.9),g:unidentified(2.9),s:Diaporthaceae_sp(2.9)
OTU15;size=66;	k:Fungi(4.5),p:Basidiomycota(2.5),c:Agaricomycetes(2.5),o:Hymenochaetales(2.5),f:Hymenochaetaceae(2.5),g:Pseudochaete(2.5),s:Pseudochaete_rigidula(2.5)

continued

Taxonomy assignment of forward reads (sample 46-60; 12th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database – continued

OTU17;siz e=43;	k:Fungi(75.9),p:Basidiomycota(75.9),c:Agaricomycetes(75.9),o:Agaricales(67.6),f:Strophariaceae(47.7),g:Gymnopilus(47.7),s:Gymnopilus_junonius(47.7)
OTU16;siz e=172;	k:Fungi(56.1),p:Ascomycota(47.1),c:Dothideomycetes(45.3),o:Incertae_sedis(45.3),f:Myxotrichaceae(45.3),g:Oidiodendron(44.7),s:Oidiodendron_truncatum(34.4)
OTU18;siz e=35;	k:Fungi(7.8),p:Ascomycota(3.9),c:Lecanoromycetes(3.9),o:Lecanorales(3.9),f:Parmeliaceae(3.9),g:Parmelina(3.9),s:Parmelina_sp_PN_2010(3.9)
OTU19;siz e=34;	k:Fungi(57.8),p:Ascomycota(57.8),c:Leotiomycetes(45.7),o:Helotiales(45.7),f:unidentified(36.1),g:unidentified(36.1),s:Helotiales_sp(36.1)
OTU20;siz e=34;	k:Fungi(8.8),p:Ascomycota(4.8),c:Leotiomycetes(4.8),o:Rhytismatales(4.8),f:Rhytismataceae(4.8),g:unidentified(4.8),s:Rhytismataceae_sp(4.8)
OTU23;siz e=17;	k:Fungi(68.0),p:Ascomycota(64.3),c:Eurotiomycetes(63.3),o:Eurotiales(63.3),f:Trichocomaceae(63.3),g:Penicillium(63.3),s:Penicillium_chrysogenum(63.3)
OTU24;siz e=18;	k:Fungi(52.0),p:Ascomycota(52.0),c:Sordariomycetes(36.9),o:Sordariales(36.9),f:Chaetomiaceae(36.3),g:Humicola(28.5),s:Humicola_sp_JZ_115(28.5)
OTU27;siz e=25;	k:Fungi(4.6),p:Ascomycota(2.7),c:Lecanoromycetes(2.7),o:Lecanorales(2.5),f:Parmeliaceae(2.5),g:Tuckermannopsis(2.5),s:Tuckermannopsis_platyphylla(2.5)
OTU25;siz e=15;	k:Fungi(58.3),p:Ascomycota(58.3),c:Sordariomycetes(30.2),o:Sordariales(30.2),f:Chaetomiaceae(30.2),g:Chaetomium(30.2),s:Chaetomium_globosum(30.2)
OTU22;siz e=24;	k:Fungi(42.6),p:Ascomycota(38.5),c:Pezizomycetes(38.3),o:Pezizales(38.3),f:Pezizaceae(38.3),g:unidentified(38.3),s:Pezizaceae_sp(38.3)
OTU28;siz e=9;	k:Fungi(60.2),p:Ascomycota(48.0),c:Leotiomycetes(47.5),o:Helotiales(46.1),f:Incertae_sedis(45.9),g:Scytalidium(43.6),s:Scytalidium_lignicola(41.8)
OTU30;siz e=8;	k:Fungi(46.9),p:Ascomycota(36.4),c:Eurotiomycetes(36.0),o:Eurotiales(36.0),f:Trichocomaceae(35.2),g:unidentified(30.0),s:Trichocomaceae_sp(30.0)
OTU29;siz e=33;	k:Fungi(58.4),p:Ascomycota(58.4),c:Dothideomycetes(37.7),o:Incertae_sedis(37.7),f:Pseudeurotiaceae(37.7),g:Pseudeurotium(37.7),s:Pseudeurotium_sp(30.9)
OTU32;siz e=5;	k:Fungi(65.1),p:Ascomycota(65.1),c:Eurotiomycetes(65.1),o:Eurotiales(45.9),f:Trichocomaceae(45.9),g:Talaromyces(35.5),s:Talaromyces_flavus(35.5)
OTU33;siz e=7;	k:Fungi(59.8),p:Ascomycota(59.8),c:Eurotiomycetes(44.0),o:Eurotiales(44.0),f:Trichocomaceae(44.0),g:Neosartorya(29.9),s:Neosartorya_aurata(29.9)

continued

Taxonomy assignment of forward reads (sample 46-60; 12th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database – continued

OTU31;size=8;	k:Fungi(5.5),p:Ascomycota(3.0),c:Sordariomycetes(3.0),o:Diaporthales(3.0),f:Diaporthaceae(3.0),g:unidentified(3.0),s:Diaporthaceae_sp(3.0)
OTU34;size=20;	k:Fungi(9.9),p:Basidiomycota(5.4),c:Agaricomycetes(5.4),o:Atheliales(5.2),f:Atheliaceae(5.2),g:unidentified(5.2),s:Atheliaceae_sp(5.2)
OTU36;size=9;	k:Fungi(37.7),p:Ascomycota(24.4),c:Leotiomyces(23.8),o:unidentified(18.9),f:unidentified(18.9),g:unidentified(18.9),s:Leotiomyces_sp(18.9)
OTU35;size=8;	k:Fungi(3.7),p:Ascomycota(1.9),c:Lecanoromycetes(1.9),o:Teloschistales(1.9),f:Physciaceae(1.9),g:Physcia(1.9),s:Physcia_magnussonii(1.9)
OTU37;size=4;	k:Fungi(21.8),p:Ascomycota(17.9),c:Pezizomycetes(15.8),o:Pezizales(15.8),f:Pezizaceae(15.8),g:unidentified(15.8),s:Pezizaceae_sp(15.8)
OTU38;size=4;	k:Fungi(21.7),p:Basidiomycota(17.0),c:Agaricomycetes(17.0),o:Russulales(12.9),f:Stephanosporaceae(12.9),g:Cristinia(12.3),s:Cristinia_helvetica(12.3)
OTU39;size=6;	k:Fungi(63.1),p:Ascomycota(55.2),c:Pezizomycetes(55.2),o:unidentified(48.5),f:unidentified(48.5),g:unidentified(48.5),s:Pezizomycetes_sp(48.5)
OTU40;size=7;	k:Fungi(42.1),p:Zygomycota(42.1),c:Incertae_sedis(42.1),o:Mucorales(42.1),f:Umbelopsidaceae(42.1),g:Umbelopsis(42.1),s:Umbelopsis_ramanniana_var_angulisporea(29.5)
OTU42;size=3;	k:Fungi(63.9),p:Basidiomycota(60.0),c:Tremellomycetes(58.8),o:Filobasidiales(57.0),f:Filobasidiaceae(57.0),g:Cryptococcus(57.0),s:Cryptococcus_terricola(35.2)
OTU41;size=3;	k:Fungi(10.0),p:Ascomycota(6.0),c:Lecanoromycetes(5.6),o:Teloschistales(5.6),f:Physciaceae(5.6),g:Physcia(5.6),s:Physcia_magnussonii(5.6)
OTU43;size=3;	k:Fungi(6.6),p:Zygomycota(3.3),c:Incertae_sedis(3.3),o:Mucorales(3.3),f:Cunninghamellaceae(3.3),g:Gongronella(3.3),s:Gongronella_butleri(3.3)
OTU44;size=7;	k:Fungi(7.8),p:Ascomycota(4.3),c:Lecanoromycetes(3.9),o:Lecanorales(3.9),f:Ramalinaceae(3.9),g:Ramalina(3.9),s:Ramalina_confirmata(3.9)
OTU45;size=5;	k:Fungi(42.6),p:Ascomycota(42.6),c:Leotiomyces(31.4),o:Helotiales(31.4),f:Incertae_sedis(23.6),g:Scytalidium(23.6),s:Scytalidium_sp(23.6)
OTU46;size=2;	k:Fungi(10.5),p:Ascomycota(6.4),c:Lecanoromycetes(5.7),o:Lecanorales(5.5),f:Lecanoraceae(5.5),g:Lecanora(5.5),s:Lecanora_sp_2_SPO_2012(5.5)
OTU47;size=2;	k:Fungi(8.2),p:Ascomycota(5.1),c:Leotiomyces(4.3),o:Rhytismatales(4.3),f:Rhytismataceae(4.3),g:unidentified(4.3),s:Rhytismataceae_sp(4.3)
OTU48;size=2;	k:Fungi(8.2),p:Ascomycota(4.5),c:Sordariomycetes(4.3),o:unidentified(4.3),f:unidentified(4.3),g:unidentified(4.3),s:Sordariomycetes_sp(4.3)

continued

Taxonomy assignment of forward reads (sample 46-60; 12th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database – continued

