INTERACTION BETWEEN ARBUSCULAR MYCORRHIZAL FUNGI AND SOIL MICROBIAL POPULATIONS IN THE RHIZOSPHERE

By

Nnenna Esther Ike-Izundu

April 2007

INTERACTION BETWEEN ARBUSCULAR MYCORRHIZAL FUNGI AND SOIL MICROBIAL POPULATIONS IN THE RHIZOSPHERE

A thesis submitted in fulfillment of the requirements for the degree of

Masters of Science in Microbiology

Of

Rhodes University

In the
Department of Biochemistry, Microbiology & Biotechnology

Faculty of Science

By

Nnenna Esther Ike-Izundu

April 2007

ABSTRACT

This study examined the rehabilitation potential of AM fungi with organic and inorganic fertilisers under pot and field trial conditions as well as their interaction with rhizospheric organisms and specific functional groups. In addition, the study highlighted the effects of land-use management on AM fungal populations in soil and the mycorrhizal status of some selected plants from one of the study sites. The study focussed on two sites that differ in operational activities and these included a mined area that was to be rehabilitated and a commercial farming site.

A pot trial was conducted using an overburdened soil resulting from kaolin clay mining. Pots were seeded with *Cynodon dactylon* and treated with either Organic Tea or NPK (3:1:5) fertiliser, with or without AM fungal inoculum. The compatibility of these fertilisers with AM fungi was assessed by plant growth and percentage root colonisation. Maximum shoot height and plant biomass were observed at the 28th week with NPK (3:1:5) fertiliser supporting mycorrhizal colonisation by 80%. The result indicated the potential of AM fungi to be used in rehabilitation with minimal phosphate fertiliser. Similarly, a field trial was set-up using 17 x 17 m^2 plots in the mining site that were treated with the same organic and inorganic fertilisers as well as with AM fungal inoculum in different combinations. The interaction between AM fungi and soil microbial population was determined using culture dependent and culture independent techniques. The culture dependent technique involved the use of soil dilution and plating on general purpose and selective media. The result showed that there was no change in the total culturable bacterial number in the untreated and AM fungal treated plots, while a change in species composition was observed in the functional groups. Different functional groups identified included nitrogen fixing bacteria, pseudomonads, actinomycetes, phosphate solubilisers and the fungal counterparts. Gram-positive bacteria were observed as the predominant phenotypic type, while nitrogen fixers and actinomycetes were the predominant functional groups. Species identified from each functional group were *Pseudomonas fulva*, *Bacillus megaterium*, *Streptomyces* and actinomycetales bacteria. Meanwhile, fungi such as *Ampelomyces*, *Fusarium*, *Penicillium*, *Aspergillus*, *Cephalosporium* and

Exserohilium were identified morphologically and molecularly. Furthermore, the mining site had a significantly higher bacterial number than the farming site thereby indicating the effects of land-use management on culturable bacterial numbers. The culture independent technique was carried out by cloning of the bacterial 16S rDNA and sequencing. Identified clones were *Bradyrhizobium*, *Propionibacterium* and *Sporichthya*. A cladogram constructed with the nucleotides sequences of identified functional species, clones and closely related nucleotide sequences from the Genbank indicated that nucleotide sequences differed in terms of the method used.

The activity and establishment of the introduced AM fungal population was determined by spore enumeration, infectivity assay, percentage root colonisation and assessment of glomalin concentrations. The results indicated that the two land use types affected AM fungal populations. However, the establishment of AM fungi in the farming site was more successful than in the mining site as indicated by the higher infectivity pontential. Selected host plants, which were collected around the mine area, were observed to be mainly colonised by AM fungi and these were identified as *Pentzia incana*, *Elytropappus rhinocerotis*, *Euphorbia meloformis*, *Selago corymbosa*, *Albuca canadensis* and *Helichrysum rosum*. These plant species were able to thrive under harsh environmental conditions, thereby indicating their potential use as rehabilitation host plants.

Generally, the findings of this study has provided an insight into the interaction between arbuscular mycorrhizal fungi and other soil microorganisms in two fields with differing land use management practices.

I dedicate this thesis to God almighty, for all His mercies and strength to persevere through to the end of the road; and to my father Late Prof. I.F Ike for inspiring me in all my years of study.

Most of the important things in the world have been accomplished by people who have kept on trying when there seemed to be no hope at all. **-Dale Carnegie**

That's why...

Our greatest glory is not in never falling, but in rising every time we fall.
- **Confucius**

ACKNOWLEDGEMENTS

My most sincere gratitude goes to my Supervisor, Dr. Joanna Dames for her guidance through this research and for her patience and humour.

I would also like to thank my Family members, my Mum Mrs F.C. Ike, Nkechi, Linda, Emeka and Kenechukwu for their support and financial assistance toward the completion of my work by ensuring that I was comfortable and focussed. Also to Dr. K.I Ozoemena and wife who gave me a home here in South Africa.

To my friends Rasheed, Tembisa, Tom, the Nigerian community at Rhodes and my church members for being there for me in times when a little outing and chatting was indeed necessary.

To Greer Hawley and Anna Clark for offering assistance in whatever way they could without hesitation.

To Prof. Randloff for her guidance with the statistical analysis, her time and patience.

To all the members of the Mycorrhizal Research group who really took away the tension in the lab with their good sense of humour.

To the management of Makana Brick and Tile (Pty) and Limestone Hill Farm for providing the working area for this project.

To all my friends and well-wishers for your moral support and understanding when I never communicated.

Finally, I will wish to thank my heartthrob Kingsley O. for his encouragement, patience, love and support. He was indeed my better half.

TABLE OF CONTENTS

ABSTRACT I

ACKNOWLEDGEMENTS IV

TABLE OF CONTENTS V

LIST OF FIGURES VIII

LIST OF TABLES X

LIST OF ABBREVIATIONS XI

1 GENERAL INTRODUCTION 1

1.1 Fungus-root 1

1.2 Soil as a living habitat 4

1.3 Arbuscular mycorrhiza 6
1.3.1 Taxonomy and Description 6
1.3.2 Distribution 10
1.3.3 Reproduction and life cycle 11
1.3.4 Symbiotic benefits 16

1.4 Factors affecting growth of arbuscular mycorrhizal fungi 23

1.5 Interaction of arbuscular mycorrhizal fungi with other soil microorganisms 28
1.5.1 Interaction with bacteria 28
1.5.2 Endosymbiotic bacteria of AM fungi 33
1.5.3 Interaction with fungi 34

1.6 The ecological roles of arbuscular mycorrhizal fungi 36

1.7 Current methods used to study microbial populations and interactions 40
1.7.1 Plate counts 41
1.7.2 Denaturing gradient gel electrophoresis and temperature-GGE 43
1.7.3 Other molecular techniques 43

1.8 Motivation for the study 46

1.9 Hypothesis and Objectives 46
1.9.1 Hypothesis 46
1.9.2 Objectives 46

2 MATERIALS AND METHODS 50
2.1 Site description 50
2.2 Field experimental establishment and sampling 52
2.2.1 Site 1 52
2.2.2 Experimental setbacks 55
2.2.3 Site 2 56
2.3 Soil texture and nutrient analysis 58
2.4 Pot trial analysis 59
2.4.1 Experimental design 59
2.4.2 Experimental establishment 60
2.4.3 Sampling and processing 60
2.5 Microbial population analysis 61
2.5.1 Estimating the number of culturable microbial populations in the rhizosphere 62
2.5.2 Determination of the unculturable microbial populations in the rhizosphere 65
2.6 Arbuscular mycorrhizal fungal population assessment in soils 73
2.6.1 Spore extraction and enumeration 73
2.6.2 Root colonisation 74
2.6.3 Glomalin extraction and quantification 75
2.6.4 Most Propable Number 75
2.7 Alternative host plants from around the mine area 76
2.8 Statistical analysis 77
3 RESULTS 78
3.1 Soil texture and nutrient analysis 78
3.2 Pot trial analysis 79
3.3 Microbial population analysis 84
3.3.1 Estimating the number of culturable microbial populations in the rhizosphere 84
3.3.2 Determination of the unculturable microbial populations in the rhizosphere 95
3.4 Arbuscular mycorrhizal fungal population assessment in soils 103
3.5 Alternative host plants from around the mine area 106
4 DISCUSSION 112
4.1 Pot trial analyses 113
4.2 Microbial population analyses 117
4.2.1 Estimating the number of culturable microbial populations in the rhizosphere 117
4.2.2 Determining specific functional groups in the rhizosphere 122
4.2.3 Determination of the unculturable microbial populations in the rhizosphere 130
4.3 Arbuscular mycorrhizal fungal population assessment in soil 135
4.4 Alternative host plants from around the mine area 144

5 SUMMARY AND CONCLUSIONS 148

5.1 The potential of arbuscular mycorrhizal fungi in rehabilitation of mine spoils 148

5.2 Effect of introduced AM fungal inoculum on soil microbial populations 149

5.3 Functional groups of soil microbial populations and their interaction with arbuscular mycorrhizal fungi 150

5.4 Effect of land use management on arbuscular mycorrhizal fungal population and infectivity 151

5.5 Mycorrhizal status of selected plants growing around the mine area 152

5.6 Recommendations 152

REFERENCES 154

APPENDICES 1

LIST OF FIGURES

Figure 1.1 Root cross section illustrating different types of mycorrhizal relationships that exist within plants **3**

Figure 1.2 Taxonomic structures of arbuscular myocrrhizal fungi in the phylum Glomeromycota and related fungi based on SSU rRNA gene sequences.. **8**

Figure 1.3 Diagrammatic representation of the characteristic structures of arbuscular mycorrhizal fungi as identified in the cortical cell of a plant host when viewed under a microscope......................... **9**

Figure 1.4 Life cycle of arbuscular mycorhizal fungi showing asymbiotic, presymbiotic and symbiotic stages of colonisation. **13**

Figure 2.1 Grahamstown and Bathurst areas in a section map of Eastern Cape Province, South Africa.. .... **51**

Figure 2.2 An overview of the landscaped overburdened soil for rehabilitation at Makana Brick and Tile (Pty) Ltd.. .... **52**

Figure 2.3 Plot layouts with designated treatments and reference reading numbers of the rehabilitation Site situated at Makana Brick and Tile Pty **54**

Figure 2.4 Limestone Hill farm cultivated with *Pelargonium graveolens* cuttings in rows **58**

Figure 2.5 Diagrammatic representation of one phase of the pot layout with replicates as setup in a plastic tunnel. **60**

Figure 3.1 Pot trial of *Cynodon dactylon* plants at 6 and 28 weeks with varying treatments. **80**

Figure 3.2 Plant shoot height of *Cynodon dactylon* treated with Organic Tea and 3:1:5 NPK fertiliser with or without arbuscular mycorrhizal fungal (AMF) inoculum.. .... **82**

Figure 3.3 Plant biomass of *Cynodon dactylon* treated with Organic Tea and 3:1:5 NPK fertiliser with or without arbuscular mycorrhizal fungal (AMF) inoculum.. .... **82**

Figure 3.4 Colonisation of root cortical cells by arbuscular mycorrhizal fungi.................................... **83**

Figure 3.5 Percentage root colonisation of *Cynodon dactylon* at 28 weeks from pot trial. A. Organic Tea + arbuscular mycorrhizal fungal (AMF) inoculum and control with fertiliser only............... **83**

Figure 3.6 Total culturable bacteria from the rhizosphere of arbuscular mycorrhizal fungal inoculated and control plots at the mining (MBT) Site over time. **85**

Figure 3.7 Total culturable bacteria from the rhizosphere of the sampled Sites indicating land usage at the MBT- mining (9 months) and LHF- farming (5 months) Site. **85**

Figure 3.8 Total microbial numbers of different functional groups from replicate plots at the MBT (1) and LHF (2) Site. **88**

Figure 3.9 Ethidium bromide stained agarose gel (1%) showing PCR amplification of 16S rDNA of bacterial isolates obtained from the mining and farming Site on various selective media............ **89**

Figure 3.10 Ethidium bromide stained agarose gel (1%) showing PCR amplification of 18S rDNA of fungal pure cultures isolated from the mining Site and the farm Site, which could not be identified morphologically.. .... **91**

Figure 3.11 PCR-RFLP analysis of 16S rDNA of identified bacterial isolates from functional groups compared with the pure bacterial isolates using restriction enzymes *Eco*R 1, *Pst* 1 and *Hinc* III **94**

Figure 3.12 PCR-DGGE amplification of 16S bacterial soil DNA obtained from four locations at Makana Brick and Tile prior to plot layout. **95**

Figure 3.13 PCR amplification of 16S bacterial soil DNA obtained from two different Sites. **96**

Figure 3.14 pGEM –T easy vector circle map and sequence reference points with restriction enzymes indicating positions at which the vector is cleaved. **97**

Figure 3.15 A and B. Agarose gel electrophoresis of restriction fragments of plasmid isolated products digested with *EcoR* 1 enzyme **98**

Figure 3.16 A and B. Plasmid PCR amplification of all clones isolated from the mining and farm Site using M13 forward and reverse primers.. .... **99**

Figure 3.17 Clustal W cladogram showing sequence similarities of all identified bacteria obtained from culture dependent and independent techniques.. .... **102**

Figure 3.18 Glomalin concentrations at various sampling periods (3, 6 and 9 months) at Makana Brick and Tile.. .... **105**

Figure 3.19 Glomalin concentrations of soil samples from Makana Brick and Tile (9 months) and Limestone Hill farm (5 months). **106**

Figure 3.20 Identified host plant growing around Makana Brick and Tile mine area. **107**

Figure 3.21 Arbuscular mycorrhizal and endomycorrhizal colonisation types observed in selected plant species obtained from the mine area. **109**

LIST OF TABLES

Table 1.1 Nutrients and elements that were absorbed and transported to plant shoots by AM fungi when present in the soil either naturally, as contaminants or when induced................................**18**

Table 1.2 Advantages and disadvantages of biochemical-molecular based method for soil microbial diversity studies. **44**

Table 2.1 Accumulated monthly rainfall data for Grahamstown from August 2005 to June 2006........**56**

Table 2.2 Single plot layout with three replicates of treatments in rows and number of plants per treatment at the Limestone Hill Farm, Bathurst. **57**

Table 2.3 PCR cycling conditions used for amplification of 16S rDNA. **67**

Table 2.4 PCR reaction mixture as used for plasmid amplification of cloned samples. **70**

Table 2.5 Recognition sequences of the restriction enzymes used for restriction fragment length polymorphism. **71**

Table 2.6 Restriction digests parameters used in RFLP analysis of plasmid extracted DNA samples. . **72**

Table 2.7 Primers used in the study for the amplification of 16S rDNA, ITS regions and plasmid inserts. **73**

Table 3.1 Chemical and physical characteristics of soil samples obtained from the field Sites: Makana Brick and Tile Pty and Limestone Hill Farm. **78**

Table 3.2 Shoot height and plant biomass of *Cynodon dactylon* treated with Organic Tea and 3:1:5 NPK fertilisers either with or without arbuscular mycorrhizal fungal inoculum **81**

Table 3.3 Phenotypic characteristic as determined by Gram staining of randomly selected bacterial isolates obtained from both mining and farming Sites. **86**

Table 3.4 Partial sequence analysis on 16S rDNA gene of bacterial isolates obtained from different functional groups and their affiliation to related nucleotide sequences **90**

Table 3.5 Partial sequence analysis on 18S rDNA gene of fungal isolates obtained from the mining and the farm Site with their affiliation to related nucleotide sequences.......................................**91**

Table 3.6 Fungal genera of morphological identified fungal isolates growing on Potato Dextrose Agar and examined using tape mounts. **92**

Table 3.7 Partial sequence analysis on 16S rDNA gene of bacterial clones obtained from the mining and farming Sites and their affiliation to related nucleotide sequences **101**

Table 3.8 Arbuscular mycorrhizal fungal spore density in the soil and percentage root colonisation of *Cynodon dactylon* root samples obtained from Makana Brick and Tile Site. **103**

Table 3.9 Arbuscular mycorrhizal fungal spore density in the soil and percentage root colonisation of *Pelargonium graveolens* root samples obtained from Limestone Hill Farm Site. **104**

Table 3.10 Mycorrhizal status of indigenous host plants, their families and habitat-flowering season. **108**

LIST OF ABBREVIATIONS

°E	Degree East
°S	Degree South
<	Less than
µ	Micron (10^{-6})
AM	arbuscular mycorrhizal
ANOVA	analysis of variance
APS	ammonium persulphate
BLAST	basic local alignment search tool
Bp	base pairs
BSA	bovine serum albumin
CEC	cation exchange capacity
cm	centimetre
CTAB	hexadecyltrimethyl ammonium bromide
DGGE	denaturing gradient gel electrophoresis
dH2O	distilled and deionized water
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
dNTP	deoxyribose nucleotide triphosphate
e.g.	for example
EDTA	ethylene diamine tetra acetic acid
ERH	extraradical hyphae
FAME	fatty acid methyl ester
g	gram
hr(s)	hour(s)
i.e.	that is
IAA	indole acetic acid
IRH	intraradical hyphae
ITS	internal transcribed spacer
Kb	kilobase
l/L	litre
LB	luria bertani
mg	milligram
mg/ml	milligram/millilitre
mM	milli (10^{-3}) molar
MHB	mycorrhizal helper bacteria

Min(s)	minute(s)
MPN	most probable number
N	nitrogen
nm	nanometer
NCBI	National Centre for Biotechnology Information
P	phosphorus
PCR	polymerase chain reaction
PGPR	plant growth promoting bacteria
PLFA	phospholipid fatty acid
ppm	part per million
PSB	phosphate solubilising bacteria
rDNA	ribosomal deoxynucleic acid
RFLP	restriction fragment length polymorphism
RNA	ribose nucleic acid
rpm	rotation per min
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
Sec	seconds
SOM	soil organic matter
sp.	species (singular)
spp.	species (plural)
SSU	small subunit
rRNA	ribosomal riboxynucleic acid
TAE	tris-acetic acid-EDTA
TE	tris-HCl EDTA
TEMED	tetramethylethylenediamine
Tm	melting temperature
T**-RFLP**	terminal restriction fragment length polymorphism
V	volts
vol/vol	volume / volume
wt/vol	weight / volume

CHAPTER 1

GENERAL INTRODUCTION

1 General introduction

1.1 Fungus-root

The success of mycorrhizal evolution has been attributed to the role that mycorrhizal fungi play in the capture of nutrients from the soil of all ecosystems (Bonfante and Perotto, 2000). Literally, "mycorrhiza" means fungus root and is derived from the Greek word "Mykes" meaning fungus and "Rhizo" meaning root (Friberg, 2001). This term was first used by Frank, a German Plant Pathologist in 1855 to describe the symbiotic relationship between plant roots and fungi. The symbiosis is characterised by the exchange of nutrients where carbon in the form of hexose sugars flows to the fungus and inorganic nutrients are passed to the plant, thereby providing a linkage between the plant root and the soil (Sylvia *et al*., 1998). Mycorrhizal fungi provide inorganic nutrients mainly phosphorus and other complexed compounds to the plant through the extensive network of their hyphae that forage for soil nutrients more effectively than plant roots (Van der Heijden *et al*., 1998b). For this association to occur there must be a host plant (the phytobiont), an ecological habitat (the soil) and a suitable fungus (the mycobiont). Mycorrhizal fungi differ from other plant–fungus associations because of their ability to create an interface for nutrient exchange which occurs within living cells of the plant (Brundrett, 2004; Brundrett, 2002).

Over 80% of plant species are associated with mycorrhizal fungi, amongst which are vascular and non-vascular plants and some important crops such as carrots, maize, leek, coffee, cocoa, soybeans, apples, citrus fruits, tomatoes and pepper to mention a few (Muchovej, 2004; Bonfante and Perotto, 2000). Mycorrhizal fungi interact with plants at different levels and can be grouped into obligately mycorrhizal, facultatively mycorrhizal and non-mycorrhizal plants (Brundrett, 2004). Facultative mycorrhizal plants as the name denotes, are not solely dependent on the fungus for phosphorus or other nutrients, but can also derive their nutrients from the soil when soil phosphorus levels are high. Thus, this level of association is dependent on soil fertility as mycorrhizal plants can reduce their association with the fungus in cases were the association provides little benefit (Brundrett, 2004; Koide and Schreiner, 1992).

Obligate mycorrhizal plants are solely dependent on mycorrhizal fungi for their phosphorus nutrition, as such both plant, and the fungus associate closely with each other. Some non-mycorrhizal plants belonging to the families *Amaranthaceae*, *Brassicaceae* and *Caryophyllaceae* are less attractive to mycorrhizal fungi but at times, attempts are still made to colonise their roots (Brundrett, 2002, Ocampo *et al*., 1980). The inability of these plants to support mycorrhizal colonisation may be due to the accumulation of chemicals like alkaloids, cyanogenic glucosinolates and antifungal compounds in the roots which fail to elicit differential hyphal branching (Brundrett, 2002; Giovannetti and Sbrana, 1998). Some of these plants like mangel and canola function independently in terms of nutrient acquisition through the use of their root system to modify the pH of the rhizosphere and increasing nutrient availability in the soil (Brundrett, 2002; Brundrett, 2004).

There are different types of mycorrhizal interactions which have been classified into ectomycorrhiza and endomycorrhiza based on the presence of various extraradical or intraradical hyphal structures (Bonfante and Perotto, 2000). Seven mycorrhizal types have been identified but the most common endomycorrhizas are the arbuscular mycorrhizas (Brundrett *et al*., 1996). Other types such as Ectendomycorrhiza, Arbutoid and Monotropoid mycorrhizas are grouped under ectomycorrhizas. These are characterised by the formation of a hartig net and a mantle or sheath around the plant roots (Fig. 1.1). Orchid and Ericoid mycorrhizas are other forms of endomycorrhizas that are known for their ability to penetrate the outer root cells to form intracellular hyphal coils, swellings, or branching (Fig. 1.1). They differ from the arbuscular mycorrhizal fungi by having septate hyphae that are restricted to the epidermal cells of plant roots (Molina *et al*., 1992). All these mycorrhizal types differ from each other by the characteristic host plant that they associate with, fungal species involved and morphology within roots (Prescott *et al*., 2005; Brundrett, 2002).

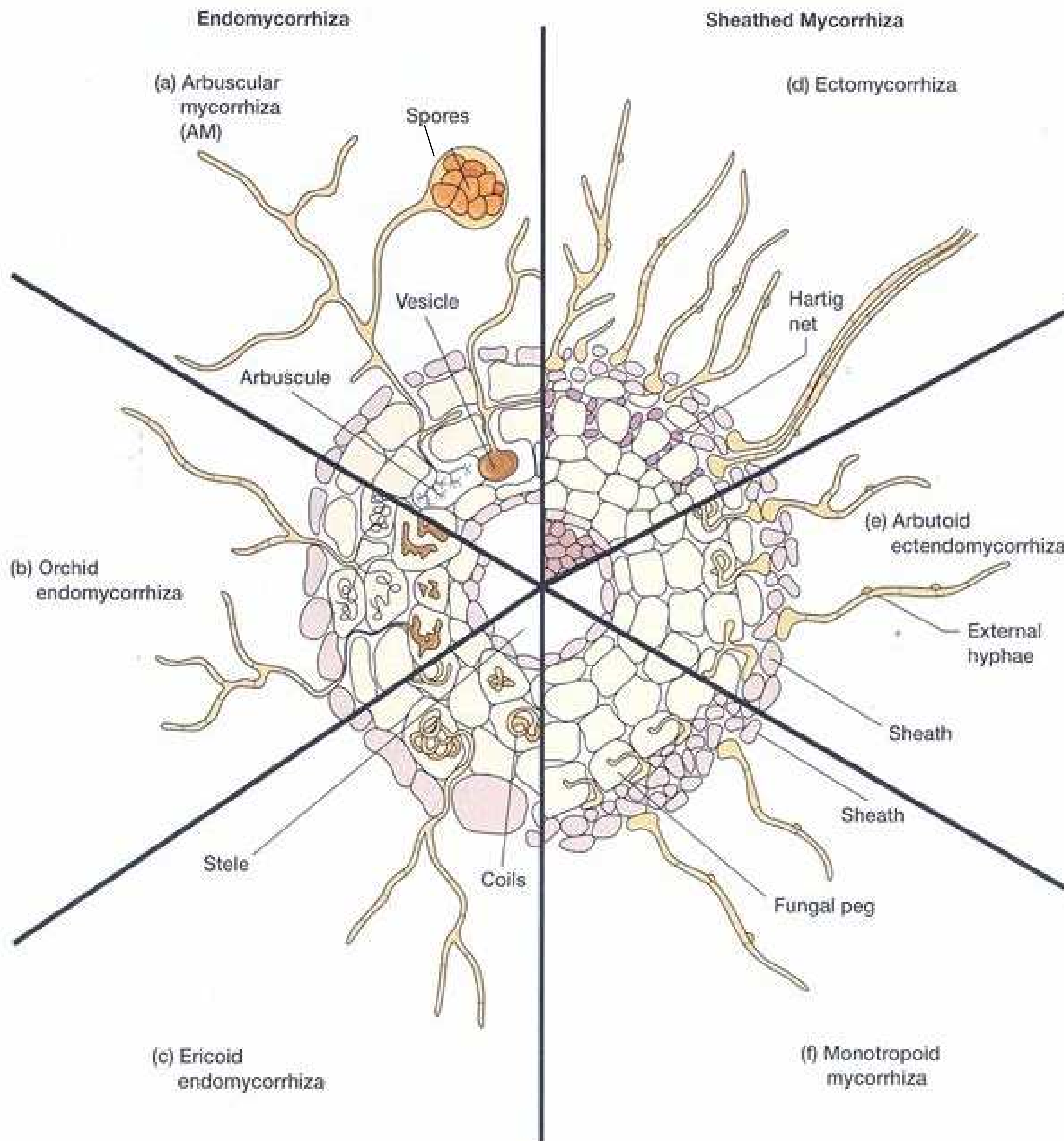


Figure 1.1 Root cross section illustrating different types of mycorrhizal relationships that exist within plants (Prescott *et al*., 2005).

Mycorrhizal fungi play a key role in terrestrial ecosystem functioning along with environmental factors such as climate, disturbances, food web interactions, mutualism and ecological history (Wardle and Van der Putten, 2002). This can be through nutrient acquisition, carbon cycling, plant diversity and plant productivity, but the

mechanism of how these functions are regulated is poorly understood (Van der Heijden *et al*., 1998b).

1.2 Soil as a living habitat

Soil is a complex heterogeneous habitat for a wide variety of organisms, which include bacteria, fungi, protozoan, nematodes and earthworms that play many functional roles in the ecosystem in which they exist. They function as populations or assemblages of similar organisms that interact with each other and their physical environment thereby contributing to plant nutrition, soil structure, soil fertility, decomposition of organic matter, cycling of nutrients, suppression of soil borne pathogens and removal of toxins (Prescott *et al*., 2005; Kirk *et al*., 2004, Kozdroj and van Elsas, 2000).

Soil organic matter (SOM), which is responsible for soil aggregate stability is composed of a fraction of non or partially degraded litter and humus that accounts for 30% cation exchange capacity of soils (Gobat *et al*., 2004). Cation exchange capacity (CEC) of soil refers to the measure of positive exchangeable ions such as Ca^{2+}, Mg^{2+} Al^{+3}, K^{+}, H^{+} and Na^{+} that the soil can hold (Rowell, 1994). Thus, a soil that is rich in organic matter content will have a high CEC with the ability to retain nutrients (Okalebo *et al*., 1993). As such, SOM which brings about high carbon content in combination with other soil factors such as moisture, soil minerals, pH and climate will influence and support the soil microbial community (Courtright *et al*., 2001; Wall and Virginia, 1999).

Soil microorganisms are involved in a wide variety of metabolic and physiological activities that influence the microhabitat. The plant's root with zone of intense microbial metabolic activity occurring where there is a high concentration of carbon is called the rhizosphere. The rhizosphere can be categorised into three sections: the endorhizosphere (interior of the root), the rhizoplane (root surface) and the soil directly adjacent and adhering to the root surface (Barea *et al*., 2005). The volume of soil that is not directly influenced by the root is called the bulk soil. Properties of the rhizosphere differ from those of bulk soil. The rhizosphere is characterised as having

a lower pH, lower water potential, low oxygen pressure and high levels of carbon dioxide (Suresh and Bagyaraj, 2002). Because the rhizosphere is rich in organic compounds due to the influence of plant roots, this zone favours microbial growth rather than the bulk soil that is carbon-limited (Sliwinski and Goodman, 2004; Gryndler, 2000; Andrade *et al.*, 1997). The bulk soil is rather low in nutrient due to soil permeability and so supports limited microbial activity (Gryndler, 2000).

Root activities can modify the soil physio-chemical properties through the release of organic or inorganic root compounds into the soil. This process is referred to as rhizodeposition or root exudation and is influenced by plant and soil biotic and abiotic factors (Jones *et al.*, 2004). Some plant biotic and abiotic factors that influence rhizodeposition include mycorrhizas, root architecture, nutrient deficiency, photosynthesis, temperature, light intensity and physical disturbance. While soil biotic and abiotic factors comprise of pathogens, biocontrol agents, root herbivory, metal toxicity, soil pH, soil texture and water availability (Jones *et al.*, 2004). Rhizodeposition corresponds to 15-30% of total carbon produced by plants during photosynthesis, which is transferred along with other organic compounds such as sugars or amino acids towards the microorganisms of the rhizosphere (Lynch and Whipps, 1990). Root exudates constitute a major part of rhizodeposition and mainly compose of soluble molecular weight molecules like flavonoids, phenolic compounds, carbohydrate monomers, organic acids and plant hormones (Farrar *et al.*, 2003; Lynch and Whipps, 1990). Therese substances directly or indirectly influence changes in rhizosphere as soil organisms utilise these compounds for nutrition and growth.

Rhizosphere microbial communities can influence ecological processes such as nutrient acquisition and fitness of plants through interaction with each other. Mycorrhizal fungi and plant growth promoting rhizobacteria (PGPR) are typical beneficial organisms that are capable of influencing changes in rhizosphere functioning (Barea *et al.*, 2002b; Suresh and Bagyaraj, 2002; Azcón-Aguilar and Barea, 1992). Mycorrhizal fungi provide an essential link between plants and the soil environments, therefore, are critical to any rhizosphere studies (Timonen and Marschner, 2005). Mycorrhiza formation modifies the root system metabolism by changing the chemical and mineral composition of root exudates that are released into

the soil (Timonen and Marschner, 2005; Azcón-Aguilar and Barea, 1992). This mycorrhiza-induced change can affect microbial populations in the rhizosphere or rhizoplane (Barea *et al*., 2002b; Azcón-Aguilar and Barea, 1992). Söderberg *et al.,* (2002) stated that the effect of mycorrhizal fungi on rhizosphere bacterial population varied with different plant species because of differential plant exudation patterns in the soil. Hence mycorrhizal fungi form a unique part of the rhizosphere and contribute to rhizodeposition dynamics (Fillion *et al*., 1999).

1.3 Arbuscular mycorrhiza

1.3.1 Taxonomy and Description

Arbuscular mycorrhizal (AM) fungi are obligate symbiotic fungi and endosymbionts of a variety of plants within the Angiosperms, Gymnosperms and Pteridophytes (Steinberg and Rillig, 2003; Smith and Read, 1997). AM fungi have three major components: the root itself which provides carbon in the form of sugars to the fungus, fungal structures within cortical cells of plant root that provide contact between fungus and the plant cytoplasm and the extraradical hyphae that aid uptake of nutrients and water (Smith and Read, 1997). The evolution of AM fungi can be dated back 460 million years ago from fossil records of the Ordovician age. These records suggest that AM fungi may have played a crucial role in colonisation of most terrestrial plants (Brundrett, 2002; Redecker *et al*., 2000; Smith and Read; 1997). The taxonomy of AM fungi has been based on morphological and anatomical characteristics of their spores. Other modern techniques such as serology, isozyme variation revealed by electrophoresis (Hepper *et al*., 1988), fatty acid variation (Bentivenga and Morton, 1994) and DNA based methods (Helgason *et al.,* 1999, Schüβler *et al.*, 2001, Morton and Redecker, 2001) have aided in a clearer phylogenetic analysis than was possible using morphological and microscopic identification (Fig. 1.2).

Arbuscular mycorrhizas were formerly classified in the phylum Zygomycota under the family Endogonaceae due to their resemblance with *Endogone* species. But this was later re-evaluated when it was found that AM fungi produced asexual spores rather than sexual spores like other *Endogone* species. The relationship between AM

fungi and other fungi as detected by molecular analysis elevated the group to the phylum Glomeromycota (Koide and Mosse, 2004). This new phylum is divided into four orders, eight families and ten genera (Walker and Schüßler, 2004; Schüβler *et al.*, 2001). The major distinguishing characters of their genera are their differences in spore wall, spore formation (e.g. spores developing as saccules, or from a cylindrical fertile hyphae or forming on a sporogenous wall), root colonisation patterns and tolerance to biotic and abiotic factors (Brundrett *et al.,* 1996; Morton and Benny, 1990). Two families, Archaeosporaceae and Paraglomaceae (Fig. 1.2) with one genus, *Archaeospora* and *Paraglomus* respectively, were added to the sub-order Glomineae due to similar morphological and phylogenetical characters between Glomaceae and Acaulosporaceae. However, to clearly differentiate the former two genera from the latter, analysis of their DNA sequences (SSU rRNA) and fatty acid profiles had to be analysed to determine their phylogenetic relationship with the two families (Morton and Redecker, 2001). The genus *Glomus* is said to be the largest within the Glomales and has been separated into two groups (Fig. 1.2) *Glomus* A and B based on phylogenetic analysis of their SSUrRNA to a family level (Schwarzott, *et al*., 2001). The *Glomus* A clade is sub categorized into G1GrAa which comprises of species like *Gl. geosporum* (Nicol. & Gerd.) Walker, *Gl. mosseae* (Nicol. & Gerd.), *Gl. caledonium* (Trappe & Gerd.), *Gl. fragilistratum* (Skou & Jakobsen) and G1GrAb comprising *Gl. intraradices* (Schenck & Smith), *Gl. fasciculatum* (Gerd. & Trappe) and *Gl. coremioides* (Redecker & Morton). *Glomus* B has only one sub group (G1GrB) that comprises of species *Gl. claroideum* (Schenck & Smith), *Gl. lamellosum* (Dalpe, Koske & Tews), *Gl. manihotis* (Sieverding & Schenck), *Gl. etunicatum* (Becker & Gerdemann) and *Gl. viscosum* (Nicol.). These groups were found to be genetically different but still form a monophyletic group in rDNA phylogenectic trees (Redecker, 2005; Redecker, 2002; Schüβler *et al.*, 2001; Schwarzott, *et al*., 2001). Recently, another new family Pacisporaceae (Fig. 1.2) which has one genus and seven species has been included in the order *Diversisporale*s e.g. *Pacispora chimonobambusae* (Oehl & Sieverd), *P. franciscana* (Oehl & Sieverd), *P. robigina* (Oehl & Sieverd), *P. boliviana* (Oehl & Sieverd), *P. coralloidae* (Redecker, 2005; Oehl and Sieverding, 2004). These species were formerly placed in the genus *Glomus* due to their shared spore formation, similarity with *Glomus* and *Paraglomus*. Conversely, it was found that these groups differed in spore germination

characteristics which they rather shared with *Scutellospora*, *Acaulospora* and *Enthrophospora* (Oehl and Sieverding, 2004).

