Status and molecular identification of arbuscular mycorrhizal (AM) fungi associated with *Acacia* spp. on rehabilitated gold and uranium mine tailings

Michelle Toni Buck

A Dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science.

Declaration

I, Michelle Toni Buck (Student number: 322062), declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

Buck

Michelle Toni Buck

27th day of August 2014

Abstract

Phytoremeditation of mine tailings provides the most cost-effective means of alleviating their pollutant effects. Research has shown that successful revegetation of mine tailings can be optimised by providing appropriate microbial symbionts for the plants. The aim of this study was to assess the arbuscular mycorrhizal (AM) status of trees currently being used for phytoremediation trials of mine tailings in the Welkom gold fields, and to determine the AM fungal diversity of these sites. The *Acacia* spp. analysed were growing on rehabilitated gold and uranium mine tailings which had undergone different rehabilitation regimes. Planted acacia trees which had been inoculated with crude AM fungal inocula were present on one mine tailing site as compared to the second mine tailing site on which the acacias were naturally colonisers and the site had been ameliorated with garden refuse. Root and slime samples were collected in early spring and half if each initial sample was used immediately for colonisation analysis and to identify AM fungi through molecular analysis of the small subunit rRNS gene sequences; the other half of each sample was used to produce trap cultures which were used later for colonisation and molecular analysis. Total AM fungal colonisation of initial samples for planted acacies was 19 % and for naturally colonising acacias was 66 %. The total AM fungal colonisation of trap culture samples for planted acacias increased to 32 % and for naturallhy colonising acacias it increased to 78 %. Spore counts of initial samples averaged 402 spores per 100 g^{-1} soil for planted acacias and 455 spores per 100 g^{-1} soil for naturally colonising acacias. For trap culture samples, spore counts decreased by approximately 50 %. The AM fungi identified fell within 8 genera, namely, *Diversispora*, *Rhizophagus*, *Scutellospora*, *Claroideoglomus*, *Cetraspora*, *Sclerocystis*, *Glomus* and *Redecker*. The study represents a first report utilising molecular biosystematics with AM fungal DNA from colonised roots as the template. The results will assist in making decisions about future AM fungal surveys and applying AM fungal inoculum in phytoremediation trials of mine waste sites.

Key words: Phytoremediation, mine tailings, arbuscular mycorrhizal (AM) fungus, *Acacia*, molecular identification, SSU rRNA gene sequence

Acknowledgements

I would like to extend my appreciation to my supervisors Professor C. J. Staker and Dr D. Mavri-Damelin for all their help and support throughout the duration of my project and for always being available to assist in resolving problems as the project progressed. I would also like to thank Brian Dawson, Prof. C. J. Straker and Alexander Spruyt for all their assistance in the field. Furthermore, I would like to thank the South African Weather Service for the information they provided for my project. I would also like to extend my thanks to the ABDC barcoding unit at the University of Johannesburg for the use of their laboratory and their facilities. Finally I would like to thank the National Research Foundation (NRF) and Anglo Gold Ashanti for financial assistance.

Research output

Original publications

A. Spruyt, M. T. Buck, A. Mia, C. J. Straker (2014) Arbuscular mycorrhiza (AM) status of rehabilitation plants of mine wastes in South Africa and determination of AM fungal diversity by analysis of the small subunit rRNA gene sequences. *South African Journal of Botany* **94**: 231-237.

Conference attendance

Oral presentations

University of the Witwatersrand 5th Cross-Faculty Graduate Symposium
1 August 2013
Wits Professional Development Hub, Empire Road, Johannesburg
Title: Status and molecular identification of arbuscular mycorrhizal fungi (AM fungi) on rehabilitated gold and uranium mine tailings.
Authors: M. T. Buck, C. J. Straker and D. Mavri-Damelin

South African Society of Microbiology 18th Biennial Conference
27 November 2013
Forever Resorts Warmbaths – Bela Bela
Title: Status and molecular identification of arbuscular mycorrhizal fungi (AM fungi) associated with *Acacia* species growing on rehabilitated gold and uranium mine tailings.
Authors: M. T. Buck, C. J. Straker and D. Mavri-Damelin

Table of contents

List of figures

List of tables

Chapter 1

Literature review

1.1 Introduction and background to project

Heavy metal (HM) pollution is toxic not only for the environment but to people as well. Examples of toxic effects for plants include growth inhibition and protein alteration leading to the disruption of enzymatic reactions (Clemens, 2006; Göhre and Paszkowski, 2006). Mining in South Africa has led to the creation of mine tailings which comprise the excess slime (sludge) which is created once the ore and thus mineral, for instance gold, has been extracted from mined rock. HMs that can be found in mine tailings include uranium, mercury, lead and arsenic. Unfortunately, when the first mine tailings were created, none of the toxicity and its effects was known, and HM pollution has more recently become a major concern. Mine tailings are wind-dispersed and leach HMs and other toxic trace elements into underground water supplies, thus leaving a large contaminated footprint (Straker *et al.* 2007; Weiersbye and Witkowski, 2007). The present research is critical for the successful rehabilitation of existing and newly formed mine tailings.

Mine tailing rehabilitation research strategies must focus on developing sustainable, accessible, environmentally-friendly and affordable techniques. The use of plants (phytoremediation) is one such inexpensive, sustainable on-site method (Krämer, 2005; Peuke and Rennenberg, 2005) as opposed to other expensive, impractical off-site methods such as physical displacement or transport and storage. These methods ultimately leave the site lacking any soil microflora as well as just translocating the problem (Göhre and Paszkowski, 2006). Phytoremediation detoxifies soil using two main strategies, namely phytostabilization, whereby plants bind HMs in the soil, and phytoextraction, whereby the plants take-up and store HMs in their aerial parts (Göhre and Paszkowski, 2006).

Until fairly recently, mine tailings have been rehabilitated with HM-intolerant, exotic (to South Africa, the area or the biome), grassland plant species, which only survive over the period of irrigation and fertilisation, for example only 5% of the 400 species introduced to two mine fields in the North-West and Free State Provinces survived after 6 years (Straker *et al.*, 2007). The predominant plant types that naturally colonise mine tailings are slow-growing, woody, semi-woody and perennial (Weiersbye *et al.*, 2006). *Acacia* spp. were chosen for this study as they are indigenous, are able to colonise mine tailings and are useful

in increasing soil fertility through their decomposition of foliage and through nitrogen fixation (Smit, 1999).This latter feature should aid in the self maintenance of the plant community as well as support colonisation by other plant species. However, *Acacia* spp. are slow growing plants and thus it is necessary to identify methods that optimise their growth rate. One such method is the colonisation of the *Acacia* spp. root material with compatible and HM-resistant arbuscular mycorrhiza (AM) fungal species. AM fungi provide the host plant with an increased capacity to absorb water and nutrients, such as phosphorus, from the soil (Entry *et al.*, 2002), thus AM fungal colonisation alleviates nutrient stress of the host plant and consequently increases plant growth (Smith and Read, 2008). AM fungi can have wide host ranges (Ruiz-Lozano *et al.*, 1995) but the effects of AM fungal colonisation on the plant are very plant species and even cultivar (of the same plant species) specific. AM fungi also differ in their sensitivities and/or tolerances to various ecosystems, soil types, pH and the levels of different soil components. It is noteworthy to mention that the percentage of colonisation, spore density and species diversity of AM in the host plant is directly proportional to the accessible root surface area and thus the length of the root (Straker *et al.*, 2007). Host roots also tend to stimulate the sporulation and growth of AM fungi (Straker *et al.*, 2008). It is imperative that data regarding the species identity of AM fungi associated with different host plant species and in different soil types is collated and communicated between researchers (especially those working in different geographical locations) (Schenck and Peréz, 1990).

It is expected that the use of effective growth promoting AM fungi combined with naturally colonising rhizobia (nitrogen-fixing bacteria) will enhance the growth rate as well as improve the host plants' resistance to low nutrient soils and HM pollution. Mine tailing land restoration or erosion control programmes can be implemented using the benefits of mycorrhizal symbioses which allow for increased soil aggregation by the extramatrical hyphal network (Tisdall, 1994) which will increase plant diversity (Dodd and Thomson, 1994). Thus identification of natural AM fungal colonisers of *Acacia* spp. is necessary in order to select for AM fungal species tolerant to the conditions found in mine tailings so as to optimise the growth of planted *Acacia* spp. through inoculation and thus enhance the rehabilitation of mine tailings.

The rationale behind this project is to improve rehabilitation of mine tailings through the application of biological methods and to stimulate further research activity leading to

practical applications. The project objectives required the collection of samples pertaining to two differently rehabilitated gold and uranium mine tailings in the Free State Province, where important biological data is being lost due to re-processing of mine tailings, and the identification of AM fungi naturally associated with *Acacia* spp. The production of the AM fungi from the sites using trap cultures with non-specific host plant species was also required in order to identify all AM fungi from the sites. The soil chemistry and characteristics were also analysed to provide background information.

1.2 Heavy metal pollution and the consequences

Heavy metals (HM) are considered to be metals with a density/specific mass above 5 $g.cm^{-3}$ (Nies, 1999; Hooda, 2010) and include copper (Cu), zinc (Zn), cadmium (Cd), lead (Pd), iron (Fe), nickel (Ni), silver (Ag), thorium (Th), uranium (U), mercury (Hg) and arsenic (As) (Kapoor and Virarghavan, 1995; Nies, 1999; Khan, 2005; Clemens, 2006; Hooda, 2010; Kabata-Pendias, 2011) with the total being 53 of the periodic elements (Nies, 1999). Several of these metals are essential for plants and most other organisms; unfortunately, they become toxic when present in excess (Weiersbye *et al.*, 1999; Clemens, 2006; Göhre and Paszkowski, 2006; Hooda, 2010).

Trace metals typically occur in soils with concentrations lower than 100 $mg.kg^{-1}$ or < 0.1% and in general many of the metals occur in even lower concentrations (Leyval *et al.*, 1997; Hooda, 2010); for instance, the Zn concentration tends to be 80 ppm or 36.6 $mg.kg^{-1}$, Cd occurs in concentrations of 0.1-0.2 ppm or 0.4 $mg.kg^{-1}$ and Pb concentration is often 15 ppm or 33.6 $mg.kg^{-1}$ (Göhre and Paszkowski, 2006; Oliveira and Pampulha, 2006). When trace elements occur in excess and the chemical state of soil deviates from its normal composition and causes negative effects on organisms, the soil is considered to be polluted (Kabata-Pendias, 2011). Examples of HM concentrations when found in polluted soils include Zn > 20 000 ppm, 8.3% or 165.5 $mg.kg^{-1}$; Cd > 14 000 ppm, 863 $\mu g.g^{-1}$ or 1.1 $mg.kg^{-1}$ and Pb > 7 000 ppm or 270.8 $mg.kg^{-1}$ (Leyval *et al.*, 1997; Göhre and Paszkowski, 2006; Oliveira and Pampulha, 2006).

The relevant areas of pollution for agriculture and thus humans involve soil and water; however, soil will be the main target in this study as there is limited new arable land with the increase in the human population (Xavier and Boyetchko, 2002; Clemens, 2006). Soils,

which are a target for a large variety of various contaminants, are polluted through a range of actions, which may either be anthropogenic or natural in origin (Clemens, 2006; Oliveria and Pampulha, 2006; Kavamura and Esposito, 2010). Anthropogenic actions include mining activities, liming materials, application of sewage sludge in agriculture, chemicals used in agriculture and industrial activities (FließBach *et al.*, 1994; Valsecchi *et al.*, 1995; Del Val *et al.*, 1999; Rösner *et al.*, 2001; Khan, 2005; Göhre and Paszkowski, 2006; Oliveira and Pampulha, 2006; Tack, 2010) and natural sources include proximity to an ore body (Glick, 2003). More specifically, industrial activities include waste disposal, waste incineration and vehicle exhausts (Khan, 2005) and mining activities include mine tailings storage facilities (TSFs) (Weiersbye and Wikowski, 2007), uncontrolled release of acid mine drainage (AMD) and wind-blown tailings material, as well as a large contaminated footprint on the subsurface from reclaimed tailings material (Rösner *et al.*, 2001; Weiersbye and Witkowski, 2007). All these sources cause accumulation of HMs in soils and present a threat to food safety and possible health risks as a result of soil-to-plant transfer of the HMs (Khan, 2005).

HM loadings of soil particularly affect biological processes (Kelly and Tate, 1998). The effects on organisms from HMs in the soil depend on several factors including HM solubility and organism sensitivity. The physical and chemical characteristics of soil determine the mobility, solubility and speciation of HMs (Kabata-Pendias, 1992; Borůvka and Drábek, 2004; Khan, 2005; Khan, 2006; Tack, 2010, Kabata-Pendias, 2011). In general HMs have a low solubility due to their strong interaction with soil particles (Clemens *et al.*, 2002; Clemens, 2006). Chemical changes in the soil, such as acidification or organic matter content, increase the solubility of HMs (Del Val *et al.*, 1999; Borůvka and Drábek, 2004; Kabata-Pendias, 2011). However, HMs can become irreversibly immobilized within the different soil components (Valsecchi *et al.*, 1995) and are non-biodegradable (Leyval *et al.*, 1997; Kelly and Tate, 1998; Göhre and Paszkowski, 2006) and thus linger in the soil for extended periods (FließBach *et al.*, 1994) depending on the metal (Kabata-Pendias, 2011).

Some of the negative effects on plants of high levels of HMs involve protein modification affecting enzymatic reactions, replacement of other vital elements leading to deficiency, changes in plasma membrane permeability and functionality (Göhre and Paszkowski, 2006), oxidative stress (Clemens, 2006) and any of the heavy metal ions may form unspecific complex compounds in the cell leading to toxic effects (Nies, 1999; Kavamura and Esposito, 2010). Some plant symptoms associated with excess HMs are growth retardation, chlorosis,

root browning, disturbance of plant water balance, inhibition of stomatal opening, cell cycle arrest and effects on both photosystems (Clemens, 2006; Göhre and Paszkowski, 2006). HMs in the soil also decrease the functional diversity of soil organisms and microorganisms (Arriagada *et al.*, 2007; Kavamura and Esposito, 2010). As well as these detrimental effects of HMs, they have also been found to be associated with the development of cancers (Türkdoğan *et al.*, 2002).

1.3 Gold slimes dams in South Africa

1.3.1 History

Gold bearing reefs were first discovered in the Witwatersrand basin in 1886 at Langlaagta by George Harrison (Adamson, 1972; Janisch, 1986; Foster, 1993). Full scale mining started in 1887 in the Central Rand and later, gold fields were established in the Orange Free State in 1951 (gold was discovered here late 1940s), and gold mines were established in Klerksdorp in 1952 and Evander in 1958 (Adamson, 1972; Foster, 1993). Gold in the Witwatersrand basin is comprised of conglomerate deposits, as opposed to nugget or vein formation or alluvial deposits, which tend to be low grade (Adamson, 1972). The conglomerate reef bands are characterised by the presence of ovoid pebbles, mainly vein quartz, that make up approximately 70 % of the overall volume and are embedded in a mineralogically complex matrix (Janisch, 1986). The matrix that contains the pebbles consists of a variety of minerals, including pyrite, chromite, uraninite, kerogen and secondary quartz to name a few (Janisch, 1986).

Certain reefs, for instance, the Vaal Reef, Carbon leader (Main Reef Group) and Basal Reef (Orange Free State) are characterised by thin, discontinuous layers of kerogen being present at their base (Adamson, 1972; Janisch, 1986). Kerogen is a carbonaceous material comprised of hydrocarbons, organic sulphur and oxygen compounds (Foster, 1993). Kerogen always contains uraninite and is often associated with gold, which is found on its surface, as well as filling open spaces between filaments and cleats (Janisch, 1986; Foster, 1993). Uranium, in the form of uraninite, is best preserved in the kerogen (Foster, 1993). Radioactive materials, such as uraninite, occur more frequently in the conglomerates than gold does and a large proportion of gold is associated with these materials (Adamson, 1972). Gold is often embedded in the uraninite in irregular pits and cracks and miniscule veins of gold may

sometimes traverse from surrounding masses of gold into the uraninite (Adamson, 1972; Foster, 1993).

Due to the conglomerate, the gold when mined, is intimately associated with a matrix and thus requires intensive extraction procedures whereby the ore undergoes some kind of milling in order to release the contained gold particles before concentration (Adamson, 1972). Concentration techniques include gravity concentration, amalgamation, cyanidation and flotation and these allow for the comminution of the ore in order to extract the gold from the surrounding debris (gangue) (Adamson, 1972).

The original milling in the Witwatersrand used stamp milling and the stamp-mill product was screened followed by being passed over amalgamation plates, which recovered approximately 75 % of the gold from the ore (Adamson, 1972; Janisch, 1986). Later, in the 1890s, the stamp milling was improved with the addition of screening by bar grizzleys and the screened coarse fraction was subjected to hand sorting and jaw or gyratory crushing (Janisch, 1986). The extraction process was further improved, in 1890, with the introduction and implementation of the MacArthur-Forest cyanidation and precipitation process, which improved the recovery of the gold from 75 % to 90 % (Adamson, 1972; Janisch, 1986). However, surplus slime (fine material of less than 50 μm), a result of early processes, that was slow to settle in water in the leaching tanks was impeding the flow of these tanks and thus was separated and placed on tailings (Janisch, 1986). However, the slime contained 4-5 g/t of gold and in 1894 the natural-settlement decantation process was introduced by J. R. Williams (Adamson, 1972; Janisch, 1986). In this process lime was added to assist slime settlement, which was then followed by the addition of cyanide solution to the thickened slime, which was then transferred to a second tank and circulated (Adamson, 1972; Janisch, 1986). This process dissolved most of the gold, which was then precipitated either using zinc metal or a zinc-lead couple (Adamson, 1972).

Slime dams (also referred to as mine tailings or mine dumps) are created in order to dispose of remaining slime as it has no function in industry. These slime dams have also been used to dispose of excess cyanide solution and water from underground, and manganese sludges from uranium plants (Adamson, 1972).

1.3.2 Consequences

Slimes dams have become a prominent feature in South Africa. This is problematic as soil is a biologically active surface layer that provides a medium for the roots of plants and provides most of the nutrients that they require (Bradshaw and Chadwick, 1980) and slime dams cause the land and soil to become derelict. Originally plants could not grow on the slimes dams (new methods for the restoration of slimes dams have since been developed) and wind would send clouds of dust into the air and would cause traffic problems due to poor visibility and factories that process food to halt work on windy days (Bradshaw and Chadwick, 1980). In addition to these problems, rivers also silted up and more than one lake was destroyed (Bradshaw and Chadwick, 1980). Plants could not grow on the slimes dams as they comprise of quartzite (roughly 95 % silica) and do not have any form of nutrients as well as containing 1.5-3.5 % pyrite (iron sulphide (FeS_2)) which is acidic when oxidised (Clausen, 1973; Bradshaw and Chadwick, 1980). Initially the slime dams have an alkaline pH, usually above pH 10, due to the liming step in the gold extraction process; however, this decreases over a period of a few months (roughly 2-4) as the pyrite becomes oxidised to produce sulphuric acid (H_2SO_4) (Hutnik and Davis, 1973; Bradshaw and Chadwick, 1980). The acidity is leached into the surrounding area, down underground (Clausen, 1973; Bradshaw and Chadwick, 1980) and flows into the nearest stream (Clausen, 1973). The decrease in pH increases the solubility of heavy metal compounds and minerals, for instance, Fe, Cu and Zn (Hutnik and Davis, 1973).

Early mining, before 1964, used to remove excess water from the top of the slime dams and dump it into a nearby water source (Clausen, 1973). Unfortunately, this caused large amounts of acid and dissolved solids to be loaded into the stream, which resulted in the destruction of the stream ecosystem for some distance (Clausen, 1973).

At least, the slime concentration of cyanide never exceeds 0.01 %, usually it is at 0.006 %, and at this concentration at least five litres would need to be consumed by an adult and two litres by a child in order to be a lethal dose (Adamson, 1972). With regards to the amount that animals can consume, it is proportional in the ratio of their mass to that of an adult person (Adamson, 1972). Since the dam supernatant solution is exposed to the atmosphere and sunlight, the cyanide quickly decomposes and the concentration decreases to

approximately 0.001 % during a period ranging between 24-36 hours and two months (Adamson, 1972; Bradshaw and Chadwick, 1980).

1.3.3 Welkom gold and uranium mine tailings

The Welkom gold and uranium mine tailings are located in the Free State province in South Africa. The mine tailings resulted from gold and uranium-bearing conglomerates which were associated with sediments of the Witwatersrand Basin with the primary constituents of the mine tailings being pyrophylite and unreactive quartz and virtually no organic matter (Weiersbye *et al.*, 2006). The chemical composition of the mine tailings depends on the parent substrate, the sliming method used and the age of the deposits (Weiersbye *et al.*, 2006).

Most dams in the Welkom area are situated on pans, streams or vleis, being surrounded by mesic to seasonally immersed hydromorphic grassland (Weiersbye *et al.*, 2006). The soils upon which the slimes dams were built are clayey to sandy (Weiersbye *et al.*, 2006). The mean annual precipitation is 604 mm, with the inter-annual variability being high (25-30 %), and the mean daily minima (July) and maxima (January) were < 0 °C and 27.5-30 °C between July 1996 and March 1997 (Weiersbye *et al.*, 2006). Frosts often occur in winter (roughly 150-175 days) and evaporation is 2-2.5 times higher than rainfall (Schulze, 1997). Between 2010 and 2012 (prior sampling) the mean daily minima (July) and maxima (January) were -5-7 °C and 20-34 °C with 2012 having the highest average temperature (South African Weather Service). However, the annual precipitation from 2010 to 2012 decreased from 605.4 mm in 2010 to 337.4 mm in 2011 and 269.4 mm in 2012 (South African Weather Service); unfortunately, the accuracy of this may be diminished due to some of the data being missing particularly with regards to 2012.

Several of the Welkom mine tailings have been used in phytoremediation trials.

1.4 Rehabilitation methods for slimes dams

Until recently mine tailings have been treated with non-tolerant, exotic, grassland plant species, which only survived over the period of irrigation and fertilisation (Weiersbye *et al.*, 2006; Straker *et al.*, 2007) as well as the less effective method of covering the surface of the

mine tailings with waste rock (Weiersbye *et al.*, 2006). Irrigation and fertilisation methods require extensive amounts of resources and time and are expensive (Straker *et al.*, 2007). It has since been determined that only 5% of the 400 species introduced to these sites survived after 6 years (Straker *et al*., 2007) and thus in the long run these plantings are unsuccessful post irrigation and fertilisation. 'Grassing', as the revegetation method is referred to, was selected as a result of the speed with which the grass cover is established and not on its survivability or as an efficient control of erosion (Weiersbye *et al.*, 2006). More recent techniques attempt to encourage predominant growth of perennial, mycotrophic plant species so that stable plant communities may be created (Straker *et al.*, 2008).

1.5 Phytoremediation

Phytoremediation is an alternative form of remediation that makes use of plants to extract, stabilise, sequestrate or decontaminate terrestrial environments (Leung *et al.*, 2007; Kavamura and Esposito, 2010). It is a cost effective form of remediation, is environmentally friendly (Kotrba *et al.*, 2009) and has advantages over microbial bioremediation as plants have higher biomasses with lower nutrient requirements (Kotrba *et al.*, 2009). Relatively few plants are able to grow on and tolerate the conditions of HM contaminated sites and the few that do are termed metallophytes (Hildebrant *et al.*, 2007). Plants that naturally grow on HM contaminated soils and are generally used in this method may be categorized into one of three groups, namely excluders, accumulators or indicators (Leung *et al.*, 2007). Tolerance of plants to pollution in soils depends on the bioavailability of HMs and the plants' adaptations, whether biological, physiological or chemical (Göhre and Paszkowski, 2006; Hildebrandt *et al.*, 2007; Leung *et al.*, 2007).

In the remediation of HM contaminated soils only two options are available due to the inability of HM to be degraded; namely removal of the metals (phytoextraction) or sequestration in nonbioavailable forms (phytostabilization) (Kelly and Tate, 1998; Göhre and Paszkowski, 2006). Phytoremediation can be further divided into 6 subgroups according to the method and/or nature of the contaminant; however, not all of them apply to HM remediation. These subgroups are as follows: phytoextraction, which is absorption and removal of the contaminant by the roots and concentration of the contaminant in the harvestable plant parts; phytodegradation is the degradation or mineralization of organic contaminants by specific enzyme activity of plants and associated microbes;

phytostabilisation is where the contaminants are immobilised or their mobility and bioavailability is reduced through the incorporation into lignin or soil humus, thus reducing spread of pollutants through erosion; rhizofiltration is the absorption, concentration and/or precipitation of contaminants from polluted water by plant roots; phytostimulation is the promotion of root growth to encourage the development of rhizosphere microorganisms that are able to degrade contaminants; phytovolatilisation is the volatilisation of some ions of the periodic elements of subgroups II, V and VI, such as arsenic, through absorption of these molecules by the root, conversion into less toxic forms and releasing them into the atmosphere (Leyval *et al.*, 1997; Glick, 2003; Khan, 2005; Kotrba *et al.*, 2009; Kavamura and Esposito, 2010).

The major issue with phytoremediation is the slow time by which the process is performed (Khan, 2005). This is due to the slow growth and small size of metallophytes and therefore it would be necessary to optimise the process with the current plants or select for fast growing metallophytes with extensive root systems (Khan, 2005) or the combination of both.

1.6 The genus *Acacia*

The genus *Acacia* comprises woody legumes and is the largest genus of the Mimosoideae sub-family and the second largest genus in the Leguminosae family (Ross, 1972; Carr, 1976; Smit, 1999). Acacias appear as trees (single-stemmed) or shrubs (multi-stemmed) that may be scandent (Ross, 1972; Smit, 1999). The branches have spines or curved prickles (Figure 1.1 row C images B1 and B2 and row D image B) and the foliage occurs as bipinnate leaves (Figure 1.1 row A) (Ross, 1972; Smit, 1999). Flowers (Figure 1.1 row C) may appear as racemes or panicles or may be axillary or fasciculate with the overall display either being spicate (Figure 1.1 row C image A) or capitates (Figure 2.1 row C images B1 and B2) (Ross, 1972; Smit, 1999). The fruit or pod, containing smooth, hard walled, un-winged seeds, varies in appearance and is generally dehiscent (Figure 1.1 row B) (Ross, 1972; Smit, 1999). Keys for the identification vary depending on the author and publication; however, a simple method for grouping the *Acacia* species uses the difference in shape and position of the spines (Figure 1.1 row C image B1 arrow) and/or prickles (Figure 1.1 row C image B2 arrow) to separate the genus into 5 groups, namely group 1 to group 5 (Smit, 1999).

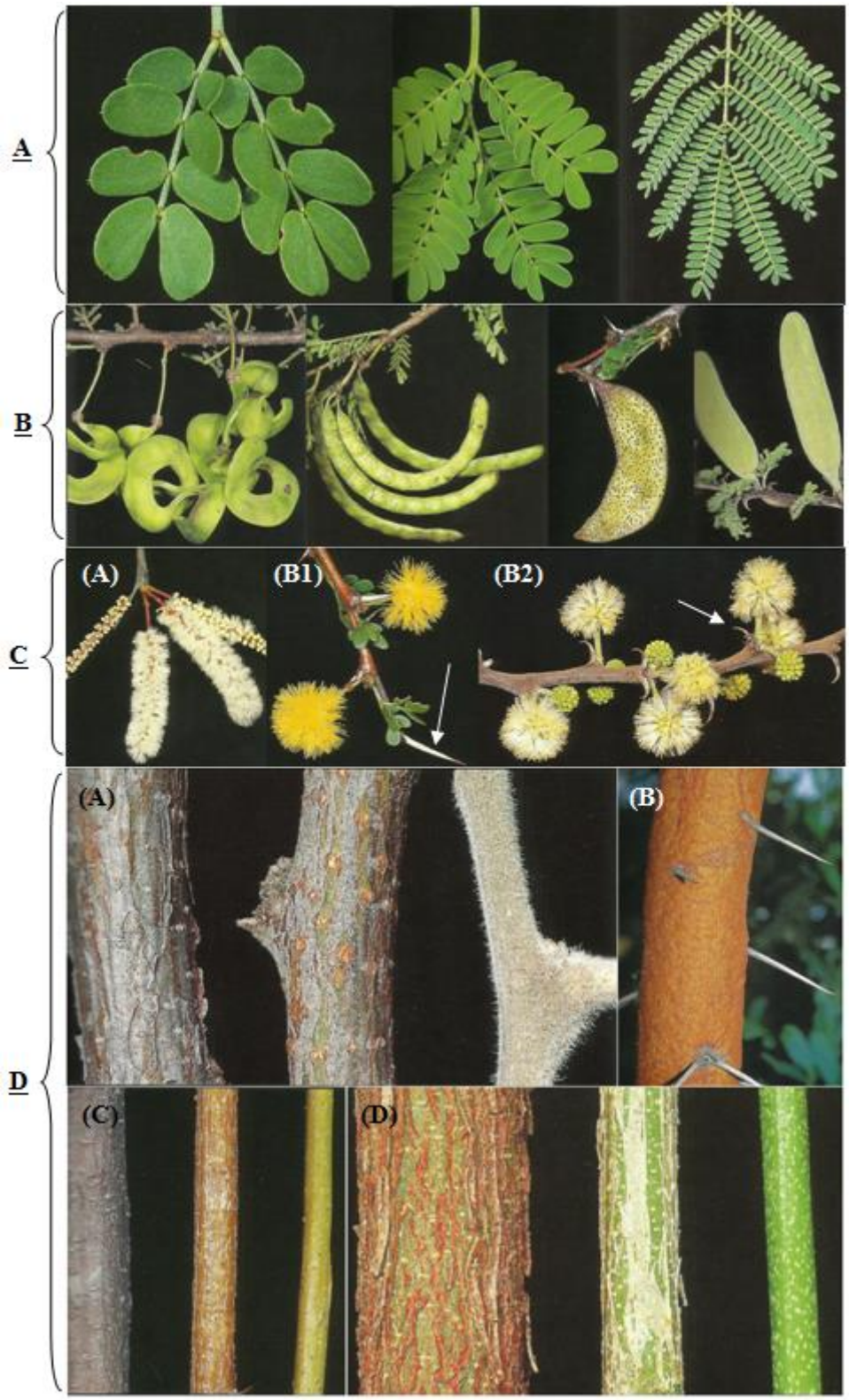

Figure 1.1: Row A shows different *Acacia* leaves; row B shows the variation in the *Acacia* pods; row C demonstrates the flower shapes as well as the difference in appearance of spikes (B1 arrow) and prickles (B2 arrow) and row D shows different appearances of trunks and branches. Illustrations taken from Smit (1999).

1.6.1 Natural habitats

The habitat of the genus depends on the species with some species able to inhabit a wide variety of habitats while others are restricted to specific ecological niches (Carr, 1976; Smit, 1999). More specifically, acacias are generally found in the tropical and sub-tropical areas of South Africa (S. A.) (Carr, 1976) with the majority being found in the savanna biome, which is the largest of the seven biomes in S. A. (Figure 1.2) covering an area of 408 876 km^2 (Smit, 1999).

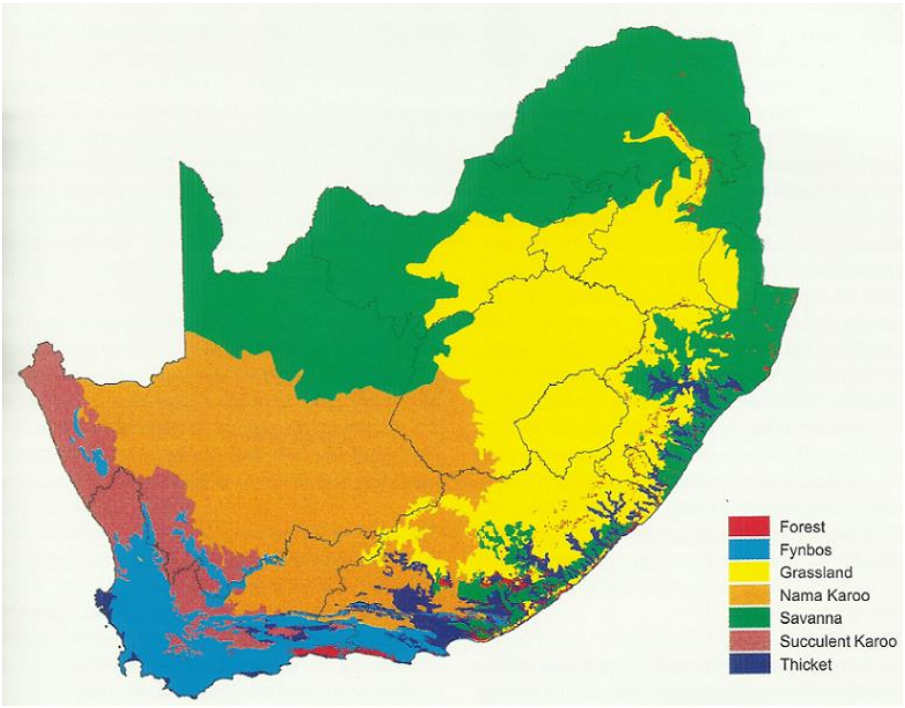

Figure 1.2: The 7 biomes of South Africa. Illustration taken from Smit (1999).

1.6.2 Uses

The tree or shrub leaves, shoots, flowers and pods act as a source of nutrients for herbivores, be it domestic live stock or game. The flowers are also useful for insects. Since acacias are legumes they tend to enhance the soil fertility as a result of nitrogen fixation by symbiotic rhizobia and also enhance soil fertility by providing litter for decomposition. Anthropogenic uses include the use of the wood as a source of fuel in cooking and heating, as fence-posts/poles, for the production of furniture and charcoal and in years gone by as railway sleepers and mining timber; the tannins are used in the leather tanning industry and parts of the trees may be used as food or medicine as well as there being several more uses for the trees (Smit, 1999).

1.7 Mycorrhizal fungi

Mycorrhizas are classified into seven classes, namely; arbuscular mycorrhizas, ectomycorrhizas, ectendomycorrhizas, arbutoid mycorrhizas, monotropoid mycorrhizas, ericoid mycorrhizas and orchid mycorrhizas (Smith and Read, 2008). Mycorrhizal fungi form symbiotic relationships in or on host plants roots and are thus ecologically significant (Entry *et al.*, 2002). In the symbiotic relationship the fungus is provided with soluble carbon sources from the host plant and in turn provides the host plant with an increased capacity to absorb water and nutrients, such as phosphorus, from the soil (Entry *et al.*, 2002). It has been reported in the literature that 90 to 95% of all land plants at some point form some sort of mycorrhizal association and thus it may be suggested that the mycorrhiza itself and not the roots are the organs for nutrient uptake by plants (Bago *et al.*, 2000; Smith and Read, 2008). It is noteworthy to mention that not only does the fungus facilitate nutrient uptake of the host plant it also improves the host plant health through improved resistance to various biotic and abiotic stresses (Lovato *et al.*, 1996; Xavier and Boyetchko, 2002).

1.7.1 Arbuscular mycorrhizal fungi (AM fungi)

AM fungi, unlike ectomycorrhizal fungi, which ensheath the host root, fail to induce obvious changes on the root surface (Norris *et al.*, 1994), but form structures within the host plant roots. The characteristic structure formed by AM fungi is the arbuscule, hence the name AM. These structures form within the cortical cells of the host plant roots. Another structure, which may be used as a diagnostic for AM symbioses along with arbuscules, is the storage vesicle, which may form within or between the cells. Other intraradical structures may be produced by the arbuscular mycorrhiza including intracellular hyphal coils, which on occasion may form in the absence of arbuscules. The formation of the various structures and overall development of the arbuscular mycorrhiza depends on and is determined by both the host plant and the fungus (Smith and Read, 2008). Figures 1.3 and 1.4 demonstrate the various structures that may be formed within the tissue of the host plant post colonisation.