OTU49;siz e=2;	k:Fungi(38.8),p:Ascomycota(30.2),c:Dothideomycetes(27.9),o:Capnodiales(27.9),f:Teratosphaeriaceae(27.5),g:Devriesia(27.5),s:Devriesia_sp(27.5)
OTU50;siz e=4;	k:Fungi(62.6),p:Ascomycota(49.8),c:unidentified(48.6),o:unidentified(48.6),f:unidentified(48.6),g:unidentified(48.6),s:Ascomycota_sp(48.6)
OTU53;siz e=2;	k:Fungi(63.0),p:Ascomycota(58.8),c:unidentified(54.1),o:unidentified(54.1),f:unidentified(54.1),g:unidentified(54.1),s:Ascomycota_sp(54.1)
OTU51;siz e=2;	k:Fungi(62.3),p:Ascomycota(55.9),c:Eurotiomycetes(54.1),o:Eurotiales(51.4),f:Trichomaceae(51.4),g:Talaromyces(51.2),s:Talaromyces_sp(51.2)
OTU54;siz e=2;	k:Fungi(88.9),p:Basidiomycota(52.7),c:Agaricomycetes(52.7),o:Agaricales(52.7),f:Entolomataceae(52.7),g:Entoloma(52.7),s:Entoloma_conferendum(52.7)
OTU56;siz e=2;	k:Fungi(4.5),p:Ascomycota(2.7),c:Sordariomycetes(2.5),o:Sordariales(2.5),f:unidentified(2.5),g:unidentified(2.5),s:Sordariales_sp(2.5)
OTU55;siz e=4;	k:Fungi(21.6),p:Ascomycota(14.1),c:Sordariomycetes(12.0),o:Sordariales(12.0),f:Lasiochaeraceae(11.2),g:unidentified(11.0),s:Lasiochaeraceae_sp(11.0)
OTU57;siz e=2;	k:Fungi(68.3),p:Ascomycota(40.1),c:Sordariomycetes(40.1),o:Hypocreales(40.1),f:Hypocreaceae(40.1),g:Hypocrea(34.2),s:Hypocrea_virens(34.2)
OTU59;siz e=2;	k:Fungi(7.3),p:Basidiomycota(3.6),c:Agaricomycetes(3.6),o:Agaricales(3.6),f:unidentified(3.6),g:unidentified(3.6),s:Agaricales_sp(3.6)
OTU60;siz e=2;	k:Fungi(9.2),p:Ascomycota(5.9),c:Leotiomycetes(5.5),o:Helotiales(5.3),f:unidentified(5.3),g:unidentified(5.3),s:Helotiales_sp(5.3)
OTU61;siz e=2;	k:Fungi(52.7),p:Ascomycota(47.7),c:Saccharomycetes(47.7),o:Saccharomycetales(47.7),f:Incertae_sedis(47.7),g:unidentified(47.7),s:Saccharomycetales_sp(47.7)

Taxonomy assignment of forward reads (sample 1-15; 24th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database

OTU1;size =4211;	k:Fungi(70.4),p:Ascomycota(36.6),c:Sordariomycetes(36.6),o:Hypocreales(36.6),f:Hypocreaceae(36.6),g:unidentified(35.2),s:Hypocreaceae_sp(35.2)
OTU3;size =287;	k:Fungi(64.2),p:Ascomycota(64.2),c:Eurotiomycetes(35.2),o:Eurotiales(35.2),f:Trichocomaceae(35.2),g:unidentified(35.2),s:Trichocomaceae_sp(35.2)
OTU4;size =265;	k:Fungi(8.8),p:Ascomycota(4.8),c:Leotiomycetes(4.8),o:Rhytismatales(4.8),f:Rhytismataceae(4.8),g:unidentified(4.8),s:Rhytismataceae_sp(4.8)
OTU6;size =740;	k:Fungi(13.3),p:unidentified(6.8),c:unidentified(6.8),o:unidentified(6.8),f:unidentified(6.8),g:unidentified(6.8),s:Fungi_sp(6.8)
OTU7;size =579;	k:Fungi(61.7),p:Basidiomycota(61.7),c:Tremellomycetes(61.7),o:Filobasidiales(40.1),f:Filobasidiaceae(40.1),g:Cryptococcus(40.1),s:Cryptococcus_terreus(40.1)
OTU2;size =278;	k:Fungi(83.5),p:Basidiomycota(63.6),c:Agaricomycetes(63.6),o:Agaricales(63.6),f:Entolomataceae(48.1),g:Clitopilus(48.1),s:Clitopilus_sp(48.1)
OTU5;size =403;	k:Fungi(5.5),p:Ascomycota(2.9),c:Sordariomycetes(2.9),o:Diaporthales(2.9),f:Diaporthaceae(2.9),g:unidentified(2.9),s:Diaporthaceae_sp(2.9)
OTU8;size =117;	k:Fungi(44.9),p:Ascomycota(44.9),c:unidentified(25.5),o:unidentified(25.5),f:unidentified(25.5),g:unidentified(25.5),s:Ascomycota_sp(25.5)
OTU9;size =86;	k:Fungi(7.8),p:Ascomycota(3.9),c:Lecanoromycetes(3.9),o:Lecanorales(3.9),f:Parmeliaceae(3.9),g:Parmelina(3.9),s:Parmelina_sp_PN_2010(3.9)
OTU10;size =155;	k:Fungi(52.0),p:Ascomycota(52.0),c:Sordariomycetes(36.9),o:Sordariales(36.9),f:Chaetomiaceae(36.3),g:Humicola(28.5),s:Humicola_sp_JZ_115(28.5)
OTU11;size =58;	k:Fungi(63.0),p:Ascomycota(63.0),c:Eurotiomycetes(42.6),o:Eurotiales(42.6),f:Trichocomaceae(42.6),g:Penicillium(42.6),s:Penicillium_sp(36.0)
OTU12;size =76;	k:Fungi(40.1),p:Zygomycota(40.1),c:Incertae_sedis(40.1),o:Mucorales(40.1),f:Umbelopsidaceae(40.1),g:Umbelopsis(40.1),s:Umbelopsis_ramanniana_var_angulispora(25.8)
OTU14;size =109;	k:Fungi(58.4),p:Ascomycota(58.4),c:Dothideomycetes(37.7),o:Incertae_sedis(37.7),f:Pseudeurotiaceae(37.7),g:Pseudeurotium(37.7),s:Pseudeurotium_sp(30.9)
OTU13;size =43;	k:Fungi(59.0),p:Ascomycota(43.6),c:Sordariomycetes(43.6),o:Hypocreales(39.3),f:Cordycipitaceae(39.3),g:Simplicillium(39.3),s:Simplicillium_lamelicola(39.3)
OTU15;size =27;	k:Fungi(42.6),p:Ascomycota(38.5),c:Pezizomycetes(38.3),o:Pezizales(38.3),f:Pezizaceae(38.3),g:unidentified(38.3),s:Pezizaceae_sp(38.3)

continued

Taxonomy assignment of forward reads (sample 1-15; 24th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database – continued