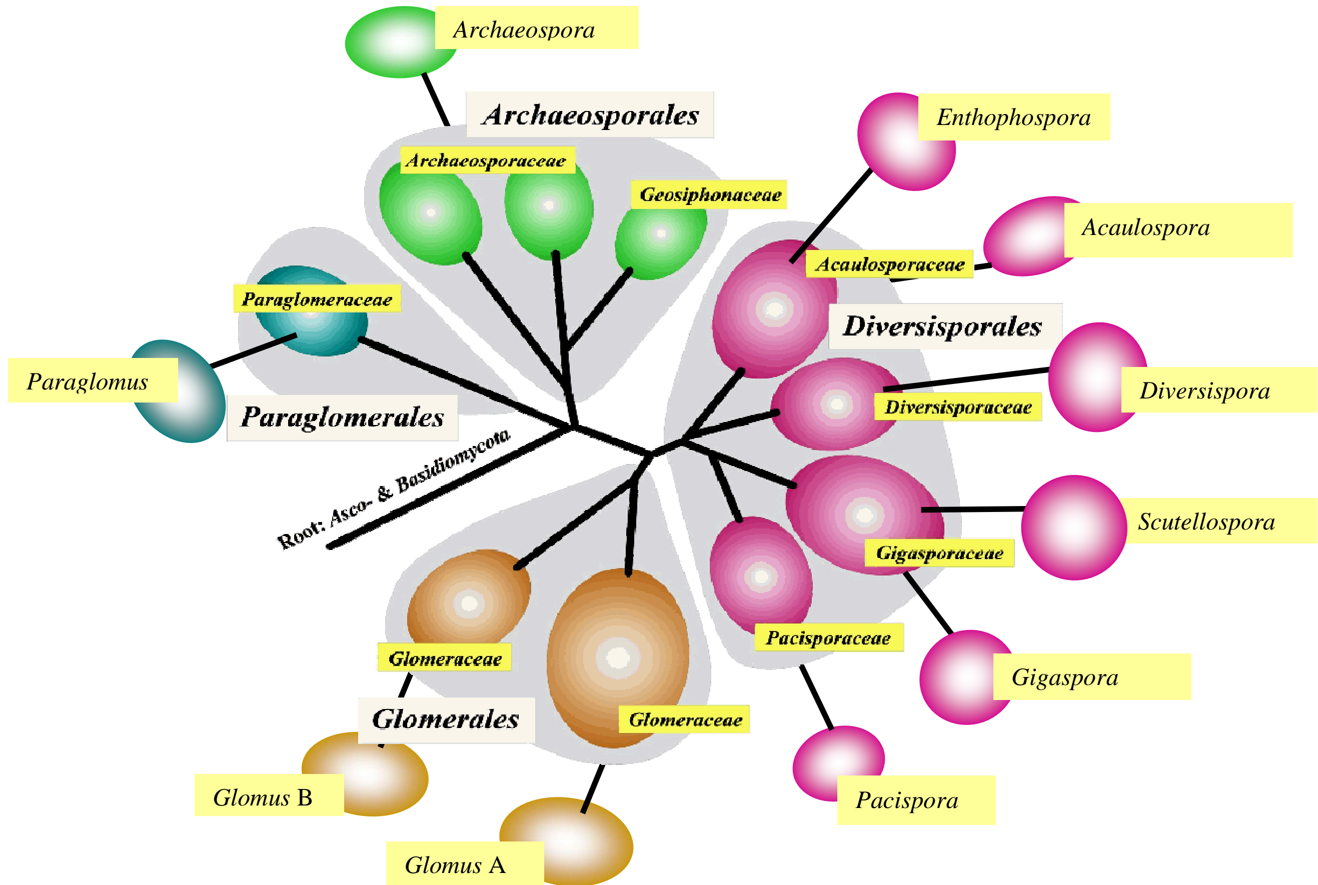


Figure 1.2 Taxonomic structures of arbuscular myocrrhizal fungi in the phylum Glomeromycota and related fungi based on SSU rRNA gene sequences (Walker and Schüßler, 2004).

Arbuscular mycorrhizal fungi are so named because they produce fine tree-like hyphal structures (Fig. 1.3) termed "arbuscles" that occur within the root cortical cells of plants. They are responsible for the exchange of carbon needed for energy and nutrients after close contact is made with the host cell. Arbuscules are similar to other fungal haustoria that are found in pathogenic associations. Except that these structures are highly branched and separated from plant cell contents by an unbreached plant plasma membrane (Isaac, 1992). Formerly, AM fungi were referred to as vesicular-

arbuscular mycorrhizal fungi but since not all genera produce vesicles, the name arbuscular mycorrhizal fungi was adopted (Friberg, 2001). Vesicles serve as carbon storage compartments for the fungi and are rich in lipids (Fig.1.3). They are found in three genera of the Glomeromycota: *Glomus*, *Acaulospora and Enthrophospora* (Isaac, 1992). However, their formation depends on environmental conditions such as high or low P levels that affect vesicle development (Smith and Read, 1997).

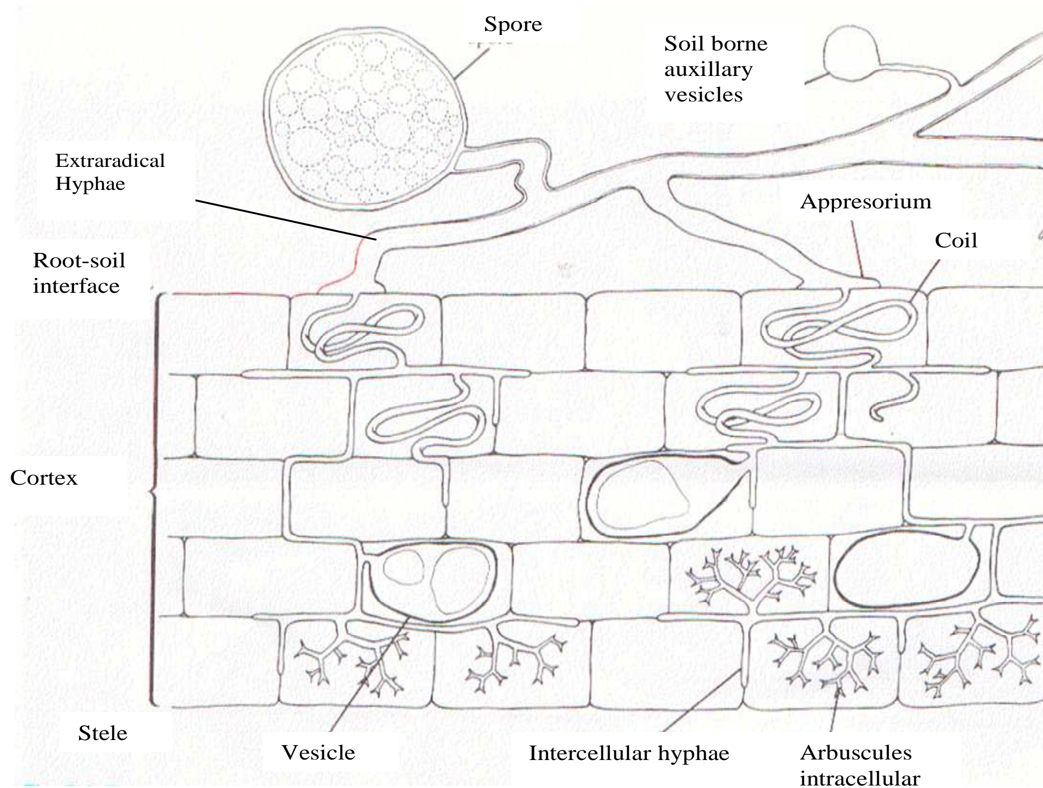


Figure 1.3 Diagrammatic representation of the characteristic structures of arbuscular mycorrhizal fungi as identified in the cortical cell of a plant host when viewed under a microscope (Modified from Isaac, 1992).

Other important structures of AM fungi that are involved in the colonisation of roots are intraradical hyphae (IRH), extraradical hyphae (ERH) and extraradical auxiliary cells. IRH provide means for the fungi to spread its hyphae within short distances of the root cortical cells forming colonisation units such as arbuscules and vesicles (Morton and Benny, 1990). ERH are distinguished as the branching absorptive hyphae (Fig. 1.3) that colonise the rhizosphere in search of nutrients, infective hyphae which run towards and along root surfaces establishing new entry points and the

reproductive hyphae that develop fertile spores after colonisation of roots (Nagashi, 2000). Extraradical auxiliary vesicles are found mostly among the family Gigasporaceae. They act as lipid storage compartments and are involved in partitioning of nuclei and nutrients (phosphorus/carbon) prior to sporulation. Their presence begins to decline as sporulation increases (Dodd *et al*., 2002; Morton and Benny, 1990).

1.3.2 Distribution

Although AM fungi are widespread and are distributed in different parts of the world especially in the tropics, little functional information was learned about them, until the mid 1950s (Smith and Read, 1997). They are reported to be found in diverse land areas such as calcareous grasslands, arid/semi arid grasslands, several temperate forests, tropical rain forests and shrub lands in diverse parts of the world (Renker *et al*., 2005; Oehl *et al*., 2003; Muthukumar and Udaiyan, 2002). Recently, AM fungi have received more attention especially in African countries such as Namibia, Cameroon, Kenya, Morocco, Nigeria, Senegal, Zambia and South Africa. These studies have concentrated on AM fungal diversity in various regions and soil types or the mycorrhizal status of indigenous crop and plant species (Bouamri *et al*., 2006; Hawley and Dames, 2004; B*â et al*., 2000; Dalpé *et al*., 2000; Stutz *et al*., 2000; Diop *et al*., 1994). Results from these studies reveal that different species of AM fungi are obtained depending on plant species and geographic location. Amongst AM fungal species, *Glomus* sp. were consistently isolated while others species belonging to the genera *Acaulospora*, *Gigaspora* and *Scutellospora* were either absent or found in few numbers (Bouamri *et al*., 2006; Uhlmann *et al*., 2006; Stutz *et al*., 2000; Dames, 1991).

The occurrence of arbuscular mycorrhizal fungi in South Africa (old name: Endogonaceae) was first reported by Hattingh (1972) when he discovered a honey coloured sessile spore attached to the stalk of an empty mother spore. This was found in large numbers in the rhizosphere soil of maize from the Outeniqua Farm, George, Cape Province and was later designated as *Acaulospora laevis* (Coetzee, 1982; Hattingh, 1972). Since then similar species and others like *Glomus fasciculatum*, *Gl.*

intraradices, *Gl. etunicatum* and *Gigaspora* sp. have been found present in Fouriesberg in the Free State Province, Nylsvley Nature Reserve as well as in association with indigenous plants such as *Vangueria infausta*, *Acacia saligna* and *Acacia cyclops* (Dames, 1991; Hoffman and Mitchell, 1986; Coetzee, 1982). Previously, there was lack of detailed information on the influence of abiotic factors on indigenous AM fungal species. However, Uhlmann *et al*., (2004) carried out a comparative study on species diversity of AM fungi in different seasonal areas of South Africa and Namibia. Results revealed that geographical distance and rainfall to a lesser extent, influenced species diversity. A consideration of seasonal changes was also suggested by Dames (1991) when AM fungal species responded differently to soil fertility factors such as pH, moisture, percentage carbon, phosphorus and cations.

Other studies on AM fungi in South Africa have recently focused on the mycorrhizal status of indigenous plants and trees (Hawley and Dames, 2004; Skinner, 2001; Allsopp and Stock, 1993). Hence, AM fungi have become a subject of interest for many scientists and have led to the realisation that members of this group are the most common soil fungi that can be obtained from any soil type (Koide and Mosse, 2004; Smith and Read, 1997).

1.3.3 Reproduction and life cycle

Spores of AM fungi are unique from other fungal spores and can perform differential functional roles such as mitosis of rich nuclei. Additionally, the presence of rough endo-reticulum and balloon-like golgi equivalents act as storage compartments (María-Laura, 2002). Reproduction in AM fungal spores is thought to be solely asexual, given the fact that there is no evidence to prove that it reproduces sexually (Pawlowska and Taylor, 2004; Smith and Read, 1997). Their reproduction mode is based on the organisation of genetic variation in the rDNA coding genes that exist within spores. The process of homokaryosis, which is the presence of genetically similar nuclei in a cell and heterokaryosis that is the coexistence of genetically different nuclei in cells are the two possible models responsible for how this genetic variation is organised (Hijri and Sanders, 2005; Pawlowska, 2005; Pawlowska and Taylor, 2004). However, the controversy as to which process is actually involved in

reproduction remains unresolved. Studies conducted to analyse these processes using Pol-like sequences and amplification of rDNA of *Gl. etunicatum* nuclei in spores, revealed heterokaryosis to be the process involved in reproduction (Hjiri and Sander, 2005; Pawlowska and Taylor, 2004). Kuhn *et al.*, (2001) observed homokaryosis in *Scutellospora castanea* nuclei using the DNA-DNA fluorescent *in situ* hybridization method. But, it was concluded, that mycorrhizal fungi have evolved to be multi-genomic given that different species exhibited different modes of reproduction (Kuhn *et al.*, 2001). The implication of this multi-genomic existence has been linked to the ability of AM fungi to undergo anastomosis (Giovannetti *et al.*, 2001; Giovannetti *et al.*, 1999), which refers to the form of network where two organisms branch out and are reconnected to form a single organism. This is to say that nuclei migration can occur through the AM fungal hyphal network and as such it will be difficult to determine the genetic composition, phenotype and symbiotic function of the fungus at a fixed point (Sanders, 2002).

Spores of AM fungi under favourable environmental conditions germinate and undergo a sequence of steps that are based on structural morphogenesis that are poorly understood biochemically (Barker *et al.,* 1998). These stages have been categorised into the asymbiotic, presymbiotic and the symbiotic stages (Bago and Bécard, 2002).

In the asymbiotic stage, AM fungal spores are produced in the soil naturally by the extraradical hyphae after symbiotic association with the host plant (Bago and Becard, 2002; Nagahashi, 2000). The asymbiotic stage is sometimes referred to as the resting stage of the AM fungal cycle (Fitter and Garbaye, 1994). These dormant spores (Fig. 1.4) may remain alive in the soil for one or even two years and dormancy periods of spores differ between species and genera (Giovannetti, 2000). For example, spores of *Gigaspora margarita*, when collected from sand dunes, showed no dormancy and were able to germinate after 3-5 days incubation on water agar or on any media without storage preservative (Giovannetti, 2000; Sward, 1981). The differences between genera are characterised by changes in cellular events such as cytoplasm activity and biochemical changes in the fungus metabolism. This results in varying modes of germination, which may be through the spore wall or from a germination shield (Giovannetti, 2000). Factors such as pH, temperature, moisture, CO_2 and

organic nutrients are likely triggers that relieve spore dormancy. This stage is reported to be host independent as AM fungal spores contain energy reserves (stored lipids and carbohydrates) and as such are not only carriers of genetic material (Giovannetti and Sbrana, 1998; Giovannetti, 2000; Bago and Bécard, 2002; Smith and Read, 1997). These energy reserves, which occur in the form of lipid droplets and trehalose, are put into action during spore germination to sustain the initial growth of the germ tube (Smith and Read, 1997). When the presence of a host is delayed, germination ceases rapidly before the energy reserves are depleted or the cytoplasm is retracted within the spore (Redecker, 2005; Bago and Bécard, 2002).

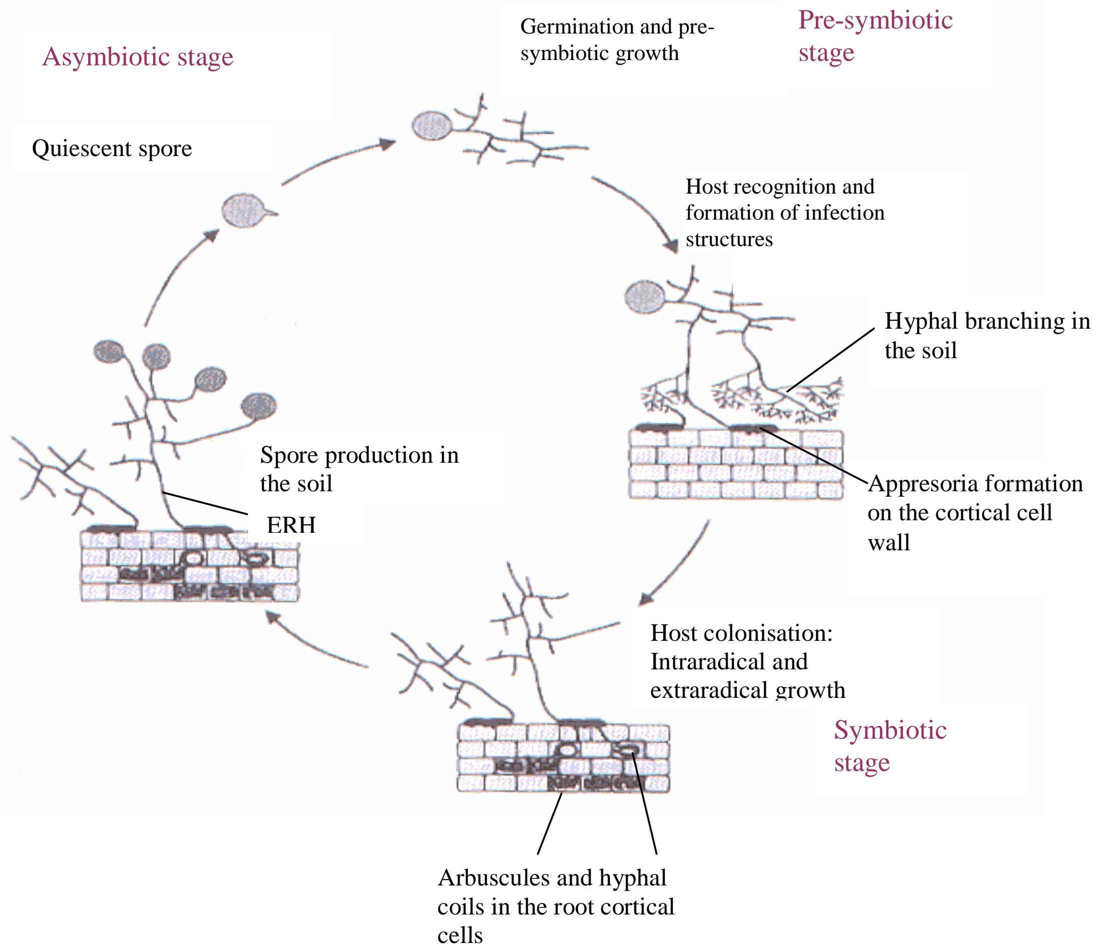


Figure 1.4 Life cycle of arbuscular mycorhizal fungi showing asymbiotic, presymbiotic and symbiotic stages of colonisation (modified from Giovannetti, 2000).

In the presymbiotic stage, germinated spores grow toward the host root by producing hyphal branches. This occurs before the formation of structures such as appressoria (Fig.1.3 and 1.4) that occur on the host root epidermal cell walls (Nagahashi, 2000; Giovanetti, 2000). An appressorium is a term used to describe hyphal tip enlargement that attaches to the root surface of the host (Nagahashi and Douds, 1997). This stage is referred to as presymbiotic because a one-on-one contact between the root and fungus is not required for stimulation of hyphal branches. But rather, the influence of some root exudates such as organic acids, amino acids, carbohydrate monomers, phenolics, or volatiles compounds (Jones *et al.*, 2004).

Plant hormones such as auxins are thought to play a vital role at this developmental stage of mycorrhizal colonisation because auxins are found in high concentrations during appressoria formation (Ludwig-Müller, 2000). Gryndler *et al*., (1998) studied the effects of other plant hormones and compounds like indole-3-acetic acid (IAA), cytokinins, gibberellic acid and Jasmonic acid on *in vitro* proliferation of *Glomus fistulosum* using maize. They observed that IAA had an inhibitory effect on the growth of *Gl. fistulosum* hyphae at a concentration range of 3 -30μM, while the others though they did not directly affect hyphae proliferation were neither signal molecules. Flavonoids are compounds that are found present as root exudates in a variety of plants and as such they differ between plants that are mycorrhizal or non-mycorrhizal (Scervino *et al*., 2005; Bago and Bécard, 2002). Flavonoids such as quercitin, flavanones, hesperetin and narigenin are among root exudates that stimulate pre-contact and hyphal branching (Scervino *et al*., 2005). They are also known to be involved in stimulation of other plant–microbe interactions such as legume-*Rhizobium* symbiosis (Singh and Adholeya, 2002; Giovannetti and Sbrana, 1998). Scervino *et al*., (2005) pointed out that the effect of flavonoids at different developmental stages of AM fungal growth is dependent on the type of flavonoid present and its concentration. Studies investigating the effect of 3 flavonoids, obtained from a mutant plant strain of clover concluded that the flavonoids, RR4 and RR4-2, were found to stimulate the presymbiotic stage of *Gigaspora* species while NM7 (5, 6, 7, 8, 9-hydroxy chalone) showed an inhibitory effect at this developmental stage for all the *Gigaspora* species tested (*G. margarita and G. rosea*).

The symbiotic stage refers to the penetration and development of the IRH and the formation of arbuscles in the cortex of roots (Fig. 1.4). The ERH growth arises after arbuscule formation and is characterised by the release of spores into the soil. Though the AM fungal hyphae are involved in different synthesis and phases, it is at this stage that there is a bidirectional exchange of carbon (C) and nutrients between the fungus and the plant (Saito, 2000; Nagashi, 2000). In the IRH phase, the intraradical hyphae are surrounded by the host plasma membrane and have specificity for hexose (carbon source) uptake that is transported from the plants to the fungus. While in the ERH phase, the hypha develops within the soil substrate and facilitates the uptake of phosphorus (P) and other nutrients. Lipid synthesis carried out in the internal hyphae are metabolised and transported to the ERH phase, where they will be utilised and stored in newly formed spores (Bago *et al*., 2000; Douds *et al*., 2000). These new spores, when mature germinate and use stored C as an energy source to re-initiate the AM fungal life cycle (Fig. 1.4). However, when there is a non-existent carbon metabolism due to the absence of a host for a long period of time, the fungus fails to complete the cycle and enters a sporulation phase where reproduction is carried out asexually pending favourable germination conditions and maturity (Azcón-Aguilar *et al*., 1999; Bago and Bécard, 2002).

Root colonisation brings about the symbiotic interaction; but the benefits of root colonisation are dependent on the survival of the AM fungal propagule particularly, the spores (Xavier and Germida, 2003). There are two types of AM colonisation strategies as described by Gallaud in 1905 based on the structures of the intraradical hyphae and these are referred to as the *Paris*-type and the *Arum*-type (Brundrett, 2004; Brundrett *et al*., 1996). In the *Arum*-type colonisation intercellular hyphae run along longitudinal channels between cortical cells in a linear form before entering the cortical cells to form arbuscules while in the *Paris*-type of colonisation, the intracellular hyphae grow as coils within cortical cells. It has been suggested that these morphological types of AM structures, though they have similar percentage root colonisation, differ in the sites where metabolic activity is carried out. In the *Arum*-type of colonisation, the arbuscules are the main sites for nutrient release while in the *Paris*-type both hyphal coils and arbuscules may be involved but this has not been fully ascertained (Van Aarle *et al.*, 2005). AM fungal species are non-specific in their relations with plants. However, different species can colonise a vast range of both

herbaceous and woody plants but not all of these species have the same effect (Smith and Read, 1997). Specificity, infectivity and effectivity are the three major parameters in determining root colonisation. Specificity refers to the ability of the fungus to colonise root cells of particular plant species, infectivity, the amount of colonisation and effectivity, the plant's response to colonisation (Sylvia *et al.,* 1998).

The presence of AM colonisation is usually undetected by the naked eye because there are no morphological root changes, mycelial mantle or large fungal fruit bodies but when cleared, stained and examined microscopically (Fig.1.3) visible root colonisation is observed (Kendrick, 1992). Detection of AM fungal colonisation in roots is essential for mycorrhizal research. Hence, ranges of methods have been employed to achieve this, such as light microscope based techniques, biochemical methods and molecular techniques. However, the standard technique for visualisation and quantification of root colonisation by AM fungi remains the non-vital staining technique with various stains such as Trypan and Cotton Blue, Chlorazol Black E or an appropriate ink (Vierheilig *et al*., 2005).

1.3.4 Symbiotic benefits

When it was demonstrated that AM fungi increased productivity in AM plants compared to non-mycorrhizal plants, interest in AM fungal symbioses arose in agriculture, forestry, rehabilitation and in environments were managerial practices have altered the soils native state (Friberg, 2001; Cuenca *et al*., 1998; Thompson, 1994a). The major benefits of AM fungi to symbionts includes enhanced nutrient uptake, increased tolerance to root pathogens, drought resistance, tolerance to toxic heavy metals and improved soil aggregation and structure.

Nutrient uptake

Macro and micronutrients are required for plant growth in varying amounts. Micronutrients are required in moderate quantities and could result in toxicity disorders when present in high levels or deficiencies when present in very low levels (Ashman and Puri, 2002). Various levels of micronutrient have been reported to affect the yield of crops such as rice, wheat and legumes (Johnson *et al*., 2005; Dhillon *et*

al., 1983). Heavy metals on the other hand are soil pollutants that are present in quantities greater than 5-6g / cm^3. But at very low concentrations, some of these heavy metals become micronutrients that are essential for plant growth e.g. lead and nickel (Ashman and Puri, 2002). AM fungi are known to enhance mainly the uptake of the macronutrient phosphorus P from the soil, which is then translocated to the host plant through hyphal networks in the soil. Their ability to also take up other micronutrients such as Cu, Zn, Ni, Pb and Fe etc; has been demonstrated by researchers (Table 1.1) using different host plants and soil type management. Furthermore, it has been proposed that AM fungi also have the ability to sequester these nutrients and minimise transfer to the plant roots when nutrients are in high concentrations. However, the mechanism of this ability has not been proved (Turnau *et al*., 1993). Phosphorus is the second essential nutrient after nitrogen (N) required for plant growth and is found in many soils in organic and complex inorganic forms (phytic acid). Due to its low solubility and mobility, plants cannot readily utilise P in an organic or complex inorganic form (Schachtman *et al*., 1998). Inorganic phosphate present in soluble forms in the soil can be readily utilised by plants but usually in limited amounts. Thus, AM fungi intervene to enhance nutrient uptake through the spread of extraradical hyphae into the surrounding soil and hydrolysing any unavailable sources of P with the aid of secreted enzymes such as phosphatase (Carlile *et al*., 2001; Koide and Kabir, 2000; Amaranthus, 1999; Schachtman *et al*., 1998). The enzyme phosphatase, produced by AM fungal extraradical hyphae hydrolyses and releases P from organic P complexes and facilitates the absorption of P and other nutrients thereby creating a depletion zone around the roots (Li *et al*., 1991). These depletion zones limits the rate of phosphorus uptake by non-mycorrhizal plants but gives mycorrhizal plants a greater advantage because of the ability of the AM fungal ERH to extend past this nutrient depletion zone to enhance absorption (Sylvia *et al*., 2001; Liu *et al*., 2000). Sylvia *et al*., (2001) reported that under nutrient deficient conditions the effectiveness of AM fungi is exercised by the ability of the ERH to bridge the nutrient depletion zones of host plant roots. But when nutrients are available to the plant, root length growth is increased and the mycorrhizal dependency of the plant to take up nutrient is reduced.

Table 1.1 Nutrients and elements that were absorbed and transported to plant shoots by AM fungi when present in the soil either naturally, as contaminants or when induced as reported by authors.

Nutrients	References
Macronutrients	
Phosphorus	Bucking and Sachar-Hill, 2005; Giri and Mukerji, 2004; Carlile *et al*., 2001; Koide and Kabir, 2000; Schachtman *et al*., 1998; Smith and Read, 1997; Smith and Gianinazzi- Pearsons, 1990; Kothari *et al*., 1991
Nitrogen	Hawkes, 2003; Giri and Mukerji, 2004; Bothe and Hildebrandt, 2002; Hawkins *et al*., 2000; Frey and Schüepp, 1993; Ames *et al*., 1983.
Magnesium	Giri and Mukerji, 2004.
Micronutrients	
Nickel	Jamal *et al*., 2002.
Zinc	Pawlowska and Charvat, 2004; Jamal *et al*., 2002; Weissenhorn *et al*., 1995 ; Kothari *et al*., 1991
Copper	Gonzalez-Chavez *et al*., 2002; Weissenhorn *et al*., 1995.
Lead	Pawlowska and Charvat, 2004; Weissenhorn *et al*., 1995.
Iron	Caris and Hördt, 1998.
Manganese	Weissenhorn *et al*., 1995.
Heavy metals	
Arsenic	Turnau *et al*., 2001.
Uranium	Rufyikiri *et al*., 2002.
Aluminium	Rufyikiri *et al*., 2000.
Cadium	Pawlowska and Charvat, 2004.

Although P is the main nutrient transported by AM fungi to plants, N is of great importance for plant growth and should not be over-looked (Onguene and Habte, 1995). Nitrogen is obtained by the extraradical hyphae of AM fungi in different forms

ranging from amino acids, peptides, ions (NO_3^- or NH_4^+) to recalcitrant organic nitrogen forms (Hawkins *et al*., 2000; Lipson *et al*., 1999; Tobar *et al*., 1994; Ames *et al*., 1983). It has been recorded that the extraradical hyphae of different *Glomus sp.* can assimilate and metabolise both organic and inorganic sources of nitrogen perhaps by glutamate synthetase activity (Hawkins *et al*., 2000; Johansen *et al*., 1996; Ames *et al*., 1983). It can be stated therefore that the concentration of P and N in the soil can determine the rate of other micro (Fe, Cu, Mn, Zn) and macronutrient (K, Ca) uptake by mycorrhizal plants (Azcón *et al*., 2003). Liu *et al*., (2000) confirmed this in their study which determined the role of AM fungi in the uptake of Cu, Zn, Mn and Fe in maize which showed that the uptake of these nutrients was significantly influenced by soil P nutrition.

Due to the potential of mycorrhizal fungi to enhance nutrient uptake, this benefit has however brought about the suggested use of AM inoculum instead of some chemical fertilisers for plant productivity, growth and restoration of polluted soils or in revegetation (Cardoso and Kuyper, 2006; Khan, 2006; Quilambo, 2003).

Drought tolerance

Along with accessing soil nutrients, the hyphae of AM fungi allows greater access to water through mechanisms such as stomatal regulations, increased root hydraulic conductivity, osmotic adjustments and maintenance of cellular water pressure and cell wall elasticity changes (Augé, 2000; Davies *et al.,* 1993). Recent studies observed that the mycorrhizal infection of maize with *Gl. mosseae* and *Gl. intraradices* helped the plant to maintain higher leaf water potential compared to non-mycorrhizal plants (Amerian and Stewart, 2001). The ability of AM fungi to effectively alleviate drought stress has been studied in terms of nutrient uptake of N and P, photosynthesis and cytokinins (Goicoechea *et al*., 1997: Tobar *et al*., 1994; Busse and Ellis, 1984; Allen *et al*., 1981). However, due to the possible interference of drought in the mobility of NO_3^- to the root surface, the role of nitrogen uptake by AM fungi (*Gl. fasciculatum)* under such conditions was tested using a radio labeled nitrogen (^{15}N). Results showed that under optimal water supply the amount of ^{15}N was the same in both mycorrhizal inoculated and non-mycorrhizal inoculated plants, but four times higher in the mycorrhizal inoculated plants under water stressed conditions (Tobar *et al*., 1994). Allen *et al*., (1981) observed that colonisation of *Bouteloua gracilis* by *Gl.*

fasciculatum enhanced water translocation, nutrient uptake and rate of photosynthesis. Goicoechea *et al*., (1997) in studies with *Gl. fasciculatum* and *Rhizobium*, investigating relationships between nutrient content and water in alfalfa observed that plants inoculated with AM fungi had the highest leaf nutrient maintenance under drought stress. Other recent studies have shown the capability of AM fungi to influence plant growth, crop quality and adaptability to stress conditions (Mena-Violante *et al*., 2006; Fagbola *et al*., 2001).

Plant pathogens

AM fungal colonisation of plant roots has been suggested to increase plants tolerance to pathogens thereby acting as a biocontrol agent (Azcón-Aguilar and Barea, 1996; Chhabra *et* al., 1992). A biocontrol agent is defined as the use of a biologically friendly resource from the ecosystem that can target and protect plants against pathogens (Azcón-Aguilar *et al*., 2002; Azcón-Aguilar and Barea, 1996). Several mechanisms or combination of mechanisms could account for the observed bio protection of plants by AM fungi. Some of these pathogens can be root-infecting fungi that are antagonistic and capable of feeding on their host as necrotrophs, wilt pathogens such as *Fusarium oxysporum*, or root rotting pathogens like *Phytophthora* and *Rhizoctonia* that are common soil borne pathogens (Smith, 1988).

Primarily, the ability of AM fungi to enhance plant vigour due to increased nutrient uptake enables it to resist pathogen infection. It was proposed by Smith (1988), that the interaction of AM fungi with soil root pathogens has everything to do with the enhanced nutritional uptake of P and other nutrients. And through this action, the fungus increases the plant's tolerance to pathogens through mechanisms such as alteration of root exudates, increased root growth and function and competition for space or infection sites. Chhabra *et al*., (1992), reported that increased nutritional status of plants with AM fungi might increase tolerance to root pathogens. But no effect on the development of leaf diseases in maize caused by *Helminthosporium maydis* and *Acremonium kiliense* was observed. Besides, AM fungi were found to increase *Zea mays* tolerance to leaf rust with control plants having 80% leaf rust as compared to AM inoculated plants, which had less than 5% leaf rust (Dames, 2006: personal communication).

AM fungi have direct access to plant photosynthetic product while pathogens, which are not obligate biotrophs can only obtain C from decomposing organic sources. This automatically gives AM fungi a growth advantage over pathogens like *Fusarium* that must access organic sources for carbon on their own. However, it is not yet confirmed if competition for carbon and other nutrients induces pathogen resistance (Linderman, 1994). Similarly, competition for colonisation sites within the roots has been suggested to occur, as some pathogenic fungal infections colonise similar plant tissues (Smith, 1988). For example, *Fusarium* infects the vascular tissues of plants, but requires passage of the hyphae through the root cortical cells. If root cortical cells are colonised by AM fungi this will limit the entry of the *Fusarium* pathogen (Agrios, 1997). However, this is a proposed localised mechanism by which AM fungi exerts biocontrol activity (Azcón-Aguilar and Barea, 1996). In addition, microbial changes in the mycorrhizosphere and anatomical changes in the root induced by AM formation may bring about stimulation of specific functional groups in the microbiota that are antagonistic towards pathogens (Azcón-Aguilar *et al*., 2002; Sylvia *et al.,* 1998; Azcón Aguilar and Barea, 1996; Linderman, 1994). However, these mechanisms are said not to be effective for all pathogens and are influenced by soil and environmental conditions (Azcón-Aguilar and Barea, 1996). A study on the biocontrol potential of AM fungi on *Fusarium* using different cultivars of maize proved to increase the plant's tolerance to the pathogen when used as an inoculant (Mukasa-Mugerwa, 2005).

The actual mechanism by which AM fungi confers localised or induced systemic protection against pathogens to plants remains unidentified (Dumas-Gaudot *et al*., 2000). Though there are indications that this mechanism is signalled by modulations such as lignifications, induction of cell wall appositions containing callose, accumulation of pathogenic related proteins or phenolic compounds (Pozo *et al*., 2002a; Dumas-Gaudot *et al*., 2000). Lignification caused by AM fungal colonisation involves the thickening of the exodermis and cortical root cell walls which makes penetration of pathogenic hyphae difficult (Cordier *et al*., 1996). As such, pathogens that target plants through this way will likely not penetrate and infect the plant root due to anatomical changes in root structure (Dumas-Gaudot *et al*., 2000). Similarly the accumulation of phenols in response to AM fungal colonisation has been reported to cause both localised and systemic induced resistance to pathogens. A study by Zhu

and Yao (2004) confirmed this when they examined the ability of *Gl. versiforme* to inhibit *Ralstonia solanacearum* when inoculated together in tomato roots. It was observed that *Gl. versiforme* increased the soluble phenol contents in the tomato roots thereby decreasing the population of *Ralstonia solanacearum* in the rhizosphere and in the xylem tissues of the plant. Pozo *et al*., (2002b) also used tomato plants and demonstrated similar effects using the pathogen *Phytophthora parasitca* and two species of AM fungi (*Gl. mosseae* and *Gl. intraradices*). They observed that *Gl. mosseae* had the ability to reduce infection of *P. parasitica* in tomato roots by inducing the mycorrhizal related hydrolytic enzymes such as chitosanases and ß-1, 3 glucanase that have lytic activity against *Phytophthora* cell walls.

Soil aggregation

Soil structure is improved by AM fungi through the secretion of a glue-like, proteinaceous, water-soluble and heat stable substance from their hyphae called glomalin (Steinberg and Rillig, 2003). This compound aids in soil aggregation by binding soil particles together thereby influencing soil porosity, which promotes aeration and water movement, essential for good root growth, root development and microbial activity (Amaranthus, 1999). Glomalin, a recalcitrant, iron-containing glycoprotein is indeed responsive to ecosystem fluctuations such as elevated atmospheric CO_2 concentrations, global warming and agricultural practices. Due to the positive correlation observed between glomalin, land-use and soil carbon-nitrogen ratio, this glycoprotein can be used to assess changes in soil C in various land-use types (Rillig *et al*., 2003). Hence, glomalin can be regarded as an indicator for soil aggregation and stability. Glomalin is easily assayed and cannot be produced from uncolonised plant roots as it is AM fungal specific. Therefore, it can be used to determine AM hyphal growth and activity in the soil (Lovelock *et al*., 2004a; Rillig *et al*., 2001; Wright and Upadhyaya, 1998).