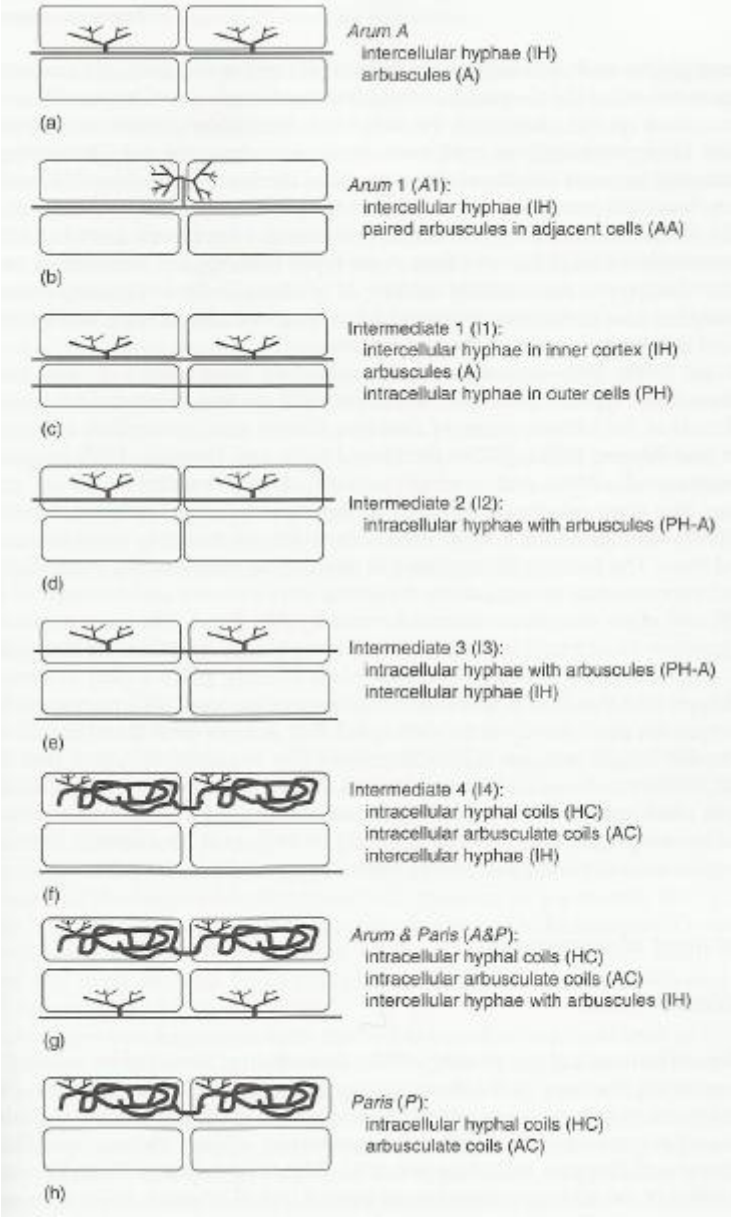

Figure 1.3: Schematic diagrams of various structures and colonisation types that may appear in the roots of different host plant species, colonised by different arbuscular mycorrhizal fungi. Illustration taken from Smith and Read (2008).

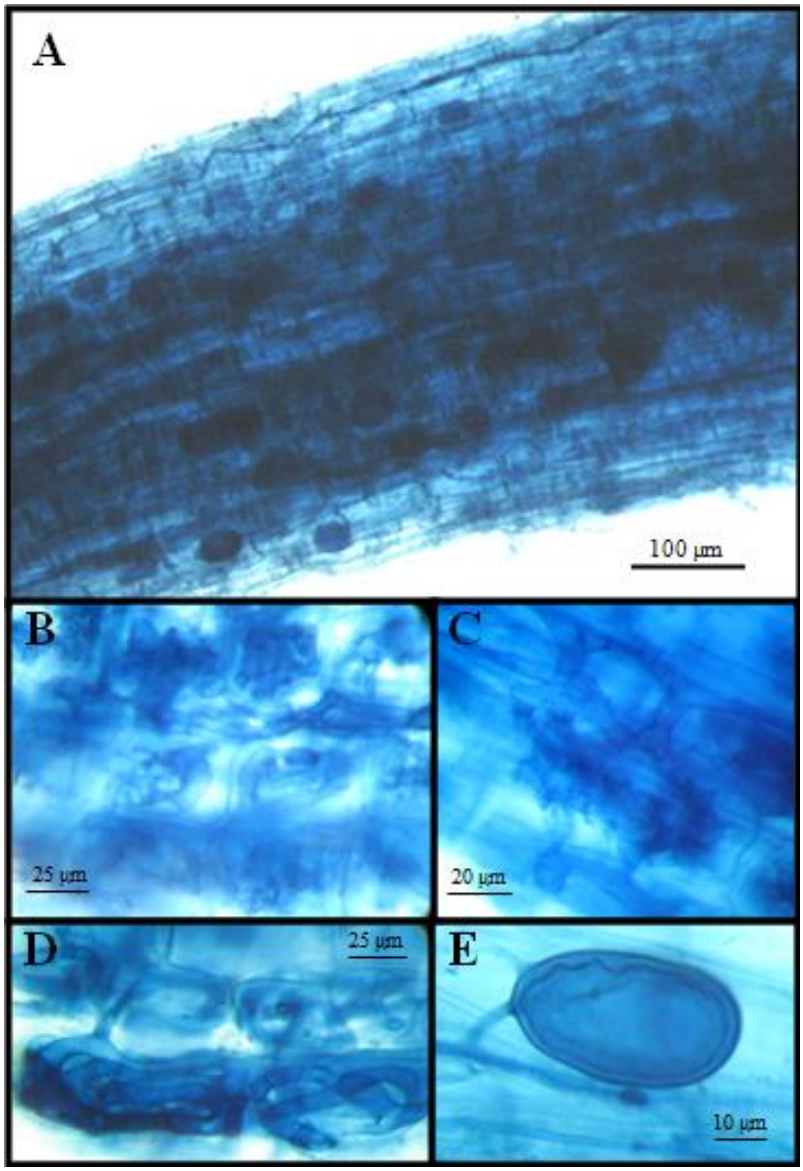

Figure 1.4: Light micrographs of intraradical fungal structures (Buck, 2011). A) Overall view of a root colonised by AM fungi. B) Root cells colonised by arbuscules. C) Young arbuscule. D) Hyphal coils. E) Vesicle attached to a hypha.

AM fungi may either be considered *Arum*-type or *Paris*-type mycorrhizas depending on the intraradical structures that they develop. *Arum*-type mycorrhizas demonstrate rapid development of intercellular hyphae that fan out from the point of penetration and extend longitudinally along intercellular spaces in the root cortex. *Arum*-type mycorrhizas form highly branched arbuscules by penetrating cortical cells with short side-branches, developing from the hyphae in the intercellular spaces, which then branch dichotomously. These arbuscules have a short life-span and the oldest arbuscules are located around the point of cortical penetration and the young and immature arbuscules are increasingly further away. This type may also produce hyphal coils when penetrating the exodermal cell layers of the

root, however, hyphal coils are not a major component of cortical colonisation (Smith and Read, 2000; Smith and Read, 2008).

Paris-type mycorrhizas are characterised by extensive growth of intracellular coiled hyphae within the cortex which develop directly from cell to cell. Arbuscules may grow from these coils and there is barely any intercellular growth (Smith and Read, 2000; Smith and Read, 2008). *Paris*-type mycorrhizal infection units grow slower than *Arum*-type infection units (Smith and Read, 2000) but are more dense and compact (Smith and Read, 2008). Vesicles may be formed by both types as the infection units age, however, members of the Gigasporaceae develop auxiliary cells on the extraradical mycelium instead of vesicles (Smith and Read, 2008).

Spores produced by the fungus are extraradical (except in the case of *Rhizophagus intraradices* which produce their spores intraradically; INVAM) and are very large being up to 500 µm in diameter and are complex consisting of abundant storage lipids, some carbohydrate, and are multinucleate containing large numbers of nuclei, with estimations ranging from 800 to 35 000 in a spore depending on the species (Hosny *et al.*, 1998; Smith and Read, 2008) (Figure 1.5). Spore walls are thick and resistant, containing chitin and in some cases ß-1,3 glucan (Gianinazzi-Pearson *et al.*, 1994; Lemoine *et al.*, 1995). The physical appearance of spores tends to vary among species of arbuscular mycorrhiza. Figure 1.6 shows the variation between spores of differing species. The degree of sporulation varies between AM fungal species (Pearson and Schweiger, 1993) as well as being influenced by environmental conditions, soil nutrient levels, host and host colonisation level (Menge *et al.*, 1978; Brundrett, 1991; Douds, 1994; Stutz and Morton, 1996).

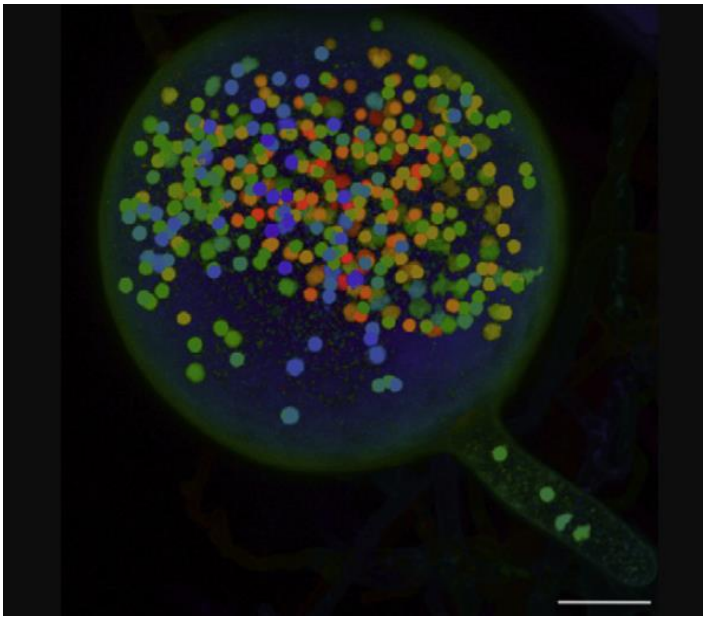

Figure 1.5: Confocal micrograph demonstrating a typical multinucleated, asexual spore of *Glomus diaphanum*. Nuclei were stained with Sytogreen fluorescent dye. Bar: 10 µm. Illustration taken from Denison and Kiers (2011).

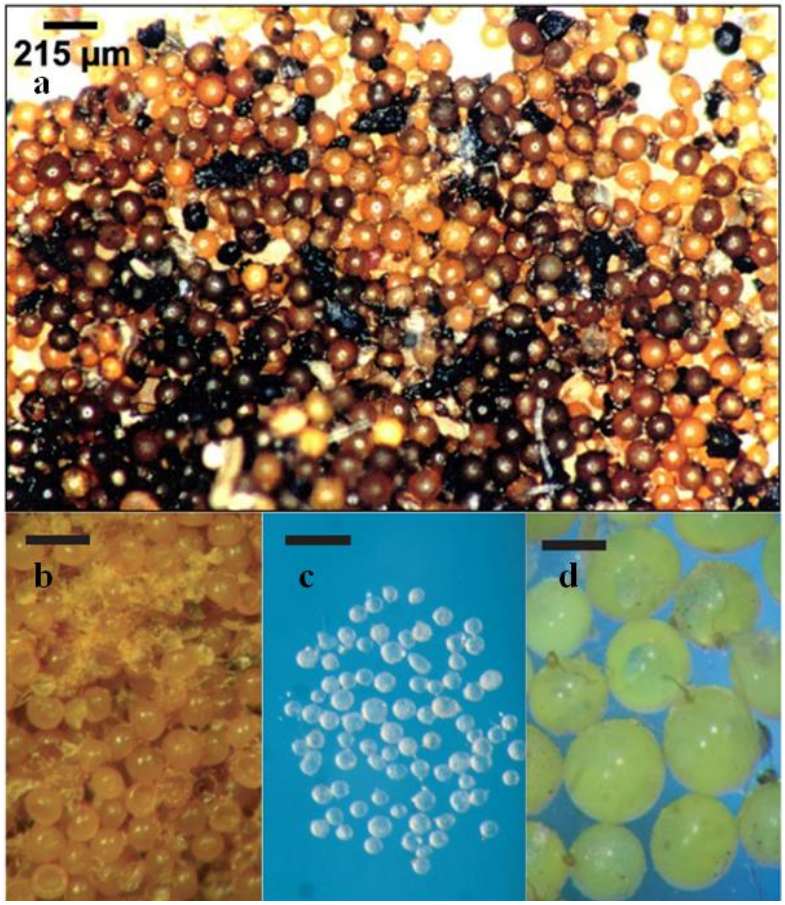

Figure 1.6: Differences in spore size, shape and overall appearance of different arbuscular mycorrhizal species. Illustrations taken from Straker *et al.* (2010) (a) and Young (2012) (b-d). (a) A collection of *Glomus etunicatum* spores of varying ages. (b) Spores of *Diversispora*. (c) Spores of *Paraglomus*. (d) Spores of *Gigaspora*. Bars for images b-d, 200 µm.

Spores are the best described source of inoculum of AM fungi and have been used in the identification and classification of AM fungi. Spore and sporocarps are able to survive passing through the gut of various mammals, birds and invertebrates and can be dispersed locally with the aid of animal movements. Spores are resistant to damage and are able to survive extended periods of time while remaining dormant and have the capacity to be dispersed by water and wind (Smith and Read, 2000; Smith and Read, 2008). Spores may also be distributed through the burrowing habits of large and small animals (Smith and Read, 2000).

1.7.1.1 Identification and classification of AM fungi

Recently the classification of AM fungi has undergone a major adjustment and many of the originally classified AM fungi have been reclassified and placed in different genera (Redecker *et al.*, 2013). This revision has been based on the fact that the original classification of these fungi was incomplete and has since been improved with the development of new DNA-based techniques and the reassessment of previous identifications and cultures.

It is crucial to note that taxonomy is a comparative science (Redecker *et al.*, 2013). Original classifications were performed using the microscopic, phenotypically distinct cell wall constituents of AM fungal spores and hyphal attachments (Schenck and Pérez, 1990) as well as the development of the spores and wall constituents (Walker, 1983, 1992; Morton and Benny, 1990; Morton and Bentivenga, 1994). This is done on broken spores mounted on microscopic slides and viewed under a light microscope.

This classification system is demonstrated with the use of murographs (Figures 1.7 and 1.8), which summarise, simplify and standardise the classification descriptions (Schenck and Pérez, 1990; Pfleger and Linderman, 1994; Smith and Read, 2000). The spore wall characteristics noted during the classification process include wall number, orientation, pigmentation, thickness, ornamentations and histochemical reactions (Schenck and Pérez, 1990; Pfleger and Linderman, 1994). Other morphological characters used during the traditional classification of AM fungi include spore shape (Figure 1.9), size and colour, hyphal attachment, shape and type of occlusions, sporocarp occurance, shape, size and

colour and peridium occurrence and characteristics (Schenck and Pérez, 1990; Giovannetti and Gianinazzi-Pearson, 1994).

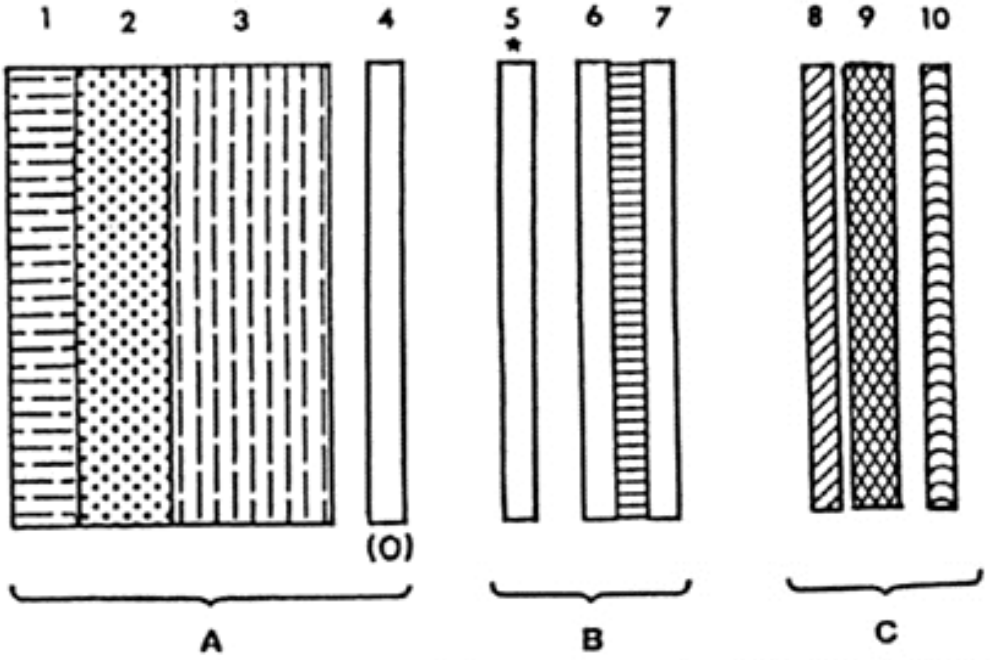

Figure 1.7: Murograph of wall type, number, group and position in an AM fungus spore. O: ornamented surface and *: difficult to see. The lines that connect two walls demonstrate a cementing layer. Walls are numbered consecutively from the outer to inner layer. 1: expanding, 2: evanescent, 3: laminated, 4-7: unit, 8: membranous, 9: coriaceous and 10: amorphous walls. Each wall group is bracketed in parentheses with consecutive letters from inner to outer wall. Illustration taken from Morton (1988).

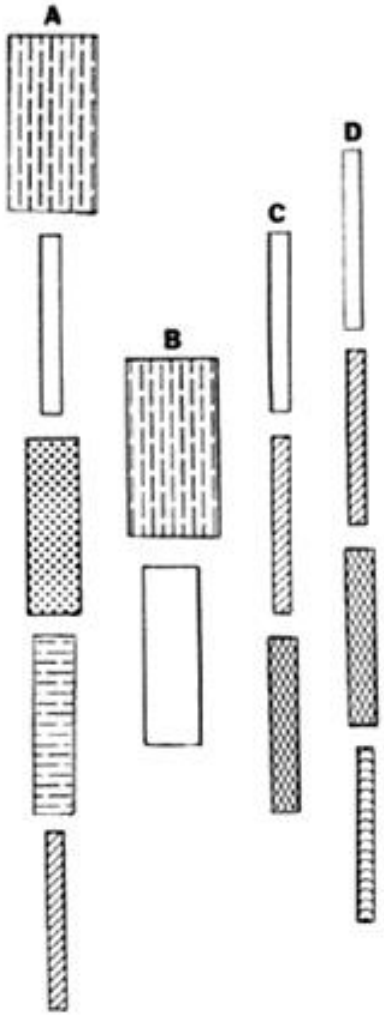

Figure 1.8: Demonstration of the relative positions of different wall types in AM fungal spores. The columns represent different wall groups with A: outer walls, B: structural walls, C: inner walls and D: innermost walls. Any combination of walls in a column can form a wall group. Illustration taken from Morton (1988).

Figure 1.9: The range of shape of spores in taxa of all genera of AM fungi. A: globose, B: sub-globose, C: ovoid, D: obovoid, E: ellipsoid, F: pyriform, G: irregular, H: oblong, I: reniform, J: fusiform and K: clavate. Illustration taken from Morton (1988).

This classification process is problematic due to limited morphological differentiation whereby some AM fungal species form more than one spore morph (Krüger *et al.*, 2012), field samples generally consist of spores in bad condition, either through association with micro-organisms that erode spore walls or cause pitting or perforations in the walls (Bhattachrjee *et al.*, 1982; Walker *et al.*, 1984), or mechanical damage either at the sampling

site or through the extraction process (Schenck and Pérez, 1990). Spore walls may also be difficult to distinguish individually as they may be colourless, wrinkled, overlapped or folded and may also remain attached to each other after being manipulated (Pfleger and Linderman, 1994), which overall may lead to misinterpretations during the classifications.

Detailed identification and classification involves long-term and intensive study of many isolates and developmental stages of an organism (Redecker *et al.*, 2013). Developmental stages or biological characters consist of spore germination, AM fungal infectivity, symbiotic efficiency, infection patterns, spore formation (ontogeny of spores), edaphic requirements and environmental tolerance (Giovannetti and Gianinazzi-Pearson, 1994). Spore germination is useful for the discrimination between *Gigaspora* and *Scutellospora*, in the family *Gigasporaceae* (Giovannetti and Gianinazzi-Pearson, 1994; Redecker *et al.*, 2013). Spore germination between isolates may vary depending on host root stimuli, pH, moisture, temperature, nutrient content, dormancy, rate and extensiveness of hyphal production (Hepper and Smith, 1976; Tommerup, 1983; Siqueira *et al.*, 1985; Gianinazzi-Pearson *et al.*, 1989; Giovannetti *et al.*, 1991). Spore and sporocarp ontogeny is used to differentiate between *Acaulospora* and *Entrophospora* (Ames and Schneider, 1979), although *Entrophospora* is not definitively categorised into a particular family as it is often referred to as a sister genus to *Acaulospora*, as it was originally placed in *Acaulosporaceae* and is still so on the INVAM website and there is insufficient evidence to be placed in an alternative family to *Entrophosporaceae* (Schüßler and Walker, 2010).

For the information from the taxonomic classification of AM fungi to be beneficial to the scientific community the information needs to be resourceful, readily available and updated regularly. Since 1990, the information has been available online on the INVAM (International Culture Collection of Arbuscular and (Vesicular) Arbuscular Mycorrhizal Fungi) website, which was created by Professor J. B. Morton of West Virginia University, USA (INVAM). This germplasm resource provides researchers with sets of voucher specimens, namely photographs, slides, vials of intact spores (Pfleger and Linderman, 1994) and this is particularly important for researchers that do not have the facilities or the time to carry out these analyses. As well as vouchers, INVAM maintains a large number of isolates, including those not described (INVAM).

In order to classify and name AM fungi in a manner that can be replicated one must use standardised recommendations and rules. These recommendations and rules are specified in the "International Code of Nomenclature for Algae, Fungi and Plants (ICN)" (Miller *et al.*, 2011; McNeill *et al.*, 2012) and are generally referred to as "The Code" (Redecker *et al.*, 2013). The Code regulates nomenclature (the application and administration of names) and provides a nomenclatural framework for taxonomy (the science of defining, classifying and arranging the names in a hierarchical system) (Redecker *et al.*, 2013). Unfortunately, names applied using the rules, which may be published, have no similarity to an ordinary phylogeny-based classification, which in turn can cause problems particularly when classification is meant to reflect evolutionary relationships (Redecker *et al.*, 2013). It is noteworthy to mention that the Code relies on concrete evidence especially if new taxa are created.

Newer classification techniques make use of molecular methods, including DNA analysis, isozyme polymorphism and protein profiles (Giovannetti and Gianinazzi-Pearson, 1994). These analyses are useful since morphologically similar isolates may be genetically and physiologically different (Krishna, 2005). DNA analyses focus mainly on ribosomal genes (Krishna, 2005). The advantage of using DNA in analyses is that there are no problems associated with gene expression because the genotypes are analysed directly (Redecker *et al.*, 2003).

The molecular method most commonly practised at present for the systematics and identification of AM fungi is DNA/PCR analysis of the ribosomal genes (Redecker, 2000; Redecker *et al.*, 2003; Krishna, 2005). This genome region has genes that are available in a high copy number and have variable as well as highly-conserved sectors, which allows for the characterisation of taxa at different levels (Redecker *et al.*, 2003). It is noteworthy to mention that the ribosomal DNA (rDNA) in single AM fungal spores is highly polymorphic (due to the spores being multinucleate), which is in contrast to other fungi in that variable rDNA sequences, for instance internal transcribed spacers (ITS), are usually identical within a species (Redecker *et al.*, 2003). ITS regions (two) are variable non-coding regions nested within the rDNA repeat between the SSU, 5.8S and LSU rRNA genes (Gardes and Bruns, 1993). The ITS regions have very high levels of polymorphism (Clapp *et al.*, 1995; Redecker *et al.*, 1997) and rarely is the same ITS sequence recovered twice from a single spore (Lanfranco *et al.*, 1999).

Originally Simon *et al.* (1992) designed the PCR primer VANS1, which was used for the analysis of three glomalean 18S (small) subunit (SSU) sequences, which were available at the time. However, later studies revealed that the VANS1 annealing site is not well conserved within the Glomerales (Clapp *et al.*, 1999; Redecker *et al.*, 2000; Schüßler *et al.*, 2001) as well as several of the newer classified ancestral lineages of the Glomerales do not have the annealing site (Redecker *et al.*, 2000). Other problems with creating primers for AM fungi include primers only being specific for single isolates and the primers may not exclude non-AM fungi (Redecker, 2000). The exclusion of other organisms is vital in the analysis of AM fungi since the fungal tissue tends to be embedded within host roots and it is likely that the host, pathogenic or saprophytic fungi will be co-detected (Redecker *et al.*, 2003). Another SSU analysis study by Helgason *et al.* (1998) involved the construction of the primer AM1, which amplified most but not all glomalean fungi. Other studies have analysed the 28S (large) ribosomal subunit (LSU), for instance van Tuinen *et al.* (1998) were able to design several specific primers which targeted the highly variable D2 region of the LSU, each for a different AM fungal species. In a study by Redecker *et al.* (1997) it was demonstrated that a minute amount of fungal biomass could be used to perform PCR/restriction analysis of ITS regions of rDNA, which allowed for species of the Glomerales to be distinguished and that the fragment patterns created were highly reproducible. Unfortunately, the variation of rDNA sequences within single spores limits the resolution of this method when closely related species are compared (Redecker, 2000). It is important to mention that as the understanding of the phylogeny of a group of organisms improves then more reliable primers can be designed (Lee *et al.*, 2008).

Currently the preferred and more advantageous PCR method used for AM phylogenetic analyses and AM fungal identification is nested PCR. It is advantageous as it has high specificity and sensitivity, which allows for the simultaneous detection of different fungi within the same root fragment and can technically allow for the detection of an unlimited number of different fungi if the sequence targeted in the nested reaction is sufficiently discriminating between AM fungi and it can also be performed on crude DNA isolates and stained root fragments. The initial PCR reaction is carried out directly on the biological material using general/non-specific primers which allows for a wide range of analyses using specific primers to be performed on a single sample, thus a small sample of colonised root can be analysed for the identification of a variety of AM fungi. The product of the initial

PCR may be diluted in order to reduce the problems associated with the presence of plant components that may reduce amplification efficiency (van Tuinen *et al.*, 1998).

At present the primer pair that is used and known to amplify most AM fungal species as well as exclude non-AM fungi is AML1 and AML2 (Lee *et al.*, 2008), which are used for the nested PCR after the initial PCR with the eukaryotic primer pair NS1 and NS4 (White *et al.*, 1990). These primers amplify the SSU rRNA gene which has a low polymorphism (Simon *et al.*, 1993) and allows for the differentiation between fungal genera and species. The disadvantage of these primers is the length of the product, which is insufficient for reliable species resolution of all AM fungi, particularly within certain families (Krüger *et al.*, 2009). In the study by Krüger *et al.* (2009) AM fungal primers were designed to include the ITS regions and part of the LSU region so as to provide species level resolution and reliable phylogenetic analyses from the PCR products. The primer sets that were designed consisted of a mixture of primers for both the first PCR reaction primers, SSUmAf and LSUmAr, and the nested PCR reaction primers, SSUmCf and LSUmBr, to allow for the amplification of all AM fungal taxa. The final product length of these primers is almost twice the length of the product produced by the primer pair AML1 and AML2 (Lee *et al.*, 2008; Krüger *et al.*, 2009).

The current nomenclature of AM fungi is as follows (Schüßler *et al.*, 2001; Schüßler and Walker, 2010; Redecker *et al.*, 2013) (Figure 1.10):

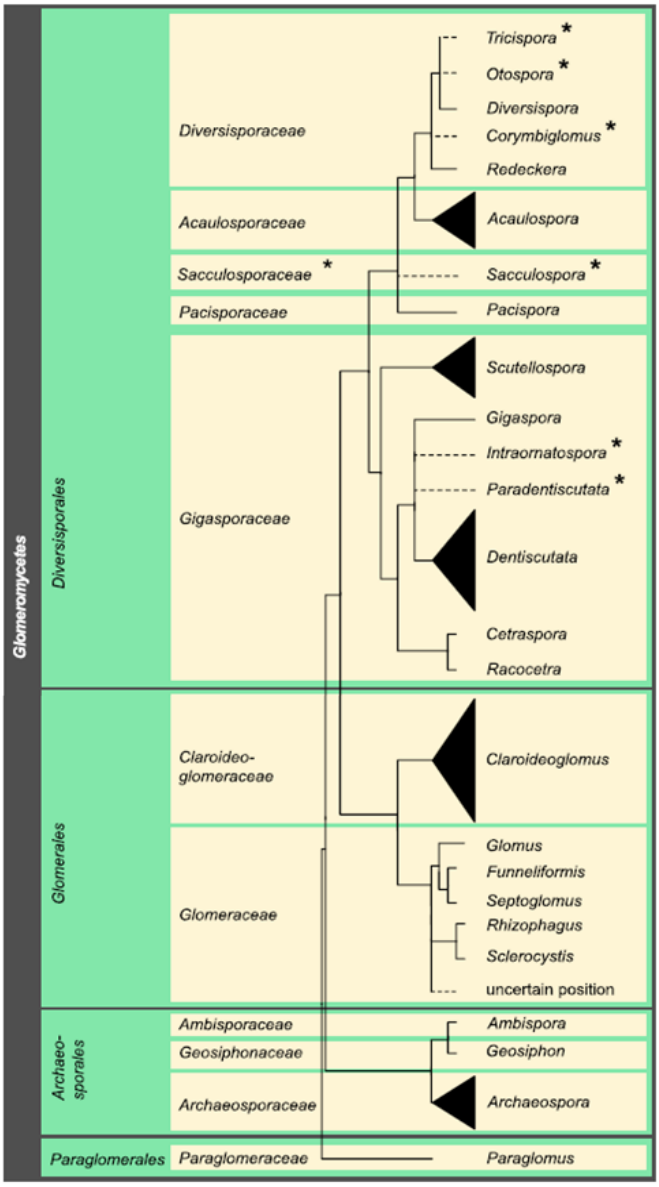

Figure 1.10: Demonstration of the consensus classification of the Glomeromycota. Dashed lines: genera of uncertain taxonomic position and *: insufficient evidence. Note: Neither Enterosporaceae nor *Enterospora* does not appear on this image. Illustration taken from Redecker *et al.* (2013).

Glomerales: The fungi are generally hypogeous, occasionally epigeous, with the symbioses being endomycorrhizal accompanied by vesicles and/or arbuscules and asexual spores (Morton and Benny, 1990; Schüßler *et al.*, 2001). Hyphae are generally coenocytic, however, septa may form in older hyphae as the cytoplasm is withdrawn or when the fungus cuts off resting spores (Morton and Benny, 1990; Schüßler *et al.*, 2001). Fungi produce asexual chlamydospores (glomoid spores; Morton and Redecker, 2001) (Morton and Benny, 1990; Schüßler, 2001). This order differs from other AM fungi by having the SSU rRNA gene sequence signature motif YTRRY}2-5}

RYYARGTYGNRCARCTTCTTAGAGGGACTATCGGTGTYTAACCGRTGG which corresponds to the homologous position 1353 of the *Saccharomyces cerevisiae* SSU rRNA sequence J01353 (Schüßler *et al.*, 2001).

Glomeraceae: (Phylogenetic *Glomus* group A; Schüßler and Walker, 2010) The fungi are mostly hypogeous, occasionally epigeous (Rani *et al.*, 2006). Fungal mycelium is generally coenocytic, rarely septate (Rani *et al.*, 2006). Mycorrhizal symbiosis develops vesicles that are generally intraradical but have the potential to become thick-walled spores in some species (Morton and Benny, 1990). This family differs from other families in this order by having the specific, but degenerate, SSU rRNA gene sequence motif TGTYADGGCAYYRCACYGG (Schüßler and Walker, 2010; INVAM)

Claroideoglomeraceae: (Phylogenetic *Glomus* group B; Schüßler and Walker, 2010) The fungus forms a mycorrhizal symbiosis with vesicle formation being varied depending on the environment and arbuscule formation (Schüßler and Walker, 2010; INVAM). Glomoid spores form in soil or, very occasionally, individually in decaying roots (Schüßler and Walker, 2010). This family differs from other families in this order by having specific SSU rRNA gene sequence motifs CAGYYGGGRAACCRACTAAA, ATTKRCACATCGGTCGTGC, CYTAAGGGGYATGAACYRGTGTAGTSA and TAAAAYRGGACGGCATGATTCTATT (Schüßler and Walker, 2010). In this family the species were separated from previous inclusion in the genus *Glomus* because they grouped in a distinct clade based on SSU rRNA sequences (INVAM). Unfortunately rDNA-based species-level resolution is not as reliable as in other glomoid clades, which may be due to the continued ancestral polymorphisms in gene repeats (VanKuren *et al.*, 2012).

Diversisporales: The fungi are hypogeous, with the symbioses being endomycorrhizal accompanied by arbscules, rarely producing vesicles, and may produce hypogeous auxiliary cells (Schüßler *et al.*, 2001; Walker and Schüßler, 2004). Fungus may produce complex spores within a sporiferous saccule (acaulosporoid spores; Morton and Redecker, 2001) or from a bulbous base on soporiferous hyphae or the fungus may produce glomoid spores (Schüßler *et al.*, 2001; Walker and Schüßler, 2004). This order differs from other AM fungi by having the specific SSU rRNA gene sequence signature motifs GGGTTTH and TYACCGGRAGGTRT which correspond to the homologous positions 234 and 1495, respectively, of the *S. cerevisiae* SSU rRNA sequence J01353 (Walker and Schüßler, 2004).

Gigasporaceae: The fungus forms a mycorrhizal symbiosis with arbuscules (Morton and Benny, 1990). The fungi produce large, globose to subglobose, asexual spores, generally

greater than 200-300 µm (Morton and Benny, 1990; Bentivenga and Morton, 1996; Rani *et al.*, 2006; INVAM), which are borne singly in the soil (Morton and Benny, 1990).

Acaulosporaceae: The fungi form an endomycorrhizal symbiosis with the production of intraradical arbuscules and ellipsoid, irregular or lobed vesicles (Morton and Benny, 1990). Glomoid spores are produced (Morton and Benny, 1990; Rani *et al.*, 2006; INVAM).

Pacisporaceae: The only genus in this family is *Pacispora* and thus the character description will be based on genus characters. The fungus forms a mycorrhizal symbiosis accompanied by vesicles and arbuscules (INVAM). The fungus produces globose, subglobose to ellipsoid, rarely irregular spores in the soil singly on the terminal ends of hyphae and the production of sporocarps is unknown at this point (Oehl and Sieverding, 2004).

Diversisporaceae: The fungus produces glomoid spores (Walker and Schüßler, 2004; INVAM). This family was constructed only due to the divergence of the SSU gene sequence divergence from other clades (INVAM). This family differs from other families in this order by having specific SSU rRNA gene sequence signature motifs GGCTCATTYGRRTYTS, ACYCATTRYCAGGCTTAAT and TTGGCATTTAGYCA which correspond to the homologous positions 487, 648 and 1389, respecitively of the *S. cerevisiae* SSU rRNA sequence J01353 (Walker and Schüßler, 2004) and the SSU rRNA gene sequence signature motifs YVRRYW}1-5}NGYYYGB AND GTYARDYHMHYY}2-4}GRADRKKYGWCRAC which correspond to the homologous positions 658 and 1346, respectively of the *S. cerevisiae* SSU rRNA sequence J01353 (Schüßler *et al.*, 2001).

Sacculosporaceae: The fungus produces spores within the hyphal neck of closely adherent terminal or intercalary soporiferous saccules and the production of sporocarps is unknown at present (Oehl *et al.*, 2011). Phylogenetic position is uncertain at present pending more sampling and sequence analyses (Redecker *et al.*, 2013).

Entrophosporaceae: The fungus has an endomycorrhizal symbiosis accompanied by vesicles and arbuscules (Oehl *et al.*, 2011). Spores produced by the fungus either follow the glomoid development or entrosporoid development (Oehl *et al.*, 2011) (which is distinguished from acaulosporoid development since the spores develop within and not laterally on the saccule subtending hypha; Kaonongbua *et al.*, 2010). Family is of uncertain phylogenetic position due to the lack of reliable evidence to substantiate its position as being its natural phylogenetic position and may in the future be combined with other groups (Schüßler and Walker, 2010).

Paraglomerales: The fungi are hypogeous, with the symbioses being endomycorrhizal forming arbuscules and intraradical mycelium and rarely producing vesicles (Schüßler *et al.*, 2001). Spores are glomoid and lack pigmentation (Schüßler *et al.*, 2001). This order differs from other AM fungi by having the SSU rRNA gene sequence signature motif GCGAAGCGTCATGGCCTTAACCGGCCGT which corresponds to the homologous position 703 of *S. cerevisiae* SSU rRNA sequence J01353 (Schüßler *et al.*, 2001). However, it is noteworthy to mention that this premise is based on only two species and may thus be subject to modification as more species are identified (Schüßler *et al.*, 2001).

Paraglomeraceae: This family is based entirely on molecular data and evidence, however, the only genus in this family is *Paraglomus* and thus the character description is based on genus characters. Molecular character studies of this family, including monoclonal antibody specificities and fatty acid profiles has not produced enough data and evidence to position the family in relation to other glomalean taxa (Rani *et al.*, 2006). The fungus forms a mycorrhizal symbiosis with arbuscules but development of vesicles is not known at present (Morton and Redecker, 2001). The fungus produces glomoid spores (Rani *et al.*, 2006; INVAM). The sequences from the SSU rRNA gene indicate that all the members of this family are an ancient group within Glomales and are distant from other glomalean families (Redecker *et al.*, 2000). This family's, and members of Archaeosporaceae, SSU rRNA is uniquely amplified by the primer ARCH 1311, sequence TGCTAAATAGCCAGGCTGY (Redecker, 2000; Morton and Redecker, 2001).