OTU16;size=28;	k:Fungi(6.4),p:Ascomycota(3.6),c:Leotiomycetes(3.4),o:unidentified(3.4),f:unidentified(3.4),g:unidentified(3.4),s:Leotiomycetes_sp(3.4)
OTU17;size=61;	k:Fungi(4.6),p:Ascomycota(2.7),c:Lecanoromycetes(2.7),o:Lecanorales(2.5),f:Parmeliaceae(2.5),g:Tuckermannopsis(2.5),s:Tuckermannopsis_platyphylla(2.5)
OTU18;size=22;	k:Fungi(3.7),p:Ascomycota(2.0),c:Sordariomycetes(1.8),o:Xylariales(1.8),f:Amphisphaeriaceae(1.8),g:Seimatosporium(1.8),s:Seimatosporium_sp(1.8)
OTU19;size=43;	k:Fungi(56.8),p:Ascomycota(56.8),c:unidentified(31.1),o:unidentified(31.1),f:unidentified(31.1),g:unidentified(31.1),s:Ascomycota_sp(31.1)
OTU20;size=16;	k:Fungi(55.8),p:Ascomycota(55.8),c:Eurotiomycetes(39.7),o:Eurotiales(39.7),f:Trichocomaceae(39.7),g:Penicillium(39.7),s:Penicillium_atrovenetum(30.0)
OTU22;size=20;	k:Fungi(63.1),p:Ascomycota(55.2),c:Pezizomycetes(55.2),o:unidentified(48.5),f:unidentified(48.5),g:unidentified(48.5),s:Pezizomycetes_sp(48.5)
OTU21;size=20;	k:Fungi(3.7),p:Ascomycota(1.9),c:Lecanoromycetes(1.9),o:Teloschistales(1.9),f:Physciaceae(1.9),g:Physcia(1.9),s:Physcia_magnussonii(1.9)
OTU23;size=14;	k:Fungi(21.7),p:Basidiomycota(17.0),c:Agaricomycetes(17.0),o:Russulales(12.9),f:Stephanosporaceae(12.9),g:Cristinia(12.3),s:Cristinia_helvetica(12.3)
OTU27;size=8;	k:Fungi(7.3),p:Basidiomycota(3.6),c:Agaricomycetes(3.6),o:Agaricales(3.6),f:unidentified(3.6),g:unidentified(3.6),s:Agaricales_sp(3.6)
OTU26;size=31;	k:Fungi(9.9),p:Basidiomycota(5.4),c:Agaricomycetes(5.4),o:Atheliales(5.2),f:Atheliaceae(5.2),g:unidentified(5.2),s:Atheliaceae_sp(5.2)
OTU24;size=9;	k:Fungi(58.3),p:Ascomycota(58.3),c:Sordariomycetes(30.2),o:Sordariales(30.2),f:Chaetomiaceae(30.2),g:Chaetomium(30.2),s:Chaetomium_globosum(30.2)
OTU28;size=9;	k:Fungi(52.5),p:Ascomycota(52.5),c:Pezizomycetes(52.5),o:Pezizales(38.5),f:Pyronemataceae(38.5),g:Scutellinia(38.5),s:Scutellinia_sp(36.5)
OTU30;size=8;	k:Fungi(46.9),p:Ascomycota(36.4),c:Eurotiomycetes(36.0),o:Eurotiales(36.0),f:Trichocomaceae(35.2),g:unidentified(30.0),s:Trichocomaceae_sp(30.0)
OTU29;size=6;	k:Fungi(8.6),p:Ascomycota(4.7),c:Lecanoromycetes(4.3),o:Teloschistales(4.3),f:Teloschistaceae(4.3),g:Caloplaca(4.3),s:Caloplaca_lenae(4.3)
OTU32;size=13;	k:Fungi(9.9),p:Basidiomycota(5.4),c:Agaricomycetes(5.4),o:Atheliales(5.2),f:Atheliaceae(5.2),g:unidentified(5.2),s:Atheliaceae_sp(5.2)

continued

Taxonomy assignment of forward reads (sample 1-15; 24th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database – continued

OTU33;siz e=6;	k:Fungi(7.8),p:Ascomycota(4.5),c:Lecanoromycetes(3.9),o:Lecanorales(3.9),f:Parmeliaceae(3.9),g:Parmelina(3.9),s:Parmelina_sp_PN_2010(3.9)
OTU34;siz e=4;	k:Fungi(5.5),p:Ascomycota(3.0),c:Sordariomycetes(3.0),o:Diaporthales(3.0),f:Diaporthaceae(3.0),g:unidentified(3.0),s:Diaporthaceae_sp(3.0)
OTU35;siz e=20;	k:Fungi(9.9),p:Basidiomycota(5.4),c:Agaricomycetes(5.4),o:Atheliales(5.2),f:Atheliaceae(5.2),g:unidentified(5.2),s:Atheliaceae_sp(5.2)
OTU36;siz e=4;	k:Fungi(65.7),p:Ascomycota(65.7),c:Eurotiomycetes(48.1),o:Eurotiales(48.1),f:Trichocomaceae(48.1),g:Penicillium(48.1),s:Penicillium_sp(45.0)
OTU37;siz e=3;	k:Fungi(9.2),p:Ascomycota(5.9),c:Leotiomyces(5.5),o:Helotiales(5.3),f:unidentified(5.3),g:unidentified(5.3),s:Helotiales_sp(5.3)
OTU39;siz e=3;	k:Fungi(41.0),p:Ascomycota(41.0),c:Pezizomycetes(31.8),o:Pezizales(31.8),f:Pyronemataceae(31.8),g:unidentified(31.8),s:Pyronemataceae_sp(31.8)
OTU38;siz e=3;	k:Fungi(42.6),p:Ascomycota(42.6),c:Leotiomyces(31.4),o:Helotiales(31.4),f:Incertae_sedis(23.6),g:Scytalidium(23.6),s:Scytalidium_sp(23.6)
OTU40;siz e=3;	k:Fungi(68.3),p:Ascomycota(38.1),c:Sordariomycetes(38.1),o:Hypocreales(38.1),f:Hypocreaceae(38.1),g:Trichoderma(34.6),s:Trichoderma_spirale(34.6)
OTU42;siz e=3;	k:Fungi(33.7),p:Ascomycota(33.7),c:Sordariomycetes(20.2),o:Coniochaetales(17.5),f:Coniochaetaceae(17.5),g:Lecythophora(16.9),s:Lecythophora_sp(16.9)
OTU41;siz e=7;	k:Fungi(37.7),p:Ascomycota(24.4),c:Leotiomyces(23.8),o:unidentified(18.9),f:unidentified(18.9),g:unidentified(18.9),s:Leotiomyces_sp(18.9)
OTU45;siz e=2;	k:Fungi(58.6),p:Ascomycota(38.5),c:Sordariomycetes(38.5),o:Sordariales(38.5),f:Chaetomiaceae(38.5),g:Humicola(32.8),s:Humicola_grisea_var._grisea(30.9)
OTU31;siz e=5;	k:Fungi(21.8),p:Ascomycota(17.9),c:Pezizomycetes(15.8),o:Pezizales(15.8),f:Pezizaceae(15.8),g:unidentified(15.8),s:Pezizaceae_sp(15.8)
OTU44;siz e=2;	k:Fungi(58.4),p:Ascomycota(58.4),c:Dothideomycetes(37.7),o:Incertae_sedis(37.7),f:Pseudeurotiaceae(37.7),g:Pseudeurotium(37.7),s:Pseudeurotium_sp(30.9)
OTU46;siz e=2;	k:Fungi(48.6),p:Ascomycota(48.6),c:Dothideomycetes(29.2),o:Incertae_sedis(29.2),f:Pseudeurotiaceae(29.2),g:Pseudeurotium(29.2),s:Pseudeurotium_hygrophilum(25.9)
OTU48;siz e=2;	k:Fungi(55.6),p:Ascomycota(55.6),c:Leotiomyces(36.0),o:Incertae_sedis(36.0),f:Incertae_sedis(36.0),g:Geomyces(36.0),s:Geomyces_auratus(36.0)

continued

Taxonomy assignment of forward reads (sample 1-15; 24th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database – continued

OTU49;siz e=2;	k:Fungi(88.9),p:Basidiomycota(52.7),c:Agaricomycetes(52.7),o:Agaricales(52.7),f:Entolomataceae(52.7),g:Entoloma(52.7),s:Entoloma_conferendum(52.7)
OTU47;siz e=2;	k:Fungi(73.8),p:Basidiomycota(61.7),c:Agaricomycetes(61.7),o:Agaricales(61.7),f:Entolomataceae(61.7),g:Entoloma(61.5),s:Entoloma_serrulatum(61.5)
OTU50;siz e=4;	k:Fungi(62.6),p:Ascomycota(49.8),c:unidentified(48.6),o:unidentified(48.6),f:unidentified(48.6),g:unidentified(48.6),s:Ascomycota_sp(48.6)
OTU52;siz e=2;	k:Fungi(34.4),p:Zygomycota(34.4),c:Incertae_sedis(34.4),o:Mucorales(34.4),f:Umbelopsidaceae(34.4),g:Umbelopsis(34.4),s:Umbelopsis_ramanniana_var_angulispora(21.6)
OTU53;siz e=2;	k:Fungi(47.7),p:Zygomycota(47.7),c:Incertae_sedis(47.7),o:Mortierellales(47.7),f:Mortierellaceae(47.7),g:Mortierella(47.7),s:Mortierella_sp(27.4)
OTU51;siz e=2;	k:Fungi(8.8),p:Basidiomycota(4.6),c:Agaricomycetes(4.6),o:Agaricales(4.4),f:Marasmiaceae(4.4),g:Marasmiellus(4.4),s:Marasmiellus_paspali(4.4)
OTU54;siz e=4;	k:Fungi(9.5),p:Basidiomycota(5.6),c:Agaricomycetes(5.3),o:Agaricales(4.9),f:Tricholomataceae(4.9),g:Clitocybe(4.9),s:Clitocybe_sp(4.9)
OTU56;siz e=2;	k:Fungi(45.6),p:Ascomycota(36.1),c:Dothideomycetes(35.1),o:Capnodiales(33.6),f:Teratosphaeriaceae(33.6),g:Devriesia(33.6),s:Devriesia_sp(33.6)
OTU55;siz e=2;	k:Fungi(50.6),p:Basidiomycota(45.1),c:Agaricomycetes(44.4),o:Agaricales(43.8),f:unidentified(41.4),g:unidentified(41.4),s:Agaricales_sp(41.4)
OTU58;siz e=2;	k:Fungi(30.2),p:Ascomycota(18.6),c:Dothideomycetes(16.9),o:Incertae_sedis(16.9),f:Pseudeurotiaceae(16.9),g:Pseudeurotium(16.9),s:Pseudeurotium_sp(16.9)
OTU59;siz e=2;	k:Fungi(56.1),p:Ascomycota(47.1),c:Dothideomycetes(45.3),o:Incertae_sedis(45.3),f:Myxotrichaceae(45.3),g:Oidiodendron(44.7),s:Oidiodendron_truncatum(34.4)