Toxic metals

The toxicity of metals lies in the concentrations in which they are present in the soil (Smith and Read, 1997). These metals can arise from a variety of sources in the form of acid rain, dust containing these metals, wash waters from polluted soils or from atmospheric factors produced as a result of mining, smelting, burning of fossil fuels,

industrial or agricultural activities and incineration of municipal waste (Gaur and Adholeya, 2004). Heavy metals have been said to affect some developmental stages of AM fungi or eliminate their establishments (Gildon and Tinker, 1983). However, it is reported that the level at which heavy metals such as Zn, Cd, Al, Cu and Pb affects plants and mycorrhizal fungi varies and is dependent on their actual concentration, oxidation state in the soil, soil pH, organic matter content, cation exchange capacity and redox potential (Entry *et al*., 2002). Pawlowska and Charvat, (2004) investigated the *in vitro* effect of Cd, Pb and Zn, on critical life stages of two AM fungi, *Gl. etunicatum* and *Gl. intraradices*. They showed that these two species differ in metal sensitivity, but generally, were able to survive metal stress. Thus, isolation of AM fungi from metal contaminated soils has proven their potential ability to thrive on such soils (Griffioen *et al*., 1994; Weissenhorn *et al*., 1995; Ietswaart *et al*., 1992). Additionally, AM fungi alleviate plant stunting caused by toxic metals by binding to these metals in the root zone with the aid of the extraradical mycelium and altering the plant cells ability to capture the metals. The polyphosphates produced by AM fungi are proposed to be the reason behind this sequestration though this has not been confirmed (Smith and Read, 1997; Turnau *et al*., 1993). Khan (2003) reported the potential use of AM fungi in detoxification of environments polluted with heavy metals and in phytoremediation. It is suggested, that although AM fungi are mycobionts that could be exploited in such processes, that the selection of AM fungal species with appropriate phytobionts needs to be considered (Entry *et al*., 2002).

1.4 Factors affecting growth of arbuscular mycorrhizal fungi

Due to the numerous benefits of AM symbioses with plants, the production and use of AM inoculants as a bio-fertiliser has encouraged a great deal of research and commercial interest in these areas (Safir, 1994). It is believed that the potential of AM fungal functioning in plant growth and yield is not maximised when naturally occurring particularly under intensive soil management, therefore the increasing demand to produce an inoculum (Safir, 1994; Fitter, 1985). The production of AM fungal inoculum has been difficult because of the inability of the fungus to grow in axenic cultures in the absence of a plant root (Brundrett *et al*., 1996). But, attempts have been made to commercially produce inoculum through nutrient film techniques,

pot cultures and tissue cultures, which require further investigation (Sylvia and Jarstfer, 1994). Different agricultural and management practices, such as fallowing, affect AM fungi in their native state, which in turn affects the establishment and improvement of AM fungal inoculum for sustainable crop production. Such practices remove potential host roots where the fungus derives its energy during mild autumn and spring weather, thereby decreasing root colonisation of the subsequent host crop (Thompson, 1994a). Crop rotation practices are also to be considered in that the colonisation potential of AM fungi in the soil will depend on the previous crops. If previous crops are non-mycorrhizal for example canola, or produce non- mycorrhizal toxic compounds as a result of root structure and physiology, this can lead to a reduction in AM fungal infective propagule density (Kling and Jakobsen, 1998; Ocampo *et al*., 1980).

Soil disturbances such as tillage and harrowing are also known to have an effect on AM fungal propagules and the extraradical hyphae. These methods involve stirring, leveling, or breaking of soil clumps in preparation for growing plants. Because AM fungal propagules, such as spores and active hyphae, are predominantly found in the topsoil, this activity hinders the ability of these propagules to germinate and colonise new host roots, which in turn affects the production and transport of nutrients to the plant at an early developmental stage (Kabir, 2005; Kling and Jakobsen, 1998). Studies by Jansa *et al*., (2002) showed that there is indeed a deleterious effect of tillage on the population and diversity of AM fungi compared to non-till soils. Therefore, the reduction in intense tillage will favour AM fungal management in soils (Thompson, 1994a) thereby enhancing colonisation potential of plants and subsequent environmental benefits.

The fate of organic or inorganic phosphate fertilisers when applied to soil is reported to be determined by biogeochemical processes. These include immobilisation, solubility and adsorption (Compton and Cole, 2001) that may be dependent on the soil pH and soil type (Rodriguez and Fraga, 1999). Immobilisation is the conversion of inorganic phosphates that are available to plants into an unavailable organic form by biochemical or microbial processes, while the reverse is termed mineralisation. P fertilisers are mainly applied to increase P levels when deficient in soils or to maximise plant growth (Xu *et al*., 2000). Fertilisers when applied, undergo

precipitation-dissociation reactions, which are then solubilised into the soil mineral solutions. These solutions bind to the soil particles which become readily available and absorbed by plant tissues. If not present in soil as solutions, it can be in the active or fixed pool P (Busman *et al*., 2002). The active P in soil is usually in the solid phase and can easily be released into soil solution. It replenishes P in the solution pool based on crop utilisation, while the fixed pool contains organic and inorganic forms of P that are insoluble and resistant to mineralisation (Busman *et al*., 2002).

Owing to the high influence of host P demand on AM fungi, application of fertilisers has a great impact on the plant-fungus relationship (Gosling *et al*., 2006). The effect of fertilisers on AM fungi has been well studied using pot trials (Xu *et al*., 2000, Braunberger *et al*., 1991). These studies have shown that the increasing use of P fertiliser led to the high P pool in soils. These high P levels affect AM fungi root colonisation and minimises growth performance by decreasing the nutrient acquisition role of AM fungi (Azcón *et al*., 2003). Braunberger *et al*., (1991) studied quantitatively the morphology of AM fungal colonisation of maize in response to variable P fertilisation. They observed that increased P fertilisation reduced the fraction root length containing arbuscules, which was due to inhibition of intraradical hyphae development. Similar results were obtained by Martensson and Calgren (1994) in a field experiment, who observed a 50% decrease over five years in spore numbers of AM fungi even when P fertilisers were applied in moderate amounts of 45kg $ha^{-1}year^{-1}$. However, when P fertilisation was excluded, spore density doubled within 5-14years and was three times the amount after 28 years of experimental establishment. A meta-analysis study which involves statistical analysis of fertiliser effects from various studies was conducted by Treseder (2004). Their results on the effect of P fertilisers such as superphoshate ($Ca(H_2PO_4)_2$) and N fertilisers (containing $NaNO_3$, NH_4NO_3 and NH_4NO_3 mixed urea) on AM fungi, reported a reduction in mycorrhizal abundance (percentage colonisation, spore counts, hyphal length) by an average of 32% and 15% respectively (Treseder, 2004). This percentage response varied with the initial soil nutrients present resulting in inconsistencies of N and P fertiliser effect. Conversely, it has been reported that some species of AM fungi such as *G. magarita* and *Scutellospora calospora* are able to survive high or low P levels (Podeszfinski *et al*., 2002). This could mean that fertilisation can lead to selective AM

species that may be of little benefit to the host in terms of effective nutrient uptake (Gosling *et al*., 2006; Kurle and Pfleger, 1994).

Organic sources of fertilisers such as farmyard manure, compost and animal feaces has been reported to have no negative effect on AM fungal colonisation (Kabir *et al*., 1998; Kurle and Pfleger, 1994). However, this fact is not entirely true as the various organic fertilisers differ in composition and are unpredictable when applied on any given soil (Douds *et al*., 1997). Studies by Douds *et al*., (1997) observed an increase in spore population of two AM fungal species (*Gl. etunicatum*, *Gl. mosseae*) using chicken or litter compost when applied alone. While Harinikumar and Bagyaraj (1989) observed that the co application of farm yard manure with varying levels of N, P or K fertilisers reduced AM fungal propagules. These varying results led to the conclusion that the use of organic manure solely or together with inorganic fertilisers is dependent on manure source, addition rate and perhaps the rate of decomposition of fertilisers (Kurle and Pfleger, 1994).

Application of pesticides such as carbendazim that is a substituted aromatic hydrocarbon, fungicides and herbicides influence the enzymatic activity in the soil (Fontanet *et al*., 1998). Test methods to access pesticide effects on AM species are contradictory and are time-consuming (Kling and Jakobsen, 1998). The predominant method for assessing the impact of these biocides on AM fungi has been through the use of inoculated and control treated plants which are grown for about 6-20 weeks, harvested and then analysed. Generally, the effect of pesticides on root colonisation and rhizosphere activity are said to vary among AM fungal species particularly the *Glomus* sp. (Fontanet *et al*., 1998). The use of this non-standardised method has led to the varying results obtained by researchers, although the inability to culture AM fungi is a good reason for following this path (Abd-All *et al*., 2000; Fontanet *et al*., 1998; Pattison *et al*., 1997; Schreiner and Bethlenfalvay, 1997). Wan *et al*., (1998) has proposed a standard bioassay method to determine the sub-lethal toxicity of pesticides to *Gl. intraradices* using root induced transferred DNA (Ri T-DNA) – transformed carrot roots. However, this technique may not always be available and optimal as only a limited number of AM fungal species can be grown in this dual culture system (Bago and Bécard, 2002).

Fungicides can be divided into systemic and non-systemic or contact types. Systemic fungicides are those absorbed by the plant root or leaf tissues and transported through the vascular system. While non-systemic are those that have direct contact with disease-causing organisms and deposit residues on plant tissues e.g captan, copper sulphate, daconil, copper oxychloride (Parvathi *et al*., 1984). Most systemic fungicides such as metalaxyl, fosetyl-Al and propamocarb are of interest due to their continual activity within the plant (enzyme activity), their effect on organisms competing with AM fungi for colonisation sites (disrupts fungal cell division) or their direct effect on AM fungi function (Fontanet *et al*., 1998; Kurle and Pfleger, 1994). Fontanet *et al*., (1998) observed that *Glomus intraradices* was not affected by metalaxyl and propamocarb. However, metalaxyl affected root colonisation of peach-almond rootstock by *Gl. mosseae* and decreased rhizosphere activity as determined by esterase activity. Other fungicides such as benomyl, pentachloronitrobenzene, terrazole and captan were observed to have affected the initial growth of AM spores, though the effect varied with different AM species, which was as a result of factors such as soil texture, composition of biocides used and method of application (Schreiner and Bethlenfalvay, 1997, Pattinson *et al*., 1997; Sukarno *et al*., 1993). Hence, it is still difficult to generalise on the effect of these compounds due to the varying formulations of compounds, mode of action and their effect on host physiology (Kurle and Pfleger, 1994).

Herbicides, insecticides and nematicides (Parvathi *et al*., 1984) are not overlooked. Even though they do not directly target AM fungi they could bring about changes in the host physiology or interfere with mycorrhizal physiological processes (Kurle and Pfleger, 1994). It was observed that paraquat, dichlobenil and simazine herbicides did not affect root colonisation of *Gl. versiforme*, but simazine affected hyphal elongation when an elongation test was carried out using *Gl. intraradices* (Hamel *et al*., 1994). Glyphosate and chlorsulfuron herbicides also had no effect on *Gl. mosseae* but when herbicides were applied in high doses, the beneficial potential of the fungus (i.e. plant biomass and nutrient acquisition) declined (Mujica *et al*., 1999). The majority of insecticides and nematicides such as fenamiphos, dichloropropene, carbofuran and aldrin do not have any deleterious effect on AM fungi when applied in low concentrations. These were found to either increase or decrease AM fungal colonisation but not hinder spore formation (Pattison *et al*., 1997; Trappe *et al*., 1984).

1.5 Interaction of arbuscular mycorrhizal fungi with other soil microorganisms

1.5.1 Interaction with bacteria

In addition to enhancing nutrient absorption capability of their host plant, the hyphae of AM fungi provide an area for the interaction of plants with other soil microorganisms that have an effect on root development and performance (Johanssson *et al.,* 2004; Ulrike, 2003). This interaction can be positive, neutral, or negative (Sylvia *et al.,* 1998). Mycorrhizal formation can directly or indirectly affect microbial communities in the rhizosphere through induced changes of root exudates, transport of energy rich carbon compounds to the mycorrhizosphere or fungal exudation of stimulatory or inhibitory compounds. This effect referred to as the mycorrhizosphere effect (Johanssson *et al.,* 2004; Suresh and Bagyaraj, 2002; Vázquez *et al.,* 2000). The term mycorrhizosphere is referred to as the zone of mycorrhizal colonisation in and outside the plant root (Andrade *et al*., 1997). In general mycorrhizal fungi, through modifications to the plant root system, interact with beneficial soil organisms such as N_2-fixing bacteria, P solubilising bacteria, fungi and root inhabiting nematodes. These interactions are important in the natural ecosystem for nutrient cycling (Gryndler, 2000; Fitter and Garbaye, 1994). Some bacteria are known to facilitate mycorrhizal formation by affecting spore germination or root colonisation (Fitter and Garbaye, 1994). An *in vitro* experiment with *Klebsiella pneumoniae* grown in compartments next to those containing *Gl. deserticola* showed no effect of the bacteria on number of spores germinated but 9 days later, there was an increased AM fungal hyphal extension away from germinated spores (Will and Sylvia, 1990). This indicated the potential of *Klebsiella pneumoniae* to influence hyphal extension through the production of volatile compounds such as long chain alcohols- 1decanol or 1dodecanol (Elgaali *et al*., 2002).

The interaction of AM fungi with *Rhizobium* has received considerable attention due to the high P demand for N_2 fixation. Studies have shown that the co-inoculation of legumes with rhizobia to fix nitrogen and AM fungi increased plant growth than when inoculated with rhizobia alone. This was attributed to the fact that under limiting conditions of N and P, AM fungi improves P uptake thereby enhancing the plants nitrogenase activity, which in turn promotes root and mycorrhizal development (Abd-

Alla *et al.,* 2000; Sylvia *et al.,* 1998; Fitter and Garbaye, 1994). Thus the symbiotic role of *Rhizobium* is said to be dependent on the beneficial nutrient effect of AM fungi. Apart from P, enhanced uptake of other nutrients such as Zn, Cu and Ca by AM fungi can influence the symbiotic effectiveness of *Rhizobium* as well as other microbial processes that occurs at root or nodule level (Barea *et al*., 2002a; Azcón-Aguilar and Barea, 1992). Though *Rhizobium* is well known inoculants for legumes, they have also been used as inoculants for non-leguminous plants (Chabot *et al*., 1996). Galal *et al*., (2003) studied the effect of P and N fertilisation on the growth and yield of wheat inoculated with AM fungi and *Rhizobium* using radiolabelled ^{15}N technique. They observed an increase in growth of wheat when both *Rhizobium* and AM fungi were inoculated together at high levels of N and P. This dual inoculation also facilitated the uptake of N and P; while the single inoculation of plants with AM fungi increased yield of wheat grain. This indicated the ability of both organisms to stimulate plant growth and accumulate P and N (Requena *et al*., 1997). Species of *Azobacter, Azospirillum, Derxia* and *Clostridium* are well known free-living diazotrophs that fix atmospheric nitrogen (Linderman, 1992). A synergistic effect was observed between *Gl. fasciculatum* and *Azotobacter chroococcum* in tomato plants. The latter helped to enhance fungal colonisation and spore production, while the former increased the bacterial population in the rhizosphere (Bagyaraj, 1984). Biró *et al*., (2000) observed an increase in nodulation of alfalfa plants with co-inoculations of *Gl. fasciculatum*, *Azospirillum* and *Rhizobium* under sterile and normal soil conditions.

Phosphate solubilising bacteria (PSB) have great prospects to improve plant growth under given conditions such as in P deficient soils when used in conjunction with AM fungi (Gryndler, 2000). They are known to mobilise phosphate ions from sparingly soluble organic and inorganic P sources. However, the released P does not reach the root surface as a result of inadequate diffusion (Barea *et al*., 2005; Azcón-Aguilar and Barea, 1992). It was proposed that AM fungi could improve the uptake of the solubilised P; hence, this combined interaction should improve P nutrition and supply to plants (Barea *et al*., 2002a). The interactive effects of AM fungi and PSB on plant use of soil P in the form of either endogenous or added rock P was studied using a soil microcosm system integrated with ^{32}P isotopic dilution. Results revealed that the PSB (*Enterobacter sp*. and *Bacillus subtilis*) promoted mycorrhizal establishment of *Gl.*

intraradices and their combined inoculation increased biomass, N and P accumulation in the onion plant tissues (Toro *et al*., 1997). Thus, the inoculation of organisms may result in utilisation of P fertilisers that quickly become unavailable in soils (Picini and Azcon, 1987). Multi-microbial interactions between AM fungi, PSB and *Azospirillum* have been reported to be synergistic when inoculated together (Muthukumar *et al*., 2001; Belimov *et al*., 1995). Muthukumar *et al*., (2001) confirmed this by inoculating the Neem tree seedlings with *Gl. intraradices*, *Gl. geosporum*, *Azospirillum brasilense* and isolated PSB individually or in various combinations under nursery conditions. Mycorrhizal colonisation, leaf area and number, plant height and biomass, nutrient content (N, P and K) and seedling quality were found to be significantly increased because of combined microbial inoculants.

Some soil bacteria isolated from the rhizosphere possess the ability to produce compounds such as antibiotics or siderophores which are Fe chelators that may act as inhibitors against pathogens or stimulate plant growth. These are referred to as plant growth promoting rhizobacteria (PGPR) and are mainly *Pseudomonas* strains that produce non-volatile diffusible compounds such as methane, acetaldehyde, acetoin and diacetyl that may or may not reduce mycorrhizal volume (Aspray *et al*., 2006; Gryndler, 2000; Linderman, 1992). Results by Vázquez *et al*., (2000) demonstrated that the incorporation of a fungus, *Trichoderma harzianum* with *Pseudomonas fluorescens*, *Azospirillum* spp and AM fungal species, *Gl. mosseae* and *Gl. deserticola* did not affect the establishment of AM fungal species in maize. However, an increase in phosphatase, esterase, trehalase and chitinase enzymatic activity was observed. These soil enzymes are mainly used as an indicator to detect microbial functioning in the rhizosphere as influenced by AM fungi and differ in their activity. Phosphatase which is produced by bacteria and AM fungi catalyses organic bound P into inorganic P (Häussling and Marschner, 1989). Esterase indicates catabolic activity in the soil which is directly correlated to microbial acivity (Vázquez *et al*., 2000). The enzyme trehalase hydrolyses trehalose a common sugar found in plant symbioses, while chitinase degrades chitin, a major component of fungal cell walls that plays a role in plant defence mechanisms (Pozo *et al*., 2002a; Vázquez *et al*., 2000). Similarly, the dual inoculation of subterranean clover and maize with *Pseudomonas putida* and different species of AM fungi were found to enhance plant growth and AM fungal colonisation (Gryndler and Vosátka, 1996; Paulitz and Lindermam, 1989, Meyer and

Linderman, 1986a). It was suggested that before commercial bacterial inoculants are considered, resultant changes in the mycorrhizosphere should be studied. Walley and Germida (1997) proposed this when they observed that the interaction of five pseudomonads selected as PGPR under laboratory conditions affected plant growth and AM fungi root colonisation when tested in the field. This effect was found to vary (positive or negative) based on the bacterial inoculant strain, harvest date and growth parameter. In addition, Ravnskov *et al*., (1999), observed under controlled conditions that the fungus *Gl. intraradices* had a negative effect on the growth and survival of *Pseudomonas fluorescens* DF57 which was likely to be due to competition for nutrients.

PGPR exert direct or indirect effects on plant growth and belong mainly to the genera *Paenibacillus*, *Burkholderia*, *Pseudomonas* and *Bacillus* sp. The direct effects are through the release of phytohormones, nitrogen fixation and mineralisation of organic phosphates into available forms for plants. While the indirect effect on plant growth is realised by decreasing or preventing deleterious effects of pathogenic organisms mainly through the synthesis of antibiotics or production of siderophores. Solubilisation of P is reported to be the most common mode of action for PGPR and studies by Singh and Kapoor (1998) showed that PSB such as *Bacillus circulans* together with AM fungi increased plant yield and P uptake of wheat. There are some inconsistencies in reports of the effects of PGPR on AM fungi as well as in their mode of action. *Bacillus substilis* and *Enterobacter* sp. were found to promote the establishment of *Gl. intraradices*, increase plant biomass and N and P contents of onion (Toro *et al*., (1997). While studies by Walley and Germida (1997) using different *Pseudomonas* strains with the co-inoculation of AM fungi observed varying effects i.e some strains of *Pseudomonas* hindered AM fungal germination. Hence it can be argued that not all PGPR are mycorrhizal helper bacteria (MHB) or vice versa. MHB are organisms that specifically promote mycorrhiza formation especially ectomycorrhizal fungi by producing growth metabolites that encourages easy proliferation of the fungal hyphae, thereby increasing the chances of the fungal hyphae to colonise plant roots with a large surface area (Schrey *et al*., 2005; Garbaye, 1994). When PGPR are found to stimulate mycorrhizal formation they can be regarded as MHB (Fitter and Garbaye, 1994), this interchangeable characteristic brings about the overlap that exists between the two groups. Similarly, not all P

solubilising PGPR promote plant growth by P availability to the host. Studies by De Freitas *et al*., (1997) revealed that a number of *Bacillus* strains and *Xanthomonas maltophilia* isolated from the rhizosphere of canola a non-mycorrhizal plant had positive effects on plant growth but not on P content of the host plant. This indicated that P solubilisation was not responsible for the plant growth response.

Generally, the microbes in the mycorrhizosphere affect mycorrhizal functioning and thus, some bacteria may interact with the mycorrhizal fungi on more than one metabolic level. For example, P solubilisers having additional functions (Sylvia *et al*., 1998; Linderman, 1988). It has been reported that some organisms especially those belonging to the genera *Bacillus* can be multifunctional. This means that they are able to perform functional roles such as being N_2 fixers, P solubilisers or grouped as PGPR or MHB (Rodriguez and Fraga, 1999). For example, Pandey *et al*., (2005) isolated an organism coded as MSSP from the root nodules of *Mimosa pudica*. This organism was found to belong to the *Burkholderia* genus and had the ability to fix N, solubilise P and had all the characteristics of PGPR. Similarly, studies and have also reported species of the genera *Bradyrhyzobium*, *Sinorhizobium*, *Rhizobium and Azorhizobium* as PGPR and phosphate solubilisers (Vessey, 2003; Rodriguez and Fraga, 1999; Antoun *et al*., 1998).

Few studies have been carried out on the interaction between AM fungi and Actinomycetes. Research conducted using both organisms to determine their effect on plant growth showed their individual enhancement, whereas dual inoculation of organisms adversely affected plant growth and exhibited antagonistic interaction towards each other. Actinomycetes was said to be responsible for the suppression of AM fungi due to its antagonism and inhibitory effect in the rhizosphere (Bagyaraj, 1984). The production of inhibitory compounds by actinomycetes could be seen as the organism's way of competing with others organisms for nutrients. However, other species of *Actinomyces* belonging to the genus *Frankia* were able to form a synergistic relationship with AM fungi when inoculated together in actinorrhizal plants such as tibetan seabuckthorn (*Hippophae tibetana*) and *Discaria trinervis* (Tian *et al*., 2002; Gryndler, 2000; Wall, 2000). *Streptomyces* is a common soil organism belonging to the actinomycetes. Their effect on AM fungi varies according to species. For example, the colonisation of finger millet roots by *Gl. fasciculatum* was shown to

be inhibited by *Streptomyces cinnamomeous* (Krishna *et al*., 1982) while *Streptomyces orientalis* produced volatile compounds that stimulated germination of the resting spores of *Gl. mosseae*, *G. margarita* and *Scutellospora heterogama* when cultured auxenically (Tylka *et al*., 1991).

Hence, the interactions of AM fungi with soil bacteria can either stimulate or inhibit each other's processes in the rhizosphere.

1.5.2 Endosymbiotic bacteria of AM fungi

Mycorrhizal fungi are the best-known examples of fungal and bacterial interactions as the hyphae offer good ecological niches for other microbes. AM fungal spores harbour Bacteria Like Organisms (BLOs) also referred to as endosymbionts in their cytoplasm and these organisms complete their life cycle within the eukaryotic cells giving rise to a further level of symbiosis (Johannson *et al.,* 2004; Minerdi *et al.,* 2002). To demonstrate this symbiosis, a combination of morphological and molecular techniques were conducted and it was concluded that the AM fungal spores of *G. margarita, Gl. versiforme* and *A. laevis* spores harboured these BLOs in their cytoplasm (Bianciotto *et al.* 1996b; Minerdi *et al.,* 2002). Analysis of the bacterial 16S rRNA gene sequence obtained from the extraction of spore DNA of *G. margarita* inferred that these bacteria are related to the genus *Burkholderia* (Minerdi *et al.,* 2002; Bianciotto *et al.,* 1996b). Investigation of two geographically separated isolates of *Gigaspora margarita* and four other isolates, *G. gigantea*, *G. rosea*, *G. margarita/rosea* and *Scutellospora persica*, showed that four out of the five species had endosymbionts, the exception being *G. rosea*. This demonstrates that BLOs are common features in the *Gigaspora* and can possibly be used as a genetic marker for members of this genus (Minerdi *et al.,* 2002, Lanfranco *et al*., 2001). Bianciotto *et al*., (2003) further analysed the morphological and molecular similarities between the endosymbionts found in *G. magarita*, *S. persica and S. castenea*. It was observed through the amplification and sequence of partially complete 16S rRNA that all endosymbionts obtained from the three AM fungal species were over 98% similar to each other. This genomic similarity in their ribosomal sequence led to their being referred to as 'Candidatus Glomeribacter gigasporarum'.

Endosymbionts in the spores of AM fungi are unculturable in cell free media and as such are determined morphologically using electron and confocal microscopy (Bianciotto and Bonafante, 2002). The most common species found is *Bulkholderia cepacia*. The complexity of this group of organisms is yet to be elucidated since some species in this family are found free living in the soil and differ phylogenetically from the AM fungal symbionts (Bianciotto *et al*., 1996a).

The ecological importance of BLO's with mycorrhiza has been questioned. Studies revealed that these endosymbionts possess *nif*HDK genes that are found in the operon of *G. margarita* spores. These genes are expressed during spore germination and because they are harboured in AM fungal spores they thought to give AM fungi the potential to fix nitrogen during the stage of the bacterial life cycle (Bianciotto and Bonafante, 2002; Minerdi *et al*., 2001; Bianciotto *et al*., 2000). Therefore, the significance of these findings lie in the interest of such a combination for a sustainable agriculture targeted to increase crop production with minimal use of chemical fertilisers (Minerdi *et al*., 2002; Minerdi *et al*., 2001).

1.5.3 Interaction with fungi

Saprotrophic fungi live on dead organic material and are common in the rhizosphere. The advantage AM fungi have over saprotrophs lie in their direct association with plants as well as the ability to utilise stored carbon-related products in their hyphae in the absence of plant photosynthates (Suresh and Bagyaraj, 2002). Saprotrophic fungi are most frequently studied for their antagonism towards AM fungi and can be classified into ecological functional groups such as P solubilisers, antagonists or synergistic organisms (Gryndler, 2000). As anatagonists, they may affect the germination of AM fungal spores and development of mycorrhizal colonisation by their competition with AM fungi for space or nutrients (Gryndler, 2000). McAllister *et al*., (1994) tested the effect of two saprophytes, *Fusarium solani* and *Trichoderma koningii*, on the growth and mycorrhiza formation in maize and lettuce. They discovered that mycorrhizal root colonisation by *Gl. mosseae* was decreased in maize when inoculated before or at the same time with *T. koningii*; while *F. solani* had no

effect on colonisation of maize. Conversely, *T. koningii* did not affect mycorrhizal colonisation when tested on lettuce.

Gliocladium virens is a saprotrophic organism that is used as a biocontrol agent. This organism produces metabolites such as the antibiotics, gliotoxin and enzymes such as endochitanase that weakening the cell wall of the pathogenic organisms. This facilitates the entry of gliotoxin that damages the cell wall of pathogenic fungi (Brimner and Boland, 2003; Di pietro *et al*., 1993). All fungi have cell walls composed of chitin which would mean that gliotoxins and endochitinase produced by *Gliocladium virens* would affect AM colonisation. To determine this effect, Paulitz and Linderman (1991) examined the effect of *Gliocladium virens* on the pathogen *Pythium ultimum* and the colonisation of AM fungus *Gl. etunicatum* in cucumber plants. They observed that *Gliocladium virens* had no deleterious effect on AM fungi as it allowed the cucumber roots to be colonised while having a biocontrol activity on *Pythium ultimum.* This indicates the synergistic interaction between *Gliocladium virens* and AM fungi. Similarly, a synergistic effect was reported by Garcia-Romera *et al*., (1998), when different strains of *Fusarium* varied remarkably in their interaction with AM fungi depending on the AM fungal species and the soil used. Their findings were that under all experimental conditions (i.e. treated soil or AM fungal inoculum treated soil), *F. oxysporum*-738, *F. oxysporum*-126 and *F. stillboide*-2169 increased plant shoot dry weight. Also a synergistic effect of the *Fusarium* strains with the AM fungus *Gl. mosseae* was observed, while the AM fungus was found to have no effect on the saprotrophic fungi. Furthermore, the inoculation of *Trichodema aureoviride* onto water agar containing *Gl. mosseae* enhanced mycelium development of AM fungi from germinating spores but did not increase the percentage germination of spores (Calvet *et al*., 1992). The above characteristic was attributed to the production of unidentified volatile compounds by these organisms in monoxenic cultures (Calvet *et al*., 1992; Will and Sylvia, 1990).

Some fungi have been reported to act as P solubilisers along with being biocontrol agents. Vassilev *et al*., (2006) reported that organisms such as *Trichoderma harzianum*, *Aspergillus niger, Penicillium variabile*, white-rot fungi and other filamentous fungi were capable of solubilising P along with exhibiting biocontrol activity. This potential was either exerted by the production of siderophores, organic

acids, lytic enzymes, glucose oxidases and melanin-degrading enzymes. The biocontrol activity of organisms such as white rot fungi remains to be confirmed (Vassilev *et al*., 2006). Souchie *et al*., (2006) in their study to isolate and identify P solubilising bacteria, fungi and AM fungal species in two reclamation areas of the Atlantic forest, Paraty Brazil, observed that the dominant P solubilising fungi were *Aspergillus* species. Kucey (1987) also confirmed a synergistic effect of P solubilising fungi (*Penicillium bilaj*) with mycorrhizal fungi to effectively increase the absorption of P by the plant root system of wheat and bean plants.

1.6 The ecological roles of arbuscular mycorrhizal fungi

Agricultural and industrial disturbances, such as mining, to the ecosystem have become a worldwide phenomenon. Opencast mining involves the removal of overburdened soil which is the surface material covering the valuable deposit of desired minerals or substance to be extracted (Internet 1). Due to the environmental effects such as underground water pollution, loss of biodiversity, erosion and formation of sinkholes caused by mining, the rehabilitation of mined and disturbed areas is a legal requirement (Wali, 1999). In South Africa, the mineral and petroleum resource department has passed a bill that mine owners must rehabilitate the surface area mined as well as integrating rehabilitation as part of their mining operations (Section 6, Act 50 of 1991, Department of Mineral and Energy, South Africa).

Rehabilitation involves the stabilisation of surface materials through appropriate landscape reconstruction, establishment of soil organic matter, nutrient availability and the establishment of long-term sustainable vegetation community (Sharma, *et al*., 2000; Singh *et al*, 2002). Reclamation, which is the process by which highly overburdened soil is developed through a nutrient cycling process, can be used interchangeably with rehabilitation. This is because both are aimed towards putting a new or altered land to use, to serve a purpose (Internet 2). Plants and microbes are now used to aid in the long-term reclamation of mine spoils (Singh *et al*., 2002).

AM fungi are found in different climates and habitats including disturbed soils from mining activities (Gaur and Adholeya, 2004). These fungi offer great benefits in the

rehabilitation of disturbed soils through inoculation and manipulation of the indigenous population (Cuenca, *et al*., 1998). However, inoculation will only be possible if AM propagule (spores, hyphae, infected root materials) density is high. The propagules of AM fungi are concentrated in the topsoil and as such, practices which disturb the soil ecosystem (section 1.4), will decrease the mycorrhizal status in the rhizosphere. Therefore, the recovery of disturbed sites using AM fungi will only be possible if these propagules are reintroduced by natural processes, long-term land management or by human intervention (Cuenca, *et al.,* 1998; Wicklow-Howard, 1994). Land management practices such as the re-spreading of topsoil from undisturbed areas on overburdened areas have proved successful in re-establishing the presence of arbuscular mycorrhiza (Wicklow-Howard, 1994). However, this is an expensive process and exposes other areas to disturbances.

The use of non-mycorrhizal plants for rehabilitation has proved successful but this has affected the establishment and population of AM propagules in the soil as well as decreasing plant succession (Wicklow-Howard, 1994). An ecosystem, with the majority of the plants being mycorrhizal, will contribute effectively toward reclamation through mechanisms such as enhancing plant growth and nutrient uptake, maintaining diversity by boosting plants resources and competitive ability, stabilising the soil through the ERH and efficient recycling of nutrients (Jasper, 1994). In addition, it has been suggested that AM fungi be used as an indicator for soil pollution and soil quality, because of their predominance in soils and plant dependability (Gaur and Adholeya, 2004; Leyval, *et al*., 2002).

The potential benefit of arbuscular mycorrhizas in rehabilitation of overburdened soil is becoming apparent due to the need to provide food, fuel wood and fibre for the increasing population through agroforestry (Kung'u, 2004). Their application for such strategies lies in their ability to interact with soil microbes and plants (Gaur and Adholeya, 2004) as well as their natural occurrence in soils (Kung'u, 2004; Quilambo, 2003). However, due to the differences in ecological adaptability of AM fungal species, it is advised that edaphic factors, nature of contaminated soil, inoculum source/density and their interaction with chosen plants be considered in order to boost reclamation efforts (Pawlowska and Charvat, 2002; Pfleger *et al*., 1994).

Arbuscular mycorrhizal fungi are ubiquitous in terrestrial ecosystems but despite their acknowledged role in ecology, most researches have focused on their interaction with plant communities with few studies at the ecosystem level (Rillig, 2004). An ecosystem by definition is a localised interdependent group of plants, animals and microbes whose activities affect the physical and chemical conditions of their environment (Naeem *et al*., 1999). The functionality of any ecosystem depends on how it exhibits biological and chemical characteristics of its type. In grassland ecosystems, AM fungi contribute towards plant diversity, carbon transfer, soil quality, nutrient cycling and plant productivity (Klironomos, *et al.,* 2000; Dell, 2002; Van der Heijden and Cornelissen, 2002). These pathways give rise to ecosystem processes such as carbon and nitrogen cycling (Hawkes, 2003; Zhu and Miller, 2003). Carbon transfer can be mediated between two plants through AM hyphal bridges due to the absence of host specificity. However, the mechanism and ecological significance of inter-plant nutrient and carbon transfer requires further investigation (Van der Heijden and Cornelissen, 2002). Klironomos *et al.,* (2000) investigated the influence of AM fungi on the relationship between plant diversity and productivity. They hypothesised that the productivity of plant communities by plant species is rendered redundant in the presence of AM fungi. This they carried out using 35 different plant species and two AM fungal species, *Gl. intraradices* and *Gl. etunicatum*. It was concluded that AM fungal diversity and species composition though positive on plant biomass reduced plant species productivity of the ecosystem. Therefore, AM fungi was suggested be considered during plant biodiversity (Klironomos, *et al.,* 2000) because of the mycorrhizal dependecy of most plants. The potential of AM fungi to influence plant community structure was also investigated by Van der Heijden *et al*., (1998a). They observed in a pot experiment that three species of plants, *Hieracium pilosella*, *Bromus erectus and Festuca ovina* differed in their mycorrhizal dependency according to AM fungal species. Also AM fungal species had significantly different effects on growth response of the plant species. Therefore it was concluded that since the plant species vary in the degree to response of various AM fungal species, that the species composition and diversity of AM fungi has the potential to influence plant community structure. This also has an important implication for growth of individual plant species to co-exist with other plant species in a community (Kiers *et al*., 2000; Van der Heijden *et al*., 1998a).