Archaeosporales: The fungi are hypogeous, with the symbioses being mycorrhizal, accompanied by arbscules and may or may not produce vesicles, or endocytosymbiotic, with the fungus associating with photoautotrophic prokaryotes (Schüßler *et al.*, 2001). These fungi produce glomoid spores in loose clusters or singly and acaulosporoid complex spores singly (Schüßler *et al.*, 2001). This order differs from other AM fungi by having the specific SSU rRNA gene sequence signature motif YCTATCYKYCTGGTGAKRCG which corresponds to the homologous position 691 of the *SD. Cerevisiae* SSU rRNA sequence J01353 (Schüßler *et al.*, 2001).

Geosiphonaceae: The only genus in this family is *Geosiphon* and the genus has only one species, *Geosiphon pyriformis* (Schüßler *et al.*, 1994; Schüßler, 2002; Rani *et al.*, 2006; INVAM) thus the character description will be based on genus and species characters. The fungus is hypogeous, however, does not form mycorrhizal structures within host roots but associates with endosymbiotic cyanobacteria in the genus *Nostoc* (Schüßler *et al.*, 1994;

Schüßler, 2002; Rani *et al.*, 2006; Smith and Read, 2008; INVAM). Molecular analyses suggest that Geosiphonaceae is more closely related to Archaeosporaceae and Paraglomaceae according to its position on phylogenetic trees (Schüßler *et al.*, 2001). The mycelium is coenocytic and unicellular multinucleated 'bladders', of about 1-2 mm in size, develop at hyphal tips, which harbour the cyanobacteria (Schüßler *et al.*, 1994; Schüßler, 2002; Rani *et al.*, 2006). The fungus produces asexual, glomoid spores (resemble AM fungal spores) individually or in loose clusters in the soil (Schüßler *et al.*, 1994; Schüßler, 2002; INVAM). The fungus has only been found occasionally (roughly five times) in the Spessart mountains in Germany (Schüßler *et al.*, 1994; Schüßler, 2002; Rani *et al.*, 2006; INVAM). Although this family is not known to form a mycorrhizal symbiosis, the rDNA evidence places it in a clade within the order Archaeosporales (Schüßler *et al.*, 2001).

Ambisporaceae: The only genus in this family is *Ambispora* and thus the character description will be based on genus characters. The fungus develops an endomycorrhizal symbiosis accompanied by arbuscules but vesicles have not yet been observed in the symbiosis (Walker *et al.*, 2007a; Walker *et al.*, 2007b; INVAM). Dimorphic spores produced by the fungus either follow the glomoid development or acaulosporoid development but both may be present and develop concurrently (Walker *et al.*, 2007a; Walker *et al.*, 2007b; INVAM). This family/genus is separated from other families/genera in the Archaeosporales by its SSU rRNA gene characteristics, for example, the gene sequence motif CAAAACCAATCTCGTCTTCGGGC (Walker *et al.*, 2007a).

Archaeosporaceae: The only genus in this family is *Archaeospora* and thus the character description will be based on genus characters. The fungus forms an endomycorrhizal symbiosis with intraradical hyphae and arbuscules and the structures are similar to the structures of Archaeosporaceae and Paraglomaceae (Walker *et al.*, 2007a). Vesicle formation has not been observed in cultures yet (Morton and Redecker, 2001). This family of AM fungi produce small, hyaline monomorphic (acaulosporoid) or dimorphic (acaulosporoid and glomoid) spores (Morton and Recker, 2001; Walker *et al.*, 2007a). Spores develop in the soil, very occasionally in roots, and appear globose to sub-globose to irregular (Walker *et al.*, 2007a). This family's, and certain members of Paraglomaceae, SSU rRNA is uniquely amplified by the primer sequence TGCTAAATAGCCAGGCTGY (Redecker, 2000).

1.7.1.2 Nutrition and influence on plants

AM fungi form part of the rhizosphere which comprises a group of organisms associated with plants that are also vital for their metabolism (Kavamura and Esposito, 2010). These organisms found in cooperation with plant roots are known as rhizosphere microorganisms (Kavamura and Esposito, 2010). The rhizosphere composition is highly tailored by the type of plant, quantity and composition of root exudates and different root zones (Yang and Crowley, 2000; Marschner *et al.*, 2004). The rhizosphere designates a specific plant interaction and interactions with other rhizosphere microorganisms and the environment (Kavamura and Esposito, 2010). An example of this is AM fungi being able to modify soil microbial communities in the rhizosphere either directly or indirectly through changes in root exudate patterns (Barea *et al.*, 2005) as well as enhancing the soil enzyme activities (Wang *et al.*, 2006).

AM fungi have different sources of nutrition, acquiring water, nitrogen (N) and phosphorus (P) (macronutrients) as well as other micronutrients (such as molybdenum (Mo) and potassium (K)) from the soil (Ames *et al.*, 1983; Harley, 1989; Li *et al.*, 1991; Chen *et al.*, 2005; Govindarajulu *et al.*, 2005; Smith and Read, 2008; Fitter *et al.*, 2011) and the majority of the carbon (C) acquisition (in the form of carbohydrates) is attributable to the host plant (Smith and Read, 2008; Fitter *et al.*, 2011). It is noteworthy to mention that C acquisition from the soil and transfer to the host plant from the fungus occurs only when the host plant has an abnormally restricted C supply, as is the case in achlorophyllous plants (Bidartondo *et al.*, 2002). However, in the case of the majority of AM fungal species the fungus cannot complete its life cycle without the symbiosis, suggesting that they are entirely dependent on the host for organic C (Smith and Read, 2008).

Nutrient transfer between the symbionts occurs across symbiotic interfaces (Smith and Read, 2008). The structure, location and composition of the interfaces vary, for instance the interface may be intracellular involving hyphal coils, arbuscules (*Paris*-type mycorrhizas) or arbusculate coils (*Arum*-type mycorrhizas) or intercellular involving hyphae growing in the intercellular spaces of the root cortical cells; however, all involve plasma membranes of both symbionts which are separated by an apoplastic interfacial compartment (Smith and Read, 2008). During the exchange of nutrients between the fungus and host plant the fungus provides the host with nutrients acquired from the soil through the external (extraradical)

hyphae such as P, Zn and N (low mobility mineral nutrients) and in return the host provides the fungus with photosynthates (Bolan, 1991; Marschner, 1995; Thingstrup *et al.*, 2000; Jakobsen *et al.*, 2001; Smith and Read, 2008; Fitter *et al.*, 2011). P has a low mobility due to its associations with soil cations, such as Ca^{2+}, Fe^{2+} and Al^{3+} (Tinker and Nye, 2000; Smith and Read, 2008) and N also has low mobility in soil as nitrate (in dry soil) and ammonium (Smith and Read, 2008). P is most readily available to plants at pH 6.5; however, at lower pH values the P associated with Al and Fe decreases in solubility and at higher pH values the P associated with Ca decreases in solubility (Smith and Read, 2008). AM fungi tend to transfer any nutrients that will result in a local sugar flux as well as any excess nutrients that they can spare, which is usually phosphate and when there is an adequate supply, N in the form of ammonium. The host tends to increasingly provide sugars to the region of elevated nutrient supply and some of the sugars diffuse into the apoplast where they may be secured by the fungus (Fitter *et al.*, 2011).

AM fungi are generally considered extensions of the host plant root system (Hodge *et al.*, 2010) by increasing the surface area of the uptake system allowing for greater nutrient assimilation and access. It is thus safe to say that due to hyphae effectively using a larger volume of soil than only roots, mycorrhizal roots are able to acquire more nutrients from nutrient-deficient soils than non-mycorrhizal roots (Entry *et al.*, 2002; Smith and Read, 2008). This is particularly important with regards to P since the transfer of P ions is faster across cell membranes than through the soil, which depends on diffusion, and as a result depletion zones develop around plant roots. Due to this, the rate of P uptake of the roots is determined by the slow diffusion rate, which is avoided by AM fungi extraradical mycelium which acquires nutrients beyond the depletion zone (Smith and Read, 2008; Fitter *et al.*, 2011). It has also been shown that nutrient availability affects AM fungal colonisation, with low nutrient levels corresponding to increased colonisation and vice versa (Douds and Chaney, 1986; Smith and Read, 2008). This may be related to fungus and host plant interactions with each other with regards to arbuscule and vesicle numbers, content of P, starch and soluble sugars in the host root, spore populations and C allocation (Douds and Chaney, 1986).

AM fungal vesicles are responsible for nutrient storage as well as for propagating AM fungi (Biermann and Linderman, 1983; Jabaji-Hare *et al.*, 1984) and are able to store and provide the host with P if there is lingering P limitation (Olsson *et al.*, 2011). Vesicles are the

preferential storage bodies over extraradical spores which may undergo predation (Olsson *et al.*, 2011). Generally P is stored as polyphosphate (a linear polymer consisting of orthophosphate residues linked together by high energy phosphoanhydride bonds) in the vesicles (Smith and Read, 2008; Olsson *et al.*, 2011). The concentration of P within vesicles is nearly double that of Ca (second most abundant element analysed in the study performed by Olsson *et al.*, 2011). Vesicles contain nearly 2-4 times the amount of P when compared with plants, namely 0.4-0.7 % and 0.2 % respectively. Larger vesicles contain more P and there is a high variation of P concentration between vesicles (Olsson *et al.*, 2011). The concentration of P in roots is decreased in soils that contain high amounts of NaCl (Allen and Cunningham, 1983), which may be due to the acidic property of Cl and polyphosphate in cells and there tends to be a negative correlation between the two elements (Olsson *et al.*, 2011). However, arbuscules in mycorrhizal roots contain high amounts of P without any detectable Cl and vice versa in non-mycorrhizal roots (Schoknecht and Hattingh, 1976).

The majority of the nutrient transfer, with regards to P and N, between AM fungi and the host plant occurs across the arbuscule-cortical cell interface. However, it is unclear as to where the carbohydrate transfer occurs (Smith and Read, 2008; Fitter *et al.*, 2011). The transfer of uncharged hexoses could be passive with direction being controlled by concentration gradients across the interface sustained by consumption and production of the hexoses. In lieu of this, the efflux from the host is passive after which influx into the fungus makes use of active proton co-transport that requires maintenance of proton motive force (PMF) across the fungal membrane (Smith and Read, 2008). Photosynthates (hexoses) transferred to AM fungi are very quickly assimilated into trehalose and glycogen, which creates a buffer against excessive glucose build-up in the cytoplasm (Shachar-Hill *et al.*, 1995; Solaiman and Saito, 1997). Lipids are the main organic C pool in AM fungi (Cooper and Lösel, 1978). Approximately 4-20 % of total photosynthate is transferred to AM fungi during the symbiosis (Smith and Read, 2008). However, it is noteworthy to mention that the production of extraradical hyphae uses less C per unit length than the production of roots (Tinker, 1975) and the small diameter of the extraradical hyphae allow for the penetration of soil pores too small for roots thus reducing the development of depletion zones (Smith and Read, 2008).

The extraradical mycelium may significantly affect plant interactions with the metal ions in the surrounding soil due to its high sorption capacity for Cd, Zn and Cu (Joner *et al.*, 2000;

Gonzalez-Chavez *et al.*, 2002; Pawlowski and Charvat, 2004). As well as influencing organic and inorganic nutrient relationships in plants, arbuscular mycorrhiza influence water relations and carbon cycling in plants (Entry *et al.*, 2002).

With regards to nutrient acquisition from the soil, namely, P, C, Cu, Zn, Fe, Mo, K, Ca and so forth, the fungus makes use of both passive and active transport mechanisms (Smith and Read, 2008). Of all the elements taken up by the extraradical mycelium P is the best documented. One mechanism of P uptake by the AM fungal extraradical mycelium includes a high affinity phosphate transporter (Harrison and van Buuren, 1995). The second best documented element taken up by AM fungi and transferred to the host plant is N. As mentioned previously N is taken up by the extraradical mycelium either as nitrite ions or ammonium but it is transported within the fungus as arginine, is then probably broken down to urea and finally transferred to the host plant as ammonia with the left over C skeletons being recycled back into the AM fungal stores (Bago *et al.*, 2001; Govindarajulu *et al.*, 2005). The ammonia may be transferred to the plant with the aid of a plant ammonium transporter (AMT), identified in *Lotus japonicas*, which is mycorrhiza-specific and is preferentially expressed in cells containing arbuscules (Guether *et al.*, 2009). N fixation and nodulation in legumes colonised by AM fungi is increased as well as N concentrations in tissues (Barea and Azcón-Aguilar, 1983). Legumes colonised with AM fungi in low P soils have increased assimilation of N_2 in rhizobial root nodules (Smith and Read, 2008).

Co-existing plants are able to increase P obtained per unit area by elevating shoot P concentration, which is attributable to mycorrhizal colonisation and the increased shoot biomass. It is noteworthy to mention that one plant species can affect other plant species within the vicinity with regards to P uptake, AM fungal colonisation and sporulation, however, this varies between plant species and the reverse is true for nonmycorrhizal plants (Chen *et al.*, 2005). Host plant responsiveness to AM fungal colonisation (mycorrhizal dependency) varies greatly between species and is largely influenced by nutrient supply (Smith and Read, 2008).

Ultimately, AM fungal colonisation and nutrient uptake results in alleviating nutrient stress of the host plant and consequently increases plant growth (Smith and Read, 2008).

1.7.1.3 Trap cultures

Generally the quality of field-collected samples of AM fungi, either spores or colonised roots, is variable and can be poor due to unfavourable environment conditions and predation (Morton *et al.*, 1993) and thus the analyses and identifications performed can be unreliable. Hence it is often favourable to establish trap/pot cultures to confirm analyses performed. Trap cultures may be initiated using field sample propagules, whether it be asexual spores, zygospores, hyphae (with or without vesicles) in living roots or in dead root fragments and extraradical hyphae (Norris *et al.*, 1994) and field sample soil diluted with sterile river sand (Liu and Wang, 2003). This particular trap culture method tends to result in the isolation of more AM fungal species than other methods (An *et al.*, 1990; Liu and Wang, 2003). Trap cultures allow for the propogation of a new generation of spores and colonised roots from which more certain identifications can be made (Smith and Read, 2008). This also allows for the detection of AM fungal species that were not detectable from initial sampling (Morton *et al.*, 1995).

Trap cultures make use of one or several plant species (Smith and Read, 2008) that can host a wide variety of AM fungi to compensate for plant-fungus compatibility but optimum trap culture plants may vary in different ecosystems (Liu and Wang, 2003).

In a study performed by Stutz and Morton (1996) it was found that most AM fungal species growing in arid environments did not sporulate with the initial trap culture and only did so after two successive trap cultures. They also found that the total number of AM fungal species spores increased with each successive trap culture and did so significantly for the second and third trap culture cycle. This finding of increased total sporulating species was found to not significantly vary between sampling sites but although the sporulation increased with each cycle, the sporulating AM fungal species varied with trap culture cycle and site. Due to this study, previous findings that there are fewer AM fungi in arid environments was found to be false and thus trap cultures may be used during the analyses of other unfavourable environments in order to correct for any bias that may occur due to sporulation levels required for detection.

1.7.1.4 Applications

AM fungi are organisms that are able to sequester toxic trace elements or exclude excessive amounts of trace elements (Khan, 2005) and hence aid mycorrhizal plants survive in polluted soils and ultimately play a role in phytostabilisation of toxic trace element (HM) polluted soils (Khan, 2006). The fungi are able to sequester toxic trace elements with the use of glomalin, an insoluble, highly stable, iron-containing glycoprotein (N-linked oligosaccharides) produced by the hyphae (Wright and Upadhyaya, 1998; González-Chávez *et al.*, 2004), which is released into the surrounding soil (Diver *et al.*, 2005), where it tends to attach to soil and helps stabilise soil aggregates (Khan, 2005) or binds HMs in the soil (Wright and Upadhyaya, 1998; González-Chávez *et al.*, 2004). Glomalin's overall structure consists of monomeric structures bound together through hydrophobic interactions (González-Chávez *et al.*, 2004). The release of glomalin is not through passive secretion and usually remains within the hyphal and spore walls (Khan, 2006), where it may immobilise HMs before they enter the fungal-plant system (Khan, 2005).

AM fungi are able to exclude HMs through adsorptions onto their chitinous cell walls (Zhou, 1999; Göhre and Paszkowski, 2006) or increased efflux with the formation of HM complexes extracellularly from organic acid release (Leyval *et al.*, 1997). An additional method that the fungus may employ as a detoxification mechanism is the use of vesicles for storage of toxic compounds (Göhre and Paszkowski, 2006) or chelation of HM intracellularly through the synthesis of ligands; for instance, polyphosphates and metallothioneins, and/or compartmentation within vacuoles (Leyval *et a.*, 1997; Göhre and Paszkowski, 2006). Since AM fungi presents a large surface area in the soil hyphal binding of HMs acts as an important HM sink (Göhre and Paszkowski, 2006).

Due to the above abilities it has been thought that AM may be used in the remediation of mine tailings as mycorrhizoremediation, which is an enhanced form of phytoremediation (Khan, 2006). This idea is further supported from studies showing AM being present on the mine tailings without introduction (Straker *et al.*, 2007; Straker *et al.*, 2008).

1.8 Rhizobia with AM fungi

AM fungi are also able to form tripartite symbiotic associations with nodule-inducing rhizobia and legumes (Ibijbijen *et al.*, 1996; Saxena *et al.*, 1997; Zhao *et al.*, 1997; Olesniewicz and Thomas, 1999). Rhizobia are soil bacteria known for being root-nodule symbionts of legumes (Denison and Kiers, 2011). However, unlike AM fungi, the symbiosis is not obligate for either the rhizobia or the host plant and some can grow endophytically in non-legumes (Ji *et al.*, 2010). There is a vast number of rhizobial cells in the soil and when a host is present only a few infect and colonise the host plant roots. These few cells proliferate to produce millions of cells within each root nodule and some of these rhizobial cells differentiate into bacteroids, which are modified forms that can convert atmospheric N_2 into N forms that the host can use (Denison and Kiers, 2011). Once the nodule senesces, many of the rhizobia escape into the soil (Moawad *et al.*, 1984; Bottomley, 1992). It is noteworthy to mention that in some hosts the bacteroids lose the ability to divide, thus the soil is repopulated with the undifferentiated rhizobial cells from the same nodules (Oono *et al.*, 2009; Oono and Denison, 2010; Oono *et al.*, 2010).

AM fungi and rhizobia both enhance mineral nutrition of the host plant in exchange for carbohydrates supplied by the host plant (Sharma and Johri, 2002). Unlike AM fungi, the rhizobia fix atmospheric N in root nodules with the use of the nitrogenase enzyme (Thorneley, 1992). A synergistic effect of co-inoculation with AM fungi and rhizobia has been reported for different crops and these effects are more noticeable in soils deficient in both P and N (Sharma and Johri, 2002). It is generally accepted that AM fungi enhance P nutrition of legumes, which improves host plant growth and N fixation (Barea and Azcon-Aguilar, 1983; Cluett and Boucher, 1983). It may be that the *Rhizobium*-plant system is assisted by the presence of AM fungi because they correct any nutrient deficiency (not just P deficiency) in the host plant that might be limiting to *Rhizobium* (Pacovsky, 1986; O' Hara *et al.*, 1988).

Nodules containing more rhizobial cells can fix more N_2, which supports more plant growth and photosynthesis, which ultimately may support more rhizobia. Rhizobial cells are also able to stockpile resources within nodules, including P and Polyhdorbybutyrate (PHB), which may boost future survival (Denison and Kiers, 2011). Nodulated AM fungal plants

have enhanced nitrogen production in comparison to non-nodulated AM fungal plants (Ibijbijen *et al.*, 1996).

The number and biomass of nodules has been shown to significantly increase when both microsymbionts are co-inoculated on to the host (Olsen and Habte, 1995; Saxena *et al.*, 1997; Zhao *et al.*, 1997). However, the exception involves plants colonised/inoculated by *Funneliformis mosseae* and *Gigaspora rosea* which have low nodule numbers, demonstrating symbiont antagonism (Schreiner *et al.*, 1997), which may be due to competition for nutrients between AM fungi and rhizobia (Bethlenfalvay *et al.*, 1992). The efficiency of a symbiosis has also been found to depend on the specific combination of *Glomus* species and *Rhizobium* strain, which indicates selective interactions between the two symbionts (Azcon *et al.*, 1991). The specific compatibility between the symbionts can exist both in terms of root colonisation by introduced AM fungi and competitive ability of introduced rhizobia (Saxena *et al.*, 1997). Simultaneous inoculation with AM fungi and rhizobia has, however, been found to control a few of the soil borne plant pathogenic fungi (Dar *et al.*, 1997).

1.9 AM fungi and mine tailing rehabilitation in South Africa

Several studies have been performed in South Africa in order to determine the species and mycorrhizal status of mine tailings. All studies were able to determine the status of AM fungi on gold and uranium mine tailings located in the North West and Free State provinces (Straker *et al.*, 2007; Straker *et al.*, 2008; Spruyt, 2010) as well as on heavy metal (HM) contaminated sites in the Gauteng province (Buck, 2011). The HM sites were ABB Zinc, a galvanising plant and Impala Platinum, a platinum refinery and the heavy metals at the sites included zinc and cadmium and platinum respectively, all in salt form (D. Furniss, *pers. comm.*).

It would appear that AM fungi colonisation is affected by substratum type; host species, with woody perennials being more mycotrophic than herbaceous annuals, and age of rehabilitation (Straker *et al.*, 2007). There were differences in AM fungal colonisation levels as well as infectivity between zones on mine tailings with the veld and toepaddocks having the highest rates and the steep slopes having the lowest rates (Straker *et al.*, 2007; Straker *et al.*, 2008).

A number of recent studies have used sequence data based on the SSU rRNA gene to identify the AM fungi associated with plants on HM sites. The study of Spruyt (2010) identified species from the *Acaulospora* and *Entrophospora* genera associated with *Tamarix usneoides*, whereas the study performed in 2011 on *T. usneoides* identified species mainly in the *Claroideoglomus* genus from two heavy metal contaminated sites with only one species matching *Acaulospora mellea* and another matching *Glomus eburneum* (Spruyt *et al.*, 2014). Since *Acaulospora mellea* was also identified from *T. usneoides* growing at Mispah in the Free State province in South Africa (Spruyt, 2010), it could be suggested that this species associates with *T. usneoides* under HM conditions. However, the differences in genera associated with *T. usneoides* at the different HM contaminated sites, suggest that HM and soil properties determine which AM fungal species are associated with *T. usneoides* (Buck, 2011).

Another study performed on *Searsia lancea* and *Searsia pendulina* at the Mispah and West Complex sites in the Free State province, identified the genera *Glomus*, *Sclerocystis*, *Diversispora*, *Scutellospora* and *Claroideoglomus* which shows greater AM fungal diversity than the studies with *T. usneoides* (Spruyt *et al.*, 2014). However, the *Claroideoglomus* (*C. lamellosum*) species identified from *S. pendulina* growing at Mispah had the same species number in the BLAST database (FR773152.1) as that identified from *T. usneoides* growing naturally in the Northern Cape (Spruyt *et al*., 2014), which demonstrates that this species can associate with different hosts as well as having the ability to tolerate HMs. This may be true of many other AM fungal species.

1.10 Aims

The overall aim of this project was to determine the AM status of *Acacia* species growing on the top of differently rehabilitated slimes dams at two sites in Welkom (one site had planted acacias and the other site had naturally-colonising acacias), and the AM fungal species diversity associated with these hosts.

1.11 Specific objectives

❶ To determine the root colonisation levels of indicator *Acacia* host species from the two sites using the magnified intersections technique.

❷ To extract spores from soil samples containing roots of the indicator host species from the two sites and perform spore counts.

❸ To induce sporulation using trap cultures.

❹ To use DNA extracted from the various AM fungal spores and host roots as a template for a nested PCR amplification of a region spanning from the SSU rRNA gene through two ITS regions to the LSU rRNA region, and a region of the highly conserved SSU rRNA gene of the fungus respectively, followed by cloning, RFLP analysis,sequencing of products and phylogenetic analysis.

Chapter 2

Materials and methods

2.1 Slimes dams sites and acacia trees sampled

The two mine tailing sites utilised in the study (Figure 2.1) were phytoremediation trials which included (i) the dumping of garden refuse on the top of the FSS5 site as well as brush packing, dryland grassing and irrigated grassing and (ii) the FSN6 site included a tree trial using trees grown from local and non local seeds in the Welkom nursery (planted in the 2001/2 to 2003/4 seasons) (I. W. Weiersbye, *pers. comm.*). The FSN6 trial comprised an *Acacia* trial of trees with and without toepaddock inocula of which only the trees with toepaddock inoculum survived (I. W. Weiersbye, *pers. comm.*). The toepaddock material served as a crude inoculum for AM fungi that are tolerant to HMs. The toepaddock is the region between the retaining wall and a toewall/trench constructed to reduce the products of erosion from the mine tailing seeping into the surrounding vegetation.

The trees sampled from FSN6 could only be one of three acacia species planted, namely, *Acacia karroo*, *A. hereroense* and *A. robusta* (I. M. Weiersbye, *pers. comm.*). Only *A. hereroense* could be positively identified as it was the only species of the three to have curved prickles. *A. karroo* and *A. robusta* could not be easily distinguished between each other as both had spines on their branches and lacked the other morphological characters, such as bipinnate leaves, flowers or pods, owing to it being so early in the growing season. The majority of the trees sampled from FSS5 were *A. karroo* based on the morphological features present.

Due to the lack of morphological features an attempt was made to identify the trees using barcoding genes. However, acacia trees have never been identified via the barcoding genes and only one sequence was analysed, MatK, which was not enough to reliably identify the trees particularly as *A. hereroense*, which had already been positively identified was used as the reference and it was not identified when a BLAST analysis was performed. In order to conclusively identify acacia trees it is required that the majority (if not all) of the barcoding genes are amplified, sequenced and analysed, but the molecular identification of the host trees was not an objective of the project.

2.2 Sampling strategy

Root and soil samples were collected from the tops of the two sites in Welkom (Figure 2.1). Sampling was performed by digging up complete root systems as well as sections of root systems that provided a representative of the complete root system (fine and course roots) of *Acacia* spp. with immediately surrounding soil from the top of each dam (Figure 2.1). Nine plants were sampled from each mine tailing and Figures 2.2 and 2.3 show the exact sites of sampling. The GPS co-ordinates were recorded for each tree (Appendix 1).

Figure 2.1: Aerial view of mine tailings in the Welkom gold fields.

Figure 2.2: Aerial view of mine tailing FSN6 in the Welkom gold fields. Dots indicate the trees that were sampled.

Figure 2.3 A: Aerial view of mine tailing FSS5 in the Welkom gold fields. Dots indicate the trees that were sampled. The image demonstrates where sampling was performed in relation to the rest of the mine tailing.

Figure 2.3 B: Aerial view of mine tailing FSS5 in the Welkom gold fields. Dots indicate the trees that were sampled. The imagedemonstrates the order of tree sampling.

2.2.1 Sample maintenance

The root samples were halved and those to be used for staining and DNA extraction were prepared by first removing the aerial parts, large particles of organic matter, debris and leaves and then gently washed in water to remove soil particles. For rapid handling, roots that were larger and had smaller roots attached or consisted of whole root systems were washed in a sieve before sampling (Norris *et al.*, 1994). The roots were then cut into 1-2 cm pieces and stored in vials containing 50% ethanol at room temperature until further processing (Norris *et al.*, 1994).

The other half of the root samples were used for pot cultures.

2.3 Trap pot culture

Pot culture was performed using a modified version of the technique developed by Straker *et al.* (2010). The modifications included course, acid-washed river sand being autoclaved at 121 °C for 1 h 20 min instead of 3 h and no potting soil was used in the sand-slime mixture.

2.3.1 Preparation of trap pot samples preceding the experiment

Half the root material from each sample was chopped into 1-2 cm fragments and thoroughly mixed with half the soil/slime extracted simultaneously. The root-slime mixture was mixed with the autoclaved river sand at a ratio of 2:1 and placed in 9 cm diameter, free-draining pots. Seeds of *Sorghum bicolor* coated in a fungicide were bubbled in water for 2 d to remove said fungicide and to promote germination. Five germinated seeds were added to each pot with each indicated by a tooth pick stuck into the soil next to each seed. The seeds were covered by 1 cm course, autoclaved river sand. One trap culture was done for each sample collected from the sites (i.e. 18 pots total).

2.3.2 Trap pot culture experiment

The pots were placed in a greenhouse, and the temperature was maintained between 25 °C and 30 °C. The *S. bicolor* seeds were allowed to grow for 2 w at which point the number of seedlings was reduced to one seedling in each pot. The pots that contained no seedlings after

roughly 4 w had *Cynodon dactylon* seeds planted over the entire pot and the 1 cm river sand replaced. This may have been due to the soil having excessive amounts of HMs or chemicals and thus the conditions were too adverse for the *S. bicolour* but not for *C. dactylon* which has been known to grow on mine tailings. *C. dactylon* was also planted in the *S. bicolor*-containing pots so as to have two general hosts so as to increase the possibility of propagating all fungal species. A few seeds were planted on the side of each pot on the opposite side to the *S. bicolor* seedling remaining in the pot.

Pots were fertilised when nutrient deficient symptoms appeared on the leaves, such as yellowing and purple streaks (Figure 2.4).

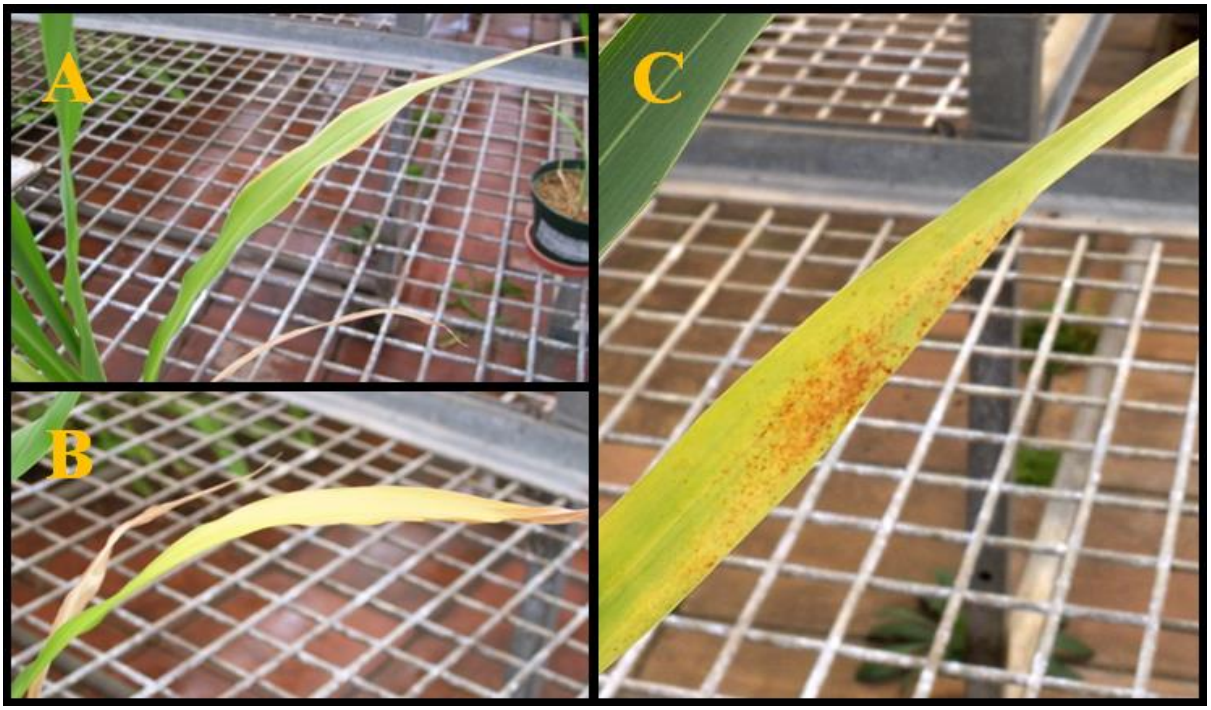

Figure 2.4: *Sorghum bicolor* leaves showing deficiency symptoms prior fertilisation. Image (A) demonstrates the initiation of deficiency symptoms with yellowing on the edge of the leaves, (B) demonstrates full deficiency symptoms and (C) demonstrates purple spots as well as yellowing indicative of potassium deficiency.

Watering of *S. bicolor* was ceased after 14 w, by which time the roots had filled the pots, and were allowed to dry in situ for 5 d. Once dry, the aerial parts of the *S. bicolor* plants were removed and the root material was separated from the slime-sand mixture. The roots were stored in 50 % ethanol and the slime-sand mixture was used for spore extractions. The pots containing both host plants had a distinct zone of inhibition between the host plant species roots, which allowed for easy separation of the roots of the *S. bicolor* from the *C. dactylon* roots and they were not confused with the *C. dactylon* roots.

Figure 2.5: Trap pot culture experiment showing the 18 free draining 9 cm diameter plastic pots with one *Sorghum bicolour* plant and some *Cynodon dactylon* per pot placed in saucers on steel-grid tables to minimise contamination and were watered once-twice a week using a measuring cylinder.

Watering of *C. dactylon* was ceased after 16 w, by which time the roots had filled the pots, and were allowed to dry in situ for 3 d. Once dry the aerial parts of the *C. dactylon* plants were removed and the root material was separated from the slime-sand mixture. The roots were stored in 50 % ethanol and the slime-sand mixture was used for spore extractions.

2.3.3 Volumetric determination of field capacity

The technique used for the determination of volumetric field capacity watering level was described by Hilditch (1999). The only change made was the use of 5 cm diameter pots as opposed to 20 cm diameter pots. Firstly, the sand-slime mixture for each pot culture was dried at 105 °C for 3 d and a specific volume measured in a measuring cylinder and then weighed. The sand-slime mixture was then put in 5 cm diameter, free draining pots of known weight. In order to prevent the sand-slime mixture from escaping and compromising the weight, muslin cloth was placed at the bottom of the pots to cover the holes. A known volume of water (50 mℓ) was added to the sand slime mixture so that it pooled on the top of the mixture. The saturated sand-slime mixture was allowed to drain through into funnels and measuring cylinders for 48 h. The volume of the water captured in the measuring cylinders

after 48 h was noted and the amount of water remaining in the soil was calculated and this amount was divided by the volume of the sand-slime mixture to give the volumetric field capacity of the sand-slime mixture for each pot sample.

2.3.4 Gravimetric determination of field capacity

The technique used for the determination of volumetric field capacity watering level was described by Hilditch (1999). The only change made was the use of 5 cm diameter pots as opposed to 20 cm diameter pots. Again the sand-slime mixture for each pot culture was dried at 105 °C for 3 d and a specific volume measured in a measuring cylinder and then weighed. The sand-slime mixture was then put in 5 cm diameter, free draining pots of known weight. In order to prevent the sand-slime mixture from escaping and compromising the weight, muslin cloth was placed at the bottom of the pots to cover the holes. A known volume of water (50 mℓ) was added to the sand slime mixture so that it pooled on the top of the mixture. The saturated sand-slime mixture was allowed to drain for 48 h and weighed to allow the mass of water retained by the soil to be calculated and this amount was then divided by the dry mass of the soil to give the gravimetric field capacity of the soil.

2.3.5 Watering procedures

All *S. bicolour* and *C. dactylon* plants were grown in free draining pots and watered once a week at roughly 60-80 % field capacity, based on the volumetric determination of field capacity, using a measuring cylinder. A few pots appeared to dry out after 3-4 days at which point they were topped up by 30-50 mℓ depending on how dry the soil was.

2.3.6 Fertiliser applications

Fertiliser solution was made according to that described by Brundett *et al.* (1995). Three stock solutions were made as per Table 2.1.

Table 2.1: A table illustrating the nutrient supplements for pot cultures grown in infertile sandy soil whilst using *Sorghum bicolour* as a host plant.

Compound	**Stock solution**	
	g/ℓ	**Stock solution number**
KH_2PO_4	10.8	A
K_2SO_4	45	A
NH_4NO_3	15	A
$CaCl_2.2H_2O$	45	B
$MgSO_4.7H_2O$	6	C
$MnSO_4.H_2O$	3	C
$ZnSO_4.7H_2O$	3	C
$CuSO_4.5H_2O$	1.5	C
H_3BO_3	0.24	C
$CoSO_4.7H_2O$	0.12	C
$(NH4)_6Mo_7O_{24}.4H_2O$	0.09	C

The compounds were dissolved in water to form the stock solutions, namely, A, B and C, which were further diluted into a nutrient solution. This was done by adding 33 mℓ of each stock solution and then making a final volume of 1 ℓ. The diluted nutrient solution was then applied to the sand-slime mixture by watering it to 60-80 % field capacity.

2.4 Assessment of host root AM fungal status

2.4.1 Clearing and staining of AM fungal roots

In order for the roots to be observed it was necessary for them to be cleared and then stained. For the clearing and staining of the roots the method described by Pitet *et al.* (2009) was used. This method involved the cytoplasm and nuclei of the host plant roots being cleared with a 1 % (w/v) solution of potassium hydroxide (KOH). The roots were then covered by the solution and left overnight at room temperature.