Taxonomy assignment of forward reads (sample 16-30; 24th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database.

OTU1;size =4827;	k:Fungi(70.4),p:Ascomycota(36.6),c:Sordariomycetes(36.6),o:Hypocreales(36.6),f:Hypocreaceae(36.6),g:unidentified(35.2),s:Hypocreaceae_sp(35.2)
OTU2;size =534;	k:Fungi(63.9),p:Ascomycota(63.9),c:Eurotiomycetes(35.1),o:Eurotiales(35.1),f:Trichocomaceae(35.1),g:unidentified(35.1),s:Trichocomaceae_sp(35.1)
OTU3;size =259;	k:Fungi(59.0),p:Ascomycota(43.6),c:Sordariomycetes(43.6),o:Hypocreales(39.3),f:Cordycipitaceae(39.3),g:Simplicillium(39.3),s:Simplicillium_lamellicola(39.3)
OTU4;size =697;	k:Fungi(61.7),p:Basidiomycota(61.7),c:Tremellomycetes(61.7),o:Filobasidiales(40.1),f:Filobasidiaceae(40.1),g:Cryptococcus(40.1),s:Cryptococcus_terreus(40.1)
OTU7;size =190;	k:Fungi(56.4),p:Ascomycota(47.3),c:Dothideomycetes(45.5),o:Incertae_sedis(45.5),f:Myxotrichaceae(45.5),g:Oidiodendron(44.9),s:Oidiodendron_truncatum(44.9)
OTU6;size =170;	k:Fungi(63.0),p:Ascomycota(63.0),c:Eurotiomycetes(42.6),o:Eurotiales(42.6),f:Trichocomaceae(42.6),g:Penicillium(42.6),s:Penicillium_sp(36.0)
OTU8;size =74;	k:Fungi(68.3),p:Ascomycota(38.1),c:Sordariomycetes(38.1),o:Hypocreales(38.1),f:Hypocreaceae(38.1),g:Trichoderma(34.6),s:Trichoderma_spirale(34.6)
OTU5;size =352;	k:Fungi(44.9),p:Ascomycota(44.9),c:unidentified(25.5),o:unidentified(25.5),f:unidentified(25.5),g:unidentified(25.5),s:Ascomycota_sp(25.5)
OTU9;size =126;	k:Fungi(5.5),p:Ascomycota(2.9),c:Sordariomycetes(2.9),o:Diaporthales(2.9),f:Diaporthaceae(2.9),g:unidentified(2.9),s:Diaporthaceae_sp(2.9)
OTU10;size =69;	k:Fungi(75.9),p:Basidiomycota(75.9),c:Agaricomycetes(75.9),o:Agaricales(67.6),f:Strophariaceae(47.7),g:Gymnopilus(47.7),s:Gymnopilus_junonius(47.7)
OTU11;size =60;	k:Fungi(53.7),p:Ascomycota(41.0),c:Sordariomycetes(41.0),o:unidentified(30.9),f:unidentified(30.9),g:unidentified(30.9),s:Sordariomycetes_sp(30.9)
OTU12;size =85;	k:Fungi(13.2),p:unidentified(6.8),c:unidentified(6.8),o:unidentified(6.8),f:unidentified(6.8),g:unidentified(6.8),s:Fungi_sp(6.8)
OTU13;size =38;	k:Fungi(5.5),p:Ascomycota(2.9),c:Sordariomycetes(2.9),o:Diaporthales(2.9),f:Diaporthaceae(2.9),g:unidentified(2.9),s:Diaporthaceae_sp(2.9)
OTU15;size =40;	k:Fungi(3.7),p:Ascomycota(2.0),c:Sordariomycetes(1.8),o:Xylariales(1.8),f:Amphisphaeriaceae(1.8),g:Seimatosporium(1.8),s:Seimatosporium_sp(1.8)

continued

Taxonomy assignment of forward reads (sample 16-30; 24th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database – continued

OTU14;size=39;	k:Fungi(83.5),p:Basidiomycota(63.6),c:Agaricomycetes(63.6),o:Agaricales(63.6),f:Entolomataceae(48.1),g:Clitopilus(48.1),s:Clitopilus_sp(48.1)
OTU16;size=39;	k:Fungi(8.8),p:Ascomycota(4.8),c:Leotiomycetes(4.8),o:Rhytismatales(4.8),f:Rhytismataceae(4.8),g:unidentified(4.8),s:Rhytismataceae_sp(4.8)
OTU17;size=35;	k:Fungi(4.5),p:Basidiomycota(2.5),c:Agaricomycetes(2.5),o:Hymenochaetales(2.5),f:Hymenochaetaceae(2.5),g:Pseudochaete(2.5),s:Pseudochaete_rigidula(2.5)
OTU18;size=25;	k:Fungi(7.8),p:Ascomycota(3.9),c:Lecanoromycetes(3.9),o:Lecanorales(3.9),f:Parmeliaceae(3.9),g:Parmelina(3.9),s:Parmelina_sp_PN_2010(3.9)
OTU19;size=24;	k:Fungi(58.0),p:Ascomycota(58.0),c:Leotiomycetes(45.9),o:Helotiales(45.9),f:unidentified(36.2),g:unidentified(36.2),s:Helotiales_sp(36.2)
OTU21;size=35;	k:Fungi(56.8),p:Ascomycota(56.8),c:unidentified(31.1),o:unidentified(31.1),f:unidentified(31.1),g:unidentified(31.1),s:Ascomycota_sp(31.1)
OTU22;size=19;	k:Fungi(55.8),p:Ascomycota(55.8),c:Eurotiomycetes(39.7),o:Eurotiales(39.7),f:Trichocomaceae(39.7),g:Penicillium(39.7),s:Penicillium_atrovenetum(30.0)
OTU23;size=24;	k:Fungi(4.6),p:Ascomycota(2.9),c:Lecanoromycetes(2.7),o:Lecanorales(2.5),f:Parmeliaceae(2.5),g:Tuckermannopsis(2.5),s:Tuckermannopsis_platyphylla(2.5)
OTU25;size=17;	k:Fungi(42.6),p:Ascomycota(42.6),c:Leotiomycetes(31.4),o:Helotiales(31.4),f:Incertae_sedis(23.6),g:Scytalidium(23.6),s:Scytalidium_sp(23.6)
OTU26;size=16;	k:Fungi(6.4),p:Ascomycota(3.6),c:Leotiomycetes(3.4),o:unidentified(3.4),f:unidentified(3.4),g:unidentified(3.4),s:Leotiomycetes_sp(3.4)
OTU27;size=11;	k:Fungi(45.6),p:Ascomycota(36.1),c:Dothideomycetes(35.1),o:Capnodiales(33.6),f:Teratosphaeriaceae(33.6),g:Devriesia(33.6),s:Devriesia_sp(33.6)
OTU29;size=15;	k:Fungi(42.1),p:Zygomycota(42.1),c:Incertae_sedis(42.1),o:Mucorales(42.1),f:Umbelopsidaceae(42.1),g:Umbelopsis(42.1),s:Umbelopsis_ramanniana_var_angulispora(29.5)
OTU28;size=21;	k:Fungi(63.1),p:Ascomycota(50.2),c:unidentified(49.0),o:unidentified(49.0),f:unidentified(49.0),g:unidentified(49.0),s:Ascomycota_sp(49.0)
OTU30;size=25;	k:Fungi(58.7),p:Ascomycota(58.7),c:Dothideomycetes(37.8),o:Incertae_sedis(37.8),f:Pseudeurotiaceae(37.8),g:Pseudeurotium(37.8),s:Pseudeurotium_sp(31.0)

continued

Taxonomy assignment of forward reads (sample 16-30; 24th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database – continued