Agriculture has repeatedly been identified as one of the largest worldwide contributors to loss of biodiversity owing to land area utilisation, high degree of physical manipulation and fertiliser usage devoted to this activity (Mclaughlin and Mineau, 1995). In agriculture, inorganic fertilisers and livestock manures are widely used to meet crop P requirements, increase productivity and to boost soil P and other nutrients (Withers *et al*., 2001). However, the environmental effects of these fertiliser types are of great importance and should be taken into consideration due to possible runoffs after application. Inorganic fertilisers are believed by farmers, to be the best source of nutrients because they are cheap, easy to handle, associated with higher product yield and are more specific in nutrient constituents. In contrast, organic fertilisers are said to be bulky, difficult to handle, vary in nutrient content and have an unpleasant odour. But to a large extent they pose less environmental hazards (Arden-Clarke and Hodges, 1988). Organic fertilisation practices enhance soil structure, improve beneficial soil fauna and flora, are less disruptive of soil chemistry and sometimes inhibit microbial pathogens. The major negative impacts of inorganic fertilisers include the loss of soil organic matter, fluctuations in soil pH, reduction in beneficial soil microbes due to high salt concentration and reduction in soil fauna, for example earthworms (Arden-Clarke and Hodges, 1988). Studies by Withers *et al*., (2001) revealed that there is a lower risk of P transfer in land runoff following the use of organic P fertilisers (sludge and manure) compared to agricultural inorganic P. This was attributed to the low solubility rate of inorganic P fertilisers which led to the suggested use of organic fertilisers that cause less harm to the environment for agricultural production (Withers *et al*., 2001).

The use of AM fungi as 'biofertilisers' in agriculture is becoming a world wide phenomenon and has successfully been in use in places like Taiwan, South Africa and United States (Juang, 2007). Their potential as a biofertiliser lies in their mycorrhizal benefits and plant-soil interactions, hence, their selection for inoculum and management in field situations are widely studied (Atkinson *et al*., 2002; Safir, 1994; Dodd and Thompson, 1994). The exact definition of biofertilisers remains unclear, however, they are commonly referred to as the use of soil microorganisms to improve availability and uptake of mineral nutrients required for plant growth (Vessey, 2003).

In order to exploit these microbes as biofertilisers, the ecological complexity of microbes in the mycorrhizosphere needs to be taken into consideration (Khan, 2006).

1.7 Current methods used to study microbial populations and interactions

The emerging interest of soil microbial ecology and diversity is due to the functional roles they play in biogeochemical functioning (Garbeva *et al*., 2004; Kirk *et al*., 2004). The importance of microbes in agro ecosystems has led to their use in land management strategies and as indicators of disturbances such as changes in agronomic practices (Kennedy *et al*., 2004; Marschner *et al*., 2004), reduced or no tillage approaches (Drijber *et al*., 2000; Ibekwe *et al*., 2002) and monocropping or crop rotation (Larkin, 2003). However, the inability to culture most environmental samples is a fundamental problem to understanding their ecological significance (Yeates *et al*., 1998; Atlas, 1984).

Several studies have reported that only about 1% of the total soil microbial population is culturable on standard media, while the other 99% though in a functional state are culturally inaccessible and differ genetically from the 1% (Prescott *et al*., 2005; Garbeva *et al*., 2004; Kirk *et al*., 2004; Kent and Triplett, 2002; Kozdroj and van Elsas, 2000; Trevors, 1998). Culturable techniques are convenient, approachable and fairly inexpensive, but factors such as incomplete understanding of growth conditions, selectivity of media, different growth rates, soil sampling heterogeneity and cell size or viability are some of the problems which face soil microbial diversity studies (Kirk *et al*., 2004, Kozdroj and van Elsas, 2000). To overcome these limitations, techniques such DNA finger-printing, proteonomics, fatty acid methyl ester analysis, community level physiological profiling (CLPP), restriction fragment length polymorphism (RFLP) and other molecular methods have been used (Kauffman *et al*., 2004; Wechter *et al*., 2003; Ibekwe and Kennedy, 1998; Cullen and Hirsch, 1998).

Molecular techniques have enabled the detection of a variety of soil organisms without prior cultivation on media. There are, however, biases in extraction and purification of soil samples regardless of its advantages over cultivation dependent

techniques (Kirk *et al*., 2004; Niemi *et al*., 2001). Prominent disadvantages of molecular based methods are efficient cell lysis dependency and co-purification of humic and fulvic acid that inhibit *Taq* polymerase during Polymerase Chain Reaction (PCR) amplification (Zhou *et al*., 1996). Several authors have put forward various rapid and cost effective methods to extract and purify soil DNA as well as efficient methods to obtain high DNA yield (Niemi *et al*., 2001; Kozdroj and van Elsas; 2000; Yeates *et al*., 1998; Cullen and Hirsch, 1998; Porteous *et al*., 1997; Zhou *et al*., 1996). It has also been suggested that there is no single method of cell lysis or purification and as such different combinations and modifications can be employed depending on the desired outcome (Zhou *et al*., 1996).

The applications of culture-independent techniques to study microbial diversity has led to studies in to the interaction between microorganisms and plants with regards to their ability to influence rhizosphere communities or plant symbiotic associations (Kent and Triplett, 2002; Söderberg *et al*., 2002; Grayston *et al*., 1998; Linderman, 1992). Arbuscular mycorrhizas are known for their interaction with other soil microorganisms and this has been detected mostly through dual inoculation with isolated microorganisms (Biró *et al*., 2000; Vázquez *et al*., 2000; Gryndler and Vosátka, 1996; Linderman, 1988). However, recent findings indicate that various AM fungal species as well as AM colonised and non-AM plants differ in bacterial community composition (Marschner and Timonen, 2005; Soderberg *et al*., 2002). Several techniques applicable in this type of study are discussed below, while other techniques are highlighted in Table 1.2.

1.7.1 Plate counts

Previously, microbial diversity studies were based on this traditional method. Though these methods are fast and inexpensive, they have various limitations (Kirk *et al*., 2004). However, they are still useful in determining the impact of anthropogenic activities as well as the phenotypic characteristics of organisms isolated. It is also argued that these culturable organisms constitute an active portion of the soil bacterial community and with advances in the design of media plate counting will be more useful (Ellis *et al*., 2003; Janssen *et al*., 2002). This argument was because the cellular

and metabolic activities of these culturable organisms could be determined thereby resulting in determination of their ecological roles in the rhizosphere (Ellis *et al*., 2003). In contrast, problems associated with plate counts that are not highlighted in Table 1.2 are the length of time required for incubation of some isolates, cell clumping that may lead to inappropriate counts, colony-colony inhibition and the predominant growth of fast and spore forming organisms (Kirk *et al*., 2004). Despite the limitations, various studies have used the plate count method successfully to determine microbial diversity or changes in the rhizosphere (Marschner and Timonen, 2005; Ellis *et al*., 2003; Wamberg *et al*., 2003; Janssen *et al*., 2002; Söderberg *et al*., 2002).

Alternatively, other biochemical methods such as fatty acid methyl ester (FAME) analysis are used effectively to determine microbial diversity. This method provides information based on the fatty acid profile of the microbial community (Øvreås, 2000) and is not dependent on cultivation. It involves methylation of samples and subsequent gas chromatography for analysis of extracted fatty acids (Ibekwe and Kennedy, 1998). Multivariate analysis such as principal component analysis is further used to compare FAME profiles from different soil mixtures (Kirk *et al*., 2004; Øvreås, 2000). Fatty acid can be used as biomarkers for interpreting community level profiles because they make up part of the cell biomass. Therefore, changes in the fatty acid profiles in a soil would indicate a change in microbial composition (Ibekwe and Kennedy, 1998; Zelles *et al*., 1995). Ibekwe and Kennedy (1998) used phospholipid fatty acid profiles and carbon utilisation patterns to determine microbial community structure of plants from the field and from green house pots. Principal component analysis showed that there was a clear distinction between the community from field and pots. The disadvantage of this method is that organisms cannot be identified to a species or strain level because individual species can have same fatty acid profile which can be found present in another species. Therefore, FAME is based on functional groupings of fatty acids (Kirk *et al*., 2004; Bossio *et al*., 1998; Ibekwe and Kennedy, 1998).

1.7.2 Denaturing gradient gel electrophoresis and temperature-GGE

Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are fingerprinting techniques with similar concepts that are used for studying microbial diversity. These techniques were originally designed to detect point mutations in DNA sequences using forward and reverse universal primers (Kirk *et al*., 2004). Separation of the amplified DNA sequence of organisms is then based on the G+C content and distribution along with the use of chemical denaturants (urea and formamide) for DGGE and temperature for TGGE to obtain a melting linear gradient (Kirk *et al*., 2004; Fromin *et al*., 2002). The fingerprinting method is effective in reducing the complexity of a microbial community and allows detection of species that are low in abundance. These methods also allow a large number of samples to be analysed simultaneously. This approach has been used to study interactions between mycorrhizal colonisation and bacterial community (Marschner and Timonen, 2005), determination of total and active bacterial communities in arable soils (Girvan *et al*., 2003) and as a molecular tool for genotypic identification of mycorrhizal species (Kwong ma, 2004).

1.7.3 Other molecular techniques

Cloning of the 16S rDNA can allow microbial diversity to be assessed with a high level of discrimination (Borneman *et al*., 1996). This involves obtaining multiple copies of genes *in vivo* and sequencing these genes for identification. Cloning is a generalised termed used for all the processes involved to achieve cell replication, which includes fragmentation, ligation, transformation and screening processes (Prescott *et al*., 2005). This method is often reliable but is mostly time and resource consuming and can be tedious when handling a large number of samples. Restriction fragment length polymorphism (RFLP) and terminal restriction fragment length polymorphism (T-RFLP) follow the same principle except that T-RFLP involves labelling of one primer with a fluorescent dye such as 4, 7 ,2', 7'- tetrachloro-6-carboxyfluorescein. The digestion of DNA fragments using restriction enzymes and subsequent agarose or polyacrylamide gel electrophoresis can be used to screen clones or measure bacterial community structure (Kirk *et al*., 2004). This method involves the use of restriction enzymes derived from bacterial organisms to restrict DNA fragments or invasion of foreign DNA (Hallberg, 2001). They are sometimes

referred to as the DNA scissors that cut nucleotide sequences into pieces to hinder functioning. Most restriction enzymes consist of 4-6bp and cut DNA at specific points called restriction sites in opposite directions (Hallberg, 2001). The size of each fragment corresponds to the distance between restriction site sequences and size of the fragmented DNA. This approach according to Liu *et al*., (1997) can only be used to determine the microbial community and not as a measure for diversity or phylogenetic analysis.

Plate counts, an attempt at DGGE, cloning and RFLP techniques were selected for use in this study and are not without their limitations. However, they were used due to availability of materials and expertise. There are other preferable methods than these (Table 1.2) but no method is without limitations and appropriate methods should be applied based on the desired outcome.

Table 1.2 Advantages and disadvantages of biochemical-molecular based method for soil microbial diversity studies (Adapted from Kirk *et al*., 2004).

Methods	Advantages	Disadvantages
Plate count	Fast, Inexpensive.	Unculturable microorganisms not detected. Bias towards fast growing individuals. Bias towards fungal species that produce large quantity of spores
Community level physiological profiling (CLPP)	Fast, highly reproducible, differentiate between microbial communities, generate large amounts of data Option of using bacterial, fungal plates or site specific carbon sources	Only represents culturable fraction of community. Favours fast growing organisms. Only represents those organisms capable of utilising available carbon sources. Potential metabolic diversity. Sensitive to inoculum density
Fatty acid methyl ester analysis (FAME)	No culturing of microorganisms, direct extraction from soil Follow specific organisms or communities	If using fungal spores a lot of material is needed. Can be influenced by external factors. Possibility of results being confounded by other microorganisms

Table 1.2 Continued

Methods	**Advantages**	**Disadvantages**
Guanin plus cytosine (G+C)	Not influenced by PCR biases. Includes all DNA extracted. Quantitative Includes rare members of community	Requires large quantities of DNA. Dependent on lysing and extraction efficiency. Coarse level of resolution
Nucleic acid reassociation and hybridization	Total DNA extracted. Not influenced by PCR biases. Study DNA or RNA. Can be studied *in situ*	Lack of sensitivity. Sequences need to be in high copy number to be detected. Dependent on lysing and extraction efficiency
DNA microarrays and DNA hybridization	Same as nucleic acid hybridization. Thousands of genes can be analysed. If using genes or DNA fragments, increased specificity	Only detect most abundant species. Need to be able to culture organism. Only accurate in low diversity systems
Denaturing and temperature gradient gel electrophoresis (DGGE and TGGE)	Large numbers of samples can be analysed simultaneously. Reliable, reproducible and rapid	PCR biases. Dependent on lysing and extraction efficiency. Sample handling can influence community i.e. if stored too long before extraction community can change. One band can represent more than one specie (co-migration) Only detects dominant species
Single strand conformation polymorphism (SSCP)	Same as DGGE/TGGE No GC clamp No gradient	PCR biases. Some ssDNA can form more than one stable conformation
Restriction fragment length polymorphism (RFLP)	Detect structural changes in the microbial community	PCR biases. Banding patterns often too complex
Terminal restriction fragment length polymorphism (T-RFLP)	Simpler banding patterns than RFLP. Can be automated; large number of samples. Highly reproducible Compare differences in microbial communities	Dependent on extraction and lysis efficiency. PCR biases Type of *Taq* can increase variability. Choice of universal primers. Choice of restriction enzymes will influence community fingerprint
Ribosomal intergenic spacer analysis (RISA)/automated ribosomal intergenic spacer analysis	Highly reproducible community profiles	Requires large quantities of DNA

1.8 Motivation for the study

There is an increasing need for plant production and mining industries to adopt a more environmentally friendly approach and to less harmful products; especially in South Africa, where it is a legal requirement that mining industries incorporate rehabilitation as part of their mining operations. AM fungi are efficient candidates for rehabilitation and agricultural management (Khan, 2006), but further investigation into their role and interaction with other rhizospheric organisms is required. Previous studies have focused on the interaction between AM fungal inoculants with microbial populations using pot trials with specific organisms but only few field trials have been conducted. Hence it was pertinent to determine the effect of AM fungal inoculum on background microbial populations using mining and agricultural sites that differed greatly in operational activities.

1.9 Hypothesis and Objectives

1.9.1 Hypothesis

The introduction of AM fungi has a positive influence on the background microbial population but this influence varies according to land-use management.

1.9.2 Objectives

To verify the hypothesis put forward, the following objectives of the study were:

I. To determine the potential of using AM fungi in rehabilitation of mine spoils with fertiliser treatments. This was achieved using a pot trial analysis from which the compatibility of fertiliser treatments with AM fungi was determined by assessing percentage AM fungal colonisation of plant roots.

II. To determine the effect of introduced AM fungal inoculum on the background microbial population using culture dependent and culture independent techniques. This was achieved by plating on media, examining the effect of AM fungi on microbial numbers and relating bacterial numbers to land use

management. The use of molecular analysis such as finger-printing methods and cloning was employed to account for both unculturable and culturable organisms with much focus on bacteria. Although. Preliminary data on fungi was assessed.

III. To examine the microbial population based on their functional capabilities in the soil. This was investigated through the isolation of microbial groups such P solubilisers, N_2 fixers, Actinomycetes, pseudomonads and fungi on selective media. This was further related to their possible roles with AM fungi in the environment.

IV. To evaluate the population and infectivity of AM fungi through the use of AM fungal specific methods such as glomalin assay, root colonisation analysis, spore enumeration and infectivity potential (most probable number) techniques. This was analysed to determine the effect of land use management of AM fungal populations.

V. To determine the mycorrhizal status of selected plant species growing around the mine site as potential host plants in rehabilitation. To achieve this, plants were identified to genus level and their root structures examined for different types of mycorrhizal colonisation.

CHAPTER 2

MATERIALS AND METHODS

2 Materials and methods

2.1 Site description

Site 1

Site 1 was a brick making industry located on farm 243 Brakkefontein, Grahamstown (Fig. 2.1), Eastern Cape Province, South Africa (33°C 18'S, 26°C 31'E). This industry, belonging to the company Makana Brick and Tile (Pty) Ltd, is known for mining kaolin, which began in 1996 as authorized by the Department of Minerals and Energy South Africa. Previously, the Site was used for grazing and left fallow for the past 10 years. The open cast mining of kaolin by the company led to a complete disturbance of the quarry topography resulting in steep slopes and overburden soils (Fig. 2.2), which were stored along the Western and Northern perimeters of the mine (EMP report, 2001). About 0.5 hectare of the overburden soil was to be rehabilitated by establishing a vegetation cover as a trial study and as part of the management strategies of the company.

Climatically, the maximum annual rainfall recorded in a 24hr interval is 185mm and 201mm in 48hrs. Mean annual evaporation is 1500-1600mm and mean annual runoffs of 20-50mm. The natural vegetation of the site is degraded and patchy. It consists of indigenous plants including a mosaic of Eastern thorn bushveld (e.g. *Acacia karro* and *Dispyros lycoides*), valley thicket (*Cassine aethiopica*, *Asparagus spp and Plumbago auriculata*) and *Renosterveld* (EMP report, 2001).

Site 2

Site 2, is a commercial agricultural farm situated South East of Site 1 in Bathurst, Eastern Cape Province, South Africa (Fig. 2.1). The farm is called Limestone Hill (33° 27.595' S, 26° 57.535' E) and has been used consistently for intensive cropping and farm produce. Fields previously planted to pineapples are presently cultivated with geranium cuttings that will be used in the production of essential oils. The natural vegetation of the area consists of indigenous plants such as yellow wild iris (*Dietes bicolor*), *Encephalartos trispinosus,* Boneseed (*Chyrsathemoides monilifera* var.*monilifera*), ragwort (*Senecio spp.*) and *Acacia karoo* (Martin and Noel, 1960).

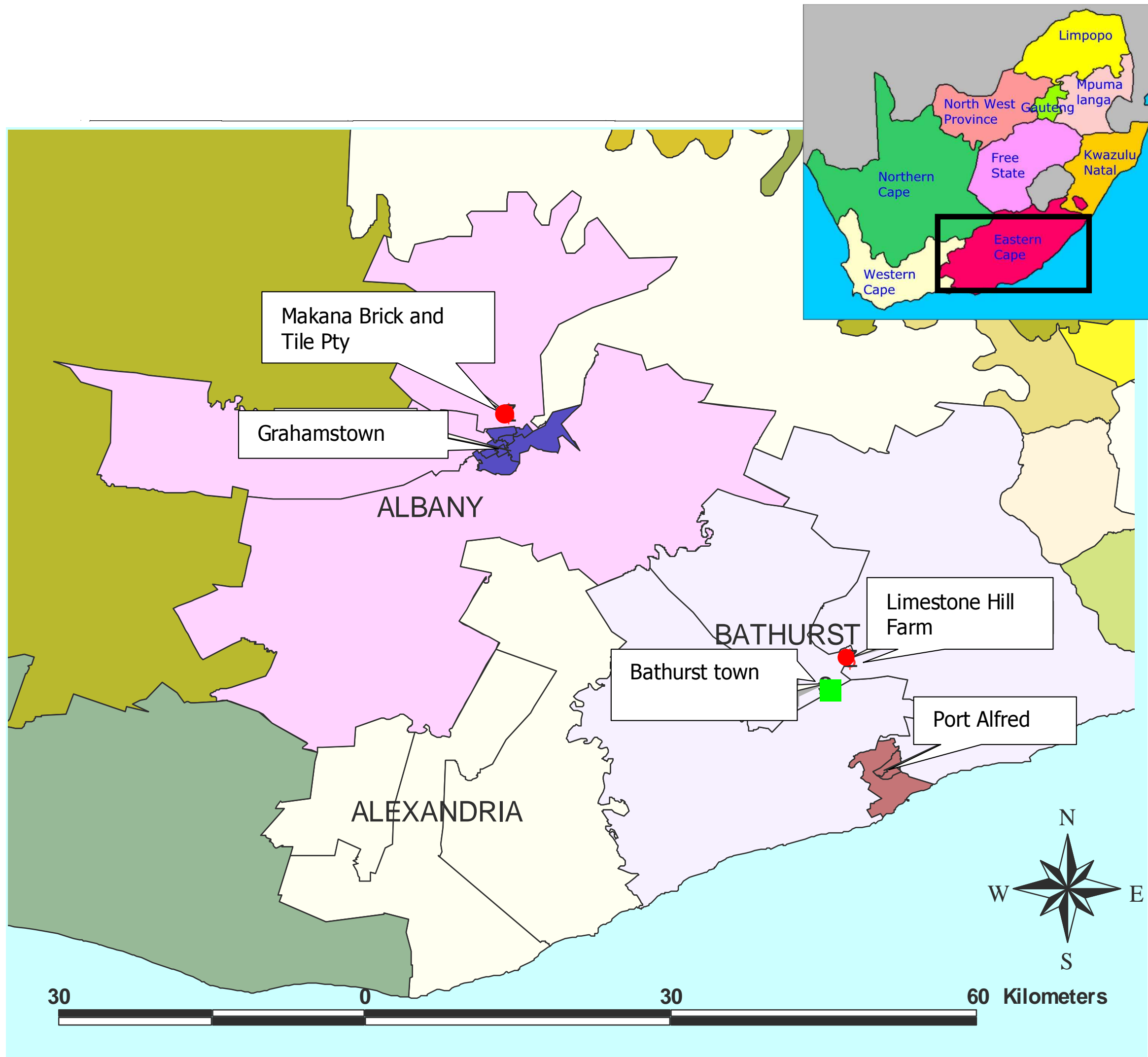


Figure 2.1 Grahamstown and Bathurst areas in a section map of Eastern Cape Province, South Africa. The experimental field Sites are highlighted as red spots. Site 1- Makana Brick and Tile (Pty) Ltd; Site 2- Limestone Hill farm. Maps indicate provinces of South Africa and location of Eastern Cape Province (Section map drawn by Gumede H.).

Figure 2.2 An overview of the landscaped overburdened soil for rehabilitation at Makana Brick and Tile (Pty) Ltd. M- kaolin mining area; R- rehabilitation area of study.

2.2 Field experimental establishment and sampling

2.2.1 Site 1

The rehabilitation Site at the Makana Brick and Tile industry was landscaped by the company with trial area between reference number 29, with coordinate 33° 15.400' S, 26° 32.964' E and reference number 97, with coordinate 33° 15.384'S, 26° 32.936' E (Fig. 2.3). Plots ($7m^2$) were marked out with 3 replicates into treatments: mycorrhizal inoculated and non-inoculated plots as well as with different fertiliser treatments in September 2005. Areas between plots called buffer zone were also seeded with no additional treatment. A total of 17 plots were allocated reference reading numbers to determine the bearing and distance between plots laid out in this study (Fig. 2.3). Designated field plots were treated with 1400g commercially available AM fungal inoculum (Mycoroot (Pty) Ltd, Grahamstown, South Africa) prior to seeding with *Cynodon dactylon* L.Perf (500g). This was done using a fertiliser spreader in a

vertical manner around each plot to ensure the inoculum and seeds were evenly spread. The fungal inoculum was applied in the same manner after which the seeds and inoculum were raked into the soil in each plot. *C. dactylon* was chosen in this study as it is a stubborn grass or creeping plant that can survive harsh conditions and intensive grazing (Van Oudtshoorn, 1992). The inoculum used was a granular solid consisting of indigenous arbuscular mycorrhizal fungi isolates containing a combination of *Gl. geosporum*, *Gl. mosseae*, *Gl. etunicatum* spores, fungal hyphae and fragments of colonised root material. The minimum infectivity potential as determined by most probable number (MPN) was 10^5 propagules/kg of product.

Application of fertilisers at Makana Brick and Tile was done using organic and inorganic fertilisers. The amount of each fertiliser treatment was determined by the results obtained from the soil nutrient analysis. The organic fertiliser called Organic Tea was produced by Guano Organic Fertiliser Company. The fertiliser was made from pure seabird guano that contains both macro and micronutrients such as N, P, K, Ca, Mg, Na, Fe, Cu, Zn and B (quantities are outlined in Appendix A). Before use a 1:40 dilution of the liquid organic fertiliser was made and sprayed over the plots (1500 ml/plot). The inorganic fertiliser used was NPK chemical fertiliser that had nitrogen, phosphate and potassium in the ratio of 3:1:5. The quantity of fertiliser applied was 1215 ml/plot measured in volume, which is equivalent to 150kg/ha. Plots were treated in six different combinations (Fig. 2.3). The period of the trial study was from September 2005 to June 2006 with a 3, 6 and 9-month sampling interval.

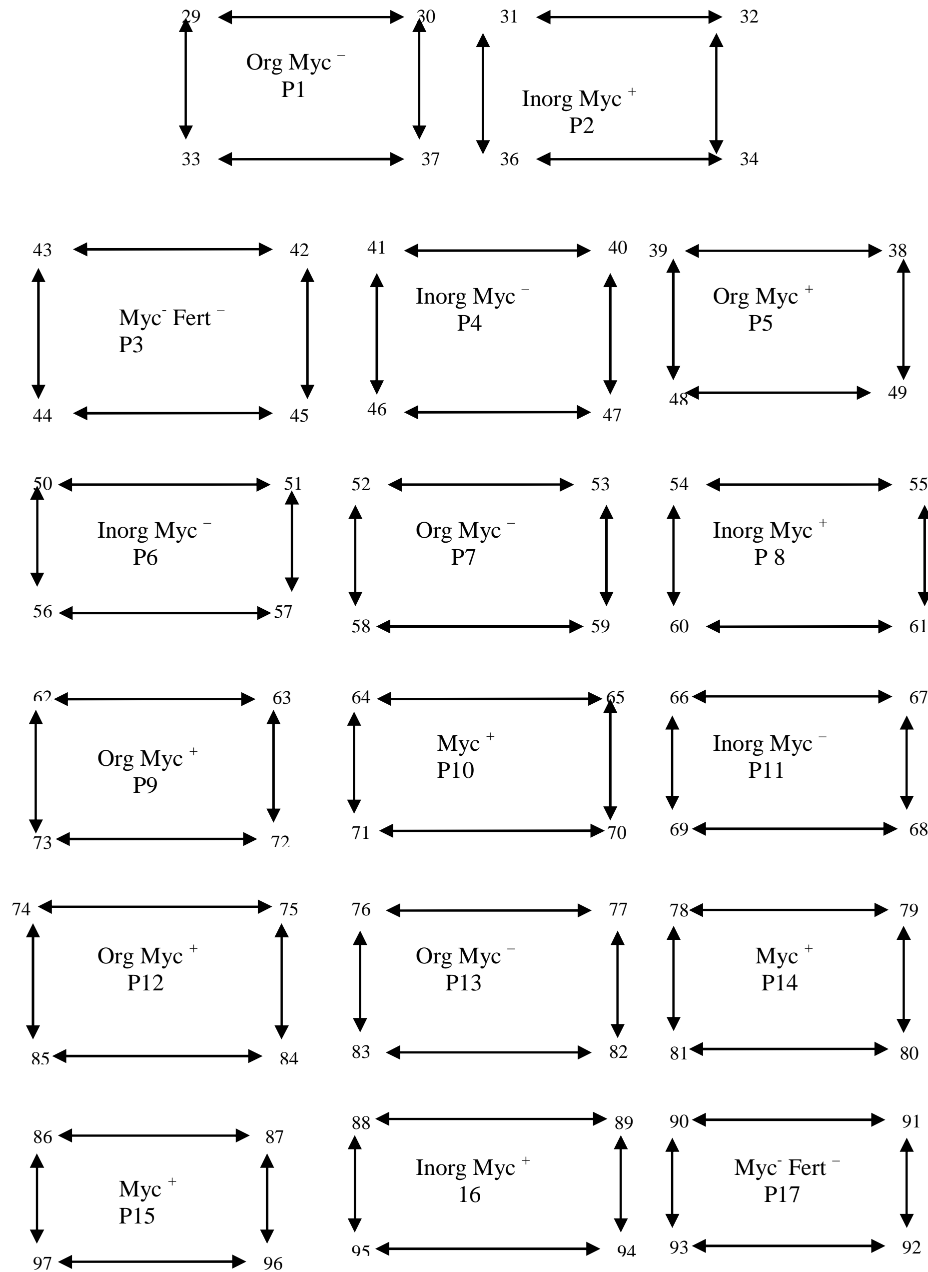


Figure 2.3 Plot layouts with designated treatments and reference reading numbers of the rehabilitation Site situated at Makana Brick and Tile Pty. Codes in plots denote the plot treatment: InorgMyc+ = inorganic fertiliser with mycorrhiza; InorgMyc- = inorganic fertiliser without mycorrhiza; OrgMyc+ = Organic Tea + mycorrhiza; OrgMyc- = Organic Tea without mycorrhiza; Myc-Fert- = No fertiliser and mycorrhiza; Myc + = Mycorrhiza only. Areas between plots are buffer zones

Soil samples were collected using a core borer of 15cm in depth and 5cm in diameter. Three samples per plot were collected randomly at 3 and 6 months sampling intervals. Samples were placed in plastic bags that were labelled appropriately. A total of nine samples were collected from each treatment plot at each sampling period. The soil samples were then air-dried, sieved through a 2mm sieve to remove stones and packed into plastic bags, each containing approximately 500g. Soil samples were stored at 4°C until they were used for further analysis (Kozdroj and van Elsas, 2000). Plant materials (i.e. shoot and roots) were removed and using a balance (Schimadzu) weighed to obtain the wet weight. This was then dried at 78˚C for 2 days in an oven and re-weighed to obtain the plant dry weight (Söderberg *et al*., 2002). Root sub-samples were removed and placed in 50% ethanol and stored at 4˚C for root staining analysis (section 2.6.2). Sub-sample wet weights were converted to dry weight and added to total biomass dry weights to ensure accuracy of data.

2.2.2 Experimental setbacks

The establishment of the trial at Site 1 was affected due to irrigation problems as well as the low rainfall (Table 2.1) experienced during growth periods of the trial (September 2005 – June 2006). The maximimum accumulated monthly rainfall experienced in November 2005 (Fig. 2.4) aided the slight germination at the beginning of the trial study. As a result, the Limestone Hill Farm was incorporated into the study as a comparative tool when germination in some plots of Site 1 had not improved after 6 months. Hence, sampling at 9 months in Makana Brick and Tile was changed to accommodate the collection of samples from the new Site, Site 2, which had no fertilisers applied. The plant material data collected at the 3 and 6 months sampling period was excluded from the results because of poor germination in some plots. Root samples in most plots were not sufficient enough to determine plant biomass as well as root colonisation. Also at Site 2, the shoots could not be collected for experimental analysis because the shoots were to be used for the commercial production of essential oil by the Farm owner. Hence determining plant biomass was excluded from the result analysis. In other words, determining the rehabilitation potential of AM fungi with chosen fertiliser treatments using the field trial was aborted. This led to the selection of 2 replicate treatment plots (mycorrhizal inoculated and un-inoculated plots) that had reasonable vegetation irrespective of the

fertiliser used from Site 1. At the 9-month sampling period, a total of 10 samples were collected from each of the selected plot and were pooled to give five sub-samples per plot. This was done to reduce spatial variability because of soil complexities (Girvan *et al.*, 2003). These samples were processed in the same manner as the initial sampling but without the weighing of plant shoots and roots. Subsequent to the period of this study, significant rainfall was recorded in this area. The vegetation cover of the mining Site has improved dramatically but further analyses were outside the scope of this study. Analyses are being continued by Mycoroot (Pty) Ltd. More importantly, it should be noted that this experimental set back, also resulted in the modification of the study objectives in order to contain the use of Site 2.

Table 2.1 Accumulated monthly rainfall data for Grahamstown from August 2005 to June 2006. This indicates the monthly rainfall obtained during the study period. (Rhodes Weather Monitoring Service, 2007).

Month	Accumulated monthly (mm)
August	10
September	10
October	20
November	90
December	20
January	60
February	60
March	40
April	30
May	80
June	20
Average	**40**

2.2.3 Site 2

At the Limestone Hill farm, the treatment plots established by Mycoroot (Pty) Ltd in February 2006 were replicated three times in rows. Each plot consisted of 20 rows with 8 treatments, 8 control and 4 buffer zones at the edges placed side-by-side (Fig. 2.4 and Table 2.2). The same commercial mycorrhizal inoculum as applied in the mining Site was used but with a different mode of application. Plots were planted with rose geranium (*Pelargonium graveolens* L. Heritier) cuttings (100 plants/row) with each plant receiving 20 ml Mycoroot applied to the bottom of the planting hole. Fertilisers were not used in this setup but have been used as well as with pesticides in previous planting seasons as part of regular farm practice. Thus treatment was either

with mycorrhizal inoculum or without inoculum. A once off sampling was conducted after a period of 5 months for inclusion in this study. A total of 10 samples were collected from each of the selected plot and were pooled to give five sub-samples per plot. Samples were sieved through a 2mm sieve to remove root materials and packed into plastic bags. Root samples were stored in 50% ethanol at 4˚C for staining until they could be further analysed.

It should be noted that henceforth Site 1 and Site 2 will be referred to as MBT or mining and LHF or agricultural Site, respectively.

Table 2.2 Single plot layout with three replicates of treatments in rows and number of plants per treatment at the Limestone Hill Farm, Bathurst.

<table>
<tr><td colspan="20">Rows</td></tr>
<tr><td>1</td><td>2</td><td>3</td><td>4</td><td>5</td><td>6</td><td>7</td><td>8</td><td>9</td><td>10</td><td>11</td><td>12</td><td>13</td><td>14</td><td>15</td><td>16</td><td>17</td><td>18</td><td>19</td><td>20</td></tr>
<tr><td>B</td><td>B</td><td>M</td><td>M</td><td>M</td><td>M</td><td>C</td><td>C</td><td>C</td><td>C</td><td>M</td><td>M</td><td>M</td><td>M</td><td>C</td><td>C</td><td>C</td><td>C</td><td>B</td><td>B</td></tr>
<tr><td colspan="20">Total plants per replication</td></tr>
<tr><td></td><td></td><td colspan="4">400</td><td colspan="4">400</td><td colspan="4">400</td><td colspan="4">400</td><td></td><td></td></tr>
<tr><td></td><td></td><td colspan="16">Total number of plants per plot</td><td></td><td></td></tr>
<tr><td></td><td></td><td colspan="16">1600</td><td></td><td></td></tr>
</table>

B = Buffer edge   M = Mycorrhiza application (20 ml/plant)   C = Un-inoculated control

Figure 2.4 Limestone Hill farm cultivated with *Pelargonium graveolens* cuttings in rows. B- buffer zone; C- control; M – mycorrhizal treated row.

2.3 Soil texture and nutrient analysis

Soil particle size of soil samples from both the mining and agricultural Sites was determined using the Bouyoucos hydrometer (ASTM 562-H) method. (Okalebo *et al*., 1993). This was done to estimate the percentage sand, silt and clay content in soil. Air dried soil (50g) from both Sites was saturated in water and 10% sodium hexametaphosphate to dispense the soil particles into the solution. This was allowed to stand for 10 mins. The solution was transferred into a measuring cylinder and water added to a final volume of 1130 ml. The suspension was inverted ten times to mix after which two or three drops of amyl alcohol was added to remove any froth. After 20 sec, a hydrometer was inserted and measurements taken after 40 sec. Temperature affects readings of the hydrometer which had been calibrated to 20°C. Hence, correction factors of ± 0.5 had to be applied when temperature readings was more or less than 20°C. The inversion process was repeated twice and then allowed to stand

for 2 hrs, after which hydrometer and temperature readings were again recorded. The readings were used to calculate the percentage sand, silt and clay (Eqn 2.1). Samples were then classified in their respective texture class using a soil textural triangle (Okalebo *et al*., 1993).

Equation 2.1

$$\text{Sand \%} = \frac{W1 - R1}{50} \times 100$$

$$\text{Clay \%} = \frac{R2}{50} X100$$

Where W1 = weight of original sample; R1= Hydrometer reading at 40 sec; R2= Hydrometer reading after 2 hrs

For soil nutrient analysis of Sites, all individual samples from each treatment plot were pooled to give three sub-samples. From the mixture, 300g samples were oven dried (78°C for 2 days) and sent to Eco Analytica Laboratory, University of Northwest, South Africa to be analysed for soil nutrients such as calcium, magnesium, phosphorus, sodium, ammonium, potassium, nitrate, nitrite, % carbon, Bray P and pH.

2.4 Pot trial analysis

The pot trial analysis was a partial replication of the field establishment at Site 1. This was done to determine the effect of fertilisers chosen on AM fungi colonisation and the rehabilitation potential of AM fungi where environmental factor effects are minimised.

2.4.1 Experimental design

A total of 36 pots ($12cm^2$ diameters) were pre-washed in 5% sodium hypochlorite to maintain a certain level of sterility. Pots were divided into three phases, each phase having 12 pots (Fig. 2. 5). In each phase treatment, pots were replicated three times.