After the roots were fully discoloured, the KOH solution was poured off and the roots rinsed with tap water at least three times. After washing, the roots that failed to be discoloured due to heavy pigmentation (*Acacia* spp) were bleached. Bleaching was not performed on the *S. bicolor* or *C. dactylon* roots. Bleaching involved the roots being placed in 10 % alkaline hydrogen peroxide (H_2O_2), containing 3 mℓ 20% ammonium hydroxide (NH_4OH) in 30 mℓ 10 % H_2O_2, and left for 1 h at room temperature. After bleaching, the roots were thoroughly rinsed using water to remove all H_2O_2.

Once the roots were rinsed they were acidified using 5 % acetic acid, completely covering the roots, for 1 h at room temperature after which the acid was poured off the roots. This step was required for the staining and thus no rinsing was performed (Norris *et al.*, 1994). Staining was performed by using a 5 % blue ink in 5% acetic acid staining solution at room temperature for 72 h. For de-staining, the acetic acid- blue ink stain was poured off and the roots were rinsed with water to remove excess stain and submerged in acidic glycerol solution (500 mℓ glycerol, 450 mℓ water, 50 mℓ 5 % acetic acid). The de-staining solution may have been changed 2-3 times in order to fully de-stain the roots. Destaining was performed at room temperature.

2.4.2 Estimation of intraradical colonisation of AM fungi

For the microscopic examination of the roots, randomly selected, stained roots, were placed in acidic glycerol mounted on microscope slides and covered with 24 x 50 cm or 22 x 50 cm coverslips. The roots were mounted so that the roots are aligned parallel to the long axis of the slide (McGonigle *et al.*, 1990). The slides were then observed under a compound microscope at 100-400 x magnification (Norris *et al.*, 1994).

The method of analysis was the magnified intersections method described by McGonigle *et al.*, (1990), which provided a statistical measure of root colonisation. The microscope stage graticule was moved so that the field of view made six to eight equidistant passes along the slide perpendicular to the long axis of the slide. The point on the root surface at which the centre of the eye piece crosshairs entered through the side of the root was taken as the point of intersection. The vertical crosshair was rotated to ensure that each intersection was at right angles to long axis of the root (Figure 2.6). Intersections were examined by moving the plane of focus through the root completely and the scoring method used was as follows; negative (where no fungal material is present at the intersection), arbuscules (where only an arbuscule structure is present at the intersection), vesicles (where only a vesicle structure is present at the intersection) and hyphae only (where only hyphae and no other structures are present at the intersection).

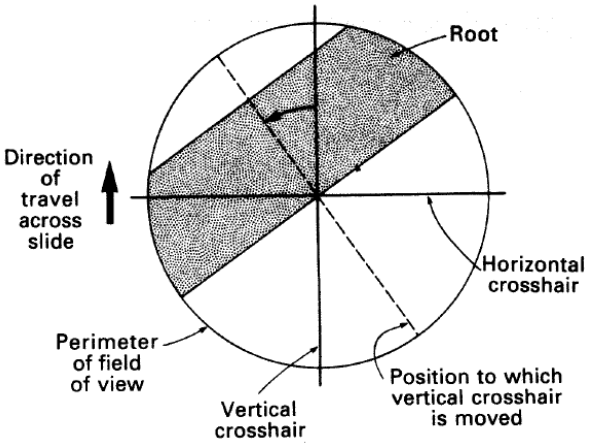

Figure 2.6: An illustration demonstrating how to make a magnified intersection perpendicular to the long axis of a root when the root is aligned with its long axis at an angle to the vertical crosshair. The stage is moved until the centre of to crosshairs is aligned with the first edge of the root reached. The vertical crosshair is rotated as shown so as to make the perpendicular intersection. Illustration taken from McGonigle *et al.* (1990).

Three root samples that were collected from each tree were each used to produce a slide containing 5 root segments. Thus at least three slides were done per tree sample so that over 100 intersects were performed for each tree.

During the magnified intersections technique any clear AM fungal structures were photographed (Canon Power Shot A640) using a camera attached to the light microscope.

2.5 Assessment of AM fungal spore density

2.5.1 Spore isolation

The protocol for the isolation of the spores was a modified version of the method developed by Gerdeman and Nicolson (1963) and improved upon by Pacioni and Rosa (1985). The isolation proceeds first by the suspension of 100 mℓ of soil in 1 ℓ of water; this was done by swirling the mixture in a 2 ℓ conical flask for 10 min. The mixture was left to stand for approximately 2 min so as to allow the heavier particles to settle. The suspension was carefully passed through a series of sieves that consist of a decreasing pore size of 1 mm to 45 µm: 1 mm, 212 µm, 125 µm, and 45µm. During the decanting process the sieves were placed on a wet shaker (Retsch) and washed under continuous running water until the water running out of the sieves ran clear.

The content of each sieve was washed and transferred into separate centrifuge tubes and centrifuged (Durafuge 100) at 3000 rpm for 3 min. The supernatant containing a fair amount of debris was discarded and the tubes were again centrifuged following the same regime. After this step, 50% sucrose (684 g sugar per litre of water) was added to each tube, and the samples were centrifuged a third time following the same regime. The sucrose served to separate the spores from denser soil constituents. The supernatant from each tube was poured back through the corresponding sieves, and the sieves were washed with water in order to remove the sucrose.

Grids of approximately 1 cm resolution were drawn on 9 cm filter paper disks to separate microscope fields for spore counting. The spores were then transferred on to the pre-wetted filter paper disks in a Büchner funnel using suction filtration.

2.5.2 Spore counts

The spores on the filter paper were ready for counting and microscopic separation according to size, shape and colour. For the quantification of spore numbers the two soil samples collected for each tree were grouped for each spore extraction. Overall there were 9 trees per site.

2.6 Soil analyses

2.6.1 pH

Water: The procedure used was a modified version of that outlined by Anderson and Ingram (1989). The modifications included the use of 10 $\pm$ 0.1 g of soil instead of 20 $\pm$ 0.1 g soil and the addition of 25 mℓ distilled water as opposed to 50 mℓ. This was done, using soil that was air dried at 105 °C for 3 days, for both original slime samples and the slime-sand mixtures used in the pot cultures. All water-soil mixtures were stirred for 10 min on a rotor shaker at 250 rpm. The mixtures were then left to stand for 30 min and stirred again for 2 min before measuring the pH (pH-meter, type 540 GLP, WTW, Germany). The pH of the supernatant was measured and noted when the reading became stable (does not change more than 0.1 unit per 30 s or 0.02 units per 5 s; van Reeuwjik, 2002).

Potassium chloride: The procedure used was that of van Reeuwjik (2002) whereby 10 ± 0.1 g of both original slime samples and slime-sand mixtures (air dried) were combined with 25 mℓ 1 M KCl. The combinations were stirred on a rotor shaker at 250 rpm for 2 h. The samples were immediately analysed using a pH meter (type 540 GLP, WTW, Germany). Just before readings were taken the samples were briefly shaken by hand, the electrode was submerged in the upper part of the suspension and the reading was recorded when the reading became stable.

2.6.2 Soil chemistry

A limited soil elemental analysis was performed by the Environmental Analytical Chemistry Unit of the School of Chemistry, University of the Witwatersrand. A summary of their protocol follows. Three slime samples from each mine tailing were used for the analysis. Around 250 mg of slime was weighed into a digestion vessel, followed by the addition of 3 mℓ nitric acid (HNO_3), 9 mℓ hydrochloric acid (HCl) and 1 mℓ hydrofluoric acid (HF). The mixture was then placed in a microwave digestion unit for 45 min. Once digested 6 mℓ boric acid was added to neutralise the HF and the digestion was transferred to a 25 mℓ flask and was then diluted to 25 mℓ. The samples were then analysed using inductively coupled plasma optical emission spectrometry (ICP OES).

2.6.3 Conductivity

The conductivity method used was a modified version by Anderson and Ingram (1989). This was performed using the water-soil mixtures from pH screening. Once the pH measurements were completed the mixtures were left to stand for 1 h before the conductivity (Thermo Scientific™ Orion™ Star A222 Conductivity Portable Meter with 0130101MD DuraProbe) of each mixture was measured.

2.6.4 Organic matter content

The method employed for the determination of soil organic matter content was that of Reddy (2014). Slime from each tree sample that had been air dried at 105 °C for 3 d was weighed into pre-weighed crucibles up to a weight of 5 ± 0.0001 g. The crucibles were then placed in a muffle furnace (Gallenkamp Instr.33) and the temperature was gradually brought up to 440

°C. The crucibles containing the sample slime were left over night at 440 °C, removed after incubation and allowed to cool. The crucibles containing the ash (burnt slime) were then weighed and the mass recorded. To determine the organic matter content first the ash weight must be determined by subtracting the crucible weight from the total weight after incubation, followed by determining the mass of organic matter by subtracting the ash from the dry soil weight (roughly 5 g), which then allows for the determination of organic matter content through the following equation:

$$\text{Organic matter content} = \frac{\text{Organic matter weight}}{\text{Dry soil weight}} \times 100$$

2.7 Statistical analysis

2.7.1 Root magnified intersection scores

The scores from each triplicate slide were added together to determine AM fungal colonisation means for each tree. The means were subjected to an Arcsine transformation (Excel 2007) and a Two-Way ANOVA (SAS Enterprise Guide 5.1) was determined in order to compare the root colonization in each category, namely arbuscular, vesicular, hyphal and total, from the three sites; followed by a Tukey-Kramer analysis (SAS Enterpriser Guide 5.1).

2.7.2 Spore counts

Spore counts from each sieve size were added together to determine means for each tree. The means were subjected to a Two-Way ANOVA; followed by a Tukey-Kramer analysis (SAS Enterprise Guide 5.1).

2.7.3 Slime chemical components and broad slime characteristics

The mean of each chemical component of the soil samples from each site was determined and subjected to a Two-Sample assuming Unequal Variances t-Test (Excel 2007). The slime characteristics pH (water and KCl) and conductivity values were subjected to a Two-Way ANOVA; followed by a Tukey-Kramer analysis (SAS Enterprise Guide 5.1). The organic matter content values were used to determine the mean from each site and subjected to a Two-Sample assuming Unequal Variances t-Test (Excel 2007).

2.8 Molecular identification of AM fungi from roots and spores

2.8.1 DNA analysis

2.8.1.1 Root DNA analysis

No manual DNA extractions were performed on any of the host plant roots. This was due to the use of the Phire® Pant Direct PCR kit that does not require DNA extraction and can amplify DNA directly from plant tissue or in this case AM fungal tissue within the plant tissue. The kit utilised a 3-5 mm punch for the sampling of tissue for the PCR reaction and this was used on one to three ± 2 mm root sections that had been positively identified to be colonised by AM fungi. For each PCR tube roughly 3 punches were performed on each root section so that a substantial amount of fungal tissue was placed in the PCR reaction mixture.

2.8.1.2 Spore DNA analysis

No manual DNA extractions were performed on any of the isolated AM fungal spores. This was due to the use of the Phire® Plant Direct PCR kit that does not require DNA extraction and can amplify DNA directly from plant seeds or in this case crushed AM fungal spores. The number of spores used depended on the size of the spores thus 10 spores from the 45 µm sieve isolation and 5 spores from the 125 µm sieve isolation were used for each PCR reaction. The 5-10 spores of similar morphology were selected using a needle moistened with glycerol and placed in sterile microcentrifuge tubes. The spores were then subjected to a freeze-thaw cycle by dipping the tubes into liquid nitrogen and allowing to thaw, repeated four times. A volume of 27 µℓ 2x Phire® Plant PCR buffer (provided in the Phire® Pant Direct PCR kit) was added to each tube and the tubes were then centrifuged (Spectrafuge 24D) at 13 000 rpm for 1-2 min. The spores were then crushed manually in the 2x Phire® Plant PCR buffer using a sterile micropestle.

2.8.2 Nested polymerase chain reaction (PCR) protocol

2.8.2.1 Root DNA

The nested PCR procedure method used was a modified version of the method outlined by Lee *et al.* (2008). The modifications entailed the use of fungal tissue as opposed to single crushed spores. Nested PCR protocol was selected as it provides increased specificity for the amplification of AM fungal DNA (Winn and Koneman, 2006). This is attributed to the fact

that two primer sets were used, with the target of the second primer set residing within that of the first (i.e. it is within the product of the first primer set) (Winn and Koneman, 2006). If the first set of primers were to amplify the wrong locus, it was unlikely that the second set of primers would be able to amplify the product thus decreasing the possibility that the incorrect sequences were amplified. Hence, the specificity of the process was increased as a greater proportion of product from the desired site was produced (Winn and Koneman, 2006).

The primary PCR reaction (Bio-Rad MyCycler™ Thermal Cycler) utilized the universal eukaryotic nuclear primers NS1 and NS4 (White *et al.*, 1990).

NS1: 5' – GTAGTCATATCGTTGTCTC – 3' (White *et al.*, 1990)
NS4: 5' – CTTCCGTCAATTCCTTTAAG – 3' (White *et al.*, 1990)

The components of the PCR reaction (20µℓ) were:
10 µℓ of 2x Phire® Plant PCR buffer
8 µℓ double distilled (nuclease free) water
0.8 µℓ NS1 stock solution 10 µM
0.8 µℓ NS4 stock solution 10 µM
0.4 µℓ Phire® Hot Start II DNA polymerase
3-4 punches of AM fungi colonised root material

The primary PCR was carried out as follows: the initial denaturation at 98 °C for 5 min; followed by 40 cycles with the denaturation steps at 98 °C for 5 s each; the primer annealing steps at 54 °C (Lee *et al.*, 2008) for 5 s each; the elongation step at 72°C for 20 s each; and after the 40 cycles a final extension step at 72 °C for 1 min. The reduced times for the PCR protocol are as a result of the Phire® Hot Start II DNA polymerase. The product produced by the first PCR was used as the template for the nested PCR reaction.

The nested PCR reaction (Applied Biosystems 2720 Thermal Cycler) utilized the AM fungal primers AML1 and AML2 (Lee *et al.*, 2008). These primers are AM fungi-specific and target the less variable SSU rRNA gene to allow for discrimination against non-AM fungi sequences and sufficient resolution down to species level in AM fungi (Lee *et al.*, 2008).

AML1: 5’ – ATC AAC TTT CGA TGG TAG GAT AGA – 3’ (Lee *et al.*, 2008)
AML2: 5’ – GAA CCC AAA CAC TTT GGT TTC – 3’ (Lee *et al.*, 2008)

The components of the PCR reaction (20 µℓ) were:
10 µℓ of 2x Phire® Plant PCR buffer
7 µℓ double distilled (nuclease free) water
0.8 µℓ AML1 stock solution 10 µM
0.8 µℓ AML2 stock solution 10 µM
0.4 µℓ Phire® Hot Start II DNA polymerase
1 µℓ primary PCR product as DNA template

The nested PCR was carried out as follows: the initial denaturation at 98 °C for 5 min; followed by 40 cycles with the denaturation steps at 98 °C for 5 s each; the primer annealing steps at 63 °C (Lee *et al.*, 2008) for 5 s each; the elongation step at 72 °C for 20 s each; and after the 40 cycles a final extension step at 72 °C for 10 min. The DNA product produced by the nested PCR reaction was analysed on an agarose gel and a few samples were selected for cloning, according to the clarity and strength of the DNA band on the agarose gels and representative samples of the different hosts in the study.

2.8.2.2 Spore DNA

The nested PCR procedure method used was a modified version of the method outlined by Lee *et al.* (2008). The modifications entailed 5-10 crushed spores (depending on spore size) as opposed to single crushed spores.

The primary PCR reaction (Bio-Rad MyCycler™ Thermal Cycler) utilised the AM fungal primers designed by Krüger *et al.* (2009) namely, SSUmAf and LSUmAr. The primers consist of a mixture of primers for both forward and reverse primer sets. This is due to different primers amplifying different AM fungal species. These primers were selected as they amplify a much larger DNA region than the NS and AML primers and can thus be used to more reliably identify AM fungal species.

SSUmAf primer mixture is comprised of SSUmAf1 and SSUmAf2:
SSUmAf1: 5’ – TGGGTAATCTTTTGAAACTTYA – 3’ (Krüger *et al.*, 2009)
SSUmAf2: 5’ – TGGGTAATCTTRTGAAACTTCA – 3’ (Krüger *et al.*, 2009)

LSUmAr primer mixture is comprised of LSUmAr1, LSUmAr2, LSUmAr3 and LSUmAr4:
LSUmAr1: 5’ – GCTCACACTCAAATCTATCAAA – 3’ (Krüger *et al.*, 2009)
LSUmAr2: 5’ – GCTCTAACTCAATTCTATCGAT – 3’ (Krüger *et al.*, 2009)
LSUmAr3: 5’ – TGCTCTTACTCAAATCTATCAAA – 3’ (Krüger *et al.*, 2009)
LSUmAr4: 5’ – GCTCTTACTCAAACCTATCGA – 3’ (Krüger *et al.*, 2009)

The SSUmAf and LSUmAr primer sets were mixed to produce working solutions with each single primer of the same concentration (10 µM).

The components of the PCR reaction (50 µℓ) were:
25 µℓ of 2x Phire® Plant PCR buffer
18 µℓ double distilled (nuclease free) water
2 µℓ SSUmAf stock solution 10 µM
2 µℓ LSUmAr stock solution 10 µM
1 µℓ Phire® Hot Start II DNA polymerase
2 µℓ of the crushed spore-buffer mixture

The primary PCR was carried out as follows: the initial denaturation at 98 °C for 5 min; followed by 40 cycles with the denaturation steps at 98 °C for 5 s each; the primer annealing steps at 60 °C (Krüger *et al.*, 2009) for 5 s each; the elongation step at 72°C for 20 s each; and after the 40 cycles a final extension step at 72 °C for 1 min. The reduced times for the PCR protocol are as a result of the Phire® Hot Start II DNA polymerase. The product produced by the first PCR was used as the template for the nested PCR reaction.

The nested PCR reaction (Applied Biosystems 2720 Thermal Cycler) utilized the AM fungal primers designed by Krüger *et al.* (2009) namely, SSUmCf and LSUmBr. The primers consist of a mixture of primers for both forward and reverse primer sets. This is due to different primers amplifying different AM fungal species. These primers were selected as they amplify a much larger DNA region than the NS and AML primers and can thus be used to more reliably identify AM fungal species.

SSUmCf primer mixture is comprised of SSUmCf1, SSUmCf2 and SSUmCf3:
SSUmCf1: 5’ – TCGCTCTTCAACGAGGAATA – 3’ (Krüger *et al.*, 2009)
SSUmCf2: 5’ – TATTGTTCTTCAACGAGGAATC – 3’ (Krüger *et al.*, 2009)
SSUmCf3: 5’ – TATTGCTCTTNAACGAGGAATC – 3’ (Krüger *et al.*, 2009)

LSUmBr primer mixture is comprised of LSUmBr1, LSUmBr2, LSUmBr3, LSUmBr4 and LSUmBr5:
LSUmBr1: 5’ – DAACACTCGCATATATGTTAGA – 3’ (Krüger *et al.*, 2009)
LSUmBr2: 5’ – AACACTCGCACACATGTTAGA – 3’ (Krüger *et al.*, 2009)
LSUmBr3: 5’ – AACACTCGCATACATGTTAGA – 3’ (Krüger *et al.*, 2009)
LSUmB4: 5’ – AAACACTCGCACATATGTTAGA – 3’ (Krüger *et al.*, 2009)
LSUmB5: 5’ – AACACTCGCATATATGCTAG – 3’ (Krüger *et al.*, 2009)

The SSUmCf and LSUmBr primer sets were mixed to produce working solutions with each single primer of the same concentration (10 µM).

The components of the PCR reaction (50 µℓ) were:
25 µℓ of 2x Phire® Plant PCR buffer
18 µℓ double distilled (nuclease free) water
2 µℓ SSUmCf stock solution 10 µM
2 µℓ LSUmBr stock solution 10 µM
1 µℓ Phire® Hot Start II DNA polymerase
2 µℓ of the crushed spore-buffer mixture

The nested PCR was carried out as follows: the initial denaturation at 98 °C for 5 min; followed by 30 cycles with the denaturation steps at 98 °C for 5 s each; the primer annealing steps at 63 °C (Lee *et al.*, 2008) for 5 s each; the elongation step at 72 °C for 20 s each; and after the 30 cycles a final extension step at 72 °C for 10 min. The DNA product produced by the nested PCR reaction was analyzed on an agarose gel and sent to Inqaba Biotec South Africa for sequencing.

2.8.3 Agarose gel electrophoresis

The DNA amplicons (3-10 µℓ) were analysed using agarose gel electrophoresis. The molecular weight marker used was the Fermentas O'GeneRuler™ 1kb DNA ladder. The expected product length of NS1-NS4 was 1100 bp, of AML1-AML2 was 795-800 bp (Lee *et al.*, 2008), of SSUmAf-SSUmAr was 1800 bp and of SSUmCf and LSUmBr was 1500 bp (Krüger *et al.*, 2009).

To make the gel, a 1x Tris/Borate/EDTA (TBE) buffer solution was prepared to a volume of 500 mℓ. To make 50 mℓ of a 1% agarose solution, 0.5 g of agarose was added to an Erlenmeyer flask and the volume made up to 50 mℓ using 1x TBE buffer. The agarose was then heated in the 1x TBE buffer in a microwave oven with gentle swirling to ensure the agarose dissolved fully. The gel solution was then allowed to cool to approximately 55 °C before adding 2.5 µℓ of 10 $mg.m\ell^{-1}$ ethidium bromide. The gel was poured into a 6 x 10 cm gel tray with an 8 or 15 well comb and allowed to set.

After setting the gel was immersed in an electrophoresis tank containing 1x TBE buffer and the comb was removed. Prior to loading of an 8 well gel, 10 µℓ of the PCR product was mixed with 2 µℓ of Fermentas 6x Orange DNA loading dye and 5 µℓ of the molecular weight marker and prior to loading of a 15 well gel, 5 µℓ PCR product was mixed with 1 µℓ loading dye – if 3 µℓ of the PCR product was used it was mixed with 2 µℓ nuclease free water before mixing with 1 µℓ loading dye. The gel was run at 100 V (LKB BROMMA 2002 Power Supply) for approximately 1 h. The PCR product was checked under a UV light and photographed using a Bio-Rad GelDoc XR Imager.

2.8.4 Cloning

Cloning was performed using the Thermo Scientific CloneJET PCR Cloning kit or the Promega pGEM®-5zf(+) vector and the *Escherichia coli* strain JM109 provided with the vector. Three root PCR reactions from each site for both the original sampling and trap pot cultures were selected for cloning so that there were a total of 12 PCR reactions to be analysed.

Bacterial competency

The protocol utilised was that of the Functional Genetics Research Laboratory at the University of the Witwatersrand. In order to make *E. coli* cells chemically competent they were first streaked onto a nutrient agar plate and grown overnight at 37 °C in order to get isolated colonies. A single colony was then grown up in 2 mℓ Luria-Bertani (LB) broth overnight at 37 °C on a shaker at 200 rpm. The 2 mℓ LB broth containing *E. coli* cells was then mixed with 53 mℓ fresh LB broth which was then incubated for 1-4 h at 37 °C on a shaker at 200 rpm. The absorbance was measured using a spectrophotometer (T60 UV Visible Spectrometer, PG Instruments) at the absorbance 600 nm. The absorbance needed to be 0.5-0.6 in order to continue with the competency protocol.

Whilst on ice, 15 mℓ of LB broth containing the bacteria was poured into a tube and centrifuged (Durafuge 100) at 3000 rpm for 5 min at 4 °C. The supernatant was discarded and another 15 mℓ of LB broth containing the bacteria was poured into a tube and centrifuged under the same conditions. This was repeated until all LB broth was used up. The pellet was resuspended with 15 mℓ 0.1 M $MgCl_2$ using 1 mℓ plastic pipettes. The suspension was then incubated on ice for 30 min and centrifuged at 3000 rpm for 5 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 2 mℓ 0.1 M $CaCl_2$ with 50 % glycerol (0.2 M $CaCl_2$ with 100 % glycerol). The competent cells were then aliquoted in 50 µℓ volumes into pre-chilled eppendorf tubes and stored at -70 °C.

The competency was tested using a HygroLacZ vector with ampicillin containing nutrient agar plates (Appendix 2). Competent cells in 2 eppendorf tubes were thawed and 2 µℓ vector (concentration of 32.8 ng/µℓ) was added to one tube, the other tube served as a negative control. The cells and vector were mixed by gentle pipetting and were then incubated on ice for 30 min. After incubation the cells were heat shocked at 42 °C for 90 s, followed by a 90 s incubation on ice. The heat shocked cells then had 300 µℓ pre-warmed SOB broth (Appendix 3) added and were then incubated at 37 °C for 1 h on a shaker at 200 rpm. After incubation 200 µℓ was spread on one ampicillin containing plate and 100 µℓ was spread on another ampicillin containing plate what was left (approximately 50 µℓ) was spread on a nutrient agar plate. The plates were then incubated at 37 °C overnight.

Cloning

Blunt end ligation of the amplicon into the Thermo Scientific CloneJET PCR Cloning kit vector was performed according to the provided intructions. The vector cloning into competent cells was done according to protocol with the amendment that higher volumes (8-10 µℓ) were added to cells due to lower vector concentration. After plating, visible colonies were used for plasmid mini-preparation.

When the Promega pGEM®-5zf(+) vector was used the vector was first restricted with Thermo Scientific FastDiget EcoRV according to the manufacturer's provided instructions and the DNA insert was ligated into the vector using Thermo Scientific T4 DNA ligase according to the manufacturer's provided instructions. To clone the vector into the competent cells the protocol used for testing competency was used except the concentration of the vector was lower thus 8-10 µℓ ligated vector was added to thawed cells and nutrient agar plates containing ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and isopropyl β-D-1-thiogalactopyranoside (IPTG) (Appendix 2) were used. Positive clones were selected by blue-white screening for plasmid mini-preparation.

Plasmid mini-preparation

The procedure used was a modified version of that outlined by Semple-Rowland Laboratory McKnight Brain Institute University of Florida. The modifications included the use 5 mℓ ampicillin (50 mg/mℓ) containing LB broth instead of 3 mℓ ampicillin (50 mg/mℓ) containing LB broth, the use of 110 µℓ resuspension buffer as opposed to 100 µℓ, no RNAse was added to the resuspension buffer, the bacterial debris was removed by centrifugation for 4 min instead of 2 min and the solution was incubated for 2 min after ethanol was added.

The positive clones were used to inoculate 5 mℓ ampicillin LB broth which was incubated at 37 °C overnight on a shaker at 200 rpm. The bacterial cells were collected by centrifuging 1.7 mℓ of LB broth in an eppendorf tube at 12 000 rpm for 1 min (Spectrafuge 24D). The supernatant was removed without disturbing the pellet and another 1.7 mℓ was centrifuged under the same conditions. This was repeated until all LB broth was used up. The pellet was resuspended in 110 µℓ resuspension buffer (25 mM Tris-HCl, pH 8.0, 10 mM EDTA) so that no lumps are visible. Once resuspended 100 µℓ lysis buffer (equal volumes of 800 mM NaOH and 4 % SDS prepared just before use) was added and mixed thoroughly by inverting the tubes 5-6 times at room temperature. Once the mixture appeared translucent and mucous-

like 120 µℓ neutralisation buffer (5 M potassium acetate, pH 5.5) was added and thoroughly by inverting the tubes 5-6 times at room temperature. The mixture was then incubated for 3 min at room temperature and the bacterial debris was then removed by centrifugation at 12 000 rpm for 4 min. The supernatant was transferred to a new eppendorf tube and 200 µℓ isopropanol was added and the solution was mixed thoroughly by inverting the tubes 10 times. The tubes were then incubated at room temperature for 1 min and then centrifuged at 14 000 rpm for 1 min. The isopropanol was poured off and 500 µℓ 70 % ethanol was added to the tubes. The tubes were incubated at room temperature for 2 min and the tubes were centrifuged at full speed for 1 min. The ethanol was poured off and the tubes were centrifuged again for 1 min and the excess ethanol was gently removed from the tubes with a 200 µℓ pipette. The tubes were then left with the lids open in a laminar flow for 10 min to allow for any remaining ethanol to evaporate. The DNA was then dissolved in 20-30 µℓ nuclease free water with the volume being determined by whether or not a pellet was visible.

Cloning products

The extracted plasmid DNA was then used for PCR analysis either using the Thermo Scientific CloneJET PCR Cloning kit primers or the T7 and SP6 primers for the Promega pGEM®-5zf(+) vector.

Analysis of the Thermo Scientific CloneJET PCR Cloning kit products

The components of the PCR reaction (20 µℓ) were:

10 µℓ of 2x Phire® Plant PCR buffer

5-7 µℓ double distilled (nuclease free) water (depending on the amount of plasmid added)

0.8 µℓ pJET1.2 Forward Sequencing primer stock solution 10 µM

0.8 µℓ pJET1.2 Reverse Sequencing primer stock solution 10 µM

0.4 µℓ Phire® Hot Start II DNA polymerase

1-3 µℓ extracted plasmid (depending on whether there was a pellet or not during miniprep)

The PCR was carried out as follows: the initial denaturation at 98 °C for 5 min; followed by 40 cycles with the denaturation steps at 98 °C for 5 s each; the primer annealing steps at 60 °C for 5 s each; the elongation step at 72°C for 20 s each; and after the 40 cycles a final extension step at 72 °C for 1 min. The product produced by the PCR was then used in a subsequent PCR using the AML primers at a volume of 50 µℓ for RFLP analysis, if

necessary, the DNA product produced by the PCR reaction was analysed on an agarose gel and sent to Inqaba Biotec South Africa for sequencing.

Analysis of the Promega pGEM®-5zf(+) vector products

The components of the PCR reaction (20µℓ) were:

10 µℓ of 2x Phire® Plant PCR buffer

5-7 µℓ double distilled (nuclease free) water (depending on the amount of plasmid added)

0.8 µℓ T7 stock solution 10 µM

0.8 µℓ SP6 stock solution 10 µM

0.4 µℓ Phire® Hot Start II DNA polymerase

1-3 µℓ extracted plasmid (depending on whether there was a pellet or not during miniprep)

The PCR was carried out as follows: the initial denaturation at 98 °C for 5 min; followed by 40 cycles with the denaturation steps at 98 °C for 5 s each; the primer annealing steps at 55 °C for 5 s each; the elongation step at 72°C for 20 s each; and after the 40 cycles a final extension step at 72 °C for 1 min. The product produced by the PCR was then used in a subsequent PCR using the AML primers at a volume of 50 µℓ for RFLP analysis, if necessary, the DNA product produced by the PCR reaction was analysed on an agarose gel and sent to Inqaba Biotec South Africa for sequencing.

2.8.5 RFLP analysis

When more than 5-6 positive clones per original sample were positively amplified by PCR, the PCR products were used for restriction fragment length polymorphism (RFLP) analysis. The restriction enzymes used were Thermo Scientific FastDigest *AluI*, Thermo Scientific FastDigest *HinfI* and Thermo Scientific FastDigest *NciI*. The PCR products of the clones were then digested using the restriction enzymes according to the manufacturer's provided instructions and the restriction digests were then analysed on 1 % agarose gels, running 8 µℓ of the digests in each lane. The samples that had different banding patterns were then sent to Inqaba Biotec South Africa for sequencing.

2.8.6 Phylogenetic analysis

The quality of the sequence electrophorograms was analysed using Chromas Lite and BioEdit; manual adjustments were made where necessary, for instance trimming the ends of the sequences for the consensus sequence construction using the forward and reverse primer sequences and a few sequences were not used for phylogenetic analysis due to poor sequence quality. The constructed consensus sequences were then used for a BLAST analysis using the megablast algorithm (Zhang *et al.*, 2000) from the Maarj*AM* and NCBI BLAST databases (http://maarjam.botany.ut.ee) (Öpik *et al.*, 2010). The species identifications were presumptive when the identities were below 97 % and in the case of genera the presumptive when the identities were below 95 %. The presumptions were made on the basis of total BLAST score and valid sequence availability within the databases. The consensus sequences of the clones were subjected to a multiple sequence alignment using MAFFT version 6 and MEGA 6 was used to perform the phylogenetic analysis to construct a bootstrapped neighbour-joining tree of the multiple sequence alignment which was then visualised using TreeExplorer.

Chapter 3

Results

3.1 Root colonisation

Objective 1:

To determine the root colonisation levels of indicator *Acacia* host species from the two sitesusing the magnified intersections technique.

During the magnified intersections technique six different intraradical structures (hyphae, vesicles, appressoria, hyphal coils, arbuscules and spores) and two extraradical structures (hyphae and spores) were observed and photographed (Figures 3.1-3.4).

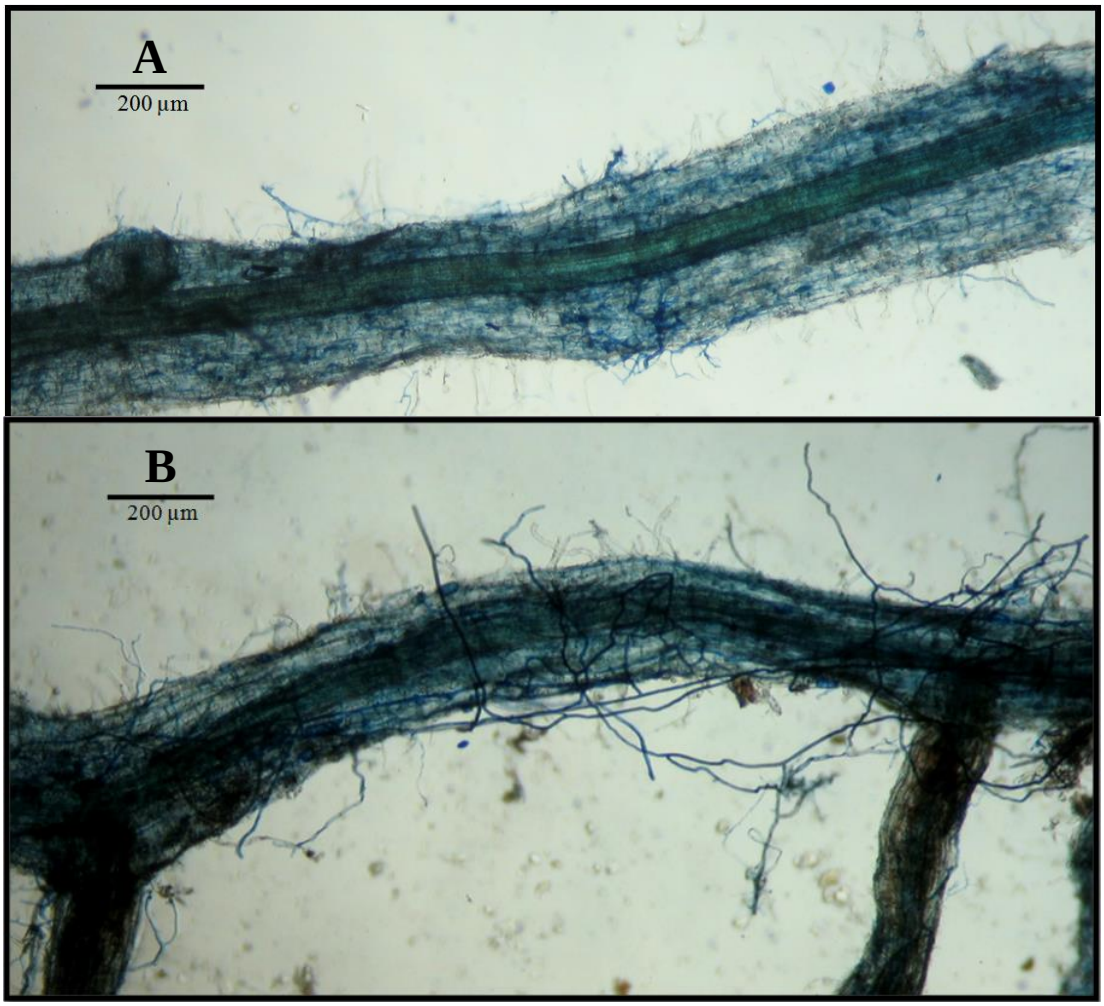

Figure 3.1: Light micrographs of intraradical and extraradical AM fungal structures observed during the magnified intersections technique. A-B) Overall view of a roots colonised by AM fungi with A) showing intraradical hyphae and B) showing extraradical hyphae.