OTU31;siz e=11;	k:Fungi(65.1),p:Ascomycota(65.1),c:Eurotiomycetes(65.1),o:Eurotiales(45.9),f:Trichocomaceae(45.9),g:Talaromyces(35.5),s:Talaromyces_flavus(35.5)
OTU33;siz e=6;	k:Fungi(21.7),p:Basidiomycota(17.0),c:Agaricomycetes(17.0),o:Russulales(12.9),f:Stephanosporaceae(12.9),g:Cristinia(12.3),s:Cristinia_helvetica(12.3)
OTU34;siz e=16;	k:Fungi(9.9),p:Basidiomycota(5.4),c:Agaricomycetes(5.4),o:Atheliales(5.2),f:Atheliaceae(5.2),g:unidentified(5.2),s:Atheliaceae_sp(5.2)
OTU20;siz e=20;	k:Fungi(42.6),p:Ascomycota(38.5),c:Pezizomycetes(38.3),o:Pezizales(38.3),f:Pezizaceae(38.3),g:unidentified(38.3),s:Pezizaceae_sp(38.3)
OTU35;siz e=10;	k:Fungi(69.1),p:Ascomycota(61.1),c:Pezizomycetes(61.1),o:unidentified(55.1),f:unidentified(55.1),g:unidentified(55.1),s:Pezizomycetes_sp(55.1)
OTU36;siz e=5;	k:Fungi(5.5),p:Ascomycota(3.0),c:Sordariomycetes(3.0),o:Diaporthales(3.0),f:Diaporthaceae(3.0),g:unidentified(3.0),s:Diaporthaceae_sp(3.0)
OTU37;siz e=8;	k:Fungi(3.7),p:Ascomycota(1.9),c:Lecanoromycetes(1.9),o:Teloschistales(1.9),f:Physciaceae(1.9),g:Physcia(1.9),s:Physcia_magnussonii(1.9)
OTU39;siz e=4;	k:Fungi(6.6),p:Zygomycota(3.3),c:Incertae_sedis(3.3),o:Mucorales(3.3),f:Cunninghamellaceae(3.3),g:Gongronella(3.3),s:Gongronella_butleri(3.3)
OTU41;siz e=3;	k:Fungi(68.0),p:Ascomycota(64.3),c:Eurotiomycetes(63.3),o:Eurotiales(63.3),f:Trichocomaceae(63.3),g:Penicillium(63.3),s:Penicillium_chrysogenum(63.3)
OTU40;siz e=3;	k:Fungi(59.3),p:Ascomycota(59.3),c:unidentified(43.6),o:unidentified(43.6),f:unidentified(43.6),g:unidentified(43.6),s:Ascomycota_sp(43.6)
OTU42;siz e=3;	k:Fungi(47.7),p:Zygomycota(47.7),c:Incertae_sedis(47.7),o:Mortierellales(47.7),f:Mortierellaceae(47.7),g:Mortierella(47.7),s:Mortierella_sp(27.4)
OTU43;siz e=2;	k:Fungi(45.6),p:Ascomycota(36.1),c:Dothideomycetes(35.1),o:Capnodiales(33.6),f:Teratosphaeriaceae(33.6),g:Devriesia(33.6),s:Devriesia_sp(33.6)
OTU44;siz e=2;	k:Fungi(43.8),p:Ascomycota(43.8),c:unidentified(22.5),o:unidentified(22.5),f:unidentified(22.5),g:unidentified(22.5),s:Ascomycota_sp(22.5)
OTU45;siz e=2;	k:Fungi(60.3),p:Ascomycota(60.3),c:Eurotiomycetes(41.1),o:Eurotiales(41.1),f:Trichocomaceae(41.1),g:Penicillium(35.7),s:Penicillium_chrysogenum(30.2)

continued

Taxonomy assignment of forward reads (sample 16-30; 24th month). The taxonomy annotations of forward reads was added to OTU sequence labels by using the utax command and Unite ITS1 Ver. 7 (http://drive5.com/usearch/manual/utax_downloads.html) as the database – continued

OTU38;siz e=8;	k:Fungi(52.3),p:Ascomycota(52.3),c:Sordariomycetes(37.0),o:Sordariales(37.0),f:Chaetomiaceae(36.4),g:Humicola(28.6),s:Humicola_sp_JZ_115(28.6)
OTU48;siz e=2;	k:Fungi(7.8),p:Ascomycota(4.3),c:Leotiomyces(4.3),o:Helotiales(4.1),f:unidentified(4.1),g:unidentified(4.1),s:Helotiales_sp(4.1)
OTU46;siz e=2;	k:Fungi(62.3),p:Ascomycota(55.9),c:Eurotiomycetes(54.1),o:Eurotiales(51.4),f:Trichocomaceae(51.4),g:Talaromyces(51.2),s:Talaromyces_sp(51.2)
OTU49;siz e=2;	k:Fungi(7.8),p:Ascomycota(4.5),c:Leotiomyces(4.1),o:Rhytismatales(4.1),f:Rhytismataceae(4.1),g:unidentified(4.1),s:Rhytismataceae_sp(4.1)
OTU47;siz e=2;	k:Fungi(15.6),p:Basidiomycota(11.1),c:Agaricomycetes(11.1),o:Cantharellales(9.3),f:unidentified(9.3),g:unidentified(9.3),s:Cantharellales_sp(9.3)
OTU51;siz e=2;	k:Fungi(21.8),p:Ascomycota(17.9),c:Pezizomycetes(15.8),o:Pezizales(15.8),f:Pezizaceae(15.8),g:unidentified(15.8),s:Pezizaceae_sp(15.8)
OTU50;siz e=2;	k:Fungi(51.0),p:Ascomycota(51.0),c:Leotiomyces(51.0),o:Helotiales(51.0),f:Sclerotiniaceae(31.1),g:unidentified(26.1),s:Sclerotiniaceae_sp(26.1)
OTU52;siz e=2;	k:Fungi(37.7),p:Ascomycota(24.4),c:Leotiomyces(23.8),o:unidentified(18.9),f:unidentified(18.9),g:unidentified(18.9),s:Leotiomyces_sp(18.9)

Appendix E. Top twenty species in soil and soil+T22 at the 12th month and the 24th month (Chapter 5)

The top twenty species in soil and soil with the addition of *T. harzianum* (T22) at the 12th month of observation. Percentages in brackets show the percentage of relative abundance of each species.

OTU Id	Soil	OTU Id	Soil+<i>T. harzianum</i> (T22)
6	<i>Fungi</i> sp. (22%) (Identified as <i>Penicillium</i> sp. by NCBI GenBank database)	6	<i>Fungi</i> sp. (25%) (Identified as <i>Penicillium</i> sp. by NCBI GenBank database)
8	<i>Cryptococcus terreus</i> (19%)	1	<i>Hypocreaceae</i> sp. (21%)
3	<i>Diaporthaceae</i> sp. (8%)	3	<i>Diaporthaceae</i> sp. (14%)
10	<i>Umbelopsis ramanniana</i> var <i>angulispora</i> (8%)	2	<i>Rhytismataceae</i> sp. (8%)
1	<i>Hypocreaceae</i> sp. (8%)	8	<i>Cryptococcus terreus</i> (7%)
2	<i>Rhytismataceae</i> sp. (7%)	7	<i>Parmelina</i> sp. (3%)
4	<i>Trichocomaceae</i> sp. (3%)	11	<i>Tuckermannopsis platyphylla</i> (3%)
7	<i>Parmelina</i> sp. (2%)	12	<i>Ascomycota</i> sp. (1%)
9	<i>Clitopilus</i> sp. (2%)	13	<i>Physcia magnussonii</i> (1%)
11	<i>Tuckermannopsis platyphylla</i> (1%)	32	<i>Atheliaceae</i> sp. (1%)
14	<i>Leotiomyces</i> sp. (1%)	15	<i>Seimatosporium</i> sp. (0.8%)
13	<i>Physcia magnussonii</i> (1%)	14	<i>Leotiomyces</i> sp. (0.8%)
12	<i>Ascomycota</i> sp. (1%)	10	<i>Umbelopsis ramanniana</i> var <i>angulispora</i> (0.7%)
27	<i>Atheliaceae</i> sp. (0.9%)	4	<i>Trichocomaceae</i> sp. (0.7%)
25	<i>Parmelina</i> sp. (0.9%)	50	<i>Leohumicola minima</i> (0.6%)
30	<i>Umbelopsis ramanniana</i> (0.9%)	34	<i>Fungi</i> sp. (0.5%)
32	<i>Atheliaceae</i> sp. (0.7%)	57	<i>Atheliaceae</i> sp. (0.4%)
20	<i>Pezizaceae</i> sp. (0.7%)	27	<i>Atheliaceae</i> sp. (0.4%)
16	<i>Humicola</i> sp. (0.5%)	23	<i>Pezizaceae</i> sp. (0.4%)
18	<i>Pseudeurotium</i> sp. (0.4%)	22	<i>Diaporthaceae</i> sp. (0.4%)

The top twenty species in soil and soil with the addition of *T. harzianum* (T22) at the 24th month of observation. Percentages in brackets show the percentage of relative abundance of each species.