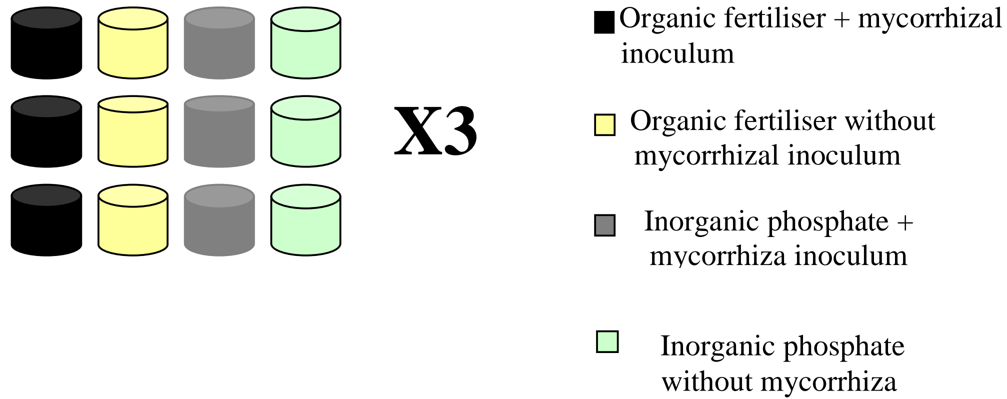


Figure 2.5 Diagrammatic representation of one phase of the pot layout with replicates as setup in a plastic tunnel.

2.4.2 Experimental establishment

Pots were half filled with sieved (2mm mesh size) overburdened soil from Makana Brick and Tile (Pty) Ltd. Designated pots were inoculated with 1.5g (equivalent to 1.5 ml) commercial Mycoroot inoculum, spread in a layer and was covered lightly with soil. Planting was conducted by weighing 0.1g *C. dactylon* seeds to limit variation in pots. This was also covered lightly with soil and watered with 100 ml distilled water. Plants were left to grow for 10 days before application of fertilisers. This was done to allow establishment of AM fungi with the plants prior to fertiliser application. The same fertilisers were used as in the field (Section 2.2.1). The Organic Tea was applied in a 1:40 dilution of the liquid and 100 ml dispensed into designated pots. The amount of NPK (3:1:5) was determined by weight (1.5g per pot). Pots were setup in a plastic mycorrhizal tunnel, with a minimum temperature of 19°C and a maximum of 35°C. Pots were irrigated daily with UV sterilized water for 5 mins. Plants were harvested after 6, 12 and 28 weeks.

2.4.3 Sampling and processing

At each phase, samples were harvested independent of the next sampling time. Soil adhering to the roots was carefully washed off to keep roots intact. Due to the inability to separate roots from the shoot without loss of root material, total plant wet weight was recorded using a balance and re-weighed after root sub-samples for

staining were removed. The whole plant was dried in an oven at 72˚C for 48 hrs before dry weights were recorded (Vàzquez *et al*., 2000). Shoot heights were measured by placing a ruler beside the whole plant and recording five random readings of plant height (cm) per pot. Values were then averaged to give the height of the plants per treatment pot. Total biomass was calculated after correcting dry weights for root sub-samples taken for staining.

2.5 Microbial population analysis

To determine the microbial population, the soil samples from the two Sites were processed using culture dependent and culture independent technique. Culture dependent, means that all bacterial and fungal samples were first obtained from culture media before being subjected to either morphological or molecular analysis to aid identification. While the culture independent technique were analyses of the bacterial population directly from soil, without culturing on media. The main focus was given to the bacterial population than the fungal population due to their fast growth rate and indications in the literature of their interaction with AM fungi (Marschner and Timonen, 2005; Fillion *et al*., 1999, Meyer and Linderman, 1986b; Linderman, 1988). The bacterial functional groups (two or three samples from each group) were selected for species identification molecularly, while fungal isolates that could not be identified morphologically were selected for further molecular analysis. Selected bacterial and fungal isolates were subjected to DNA extraction and amplification, which were sequenced for species identification. It should also be noted that though the bacterial and fungal samples were identified using molecular techniques, they are still referred to as culture dependent since they were first isolated from different respective media.

2.5.1 Estimating the number of culturable microbial populations in the rhizosphere

Plate method

Soil samples (1g) taken from each plot were mixed in 10 ml 2% saline and serially diluted (six 10 fold series). Aliquots (0.1 ml) from dilutions 10^{-3}, 10^{-4}, 10^{-5} were spread on Nutrient Agar (Biolab Cat no. HG0000C1) and Tryptone Soy Agar (Biolab Cat no. HG000C17) for total bacterial count and on Potato Dextrose Agar (Biolab HG00C100) for fungi. Other selective medium used were Pseudomonads Selective Agar with CFC supplement (Merck Cat no.107620.0500/7.001), modified phosphate medium-NBRIP (formulated by National Botanical Research Institute, Lucknow, India) for phosphate solubilisers (Mehta and Nautiyal, 2001), nitrogen free medium-N_2A (Paustian, 2006) for free living nitrogen fixers and Benedict's Modification of Lindenbein medium-BLM for Actinomycetes (Porter *et al.*, 1960). Each dilution was spread onto two replicate plates. Compositions of modified NBRIP, N_2A and BLM media are outlined in Appendix B. The number of colonies forming on each medium was counted at 2 days for fast growing organisms and 4-8 days after incubation at 37^oC for bacteria and 28^oC for fungi. Colony forming units per gram of soil (CFU/g) was calculated using Eqn 2.2 (Johnson and Case, 2007).

Equation 2.2

$$\text{CFU/g} = \frac{No.of\ colonies}{Volume\ plated\ (ml)} \times Dilution\ factor$$

From each dilution, colonies were picked at random and sub-cultured back onto selective media of isolation to obtain pure cultures. These pure isolates were stored after incubation and growth at 4^oC for further analysis and identification.

Selectivity of media

For the isolation of pseudomonads, the *Pseudomonas* selective CFC supplement was added to the pseudomonads agar base. The CFC supplement is composed of the antibiotics cephaloridine, fucidin and cetrimide. These ingredients inhibit both Gram-positive and Gram-negative accompanying microflora. The modified NBRIP medium

for the isolation of P solubilizing bacteria was differential due to the presence of an insoluble phosphide, $CaHPO_4.2H_2O$ (Illmer and Schinner, 1992). The ability of an organism to solubilise this phosphate source was observed by a clearing zone around the colony. The N_2A medium is a selective media that lacks any nitrogen source. It therefore encourages the growth of free-living N_2 fixers due to their ability to utilise nitrogen from the atmosphere. BLM on the other hand, was selective for actinomycetes due to the presence of complex nitrogen sources such as glycerol and L-arginine in the agar. The nitrogen sources are not readily utilised by most bacterial organisms thereby favouring the development of members of this genus (Porter *et al*., 1960).

2.5.1.1 Morphological identification

Gram staining

Selected pure bacterial isolates were picked from all samples and were Gram stained. Samples were heat fixed, stained with crystal violet, iodine solution, 95% ethanol and safranin solution respectively for 60 sec each with a 5 sec wash interval with water (Madigan *et al*., 1998). Samples were air dried and visualised under a compound microscope (Nixon YS100). Recipes of stains and a detailed protocol are outlined in Appendix C.

Tape mounts

Fungal isolates were identified morphologically by using a scotch tape of approximately 1cm in length. The sticky end was placed over the fungal culture to pick up mycelia and other reproductive structures of fungi. This was then placed upwards on a microscope slide. A drop of Trypan Blue in lactogylcerol was added and coverslips placed over the slide which was followed by visualisation under the compound microscope (Harris, 2000). Reproductive structures were examined and identified according to Barnett (1962). Other fungal samples that could not be identified using this method were subjected to molecular identification.

2.5.1.2 Molecular identification

DNA extraction from bacterial isolates

This was conducted for bacterial isolates obtained from the selective media to identify isolates to a genus or species level. Genomic DNA extraction from pure bacterial cultures was carried out according to a procedure outlined by Moore *et al*., (1987). Bacterial isolates were grown overnight in nutrient broth cultures. A volume of 1 ml was taken from each of the cultures and centrifuged (Heraeus Instrument) in sterile 1.5 ml microcentrifuge tubes at 13000 rpm for 2 mins. The supernatant was discarded and the pellet was re-suspended in 567µl Tris EDTA extraction buffer (10mM Tris-HCl, 1mM Sodium ethylenediamine acetic acid (pH 8.0) and 30µl, 10% sodium deodecyl sulphate (SDS). Subsequently, 3µl of 20 mg/ml proteinase K (Promega Cat no.V302B) was added to inactivate any enzymatic activities. This reaction was mixed and incubated for 1hr at 37°C.

A 100µl volume of sodium chloride (NaCl) was added and mixed thoroughly. This was followed by the addition of 80µl of Cetytrimethylammonium bromide/sodium chloride mix (1% CTAB / 0.7M $NaCl^-$) and incubated for 10 mins at 65°C. After incubation, an equal volume of chloroform/isoamyl alcohol (24:1) was added and the mixture was centrifuged for 5 mins at 13000 rpm. The aqueous phase was transferred to a sterile 1.5 ml microcentrifuge tube and an equal volume of phenol/chloroform/isoamyl alcohol (23:24:1) was added from which the aqueous phase was once again obtained. This stage denatured the proteins and kept them soluble in the organic phase, while the nucleic acid was suspended in the aqueous phase. The DNA was then precipitated with 0.6 vol of isopropanol. Pellets were washed with ice cold 70% ethanol, dried at room temperature and re-suspended in 100µl TE buffer to elute the DNA. Samples were stored at -20°C (Moore *et al*., 1987).

DNA extraction of fungal isolates

A section of the mycelial growth on the plate culture was scraped off and put into microcentrifuge tubes containing 500µl of water. Extraction method followed was modified from Lee *et al*., (1988). Samples were vortexed for 2 mins and centrifuged at 13000 rpm for 5 mins and the supernant decanted. Three freeze thaw cycles were performed with liquid nitrogen and samples were crushed in 2% CTAB with the aid

of a sterile micro pestle to ensure effective lysis of cells. Proteinase K (20µl 2 mg/ml) was added and allowed to stand at room temperature for 15 mins. Samples were incubated at 65°C for 45 mins with intermittent vortexing every 15 mins. Chloroform (400µl) was added after incubation, vortexed and centrifuged at 13000rpm for 5 mins. The aqueous phase was removed and precipitated with 800µl isopropanol overnight at -20°C. Pellets were washed with ice cold 70% ethanol, dried at room temperature and re-suspended in 100µl Tris EDTA buffer, which was stored at -20°C (Lee *et al.*, 1988).

2.5.2 Determination of the unculturable microbial populations in the rhizosphere

DNA extraction directly from soil samples

DNA was extracted directly from soil samples using a combination of protocols (Kauffman *et al.*, 2004; Yeates *et al.*, 1998; Zhou *et al.*, 1996). This was to ensure efficient cell lyses and an amplifiable extract. Soil (1g) was mixed with 2.7 ml extraction buffer (100mM Tris- HCl, pH 8.0, 100mM sodium EDTA, 1.5M NaCl, 1% CTAB) plus 1g of sterile glass beads (2mm diameter) in a 50 ml centrifuge tube. The mixture was vortexed for 5 mins and subjected to three cycles of freezing in liquid nitrogen and thawing in a water bath at 67°C was performed to lyse the cells. Proteinase K (20µl) was added and the centrifuge tube shaken horizontally at 37°C for 30 mins. Subsequently 20% SDS (300µl) was added and samples were incubated at 67°C for 2hrs. After ultra-centrifugation (Beckham) at 6000 rpm for 10 mins, supernatant was transferred to clean sterile centrifuge tubes. The pellet was then rinsed with 900µl extraction buffer and 100µl 20% SDS. Mixture was vortexed for 2 mins, incubated at 67°C for 10 mins and centrifuged at 6000 rpm for 5 mins. The supernatant was combined with the previous extraction and mixed with equal volume of 30% polyethylene glycol and 1.6 M NaCl. This was incubated for 1 hr at room temperature to precipitate DNA away from proteins and polysaccharides. The supernatant was decanted after centrifugation and the pellet was re-suspended in 100µl TE buffer and 150µl 7.5 M potassium acetate. Transferred supernatant was treated with an equal volume of phenol/chloroform (1:1). The aqueous phase containing the DNA was transferred to clean tubes and treated with an equal volume of chloroform/isoamyl alcohol (24:1). The resultant DNA was then precipitated with

0.6 vol of isopropanol and left at -20°C overnight. The precipitate was pelleted and washed with ice-cold 70% ethanol, air dried at room temperature and re-suspended in 100µl TE buffer. Samples were stored at -20°C (Kauffman, *et al*., 2004).

Optimisation of soil DNA extraction protocol

The optimisation of the combined protocol was done at two stages. The first stage was at the beginning of the extraction whereby 1g of soil was placed in a sterile mortar and crushed with liquid nitrogen. This was done to crush cells in the soil prior to further extraction with extraction buffer and glass beads. The second stage of optimisation was after re-suspending of the pellet in TE buffer, the potassium acetate and chloroform stages were left out. To remove inhibitory substances a Qiagen Dneasy® Plant Mini kit (Cat No. 69104) was used. A sample (500µl) from the previous extraction stage was mixed with 150µl AP2 buffer in a microcentrifuge tube to further precipitate detergents, proteins and polysaccharides. Samples were incubated on ice for 5 mins and centrifuged for 3 mins at 1300 rpm. Supernatant was transferred into a QIAshredder spin column that removes most precipitates and cell debris. Samples were centrifuged at 1300 rpm for 2 mins and eluate (400µl) transferred to clean tubes. Buffer AP3 (600µl) was added and mixed by inverting tubes for DNA precipitation. An aliquot (500µl) was put into a DNeasy column and spun for 1 min at 1300 rpm. The flow through was discarded and this step was repeated with the remaining 500µl sample. Subsequently, the column was washed with 500µl AW buffer containing ethanol and the flow through discarded. To remove traces of ethanol as this may interfere with subsequent reactions, columns were centrifuged for 1 min. Columns were transferred to clean microcentrifuge tubes and 50µl preheated (65°C) AE elution buffer was added. Tubes were incubated on ice for 20 mins and then centrifuged for 1 min. This step was repeated with 50µl AE buffer to give a final volume of 100µl. Samples were stored at -20°C.

Polymerase chain reaction amplification of bacterial genes

Extracted DNA from plate isolates and directly from soil samples followed the same PCR conditions. Amplification of the 16S rDNA bacterial genes was carried out using the universal bacterial primers, GM5F and R907 (Table 2.7). A reaction volume of 50µl with 3-5µl template DNA was carried out. The reaction contained 0.25ul/1.25

units *Taq* polymerase (Promega Cat no. M166B), 5µl 10X buffer (Promega Cat no. M188J), 0.2mM Promega dNTPs, 1.75-3.5 Mm $MgCl_2$, primers (0.4 mM) and 3µl of 3 mg/ml Bovine Serum Albumin (Sigma Cat no. 015K0561). Amplification was performed on a MJ Mini Personal Thermal Cycler (Bio-Rad) using the conditions in Table 2.3. The use of different annealing temperatures in decreasing order was due to the high Tm variation between primers (Table 2.7).

Table 2.3 PCR cycling conditions used for the amplification of 16S rDNA.

Steps	Conditions	Temperature (°C)	Time (s)	Cycles
Initial Step	Initial denaturing	94	120	1
Step 1	Denaturing	94	30	4
	Annealing	64	45	
	elongation	72	120	
After step 1, subsequent annealing temperatures were decreasing in the order 62, 60, 58, 56°C for the same number of cycle. The last cycle before the final step (54°C) was for 12 cycles.				
Final Step	Final elongation	72	240	1

PCR products were electrophoresed with a 100bp-1kb molecular marker on an ethidium bromide stained (0.1µg/ml) agarose gel (1% wt/vol agarose) at max 120V for 45 mins. Gels were visualised using a Uviprochem Transilluminator and a digital image was recorded.

PCR amplification of fungal genes

Amplification of the internal transcribed spacer (ITS) region of the fungal gene was carried out using universal fungal primers ITS1F and ITS4 primers (Table 2.7). A similar reaction protocol to the bacterial PCR mixture was used. The reaction was run on MJ Mini Personal Thermal Cycler (Bio-Rad) using the following conditions: initial denaturing at 94˚C for 2 mins, 35 cycles (94˚C for 30 sec, annealing at 47˚C for 30 sec, elongation at 72˚C for 60 sec) and a final elongation of 72˚C for 7 mins. Analysis of PCR products was conducted as previously stated above.

Purification of DNA

PCR product to be used for sequencing and cloning were purified using the Wizard SV gel and PCR clean up kit (Promega Cat no. A9281). DNA was purified by the gel incision and centrifugation method as instructed by the manufacturer. Incised gel was placed in micro-centrifuge tube and an equal amount of membrane bind solution was added (10µl solution per 10 mg gel slice). This was incubated at 65˚C for 2 mins to dissolve the gel. Dissolved gel mixture was transferred to a minicolumn assembly, allowed to stand for 1 min and centrifuged at 13,000 rpm for 1 min. Columns were washed with 700µl membrane wash solution, centrifuged to remove flow through, after which samples were eluted to a final volume of 50µl with the nuclease free water provided and stored at -20°C.

2.5.2.1 Sequencing and analysis

Cleaned PCR products were sent for sequencing to the Rhodes University Sequencing Facility. The product was sequenced using ABI Prism BigDye Terminator v3.1 Ready Reaction Cycle Sequencing kit (Cat no. 433619) according to manufacturer's instructions with the primer pair: GM5F and R 907 for bacteria, ITS 1 and ITS 4 for fungi samples. The sequences were visualised by electrophoresis on an AB3100 Genetic Analyser (Applied Bioinformatics). Sequences were aligned using Bioedit software (Hall, 1999) and submitted for comparative analysis to the National Centre for Biotechnology Information (NCBI) online standard Basic Local Alignment Search Tool (BLAST) program (Wheeler *et al*., 2006). The significant level of similarities with 16S rDNA and ITS sequences in the Genbank database was determined by noting the percentage identity and the expectation value (E-value). The reliability of the percentage identity was related to the expectation value (E-value). The E-value shows the equivalent or similarity of a number of alignment score to the raw alignments score that are expected to occur in a database; the lower the E-value the more significant the score (Wheeler *et al*., 2006). A significant similarity value of >95% and >98% was accepted in this study as belonging to the same genus and species, respectively.

2.5.2.2 Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis Analysis

DGGE analysis on the bacterial 16S rDNA genes was carried out using 10µl of PCR product obtained from DNA extraction directly from soil. Gels (5.4 ml final volume) contained 40% (wt/vol) polyacrylamide (37:1 acrylamide/bis-acrylamide) (Sigma Cat no. A7168), 10X Tris/acetic-acid/EDTA (TAE) buffer, 20µl 20% (wt/vol) ammonium perosulphate (Biorad Cat no.1610700) and 4µl N,N,N,N, Tetramethylethylenediamine (Sigma Cat no. T7024). The denaturant gradient was from 35% to 80%, where 100% denaturing acrylamide was defined as containing 7M urea (Sigma Cat no. 2003155) and 40% (vol/vol) formamide (Sigma Cat no. F9037). This gradient was chosen due to the nucleotide size obtained from the PCR amplification (Atkins, 2005). The gradient allows nucleotides sequences to separate based on the G-C content. All DGGE analyses were electrophoresed in a DCode System (Bio-Rad) with preheated (60˚C) 1x TAE for 10 mins at 200V, as a pre-run and later at 180V for 2 hrs after loading samples in wells. Gels were stained in 1x TAE containing 50µl of ethidium bromide for 15 mins. This was rinsed with water and using a UV Transilluminator visualized.

2.5.2.3 Cloning, ligation and transformation

Purified bacterial DNA samples were cloned using PGEM -T Easy Vectors Kit (Promega Cat no. A1360). Ligation and transformation was carried out according manufacturers instruction. Ligation reaction contained 1ul PGEM -T, 5µl, 2x ligation buffer, 1µl, T4 ligase and 4µl of purified DNA. Positive and negative controls were also used to access the performance of the PGEM -T Easy Vector system. For the positive control a control insert DNA supplied with the kit was used, while for the negative control no insert was added. This was left over night or longer for optimal ligation. Transformation into recombinant plasmids was achieved with laboratory prepared competent cells using a DH α *E. coli* colony (Appendix D). Transformation was conducted by transferring 2µl of ligation mixture into sterile 1.5 ml microcentrifuge tube on ice. Thawed competent cells (50µl) were added and the tubes flicked to mix. Tubes were incubated on ice for 20 mins after which a preheated water bath was used to heat shock cells at exactly 42ºC for 45-50 sec. Tubes were

immediately returned to ice and 900µl of SOC medium added to tubes containing transformed cells and ligation reaction. This was incubated for 1.5 hrs at 37ºC for cell growth. A volume of 100µl of the transformation culture was plated onto Luria Bertani Agar (Biolab 1023845) which contained Ampicillin (100mg/ml), 0.1M isopropyl-beta-D-thiogalactopyranoside (IPTG) (Promega Cat no. V3955) and 50µg/ml 5-bromo-4-chloro-3-indolyl-b-D-galactoside (Xgal) (Promega Cat no. V3941). Refer to Appendix E for all cloning recipes.

Screening of transformants was conducted by selecting white colonies at random and growing colonies overnight in LB broth cultures containing ampicillin (5µl Amp/5 ml of cultures) at 37˚C. With the use of experimental control, transformation was determined as whether it was sub-optimal or failed. To isolate the plasmid from the broth cultures a Smart Prep Plasmid Isolation kit (Qiagen 27104) was used according to the manufactures instructions. Plasmid extracts were stored at -20ºC pending confirmation of inserts and correct size. To confirm the size and presence of an insert, a plasmid PCR condition (Table 2.4) using primers M13 forward and M13 reverse (Table 2.7) as well as a restriction digest with *Eco*R 1 (Table 2.4) was conducted. These primers bind symmetrically to the PGEM-T Easy Vector immediately on both sides of the plasmid 249-2972 binding site of pUC/M13 forward and 176-197 pUC/M13 reverse (Promega, 2005). This means that the primers would only amplify a section of the plasmid and the insert.

Table 2.4 PCR reaction mixture as used for plasmid amplification of cloned samples.

Component	Volume per 15µl of reaction	Final concentration
Water	6.275	-
10X buffer	1.75	1x
DNTP mix, 2mM each	1.35	0.2mM each
M13 forward	1.75	1.5µm
M13 reverse	1.75	1.5µm
25mM $MgCl_2$	1	1.75mM
Taq polymerase	0.12	1.25/2.0U/50µl
Template	2	

Amplification was performed on a MJ Mini Personal Thermal Cycler (Bio-Rad) using the following conditions: an initial denaturing at 95˚C for 2 mins, 35 cycles (94˚C for 30 sec, 55˚C for 1 min, 72˚C for 2 min 30 sec) and a final elongation of 72˚C for 5 mins. PCR products were electrophoresed with a 100bp-1kb molecular marker on an ethidium bromide stained (0.1µg/ml) agarose gel (1% wt/vol agarose). Inserts with correct size of approximately (600-680 bp) were then sequenced (Section 1.5.2.1) using the plasmid extracts and the universal bacterial primers GM5F and R907 (Table 2.7). Nucleotide sequences of clones were BLASTed on NCBI website for closely related matches.

2.5.2.4 Restriction digest

Products from cloning and purified PCR products were digested using three restriction enzymes that have sites for the following recognition nucleotide sequences (Table 2.5). Restriction enzymes are proteins that cut double stranded DNA at very specific locations. Each restriction enzyme recognises a very specific DNA sequence before cutting the DNA. The enzyme recognition sites are usually the same sequence on both strands but in the reverse direction (Table 2.5). Hence it either recognises the 5´-3´ sequence or vice versa. Reactions were carried out as outlined in Table 2.6.

Table 2.5 Recognition sequences of the restriction enzymes used for restriction fragment length polymorphism.

Enzyme	Sequences
*Eco*R 1 (Promega Cat.no. R6011)	5′ G▼AATT C 3′ 3′ C TTAA▲G 5′
Pst 1 (Promega Cat no. R6111)	5′ C TGCA▼G 3′ 3′ G▲ACGT C 5′
Hinc II (Promega Cat no. R6031)	5′GT(T/C)▼(A/G)AC 3′ 3′CA(A/G)▲(T/C)TG 5′

▲ Indicates excision points

Table 2.6 Restriction digests parameters used in RFLP analysis of plasmid extracted DNA samples.

Component (20µl volume)	**Single digest**	**Triple digest**
Buffer	*2µl*	*2µl*
Enzyme(s)	*1µl*	*0.5µl each*
Distilled water	*13µl*	*12.5µl*
Template	*4µl*	*4µl*

In each reaction tube, 0.2µl BSA was added and samples were incubated at 37˚C for 1hr. Reaction mixture (8µl) was run on ethidium bromide stained agarose gel (1% wt/vol) and electrophoresed with 1kb molecular marker (Sbrana *et al*., 2002). Visualization was done using the UVprochemi Transilluminator. Agarose gel was analysed based on the comparison between known restriction patterns of identified bacterial isolates and also with unknown bacterial cultures from plates. Band size was determined using a molecular ladder.

All nucleotide sequences obtained from the molecular identification of pure cultures from selective media and clones obtained directly from soil extractions were aligned (Appendix F) together with closely related sequences obtained from the Genbank. These nucleotide sequences were used to construct a cladogram that highlights similarities between sequences. The intention was not to determine genetic or phylogenetic resemblance but rather to determine the similarity between sequences obtained through culture dependent and culture independent techniques.

Table 2.7 Primers used in the study for the amplification of 16S rDNA, ITS regions and plasmid inserts.

Primer	Sequence	Tm	Reference
GM5F	5'-CCTACGGGAGGCAGCAG-3'	58.2	Muyzer *et al.*, 1995
R907	5'CGCCCGCCGCGCCCCGCGCCCGTCCCGCCG CCCCCGCCCGCCGTCAATTCCTTTGAGTTT-3'	81.8	Muyzer *et al.*, 1995
ITS1F	5'-CTTGGTCATTTAGAGGAAGTAA-3'	49.7	Gardes and Bruns, 1993
ITS4	5'-TCCTCCGCTTATTGATATGC-3'	52.1	White *et al.*, 1990
M13 forward	5'-GTTTTCCCAGTCACGAC -3'	57.1	Promega, 2005
M13 reverse	5'-AGCGGATAACAATTTCACACAGG-3'	60.9	Promega, 2005

2.6 Arbuscular mycorrhizal fungal population assessment in soils

2.6.1 Spore extraction and enumeration

Soil samples collected from each treatment plot from both field Sites were sieved through a 2mm sieve to remove large debris. Air dried sub sample (100 g) was taken from each sample and placed in a 500 ml beaker containing 200 ml 0.08 M sodium hexametaphosphate solution to break up clay clumps. The suspension was agitated for 5 mins and left to settle for 15 secs (Smith and Dickson, 1997). The supernatant was decanted through a nest of sieves with reducing mesh sizes from 425µm, 250µm, 125µm to 45µm. This step was repeated with water twice and the debris from the 425µm was discarded. The debris on the remaining sieves, containing the AM spores was washed into 50 ml centrifuge tubes for purification. The spore suspension was centrifuged (J.P. Selecta S.A) at 3000 rpm for 5 mins, after which the supernatant was discarded. The pellet was re-suspended in 60% sucrose solution and centrifuged for another 5 mins. The supernatant containing AM fungal spores was decanted into a 45µm sieve and washed with water to remove sucrose on the spores (Smith and Dickson, 1997).

Spore enumeration

Spores were then washed onto a 9 cm grided filter paper disc (Whatman #1) in a Buchner funnel and vacuum filtered. The filter paper was transferred to clean Petri dish lids and enumerated. AM fungal spore enumeration included both dead and viable spores, although every attempt was made to count only healthy looking spores. Spores were recorded as representatives of AM fungal species present in 100g of sample (Smith and Dickson, 1997). This was done using a dissecting microscope, Leica S4E.

2.6.2 Root colonisation

Root samples from pot trials, Site 1 and Site 2, were carefully washed and cut into 1-3cm sections. Sections were covered with 5% KOH solution and incubated at 90°C for 45 mins, to remove the cytoplasm and all coloured material from the plant cells. The KOH solution was discarded and the roots were rinsed well with distilled water. Roots were covered with a freshly prepared alkaline H_2O_2 solution (Appendix G) to bleach for 60 mins. The bleaching solution was discarded and the roots were rinsed with water. Roots were acidified in a 0.1M HCl solution overnight to ensure adequate binding of stain to fungal structures. The HCl solution was discarded and roots were covered with Lactoglycerol Trypan Blue (0.05%) stain and incubated for 45 mins at 90°C. The stain was poured off and roots were covered with lactoglycerol destain (Appendix G). Roots were allowed to destain overnight before microscopic examination (Smith and Dickson, 1997). Finally, roots were mounted on microscopic slides and using a compound microscope examined. The percentage root colonisation was calculated using a modified Line Intersect Method (McGonigle *et al.,* 1990). This method involved squashing segments of stained roots on a microscope slide after it is covered with a cover slip. Roots were selected and examined for their entire length, a field of view at a time. One field of view using 40 x magnifications was scored at intersects between an eyepiece micrometer with or without mycorrhizal structures (arbuscules, vesicles or hyphae). The number of mycorrhizal structures in a 100 fields of view was equal to the percentage colonisation.

2.6.3 Glomalin extraction and quantification

Glomalin, a glycoprotein commonly produced by AM fungi was extracted from soil (1g) samples collected from trial plots as described by Wright and Upadhyaya (1996; 1998). The total protein extraction was carried out with 8 ml, 50mM sodium citrate, pH 8.0 at 121˚ C for 60 mins. Samples were centrifuged immediately at 5000g for 15 mins and were repeated twice. Supernatant containing protein was removed and stored at 4˚C for quantification.

Bradford assay

Concentration of glomalin (mg/ml) was determined with the Bradford assay, using Bovine Serum Albumin as a standard (Bradford, 1976). BSA stock solution was prepared by dissolving 16mg of BSA (Sigma Cat no. A3294) in 10 ml triple distilled water. Standard concentrations ranging from 0.1-1.6 to a final volume of 160µl (Appendix H) were prepared using stock solution and water. A volume (5µl) of standard, blank (water) and test samples were put on microtitre plates, after which 250µl of Bradfords reagent (Sigma Cat no. B6916) was added into each wells and left for 15 mins. Spectroscopy readings were taken at 595nm using a Power Wave X Spectrophotometer (Biotek). Readings from the test sample were compared to the standard curve (Appendix H), which is known to exhibit a linear relationship (Bradford, 1976). Concentrations were calculated using the equation $y = ax + bc$ derived from the standard curve (Appendix H), where x gives the value of the unknown concentration.

2.6.4 Most Propable Number

An average most probable number (MPN) was conducted using soil samples from treatment and control plots of each Site. Procedure followed was that of Smith and Dickson (1997). It involved a series of ten-fold soil-soil dilutions, using test soil and pasteurised soil. Each dilution had five replicate plants. A 10^{-1} dilution was made by adding 200g of test soil to 1800g of sterilized soil in a plastic bag. Samples were mixed thoroughly and 200g were removed for the next dilution (10^{-2}). The remaining soil sample was put into torpedo tubes that were pre-washed in 30% sodium hypochloride. The next dilution (10^{-2}) was made by using the 200g from the previous

dilution and mixing it with 1800g of sterilized soil. 200g from the mixture were also kept for the next dilution. This was repeated until the 10^{-4} dilution. Seedlings (Sorghum grain), used as trap plants, were surface sterilised in 30% sodium hypochloride for 20 mins. The seeds were then rinsed thoroughly with water and planted into the tubes starting from the highest dilution. Seeded trays were kept in a plastic tunnel where they were watered daily with UV sterilised water for 4 weeks. Plant roots were stained (section 2.6.2) and examined for the presence or absence of AM fungal colonisation under a compound microscope. A plus was given when any mycorrhizal structures were found in the entire root system.

MPN infectivity assessment

The MPN readings were calculated using equations based on the probability theory from the number of root systems in dilutions made above i.e. 10^{-1}- 10^{-4} (Smith and Dickson, 1997). From the results, the P values (P1, P2 and P3) were obtained. Where P1 was the number of mycorrhizal plants in the least concentrated dilution that had the greatest colonisation; P2 and P3 was the number of plants with mycorrhizal colonisation in the next two higher dilutions. Using the probability table (Cochran, 1950), the row to which the observed P1 and P2 corresponds to P3 was taken as the MPN of propagules in that compartment. The value was then multiplied by the dilution to give the MPN per 200g of soil.

2.7 Alternative host plants from around the mine area

Plants growing around the mine area at Makana Brick and Tile were collected using a hand spade to uproot whole plants. The shoots were separated from root materials and were stained individually following procedure in section 2.6.2. Stained roots were observed for presence of arbuscules, vesicles, hyphal coils, extra-radical hyphae and other mycorrhizal structures (sheath, mantle, Hartig net). These mycorrhizal features were grouped either into an AM fungal (*Arum* – linear hyphae in between root cortical cells or *Paris* – hyphal coils in the root cortical cells) type of colonisation or into endomycorrhizal type of colonisation (hyphal coils or intercellular hyphae with no trace of vesicles or arbuscules). Shoots were dried and taken to the Selmar Schonland

Herbarium, Grahamstown for identification. Identified plants were classified into their respective families, flowering season and habitat (Goldblatt and Mannings, 2000; Martin and Noel, 1960; Levyns 1929).

2.8 Statistical analysis

To normalise data sets, data were log transformed if expressed in CFU/g. A repeated measure Analysis of Variance (ANOVA) with 2 way effects (Zar, 1999) was used for all data sets that were obtained over a period of time. This type of analysis was chosen under the premise that members of a random sample were measured under different conditions and the dependent variable (i.e. treatment) was measured repeatedly (Statsoft, 2005; Lutgen *et al*., 2003). The use of standard ANOVA in this case did not seem appropriate because it failed to correlate the repeated measure (measurement of treatment over time) simultaneously. Comparisons between Sites were analysed using two-way ANOVA. For analysis of microbial and mycorrhizal population in Limestone Hill farm a one-way ANOVA was used due to the once off sampling. Significant difference between group means was determined using Bonferroni test of significance. Due to the nature of this study, which was to gain extrapolative information on the effect of AM fungi on bacterial population numbers, the level of significance for ANOVA and Bonferroni test was 5%. All analyses were carried out by means of StatSoft, Inc. (2005) STATISTICA (data analysis software system) Version 7. www.statsoft.com.

CHAPTER 3

RESULTS

3 Results

3.1 Soil texture and nutrient analysis

To study the influence of land use and plant species on rhizosphere micro flora, two Sites with varying soil characteristics and plant types were compared (Table 3.1). From the soil analysis conducted by Eco Analytica laboratory, the mining Site (MBT) was observed to have higher cations (Ca, Mg, Na, K) concentrations than the farm Site (LHF). The MBT Site had a pH of 7.68 that is close to alkaline and the LHF Site an acidic pH of 4.93 (Table 3.1). Though the amount of nitrate and nitrite was not determined for the LHF Site, the amount of mineral nitrogen in the form of NH_4 was greater than that of the mining Site. Available P (Bray P) measured at the LHF Site was approximately 12 times greater than the amount found at the MBT Site. In terms of soil texture, which was analysed using a textural triangle, both Sites are regarded to have relatively high soil clay contents (Table 3.1).

Table 3.1 Chemical and physical characteristics of soil samples obtained from the field Sites: Makana Brick and Tile Pty and Limestone Hill Farm.

Site		**Makana Brick Tile Pty**	**Limestone Hill Farm**
Land use		Mining	Farming
Clay %		40	65
Silt %		16	0
Sand %		44	38
Textural sum		100	103
Soil texture		Clay loam	Clay
Nutrients (mM/L)	Ca	2.65	0.14
	Mg	5.00	0.14
	K	0.68	0.47
	Na	32.00	0.53
	NO_3	2.86	ND
	NO_2	0.01	ND
	P	0.01	ND
	NH_4	0.06	0.12
	%C	1.38	1.02
	Bray P (ppm)	5.87	72.58
pH		7.68	4.93

Values of each nutrient represents mean of three replicates. ND – Not determined

3.2 Pot trial analysis

Determination of fertiliser effects on AM fungi in a plastic tunnel was successfully achieved using *C. dactylon* seedlings (Fig. 3.1). To determine this effect, the shoot height, plant biomass and percentage root colonisation were analysed (Table 3.2; Fig. 3.2; Fig. 3.3). The maximum shoot height measured at 6 weeks was 9.82 cm in pots treated with Organic Tea + AM fungi. However, shoot heights of 16.36 cm and 17.45 cm were recorded at 12 and 28 weeks respectively from plants treated with 3:1:5 NPK + AM fungi. Analysis of the overall effect observed at 6, 12 and 28 weeks using a two-way ANOVA indicated that there was a significant effect (P = <0.05) of treatment and number of weeks on plant shoot height (Table 3.2, Fig. 3.2). Differences between treatments were mostly insignificant, with an exception between control pots (4.23 cm) treated with Organic Tea only at 6 weeks and pots treated with 3:1:5 NPK + AM fungi at 12 (16.36 cm) and 28 (17.45 cm) weeks. No significant difference between treatments was observed at 6 weeks for plant biomass. But, at week 12 a significant difference was recorded between pots treated with 3:1:5 NPK + AM fungi and other treatment pots during that period (Table 3.2, Fig. 3.3). At 28 weeks, the differences between treatments were insignificant.