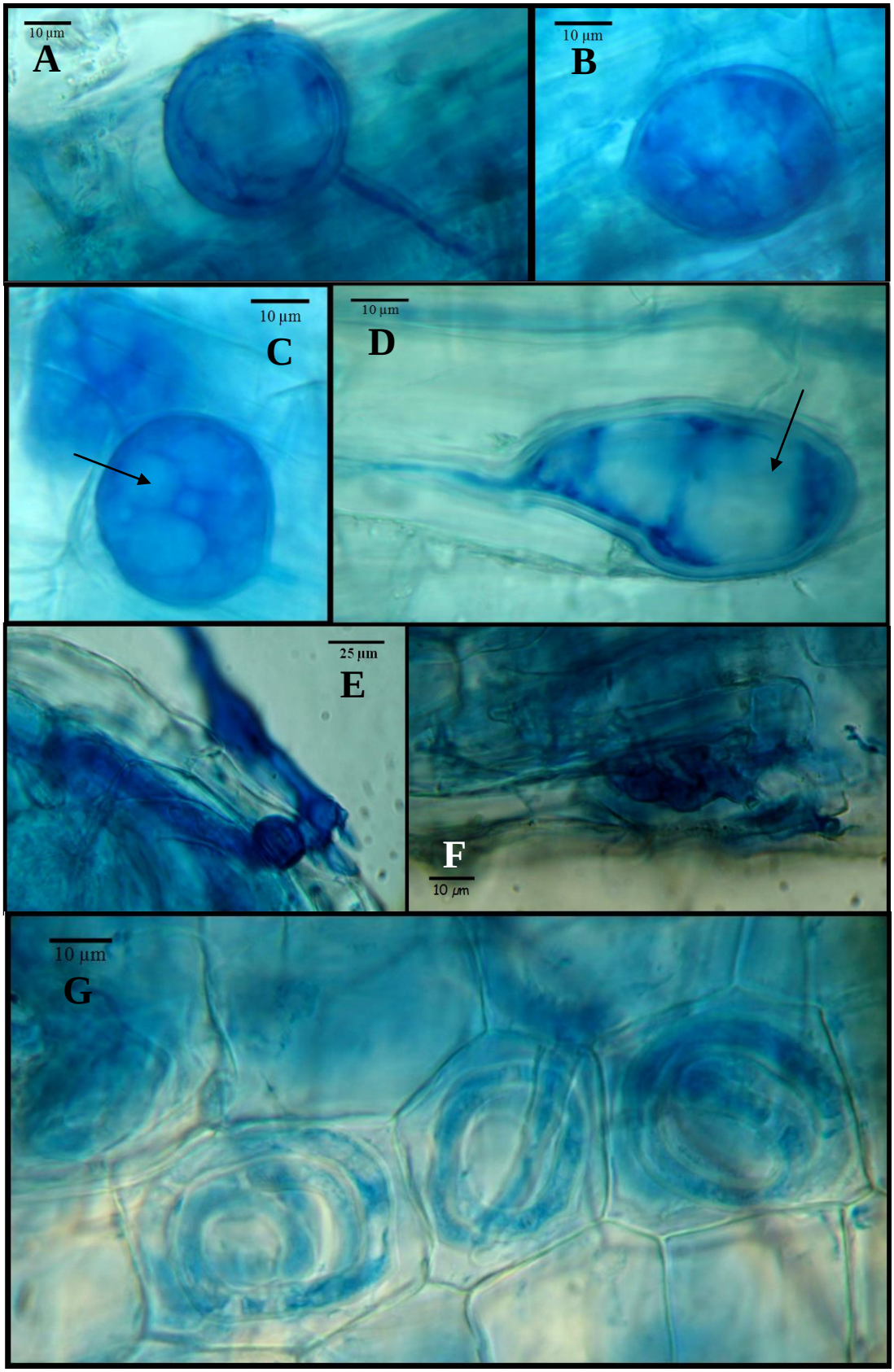

Figure 3.2: Light micrographs of intraradical AM fungal structures observed during the magnified intersections technique. A-D) differently shaped vesicles, arrows show lipid bodies; E-F) appressoria penetrating roots and G) root cells containing hyphal coils.

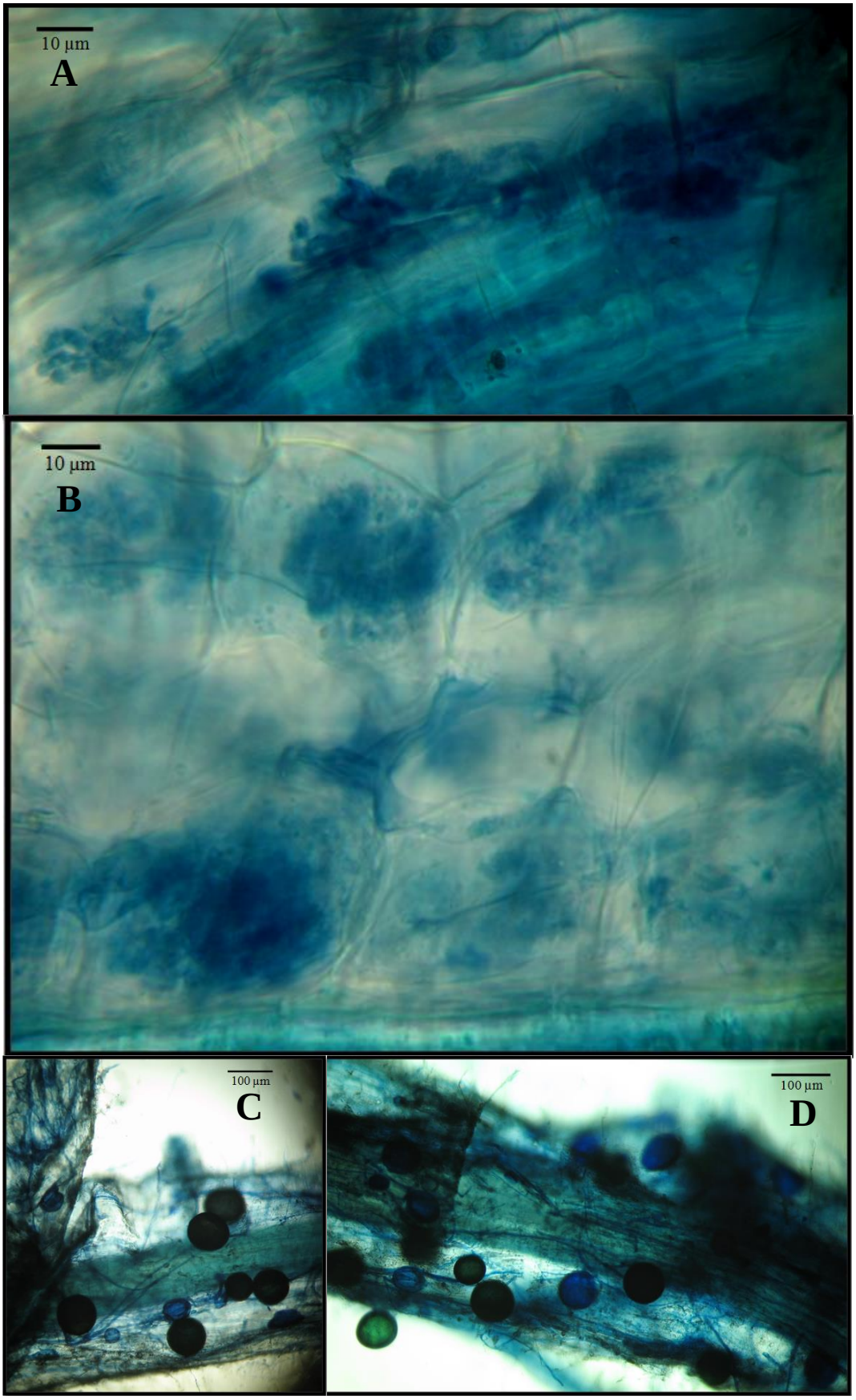

Figure 3.3: Light micrographs of intraradical AM fungal structures observed during the magnified intersections technique. A-B) young arbuscules colonising root cells and C-D) intraradical spores.

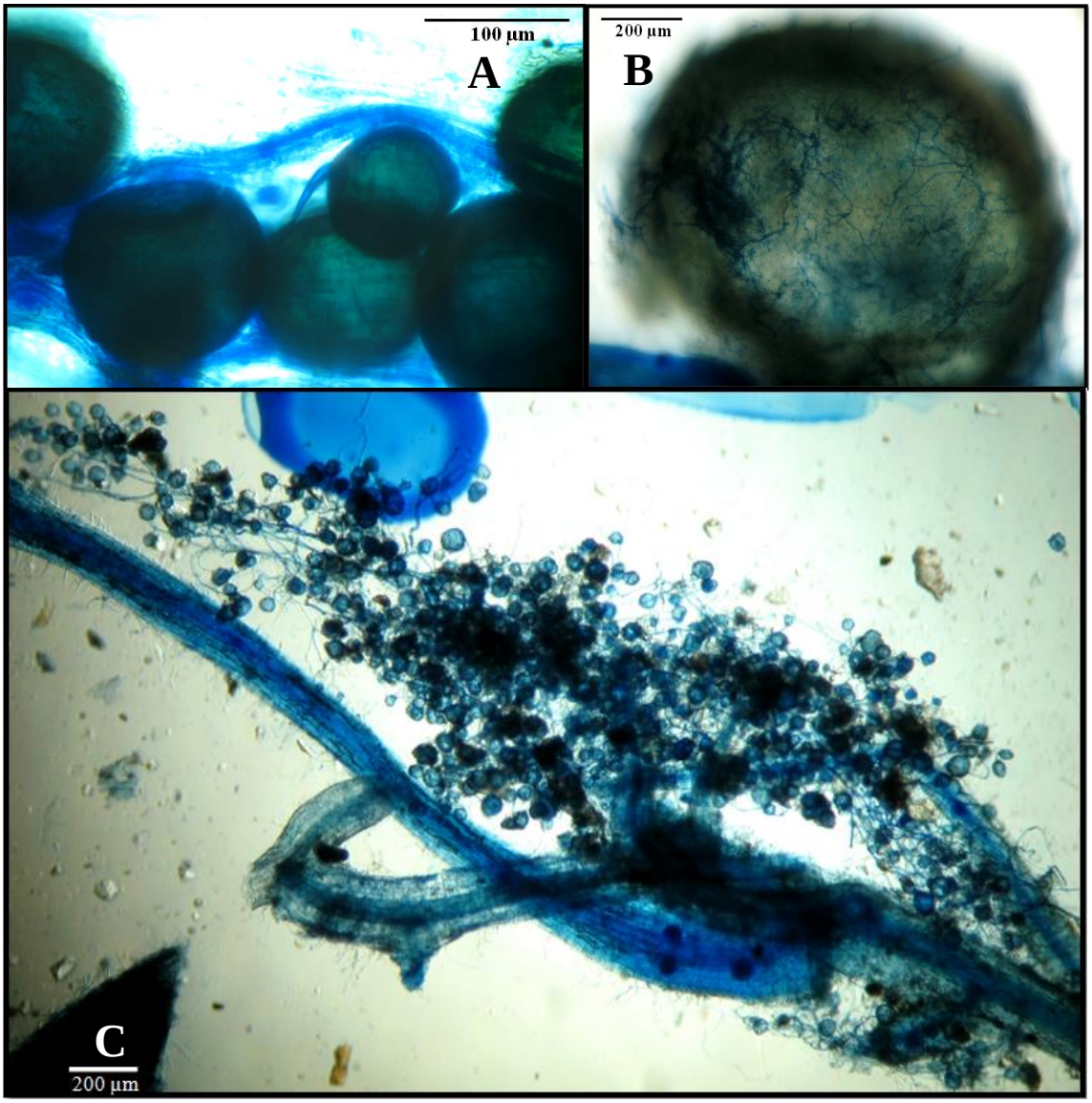

Figure 3.4: Light micrographs of intraradical and extraradical AM fungal structures observed during the magnified intersections technique. A) intraradical spores; B) *Acacia* nodule colonised with AM fungal hyphae and C) extraradical hyphae attached to spores.

The colonisation level of the intraradical AM fungal structures as well as total colonisation from the initial sampling and trap cultures of the two mine tailings were determined by the magnified intersections technique, and are represented in Figure 3.5. The acacias from FSS5, when compared with FSN6 acacias, had a higher colonisation level for all colonisation categories when initial sampling was compared with initial sampling and trap culture samples were compared with trap culture samples (except for vesicular colonisation in the trap samples). FSS5 initial sampling colonisation was significantly lower than the trap culture colonisation for the arbuscular and hyphal categories and vice versa for the vesicular colonisation. There was no significant difference between initial sampling and trap culture

samples for FSS5 total colonisation. Despite the trends, FSN6 initial sampling colonisation was not significantly lower than the trap culture samples for either arbuscular, hyphal or total colonisation categories nor significantly higher in the case of vesicle colonisation. The colonisation level for FSS5 initial sampling was significantly higher than FSN6 initial sampling in all categories except for arbuscular colonisation.

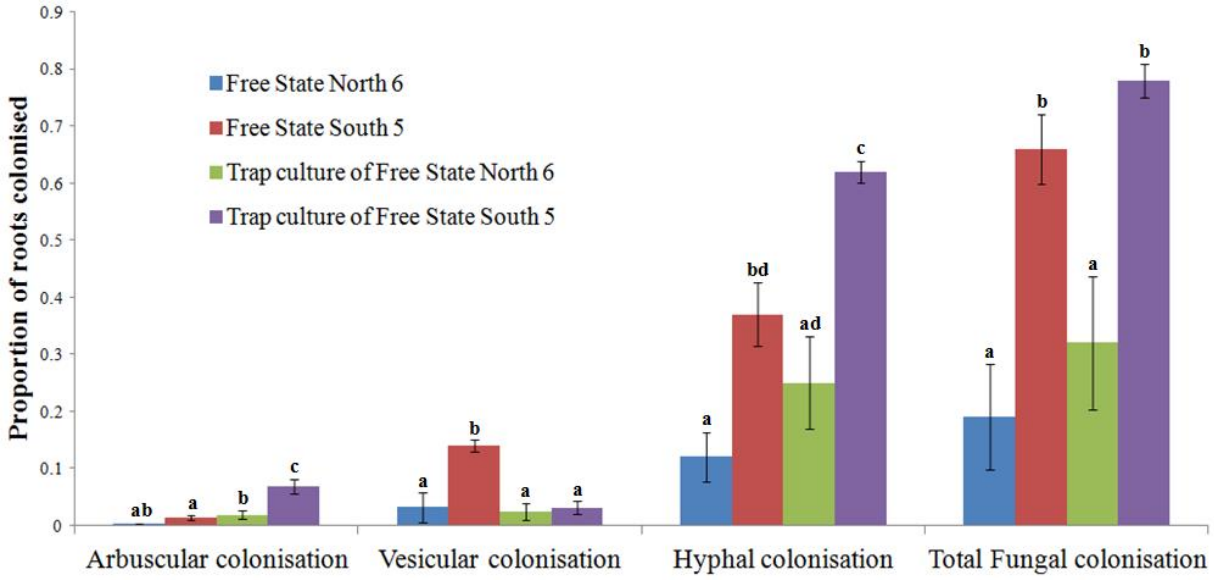

Figure 3.5: Overall comparison of the mean colonisation levels of the various AM fungal structures associated with the *Acacia* roots from the mine tailings Free State North 6 and Free State South 5 and the trap cultures of these mine tailings. The values represent the means of 9 replicates ± SE. The means with different superscripts are significantly different ($p<0.050$) between sites for each category of colonisation according to the Tukey Kramer analysis.

3.2 Spore counts

Objectives 2 and 3:

- To extract spores from soil samples containing roots of the indicator host species from the two sites and perform spore counts.
- To induce sporulation using trap cultures.

It was determined that initial sampling of FSS5 had a higher spore number than initial sampling of FSN6 and vice versa was true for trap culture spore numbers. However, the differences were not significant except for when initial sampling of FSS5 was compared with trap cultures of FSS5, in which case the initial sampling spore number was significantly higher than the trap culture spore number (Figure 3.6).

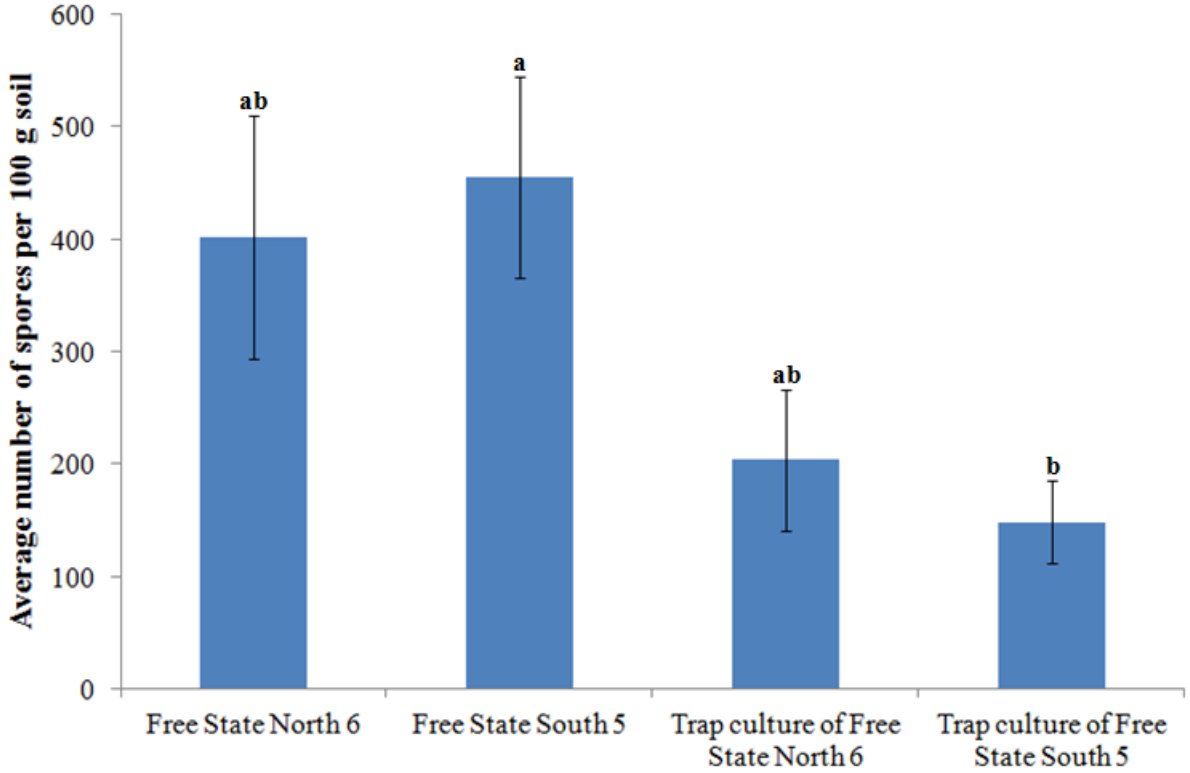

Figure 3.6: Soil spore levels of *Acacia* spp. from the mine tailings Free State North 6 and Free State South 5 and the trap cultures of these mine tailings. The values represent the means of 9 replicates ± SE. The means with different superscripts are significantly different ($p<0.050$) between sites for each category of colonisation according to the Tukey Kramer analysis.

3.3 Soil analyses

3.3.1 Slime chemical elements

The concentration of various HM or toxic elementss was determined for each mine tailing using ICP OES (Table 3.1) and it was determined that aluminium was present in the highest concentration and cadmium and yttrium were present in the lowest concentrations for both mine tailings. Chemical concentrations between the mine tailings were not significantly different except for aluminium, whereby FSN6 concentration was almost double that of FSS5. During the chemical analysis a few chemicals were not detected, either due to not being present or they were present in concentrations that were too low to be detected; these elements included lead, mercury, molybdenum, nickel, platinum and thorium.

Table 3.1: Results of the chemical analysis on slime from two differently rehabilitated mine tailings in the Welkom gold fields to give the concentration (mg/kg) (means of 3 replicates ± S. E.) of various chemicals.

Chemical	**Free State North 6**	**Free State South 5**
Aluminium	25809.8 ± 2098.95[a]	13396.2 ± 1101.21[b]
Arsenic	18.37 ± 0.50	19.52 ± 2.24
Cadmium	7.99 ± 0.13	8.86 ± 0.35
Calcium	8395.9 ± 1902.43	11457.32 ± 5268.08
Chromium	183.24 ± 16.64	173.37 ± 23.48
Copper	71.15 ± 20.12	6.14 ± 1.62
Iron	12505.67 ± 2050.67	7038.4 ± 783.41
Potassium	3153.53 ± 355.23	2274.2 ± 439.31
Titanium	1403.93 ± 158.16	1052.44 ± 141.95
Uranium	254.31 ± 73.63	83.66 ± 5.37
Yttrium	9.58 ± 1.14	5.74 ± 0.32
Zinc	131.3 ± 6.93	79.85 ± 24.75

The means with different superscripts are significantly different ($p<0.050$) between sites according to the Two-Sample assuming Unequal Variances t-Test.

3.3.2 Slime characteristics

Several soil characteristics were analysed for each mine tailing slime sample and trap culture sand-slime mixture (Table 3.2). It was determined that there were only significant differences between initial sampling and trap culture samples with the exception of organic matter content which only analysed initial slime samples and conductivity which was different between the sites for initial slime samples and trap culture samples.

Table 3.2: Broad characteristics of the slime and trap culture sand-slime mixture (means of 9 replicates ± S.E.) of two differently rehabilitated mine tailings in the Welkom gold fields.

	Free State North 6	**Free State South 5**	**Trap culture of Free State North 6**	**Trap culture of Free State South 5**
pH (water)	6.02 ± 0.26[a]	7.09 ± 0.204[a]	6.08 ± 0.18[b]	7.22 ± 0.18[b]
pH (KCl)	5.92 ± 0.28[a]	6.59 ± 0.19[a]	5.89 ± 0.22[ab]	6.80 ± 0.17[b]
Conductivity µS/cm	4268.89 ± 622.72[a]	707 ± 325.78[b]	2287.89 ± 128.51[c]	510.08 ± 240.56[b]
Organic matter content (%)*	3.42 ± 0.27[a]	3.68 ± 0.83[a]	-	-

The means with different superscripts are significantly different ($p<0.050$) between sites according to the Tukey Kramer analysis.

* Statistical analysis performed was two sample t-Test assuming unequal variances.

3.4 Molecular analysis

3.4.1 Root AM fungal analysis

Objective 4 part 1:

To use DNA extracted from host roots as a template for a nested PCR amplification of the highly conserved SSU rRNA gene of the fungus, followed by cloning, RFLP analysis, sequencing of products and phylogenetic analysis.

3.4.1.1 Initial PCR analysis

Once root fragments had been analysed using the magnified intersections technique the colonised sections (sometimes in combination) were used for direct PCR amplification and 12 sequences (Figure 3.7) were selected for cloning. The PCR amplification was performed using the NS primer pair for the primary PCR reaction and the AML primer pair was used for the nested PCR reaction, and the 12 samples selected for cloning (Figure 3.7) showed clear bands with the approximate size of 795 bp indicating the correct DNA sequence was amplified by the primer pair.

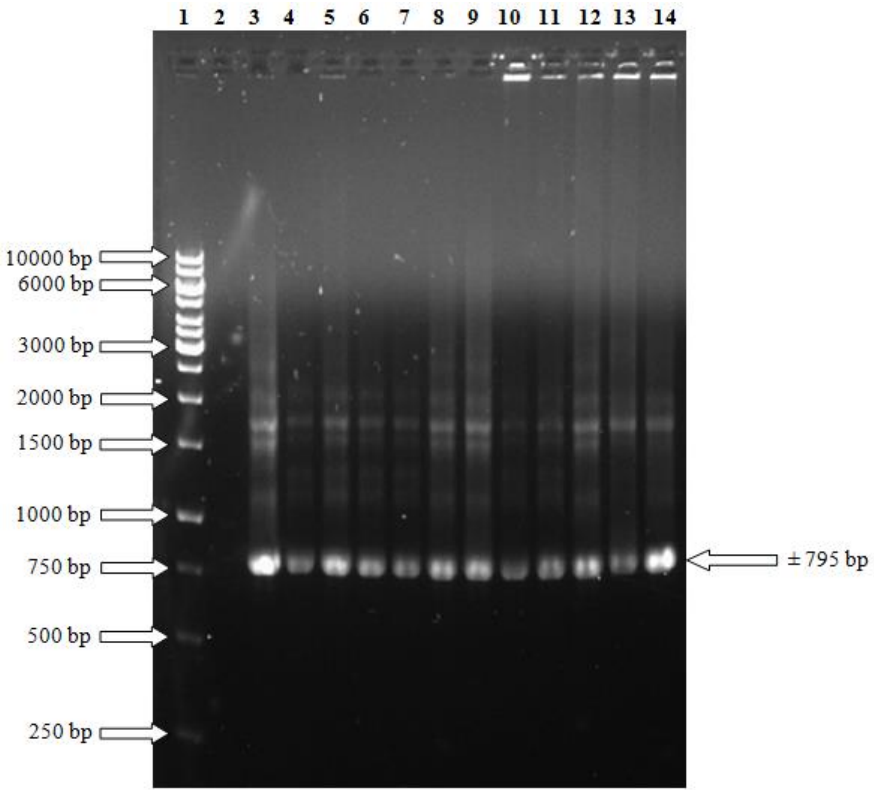

Figure 3.7: 1 % agarose gel showing nested PCR products of AM fungal SSU rDNA extracted from colonised root material from initial and trap cultures of mine tailings Free State North 6 and Free State South 5.

Lanes: (1) O' GeneRuler™ 1kb DNA ladder; (3) Trap culture FSN6 sample 1; (4) *Acacia* sp. FSN6 sample 1; (5) *Acacia* sp. FSN6 sample 2; (6) *Acacia* sp. FSN6 sample 3; (7) *Acacia* sp. FSS5 sample 1; (8) *Acacia* sp. FSS5 sample 2; (9) *Acacia* sp. FSS5 sample 3; (10) Trap culture FSS5 sample 1; (11) Trap culture FSS5 sample 2; (12) Trap culture FSS5 sample 3; (13) Trap culture FSN6 sample 2; (14) Trap culture FSN6 sample 3

3.4.1.2 Cloning and RFLP analysis

Few clones were produced per plate; thus all DNA samples were cloned two-three times. In order to avoid repetition of amplicons sequenced some samples were analysed using RFLP analysis. Only two DNA samples (*Acacia* sp. FSS5 sample 3 and trap culture FSN6 sample 2) were analysed using RFLP analysis. However, sample *Acacia* sp. FSS5 sample 1 should have been analysed using RFLP analysis as there were 11 clones produced and sequenced. RFLP analysis was performed by restricting amplicons using three restriction enzymes, *AluI*, *Hinfl* and *NciI* in order to produce banding patterns on agarose gels. Figure 3.8 demonstrates banding patterns of amplicons from one sample and where amplicons are different, banding patterns differ. Figure 3.8 illustrates the various banding patterns produced by cloned sequence FSN6 sample 2.

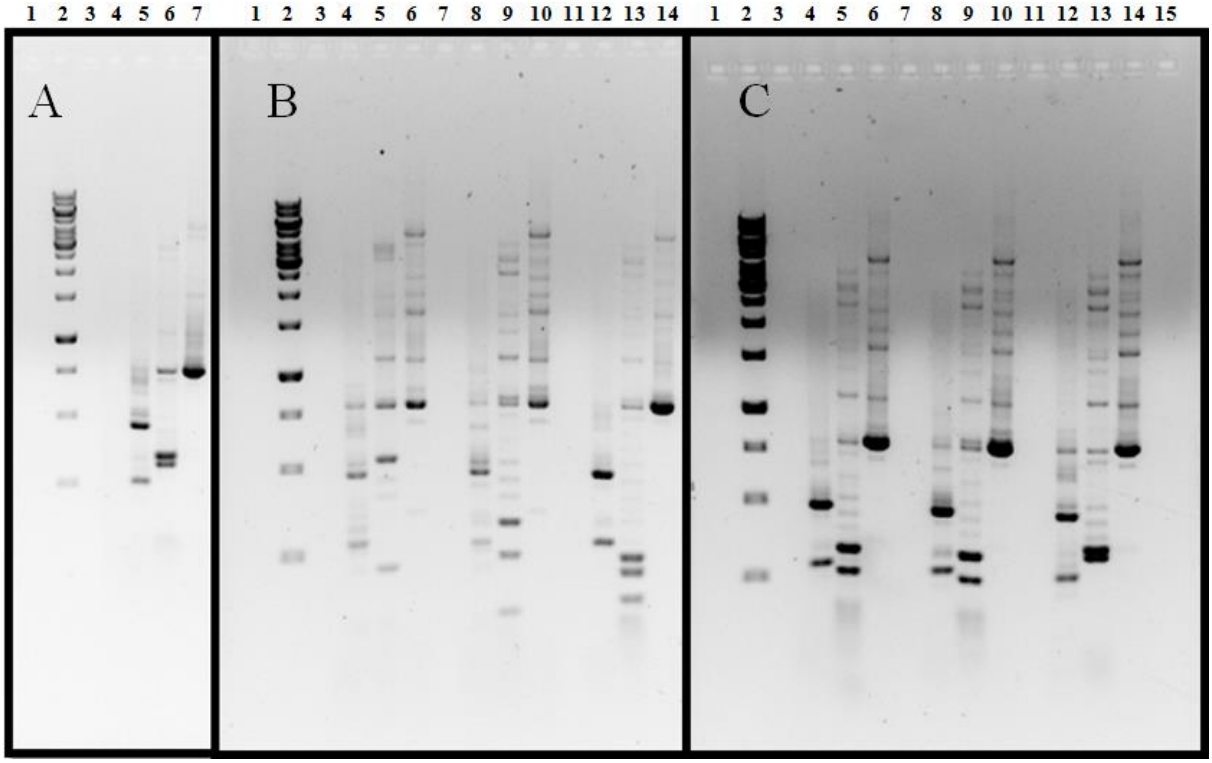

Figure 3.8: 1 % agarose gels of the RFLP analysis performed on trap culture FSN6 sample 2 clones. For each sample: the first lane contains clone DNA restricted with AluI, the second lane contains clone DNA restricted with NciI and the third lane contains clone DNA restricted with Hinfl. A) Clone 17; B) Clones 18-20 and C) Clones 21 and 22 – first clone sample (Lanes 4-6) was not sequenced.

From the gels it was observed that there were only two banding patterns that were identical [gel C sample 1 (lanes 4-6) and 2 (8-10)] thus one of the two sequences was selected for sequencing based on the strength and clarity of the DNA band on an agarose gel.

3.4.1.3 Sequencing analysis

The electropherograms (Figure 3.9, which is a representative section of the AM fungal sequence, *Acacia* FSN6 sample 1) of the majority of the sequences (forward and reverse primers) were clear and showed very little or no shadow bands demonstrating that the sequences were relatively free of contaminating nucleotides and sequences and were of high quality and had high identity (between the forward and reverse primers) when constructing consensus sequences. All sequences were used for Maarj*AM* and BLAST analyses with few adjustments.

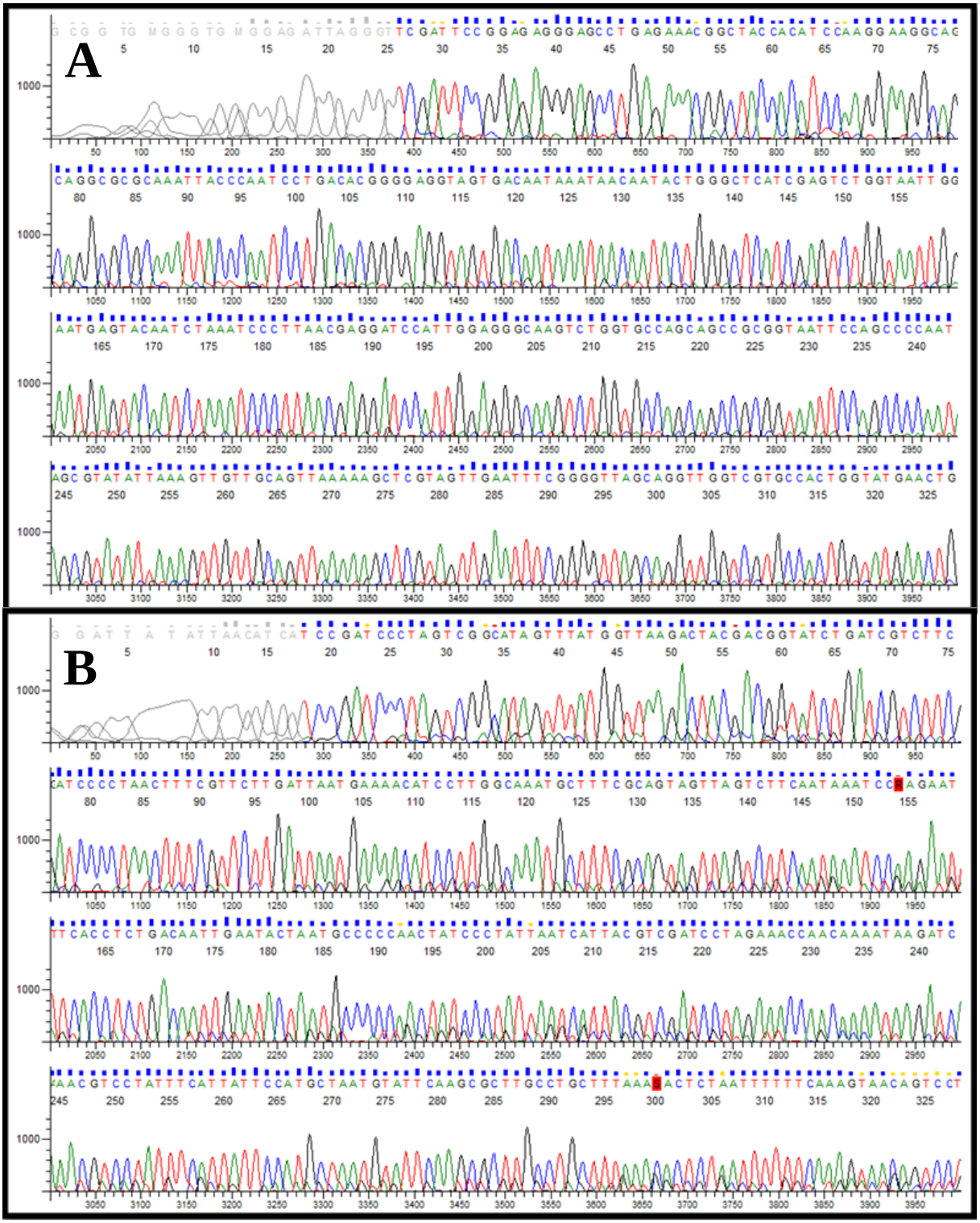

Figure 3.9: Electropherogram sections of the nested PCR forward primer (A) and reverse primer (B) product sequence of *Acacia* FSN 6 sample 1 clone DNA viewed using Sequence Scanner v1.0.

As illustrated in the electropherograms, the reverse primer product sequence was less clear than the forward primer product and this was observed for all AM fungal sequences extracted from root material.

3.4.1.4 Phylogenetic analysis

Alternate results for several sequences were provided as equally high identities were present during the BLAST analysis (Table 3.3). This may have been due to the AML primers producing insufficient amplicon lengths to reliable resolve identification beyond genus level. The sequences were then used to produce a phylogram using the Jukes Cantor algorithm for a neighbour joining tree.

Table 3.3: Results of BLAST analysis using the Maarj*AM* and NCBI BLAST databases which is based on small subunit rRNA gene sequences identifying AM fungi associated with *Acacia* spp. growing on gold and uranium mine tailings. All e values were zero except one°.