OTU Id	Soil	OTU Id	Soil+<i>T. harzianum</i> (T22)
7	<i>Cryptococcus terreus</i> (17%)	1	<i>Hypocreaceae</i> sp. (50%)
6	<i>Fungi</i> sp. (15%) (Identified as <i>Penicillium</i> sp. by NCBI GenBank database)	6	<i>Fungi</i> sp. (16%) (Identified as <i>Penicillium</i> sp. by NCBI GenBank database)
1	<i>Hypocreaceae</i> sp. (14%)	5	<i>Diaporthaceae</i> sp. (7%)
10	<i>Humicola</i> sp. (8%)	4	<i>Rhytismataceae</i> sp. (5%)
5	<i>Diaporthaceae</i> sp. (8%)	7	<i>Cryptococcus terreus</i> (5%)
3	<i>Trichocomaceae</i> sp. (6%)	9	<i>Parmelina</i> sp. (2%)
4	<i>Rhytismataceae</i> sp. (5%)	3	<i>Trichocomaceae</i> sp. (1%)
12	<i>Umbelopsis ramanniana</i> var <i>angulispora</i> (4%)	17	<i>Tuckermannopsis platyphylla</i> (1%)
9	<i>Parmelina</i> sp. (2%)	10	<i>Humicola</i> sp. (0.9%)
17	<i>Tuckermannopsis platyphylla</i> (2%)	12	<i>Umbelopsis ramanniana</i> var <i>angulispora</i> (0.7%)
8	<i>Ascomycota</i> sp. (2%)	2	<i>Clitopilus</i> sp. (0.6%)
2	<i>Clitopilus</i> sp. (1%)	16	<i>Leotiomyces</i> sp. (0.6%)
14	<i>Pseudeurotium</i> sp. (1%)	35	<i>Atheliaceae</i> sp. (0.5%)
26	<i>Atheliaceae</i> sp. (1%)	19	<i>Ascomycota</i> sp. (0.5%)
22	<i>Pezizomyces</i> sp. (0.8%)	44	<i>Pseudeurotium</i> sp. (0.5%)
15	<i>Pezizaceae</i> sp. (0.8%)	8	<i>Ascomycota</i> sp. (0.5%)
16	<i>Leotiomyces</i> sp. (0.7%)	41	<i>Leotiomyces</i> sp. (0.4%)
20	<i>Penicillium atrovenetum</i> (0.7%)	15	<i>Pezizaceae</i> sp. (0.3%)
21	<i>Physcia magnussonii</i> (0.6%)	28	<i>Scutellinia</i> sp. (0.3%)
32	<i>Atheliaceae</i> sp. (0.5%)	32	<i>Atheliaceae</i> sp. (0.3%)

Appendix F. The top twenty species in Ag contaminated soil at 100 mg kg⁻¹ with the addition of *T. harzianum* (T22) in soil (Chapter 5).

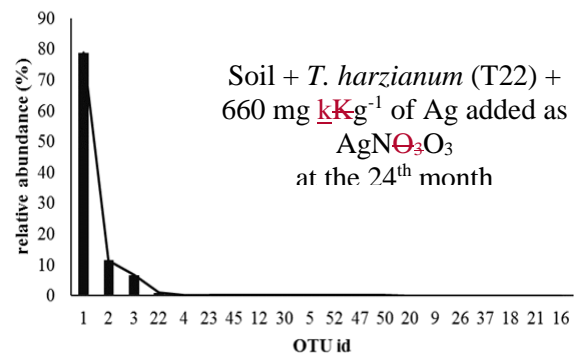
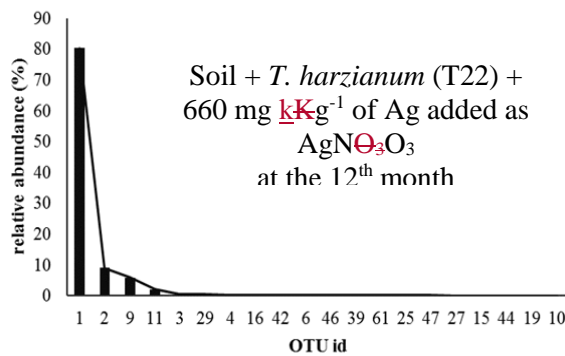
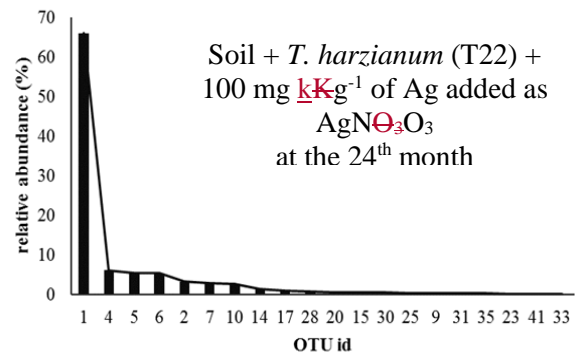
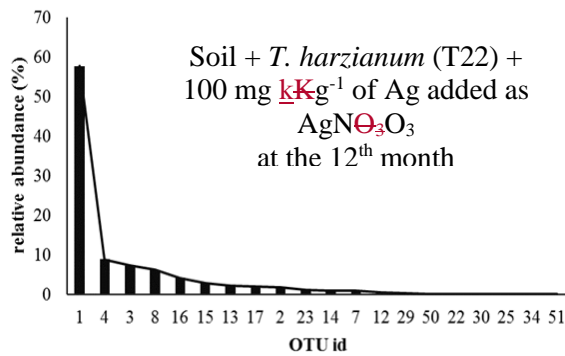
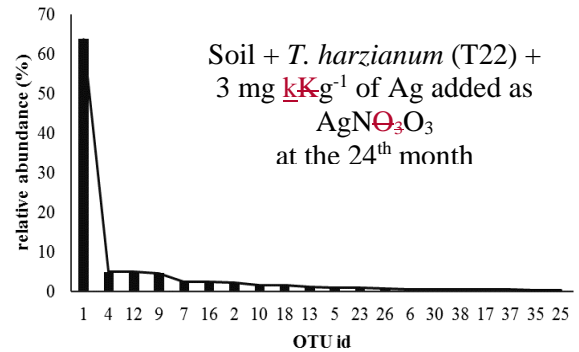
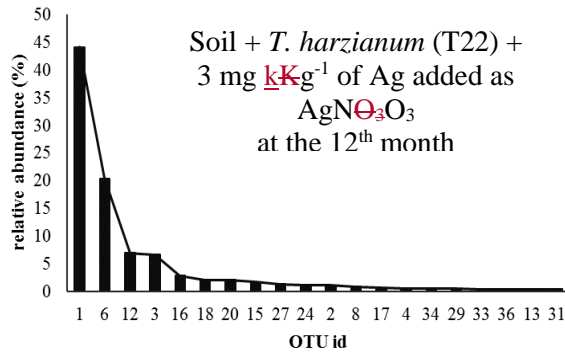
The top twenty species in AgNPs contaminated soil at 100 mg Kg⁻¹ with the addition of *T. harzianum* (T22) sampled at the 12th and 24th month. Percentages in brackets show the percentage of relative abundance each species.