The microscopic examination of stained *C. dactylon* roots sampled at 6 and 12 weeks had a colonisation percentage range of 0-10% for the treated and control pots. Colonisation became more evident at the 28th week with the majority of roots colonised by *Paris*-type hyphal coiling and formation of vesicles (Fig. 3.4). Although the differences between treatments were not clearly distinctive, the percentage root colonisation at 28 weeks showed a remarkable difference between treatments. From this result, there is an indication that the Organic Tea and 3:1:5 NPK fertilisers are capable of improving plant yield by 60% and 70%, respectively as measured by plant biomass (Fig. 3.3). However, in terms of their effect on AM fungi, the inorganic fertiliser (NPK- 3:1:5) was more compatible with the AM fungal inoculant and enhanced root colonisation (80%), while application of Organic Tea reduced colonisation to 65% (Fig. 3.5).

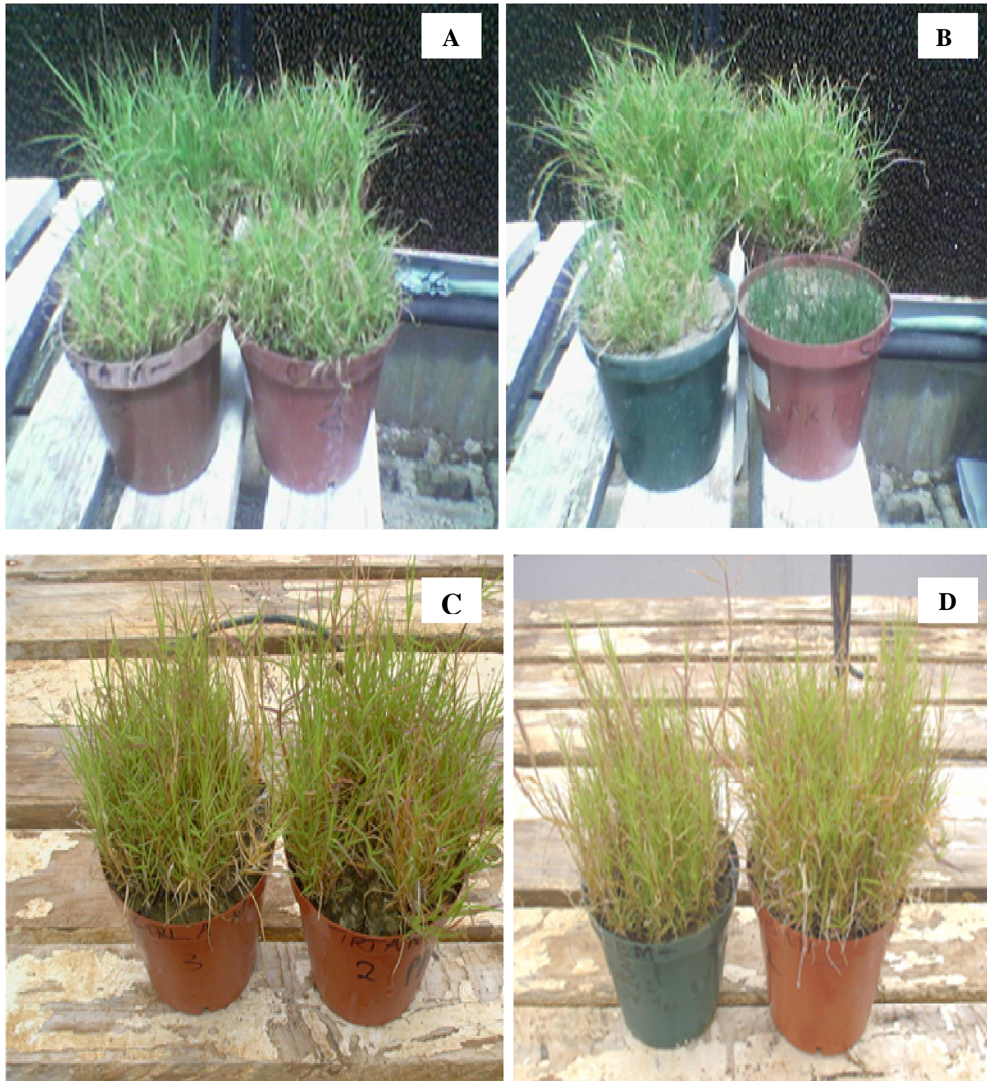


Figure 3.1 Pot trial of *Cynodon dactylon* plants at 6 and 28 weeks with varying treatments. **A**. *Cynodon* plants behind have been treated with Organic Tea + AMF; plants in front have been treated with Organic Tea only (6 weeks after planting). **B**. *Cynodon* plants behind have been treated with 3:1:5 NPK + AMF; plants in from have been treated with 3:1:5 NPK only (6 weeks after planting). **C**. From left to right plants were treated with Organic Tea only and Organic Tea + AMF. **D**. From left to right plants were treated with 3:1:5 NPK only and 3:1:5 NPK + AMF.

Table 3.2 Shoot height and plant biomass of *Cynodon dactylon* treated with Organic Tea and 3:1:5 NPK fertilisers either with or without arbuscular mycorrhizal fungal inoculum (AMF)**.**

Treatment	Shoot Height (cm)	Plant biomass (g)
6 week		
Organic Tea + AMF	9.82 ± 2.72 cd	4.26 ± 2.53 c
Control A (Organic Tea only)	4.32 ± 3.94 c	4.86 ± 4.28 dc
3:1:5 NPK + AMF	9.35 ± 6..35 bcd	5.60 ± 3.71 c
Control B (3:1:5 NPK only)	6.62 ± 2.68 cd	2.93 ± 0.85 c
12 week		
Organic Tea + AMF	11.15 ± 2.24 bc	7.56 ± 1.19 abcd
Control A (Organic Tea only)	7.86 ± 2.41 bc	6.86 ± 1.68 dc
3:1:5 NPK +AMF	16.36 ±2.47 ab	17.23 ± 4.12 a
Control B (3:1:5 NPK only)	11.23 ± 4.00 bc	6.23 ± 0.70 c
28 week		
Organic Tea + AMF	11.62 ± 1.48 bc	13.9 ± 6.64 abd
Control A (Organic Tea only)	13.80 ±2.57 bc	11.83 ± 3.65 abcd
3:1:5 NPK +AMF	17.45 ± 1.43 ab	17.49 ± 1.04 a
Control B (3:1:5 NPK only)	15.27 ± 3.15 bd	9.79 ± 1.91 abcd
***P* value/F value**		
Treatment	0.0005/4.8447	0.0089/8.2823
Weeks	<0.01/14.3948	<0.01/22.9598

Values are means of three replicates ± standard deviation. Means followed by same letters within columns are not significantly different at $P < 0.05$ by Bonferroni test.

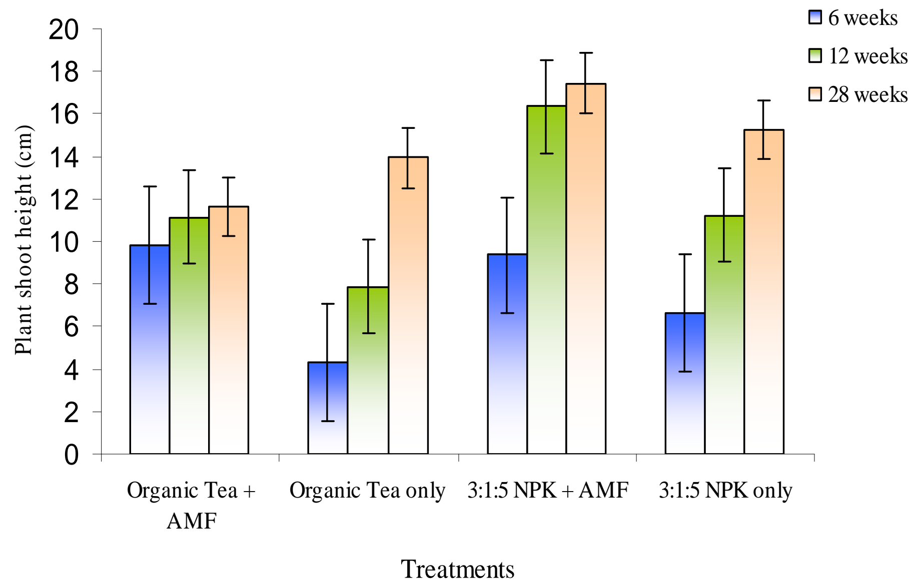


Figure 3.2 Plant shoot height of *Cynodon dactylon* treated with Organic Tea and 3:1:5 NPK fertiliser with or without arbuscular mycorrhizal fungal (AMF) inoculum. Bars represent means of three replicates ± standard deviations.

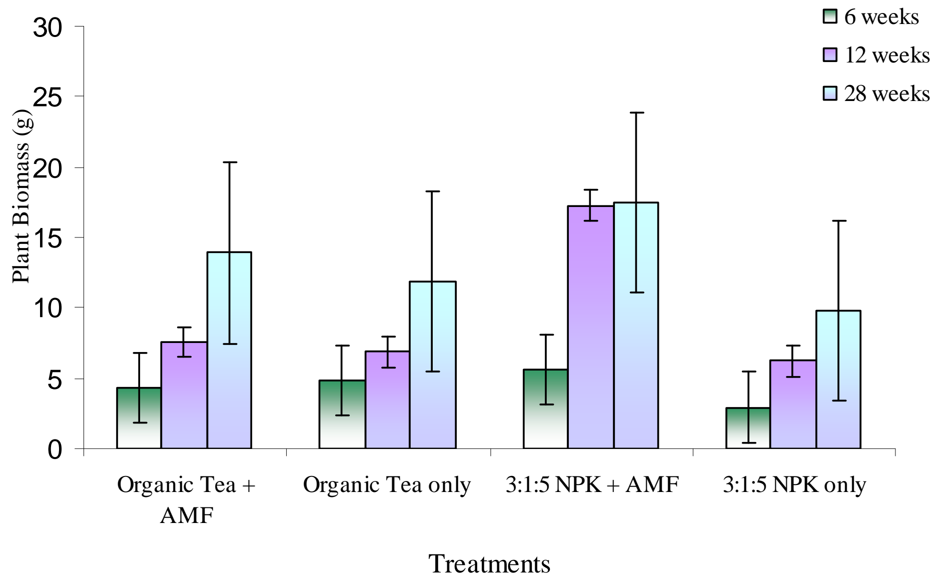


Figure 3.3 Plant biomass of *Cynodon dactylon* treated with Organic Tea and 3:1:5 NPK fertiliser with or without arbuscular mycorrhizal fungal (AMF) inoculum. Bars represent means of three replicates ± standard deviations.

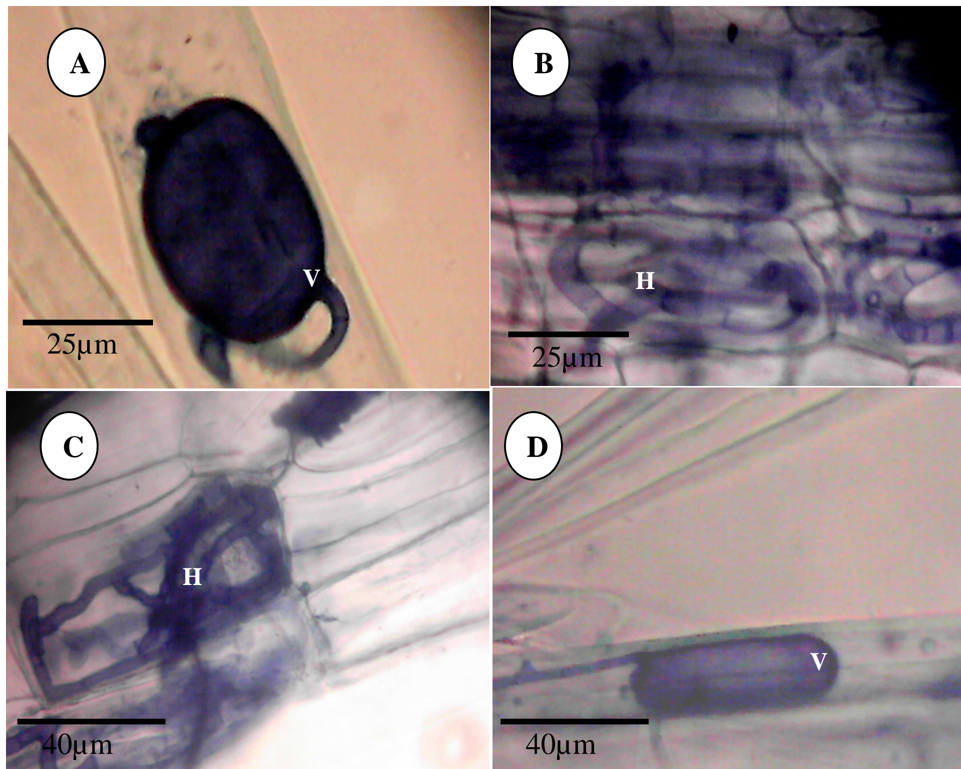


Figure 3.4 Colonisation of root cortical cells by arbuscular mycorrhizal fungi. All images were obtained from stained *Cynodon dactylon* root samples that were assessed for percentage root colonisation. **A** and **D** show vesicles (V) of different sizes, which shapes to the structural boundary of the cell. **B** and **C** show *Paris* type hyphal (H) coiling.

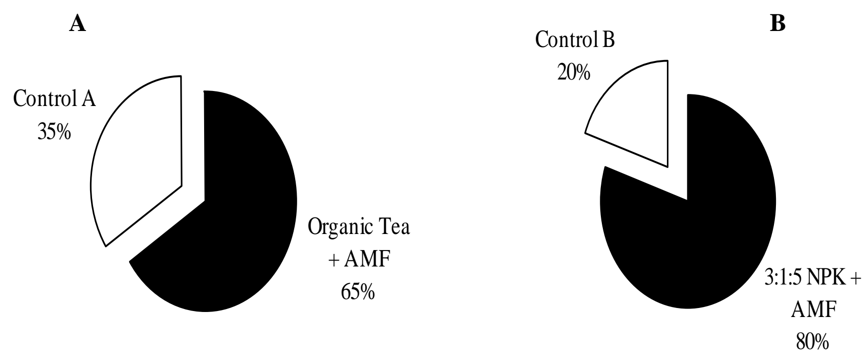


Figure 3.5 Percentage root colonisation of *Cynodon dactylon* at 28 weeks from pot trial. **A**. Organic Tea + arbuscular mycorrhizal fungal (AMF) inoculum and control with fertiliser only. Percentage values are mean of three replicates with ± standard deviations of 9.5 and 6.8 for treatment and control, respectively. **B**. 3:1:5 NPK with AMF inoculum and control with fertiliser only. Percentage values are mean of three replicates with ± standard deviations of 10.5 and 4.0 for treatment and control, respectively.

3.3 Microbial population analysis

3.3.1 Estimating the number of culturable microbial populations in the rhizosphere

In this study, greater attention was given to bacterial populations in the rhizosphere rather than to the fungal population, although preliminary data was recorded. The colony forming units (CFU) for the total bacterial population was carried out using Nutrient Agar (NA) and Tryptone Soy Agar (TSA). NA supported the growth of bacterial colonies slightly better than TSA medium by an average value of 7.08 (NA) to 6.95 (TSA) Log CFU/g soil though not statistically validated. The total culturable bacteria over time were detected on NA at 10^{-4} dilutions and analysed using repeated measure ANOVA (Fig. 3.6). The result indicated that the total microbial numbers remained stable in both treated and control plots throughout the sampling period at the MBT Site. From the univariate result at each period, there was a significant effect of treatment (P = 0.0152) on bacterial numbers at 9 months. But as determined by Bonferroni test of significance, there was no significant difference between plots treated with and without AM fungi at the same period of time. The simultaneous analyses of all sampling periods indicated a significant effect of time but with no significant effect of treatment (P = 0.6888) (Fig. 3.6). Comparing the bacterial numbers obtained at the MBT Site at 9 months to that at the LHF Site (once off sampling) after 5 months, a significant difference in bacterial numbers between the two Sites were observed statistically (Fig. 3.7). The MBT Site had a higher bacterial number of 7.08 Log CFU/g soil than the LHF Site (6.6 Log CFU/g soil) for both control and treated plots. But, the differences between treated plots with AM fungi and the control plots remained insignificant ($P > 0.05$) at both Sites. The interaction ratio of 0.725 of effects tested in Fig. 3.7 indicates that the effect of Site and treatment is independent of each other.

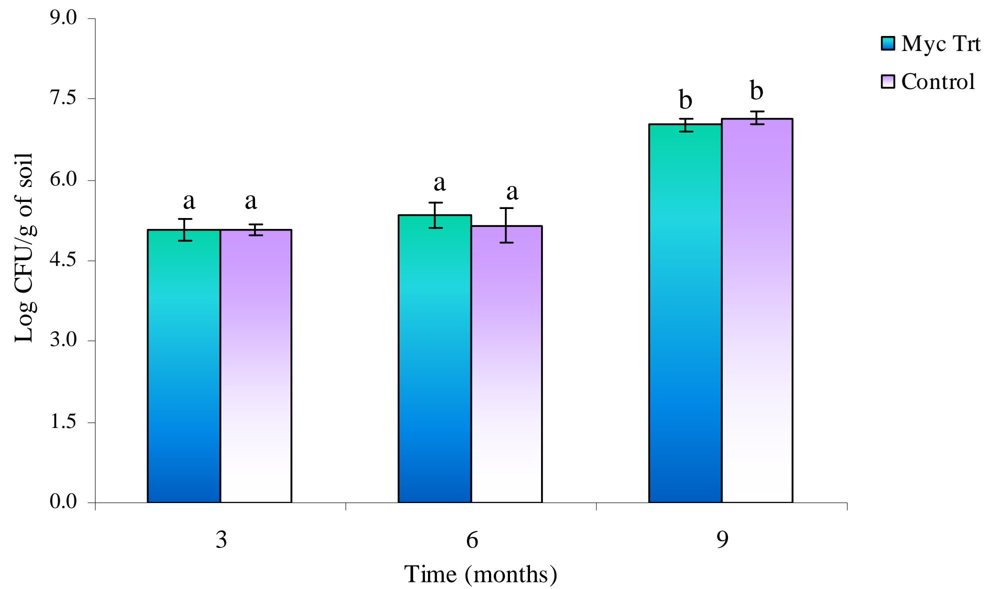


Figure 3.6 Total culturable bacteria from the rhizosphere of arbuscular mycorrhizal fungal inoculated and control plots at the mining (MBT) Site over time. Colonies counted were incubated for 4 days at 37°C on Nutrient Agar. Log CFU values are means of ten replicates, bars represents ± standard deviation. Means followed by the same letter are not significantly different at $P < 0.05$ by Bonferroni test. Significant probability value of overall time effect was $F_{2, 32} = 597.77$, $P = < 0.01$.

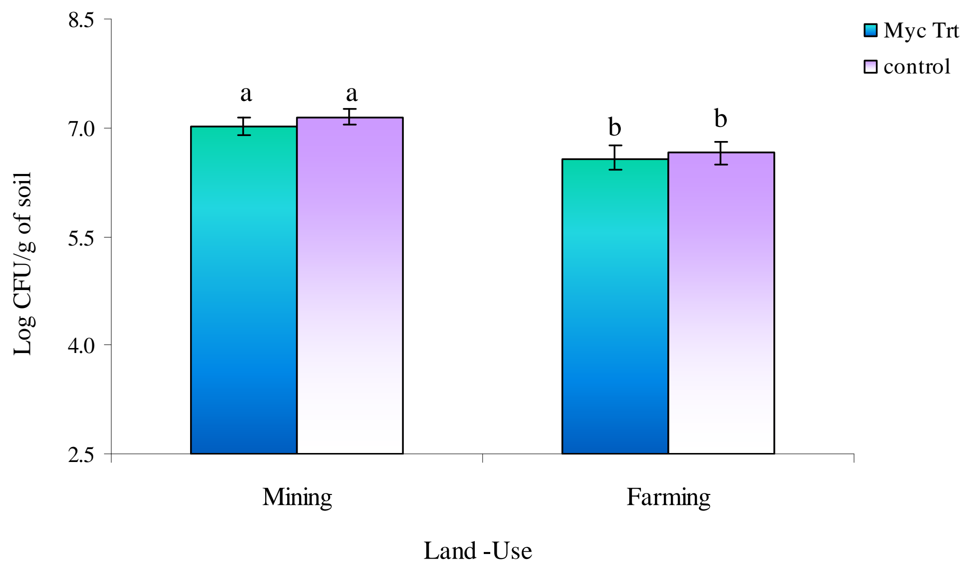


Figure 3.7 Total culturable bacteria from the rhizosphere of the sampled Sites indicating land usage at the MBT- mining (9 months) and LHF- farming (5 months) Site. Colonies counted were incubated for 4 days at 37°C on Nutrient Agar. Log CFU values are means of ten replicates, bars represent ± standard deviation. Means followed by same letter are not significantly different at $P < 0.05$ by Bonferroni test. Significant probability value of overall effects tested included: Treatment = $F_{1, 31} = 5.79$, $P = 0.0222$; Site = $F_{1, 31} = 106.47$, $P = 0.01$

From the MBT Site a total of 30 isolates were selected at random for Gram staining from NA plates at 3, 6 months and from both NA and TSA plates at 9 months stage of sampling. While from the LHF Site a total of 40 isolates were selected from NA and TSA plates. Once pure cultures of all isolates were established, Gram staining was conducted. The Gram staining of isolates was conducted to determine the phenotypic majority present in the soil. Thus, isolates were selected regardless of the treatment plots from where they were isolated. As much as possible, colonies with similar morphological features were avoided to ensure a wide range of bacterial species. The results (Table 3.3) indicated possible morphological changes in the bacterial population over time. The last stage of sampling at the MBT Site depicts Gram-positive rods (a 3 to 2 ratio) as being the dominant groups present. This could also be said for the LHF Site were the ratio of Gram-positive to negative was 6:4 with rods being the major phenotype.

Table 3.3 Phenotypic characteristic as determined by Gram staining of randomly selected bacterial isolates obtained from both mining and farming Sites.

Time (months)	No. of Gram-positive isolates	No. of Gram-negative isolates	Total Gram-positive and Gram-negative isolate	Lowest denomination ratio of Gram-positive and Gram-negative isolates	Rods:cocci: spirillum
MBT					
3	25	5	30	5:1	13:3:14
6	4	26	30	2:13	16:14:0
9	18	12	30	3:2	24:6:0
LHF	24	16	40	6:4	25:14:1

MBT- Makana Brick and Tile (mining)
LHF – Limestone Hill Farm (farming)

Determining specific functional groups in the rhizosphere

Colony forming units for the functional groups, P solubilisers, Actinomycetes, free-living N_2 fixers and pseudomonads were obtained at the 10^{-3}dilution. Seeing that the colonies were below the range (25- 300 colonies) that was used for statistical analysis as well as the zero values obtained indicating no growth, comparison between media and the effect of treatment was not analysed. Rather, the Log CFU/g for each treatment was graphically represented. On the NBRIP medium clearing zones of $CaPHO_4$ solubilisation were visible for fungal isolates. The bacterial isolates with clearing zones were sometimes difficult to determine until sub-cultured because this medium accommodated the growth of other organisms. BLM medium was selective for Actinomycetes as filamentous colonies growing on and inside the agar were observed. The majority of the actinomycete*s* isolated, when sub-cultured were non-fastidious growing organisms as they took a minimum of 4-5 days to grow. For the N_2A medium, the ability of bacterial isolates to grow on the nitrogen free media was an indication of the presence of free-living N_2 fixers that can utilise atmospheric nitrogen for growth. From Fig. 3.8, results showed that bacteria rather than fungi were in majority, with the free-living N_2 fixers and actinomycetes as the dominant functional groups. Differences in the microbial numbers in plots treated with mycorrhizal fungi and the control plots were not distinctive. At the LHF Site, the presence of P solubilisers in plots treated with AM fungal inoculum with none recorded from the control plots (Fig. 3.8) indicates that there could be a possible change in species composition, not necessarily representative in bacterial numbers. Similar numbers of actinomycetes, N_2 fixers and fungi were recorded in both Sites though not statistically validated. Slight differences between the two land use types were observed in the number of pseudomonads (Fig. 3.8). Though the fungal population was not given much attention it was observed from the preliminary analysis that they were present in an amount of 5.13 Log CFU/g soil, which was similar to the bacterial numbers (5.01 Log CFU/g soil) obtained during that period.

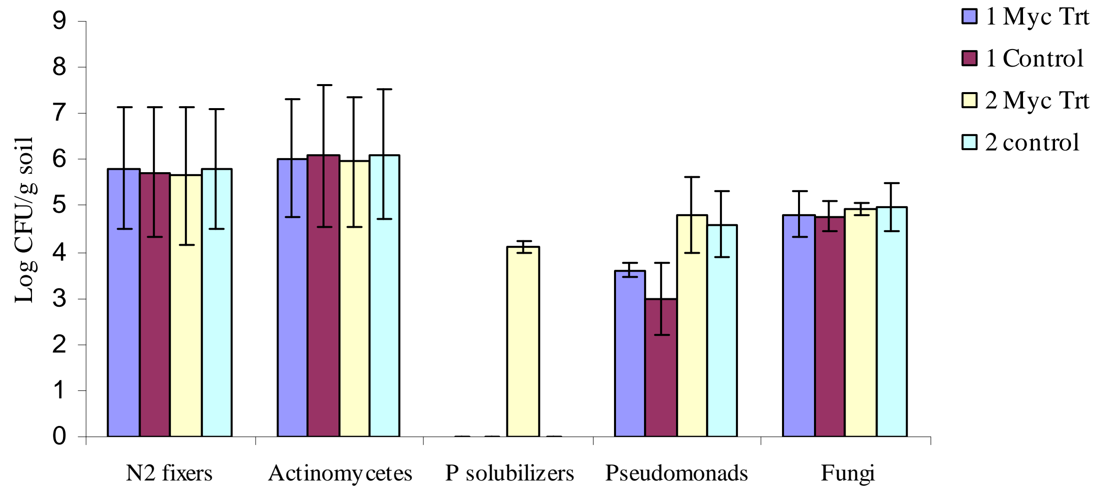


Figure 3.8 Total microbial numbers of different functional groups from replicate plots at the MBT (1) and LHF (2) Site ± SD at 10^3 dilutions. Log CFU values are means of ten replicates. Colonies counted were incubated for 4 days at 37˚C and 28˚C for bacteria and fungi, respectively.

From each functional group, representative isolates chosen for species identification were subjected to DNA extraction and PCR amplification, which were successful and efficient judging from the quality of bands obtained (Fig. 3.9). Isolates were coded as Act, PSA, N2A and NBP to represent functional group or the media from which the isolate was obtained. Letters A and B were used to denote control plots while letters C and D were used for the plots treated with AMF. Amplification of the 16S rDNA resulted in bands of between 500-700bp in size as visualised using agarose gel electrophoresis (Fig. 3.9). BLASTed nucleotide sequences of the bacterial isolates were closely related to species, which included actinomycetales bacterium, *Pseudomonas* spp., *Bacillus megaterium*, *Burkholderia* spp. *and Streptomyces* sp. (Table 3.4). Identity values of Isolate N2A A53 and NBP C33 were below the cut off, which was greater than 95% at the genus level and 98% for species level. Thus, identity of the two isolates is not conclusive. Fungi on the other hand, were morphologically identified using tape mounts and viewed under the compound microscope. Molecularly, the CTAB method was effective in the extraction of DNA from pure fungal cultures. Subsequent amplification of extracts resulted in bands of between 500-700bp (Fig. 3.10). Fungal isolates identified belonged to various genera that were either saprotrophic or potentially pathogenic (Table 3.5). Closely related species for the fungal isolates included 4 isolates of *Fusarium* spp., *Trichoderma*

harziarum, 3 isolates of *Ampelomyces* spp. and a rarely isolated soil fungi *Exserohilum rostratum* (Appendix F). The frequently isolated genera as determined morphologically, from the MBT Site were mainly *Cunninghamella* sp., *Aspergillus* sp., *Penicillum* sp. and *Cephalosporium* sp., while *Aspergillus* sp. and *Trichoderma* sp. were the majority at the LHF Site (Table 3.6). The genera *Cunninghamella*, *Aspergillus* and *Penicillium* were also found to be P solubilisers and had a high affinity to solubilise $CaHPO_4$ which was observed from the large clearing zones formed.

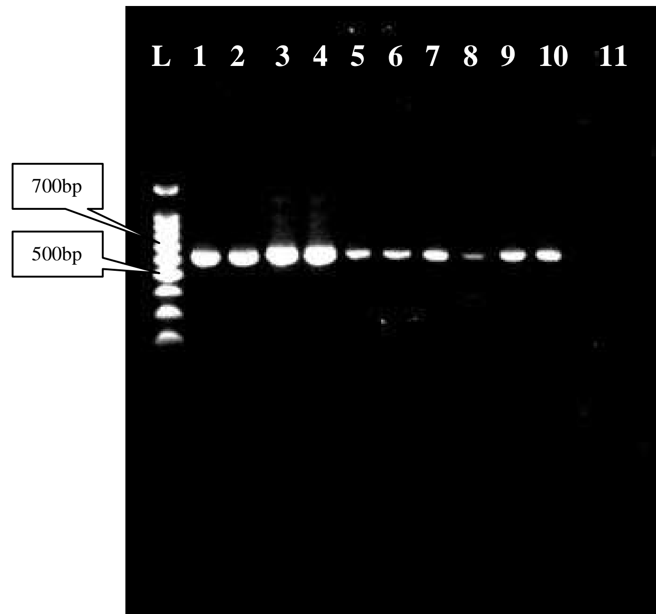


Figure 3.9 Ethidium bromide stained agarose gel (1%) showing PCR amplification of 16S rDNA of bacterial isolates obtained from the mining and farming Site on various selective media. L - molecular ladder (1kb), lanes 1- Act C29, lanes 2- Act C31, lanes 3 – Act C39, lanes 4 – N2A A52, lanes 5 – N2A A53, lanes 6 – PSA C41, lanes 7- PSA A1,lanes 8 – PSA D41, lanes 9 – NBP C31, lanes 10 – NBP C33, lanes 11 – control (water instead of template DNA). Act – Actinomycetes (BLM), N2A – free nitrogen agar, PSA – pseudomonads selective agar, NBP – phosphate solubilising agar. Code A and B denotes – control plots, C and D denotes – arbuscular mycorrhizal fungal inoculated plots. Figures are plate replicate codes.

Table 3.4 Partial sequence analyses on 16S rDNA gene of bacterial isolates obtained from different functional groups and their affiliation to related nucleotide sequences. Identity values >95% were regarded as being significant

Bacterial Isolates	**Closely related match identity (NCBI)**	**Site Isolation**	**% Identity**	**E-values**	**Accession No. of closely related sequences (NCBI)**
Actinomycetes					
Act C29	Actinomycetales bacterium	LHF	99	0.0	DQ144217
Act C31	*Streptomyces* sp.	MBT	95	0.0	DQ663172
Act C39	Unknown	MBT	-	-	-
Pseudomonads					
PSA C41	*Pseudomonas* sp.	LHF	98	0.0	DQ464386
PSA A1	*Pseudomonas fulva*	MBT	96	0.0	AM161143
PSA D41	Unknown	MBT	-	-	-
Nitrogen fixers					
N2A A52	*Bacillus megaterium*	LHF	98	0.0	DQ904608
N2A A53	*Burkholderia glathei*	LHF	90	0.0	AY154378
Phosphate solubilisers					
NBP C31	*Bacillus megaterium*	LHF	98	0.0	DQ904608
NBP C33	Burkholderiaceae bacterium	MBT	92	0.0	DQ490307

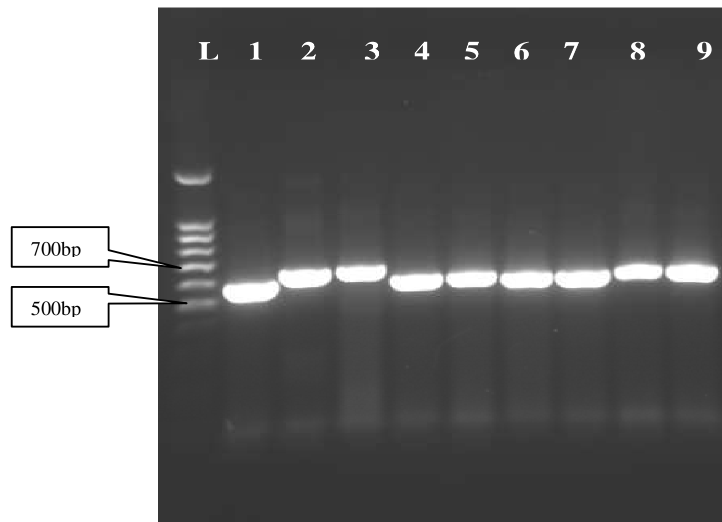


Figure 3.10 Ethidium bromide stained agarose gel (1%) showing PCR amplification of 18S rDNA of fungal pure cultures isolated from the mining Site and the farm Site, which could not be identified morphologically. L – 100bp molecular marker, lanes 1-A1, lanes 2– A4, lanes 3– A52, lanes 4– B1, lanes5- C31, lanes 6- C51, lanes 7- D11, lanes 8- D31, lanes 9- D53 fungal isolates. Code A and B denotes – control plots, C and D denotes – arbuscular mycorrhizal fungal inoculated plots. Figures are plate replicate codes.

Table 3.5 Partial sequence analysis on 18S rDNA gene of fungal isolates obtained from the mining and the farm Site with their affiliation to related nucleotide sequences. Identity values >95% were regarded as being significant

Fungal Isolate	Closely related match identity (NCBI)	Site Isolation	% identity	E-value	Accession No. of closely related sequences (NCBI)
A1	*Ampelomyces* sp.	MBT	99	0.0	AY207317
A4	*Exserohilum rostratum*	MBT	99	8e-62	AJ853741
A52	*Trichoderma harziarum*	MBT	96	0.0	AF443913
B1	*Fusarium oxysporum*	LHF	99	0.0	DQ459007
C31	*Ampelomyces* sp.	LHF	99	0.0	AY513942
C51	*Fusarium* sp.	LHF	95	0.0	DQ166549
D11	*Ampelomyces* sp.	MBT	99	0.0	AY513942
D31	*Fusarium* sp.	MBT	94	0.0	DQ166549
D53	*Fusarium* sp.	LHF	98	0.0	DQ166550

Table 3.6 Fungal genera of morphological identified fungal isolates growing on Potato Dextrose Agar and examined using tape mounts.

Location	**Fungal genera**
MBT	*Cephalosporium* (Corda)
	Microsporum (Gruby)
	Penicillium (Fr.)
	Gliocladium (Corda)
	Didymocladium (Sacc.)
	Cunninghamella (Matr.)
	Aspergillus (Micheli)
LHF	*Rhizopus* (Ehrenb.)
	Acremonium (Fr.)
	Pullularia (Berkhout)
	Humicola (Traaen)
	Penicillium (Fr.)
	Aspergillus (Micheli)
	Trichoderma (Fr.)

Restriction digests of pure cultures versus identified bacterial isolates from the functional groups

A total of 24 bacterial isolate and 8 identified bacterial isolates from the functional groups (from here on referred to as standards) were digested with the restriction enzymes *Eco*R 1, *Pst* 1 and *Hinc* III, simultaneously. All the standard isolates had no restriction digest sites for *Hinc* III when a single digest was conducted. This was confirmed by assessing the nucleotide sequences of the standard isolates for presence of the *Hinc* III recognition sites, which were absent. As such distinct patterns were expected to be picked up from the plate isolates. The three types of patterns that were observed (Fig. 3.11) were those that were cut by restriction enzymes into two clearly distinct bands, those that were cut but not clearly distinctive and those that had no recognition site for the enzymes used. The restriction digest of Act C29 and Act C31 (Fig 3.11A, Lanes 1 and 2) were not clearly visible. However, the reduction in band size from ~ 600bp to 300bp indicates that a digest had occurred. In Figure 3.11A, the standard N2A A52 (lanes 3) and NBPC31 (lanes 6) had similar digest patterns, while N2A A53 (lanes 4) and NBP C3C (lanes 5) exhibited similar digest patterns. Isolates PSA A1 and PSA C41 (Fig. 3.11A, lanes 7 and 8) had no restriction sites for the three

enzymes tested. Fragment sizes below 600bp were assumed to be digested with one or more enzymes, while thickness of the fragments with reduced band size were taken to be an indication that upon digest, same fragment sizes were obtained which would result in the clustering of both bands at the same point. Comparing the restriction digest patterns of the standards to the pure bacterial isolates, considerable similarities were observed among lanes 2, 18, 19, 21, 23 and 24 (Fig 3.11B and C). Patterns of these organisms were not digested with any of the three enzymes as they resulted in 600bp band size and were similar to what was obtained with the standard isolates PSA A1 and PSA C41(Fig. 3.11A). Lanes 1, 3, 4, 6, 8, 9, 11, 13, 14, 16 and 22 (Fig. 3.11B and C) had similar banding patterns to each other but did not seem to be similar to any of the standard isolates. Banding patterns of lanes 12 and 15 (Fig. 3.11C) were similar to each other and differed from other plate bacterial isolates and standards. This could also be said for lanes 5, 7 and 10 banding patterns.