Number on phylogenetic tree	**Site, sampling and clone number**	**Original DNA sample**	**Fungal species**	**Accession number**	**% Identity**
1	Acacia FSN6 Clone 1	Sample 1	*Sclerocystis sinuosa*[*]	AJ133706.1	95
2	Acacia FSN6 Clone 2		*Rhizophagus intraradices*	FR750209.1	87
3	Acacia FSN6 Clone 3		*Scutellospora dipurpurescens*	FM212925.1	86
4	Acacia FSN6 Clone 4		*Diversispora celata*	AM713428.1	87
5	Acacia FSN6 Clone 5		*Scutellospora calospora*[a]	FJ009671.1	79
6	Acacia FSN6 Clone 6		*Diversispora celata*[b]	AM713428.1	95
7	Acacia FSN6 Clone 7		*Sclerocystis sinuosa*[*]	AJ133706.1	92
8	Acacia FSN6 Clone 8	Sample 2	*Sclerocystis sinuosa*[*]	AJ133706.1	98
9	Acacia FSN6 Clone 9		*Claroideoglomus etunicatum*	FR750216.1	91
10	Acacia FSN6 Clone 10		*Claroideoglomus lamellosum*	FR773152.1	92
11	Acacia FSN6 Clone 11		*Glomus iranicum*	HM153420.1	96
12	Acacia FSN6 Clone 12		*Diversispora celata*[c]	AM713428.1	96
13	Acacia FSN6 Clone 13	Sample 3	*Diversispora trimerales*	FR686956.1	87
14	Acacia FSN6 Clone 14		*Redeckera fulvum*[•]	AM418543.1	94 (BLAST: 95)
15	Acacia FSN6 Clone 15		*Claroideoglomus etunicatum*	FR750216.1	91
16	Acacia FSN6 Clone 16		*Rhizophagus intraradices*	FR750206.1/FR750209.1	87
17	Acacia FSN6 Clone 17		*Diversispora celata*[d]	AM713428.1	96
18	Acacia FSN6 Clone 18		*Diversispora spurca*	Y17650.2	94
19	Acacia FSS5 Clone 1	Sample 1	*Claroideoglomus lamellosum*	FR773152.1	92
20	Acacia FSS5 Clone 2		*Rhizophagus irregularis*[e]	FR750222.1/FR750223.1	87
21	Acacia FSS5 Clone 3		*Rhizophagus fascicalatus*	Y17640.2	96 (BLAST: 98)
22	Acacia FSS5 Clone 4		*Sclerocystis sinuosa*	AJ133706.1	97

23	Acacia FSS5 Clone 5		*Claroideoglomus lamellosum*	FR773152.1	91
24	Acacia FSS5 Clone 6		*Diversispora celata*[f]	AM713428.1	96
25	Acacia FSS5 Clone 7		*Rhizophagus intraradices*	FR750206.1	96
26	Acacia FSS5 Clone 8		*Claroideoglomus lamellosum*	FR773152.1	92
27	Acacia FSS5 Clone 9		*Claroideoglomus lamellosum*	FR773152.1	92
28	Acacia FSS5 Clone 10		*Claroideoglomus lamellosum*	FR773152.1	92
29	Acacia FSS5 Clone 11		*Claroideoglomus lamellosum*	FR773152.1	91
30	Acacia FSS5 Clone 12	Sample 2	*Diversispora celata*[g]	AM713428.1	93
31	Acacia FSS5 Clone 13		*Claroideoglomus lamellosum*	FR773152.1	92
32	Acacia FSS5 Clone 14		*Glomus iranicum*	HM153420.1	91
33	Acacia FSS5 Clone 15		*Glomus iranicum*	HM153420.1	97
34	Acacia FSS5 Clone 16		*Claroideoglomus etunicatum*	FR750216.1	91
35	Acacia FSS5 Clone 17	Sample 3	*Diversispora celata*[h]	AM713428.1	96
36	Acacia FSS5 Clone 18		*Cetraspora gilmorei*	FR773142.1	97
37	Acacia FSS5 Clone 19		*Claroideoglomus etunicatum*	FR750216.1	91
38	Acacia FSS5 Clone 20		*Glomus iranicum*	HM153420.1	95
39	Acacia FSS5 Clone 21		*Claroideoglomus etunicatum*	FR750216.1	91
40	Acacia FSS5 Clone 22		*Diversispora celata*[i]	AM713428.1	95
41	Trap culture FSN6 Clone 1	Sample 1	*Claroideoglomus etunicatum*[j]	FR750217.1/FR750216.1	96
42	Trap culture FSN6 Clone 2°		*Cetraspora pellucida*[k]	FR750215.1	90
43	Trap culture FSN6 Clone 3		Could not be resolved[l]		
44	Trap culture FSN6 Clone 4		*Scutellospora reticulata*	AJ871271.1/AJ871273.1	96
45	Trap culture FSN6 Clone 5		*Claroideoglomus lamellosum*	FR773152.1	89
46	Trap culture FSN6 Clone 6	Sample 2	*Diversispora celata*	AM713428.1	95
47	Trap culture FSN6 Clone 7		*Claroideoglomus lamellosum*	FR773152.1	86
48	Trap culture FSN6 Clone 8		*Sclerocystis sinuosa*[*]	AJ133706.1	93
49	Trap culture FSN6 Clone 9		*Rhizophagus intraradices*[m]	FR750206.1/FR750209.1	90
50	Trap culture FSN6 Clone 10		*Redecker fulvum*[•]	AM418543.1	92 (BLAST: 88)

51	Trap culture FSN6 Clone 11		*Diversispora spurca*	FR686954.1/Y17650.2	86
52	Trap culture FSN6 Clone 12		*Claroideoglomus lamellosum*	FR750221.1/FR773152.1	90
53	Trap culture FSN6 Clone 13		*Diversispora celata*[n]	AM713428.1	96
54	Trap culture FSN6 Clone 14	Sample 3	*Rhizophagus intraradices*	FR750206.1	97
55	Trap culture FSN6 Clone 15		*Claroideoglomus lamellosum*	FR773152.1	92
56	Trap culture FSN6 Clone 16		*Sclerocystis sinuosa*[*]	AJ133706.1	97 (BLAST: 98)
57	Trap culture FSN6 Clone 17		*Claroideoglomus etunicatum*	FR750216.1	93
58	Trap culture FSN6 Clone 18		*Rhizophagus intraradices*	FR750206.1/FR750209.1	87
59	Trap culture FSS5 Clone 1	Sample 1	*Diversispora eburnea*[o]	AM713429.1	93 (BLAST: 94)
60	Trap culture FSS5 Clone 2		Uncultured Glomeromycota	JN009133.1	88
61	Trap culture FSS5 Clone 3		*Claroideoglomus etunicatum*	FR750216.1	91
62	Trap culture FSS5 Clone 4		*Claroideoglomus lamellosum*	FR773152.1	92
63	Trap culture FSS5 Clone 5		*Glomus iranicum*	HM153420.1	95
64	Trap culture FSS5 Clone 6		*Diversispora spurca*	Y17650.2	94 (BLAST: 95)
65	Trap culture FSS5 Clone 7	Sample 2	*Claroideoglomus lamellosum*	FR773152.1	92
66	Trap culture FSS5 Clone 8		*Diversispora trimerales*	FR686956.1	89
67	Trap culture FSS5 Clone 9		*Diversispora celata*[p]	AM713428.1	86
68	Trap culture FSS5 Clone 10		*Diversispora celata*	AM713428.1	95
69	Trap culture FSS5 Clone 11		*Diversispora trimerales*	FR686957.1	86
70	Trap culture FSS5 Clone 12	Sample 3	*Diversispora celata*[q]	AM713428.1	94
71	Trap culture FSS5 Clone 13		*Claroideoglomus lamellosum*	FR773152.1	92
72	Trap culture FSS5 Clone 14		*Sclerocystis sinuosa*[*]	AJ133706.1	96
73	Trap culture FSS5 Clone 15		*Claroideoglomus lamellosum*	FR773152.1	90
74	Trap culture FSS5 Clone 16		*Diversispora celata*	AM713428.1	93

[○] E value: 8^{-66}.

[*] *Rhizophagus sinuosus* on the INVAM website but *Sclerocystis sinuosa* according to Schüßler and Walker (2010).

[•] *Glomus fulvum* initially and the term may still be used but recent change has been to *Redeckera fulvum* (Schüßler and Walker, 2010).

[a] Equivalent NCBI BLAST result: *Dentiscutata colliculosa* (GQ376067) with a 87 % identity

[b] Equivalent NCBI BLAST result: *Diversispora aurantia* (AM713432.1) with a 95 % identity

[c] Equivalent NCBI BLAST result: *Diversispora aurantia* (AM713432.1) with a 96 % identity

[d] Alternate Maarj*AM* result: *Diversispora spurca* (Y17650.2; 96 %) and BLAST result: *Diversispora aurantia* (AM713432.1) with a 97 % identity

[e] Maarj*AM* result with lower max score but better identity: *Glomus indicum* (GU059538.1; 88 %)

[f] Equivalent NCBI BLAST result: *Diversispora aurantia* (AM713432.1) with a 97 % identity

[g] Equivalent NCBI BLAST result: *Diversispora aurantia* (AM713432.1) with a 94 % identity

[h] Equivalent NCBI BLAST result: *Diversispora aurantia* (AM713432.1) with a 97 % identity

[i] Equivalent NCBI BLAST result: *Diversispora aurantia* (AM713432.1) with a 96 % identity

[j] Alternate Maarj*AM* result: *Claroideoglomus lamellosum* (FR773152.1: 96 %)

[k] Alternate Maarj*AM* result: *Dentiscutata heterogama* (on the INVAM website and according to Redecker *et al*. (2013) but *Scutellospora heterogama* according to Schüßler and Walker (2010); *Cetraspora gilmorei* (Redecker *et al*., 2013) (*Scutellospora gilmorei*; Schüßler and Walker, 2010) (FR773142.1; 90 %) and *Dentiscutata colliculosa* (GQ376067.1; 90 %) (species phylogenetic position is still pending revision; Schüßler and Walker, 2010).

[l] Results on the Maarj*AM* database matched five genera and nine species of AM fungi with the same Max Score (470), Query Coverage (40 %) and identity (94 %).

[m] Equivalent NCBI BLAST result: *Diversispora aurantia* (AM713432.1) with a 91 % identity

[n] Equivalent NCBI BLAST result: *Diversispora aurantia* (AM713432.1) with a 97 % identity

[o] Compilation of Maarj*AM* and NCBI BLAST results

[p] Alternate Maarj*AM* result: *Diversispora trimerales* (FR686956.1; 86 %)

[q] Alternate Maarj*AM* result: *Diversispora spurca* (Y17650.2; 94 %) and the equivalent NCBI BLAST result: *Diversispora aurantia* (AM713432.1) with a 95 % identity

Figure 3.10: Jukes Cantor neighbour-joining tree based on sequences of SSU rDNA from AM fungal colonised roots. Bootstrap values (based on 1000 replicates) are shown at the nodes and *Geosiphon pyriformis* (Glomeromycota) (Y15905.3) served as the outgroup.

Overall 8 genera and 17 species were identified using the *MaarjAM* BLAST, excluding the equivalent matches on NCBI BLAST and the alternate matches on Maarj*AM*. From the mine tailing FSN6, 8 genera and 13 species were identified and from FSS5, 6 genera and 12 species were identified. Although the trap cultures did not demonstrate as high a species number as initial samples and in the case of FSS5 as high a genus number as the initial sampling, they did reveal a few additional species from the mine tailings. With regards to FSN6 two additional species were identified and from FSS5 three additional species were identified with the use of trap cultures. *Diversispora celata* was identified from all three samples from FSN6 initial sampling (*Acacia* roots); *Claroideoglomus lamellosum* was identified from all three samples from FSS5 initial sampling (*Acacia* roots); *Claroideoglomus lamellosum* was identified from all three samples from FSN6 trap culture samples (*S. bicolor* and *C. dactylon*), however, both *D. celata* and *Rhizophagus intraradices* were identified from FSN6 trap culture samples 2 and 3; and *C. lamellosum* was identified from all three samples from FSS5 trap culture samples, however, *D. celata* was identified from FSS5 trap culture samples 2 and 3.

There were inconsistencies with the grouping in the phylogram, for instance, the *Diversispora* cf. *celata* sequences did not all group together nor did the *D. celata*, with the *Diversispora aurantia* equivalent match, group together separately from the other *D. celata* sequences. Sequence 41 (Trap culture FSN6 Clone 1: *Claroideoglomus* cf. *etunicatum*) did not group with the other *C. etunicatum* sequences nor did it group with its equivalent match (*C. lamellosum*) and instead grouped with the *Scutellospora* and *Cetraspora* sequences. Sequence 49 (Trap culture FSN6 Clone 9: *Rhizophagus* cf. *intraradices*) did not group with the other *R. intraradices* identified, however, it also didn't group with the *D. celata* sequences that matched *D. aurantia* on NCBI BLAST even though it's equivalent match was also *D. aurantia*.

3.4.2 Spore analysis

The nested PCR products of the spore DNA that were successfully amplified were analysed using a 1 % agarose gel (Figure 3.11) to visualise product size. The DNA band sizes of all samples were of the expected size (± 1500 bp) for the primer mixture SSUmCf and LSUmBr.

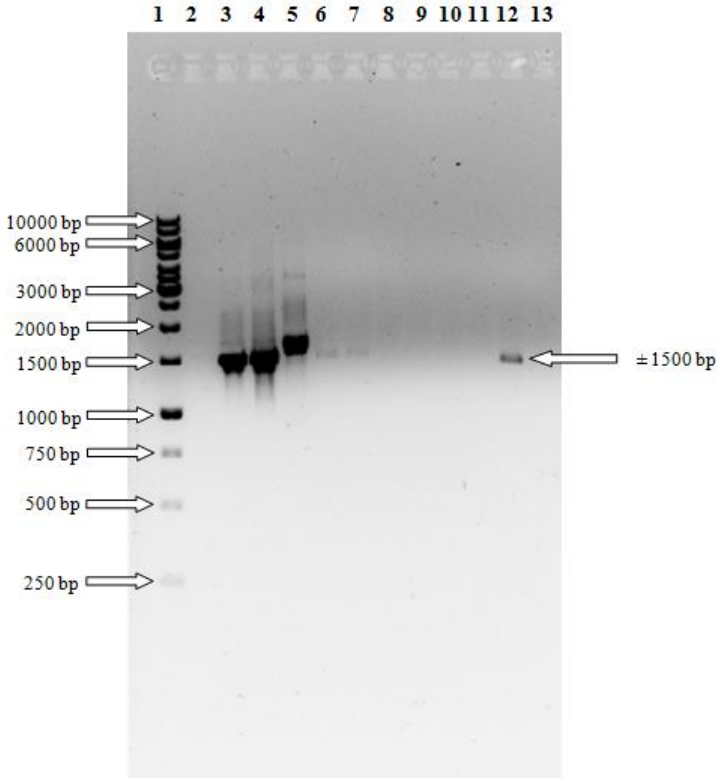

Figure 3.11: 1 % agarose gel showing nested PCR products of AM fungal DNA extracted from spores from slime from Free State South 5.

Lanes: (1) O' GeneRuler™ 1kb DNA ladder; (3) Translucent orange 45 µm; (4) Translucent yellow 45 µm; (5) Dark translucent orange 125 µm ; (6) Ginger 45 µm; (7) White 45 µm; (8) Beige 45 µm; (9) Black 45 µm; (10) Ginger 45 µm; (11) Beige 45 µm; (12) Translucent orange-yellow 45 µm; (13) Clear to white 45 µm

4.4.2.1 Sequencing analysis

The electropherograms (Figure 3.12) of the sequences using the forward primers were clear and showed very little or no shadow bands demonstrating that the sequences were relatively free of contaminating nucleotides and sequences and were of high quality. The reverse primers showed shadow bands demonstrating that the sequences contained contaminating nucleotides and the sequences were of poor quality and thus it was not possible to create consensus sequences as the identities were very low, nor could the sequences be used for identification.

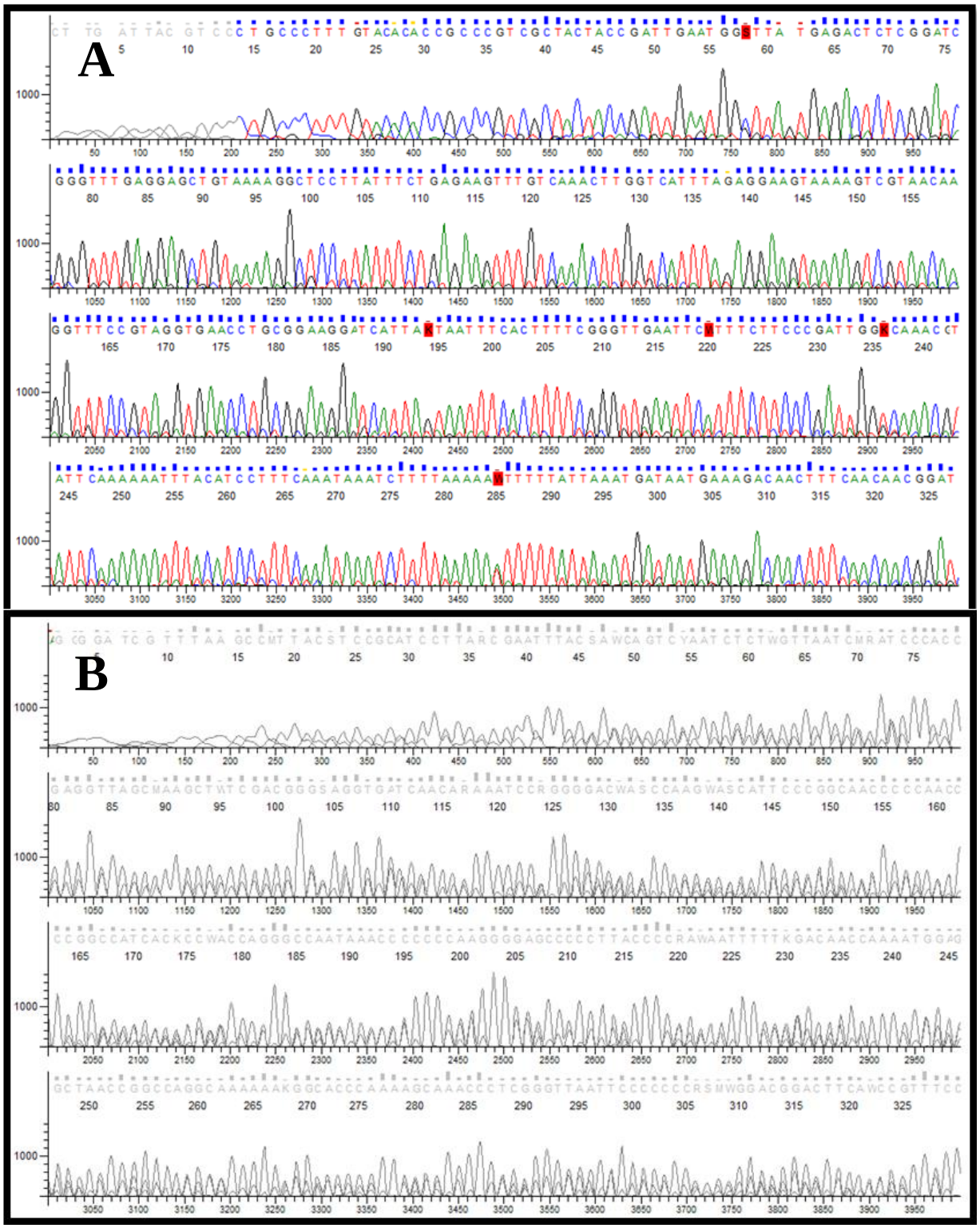

Figure 3.12: Electropherogram sections of the nested PCR forward primer (A) and reverse primer (B) product sequence of spore DNA viewed using Sequence Scanner v1.0.

Chapter 4

Discussion

4.1 Root analysis

4.1.1 Root colonisation

It is vital that the interactions between specific AM fungal species (as well as other soil microorganisms) and specific plant species is understood as not all relationships are synergistic or beneficial for every environment (Khan, 2006). In this study it was found that the self-cultivated *Acacia* spp. (FSS5) had a significantly higher total colonisation level than planted *Acacia* spp. (FSN6) which may reflect the rehabilitation technique employed. It has also been indicated that acacia trees cannot, in fact, survive on bare mine tailings without the appropriate AM fungal inoculum since only acacia trees inoculated with toepaddock material, which served as a crude AM fungal inoculum, survived introduction to FSN6, a bare mine tailing without top soil (I. M. Weiersbye, *pers. comm.*). It could be speculated that colonisation is favoured when slimes are ameliorated with garden refuse to create a layer of topsoil and that pre-inoculation of trees with HM resistant AM fungi combined with the rehabilitation technique of dumping garden refuse on the mine tailing would be an effective best phytoremediation method. This proposed method of rehabilitation was previously suggested in a study by Straker *et al.* (2008) where the researchers ascertained that the presence of organic matter and AM fungi aid in increasing plant survival on gold and uranium mine tailings. This amendment regime was also proposed by Celik *et al.* (2004), where the addition of compost (a common rehabilitation technique) to soil enhances degraded soil physical properties and AM fungi inoculated with the organic material proved more effective in improving the physical properties of semi-arid Mediterranean soil than inorganic or other organic treatments plus AM fungi.

In previous studies it has been shown that AM fungi attain a tolerance to HMs (Gildon and Tinker, 1983) and these tolerant AM fungi are required for acacia trees to better withstand harsh environments. In our study, the AM fungal root colonisation levels of *Acacia* spp. were found to be high enough to consider *Acacia* spp. as a mycorrhizal phytoremediation agent in conjunction with compost amelioration.

The arbuscular colonistaion was low for initial sampling and trap culture for both mine tailings. The same can be said for vesicular colonisation except for initial sampling for FSS5,

which was relatively low and significantly different from the other vesicle colonisation levels (14 % in comparison to 2-3 %). The low arbuscular colonisation has been recognised as a common trait in the natural plant-mycorrhizal symbiosis in toxic metal environments (Oryowska *et al.*, 2005). Oryowski *et al.* (2005) also observed that plants inoculated with AM fungi in toxic metal environments increased their arbuscular richness; however, this was not seen with the *Acacia* spp. on FSN6. Nonetheless, this could be one of the other benefits of inoculating the selected plants in a mycorrhizal remediation strategy. The low levels of root colonisation may have been due to the time of sampling as in the early spring colonisation is typically lower than that of late spring and summer (AM fungal intraradical formation depends on the stage of the host plant's stage in its lifecycle (Orlowski *et al.*, 2002)). Overall, colonisation levels were improved in trap cultures with the exception of vesicular colonisation for FSS5, however, the difference was not significantly improved except for FSS5 hyphal colonisation.

In a study by Straker *et al.* (2007), which was performed during a different season (late summer), the AM fungal status of plants growing on South African mine tailings and their footprints was determined. This is the only other study published on this subject. The authors analysed five host plant species on mine tailings and polluted veld surrounding the tailings and they found the colonisation levels were low to moderate and that hosts had strong effects on the degree of colonisation. Unpublished studies, Spruyt (2010) and Buck (2011), performed AM fungal colonisation level analysis on *T. usneoides* on mine tailings and HM contaminated sites respectively. Their studies revealed that the colonisation levels were either low or moderate and low, respectively.

4.1.2 Phylogenetic analysis

This study, unlike previous studies, utilised colonised roots for AM fungal DNA analysis, rather than spores, and it was shown that the AM fungal diversity of the sites was high; higher than previous studies on mine tailings and HM contaminated sites (Spruyt *et al.*, 2014). This supports the point made by Stutz and Morton (1996) that the level of AM fungal diversity is masked by low levels of sporulation. The high AM fungal diversity contradicts previous findings that polluted environments tend to negatively impact the diversity of mycorrhizal fungi (Zarei *et al.*, 2008; Zarei *et al.*, 2010) and that undisturbed areas tend to demonstrate a large number of AM fungal taxa (Öpik *et al.*, 2008). In addition to this,

several AM fungal species associated with *Acacia* spp. identified have not been previously identified in South African soils. These taxa include *Diversispora trimerales, D. spurca, Scutellospora reticulata, Rhizophagus irregularis, R. fascicalatus, Glomus iranicum, Cetraspora pellucida, Redeckera fulvum.*

A higher AM fungal genus and species number (by two and one, respectively) associated with *Acacia* spp. on FSN6 may be due to the addition of the crude inoculum prior to planting the *Acacia* trees. The species number that overlap between the sites (eight) was slightly less than the number of the species that were specific to each site (nine) with five species being specific to FSN6, namely, *Scutellospora dipurpurenscens*, *S. calospora, S. reticulata*, *Redeckera fulvum* and *Cetraspora pellucida* and four being specific to FSS5, namely, *Diversispora eburnean*, *Rhizophagus irregularis, R. fascilatus* and *Cetraspora gilmorei*. Two genera were specific to FSN6 (*Scutellospora* and *Redeckera*) while no genus was specific to FSS5. These differences in site specific AM fungal species may be due to the differences in the conditions at the sites due to the rehabilitation technique employed at each site.

The lower species levels from the trap cultures for both sites could be due to the analyses being performed on only one cycle of culturing, which was also observed in a study by Stutz and Morton (1996). Their study demonstrated that at least three successive cultures were required to ascertain a better representation of the species diversity from a site; however, their study was based on a spore analysis. It was noted by Redecker *et al.* (2003) that analysing the samples taken from the field (initial sampling) and the samples from trap cultures complement each other, which was demonstrated by the phylogenetic analysis in this study. Notably, the host plant species utilised in trap cultures may influence the AM fungi growth and thus its detection (Jansa *et al.*, 2002). It is safe to say that not all fungi present in the initial slime sample may be able to colonise the respective trap culture plants (Redecker *et al.*, 2003) as a result of environmental factors and AM fungal host preference (Bidartondo *et al.*, 2002; Helgason *et al.*, 2002). Jansa *et al.* (2002) suggested that this may affect the AM fungal species isolated from trap cultures and, according to the molecular analysis, the species richness of the trap cultures was not as high as initial samples, which indicates that the suggestion may be correct.

The molecular results not only aid in revealing the AM fungal diversity of HM polluted mine tailing environments but also contributes to our knowledge of their diversity in South Africa. This is of particular importance as at present the understanding of the distribution of AM fungi geographically is hampered by low levels of molecular data from specific areas, for instance Africa (Öpik *et al.*, 2010). The results can be compared to previous AM fungal community investigations that used either morphological or molecular identification methods. A morphological identification analysis of AM fungi associated with *Manihot esculenta* (cassava) in the Mpumalanga and Limpopo provinces was performed by Straker *et al.* (2010). In that study, *Glomus etunicatum* (now *Claroideoglomus etunicatum*), *Glomus rubiforme* (now *Sclerocystis rubiformis*), three species from the *Acaulospora* genus and the genera *Gigaspora* and *Scutellospora* were identified, however, only *C. etunicatum* and species from the genus *Scutellospora* were identified in this study. In an unpublished study which analysed the diversity and distribution of AM fungi associated with three different tree species currently being used as phytoremedial agents in environmental rehabilitation trials, it was found that seven AM fungal species were identified from four HM sites (Spruyt *et al.*, 2014). Only one of the species identified, *Acaulospora mellea*, was not identified in this study. Due to the higher number of species identified in this study, the overlap of AM fungal species identified in these various investigations appears to be moderate, which suggests that the previous analyses may under-represent AM fungal diversity. As previous analyses were performed on spores the AM fungal community in South Africa may be even more diverse than has been revealed in this study. Further avenues for research in this field would be to analyse the AM fungal diversity using colonised roots of other plant species at mine tailings, HM metal polluted sites and undisturbed environments. From this study and the unpublished study by Spruyt *et al.* (2014) it may be proposed that when selecting a phytoremedial technique it is vital to select AM fungal species based on host species and site instead of employing particular AM fungal taxa or group as generic inocula.

In a recently published study by Hazarika *et al.* (2014) the analysis of a variety of plants growing on coal overburden mine dumps in India revealed an AM fungal species diversity of five genera namely, *Glomus*, *Gigaspora*, *Acaulospora*, *Scutellospora*, and *Entrophospora*. Unfortunately, spore identification was not resolved down to species level. Two of the genera in their study were not identified in this study, that is, *Gigaspora* and *Acaulospora*.

According to the phylogram (Figure 3.10) several of the sequences identified as the same species did not group together; one reason for this may be due to mutations in the SSU rRNA gene sequence due to harsh environmental conditions (Clapp *et al.*, 2001). Another reason could be that although the sequences matched the same species, the sequences were not complete rRNA gene sequences and the partial sequences matched only sections of the rRNA gene in the database thus allowing for the discrepancies.

The primers used in the analysis of the colonised *Acacia* roots exclude non-AM fungal sequences; however, the product length that is generated may be insufficient for reliable species identification, particularly within certain families (Krüger *et al.*, 2009). Due to the small size of the AML primer pair amplicon, equally high BLAST scores can be obtained for multiple species within a genus for one sequence (Krüger *et al.*, 2009) as was seen in Table 3. Unfortunately, the new set of primers (SSUmCr and LSUmBr nested within SSUmAf and LSUmAr; Krüger *et al.*, 2009) did not successfully amplify AM fungal DNA directly from colonised roots, thus for future analyses AM fungal DNA should be extracted from colonised roots prior to PCR amplification and it would be prudent to use the new set of primers in future studies in this field. An additional option for future research would be to analyse the relationship between the AM fungal spore community composition with that of the colonised roots of the host plants so that the extent of colonisation by individual AM fungal taxa can be made.

4.2 Soil analysis

It has been speculated that the level and type of HM contamination and environmental conditions serve as selective pressures for different AM fungal taxa and this might apply to the differences in soil chemico-physical characteristics between FSN6 and FSS5. It is noteworthy to mention that speciation and mobility of HMs is determined by the physical and chemical characteristics of soil (Kabata-Pendias and Pendias, 1992). In other words the availability and toxicity of HMs to AM fungi and plants varies, based on pH, texture, organic matter content, the concentration and oxidation state of HMs, cation exchange capacity and redox potential of soil (Adriano, 1986).

There was no significant difference in pH levels between sites and trap cultures, however, the slime and trap culture sand-slime mixture of FSN6 was slightly more acidic than FSS5

for both water and KCl measurements. The study performed by Straker *et al.* (2008) found that there was a positive response of AM fungi to increasing pH. The plant species diversity is affected by pH and grasses grow less prominently on acidic soils but the diversity of woody species is greater on acidic soils (Weiersbye *et al.*, 2006). However, AM fungi vary in their response to pH (Entry *et al.*, 2002) and may distinguish between different ecotypes (Straker *et al.*, 2008). Some AM fungi do poorly in limed acidic soils whilst others do poorly in acidic soils (Mosse, 1972a,b) and some AM fungi promote plant growth in limed soils, while other AM fungi perform equally well in acidic soils. It has not been ascertained definitively whether AM fungi protect host plants from harmful effects of unfavourable pH conditions (Sylvia and Williams, 1992). It is important to note that several AM fungi are capable at tolerating low pH conditions (Entry *et al.*, 2002).

The conductivity of tailings solution extracts tend to be extremely high (Weiersbye *et al.*, 2006), which was observed from slime sampled from FSN6 but not from FSS5. Straker *et al.* (2008) found that there was a weak, but significant, negative correlation between slime/soil conductivity and percentage infectivity of roots by AM fungi spore levels in the soil, which suggests that there is a harmful effect of conductivity on AM fungal inoculum potential.

Upon observation the soil on the mine tailing FSS5 visually appeared more fertile due to the layer of topsoil and lack of barreness when compared to the sandy slimes and barren landscape of FSN6. However, this was not validated as fertility parameters such as the C:P or C:N ratios were not determined. For future studies it would be advisable that these ratios be determined.

ICP OES analysis does not determine chemical speciation but only elemental concentrations and those relating to the soils/slimes of this study are discussed in alphabetical order. The consideration of the concentrations of these elements is important because at sufficiently high concentrations they present a risk to both human and animal health in the regions they are found.

Aluminium (Al), a heavy metal, can affect roots by increasing cell wall rigidity, impairing cell division, altering root respiration, interfering with the uptake and transport of calcium (Ca), magnesium (Mg), phosphorous (P) and iron (Fe) and precipitating nucleic acids (Foy, 1983). The total average concentration of Al globally is 80 000 $mg.kg^{-1}$ (Koljonen, 1992)

and FSN6 slime contained 3-fold less than this average and FSS5 slime contained 6-fold less than this average.

Arsenic (As) sometimes acts as a metal but is generally a heavy metalloid and it predominantly occurs as As(V) in AsO_4^{3-} (arsenate) and as As (III) in AsO_2^- (arsenite) (Nies, 1999). Arsenate is related to phosphate (PO_4^{3-}) structurally; hence its major toxicity arises from its interference with the vital bioelement P (Nies, 1999). However, bacteria can use it in anaerobic respiration as an electron acceptor (Laverman *et al.*, 1995). Organic arsenicals are microbially degraded, however, it is often done slowly, which complicates analyses of food-chain transfer and plant availability (McLaughlin *et al.*, 1999). As is not required by plants and tends to be toxic (Finnegan and Chen, 2012). As inhibits root development and proliferation; it also inhibits plant growth and reduces the plant's capacity to reproduce (Finnegan and Chen, 1012). If the concentration of As is high enough critical metabolic processes are interfered with and the plant may die (Finnegan and Chen, 2012). The average concentration range of As in the earth's crust is 1.0 $mg.kg^{-1}$ to 1.8 $mg.kg^{-1}$ (Mielke, 1979; McLennon and Taylor, 1999) but can be as high as 20 $mg.kg^{-1}$ depending on the parent material and on anthropogenic activities (Tremearne and Jacob, 1941; Wedepohl, 1978; Davies, 1980; Ure and Berrow, 1982). The slime from FSN6 contained 10-fold more As than the average concentration range and FSS5 slime contained almost 11-fold more As than the average concentration range, which indicates that the slime has an excessive amount of As.

Of the chemicals identified from the slimes cadmium (Cd) is the best known toxic heavy metal (Nies, 1999). Cd is more toxic (Ragan and Mast, 1990) than zinc (Nies, 1999) since the solubility product of cadmium sulphide (CdS) is 1.4×10^{-29} and for zinc sulphide (ZnS) the solubility product is 2.91×10^{-25} (Weast, 1984). There are several effects on biological processes caused by Cd, namely, membrane damage, interaction with Ca metabolism, interaction with Zn metabolism, loss of protective function, thiol-binding and protein denaturation (Nies, 1999). Very rarely does Cd effect a single biological process listed at one time (Nies, 1999). Stunted plant growth, leaf rolling and chlorosis are the predominant symptoms of Cd toxicity (Benavides *et al.*, 2005). These symptoms may be explained by the Cd effect and interference of the plant uptake, transport and metabolism of water and several elements, such as Ca, P, K and Mg (Das *et al.*, 1997). The average concentration of Cd in the soil is 0.098 $mg.kg^{-1}$ (Heinrichs *et al.*, 1980), or according to Göhre and Paszkowski (2006)

and Oliveira and Pampulha (2006) 0.1-0.2 ppm or 0.4 $mg.kg^{-1}$ which is about 80-82 fold less than that which was detected in the slime samples.

Of all the elements Ca is the fifth most abundant and constitutes about 3 % - 5.29 % by weight of the earth's crust (McLennon and Taylor, 1999). Ca forms several common minerals in the soil for instance anhydrite ($CaSO_4$), calcite ($CaCO_3$), dolomite ($CaMg(CO_3)_2$), fluorite (CaF_2) and gypsum ($CaSO_4$) and distributed widely in other minerals (Zupančič and Pirc, 1999). Ca easily infiltrates a plant's apoplasm and is bound to the cell walls and on the exterior plasma membrane surface in an exchangeable form (Marschner, 1986). However, the uptake rate of Ca into the cytoplasm is extremely restricted and appears to be only loosely connected to metabolic processes (Marschner, 1986). The mobility of Ca^{2+} within the plant is also slow (Marshner, 1986). Ca is non-toxic even at high concentrations and can be decidedly effective in the detoxification of high concentrations of mineral elements in plants (Marschner, 1986). The concentration of Ca present in soil ranges from 5100-76000 $mg.kg^{-1}$ (Mielke, 1979) with an average of 24 000 $mg.kg^{-1}$ (Shacklette and Boerngen, 1984). The slime samples from FSN6 and FSS5 both contained amounts of Ca that fell within the average concentration range and fell below the average amount present in soil. These low are levels are important to consider as they may impair the ability of phytoremediation agents to persist in these environments.

Chromium (Cr) generally occurs as the oxyanion chromate, Cr(VI), and the trivalent cation, Cr(III) (Nies, 1999). Cr(VI) is more toxic than Cr(III), thus only Cr(III) can perform favourable functions (Nies, 1999). However, Cr has no beneficial effect on microorganisms (Nies, 1999). Cr is extremely toxic to plants and severly effects plant development and growth (Shanker *et al.*, 2005). However, the degree of toxicity of Cr on plants varies at different concentrations of Cr and at different stages of the plant's development and growth (Shanker *et al.*, 2005). Cr(VI) toxicity is due to it acting as an oxidising agent and the fact that it forms free radicals when it is reduced to Cr(III) inside the cell (Shanker *et al.*, 2005). Cr(III) may be toxic through its coordination of various organic compounds which results in the inhibition of some metalloenzyme systems, when Cr(III) is present in high concentrations (Shanker *et al.*, 2005). Total Cr concentrations in United States soils average 37 $mg.kg^{-1}$ (Shacklett and Boerngen, 1984) and the slime samples from FSN6 contained almost 5-fold more Cr and the samples from FSS5 contained 4.5-fold more Cr than the average. The higher concentrations may impair the ability of phytoremediation agents to

persist in these environments as Cr toxicity is an established cause for the impairment of plant health (Shanker *et al.*, 2005).

Copper (Cu) has an electrochemical potential of -268 mV for Cu^{2+}/Cu^{+}, which is within the physiological range (Nies, 1999). Cu^{2+} (divalent Cu ion) strongly binds to humic and fulvic acids in soils and forms Cu-organic matter complexes (Stevenson and Fitch, 1981). Cu readily interacts with radicals, ideally with molecular oxygen (Nies, 1999). Due to the radical character of Cu it is very toxic with many organisms being more sensitive (Gordon *et al.*, 1994) to Cu than *Escherichia coli* (Nies, 1999). The toxicity depends on hydroperoxide radical production (Rodriguez Montelongo *et al.*, 1993) and on interaction with cell membranes (Suwalsky *et al.*, 1998). Cu toxicity in plants may cause Fe deficiency (Woolhouse, 1983), however, this depends on the iron supply source (Rahimi and Bussler, 1973). High Cu concentrations act on lipid peroxidation, destroying the thylakoid membranes and causing chlorosis (Sandmann and Böger, 1983). Roots are generally affected by high Cu concentrations with the inhibition of elongation and development (Marschner, 1986). The concentration of Cu present in soil ranges from 12-120 $mg.kg^{-1}$ (Wedepohl, 1978; Ure and Berrow, 1982) with an average of 68 $mg.kg^{-1}$ (Mielke, 1979). The amount of Cu in slime from FSN6 fell within the average range within soils and was almost the same amount as the average but the amount of Cu in FSS5 slime was below (half) the lowest value in the range which could indicate that the slime from FSS5 is Cu deficient. Cu is an essential micronutrient for plants and thus low concentrations may affect down stream metabolic processes thus causing developmental problems.