Fungal species in soil+AgNPs at 100 mg kg⁻¹+T22	
12th month	24th month
<i>Hypocreaceae</i> sp. (69%)	<i>Hypocreaceae</i> sp. (62%)
<i>Cryptococcus terreus</i> (9%)	<i>Cryptococcus terreus</i> (10%)
<i>Trichocomaceae</i> sp. (3%)	<i>Trichocomaceae</i> sp. (4%)
<i>Pseudeurotium</i> sp. (2%)	<i>Diaporthaceae</i> sp. (3%)
<i>Diaporthaceae</i> sp. (2%)	<i>Penicillium</i> sp. (3%)
<i>Humicola</i> sp. (1%)	<i>Pseudeurotium</i> sp. (2%)
<i>Penicillium</i> sp. (1%)	<i>Ascomycota</i> sp. (1%)
<i>Tuckermannopsis platyphylla</i> (1%)	<i>Clitopilus</i> sp. (1%)
<i>Ascomycota</i> sp. (0.9%)	<i>Seimatosporium</i> sp. (1%)
<i>Seimatosporium</i> sp. (0.8%)	<i>Ascomycota</i> sp. (0.8%)
<i>Ascomycota</i> sp. (0.8%)	<i>Tuckermannopsis platyphylla</i> (0.7%)
<i>Pezizaceae</i> sp. (0.7%)	<i>Humicola</i> sp. (0.7%)
<i>Clitopilus</i> sp. (0.5%)	<i>Atheliaceae</i> sp. (0.7%)
<i>Atheliaceae</i> sp. (0.3%)	<i>Fungi</i> sp. (0.4%)
<i>Physcia magnussonii</i> (0.3%)	<i>Pezizaceae</i> sp. (0.4%)
<i>Diaporthaceae</i> sp. (0.3%)	<i>Oidiodendron truncatum</i> (0.3%)
<i>Oidiodendron truncatum</i> (0.3%)	<i>Trichocomaceae</i> sp. (0.3%)
<i>Ramalina confirmata</i> (0.2%)	<i>Caloplaca lenae</i> (0.3%)
<i>Ascomycota</i> sp. (0.2%)	<i>Cristinia Helvetica</i> (0.3%)
<i>Pezizaceae</i> sp. (0.2%)	<i>Pezizomycetes</i> sp. (0.3%)

The top twenty species in AgNO₃ contaminated soil at equal dose of 100 mg kg⁻¹ of AgNPs with the addition of *T. harzianum* (T22) sampled at the 6th, 12th and 24th month. Percentages in brackets show the percentage of relative abundance each species.

Fungal species in soil+Ag at 100 mg kg⁻¹ (added as AgNO₃)+T22		
6th month	12th month	24th month
<i>Gymnopilus junonius</i> (23%)	<i>Hypocreaceae</i> sp. (57%)	<i>Hypocreaceae</i> sp. (66%)
<i>Penicillium</i> sp. (18%)	<i>Ascomycota</i> sp. (8%)	<i>Cryptococcus terreus</i> (6%)
<i>Hypocreaceae</i> sp. (17%)	<i>Cryptococcus terreus</i> (7%)	<i>Ascomycota</i> sp. (5%)
<i>Cryptococcus terreus</i> (13%)	<i>Penicillium</i> sp. (6%)	<i>Penicillium</i> sp. (5%)
<i>Clitopilus</i> sp. (11%)	<i>Oidiodendron truncatum</i> (4%)	<i>Trichocomaceae</i> sp. (3%)
<i>Trichocomaceae</i> sp. (6%)	<i>Pseudochaete rigidula</i> (3%)	<i>Oidiodendron truncatum</i> (3%)
<i>Oidiodendron truncatum</i> (2%)	<i>Ascomycota</i> sp. (2%)	<i>Gymnopilus junonius</i> (2%)
<i>Pseudeurotium</i> sp. (1%)	<i>Gymnopilus junonius</i> (2%)	<i>Clitopilus</i> sp. (1%)
<i>Atheliaceae</i> sp. (1%)	<i>Trichocomaceae</i> sp. (1%)	<i>Pseudochaete rigidula</i> (0.9%)
<i>Ascomycota</i> sp. (1%)	<i>Penicillium chrysogenum</i> (1%)	<i>Ascomycota</i> sp. (0.7%)
<i>Pezizaceae</i> sp. (0.6%)	<i>Seimatosporium</i> sp. (1%)	<i>Pezizaceae</i> sp. (0.6%)
<i>Ascomycota</i> sp. (0.3%)	<i>Clitopilus</i> sp. (0.9%)	<i>Seimatosporium</i> sp. (0.5%)
<i>Cryptococcus terricola</i> (0.3%)	<i>Diaporthaceae</i> sp. (0.5%)	<i>Pseudeurotium</i> sp. (0.5%)
<i>Pseudochaete rigidula</i> (0.2%)	<i>Pseudeurotium</i> sp. (0.3%)	<i>Scytalidium</i> sp. (0.4%)
<i>Pezizomycetes</i> sp. (0.2%)	<i>Ascomycota</i> sp. (0.2%)	<i>Diaporthaceae</i> sp. (0.4%)
<i>Clitocybe</i> sp. (0.2%)	<i>Pezizaceae</i> sp. (0.2%)	<i>Talaromyces flavus</i> (0.3%)
<i>Botryosphaeriaceae</i> sp. (0.1%)	<i>Trichocomaceae</i> sp. (0.2%)	<i>Pezizomycetes</i> sp. (0.3%)
<i>Mortierella exigua</i> (0.1%)	<i>Chaetomium globosum</i> (0.1%)	<i>Tuckermannopsis platyphylla</i> (0.2%)
<i>Atheliaceae</i> sp. (0.1%)	<i>Atheliaceae</i> sp. (0.1%)	<i>Penicillium chrysogenum</i> (0.1%)
<i>Seimatosporium</i> sp. (0.1%)	<i>Talaromyces</i> sp. (0.1%)	<i>Cristinia Helvetica</i> (0.1%)

Appendix G. Impact of Ag concentration at 3, 100, 660 mg kg⁻¹ (added as AgNO₃) and *T. harzianum* (T22) addition into soil on fungal species evenness at the 12th and 24th month (Chapter 5).



Appendix H. The top twenty species in Ag contaminated soil at 3, 100, and 660 mg kg⁻¹ (added as AgNPs) with the addition of *T. harzianum* (T22) at the 24th month observation. Percentages in brackets show the percentage of relative abundance each species (Chapter 5).

3 mg kg⁻¹ of Ag	100 mg kg⁻¹ of Ag	660 mg kg⁻¹ of Ag
<i>Hypocreaceae</i> sp. (55%)	<i>Hypocreaceae</i> sp. (62%)	<i>Hypocreaceae</i> sp. (71%)
<i>Fungi</i> sp. (0.05%) (Identified as <i>Penicillium</i> sp. by NCBI GenBank database)	<i>Cryptococcus terreus</i> (10%)	<i>Clitopilus</i> sp. (13%)
<i>Diaporthaceae</i> sp. (7%)	<i>Trichocomaceae</i> sp. (4%)	<i>Trichocomaceae</i> sp. (3%)
<i>Rhytismataceae</i> sp. (5%)	<i>Diaporthaceae</i> sp. (3%)	<i>Simplicillium lamellicola</i> (3%)
<i>Cryptococcus terreus</i> (4%)	<i>Penicillium</i> sp. (3%)	<i>Ascomycota</i> sp. (3%)
<i>Trichocomaceae</i> sp. (3%)	<i>Pseudeurotium</i> sp. (2%)	<i>Pseudeurotium</i> sp. (2%)
<i>Parmelina</i> sp. (1%)	<i>Ascomycota</i> sp. (1%)	<i>Chaetomium globosum</i> (0.2%)
<i>Ascomycota</i> sp. (1%)	<i>Clitopilus</i> sp. (1%)	<i>Cryptococcus terreus</i> (0.2%)
<i>Ascomycota</i> sp. (0.7%)	<i>Seimatosporium</i> sp. (1%)	<i>Trichoderma spirale</i> (0.2%)
<i>Pseudeurotium</i> sp. (0.5%)	<i>Ascomycota</i> sp. (0.8%)	<i>Tuckermannopsis</i> <i>platyphylla</i> (0.1%)
<i>Tuckermannopsis</i> <i>platyphylla</i> (0.5%)	<i>Tuckermannopsis</i> <i>platyphylla</i> (0.7%)	<i>Mortierella</i> sp. (0.1%)
<i>Leotiomyces</i> sp. (0.4%)	<i>Humicola</i> sp. (0.7%)	<i>Diaporthaceae</i> sp. (0.05%)
<i>Humicola</i> sp. (0.3%)	<i>Atheliaceae</i> sp. (0.7%)	<i>Fungi</i> sp. (0.05%) Identified as <i>Penicillium</i> sp. by NCBI GenBank database)
<i>Atheliaceae</i> sp. (0.3%)	<i>Fungi</i> sp. (0.4%) (Identified as <i>Penicillium</i> sp. by NCBI GenBank database)	<i>Rhytismataceae</i> sp. (0.05%)
<i>Seimatosporium</i> sp. (0.2%)	<i>Pezizaceae</i> sp. (0.4%)	<i>Penicillium atrovenetum</i> (0.05%)
<i>Pezizaceae</i> sp. (0.2%)	<i>Oidiodendron truncatum</i> (0.3%)	<i>Atheliaceae</i> sp. (0.05%)
<i>Atheliaceae</i> sp. (0.2%)	<i>Trichocomaceae</i> sp. (0.3%)	<i>Penicillium</i> sp. (0.05%)
<i>Penicillium atrovenetum</i> (0.1%)	<i>Caloplaca lenae</i> (0.3%)	<i>Penicillium</i> sp. (0.05%)
<i>Umbelopsis ramanniana</i> var <i>angulispora</i> (0.1%)	<i>Cristinia Helvetica</i> (0.3%)	<i>Clitocybe</i> sp. (0.05%)
<i>Clitopilus</i> sp. (0.1%)	<i>Pezizomycetes</i> sp. (0.3%)	<i>Physcia magnussonii</i> (0.05%)