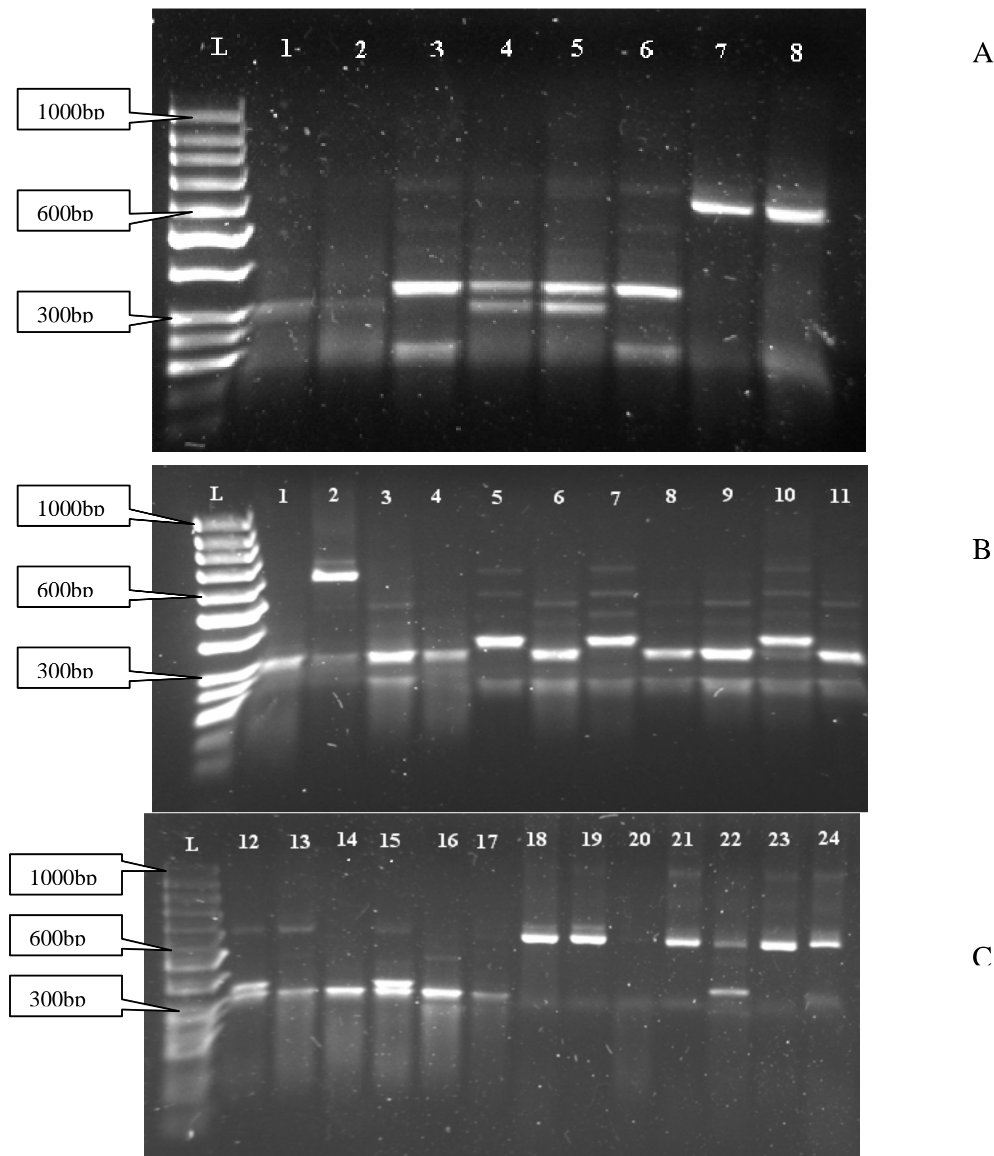


Figure 3.11 PCR-RFLP analysis of 16S rDNA of identified bacterial isolates from functional groups compared with the pure bacterial isolates using restriction enzymes *Eco*R 1, *Pst* 1 and *Hinc* III. A. L - molecular ladder (1kb), lanes 1- Act C29, lanes 2- Act C31, lanes 3 – N2A A52, lanes 4 – N2A A53, lanes 5 – NBP C33, lanes 6 – NBP C31, lanes 7 - PSA A1, lanes 8 – PSA C41. **B**. Lanes 1-16 isolates selected at random from the mining Site at 6 and 9 months period. **C**. Lanes 17-24 isolates selected from the farming Site at 5 months. Act – *Actinomycetes* agar, N2A – free nitrogen agar, PSA – pseudomonads selective agar, NBP – phosphate solubilising agar. Code A and B denotes – control plots, C and D denotes – arbuscular mycorrhial fungal inoculated plots. Figures are plate replicate codes.

3.3.2 Determination of the unculturable microbial populations in the rhizosphere

The extraction protocol described in section 2.5.1 was successfully used in the extraction of DNA directly from soil with subsequent amplification of samples (Fig. 3.12). However, the success of this protocol was only achieved from four samples collected from strategic points at the trial Site of MBT before plots were laid out. Products obtained from this extraction were subjected to an attempt of DGGE analysis (Fig. 3.12) to determine microbial diversity in the soil prior to landscaping and experimental establishment. Visual inspection of the result generated by PCR-DGGE analysis showed similar patterns to each other and resulted in a total of 12 bands. Conversely the intensity of each band which is an indication of species quantity was quite different. As the goal of this exercise was to analyse the effect of AM fungal inoculum on rhizospheric organisms, incision of dominant bands for sequencing at this stage was not conducted.

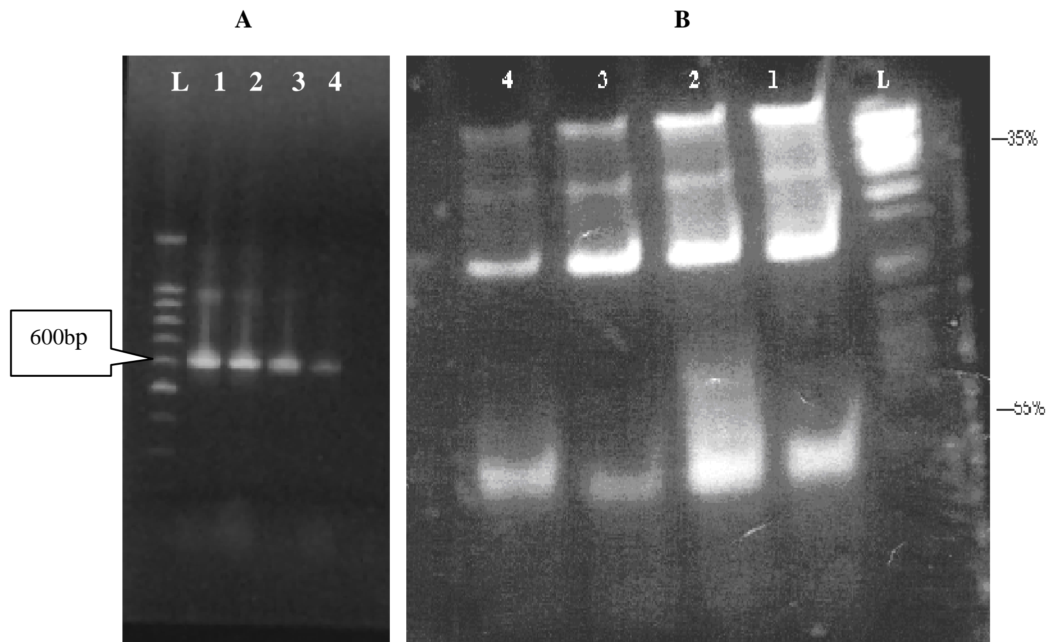


Figure 3.12 PCR-DGGE amplification of 16S bacterial soil DNA obtained from four locations at Makana Brick and Tile prior to plot layout. **A.** PCR amplification using primers GM5F and R907. L- non DGGE molecular marker used to indicate how samples were loaded, lanes 1- location A, lanes 2 – location B, lanes 3 – location C, lanes 4 – location D. **B**. PCR-Denaturing gradient gel electrophoresis of soil samples collected from the 4 locations at Makana Brick and Tile. Percentage values indicates the denaturing gradient used.

Subsequently, the extraction of DNA directly from soil samples using this method became problematic and non-reproducible. Gels resulted in smears and faint bands, which were probably due to presence of inhibitors or polysaccharides. The addition of solvents such as dimethyl sulfoxide and BSA was not helpful. Thus the use of DGGE for further analysis became unsuccessful. This protocol, which was optimised using Qiagen DNeasy plant extraction kit, from which products were obtained, was finally analysed by cloning and sequencing.

Due to the complexities and expenses in DNA extraction, replicate samples were combined for each treatment (mycorrhizal treated and control) plot into 2 samples per Site and were only limited to the last stage of sampling. Judging from the intensities of bands on the ethidium bromide stained agarose gel (Fig. 3.13), it can be said that despite the optimisation, the quality of DNA extracted was poor, particularly for the MBT Site. Cloning of PCR product (~ 600bp in size) into pGEM-T easy vector was optimal when left for 3 days at 4°C. Transformation of clones was successful and randomly selected from replicate Luria Bertani (LB) Agar plates. A total of seven clones were selected for each treatment to give a final total of 28 clones for the two Sites. The use of Plasmid Isolation Kit allowed easy and fast extraction of plasmids from starter cultures

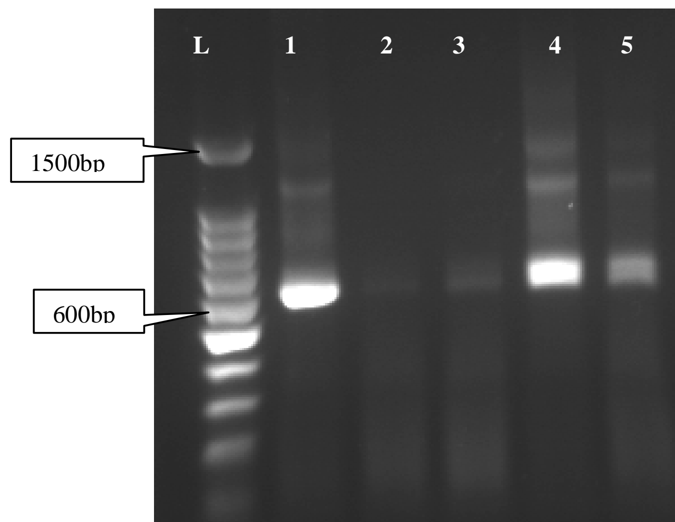


Figure 3.13 PCR amplification of 16S bacterial soil DNA obtained from two different Sites. L- Molecular marker, Lanes 1 – Control DNA, Lanes 2 – 1MBT, lanes3 – 2MBT, lanes 4 – 3LHF, lanes 5 – 4LHF. 1MBT denotes plots treated with arbuscular mycorrhizal fungal (AMF) inoculum and 2MBT the control plots both in Makana Brick and Tile Site. 3LHF denotes plots treated with AMF inoculum and 4LHF the control plots both from Limestone Hill farm Site.

Inserts were verified by restriction digest of isolated plasmid with *Eco*R 1 (Fig. 3.15), which cleaves the two ends of the T-T overhang of the vector releasing the insert (Fig. 3.14). Thick super-coiled bands of above 3000bp are the plasmids while the linear bands are the inserts. Analysing the bands obtained on the agarose gel revealed the presence of non-specific inserts that were <600bp. This resulted in the use of plasmid PCR to ascertain sizes of inserts from which results confirmed 10 clones of specific sizes (2 clones from the MBT Site and 8 clones from the LHF Site) (Fig. 3.16).

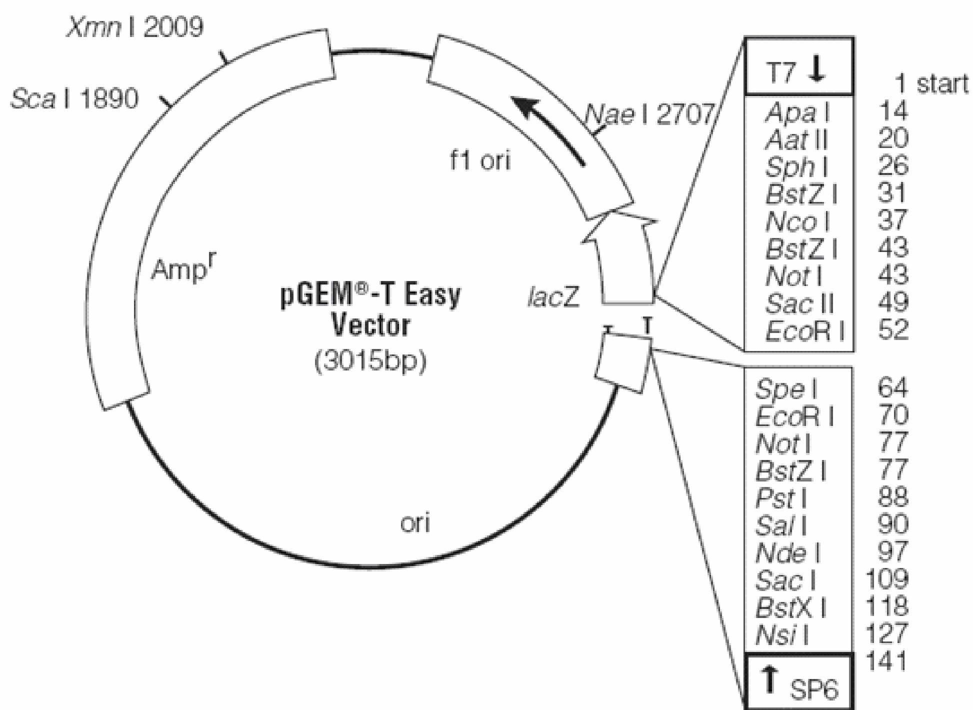


Figure 3.14 pGEM –T easy vector circle map and sequence reference points with restriction enzymes indicating positions at which the vector is cleaved (Promega, 2006).

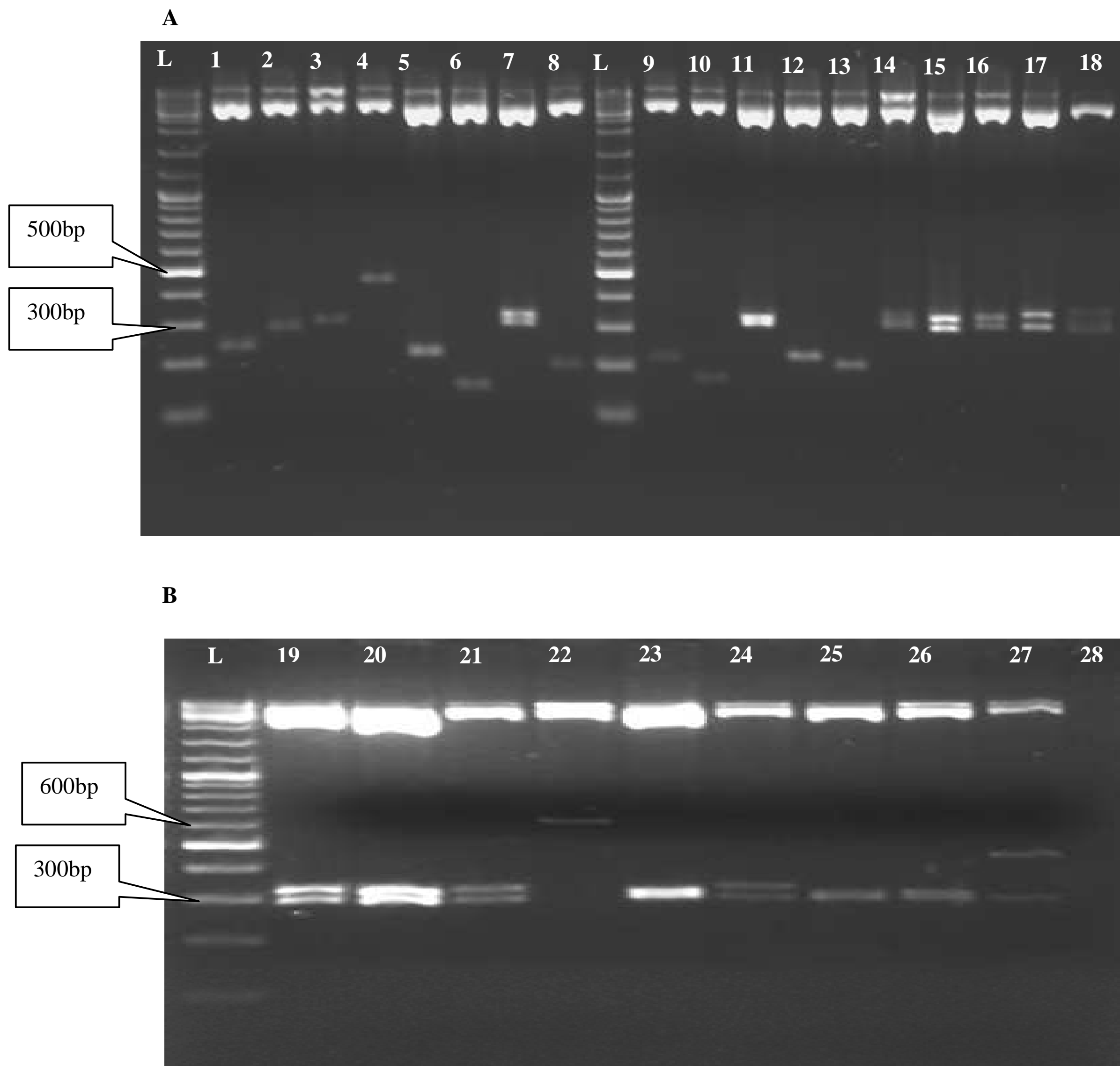


Figure 3.15 A and **B**. Agarose gel electrophoresis of restriction fragments of plasmid isolated products digested with *EcoR* 1 enzyme. L- Molecular marker, Lanes 1-7 are clones 1MBT1, 1MBT2, 1MBT3, 1MBT4, 1MBT5, 1MBT6, 1MBT7. Lanes 8-14 are clones 2MBT1, 2MBT2, 2MBT3, 2MBT4, 2MBT5, 2MBT6 and 2MBT7. Lanes 15-21 are clones 3LHF1, 3LHF2, 3LHF3, 3LHF4, 3LHF5, 3LHF6 and 3LHF7. Lanes 22-28 are clones 4LHF1, 4LHF2, 4LHF3, 4LHF5, 4LHF6 and 4LHF7. 1MBT denotes plots treated with arbuscular mycorrhiazl fungal (AMF) inoculum and 2MBT the control plots both in Makana Brick Tile Site. 3LHF denotes plots treated with AMF inoculum and 4LHF the control plots both from Limestone Hill farm Site. Subsequent Figures are clone numbers.

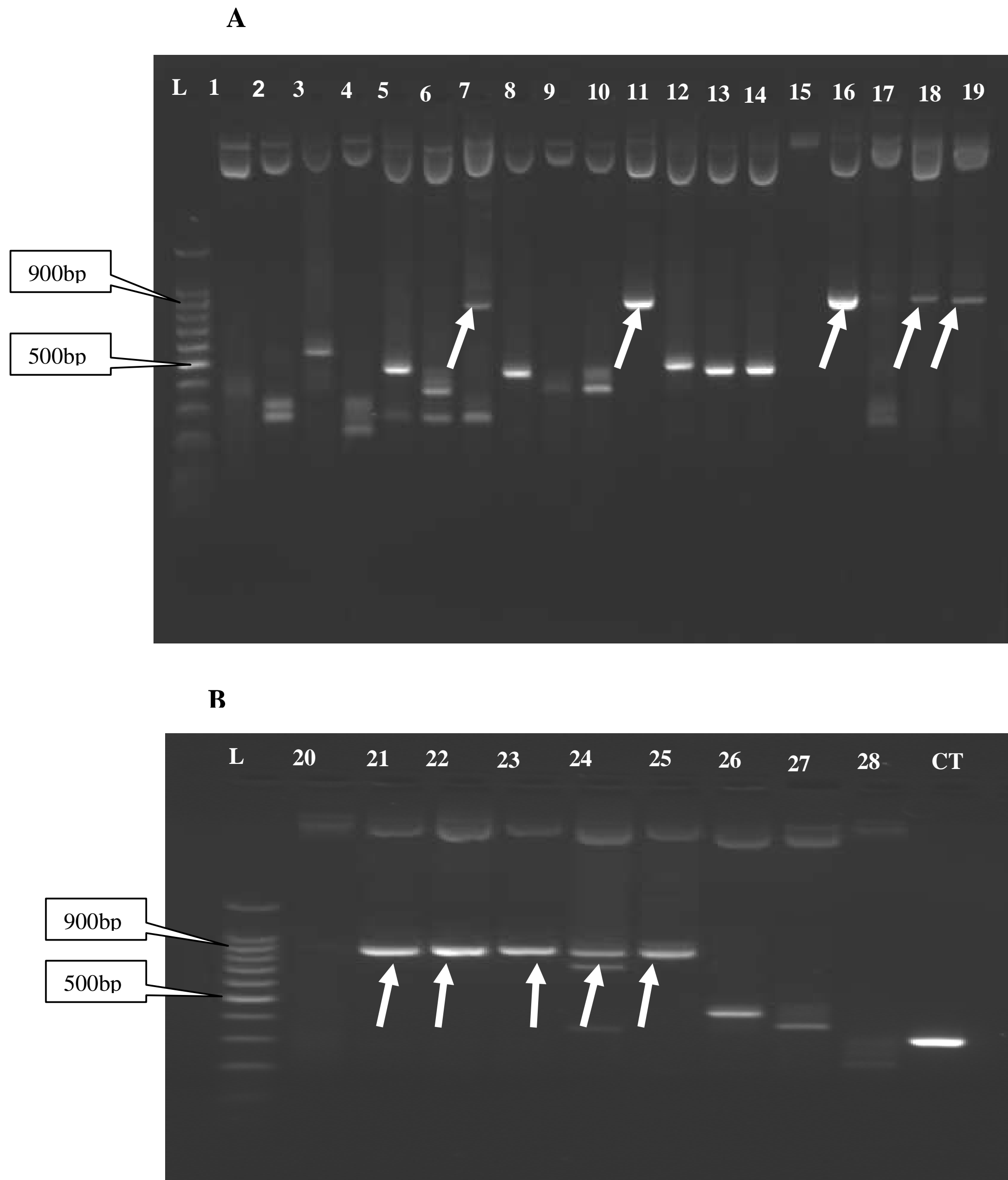


Figure 3.16 A and **B.** Plasmid PCR amplification of all clones isolated from the mining and farm Site using M13 forward and reverse primers. L – Molecular marker, Lanes 1-7 are clones 1MBT1, 1MBT2, 1MBT3, 1MBT4, 1MBT5, 1MBT6, 1MBT7. Lanes 8-14 are clones 2MBT1, 2MBT2, 2MBT3, 2MBT4, 2MBT5, 2MBT6 and 2MBT7. Lanes 15-21 are clones 3LHF1, 3LHF2, 3LHF3, 3LHF4, 3LHF5, 3LHF6 and 3LHF7. Lanes 22-28 are clones 4LHF1, 4LHF2, 4LHF3, 4LHF5, 4LHF6 and 4LHF7. 1MBT denotes plots treated with arbuscular mycorrhizal fungal (AMF) inoculum and 2MBT the control plots both in Makana Brick Tile Site. 3LHF denotes plots treated with AM fungi and 4LHF the control plots both from Limestone Hill farm Site. Subsequent Figures are clone numbers. Arrows indicate selected clones of correct size.

Restriction analysis of amplified selected clones using the restriction enzyme *Eco*R 1 produced slightly similar band patterns to each other. Comparing the patterns obtained from the standards when digested with *EcoR* 1 revealed some similarity to some clones. However, when a triple digest was performed with all three restriction enzymes, band patterns varied. Clones of actual size, after sequencing and BLAST analysis were identified to belong to the genera *Bradyrhizobium*, *Sporichthya*, *Propionibacterium*, alpha *Proteobacterium*, *Acidobacterium* and *Actinobacterium* with one organism being an uncultured bacterial clone. Significant identity values greater than 95% for genus level and 98% for species level were chosen as the cut off (Table 3.7). Identification of clones 3MBT3 and 4LHF4 were not ascertained. To ascertain the similarities or differences between the clones and standards, a cladogram was constructed. Clones differed in their similarity to the plate cultures but were similar to each other. Six groups were formed from the cladogram. Group A consisted of all *Actinomyces* sp. from plate cultures and those obtained from the Genbank. Group B comprised of 2 subgroups, one with all the *Pseudomonas* species and the other with the *Bacillus* and *Burkholderia* species that were obtained from the Genbank. Group C mainly consisted of the *Bacillus* and *Burkholderia* species that were isolated from different media and Sites. Group D, E and F are the selected clones obtained from the two study Sites. It should be emphasized that groups A to F are only based on pair wise comparison of aligned nucleotide sequences which included closely related sequences to the standard from the Genbank (Appendix F).

Table 3.7 Partial sequence analysis on 16S rDNA gene of bacterial clones obtained from the mining and farming Sites and their affiliation to related nucleotide sequences. Identity values >95% were regarded as being significant.

Fungal Isolate	Closely related match identity (NCBI)	% Identity	E-value	Accession No. of closely related sequences (NCBI)
1MBT7	*Propionibacterium* sp.	98	0.0	AM410900
2MBT4	*Propionibacterium* sp.	99	0.0	AM410900
3LHF3	Uncultured *Actinobacterium*	92	2e-54	AB265832
3LHF5	*Sporichthya polymorpha*	97	0.0	AB025317
3LHF6	*Bradyrhyzobium* sp.	99	0.0	AF510604
3LHF	Uncultured alpha *Proteobacterium* clone	98	0.0	EF074956
4LHF1	*Propionibacterium* sp.	100	0.0	AM161153
4LHF2	Uncultured *Actinobacterium*	97	0.0	EF135068
4LHF3	Uncultured Acidobacteria bacterium	100	6e-49	AB265935
4LHF4	Uncultured bacteria clone	86	1e-93	DQ990936

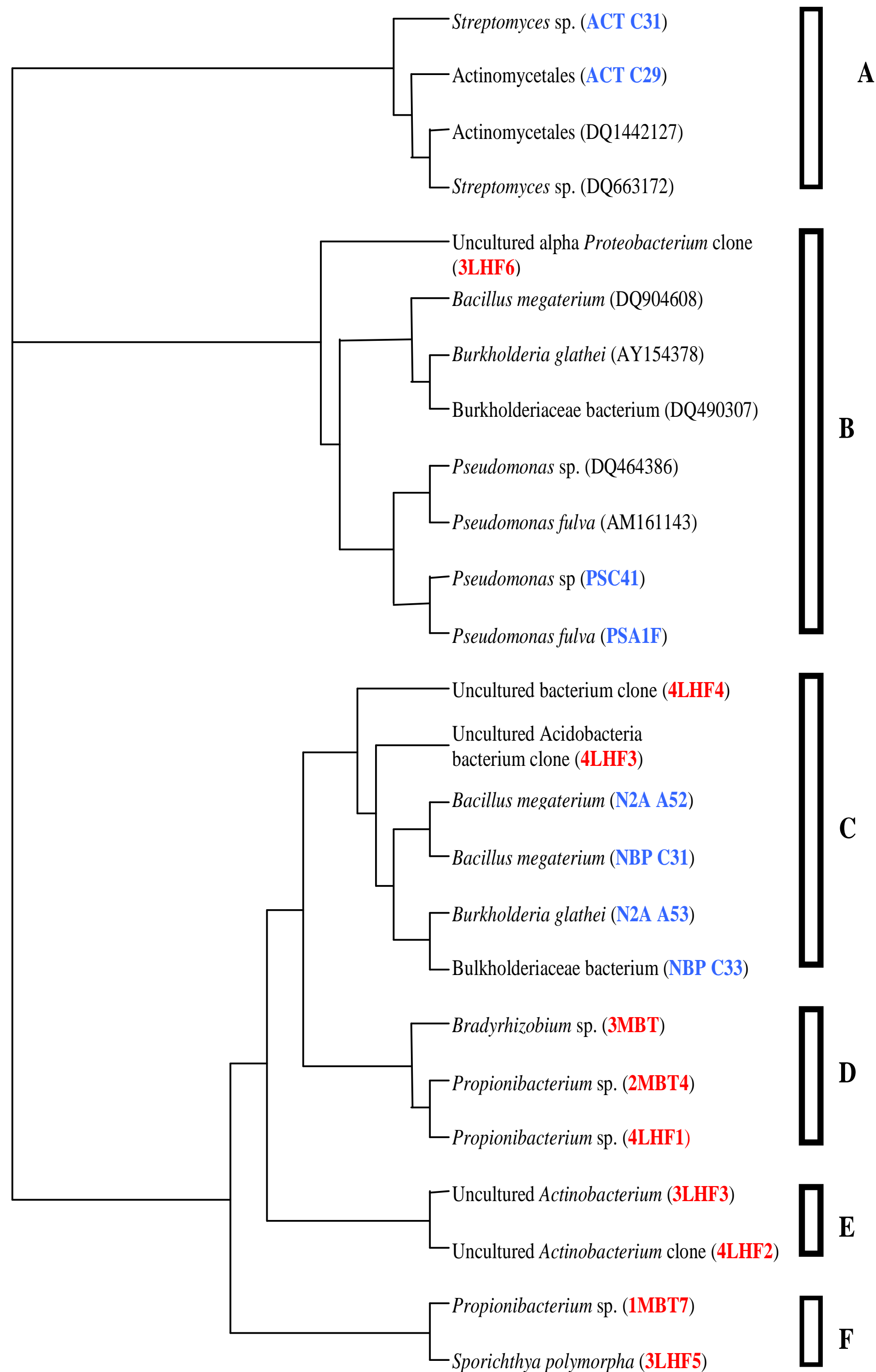


Figure 3.17 Clustal W cladogram showing sequence similarities of all identified bacteria obtained from culture dependent and independent techniques. Names of isolated organisms from the study are denoted with isolate codes in parenthesis. Letters in blue are isolates from plate cultures, while letter in red are clones. Genbank accession numbers are listed in parenthesis with organism identity.

3.4 Arbuscular mycorrhizal fungal population assessment in soils

Mycorrhizal fungal populations at the MBT Site as determined by spore enumeration revealed a decreasing trend (80, 56 and 41) in the amount of spores/100g of soil present in plots treated with AM fungal inoculum at 3, 6 and 9 months sampling periods, respectively (Table 3.8). From the preliminary analysis, AM fungal spore density of soil samples collected from the 4 strategic points at the MBT Site was found to be an average of 71 spores/100g of soil. This indicates that any decrease in the mycorrhizal spore density began after the 3rd month of sampling since at 3 months the spore density increased to 80 spore/100g of soil (Table 3.9). Similarly, in the control plots a decreasing trend was observed at 6 and 9 months of sampling. The high mean value (112 spores/ 100g soil) in the control plots at 3 months when compared to the treated plots was of concern and was therefore re-enumerated for confirmation, which yielded similar results. Inspite of decreasing numbers, statistically it was observed that the effect of treatment on spore density at the MBT Site significantly increased at 6 ($P = 0.04$) and 9 ($P = 0.01$) month period. Comparing the values of AM fungal spore density at the LHF Site (Table 3.9) with the MBT Site, it was observed that there was a statistical difference between spore numbers at the two Sites as well as a high significant effect of treatment at the LHF Site ($P = < 0.01$).

Table 3.8 Arbuscular mycorrhizal fungal spore density in the soil and percentage root colonisation of *Cynodon dactylon* root samples obtained from Makana Brick and Tile Site.

Makana Brick & Tile	Time (months)	Mycorrhizal treated	Control	*P* value	F value
AM fungal[x] Spores/100g soil	3	80 ± 38 a	112 ± 43 a	0.08	3.6147
	6	56 ± 31 b	35 ± 21 b	0.04	5.2038
	9	41 ± 14 b	23 ± 10 b	0.01	7.7576
% root colonisation[y]	6	4.5 ± 2.7 a	3.6 ± 3.3 a	0.54	0.3894
	9	9.4 ± 4.7 b	5.20 ± 2.2 ab	0.02	6.2887

[x] Values are mean ± standard deviation of 9 replicates. Means followed by the same alphabets across columns are not significantly different from each other.
[y] Values are mean ± standard deviation of 10 replicates. Means followed by the same alphabets across rows are not significantly different from each other.

Table 3.9 Arbuscular mycorrhizal fungal spore density in the soil and percentage root colonisation of *Pelargonium graveolens* root samples obtained from Limestone Hill Farm Site.

Limestone Hill Farm	Mycorrhizal treated	Control	*P* value	F value
AM fungal[x] Spores/100g soil	257 ± 70 a	96 ± 38 b	< 0.01	36.1409
% root colonisation[y]	11.3 ± 5.5 a	6.5 ± 2.8 b	0.03	5.3957

[x] Values are mean ± standard deviation of 9 replicates
[y] Values are mean ± standard deviation of 10 replicates.
Means followed by the same letter across rows are not significantly different frome each other.

Staining of the respective plant roots from the two Sites with lactoglycerol trypan blue aided in a clear distinction between fungal and plant structures. Roots were mainly colonised by the formation of vesicles and hyphal coils in the root cortical cells. At the MBT Site, little root material was obtained at 3 months and AM fungal colonisation was not evident. Colonisation of plant roots increased with time in the inoculated and control plots at a slow rate (Table 3.8). The percentage root colonisation of *C. dactylon* plants at the MBT Site ranged between 4%-9% at 6 and 9-month sampling periods. Statistical analysis using a two-way ANOVA indicated a significant effect of treatment at 9 months (Table 3.8) with an increase in percentage colonisation. The percentage root colonisation values obtained at 9 months at the MBT Site to the LHF Site at 5 months (Table 3.9) was compared. It was observed that though there was a significant effect of treatment at the two Sites (P = 0.02 and 0.03 respectively), that the difference in terms of values is not as distinctive as in the spore density values.

The most probable number (MPN) technique, which determines infectivity of propagules, was used to determine the overall AM fungal infectivity potential at the two Sites. The MPN of inoculum used was 100000 propagules/1kg that is equivalent to 10000 propagules/100g. From the results of the preliminary analysis carried out on the MBT samples, no infectivity was observed when the stained root materials were examined for AM fungal colonisation features; but at the last sampling period infectivity potential was determined to have increased to 850 progagules/100g of soil.

From the Limstone Hill farm soil an MPN value of 5500 propagules/100g of soil was calculated.

The total extractable glomalin (TEG) quantified by the Bradford assay was analysed using a two-way ANOVA (Fig.3.18). From the analysis, an overall mean concentration of 0.211, 0.121 and 0.106 mg/ml at the three sampling periods at the MBT Site indicates that the TEG concentration decreased over time. However, the complexity of analysing this result lies in the fact that a concentration of 0.12mg/ml was obtained before trial plots were treated. The simultaneous effect of treatment at various sampling times showed a significant effect of treatment at 9 months only (P = 0.0026). Also, there was no interaction between treatment and time (P = 0.0695), which is an indication of independent effects. The glomalin concentrations at the two Sites (Fig. 3.19) when compared showed that the LHF Site had a greater concentration of glomalin in the soil than the MBT Site. Significant differences in the treated and control plots were recorded at the two study Sites (Fig. 3.19).