Of the HMs, Fe, is the only macro-element (Nies, 1999). Fe^{2+} is not toxic to aerobic bacteria due to its low solubility (Nies, 1999). Fe toxicity displays itself as bronzing; brown spots developing on the tips of older leaves and progressing to younger leaves, where the leaves eventually turn grey or white; foliar injury (leaf damage); inhibited root and shoot growth and sometimes thickened roots (Foy *et al.*, 1978). Generally Fe concentrations in soil range from 3300 to 550 000 $mg.kg^{-1}$ (Ure and Berrow, 1982; Bodek *et al.*, 1988) with an average of 21 000 $mg.kg^{-1}$ (Rose *et al.*, 1979). The Fe present in the slime samples from the two sites were within the average range but fell below the average amount and FSS5 contained 3 fold less Fe than the average amount.

Potassium (K) has one oxidation state, K^+, and belongs to the periodic table group 1 along with lithium (Li), sodium (Na), rubidium (Rb) and caesium (Cs) (Canney, 1953). Excessive amounts of K influences the uptake of Mg, Ca, Mn and Fe by plants and may cause nitrogen deficiency (Evans, 2003). The average concentration of K present in soil is 15 000 $mg.kg^{-1}$ (Shacklette and Boerngen, 1984) and both mine tailings contained almost 5-fold and 6.5-fold less K from FSN6 and FSS5 respectively, which may be too low to appropriately support plants.

Titanium (Ti) is a typical constituent of rocks and it forms a variety of minerals, for instance, oxides, silicates and titanates and three of the most prevalent titanium minerals include titanomagnetite (Fe_2TiO_4), sphene ($CaTiSiO_5$), rutile (TiO_2) and ilmenite ($FeTi_3$) (Goldschmidt, 1954; Kabata-Pendias and Pendias, 1984). Dumon and Ernst (1988) reported that Ti does not appear to be essential for plant growth nor is there any evidence that it is toxic to plants. The concentration of Ti present in soil ranges from 330-14 000 $mg.kg^{-1}$ (Kabata-Pendias and Pendias, 1984) with an average of 2 400 $mg.kg^{-1}$ (Shacklette and Boerngen, 1984). Both mine tailing slime samples contained Ti amounts that were on the high side of the range, well above the average amount but FSN6 slime has 3 $mg.kg^{-1}$ more Ti than the maximum in the range.

Of the natural elements, uranium (U) has the highest atomic number, is an actinide and generally occurs as U(IV) in UO_2^{2-} (Nies, 1999). U(IV) has low toxicity to bacteria (Pavlakis *et al.*, 1996). U causes plant death when present at suffiently high concentrations in the soil (300-10 000 $mg.kg^{-1}$) but otherwise stunts plant growth and seed production (Sheppard *et al.*, 1992). The average concentration of U in soil is 2.3 $mg.kg^{-1}$ (Mielke, 1979; Shacklette and Boerngen, 1984). FSN6 slime contained 110.5-fold more U than the average indicating that the level of U present is excessive and could be the main cause of toxicity of the site; however, FSS5 slime contained 36-fold more U than the average, which is still an excessive amount but not as toxic as slime from FSN6.

Yttrium (Y) has one oxidation state, Y^{3+}, and belongs to the periodic table group 3 along with scandium (Sc), lanthanum (La) and actinium (Ac) (McLennon, 1999). Y tends to accumulate within plant roots and leaves but is less concentrated in the seeds, fruit and edible roots but otherwise Y in the soil is strongly bound and the uptake and translocation is restricted (Kastori *et al.*, 2010). The average concentration range of Y present in soil is 21-31

$mg.kg^{-1}$ (Mielke, 1979; Shacklette and Boerngen, 1984). The slime samples from both sites contained amounts of Y that fell below the range by 2 fold to almost 4 fold for FSN6 and FSS5 respectively.

Zn exists solely as the divalent cation Zn^{2+} (Nies, 1999). The Zn cation, with completely filled d orbitals, cannot go through redox changes under biological conditions (Nies, 1999). Zn is used to complex polypeptide chains (Fosmire, 1990) and the toxicity of excess Zn in plants appears as chlorosis of young leaves (Marschner, 1986) and in non-tolerant plants, root growth inhibition (Godbold *et al.*, 1983); however, Zn is less toxic than Cu (Nies, 1999). The average concentration of Zn in the soil is 70 $mg.kg^{-1}$ (Heinrichs *et al.*, 1980). However, according to Göhre and Paszkowski (2006) and Oliveira and Pampulha (2006) Zn concentration tends to be 80 ppm or 36.6 $mg.kg^{-1}$. So according to Heirichs *et al.*, (1980) the slime at FSN6 contains almost 2 fold more Zn and the slime at FSS5 contains just over the average amount of Zn present in soil.

Chemicals analysed in the study due to being toxic to people and animals, are known soil contaminants, and/or because they are necessary for metabolism but were not found during analysis, include lead (Pb), mercury (Hg), molybdenum (Mo), nickel (Ni), platinum (Pt) and thorium (Th).

Briefly, the elements that were within the usual range but fell below the average amount present in soils include Ca, Cu and Fe; those that were above the average amount include As, Cd, Cr, Ti, U and Zn and those that were below the average amount include Al, K and Y. For mycorrhizoremediation it is vital to use indigenous AM fungal strains from the site so that the strains best adapted to the soil and climatic conditions are used to produce site-specific AM fungal inoculum (Khan, 2005). From analyses performed by Gildon and Tinker (1983) on *Glomus* spp. with HM it was noted that there were differences in their sensitivities and isolates collected from HM-contaminated soils had more tolerant germination and growth patterns in agar media containing higher concentrations of metals. Leyval *et al.* (1995) used greenhouse and field studies to analyse spore numbers, spore germination and mycorrhizal infectivity potential of spores collected from contaminated soils and it was found that these parameters were lower than uncontaminated soils. They also found that the colonisation in soils with higher HM content was delayed. Plants and AM fungi may vary in their tolerance to HMs individually or in combination (Entry *et al.*, 2002). It is required that specific

combinations of AM fungi and host plant are meticulously selected for particular soil conditions in order to optimise the use of AM fungi to promote the growth of host plants in HM contaminated soils (Entry *et al.*, 2002). Notably, the inoculum should contain a combination of AM fungal strains (Straker *et al.*, 2008) as AM fungi demonstrate a vast functional diversity (Johnson *et al.*, 2003; Munkvold *et al.*, 2004) and this would be more favourable than individual strains.

4.3 Spore analysis

4.3.1 Spore numbers

Spore production varies between locations as well between hosts colonised, AM fungal species, time period (season) and environmental conditions (Redecker *et al.*, 2003). The conditions may warrant mass spore production or may cause sporulation to be inhibited to the point where no spores are produced and thus not present (Redecker *et al.*, 2003). In this study it was shown that spore production was possible and favoured at the sites as opposed to trap cultures. The spore numbers suggest that the conditions at the sites promoted sporulation, which could be due to the harsher conditions causing the AM fungi to sporulate as a survival mechanism or it could be due to the sampling time being early spring, thus the spores were present from the previous season. The trap culture for FSN6 produced roughly 50 % of the spore number recovered from initial samples whereas the trap culture for FSS5 produced only a third of the number of spores in comparison to initial samples; this could be due to the trap cultures only going through one cycle instead of successive cycles. Stutz and Morton (1996) found that 75 % of the species richness determined after three trap culture cycles was not detected in the first trap culture cycle. Thus future analyses using trap cultures should perform at least three successive propagation cycles. Another explanation for the lower spore numbers in the trap cultures is that the pots were fertilised whenever nutrient deficient symptoms appeared in the host plants and mycorrhizal activity is often lower in agricultural soils due to the soil P content being high because of the addition of fertilisers (Straker *et al.*, 2010). This would require a delicate process balancing the needs of plants for growth and promotion of mycorrhizal colonisation and activity.

The apparent lack of correlation between number of spores and colonisation level demonstrates that the relationship between sporulation and AM fungal colonisation is an approximation and tends to vary between different species of AM fungi (Pearson and

Schweiger, 1993), as well as between hosts and nutrient levels in the soil (Menge *et al.*, 1978; Brundrett, 1991; Douds, 1994). In order to bring about sporulation in some AM fungal taxa, a minimum amount of root length colonisation must be reached and this is either coordinated by AM fungal genotype alone or by a combination of this with host factors (Gazey *et al.*, 1992; Franke and Morton, 1994). Sporulation depends on environmental conditions and on the physiological parameters of the AM fungus (Redecker *et al.*, 2003). Some AM fungi can produce large quantities of spores during certain seasons of the year and under specific conditions thus appearing as the chief root colonisers but under alternate conditions AM fungi may not produce spores at all (Redecker *et al.*, 2003). Some AM fungi sporulate in late spring while others sporulate at the end of summer, which indicates that AM fungi have contrasting seasonalities (Schultz *et al.*, 1999). AM fungal species that do not sporulate will thus not be detected and the sporulating AM fungi then become the basis for AM fungal ecology (Redecker *et al.*, 2003).

It has been shown that spore number and colonisation level dynamics differ between AM fungal species (Bever *et al.*, 1996) and thus one or the other individually cannot be used as an indication of the fungal diversity, however, it is best to use both together simultaneously as the one is not an indication of the other (Allen and Allen, 1980; Morton *et al.*, 1995) Previous studies have shown that AM fungal surveys that have been based only on spore observations are inaccurate as some species may not produce their spores in the soil (Brundrett *et al.*, 1999; Smith and Read, 1997, Liu and Li, 2000; Smith and Read, 2008).

In relation to the interaction between metal contaminants and spore production, a study by Del Val *et al.* (1999) determined that the total number of AM fungal spores drastically decreased when increasing loads of HMs were added; however, AM fungal spores did not completely disappear when the highest amounts of HMs were added to the soils (in the form of sludge), suggesting that the indigenous AM fungi develop a certain adaptation/tolerance to this environmental stress or there is selection for tolerant populations. Overall, the study found that the total number of AM fungal spores negatively correlated with the HM content of the soil, although the correlation coefficient was higher for the concentration of free cations present in the soil (Del Val *et al.*, 1999). In the study by Weiersbye *et al.* (1999) it was established that AM fungal spores obtain and accumulate transition metals and radionuclides at higher levels than plant tissues. Clearly though, further research into the negative effects of HMs on spore fecundity is needed.

4.3.2 Phylogenetic analysis

Although attempted, it was difficult to amplify DNA directly from crushed spores using the primers SSUmCr and LSUmBr nested within SSUmAf and LSUmAr, and DNA amplification may be less challenging if a DNA extraction from the spores is performed prior to PCR amplification. In individual spores, the rDNA is highly polymorphic (Redecker *et al.*, 2003) thus DNA analysis from spores is problematic due to mixed templates occurring during sequencing, as illustrated in Figure 4.9. In order to ameliorate the problem one would need to clone sequences amplified from spores which should be performed in future studies.

4.4 Summary and Conclusions

Acacia trees have the potential to be phytoremedial agents for mine tailings as they are capable of growing on HM contaminated sites as well as being mycorrhizal (established in this study) and have the ability to fix nitrogen thus increasing the fertility of the soil. *Acacia* trees are not only mycorrhizal but a variety of identified AM fungal taxa were associated with the trees with only a few differences between the sites. The AM fungal identification method in the study was unique in that it was performed exclusively on colonised root material. Although FSN6 was found to have a higher species number, FSS5 was found to have a higher colonisation level indicating different rehabilitation techniques may have different effects on the hosts and mycorrhizal symbionts.

Although the spore molecular identification and phylogenetic analysis using the new primer set was unsuccessful it did pinpoint fields that require further analysis to improve future knowledge in the mycorrhizal field.

This study did not examine the chemical speciation of the elements present in the slimes which would have required a much larger experimental design, but the limited elemental analysis provides background information pertaining to site differences (with possible causal relationshhips with biological paramenters) and a future study might include a more rigorous correlation between abiotic factors and biological phenomena.

In a rehabilitation sense, it is still vital to know the various parameters of a contaminated site, for instance the soil charactereistics and chemistry, in order to rapidly and successfully

rehabilitate the site with appropriate plants and associated symbionts. Extreme environments would select for specific tolerant strains of AM fungi and if these are to be introduced as inocula they need to be matched to the prevailing abiotic stresses of the site for successful rehabilitation.

References

- Adamson, R. J. (1972) *Gold metallurgy in South Africa.* Chamber of Mines of South Africa, South Africa. pp. ix, x, 152, 154, 357 and 364-366.
- Adriano, D. C. (1986) *Trace elements in the terrestrial environment.* Springer-Verlag, New York. pp. 533.
- Allen, E. B., Allen, M. F. (1980) Natural re-establishment of vesicular- arbuscular mycorrhizae following stripmine reclamation in Wyoming. *Journal of Applied Ecology* **17**: 139-147.
- Allen, E. B., Cunningham, G. L. (1983) Effects of vesicular-arbuscular mycorrhizae on *Distichlis spicata* under three salinity levels. *New Phytologist* **93**: 227-236.
- Ames, R. N., Reid, C. P. P., Porter, L., Cambardella, C. (1983) Hyphal uptake and transport of nitrogen from two ^{15}N-labeled sources by *Glomus mosseae*, a vesicular-arbuscular mycorrhizal fungus. *New Phytologist* **95**: 381-396.
- Ames, R. N., Schneider, R. W. (1979) *Entrophospora*, a new genus in the Endogonaceae. *Mycotaxon* **8**: 347-352.
- An Z.-Q., Hendrix, J. W., Hershman, D. E., Henson, G. T. (1990) Evaluation of the "most probable number (MPN)" and wet-sieving methods for determining soil-borne populations of endogonaceous mycorrhizal fungi. *Mycologia* **82**: 576-581.
- Anderson, J. M., Ingram, J. S. I. (1989) *Tropical soil biology and fertility: A handbook of methods.* C.A.B. International, Michigan. pp. 33.
- Arriagada, C. A., Herrera, M. A., Ocampo, J. A. (2007) Beneficial effect of saprobe and arbuscular mycorrhizal fungi on growth of *Eucalyptus globules* co-cultured with *Glycine max* in soil contaminated with heavy metals. *Journal of Environmental Management* **84**: 93-99.
- Azcon, R., Rubio, R., Barea, J. M. (1991) Selective interactions between different species of mycorrhizal fungi and *Rhizobium meliloti* strains, and their effects on growth, N_2 fixation (^{15}N) and nutrition of *Medicago sativa* L. *New Phytologist* **117**: 399-404.
- Bago, B., Pfeffer, P. E., Shachar-Hill, Y. (2000) Carbon metabolism and transport in arbuscular mycorrhizas. *Plant Physiology.* **124**: 949-957.
- Bago, B., Pfeffer, P. E., Shachar-Hill, Y. (2001) Could the urea cycle be translocating nitrogen in the arbuscular mycorrhizal symbiosis? *New Phytologist* **149**: 4-8.

- Barea, J. M., Azcon-Aquilar, C. (1983) Mycorrhizas and their significance in nodulating nitrogen-fixing plants. *Advances in Agronomy* **36**: 1-54.
- Barea, J. M., Pozo, M. J., Azcón, R., Azcón-Aguilar, C. (2005) Microbial co-operation in the rhizosphere. *Journal of Experimental Botany* **56**: 1761-1778.
- Benavides, M. P., Gallego, S. M. and Tomaro, M. L. (2005) Cadmium toxicity in plants. *Brazilian Journal of Plant Physiology* **17**: 21-34.
- Bentivenga, S. P., Morton, J. B. (1996) Congruence of fatty acid methyl ester profiles and morphological characters of arbuscular mycorrhizal fungi in Gigasporaceae. *Proceedings of the National Academy of Sciences* **93**: 5659-5662.
- Berrow, M. L., Reaves, G. A. (1984) Background levels of trace elements in soils. *Proceedings of the International Conference on Environmental Contamination*, London. pp. 333-340.
- Bethlenfalvay, G. J. (1992) Vesicular-arbuscular mycorrhizal fungi in nitrogen-fixing legumes: problems and prospects. *Methods in Microbiology* **24**: 375-389.
- Bever, J. D., Morton, J. B., Antonovics, J., Schultz, P. A. (1996) Host-depentent sporulation and species diversity of arbuscular mycorrhizal fungi in a mown grassland. *Journal of Ecology* **84**: 71-82.
- Bever, J. D., Schultz, P. A., Pringle, A., Morton, J. B. (2001) Arbuscular mycorrhizal fungi: more diverse than meets the eye, and the ecological tale of why. *BioScience* **51**: 923-932.
- Bhattachrjee, M., Mukerji, K. G., Tewari, J. P., Skoropad, W. P. (1982) Structure and hyperparasitism of a new species of *Gigaspora*. *Transactions of the British Mycological Society* **78**: 184-188.
- Bidartondo, M. I., Redecker, D., Hijri,I., Wiemken, A., Bruns, T. D., Domínguez, L., Sérsic, A., Leake, J. R., Read, D. J. (2002) Epiparasitic plants specialised on arbuscular mycorrhizal fungi. *Nature* **419**: 389-393.
- Bierman, B., Linderman, R. G. (1983) Use of vesicular-arbuscular mycorrhizal roots, intraradical vesicles and extraradical vesicles as inoculum. *New Phytologist* **95**: 97-105.
- Bodek, I., Lyman, W. J., Reehl, W. F., Rosenblatt, D. H. (1988) *Environmental inorganic chemistry: properties, processes, and estimation methods.* Society of environmental toxicology and chemistry. SETAC special publications series. Pergamon Press, New York. pp. 29.

- Bolan, N. S. (1991) A critical review of the role of mycorrhizal fungi in the uptake of phosphorus by plants. *Plant and Soil* **134**: 189-208.
- Borůvka, L., Drábek, O. (2004) Heavy metal distribution between fractions of humic substances in heavily polluted soils. *Plant, Soil and Environment* **50**: 339-345.
- Bottomley, P. J. (1992) Ecology of *Bradyrhizobium* and *Rhizobium*. *In*: Stacey, G., Burris, R. H., Evans, H. J. (Eds). *Biological nitrogen fixation.* Chapman and Hall, New York. pp. 293-348.
- Bradshaw, A. D., Chadwick, M. J. (1980) *The restoration of land: The ecology and reclamation of derelict and degraded land.* Studies in Ecology Vol. 6. Blackwell Scientific Publications, London. pp. 4, 136, 137.
- Brundrett, M. (1991) Mycorrhizas in natural ecosystems. *Advances in Ecological Research* **21**: 171-313.
- Brundrett, M. C., Abbott, L. K., Jasper, D. A. (1999) Glomalean mycorrhizal fungi from tropical Australia. I. Comparison of the effectiveness and specificity of different isolation procedures. *Mycorrhiza* **8**: 305-314.
- Brundett, M., Bougher, N., Dell, B., Grove, T., Malajczuk, N. (1995) *Working with mycorrhizas in forestry and agriculture.* Australian Centre for International Agricultural Research, Canberra. pp. 165-170.
- Buck, M. T. (2011) Status and molecular identification of arbuscular mycorrhizal fungi (AMF) associated with wild populations of *Tamarix usneoides* E.Mey. ex Bunge growing in an arid environment and heavy metal contaminated sites. Honours project, School of Molecular and Cell Biology. University of the Witwatersrand, Johannesburg.
- Canney, F. C. (1953) Some aspects of the geochemistry of potassium, rubidium, caesium and thallium in sediments. *American Minerologist* **38**: 331.
- Carr, J. D. (1976) *The South African Acacias.* Conservation Press, Johannesburg. pp. 7
- Celik, I., Ortas, I., Kilic, S. (2004) Effects of compost, mycorrhiza, manure, fertiliser on some physical properties of a Chromoxerert soil. *Soil and Tillage Research* **78**: 59-67.
- Chen, X., Tang, J., Zhi, G., Hu, S. (2005) Arbuscular mycorrhizal colonisation and phosphorus acquisition of plants: effects of coexisting plant species. *Applied Soil Ecology* **28**: 259-269.
- Clapp, J. P., Fitter, A. H., Young, J. P. W. (1999) Ribosomal small subunit sequence variation within spores of an arbuscular mycorrhizal fungus, *Scutellopora* sp. *Molecular Ecology* **8**: 915-921.

- Clapp, J. P., Rodriguez, A., Dodd, J. C. (2001) Inter- and intra-isolate rRNA large subunit variation in *Glomus coronatum* spores. *New Phytologist* **149**: 539-554.
- Clapp, J. P., Young, J. P.W., Merryweather, J. W., Fitter, A. H. (1995) Diversity of fungal symbionts in arbuscular mycorrhizas from a natural community. *New Phytologist* **130**: 259-265.
- Clausen, H. T. (1973) Ecological aspects of slimes-dam construction. *Journal of the South African Institute of Mining and Metallurgy*: 178-183.
- Clemens, S., Palmgren, G., Krämer, U. (2002) A long way ahead: Understanding and engineering plant metal accumulation. *TRENDS in Plant Science* **7**: 309-315
- Clemens, S. (2006) Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. *Biochimie* **88**: 1707-1719.
- Cluett, H. C., Boucher, D. H. (1983) Indirect mutualism in the legume – *Rhizobium* – mycorrhizal fungus interaction. *Oecologia* **59**: 405-408.
- Cooper, K. M., Lösel, D. (1978) Lipid physiology of vesicular-arbuscular mycorrhiza. I. Composition of lipids in roots of onion, clover and ryegrass infected with *Glomus mosseae*. *New Phytologist* **80**: 143-151.
- Costa, M. (1997) Toxicity and carcinogenicity of Cr(VI) in animal models and humans. *Critical Reviews in Toxicology* **27**: 431-442.
- Dar, G. H., Zargar, M. Y., Beigh G. M. (1997) Biocontrol of *Fusarium* root rot in the common bean (*Phaseolus vulgaris* L.) by using sumbiotic *Glomus mosseae* and *Rhizobium leguminosarum*. *Microbial Ecology* **34**: 74-80.
- Das, P., Samantaray, S., Rout, G. R. (1997) Studies on cadmium toxicity in plants: a review. *Environmental Pollution* **98**: 29-36.
- Davies, B. E. (1980) Trace element pollution. *In*: Davies, B. E. (Ed.) *Applied soil trace elements.* Wiley & Sons, Chichester. pp. 482.
- Del Val, C., Barea, J. M., Azcón-Aquilar, C. (1999) Diversity of arbuscular mycorrhizal fungus populations in heavy-metal-contaminated soils. *Applied and Environmental Microbiology* **65**: 718-723.
- Denison, R. F., Kiers, E. T. (2011) Life histories of symbiotic rhizobia and mycorrhizal fungi. *Current Biology* **21**: R775-R785.
- Diver, J. D., Holben, W. E., Rilling, M. C. (2005) Characterization of glomalin as a hyphal wall component of arbuscular mycorrhizal fungi. *Soil Biology and Biochemistry*. **37**(1):101-106.

- Dodd, J. C., Thomson, B. D. (1994) The screening and selection of inoculants arbuscular-mycorrhizal and ectomycorrhizal fungi. *Plant and Soil* **159**: 149-158.
- Douds, D. D. Jr. (1994) Relationship between hyphal and arbuscular colonisation and sporulation in mycorrhiza of *Paspalum notatum* Flugge. *New Phytologist* **126**: 233-237.
- Douds, D. D. Jr., Chaney, W. R. (1986) The effect of high nutrient addition upon seasonal patterns of mycorrhizal development, host growth, and root phosphorus and carbohydrate content in *Fraxinus pennsylvanica* Marsh. *New Phytologist* **103**: 91-106.
- Dumon, J. C., Ernst, W. H. O. (1988) Titanium in plants. *Journal of Plant Physiology* **133**: 203-209.
- Entry, J. A., Rygiewicz, P. T., Watrud, L. S., Donnelly, P. K. (2002) Influence of adverse soil conditions on the formation and function of arbuscular mycorrhizas. *Advances in Environmental Research* **7**: 123-138.
- Evans, E. (2003) *Nutrient deficiency: Action mode, deficiency and toxicity symptoms of the 17 essential nutrients.* North Carolina State University. http://www.ces.ncsu.edu/depts/hort/consumer/quickref/fertilizer/nutri_def.html (Accessed 07/06/2014)
- Finnegan, P. M., Chen, W. (2012) Arsenic toxicity: the effects on plant metabolism. *Frontiers in Physiology* **182**: 1-18.
- Fitter, A. H., Helgason, T., Hodge, A. (2011) Nutritional exchanges in the arbuscular mycorrhizal symbiosis: Implication for sustainable agriculture. *Fungal Biological Reviews* **25**: 68-72.
- FließBach, A., Martens, B., Reber, H. H. (1994) Soil microbial biomass and microbial activity in soils treated with heavy metal contaminated sewage sludge. *Soil Biology and Biochemistry* **26**: 1201-1205.
- Fosmire, G. J. (1990) Zinc toxicity. *The Amercian Journal of Clinical Nutrition* **51**: 225-227.
- Foster, R. P. (1993) *Gold metallurgy and exploration.* Chapman & Hall, London. pp. 300-302 and 405-407.
- Foy, C. D. (1983) Physiological effects of hydrogen, aluminium and manganese toxicities in acid soil. *In*: Adams, F. (Ed) *Soil acidity and liming.* American Society of Agronomy, Wisconsin. pp. 57-79.
- Foy, C. D., Chaney, R. L. and White, M. C. (1978) The physiology of metal toxicity in plants. *Annual Review of Plant Physiology* **29**: 511-566.

- Franke, M., Morton, J. B. (1994) Ontogenetic comparisons of the endomycorrhizal fungi *Scutellospora heterogama* and *Scutellospora pellucid*: revision of taxonomic character concepts, species descriptions, and phylogenetic hypotheses. *Canadian Journal of Botany* **72**: 122-134.
- Gardes, M., Bruns, T. D. (1993) ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**: 113-118.
- Gazey, C., Abbott, L. K., Robson, A. D. (1992) The rate of development of mycorrhizas affects the onset of sporulation and production of external hyphae by two species of *Acaulospora*. *Mycological Research* **96**: 643-650.
- Genney, D. R., Hartley, S. H., Alexander, I. J. (2001) Arbuscular mycorrhizal colonisation increases wth host density in a heathland community. *New Phytologist* **152**: 355-363.
- Gerdeman, J. W., Nicolson, T. H. (1963) Spores of mycorrhizal *Endogone* species extracted from soil by wet-sieving and decanting. *Transaction of the British Mycological Society* **46**: 235-244.
- Gianinazzi-Pearson, V., Branzanti, B., Gianinazzi, S. (1989) *In vitro* enhancement of spore germination and early hyphal growth of a vesicular-arbuscular mycorrhizal fungus by host root exudates and plant flavinoides. *Symbiosis* **7**: 243-255.
- Gianinazzi-Pearson, V., Lemoine M.-C., Arnould, C., Gollotte, A., Morton, J. B. (1994) Localization of ß 1-3 glucans in spore and hyphal walls of fungi in the Glomales. *Mycologia* **86**: 478-485.
- Gildon, A., Tinker, P. B. (1983) A heavy metal-tolerant strain of mycorrhizal fungus. *Transactions of the British Mycological Society* **77**: 648-649.
- Giovannetti, M, Avio, L., Salutini, L. (1991) Morphological, cytochemical, and ontogenetic characteristics of a new species of vesicular-arbuscular mycorrhizal fungus. *Canadian Journal of Botany* **69**: 161-167.
- Giovannetti, M., Gianinazzi-Pearson, V. (1994) Biodiversity in arbuscular mycorrhial fungi. *Mycological Research* **98**: 705-715.
- Glick, B. R. (2003) Phytoremediation: Synergistic use of plants and bacteria to clean up the environment. *Biotechnology Advances* **21**: 383-393.

- Godbold, D. L., Horst, W. J., Marschner, H., Collins, J. C., Thurman, D. A. (1983) Root growth and Zn uptake by two ecotypes of *Deschampsia caespitose* as affected by high Zn concentrations. *Zeitschrift für Pflanzenphysiologie* **112**: 315-324
- Göhre, V., Paszkowski, U. (2006) Contribution of the arbuscular mycorrhizal symbiosis to heavy metal phytoremediation. *Planta* **223**: 1115-1122.
- Goldschmidt, V. M. (1954) *Geochemistry.* Oxford University Press, Oxford. pp. 730.
- González-Chávez, M. C., Carrillo-González, R., Wright, S. F., Nichols, K. A. (2004) The role of glomalin, a protein produced by arbuscular mycorrhizal fungi, in sequestering potentially toxic elements. *Environmental Pollution* **130**: 317-323.
- Gonzalez-Chavez, C., D'Haen, J., Vangronsveld, J., Dodd, J. C. (2002) Copper sorption and accumulation by the extraradical mycelium of different *Glomus* spp. (arbuscular mycorrhizal fungi) isolated from the same polluted soil. *Plant and Soil* **240**: 287-297.
- Gordon, A. S., Howell, L. D., Harwood, V. (1994) Response of diverse heterotrophic bacteria to elevated copper concentrations. *Canadian Journal of Microbiology* **40**: 408-411.
- Govindarajulu, M., Pfeffer, P. E., Jin, H., Abubaker, J., Douds, D. D., Allen, J. W., Büking, H., Lammers, P. J., Shachar-Hill, Y. (2005) Nitrogen transfer in the arbuscular mycorrhizal symbiosis. *Nature* **435**: 819-823.
- Goyer, R. A. (1993) Lead toxicity: current concerns. *Environmental Health Perspectives* **100**: 177-187.
- Guether, M., Neuhäuser, B., Balestrini, R., Dynowski, M., Ludewig, U., Bonfante, P. (2009) A mycorrhizal-specific ammonium transporter from *Lotus japonicas* acquires nitrogen released by arbuscular mycorrhizal fungi. *Plant Physiology* **150**: 73-83.
- Harley, J. L. (1989) The significance of mycorrhiza. *Mycological Research* **92**: 129-139.
- Harrison, M. J., van Buuren, M. L. (1995) A phosphate transporter from the mycorrhizal fungus *Glomus versiforme*. *Nature* **378**: 626-629.
- Hazarika, P., Singh, Y. P., Talukdar, N. C. (2014) Arbuscular mycorrhizal fungi (AMF) in revegetated coal mine overburden dumps of Margherita, Assam, India. *Life Sciences Leaflets* **51**: 40-58.
- Heinrichs, H., Schulz-Dobrick, B., Wedepohl, K. H. (1980) Terrestrial geochemistry of Cd, Bi, Ti, Pb, Zn and Rb. *Geochimica et Cosmochimica Acta* **44**: 1519-1532.
- Helgason, T., Daniell, T. J., Husband, R., Fitter, A. H., Young, J. P. W. (1998) Ploughing up the wood-wide web? *Nature* **394**: 431.

- Helgason, T., Merryweather, J. W. Denison, J., Wilson, P., Young, J. P. W., Fitter, A. H. (2002) Selectivity and functional diversity in arbuscular mycorrhizas of co-ocurring fungi and plants from a temperate deciduous woodland. *Journal of Ecology* **90**: 371-384.
- Hepper, C. M., Smith, G. A. (1976) Observations on the germination of *Endogone* spores. *Transactions of the British Mycological Society* **66**: 189-194.
- Hildebrandt, U., Regvar, M., Bothe, H. (2007) Arbuscular mycorrhiza and heavy metal tolerance. *Phytochemistry* **68**: 139-146.
- Hilditch, A. J. (1999) Effects of arbuscular mycorrhizal fungi and the rhizobacterium, *Enterobacter cloacae* 6.11 pDTC on growth of cassava (*Manihot esculenta* L. Crantz) in greenhouse trials under different phosphorus and water conditions. Master's project, School of Molecular and Cell Biology. University of the Witwatersrand, Johannesburg.
- Hodge, A., Helgason, T., Fitter, A. H. (2010) Nutritional ecology of arbuscular mycorrhizal fungi. *Fungal Ecology* **3**: 267-273.
- Hooda, P. S. (2010) *Trace elements in soils.* Blackwell Publishing, John Wiley & Sons Ltd., Chichester. pp. 3.
- Hosny, M., Gianinazzi-Pearson, V., Dulieu, H. (1998) Nuclear DNA content of 11 fungal species in Glomales. *Genome* **41**: 422-428.
- Hutnik, R. J., Davis, G. (1973) *Ecology and reclamation of devastated land.* Vol 1. Gordon and Breach, New York. pp. 16 and 17.
- Ibijbijen, J., Urquiaga, S., Ismaili, M., Alves, B. J. R., Boddey, R. M. (1996) Effect of arbuscular mycorrhizal fungi on growth, mineral nutrition and nitrogen fixation of three varieties of common beans (*Phaseolus vulgaris*). *New Phytologist* **134**: 353-360.
- INVAM: International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi. (Accessed 21/028/2014). West Virginia University. (Updated 13/07/2013) http://invam.wvu.edu/.
- Jabaji-Hare, S., Deschene, A., Kendrick, B. (1984) Lipid content and composition of vesicles of a vesicular-arbuscular mycorrhizal fungus. *Mycologia* **76**: 1024-1030.
- Jakobsen¸ I., Gazey, C., Abbott, L. K. (2001) Phosphate transport by communities of arbuscular mycorrhizal fungi in intact soil cores. *New Phytologist* **149**: 95-103.
- Janisch, P. R. (1986) Gold in South Africa. *Journal of the South African Institute of Mining and Metallurgy* **86**: 273-316.

- Jansa, J., Mozafar, A., Anken, T., Ruh, R., Sanders, J. R., Frossard, E. (2002) Diversity and structure of AMF communities as affected by tillage in a temperate soil. *Mycorrhiza* **12**: 225-234.
- Ji, K. X., Chi, F., Yang, M. F., Shen, S. H., Jing, Y. X., Dazzo, F. B., Cheng, H. P. (2010) Movement of rhizobia inside tobacco and lifestyle alteration from endophytes to free-living rhizobia on leaves. *Journal of Microbiology and Biotechnology* **20**: 238-244.
- Johnson D., Vandenhoornhuyse, P. J., Leake, J. R., Gilbert, L., Booth, R. E., Grime, J. P., Young, J. P. W., Read, D. J. (2003) Plant communities affect arbuscular mycorrhizal fungal diversity and community composition in grassland microcosms. *New Phytologist* **161**: 503-515.
- Joner, E. J., Briones, R., Leyval, C. (2000) Metal-binding capacity of arbuscular mycorrhizal mycelium. *Plant and Soil* **226**: 227-234.
- Kabata-Pendias, A. (1992) Trace metals in soils in Poland – Occurrence and behaviour. *Soil Sciences* **140**: 53-70.
- Kabata-Pendias, A. (2011) *Trace elements in soils and plants.* 4th ed. CRC Press Taylor & Francis Group, Boca Raton. pp. 14, 15, 20 and 65.
- Kabata-Pendias, A., Pendias, H. (1984) *Trace elements in soils and plants.* CRC Press, Florida. pp. 315.
- Kabata-Pendias, A., Pendias, H. (1992) *Trace elements in soils and plants.* 2nd ed. CRC Press, Florida. pp. 365.
- Kaonongbua, W., Morton, J. B., Bever, J. D. (2010) Taxonomic revision transferring species in *Kuklospora* to *Acaulospora* (Glomeromycota) and a description of *Acaulospora colliculosa* sp. nov. from field collected spores. *Mycologia* **102**: 1497-1509.
- Kapoor, A., Viraraghavan, T. (1995) Fungal biosorption – An alternative treatment option for heavy metal bearing wastewaters: A review. *Bioresource Technology* **53**: 195-206.
- Kastori, R. R., Maksimović, I. V., Zeremski-Škorić, T. M., Putnik-Delić, M. I. (2010) Rare earth elements – Yttrium and higher plants. *Proceedings of the National Academy of Sciences*, Matica Srpska building in Novi Sad **118**: 87-98.
- Kavamura, V. N., Esposito, E. (2010) Biotechnological strategies applied to the decontamination of soils polluted with heavy metals. *Biotechnology Advances* **28**: 61-69.