Appendix I. The top twenty species in Ag contaminated soil at 3, 100, and 660 mg kg⁻¹ (added as AgNO₃) with the addition of *T. harzianum* (T22) at the 12th and 24th month observation. Percentages in brackets show the percentage of relative abundance each species.

Fungal species in AgNO₃ contaminated soil at the 12th month sampling time		
3 mg kg⁻¹ of Ag	100 mg kg⁻¹ of Ag	660 mg kg⁻¹ of Ag
<i>Hypocreaceae</i> sp. (44%)	<i>Hypocreaceae</i> sp. (57%)	<i>Hypocreaceae</i> sp. (80%)
<i>Fungi</i> sp. (20%) (Identified as <i>Penicillium</i> sp. by NCBI GenBank database)	<i>Ascomycota</i> sp. (8%)	<i>Trichocomaceae</i> sp. (9%)
<i>Diaporthaceae</i> sp. (7%)	<i>Cryptococcus terreus</i> (7%)	<i>Penicillium atrovenetum</i> (5%)
<i>Cryptococcus terreus</i> (6%)	<i>Penicillium</i> sp. (6%)	<i>Simplicillium lamellicola</i> (2%)
<i>Oidiodendron truncatum</i> (2%)	<i>Oidiodendron truncatum</i> (4%)	<i>Cryptococcus terreus</i> (0.4%)
<i>Parmelina</i> sp. (2%)	<i>Pseudochaete rigidula</i> (3%)	<i>Pseudeurotium</i> sp. (0.3%)
<i>Rhytismataceae</i> sp. (2%)	<i>Ascomycota</i> sp. (2%)	<i>Ascomycota</i> sp. (0.3%)
<i>Pseudochaete rigidula</i> (1%)	<i>Gymnopilus junonius</i> (2%)	<i>Oidiodendron truncatum</i> (0.2%)
<i>Tuckermannopsis platyphylla</i> (1%)	<i>Trichocomaceae</i> sp. (1%)	<i>Cryptococcus terricola</i> (0.1%)
<i>Humicola</i> sp. (1%)	<i>Penicillium chrysogenum</i> (1%)	<i>Fungi</i> sp. (0.1%) (Identified as <i>Penicillium</i> sp. by NCBI GenBank database)
<i>Trichocomaceae</i> sp. (1%)	<i>Seimatosporium</i> sp. (1%)	<i>Lecanora</i> sp. (0.1%)
<i>Penicillium</i> sp. (0.8%)	<i>Clitopilus</i> sp. (0.9%)	<i>Pezizomycetes</i> sp. (0.1%)
<i>Gymnopilus junonius</i> (0.7%)	<i>Diaporthaceae</i> sp. (0.5%)	<i>Saccharomycetales</i> sp. (0.1%)
<i>Ascomycota</i> sp. (0.6%)	<i>Pseudeurotium</i> sp. (0.3%)	<i>Chaetomium globosum</i> (0.1%)
<i>Atheliaceae</i> sp. (0.5%)	<i>Ascomycota</i> sp. (0.2%)	<i>Rhytismataceae</i> sp. (0.1%)
<i>Pseudeurotium</i> sp. (0.5%)	<i>Pezizaceae</i> sp. (0.2%)	<i>Tuckermannopsis platyphylla</i> (0.05%)
<i>Neosartorya aurata</i> (0.5%)	<i>Trichocomaceae</i> sp. (0.2%)	<i>Pseudochaete rigidula</i> (0.05%)
<i>Leotiomyces</i> sp. (0.4%)	<i>Chaetomium globosum</i> (0.1%)	<i>Ramalina confirmata</i> (0.05%)
<i>Ascomycota</i> sp. (0.4%)	<i>Atheliaceae</i> sp. (0.1%)	<i>Helotiales</i> sp. (0.05%)
<i>Diaporthaceae</i> sp. (0.4%)	<i>Talaromyces</i> sp. (0.1%)	<i>Trichoderma spirale</i> (0.05%)

Fungal species in AgNO₃ contaminated soil at the 24th month sampling time		
3 mg kg⁻¹ of Ag	100 mg kg⁻¹ of Ag	660 mg kg⁻¹ of Ag
<i>Hypocreaceae</i> sp. (63%)	<i>Hypocreaceae</i> sp. (66%)	<i>Hypocreaceae</i> sp. (78%)
<i>Cryptococcus terreus</i> (5%)	<i>Cryptococcus terreus</i> (6%)	<i>Trichocomaceae</i> sp. (11%)
<i>Fungi</i> sp. (5%) (Identified as <i>Podospora ellisiana</i> by NCBI GenBank database)	<i>Ascomycota</i> sp. (5%)	<i>Simplicillium lamellicola</i> (6%)
<i>Diaporthaceae</i> sp. (4%)	<i>Penicillium</i> sp. (5%)	<i>Penicillium atrovenetum</i> (0.9%)
<i>Oidiodendron truncatum</i> (2%)	<i>Trichocomaceae</i> sp. (3%)	<i>Cryptococcus terreus</i> (0.3%)
<i>Rhytismataceae</i> sp. (2%)	<i>Oidiodendron truncatum</i> (3%)	<i>Tuckermannopsis platyphylla</i> (0.2%)
<i>Trichocomaceae</i> sp. (2%)	<i>Gymnopilus junonius</i> (2%)	<i>Penicillium chrysogenum</i> (0.2%)
<i>Gymnopilus junonius</i> (1%)	<i>Clitopilus</i> sp. (1%)	<i>Fungi</i> sp. (0.2%) (Identified as <i>Podospora ellisiana</i> by NCBI GenBank database)
<i>Parmelina</i> sp. (1%)	<i>Pseudochaete rigidula</i> (0.9%)	<i>Pseudeurotium</i> sp. (0.2%)
<i>Diaporthaceae</i> sp. (1%)	<i>Ascomycota</i> sp. (0.7%)	<i>Ascomycota</i> sp. (0.2%)
<i>Ascomycota</i> sp. (1%)	<i>Pezizaceae</i> sp. (0.6%)	<i>Leotiomyces</i> sp. (0.1%)
<i>Tuckermannopsis platyphylla</i> (1%)	<i>Seimatosporium</i> sp. (0.5%)	<i>Cantharellales</i> sp. (0.1%)
<i>Leotiomyces</i> sp. (0.7%)	<i>Pseudeurotium</i> sp. (0.5%)	<i>Sclerotiniaceae</i> sp. (0.1%)
<i>Penicillium</i> sp. (0.7%)	<i>Scytalidium</i> sp. (0.4%)	<i>Pezizaceae</i> sp. (0.05%)
<i>Pseudeurotium</i> sp. (0.6%)	<i>Diaporthaceae</i> sp. (0.4%)	<i>Diaporthaceae</i> sp. (0.05%)
<i>Humicola</i> sp. (0.5%)	<i>Talaromyces flavus</i> (0.3%)	<i>Leotiomyces</i> sp. (0%)
<i>Pseudochaete rigidula</i> (0.5%)	<i>Pezizomyces</i> sp. (0.3%)	<i>Physcia magnussonii</i> (0%)
<i>Physcia magnussonii</i> (0.5%)	<i>Tuckermannopsis platyphylla</i> (0.2%)	<i>Parmelina</i> sp. (0%)
<i>Pezizomyces</i> sp. (0.4%)	<i>Penicillium chrysogenum</i> (0.1%)	<i>Ascomycota</i> sp. (0%)
<i>Scytalidium</i> sp. (0.3%)	<i>Cristinia Helvetica</i> (0.1%)	<i>Rhytismataceae</i> sp. (0%)