- Kelly, J. J., Tate III, R. L. (1998) Effects of heavy metal contamination and remediation on soil microbial communities in the vicinity of a zinc smelter. *Journal of Environmental Quality* **27**: 609-617.
- Khan, A. G. (2005) Role of soil microbes in the rhizospheres of plants growing on trace metal contaminated soils in phytoremediation. *Journal of Trace Elements in Medicine and Biology* **18**: 355-364.
- Khan, A. G. (2006) Mycorrhizoremediation – an enhanced form of Phytoremediation. *Journal of Zheijang University SCIENCE B* **7**: 503-514.
- Koljonen, T. (1992) The geochemical atlas of Finland. Part 2: Till. *Geological Survey of Finland*, Espoo. pp. 218.
- Kotrba, P., Najmanova, J., Macek, T., Ruml, T., Mackova, M. (2009) Genetically modified plants in phytoremediation of heavy metal and metalloid soil and sediment pollution. *Biotechnology Advances* **27**: 799-810.
- Krämer, A. G. (2005) Phytoremediation: novel approaches to cleaning up polluted soils. *Current Opinion in Biotechnology* **16**: 133-141.
- Krishna, K. R. (2005) *Mycorrhizas: A molecular analysis.* Science Publishers, Inc. Enfield, New Hampshire. pp. 116-120.
- Krüger, M., Krüger, C., Walker, C., Stockinger, H., Schüßler, A. (2012) Phylogenetic reference data for systematics and phylotaxonomy of arbuscular mycorrhizal fungi from phylum to species level. *New Phytologist* **193**: 970-984.
- Krüger, M., Stockinger, H., Krüger, C., Schüßler, A. (2009) DNA-based species level detection of Glomeromycota: one PCR primer set for all arbuscular mycorrhizal fungi. *New Phytologist* **183**: 212-223.
- Lanfranco, L., Delpero, M., Bonfante, P. (1999) Intrasporal variability of ribosomal sequences in the endomycorrhizal fungus *Gigaspora margarita*. *Molecular Ecology* **8**: 37-45.
- Laverman, A. M., Blum, J. S., Schaefer, J. K., Philips, E. J. P., Lovley, D. R., Oremland, R. S. (1995) Growth of strain SES-3 with arsenate and other diverse electron acceptors. *Applied and Environmental Microbiology* **61**: 3556-3561.
- Lee, J., Lee, S., Young, P. W. (2008) Improved PCR primers for the detection and identification of arbuscular mycorrhizal fungi. *FEMS Microbiology Ecology* **65**: 339-349.

- Lemoine, M. C., Gollotte, A., Gianinazzi-Pearson, V. (1995) ß 1-3 Glucan localization in the walls of the endomycorrhizal fungi *Glomus mosseae* and *Acaulospora laevis* during colonization of host roots. *New Phytologist* **129**: 97-105.
- Leung, H. M., Ye, Z. H., Wong, M. H. (2007) Survival strategies of plants associated with arbuscular mycorrhizal fungi on toxic mine tailings. *Chemosphere* **66**: 905-915.
- Leyval, C., Singh, B. R., Joner, E. J. (1995) Occurrence and infectivity of arbuscular mycorrhizal fungi in some Norwegian soils influenced by heavy metals and soil properties. *Water, Air and Soil Pollution* **84**: 203-216.
- Leyval, C., Turnau, K., Haselwandter, K. (1997) Effect of heavy metal pollution on mycorrhizal colonization and function: Physiological, ecological and applied aspects. *Mycorrhiza* **7**: 139-153.
- Li, X.-L., Marschner, H., George, E. (1991) Acquisition of phosphorus and copper by VA-mycorrhizal hyphae and root-to-shoot transport in white clover. *Plant and Soil* **136**: 49-57.
- Liu, R. J., Li, X. L. (2000) *Arbuscular mycorrhiza and its application*. Science Press, Beijing. pp. 1-224.
- Liu, R., Wang, F. (2003) Selection of appropriate host plants used in trap culture of arbuscular mycorrhizal fungi. *Mycorrhiza* **13**: 123-127.
- Lorscheider, F. L., Vimy, M. J., Summers, A. O. (1995) Mercury exposure from silver tooth fillings – emerging evidence questions a traditional dental paradigm. *FASEB Journal* **9**: 504-508.
- Lovato, P. E., Gianinazzi-Pearson, V. Trouvelot, A., Gianinazzi, S. (1996) The state of art mycorrhizas and micropropagation. *Advances in Horticultural Science* **1**: 46-52.
- MacLean, A. J. (1974) Mercury in plants and retention of mercury by soils in relation to properties and added sulphur. *Canadian Journal of Soil Science* **54**: 287-292.
- Marschner, H. (1986) *Mineral nutrition of higher plants.* Academic Press, London. pp. 243, 254-255, 269, 287-288, 298-299, 301, 311-312 and 366-367.
- Marschner, H. (1995) *Mineral nutrition of higher plants.* 2nd ed. Academic Press, London. pp. 566-595.
- Marschner, P., Crowley, D., Yang, C-H. (2004) Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type. *Plant Soil* **261**: 199-208.

- Mason, B., Moore, C. B. (1982) *Principles of Geochemistry.* John Wiley, New York. pp. 344.
- McGonigle, T. P., Miller, M. H., Evans, D G., Fairchild, G. L., Swan, J. A. (1990) A new method which gives an objective measure of colonisation of roots by vesicular-arbuscular mycorrhizal fungi. *New Phytologist* **115**: 495-501.
- McLaughlin, M. J., Parker, D. R., Clarke, J. M. (1999) Metals and micronutrients – food safety issues. *Field Crops Research* **60**: 143-163.
- McLennon, S. M. (1999) Yttrium. *In*: Marshall, C. P., Fairbridge, R. W. (Eds) *Encyclopaedia of Geochemistry.* Kluwer Academic Press, Dordrecht. pp. 673.
- McLennon, S. M., Murray, R. W. (1999) Geochemistry of sediments. *In*: Marshall, C. P., Fairbridge, R. W. (Eds) *Encyclopaedia of Geochemistry.* Kluwer Academic Press, Dordrecht. pp. 282-292.
- McLennon, S. M., Taylor, S. R. (1999) Earth's continental crust. *In*: Marshall, C. P., Fairbridge, R. W. (Eds) *Encyclopaedia of Geochemistry.* Kluwer Academic Press, Dordrecht. pp. 145-150.
- McNeill, J., Barrie, F. R., Buck, W. R., Demoulin, V., Greuter, W., Hawksworth, D. L., Herendeen, P. S., Knapp, S., Marhold, K., Prado, J., Prud'homme van Reine, W. F., Smith, G. F., Weirsma, J. H., Turland, N. (2012) International Code of Nomenclature for algae, fungi, and plants (Melbourne Code). Regnum Vegetabile 154. Koeltz Scientific Books. (Available at: http://www.iapt-taxon.org/nomen/main.php)
- Menge, J. A., Steirle, D., Bagyaraj, D. G., Johnson, E. L. V., Leonard, R.T. (1978) Phosphorus concentration in plants responsible for inhibition of mycorrhizal infection. *New Phytologist* **80**: 575-578.
- Mielke, J. E. (1979) Composition of the Earth's crust and distribution of the elements. *In*: Siegel, F. R. (Ed) *Review of research on modern problems in geochemistry.* International Association for Geochemistry and Cosmochemistry. Earth Science Series No. 16. UNESCO report SC/GEO/544/3, Paris. pp. 13-37.
- Mihaljevic, M. (1999) Mercury. *In*: Marshall, C. P., Fairbridge, R. W. (Eds) *Encyclopaedia of Geochemistry.* Kluwer Academic Press, Dordrecht. 387-389.
- Miller, J. S., Funk, V. A., Wagner, W. L., Barrie, F., Hoch, P. C., Herendeen, P. (2011) Outcomes of the 2011 Botanical Nomenclature Section at the XVIII International Botanical Congress. *Phytokeys* **5**: 1-3.

- Moawad, H. A., Ellis, W. R., Schmidt, E. L. (1984) Rhizosphere response as a factor in competition among three serogroups of indigenous *Rhizobium japonicum* for nodulation of field-grown soybeans. *Applied and Environmental Microbiology* **47**: 607-612.
- Morton, J. B. (1988) Taxonomy of VA mycorrhizal fungi: Classification, nomenclature, and identification. *Mycotaxon* **32**: 267-324.
- Morton, J. B., Benny, G. L. (1990) Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): a new order, Glomales, two new suborders, Glomineae and Gigasporineae, and two new families, Acaulosporaceae and Gigasporaceae, with an emendation of Glomaceae. *Mycotaxon* **37**: 471-491.
- Morton, J. B., Bentivenga, S. P. (1994) Levels of diversity in endomycorrhizal fungi (Glomales, Zygomycetes) and their role in defining taxonomic and non-taxonomic groups. *Plant and Soil* **159**: 47-59.
- Morton, J. B., Bentivenga, S. P., Bever, J. D. (1995) Discovery measurement and interpretation of diversity in arbuscular endomycorhizal fungi (Glomales, Zygomycetes). *Canadian Journal of Botany* **73**: 25-32.
- Morton, J. B., Bentivenga, S. P., Wheeler, W. W. (1993) Germ plasma in the International Collection of Arbuscular and Vesicular-arbuscular mycorrhizal Fungi (INVAM) and procedures for culture development, documentation and storage. *Mycotaxon* **48**: 491-528.
- Morton, J. B., Redecker, D. (2001) Two new families of Glomales, Archaeosporaceae and Paraglomacea, with two new genera *Archaeospora* and *Paraglomus*, based on concordant molecular and morphological characters. *Mycologia* **93**: 181-195.
- Mosse, B. (1972a) The influence of soil type and Endogone strain on the growth of mycorrhizal plants in phosphate deficient soils. *Revue d'Ecologie et de Biologie du Sol* **9**: 529-537.
- Mosse, B. (1972b) Effects of different Endogone strains on the growth of *Paspalum notatum*. *Nature* **229**: 221-223.
- Munkvold, L., Kjøller, R., Vestberg, M., Rosendahl, S., Jokobsen, I. (2004) High functional diversity within species of arbuscular mycorrhizal fungi. *New Phytologist* **164**: 357-364.
- Nemery, B., Lewis, C. P., Demedts, M. (1994) Cobalt and possible oxidant-mediated toxicity. *Science of the Total Environment* **150**: 57-64.

- Nies, D. H. (1999) Microbial heavy-metal resistance. *Applied Microbiology and Biotechnology* **51**: 730-750.
- Norris, J. R., Read, D J., Varma, A. K. (1994) *Techniques for mycorrhizal research.* Academic Press, London. pp. 763-767 and 778-782.
- Nriagu, J. O. (1978) Lead in soils, sediments and major rock types. *In*: Nriagu, J. O. (Ed) *The biogeochemistry of lead in the environment, part A.* Elsevier, Amsterdam. pp. 15-72.
- Oehl, F., da Silva, G. A., Sánchez-Castro, I., Goto, B. T., Maia, L. C. Veiera, H. E. E., Barea, J.-M., Sieverding, E., Palenzuela, J. (2011) Revision of *Glomeromycetes* with entrosporoid and glomoid spore formation with three new genera. *Mycotaxon* **117**: 297-316.
- Oehl, F., Sieverding, E. (2004) *Pacispora*, a new vesicular arbuscular mycorrhizal fungal genus in the Glomeromycetes. *Journal of Applied Botany and Food Quality* **78**: 72-82.
- O'Hara, E. W., Boonkerd, N., Dilworth, M. J. (1988) Mineral constraints to nitrogen fixation. *Plant and Soil* **108**: 93-110.
- Olesniewicz, K. S., Thomas, R. B. (1999) Effects of mycorrhizal colonisation on biomass production and nitrogen fixation of black locust (*Robinia pseudoacacia*) seedlings grown under elevated atmospheric carbon dioxide. *New Phytologist* **142**: 133-140.
- Oliveira, A., Pampulha, M. E. (2006) Effects of long-term heavy metal contamination on soil microbial characteristics. *Journal of Bioscience and Bioengineering* **102**: 157-161.
- Olsen, T., Habte, M. (1995) Mycorrhizal inoculation effect on nodulation and N accumulation in *Cajanus cajan* at soil P concentrations sufficient or inadequate for mycorrhiza free growth. *Mycorrhiza* **5**: 395-399.
- Olsson, P. A., Hammer, E. C., Pallon, J., Van Aarle, I. M., Wallander, H. (2011) Elemental composition in vesicles of an arbuscular mycorrhizal fungus, as revealed by PIXE analysis. *Fungal Biology* **115**: 643-648.
- Oono, R., Denison, R. F., Kiers, E. T. (2009) Controlling the reproductive fate of rhizobia: how universal are legume sanctions? *New Phytologist* **183**: 967-979.
- Oono, R., Denison, R. F. (2010) Comparing symbiotic efficiency between swollen versus nonswollen rhizobial bacteroids. *Plant Physiology* **154**: 1541-1548.
- Oono, R., Schmitt, I., Sprent, J. I., Denison, R. F. (2010) Multiple evolutionary origins of legume traits leading to extreme rhizobial differentiation. *New Phytologist* **187**: 508-520.

- Öpik, M., Moora, M., Zobel, M., Saks, U., Wheatley, R., Wright, F., Danielli, T., (2008) High diversity of arbuscular mycorrhizal fungi in a boreal herb-rich coniferous forest. *New Phytologist* **179**: 867-876.
- Öpik, M., Vanatoa, A., Vanatoa, E., Moora, M., Davison, J., Kalwij, J.M., Reier, U., Zobel, M. (2010) The online database Maarj*AM* reveals global and ecosystemic distribution patterns in arbuscular mycorrhizal fungi (Glomeromycota). *New Phytologist* **188**: 223-241.
- Orlowska, E., Zubeck, S. Z., Jurkiewicz, A., Szarek-Lukaszewska, G., Turnau, K. (2002) Influence of restoration of arbuscular mycorrhiza of *Biscutella laevigata* L. (Brassicaceae) and *Plantago lanceolata* L. (Plantaginaceae) from calamine spoil mounds. *Mycorrhiza* **12**: 153-160.
- Oryowska, E., Ryszka, P., Jurkiewicz, A., Turnau, K. (2005) Effectiveness of arbuscular mycorrhizal fungal (AMF) strains in colonisation of plants involved in phytostabilisation of zinc wastes. *Geoderma* **129**: 92-98.
- Pacioni, G., Rosa, S. (1985) An improved filtering apparatus for the 'wet-sieving and decanting' technique for extraction of endogenous spores. *Bulletin of the British Mycological Society* **19**: 66-68.
- Pacovsky, R. S. (1986) Micronutrient uptake and distribution on mycorrhizal and phosphorus fertilized soybeans. *Plant and Soil* **95**: 379-385.
- Pavlakis, N., Pollock, C. A., McLean, G., Bartrop, R. (1996) Deliberate overdose of uranium: toxicity and treatment. *Nephron* **72**: 313-317.
- Pawlowski, T. E., Charvat, I. (2004) Heavy-metal stress and development patterns of arbuscular mycorrhizal fungi. *Applied and Environmental Microbiology* **70**: 6643-6649.
- Pearson, J. N., Schweiger, P. (1993) *Scutellospora calospora* (Nicol. & Gerd.) Walker and Sanders associated with subterranean clover: dynamics of colonisation, sporulation, and soluble carbohydrates. *New Phytologist* **124**: 215-219.
- Peuke, A. D., Rennenberg, H. (2005) Phytoremediation. *EMBO Reports* **6**: 497-501.
- Pfleger, F. L., Linderman, R. G. (1994) *Mycorrhizae and plant health*. The American Phytopathological Society, United States of America. pp. 283-309.
- Pirozynski, K. A., Dalpe, Y. (1989) Geological history of the Glomaceae with particular reference to mycorrhizal symbiosis. *Symbiosis* **7:** 1–36.
- Pitet, M. Camprubí, M., Calvet, C., Estaún, V. (2009) A modified staining technique for arbuscular mycorrhiza compatible with molecular probes. *Mycorrhiza* **19**: 125-131.

- Ragan, H. A., Mast, T. J. (1990) Cadmium inhalation and male reproductive toxicity. *Reviews of Environmental Contamination and Toxicology* **114**: 1-22.
- Rahimi, A., Bussler, W. (1973) Physiologische Voraussetzungen für die Bildung der Kupfermangelsymptome. *Journal of Plant Nutrition and Soil Science* **136**: 25-32.
- Rani, S. S., Kunwar, I. K., Manoharachary, C. (2006) Arbuscular mycorrhizal fungal taxonomy: An overview. *In*: Mukerji, K. G., Manoharachary, C. (Eds) *Current concepts in botany.* I. K. International Publishing House Pvt. Ltd., New Deli. pp. 37-47.
- Reddy, K. R. (Accessed 12/01/2014) Experiment 2: Organic matter determination. *In*: *Engineering properties of soils based on laboratory testings.* University of Illinois, Chicago. (Available at: CME315 soil mechanics laboratory; http://www.uic.edu/classes/cemm/cemmlab/)
- Redecker, D. (2000) Specific PCR primers to identify arbuscular mycorrhizal fungi within colonised roots. *Mycorrhiza* **10**: 73-80.
- Redecker, D., Hijri, I., Wiemken, A. (2003) Molecular identification of arbuscular mycorrhizal fungi in roots: Perspectives and problems. *Folia Geobotanica* **38**: 113-124.
- Redecker, D., Morton, J. B., Bruns, T. D. (2000) Ancestral lineages of arbuscular mycorrhizal fungi (Glomales). *Molecular Phylogenetics and Evolution* **14**: 276-284.
- Redecker, D., Schüßler, A., Stockinger, H., Stürmer, S. L., Morton, J. B., Walker, C. (2013) An evidence-based consensus for the classification of arbuscular mycorrhizal fungi (*Glomeromycota*). *Mycorrhiza* **23**: 515-531.
- Redecker, D., Thierfelder, H., Walker, C., Werner, D. (1997) Restriction analysis of PCR-amplified internal transcribed spacers of ribosomal DNA as a tool for species identification in different genera of the order Glomales. *Applied and Environmental Microbiology* **63**: 1756-1761.
- Rodriguez Montelongo, L., de la Cruz Rodriguez, L. C., Farias, R. N., Massa, E. M. (1993) Membrane-associated redox cycling of copper mediates hydroperoxide toxicity in *Escherichia coli*. *Biochima et Biophysica Acta* **1144**: 77-84.
- Rose, A. W., Hawkes, H. E., Webb, J. S. (1979) *Geochemistry in mineral exploration.* Academic Press, New York. pp. 657.
- Rösner, T., Boer, R., Reyneke, R., Aucamp, P., Vermaak, J. (2001) A preliminary assessment of pollution contained in the unsaturated and saturated zone beneath reclaimed gold mined residue deposits. *Water Research Commission Report No. 797/1/01.*

- Ross, J. H. (1972) *The Acacia Species of Natal.* 2nd ed. The natal branch of the wildlife protection and conservation society of South Africa. pp. 4-5.
- Ruiz-Lozano, J. M., Azcon, R., Gomez, M. (1995) Effects of arbuscular-mycorrhizal Glomus species on drought tolerance: Physiological and nutritional plant responses. *Applied and Environmental Microbiology* **61**: 456-460.
- Sandmann, G., Böger, P. (1983) The enzymatology function of heavy metals and their role in electron transfer processes of plants. *In*: Läuchli, A., Bieleski, R. L. (Eds) *Encyclopaedia of plant physiology, New series.* Vol 15A. Springer-Verlag, New York and Berlin. pp. 563-596.
- Savolainen, H. (1996) Biochemical and clinical aspects of nickel toxicity. *Reviews on Environmental Health* **11**: 167-173.
- Saxena, A. K., Rathi, S. K., Tilak, K. V. B. R. (1997) Differential effect of various endomycorrhizal fungi on nodulating ability of green gram by *Bradyrhizobium* sp. (*Vigna*) strain S24. *Biology and Fertility of Soils* **24**: 175-178.
- Schenck, N. C., Pérez, Y. (1990) *Manual for the identification of VA myocorrhizal fungi.* 3rd ed. Synergistic Publications, Florida. pp. 17-40.
- Schoknecht, J. D., Hattingh, M .J. (1976) X-ray microanalysis of elements in cells of VA mycorrhizal and nonmycorrhizal onions. *Mycologia* **68**: 296-303.
- Schreiner, R. P., Mihara, K. L., McDaniel, H., Bethlenfalvay, G. J. (1997) Mycorrhizal fungi influence plant and soil functions and interactions. *Plant and Soil* **188** (2): 199-209.
- Schüßler, A. (2002) Molecular phylogeny, taxonomy and evolution of *Geosiphon pyriformis* and arbuscular mycorrhizal fungi. *Plant and Soil* **244**: 75-83.
- Schüßler, A., Mollenhauer, D., Schnepf, E., Kluge, M. (1994) *Geosiphon pyriforme*, an endosymbiotic association of fungus and cyanobacteria: the spore structure resembles that of arbuscular mycorrhizal AM fungi. *Botanica Acta* **107**: 36-45.
- Schüßler, A., Schwarzott, D., Walker, C. (2001) A new fungal phylum, the *Glomeromycota*: phylogeny and evolution. *Mycological Research* **105**: 1413-1421.
- Schüßler, A., Walker, C. (2010) The *Glomeromycota*. A species list with new families and new genera. Published in libraries at Royal Botanic Garden Edinburgh, Kew, Botanische Staatssammlung Munich, and Oregon State University. http://www.amf-phylogeny.com

- Schultz, P. A., Bever, J. D., Morton, J. (1999) *Acaulospora colossica* sp. nov. from an old field in North Carolina and morphological comparisons with similar species, *A. laevis* and *A. koskei*. *Mycologia* **91**: 676-683.
- Schulze, R. E. (1997) Climate. *In*: Cowling, R. M., Richardson, D. M., Pierce, S. M. (Eds) *Vegetation of Southern Africa.* Cambridge University Press, Cambridge. pp. 21-42.
- Shachar-Hil, Y. Pfeffer, P. E., Douds, D., Osman, S. F., Doner, L. W., Ratcliff, R. G. (1995) Partioning of intermediary carbon metabolism in vesicular-arbuscular mycorrhizal leek. *Plant Physiology* **108**: 7-15.
- Shacklette, H. T., Boerngen, J. G. (1984) Element concentrations in soils and other surficial materials of the conterminous United States. *U. S. Geological Survey Professional Paper 1270.* United States Government Printing Office, Washington. pp. 4.
- Shanker, A. K., Cervantes, C., Loza-Tavera, H., Avudainayagam, S. (2005) Chromium toxicity in plants. *Environment International* **31**: 739-753.
- Sharma, A. K., Johri, B. N. (2002) *Arbuscular mycorrhizae: Interactions in plants, rhizosphere and soils.* Science Publishers, Inc., United State of America. pp. 48-55.
- Sheppard, S. C., Evenden, W. G., Anderson, A. J. (1992) Multiple assays of uranium toxicity in soil. *Environmental Toxicology and Water Quality* **7**: 275-294.
- Simon, L., Lalonde, M., Bruns, T. D. (1992) Specific amplification of 18S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungi colonising roots. *Applied and Environmental Microbiology* **58**: 291-295.
- Simon, L., Lévesque, R. C., Lalonde, M. (1993) Identification of endomycorrhizal fungi colonising roots by fluorescent single-strand conformation polymorphism-polymerase chain reaction. *Applied and Environmental Microbiology* **59**: 4211-4215.
- Siqueira, J. O., Sylvia, D. M., Gibson, J., Habbell, D. H. (1985) Spores, germination, and germ tubes of vesicular-arbuscular mycorrhizal fungi. *Canadian Journal of Microbiology* **31**: 965-972.
- Smit, N. (1999) *Guide to the Acacias of South Africa.* Briza Publications, Pretoria. pp. 7, 11, 18, 21, 31, 67 and 111.
- Smith, S. E., Read, D. J. (1997) *Mycorrhizal symbiosis.* 2nd ed. Academic Press, London.
- Smith, S. E., Read, D. J. (2000) *Mycorrhizal symbiosis.* 2nd ed. Academic Press, San Diego. pp. 13-80.
- Smith, S. E., Read, D. J. (2008) *Mycorrhizal symbiosis.* 3rd ed. Academic Press, San Diego. pp. 11-187.

- Solaiman, M. Z., Saito, M. (1997) Use of sugars by intraradical hyphae of arbuscular mycorrhizal fungi revealed by radiorespirometry. *New Phytologist* **136**: 533-538.
- Spruyt, A. (2010) Molecular identification of arbuscular mycorrhizal fungi (AMF) associated with *Tamarix usneoides* growing on high metal sites. Honours project, School of Molecular and Cell Biology. University of the Witwatersrand, Johannesburg.
- Spruyt, A., Buck, M. T., Mia, A., Straker, C. J. (2014) Arbuscular mycorrhiza (AM) status of rehabilitation plants of mine wastes in South Africa and determination of AM fungal diversity by analysis of the small subunit rRNA gene sequences. *South African Journal of Botany* **94**: 231-237.
- Stevenson, F. J., Fitch, A. (1981) Reactions with organic matter. *In*: Loneragan, J. F., Robson, A. D., Graham, R. D. (Eds) *Copper in soils and plants.* Academic Press, New York. pp. 69-95.
- Straker, C. J., Weiersbye, I. M., Witkowski, E. T. F. (2007) Arbuscular mycorrhiza status of gold and uranium tailings and surrounding soils of South Africa's deep level gold mines: I. Root colonization and spore levels. *South African Journal of Botany* **73**: 218-225.
- Straker, C. J., Freeman, A. J., Witkowski, E. T. F., Weiersbye, I. M. (2008) Arbuscular mycorrhiza status of gold and uranium tailings and surrounding soils of South Africa's deep level gold mines. II. Infectivity. *South African Journal of Botany* **74**: 197-207.
- Straker, C. J., Hilditch, A. J., Rey, M. E. C (2010) Arbuscular mycorrhizal fungi associated with cassava (*Manihot esculenta* Crantz) in South Africa. *South African Journal of Botany* **76**: 102-111.
- Stutz, J. C., Morton, J. B. (1996) Successive pot cultures reveal high species richness of arbuscular endomycorrhizal fungi in arid ecosystems. *Canadian Journal of Botany* **74**: 1883-1889.
- Suwalsky, M., Ungerer, B., Quevedo, L., Aguilar, F., Sotomayor, C. P. (1998) Cu^{2+} ions interact with cell membranes. *Journal of Inorganic Biochemistry* **70**: 233-238.
- Sylvia, D. M., Williams, S. E. (1992) Vesicular-arbuscular mycorrhizae and environmental stress. *In*: Bethlenfalvay, G. J., Linderman, R. G. (Eds) *Mycorrhizae in sustainable agriculture.* American Society of Agronomy Special Publication No. 54, Madison, Wisconsin. pp. 101-124.

- Tack, F. M. G. (2010) Trace elements: General soil chemistry, principles and processes. *In*: Hooda, P. S. (Ed). *Trace elements in soils.* Blackwell Publishing, Wiley & Sons, Ltd., Chichester. pp. 9-37.
- Tisdall, J. M. (1994) Possible role of sol microorganisms in aggregation in soils. *Plant and Soil* **159**: 115-121.
- Thingstrup, I., Kahiluoto, H., Jakoben, I. (2000) Phosphate transport by hyphae of field communities of arbuscular mycorrhizal fungi at two levels of P fertilization. *Plant and Soil* **221**: 181-187.
- Thorneley, R. N. F. (1992) New light on nitrogenase. *Nature* **360**: 532-533.
- Tinker, P. B. (1975) Soil chemistry of phosphorus and mycorrhizal effects on plant growth. *In*: Sanders, F. E., Mosse, B., Tinker, P. B. (Eds). *Endomycorrhizas*. Academic Press, United Kingdom. pp. 353-371.
- Tinker, P. B., Nye, P. H. (2000) *Solute movement in the rhizosphere.* Oxford University Press, United Kingdom. pp. 173.
- Tommerup, I. C. (1983) Spore dormancy in vesicular-arbuscular mycorrhizal fungi. *Transactions of the British Mycological Society* **81**: 37-45.
- Tremearne, T. H., Jacob, K. D. (1941) *Arsenic in natural phosphates and phosphate fertilisers.* United States Department of Agriculture Technical Bulletin No. 781, Washinton, D. C. pp. 39.
- Türkdoğan, M .K., Kilicel, F., Kara, K., Tuncer, I., Uygan, I. (2002) Heavy metals in soil, vegetables and fruits in the endemic upper gastrointestinal cancer region of Turkey. *Environmental Toxicology and Pharmacology* **13**: 175-179.
- Ure, A. M., Berrow, M. L. (1982) The elemental constituents of soils. *In*: Bowen, H. J. M. (Ed.) *Environmental chemistry.* Royal Society of Chemistry Special Report Series, London. pp. 94-204.
- Valsecchi, G., Gigliotti, C., Farini, A. (1995) Microbial biomass, activity, and organic matter accumulation in soils contaminated with heavy metals. *Biology and Fertility of Soils* **20**: 253-259.
- VanKuren, N. W., den Bakker, H. C., Morton, J. B., Pawlowska, T. E. (2012) Ribosomal RNA gene diversity, effective population size, and evolutionary longevity in asexual Glomeromycota. *Evolution* **67**: 207-224.
- van Reeuwjik, L. P. (2002) *Procedures for soil analysis.* 6th ed. International Soil Reference and Information Centre, Netherlands. pp. 4-1.

- van Tuinen, D., Jacquot, E., Zhao, B., Gollotte, A., Gianinazzi-Pearson, V. (1998) Characterisation of root colonisation profiles by a microcosm community of arbuscular mycorrhizal fungi using 25 rDNA-targetted nested PCR. *Molecular Ecology* **7**: 879-887.
- Walker, C. M. (1983) Taxonomic concepts on the Endogonaceae: spore wall characteristics in species description. *Mycotaxon* **18**: 443-445.
- Walker, C., Reed, L. E., Sanders, F. E. (1984) *Acaulospora nicolsonii*, a new Endogonaceaes species from Great Britain. *Transactions of the British Mycological Society* **83**: 360-364.
- Walker, C. M. (1992) Systematics and taxonomy of the arbuscular endomycorrhizal fungi (Glomales) – a possible way forward. *Agronomie* **12**: 887-897.
- Walker, C., Schüßler, A. (2004) Nomenclatural clarifications and new taxa in the glomeromycota. Mycological Research **108** (9): 981-98
- Walker, C., Vestberg, M, Demircik, F. Stockinger, H., Saito, M., Sawaki, H., Nishmura, I., Schüßler, A. (2007a) Molecular phylogeny and new taxa in the *Archaeosporales* (*Glomeromycota*): *Ambispora fennica* gen. sp. nov., *Ambisporaceae* fam. nov., and emendation of *Archaeospora* and *Archaeosporaceae*. *Mycological Research* **111**: 137-153.
- Walker, C., Vestberg, M., Schüßler, A. (2007b) Nomenclatural clarification in Glomeromycota. *Mycological Research* **111**: 253-256.
- Wang, F. Y., Lin, X. G., Yin, R., Wu, L. H. (2006) Effects of arbuscular mycorrhizal inoculation on growth of *Elsholtzia splendens* and *Zea mays* and the activities of fungal characteristics and soil enzymatic activities in Cd polluted soil. *Environmental Pollution* **134**: 257-266.
- Weast, R. C. (1984) *CRC handbook of chemistry and physics.* 64 ed. CRC, Florida.
- Wedepohl, K. H. (1978) *Handbook of geochemistry.* Vol II/5. Springer, Berlin.
- Weiersbye, I. M., Straker, C. J., Przybylowicz, W. J. (1999) Micro-PIXE mapping of elemental distribution in arbuscular mycorrhizal roots of the grass, *Cynodon dactylon*, from gold and uranium mine tailings. *Nuclear Instruments and Methods in Physics Research B* **158**: 335-343.
- Weiersbye, I. M., Witkowski, E. T. F., Reichardt, M. (2006) Floristic composition of gold and uranium tailings dams, and adjacent polluted areas, on South Africa's deep-level mines. *Bothalia* **36**: 101-127.

- Weiersbye, I. M., Witkowski, E. T. F. (2007) Impacts of acid mine drainage on the regeneration of potential Highveld phreatophyte plants. In: Bester, J. J., Seyback, A. H. W., Vorster, T., Van der Merwe, I. J. and Deivhani, S. (Eds) *Multiple use management of natural forests and woodlands: Policy refinements and scientific progress IV.* Department of Water Affairs and Forestry, South Africa. pp. 224-237.
- White T. J., Bruns, T., Lee, S., Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics: *In*: Innis, M. A., Gelfand, D. H., Sninsky, J. J., White, T. J. (Eds) *PCR protocols: A guide to methods and applications.* Academic Press, San Diego. **99**: 315-322.
- Winn, W. C., Koneman, C. W. (2006) *Koneman's colour atlas and textbook of diagnostic microbiology.* 6th ed. Lippincott Wilkins, Baltimore. pp. 1151-1243.
- Woolhouse, H. W. (1983) Toxicity and tolerance in responseof plants to metals. *In*: Lange, O. L., Nobel, P. S., Osmond, C. B., Ziegler, H. (Eds) *Encyclopaedia of plant physiology, New series.* Spriner-Verlag, New York and Berlin. pp. 246-300.
- Wright, S. F., Upadhyaya, A. (1998) A survey of soils for aggregate stability and glomalin, a glycoprotein produced by hyphae of arbuscular mycorrhizal fungi. *Plant and Soil*. **198**: 97-107.
- Xavier, L. J. C., Boyetchko, S. M. (2002) Arbuscular mycorrhizal fungi as biostimulants and bioprotectants of crops. *Applied Mycology and Biotechnology* **2**: 311-340.
- Yang, C-H., Crowley, D. E. (2000) Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. *Applied Environmental Microbiology* **66**: 345-351.
- Young, J. P. W. (2012) A molecular guide to the taxonomy of arbuscular mycorrhizal fungi. *New Phytologist* **193**: 823-826.
- Zarei, M., Hempel, S., Wubet, T., Schäfer, T., Savaghebi, G., Jouzani, G.S., Nekouei, M. K., Buscot, F. (2010) Molecular diversity of arbuscular mycorrhizal fungi in relation to soil chemical properties and heavy metal contamination. *Environmental Pollution* **158**: 2757-2765.
- Zarei, M., König, S., Hempel, S., Nekouei, M.K., Savaghebi, G., Buscot, F. (2008) Community structure of arbuscular mycorrhizal fungi associated to *Veronica rechingeri* at the Anguran zinc and lead mining region. *Environmental Pollution* **156**: 1277-1283.
- Zhang, Z., Schwartz, S., Wagner, L., Miller, W. (2000) A greedy algorithm for aligning DNA sequence. *Journal of Computational Biology* **7**: 203-214.

- Zhao, B., Trouvelot, A. Gianinazzi, S., Gianinazzi-Pearson, V. (1997) Influence of two legume species on hyphal production and activity of two arbuscular mycorrhizal fungi. *Mycorrhiza* **7**: 179-185.
- Zhou, J. L. (1999) Zn biosorption by *Rhizopus arrhizus* and other fungi. *Applied Microbiology and Biotechnology* **51**(5): 686-693.
- Zupančič, N., Pirc, S., 1999. Calcium distribution in soil and stream sediments in Istria (Croatia) and the Slovenian littoral. *Journal of Geochemical Exploration* **65**: 205-218.

Appendices

Appendix 1 – Tree GPS co-ordinates

A table illustrating the trees and their respective GPS co-ordinates selected on the mine tailings Free State North 6 and Free State South 5.

Site	Tree number	GPS co-ordinates
Free State North 6	1	S 27° 57.869’ E 26° 41.261’
	2	S 27° 57.877’ E 26° 41.260’
	3	S 27° 57.961’ E 26° 40.623’
	4	S 27° 57.959’ E 26° 40.632’
	5	S 27° 58.062’ E 26° 40.511’
	6	S 27° 58.082’ E 26° 40.535’
	7	S 27° 58.084’ E 26° 40.528’
	8	S 27° 58.072’ E26° 40.527’
	9	S 27° 58.073’ E 26° 40.538’
Free State South 5	1	S 28° 00.619’ E 26° 48.350’
	2	S 28° 00.633’ E 26° 48.350’
	3	S 28° 00.645’ E 26° 48.358’
	4	S 28° 00.684’ E 26° 48.369’
	5	S 28° 00.684’ E 26° 48.375’
	6	S 28° 00.576’ E 26° 48.414’
	7	S 28° 00.575’ E 26° 48.416’
	8	S 28° 00.573’ E 26° 48.416’
	9	S 28° 00.571’ E 26° 48.417’

Appendix 2 – Agar plates for cloning

Ampicillin containing agar plates

For 100 mg/mℓ ampicillin solution 0.5 g ampicillin was added to 5 mℓ sterile distilled water. Of this solution 0.5 mℓ was added to 500 mℓ autoclaved, precooled nutrient agar and poured into petridishes and allowed to cool.

Ampicillin, X-gal and IPTG agar plates

For 3 % X-gal solution 0.15 g was added to 5 mℓ in dimethyl formamide and stored at -20 °C.

For 100 mM IPTG solution 0.12 g was added to 5 mℓ sterile distilled water and stored at -20 °C.

To make the ampicillin, X-gal and IPTG plates 0.5 mℓ of each solution (including 100 mg/mℓ ampicillin) was added to 500 mℓ autoclaved, precooled nutrient agar and poured into petridishes and allowed to cool.

Appendix 3 – SOB Media

Reagents

Tryptone

Yeast extract

NaCl

250 mM KCl

2 M $MgCl_2$

Procedure

The KCl solution was made by adding 1.6 g KCl to 100 mℓ distilled water.

The $MgCl_2$ solution was made by adding 19 g to 100 mℓ distilled water.

The SOB media was made by first disolving 20 g tryptone, 5 g yeast extract and 0.5 g NaCl into 950 100 mℓ distilled water, then 10 mℓ of the KCl solution was added and the pH was adjusted to 7.0 with 5 N NaOH. The solution volume was adjusted to 1 ℓ and autoclaved. Finally 5 mℓ of the $MgCl_2$ solution was added to the media.