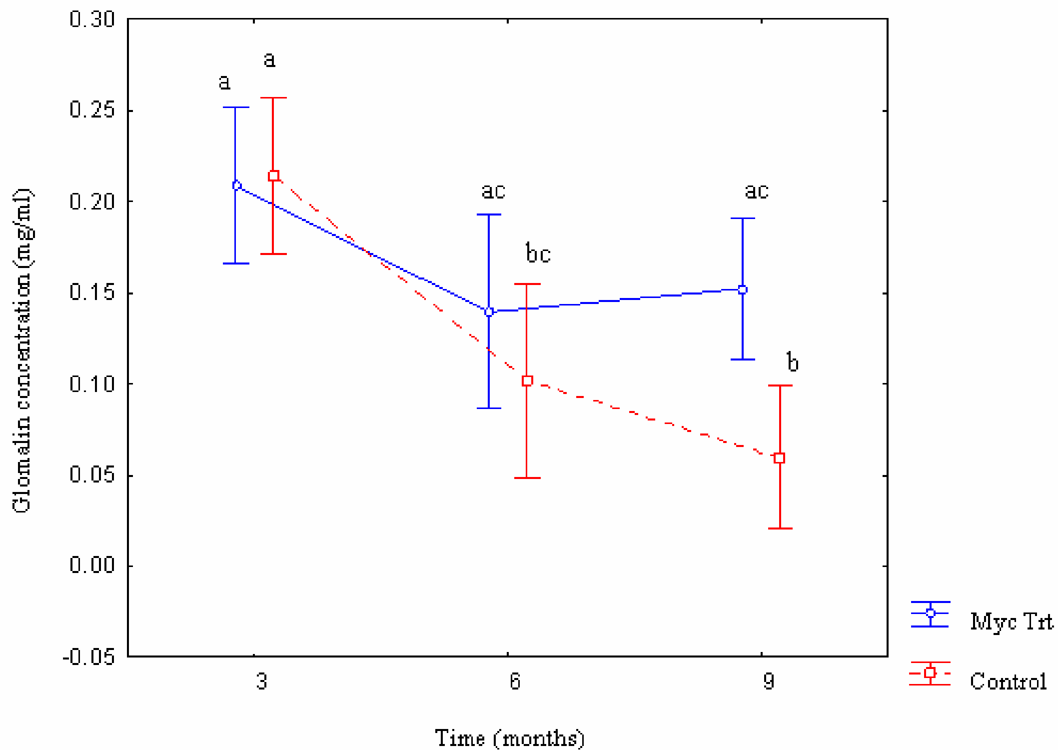


Figure 3.18 Glomalin concentrations at various sampling periods (3, 6 and 9 months) at Makana Brick and Tile. Each point represents mean of 9 replicates ± standard deviation. Same letters are not significantly different at $P < 0.05$ by Bonferroni test. Significant probability value of overall effects tested include: Treatment = $F_{1, 32}$= 4.7528, P = 0.0445; Time = $F_{2, 32}$=15.597, P = 0.0002.

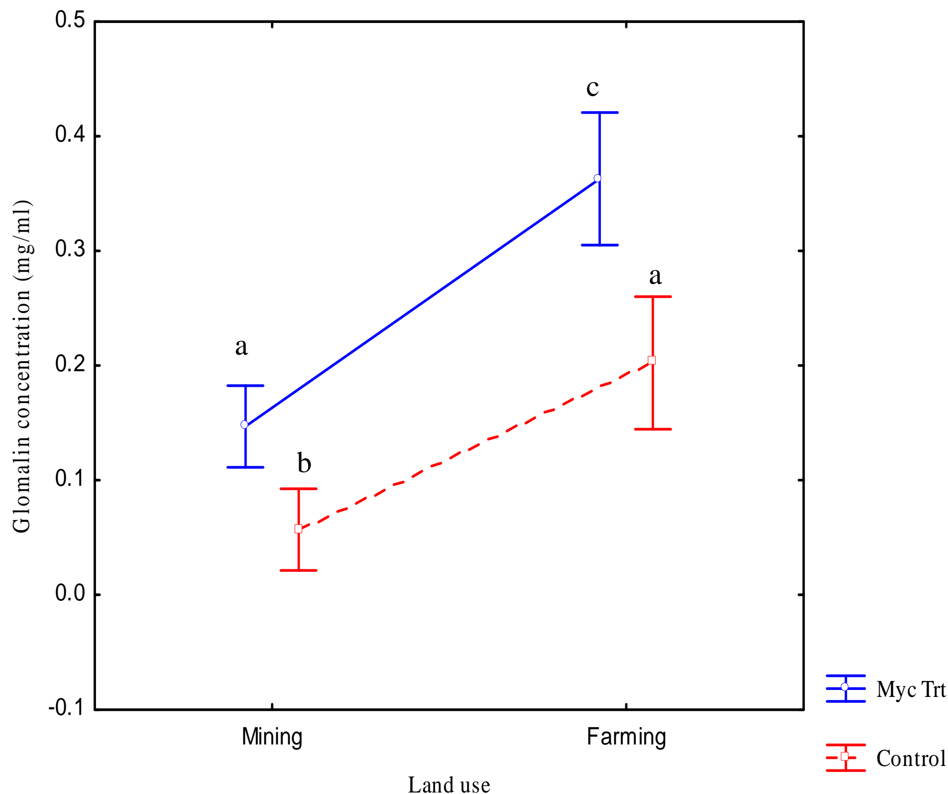


Figure 3.19 Glomalin concentrations of soil samples from Makana Brick and Tile (9 months) and Limestone Hill farm (5 months). Bars represent mean of 10 replicates ± standard deviation. Same letters are not significantly different at $P < 0.05$ by Bonferroni test. Significant probability values of overall effects tested include: Treatment = $F_{(1,32)}$ =26.241, $P < 0.01$; Site = $F_{(1,32)}$ =76.057, $P < 0.01$.

3.5 Alternative host plants from around the mine area

Plants obtained from the mine areas as identified by Selmar Schonland Herbarium (Table 3.10) were found to be indigenous, common to the Grahamstown and Bathurst regions. The flowering season of selected plants was found to vary throughout the year. Plant species habitat was mostly found in dry places and stony slopes. Analysis of their roots when stained revealed that they were colonised by arbuscular mycorrhizal fungi, of which 2 plants were colonised by endomycorrhizal fungi. The AM fungal roots of *Pentzia incana* and *Elytropappus rhinocerotis* showed *Paris* type of colonisation, while in the other plant species roots, spores and/or vesicles were present with a trace of *Arum* type colonisation (Fig. 3.21).

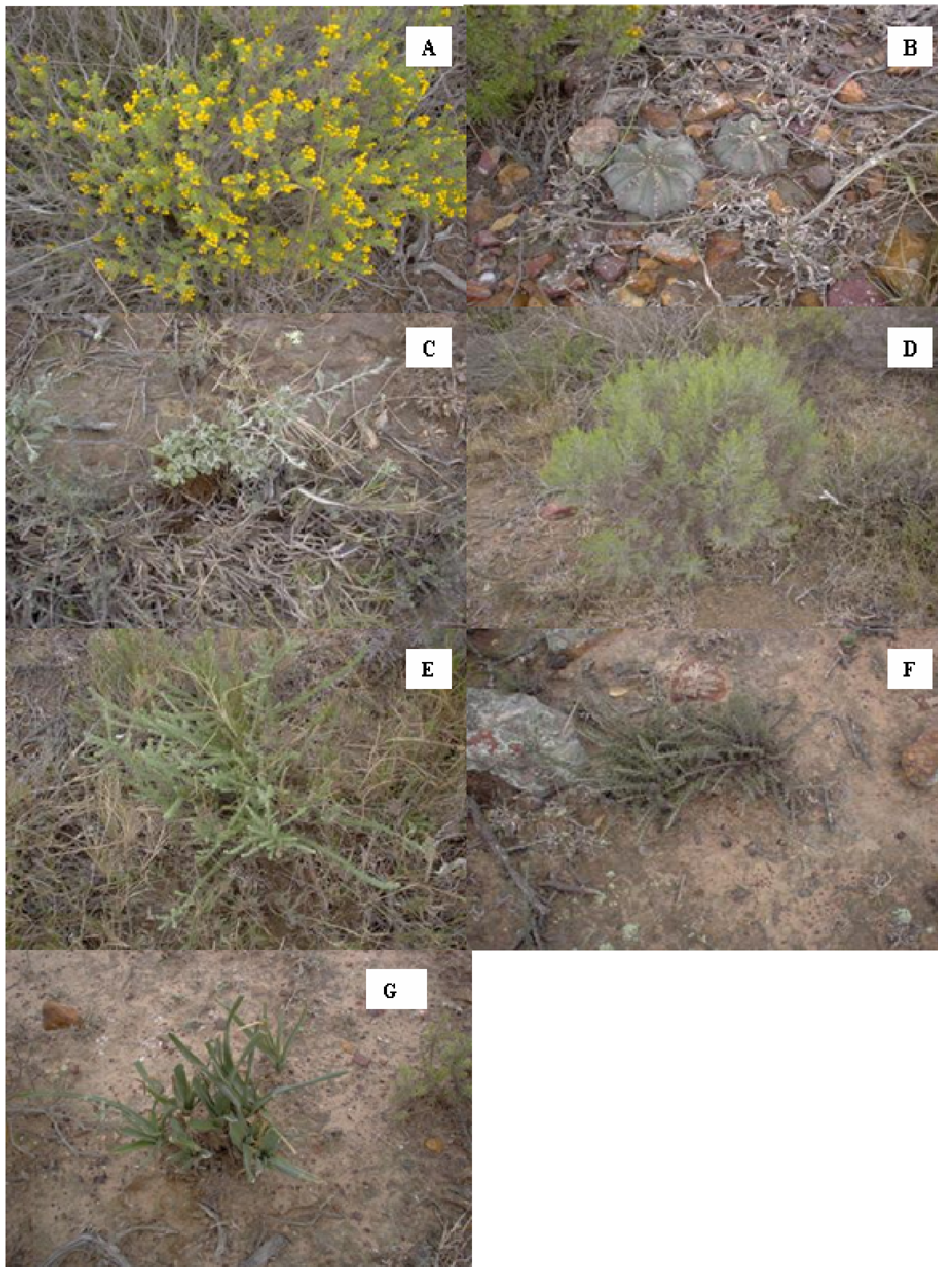


Figure 3.20 Identified host plant growing around Makana Brick and Tile mine area. **A**. *Pentzia incana* **B**. *Euphorbia meloformis*, **C**. *Indigofera sp.*, **D**. *Elytroppapus rhinocerotis*, **E**. *Selago corymbosa*, **F**. *Helichrysom rosum*, **G**. *Albuca canadensis.*

Table 3.10 Mycorrhizal status of indigenous host plants, their families and habitat-flowering season.

Identification	Family	Vesicles	Arbuscules	Spores	Hyphal Coiling	Mycorrhizal status	Habitat/flowering season
Pentzia incana (kuntze)	Asteracea	-	-	-	+	*Paris* type AM	Dry places areas. Sept-Nov
Euphorbia meloformis (Aiton)	Euphorbiaceae	+	-	+	+	AM	Dry gravely soil Nov-March
Indigofera sp. (Scrire)	Fabaceae	+	+	+	+	*Arum* type AM	Common on flants and mountains. No defined flowering season
Elytropappus rhinocerotis (Less)	Asteracea	+	-	-	+	*Paris* type AM	Dry shale and sand slopes. Feb-April.
Selago corymbosa (L.)	Scrophulariaceae	-		-	+	Endo	Stony slops and flats. Feb-Apr
Helichrysum rosum sp. (Berg.)	Asteraceae	-	-	-	+	Endo	Stony slopes and flats. Sept –March
Albuca canadensis (L.)	Hyacinthaceae	+		+	+	AM	Sandy stony slopes. Aug – Oct.

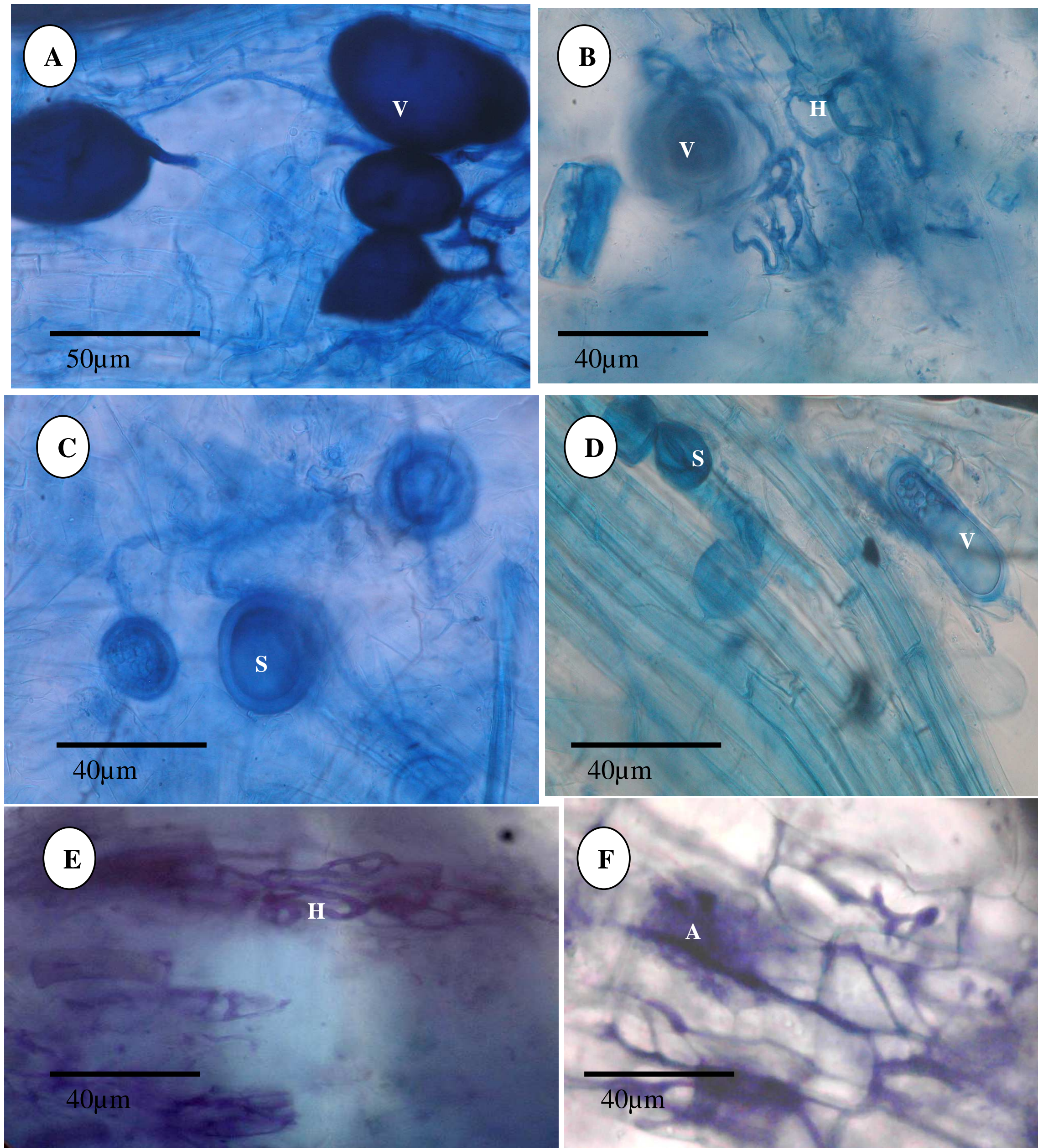


Figure 3.21 Arbuscular mycorrhizal and endomycorrhizal colonisation types observed in selected plant species obtained from the mine area. **A**. Large and small globose shaped vesicles (V) in the stained roots of *Elytropappus* rhinocerotis. **B**. *Paris* type hyphal coiling (H) with attached vesicle in the stained roots of *Indigofera* sp. **C**. Spores with intact spore walls connected to an extraradical hyphae in the stained roots of *Euphorbia meloformis*. **D**. Oblong shaped vesicles and spores (S) in the stained roots of *Albuca canadensis*. **E**. Hyphal coiling in the cortical cell in stained roots of *Pentzia incana*. **F**. *Arum* type colonisation with formation of arbuscule (A) in the cortical cells in stained *Indigofera* sp. roots.

CHAPTER 4

DISCUSSION

4 Discussion

In this study, the field trial aimed to determine the effect of AM fungi on background microbial population as well as to establish a vegetation cover on the overburdened soil resulting from kaolin clay mining. However, the latter goal could not be achieved due to the inability of the company to provide an efficient irrigation system at the MBT Site. This study which was conducted during a low rainfall period (Table 2.1) relied on artificial watering of the plots. Efforts to water plots manually by the company were made, but were inefficient. The inefficiency of the manual irrigation can be attributed to soil factors such as water retention, infiltration, permeability and soil variability which are characteristics that are relevant for a proper irrigation management (Uphoff *et al*., 1991). Therefore, it was either that the water retention capability or permeability of the soil at the mining Site was low or the amount of water applied was insufficient for proper germination of *C. dactylon* seedlings (Section 4.1).

In plant or field related studies proper irrigation is necessary, as water is essential for plants to attain maximum growth. Water is required for plant transpiration and through this process translocation of nutrients from the roots to leaves for photosynthesis is facilitated (Uphoff *et al*., 1991). However, this will be greatly affected if water loss, as a result of permeability in soil does not match up to transpiration rates. Therefore, it is advised that when field studies are to be conducted, irrigation is one parameter that should be adequately provided to ensure success of the study (Uphoff *et al*., 1991). In the present study, this problem could not be corrected due to the period of the study and so a contingency plan was implemented. This involved the use of the Limestone Hill Farm which was an irrigated field and so used obtain additional information.

The two study Sites differed in soil texture with the mining Site soil texture being a clay loam and the farming Site a soil rich in clay content (Table 3.1). Soil texture is an important soil property as it determines soil pore size, which in turn influences nutrient retention capacity in soils as well as soil physical and chemical characteristics

(Ashman and Puri, 2002). These properties in turn exert an influence on soil microorganisms through modifications in soil pH, soil minerals and soil temperature (Gray and Williams, 1971).

4.1 Pot trial analyses

Pot trials have predominantly been used to study a variety of effects and parameters first before a field trial is conducted. Frequently, generalisations are made from these trials without conducting field validation trials. In this study the use of a pot trial was conducted to determine the potential of using AM fungi in the rehabilitation of mine spoils (the MBT Site) with co-application of fertilisers. This setup was similar to the initial proposed objective for the field analysis from which comparisons would have been drawn from a field and pot trial perspective. However this comparison can now be done indirectly.

AM fungi have become a well-known bio-inoculant due to their numerous benefits to plants in agriculture and its uses in phytoremediation with no known environmental risks, unlike the use of chemical fertilisers that is a common practice. The use of fertilisers has been reported to affect AM fungal functionality (Ryan and Graham, 2002) as inorganic P fertilisers and animal manures are routinely applied to improve or meet crop P demand. The present study used Organic Tea and 3:1:5 NPK to boost the nutrient levels of the soil obtained from the mine spoil as well as to determine its effect on AM fungal root colonisation. This study demonstrated that in spite of the treatment and time period, the shoot height remained insignificantly different in some treatment pots (Table 3.2). The measurement of few numbers of plant shoot in each pot may have accounted for the insignificant difference in shoot heights among treatments. Therefore, in subsequent pot trials, thinning out of plants should be considered, especially in cases were small seedlings such as *C. dactylon* are difficult to count. Otherwise, attempts should be made to measure at least 50% of the shoots grown. The observed difference in shoot heights, between pots treated with Organic Tea only at 6 weeks and those treated with 3:1:5 NPK + AMF at 6 and 28 weeks (Table 3.2) is an indication that the overall significant effect of treatment obtained for shoot height was largely attributed to the presence of AM fungi. Also, the

insignificant difference between pots with each fertiliser + AM fungal treatment in terms of plant biomass (Table 3.2) indicates that the two fertilisers used are equally capable of improving plant growth and could be used in rehabilitation, if the desired outcome is to establish a vegetation cover over a short period of time. The rate of fertiliser absorption by plants can be a contributing factor to the observed treatment differences, as organic fertilisers are believed to be slow releasing compounds as opposed to inorganic fertilisers (Arden-Clarke and Hodges, 1988). Contrary to this generalisation, the Organic Tea + AMF increased shoot height at 6 weeks than the 3:1:5 NPK fertiliser, but reduced subsequently at 12 and 28 weeks period. This could mean that the Organic Tea was taken up by the plant at a higher rate than the 3:1:5 NPK fertiliser and would require more than one application for plant growth to be maintained.

The choice of fertilisers in most studies or systems is probably determined by the ease of application (Arden-Clarke and Hodges, 1988). Organic fertilisers such as the Organic Tea used in this study may not be desirable as compared to the 3:1:5 NPK due to its liquid nature, as application would require good spraying equipment or irrigation (fertigation). If no suitable irrigation is available, the Organic Tea will unlikely be used but the granular 3:1:5 NPK can be easily broadcast on Sites. According to Rosen and Bierman (2005) some organic fertilisers such as manure or compost when not stored properly loose nutrients such as P and K through leaching. Therefore, consideration into these factors may be the reason why inorganic fertilisers remain widely accepted. Studies have reported that different fertilisers such as superphosphate, NPK, urea, N_2 and organic fertilisers increased biomass, shoot height and crop yield (Fletcher *et al*., 2004). Increasing attention is now being paid to organic farming practices whereby the use of compost, green manures and animal droppings as fertilisers are encouraged (Ashman and Puri, 2002).

The effects of fertilisers on AM fungi using field and pot trials have been well documented. Studies have reported that fertilisers that are high in P or N reduce AM fungal colonisation (Azcón *et al*., 2003; Xu *et al*., 2000; Kurle and Pfleger, 1994; Thompson, 1994a). Azcón *et al*., (2003) demonstrated this when they evaluated how levels of N and P nutrients affected the rate of micronutrient uptake by *Gl. mosseae* using lettuce plants. They observed that the use of 0.1mM P increased colonisation of

Gl. mosseae, which contributed to the accumulation of micronutrients than when 0.5mM of P was applied. From the present study, the root colonisation analysis (Fig. 3.5), showed that 3:1:5 NPK fertiliser allowed 80% colonisation, while Organic Tea allowed 65% colonisation of *C. dactylon* roots by AM fungi. This study anticipated that the use of Organic Tea would result in a greater percentage colonisation because, if P was in a form unavailable to plants, mycorrhizal fungi could play a vital role in mineralisation of organic constituents. However, it was observed that the amount of P in the Organic Tea was 0.5% = 5000ppm (Appendix A1), which is equivalent to 125ppm when a 1:40 dilution was made. According to Swift (2006), mycorrhizal fungi exercise a greater benefit when soil P levels are at or below 50ppm (50mg/kg). This means that the lower percentage colonisation observed with Organic Tea was as a result of high P level rather than the form of P present. Similar results were obtained by Maropula (2006) who determined the compatibility of four organic fertilisers (Organic Tea, Compost, Kelp and Nitrosol) with the same mycorrhizal inoculum used in this study. Furthermore, her study confirmed the reduced mycorrhizal colonisation (32%) caused by Organic Tea in Spinach roots, while the other organic fertilisers evaluated were non-compatible with AM fungi with the exception of Nitrosol; but were able to improved plant growth. In spite of this similarity in the reduced effect of Organic Tea, the difference between studies was that the Organic Tea was applied on a weekly basis as opposed to a once off application in this study. This could, account for a higher colonisation obtained with Organic Tea (65%) in this study compared to the 35% obtained by Maropula (2006). One of the disadvantages of using organic fertiliser is the inability to single out a particular compound that may be detrimental to AM fungal establishment. Contrary to this, the use of chemical fertilisers allows the selection of a compound that may be required in a reduced amount. An example is the 3:1:5 NPK as opposed to 2:3:2 NPK that was chosen in this study in order to reduce the P content. Besides the environmental hazards of these fertilisers such as leaching and runoffs, this study suggests that the use of a reduced P fertiliser in conjunction with an AM fungal inoculant for plant growth will be more efficient and cost effective for crop yield or establishing a vegetation cover (Khan, 2006; Xu *et al*., 2000).

The pot trial can be compared indirectly to the field trial at the MBT Site in terms of percentage root colonisation. It was observed that the field trial, which had 9.4% root colonisation at 9 months (Table 3.8), was approximately 7 to 8 times less than the

percentage colonisation obtained in the pot trial with the two-fertiliser treatments. This high variation between the results could be associated to different growth systems. In a controlled environment plant growth and subsequent AM fungal colonisation will not be affected by any environmental factors such as rainfall, temperature, humidity, or wind. Therefore, the problem of inadequate irrigation encountered in this study could have affected the AM fungal colonisation in the field. Although AM fungi are known to improve drought tolerance of plants, germination of spores and fungal propagules prior to colonisation requires moisture (Al-karaki *et al*., 2004). Al-karaki *et al*., (2004) demonstrated this by determining the colonisation of *Gl. mosseae* and *Gl. etunicatum* in a field grown wheat under well-watered and poorly watered conditions. It was observed that the colonisation of these AM fungal species was higher (41.3%) than the percentage colonisation in water stressed plant (18.8%), confirming the need for water and its influence on AM fungal colonisation. This is to say that it is only when the relationship has become established that AM fungi can improve drought tolerance (Augé, 2001).

Kurle and Pfleger (1994) stated that species of AM fungi differ in their response to chemical fertilisers. To determine this, Caravaca *et al*., (2006) found that among three species of AM fungi (*Gl. mosseae*, *Gl. intraradices* and *Gl. deserticola*), the latter two species produced an increased effect on shoot biomass of *Retama sphaerocarpa* seedlings in combination with medium dosage of liquid organic amendment obtained from dry olive residues. *Gl. mosseae* produced an increased effect on plant biomass when inoculated alone. Also Podeszfinski *et al*., (2002) observed the predominance of *G. margarita* and *S. calospora* at Sites that were high and low in P, respectively. This means that the higher percentage colonisation of plant roots obtained with 3:1:5 NPK rather than the Organic Tea could be as a result of AM fungal species tolerance to fertiliser types. These results highlight the potential use of AM fungal inoculant for a rehabilitation of an overburdened soil with reduced P fertiliser.

4.2 Microbial population analyses

4.2.1 Estimating the number of culturable microbial populations in the rhizosphere

Mycorrhizal fungi have often been reported to influence bacterial communities in the rhizosphere quantitatively or in species composition, which have been determined using various techniques (Marschner *et al*., 2001b; Marschner *et al*., 2001a; Grayston *et al*., 1998; Meyer and Linderman, 1986b). The rhizosphere is a dynamic environment and as such the colonisation of this area by mycorrhizal fungi integrates this fungus as part of the soil microflora that is capable of contributing to the stability of the rhizosphere (Fillion *et al.,* 1999; Meyer and Linderman, 1986b). In this study a combined approach integrating traditional and molecular (PCR-based) techniques were used to examine qualitatively and quantitatively, the effects of AM fungi and land-use management practices on the soil microbial community.

This study highlighted the relationship between bacterial numbers obtained using full strength NA and TSA (Appendix B). This was done to determine the most suitable media in microbial diversity studies. These two media were used in an effort to increase the chances of isolating more culturable bacterial species (Balestra and Misaghi, 1997). NA and TSA are universal media that differ in compositions such as meat extract, yeast extract, peptone, tryptone and soy meal (Appendix B). These constituents are complex compounds that supply organisms with nutrients required for growth. The amount of constituents present in media types can either be sufficient or minimal for the growth of some organisms (Balestra and Misaghi, 1997). The higher number of colonies recorded on NA compared to TSA, is an indication that the use of different media and its composition may have important effects on the growth of organisms. Furthermore, the low colony count and poor growth obtained on TSA was probably because of the full strength concentration that was used (Elliot and Des Jardin, 1999). This could perhaps be why other studies that determined the microbial populations in the rhizosphere used TSA at reduced strengths such as 0.1% and 0.3% (Mansfeld-Giese *et al.,* 2002; Garbeva *et al*., 2001; Chiarini *et al.,* 1998; Grayston *et*

al., 1998; Meyer and Linderman, 1986b). As a result, the full strength concentration of TSA could account for the lower isolated bacterial numbers compared to NA.

At the MBT Site, the total number of culturable bacteria isolated on NA as observed over time was not affected by the presence of AM fungi (Fig. 3.6). This means that the presence of AM fungi did not stimulate or inhibit the numbers of rhizospheric organisms as estimated by the plate counts (CFU). However, from the statistical result there was a significant effect of this treatment at the 9th month which was indicated in the overall increase of bacterial numbers at that period (Fig. 3.6). Despite this overall significant effect of treatment, the insignificant difference between the control and plots treated with AM fungal inoculum reduces the importance of the treatment effect. This is because the significant difference between plots provides a more direct evaluation between control and inoculated plots than the Univariate result. Furthermore, the change in species composition in the control and treated plots observed from analysis of the functional groups (Fig. 3.8) indicated that the effect of AM fungi on culturable rhizospheric organisms might not be a quantitative one but rather an influence on species composition. This can be attributed to either plant root or AM hyphal exudates that are released in the rhizosphere or a combination of both. This result is in accordance with the findings of Andrade *et al*., (1997) who observed a qualitative response in the rhizosphere when roots of Sorghum were colonised by native and inoculated AM fungal species. AM fungal hyphae have been reported to exude simple carbohydrates in the form of glucose and low molecular weight aliphatic acids that attract particular groups of organisms that utilise these molecules as a source of carbon (Toljander, 2006). Hence, these organic compounds may either stimulate (Meyers and Linderman, 1986b) or inhibit (Christensen and Jakobsen, 1993) certain rhizospheric groups of organisms that will compete for these substrates in the soil. Fillion *et al*., (1999) confirmed this stimulatory-inhibitory effect of AM fungi in an *in vitro* system using transformed carrot plants and test organisms. They observed that the extract obtained from AM fungal hyphae stimulated the growth of *Pseudomonas chlororaphis* and *Trichoderma harzianum*, while inhibiting the growth of *Fusarium oxysporum* and *Fusarium chrysanthemi*. This means that it is possible for AM fungal hyphal exudates to favour qualitatively the presence of a particular functional group of organisms in the rhizosphere. Furthermore, changes in rhizosphere composition could be as a result of varying root exudates of plant species,

or changes in root exudate composition due to mycorrhizal formation (Yang and Crowley, 2000). This was reported by Benabdellah *et al*., (1998) in their study to determine the effect of AM fungal colonisation on the expression of polypeptides in mycorrhizal and non-mycorrhizal tomato roots. They identified 44 different polypeptides present in tomato roots due to AM fungal colonisation, thereby indicating the possible effect of AM fungi on protein metabolism in plant roots.

The effect of mycorrhizal fungi on rhizosphere organisms or microbial diversity has been studied based on plant species, soil type and land use management (Söderberg *et al.,* 2002; Marschner and Timonen, 2005; Marschner *et al*., 2001a; Buckley and Schmidt, 2001). Plant species and soil type interact with each other, as one factor can influence the other (Marschner *et al*., 2004). In this study, the two Sites differed in plant species, which can influence rhizospheric organisms through other plant related factors such as plant age and root zone location. This is indicated in the increase of bacterial numbers observed at the 9th month at the MBT Site (Fig. 3.6), which can be attributed to plant age. This is because at a certain plant developmental stage, specific root exudates are released and soil microorganisms will need time to adapt to the present conditions in the rhizosphere before an effect is exerted on the bacterial numbers (Marschner *et al*., 2001a). Hence, the reason for the insignificant difference in the bacterial numbers recorded at the 3rd and 6th months at the MBT Site. Studies have also reported more effects of plant species on microbial community than soil type. Grayston *et al*., (1998) using community level physiological profiling (CLPP) method, compared the effect of plant types: ryegrass, bentgrass and clover, grown in two different soils with similar crop history. They observed that the rhizosphere activity differed according to plant type with no differentiation in the soil type effects. Similarly, Marschner *et al*., (2001b), examined using PCR-DGGE of soil rRNA the effect of plant species: chicken pea, rape and sudangrass, soil type (sandy, sandy loam and clay soil) and root zone location on the bacterial community structure in the rhizosphere. They observed that these factors interacted with each other to bring about variations in the rhizosphere community structure. The rhizosphere bacterial community of chicken pea plants was influenced by soil type, while that of sudangrass and rape were affected by root zone. Thereby, they concluded, that the effect of plant species to an extent was controlled by soil type. The MBT Site had a higher record in bacterial numbers at 9 months than the LHF Site at 5 months (Fig.

3.7), which could be as a result of these mentioned factors. However, this difference was expected given the fact that different plant species were used and the sampling time, which is an indication of plant age, varied.

Furthermore, it has been reported that soil properties such as soil texture, microaggregates, pH, presence of key cations and organic matter can affect rhizospheric community directly or indirectly (Garbeva *et al*., 2004). The direct effect is through the selection of specific microorganisms in a habitat or indirectly by modifying the plant root exudation pattern in a soil-specific manner (Garbeva *et al.,* 2004). Girvan *et al*., (2003) supported this when they examined the rhizosphere community based on 16S rRNA, as affected by plant type (ryegrass, sugarbeet, clover, wheat and barley) and land use history (organic versus conventional farming and manure treated versus non-manure treated). They revealed that there was a reduced effect of plant species with a major rhizospheric activity due to different soil types. In an analysis to determine the diversity of *Paenibacillus* populations in the rhizosphere of maize grown in two different soils, da Silva *et al*., (2003) observed that the soil type, rather than cultivar, had a domineering effect on the *Paenibacillus* community. In the present study, the Sites differed greatly in soil characteristics and could account for the differences in bacterial numbers (Fig. 3.7). The MBT Site was observed to have high cation content (Table 3.1), which could be as a result of the clay mineral kaolinite present in the soil after mining of kaolin. This clay mineral is negatively charged and so attracts cations to form positively charged soil colloids (Ahn, 1970). Calcium, potassium, sodium and magnesium ions are involved in the following bacterial activities in the order listed: ion transport, increasing cell osmolarity, serves as a link in exergonic and endogonic reactions during bioenergetics (i.e. flow and transformation of energy in between the organism and the environment) and stabilisation of the cell membrane (Norris, 1996; Dimroth, 1994). Hence, a soil rich in these cations would promote bacterial growth (Chiarini *et al.,* 1998). However, soil pH has been reported to not only determine H^+ ion concentration but also to influence the concentration of these cations (Ashman and Puri, 2002). According to Ashman and Puri (2002), a soil with pH range of 6.5-7.5 will have high concentrations of Ca^{2+}, Mg^{2+} and K^+ compared to an acidic soil (4.0-5.5), which they reported as being characteristic of agricultural soils. It was explained that agricultural soils are prone to gradual acidification after planting because cations which balance excess acidity are

not returned to the soil, thereby resulting in a high concentration of Al^{3+}. This ion binds firmly to the colloidal exchange site making it impossible for other cations to be retained (Ashman and Puri, 2002; Ahn, 1970). This explains the high cation concentrations at the MBT Site that had a pH of 7.5 as opposed to the farming Site with a pH of 4.5, which also corresponds to the difference in bacterial numbers between Sites. This can be related to the study of Marschner *et al*., (2004), who reported differences in microbial community structure of Sorghum as a result of varying pH (5.9, 6.8, 7.5, 7.0 and 8.1). On the other hand, soil microorganisms can modify the physio-chemical properties of the rhizosphere (Garbaye, 1994) through their metabolic activities. For example, siderophore-producing pseudomonads may influence soil pH, in an iron rich soil. In this study, the isolation of microbial functional groups, which are known to be involved in biological processes such as nitrogen fixation, decomposition of organic matter and contributors to the P cycle (Table 3.4) indicates the possible effects of these organisms on soil fertility. However, the influence of soil organisms on soil properties was outside the scope of this study.

Land use history has been reported to affect the microbial diversity in the rhizosphere. This effect can be long lasting depending on the purpose for which the Site was previously used (Buckley and Schmidt, 2001). Such effect was analysed in a study using 16S rRNA targeted probes to quantify the abundance of specific microbial groups and subsequent land use history effect (Buckley and Schmidt, 2001). The Sites that they used included active and abandoned agricultural fields as well as uncultivated fields. They observed that the microbial community structures were remarkably similar with plots that had the same long-term history of agricultural management even when different plant species and recent land management practices were maintained in the plots, while the microbial community structure significantly differed between fields that had never been cultivated. In the present study the effect of land use on the microbial population as analysed using ANOVA revealed that there was a significant Site effect on the number of culturable microorganisms (Fig. 3.7). Considering the land history of the Sites, which was mining and farming, it was expected that the farming Site would have a higher bacterial number than the mining Site but this was not the case. Besides soil pH, this difference can be related to the long-term use of pesticides and fertilisers on the farm Site that may have directly or indirectly decreased the microbial population (Van Zwieten, 2006, Nicholson and

Hirsch, 1998). Steenwerth *et al*., (2003) studied the soil microbial structure of 9 land types which had the same soil texture; sandy loam but were categorised into irrigated agricultural soil, non-irrigated agricultural soil, annual grassland and perennial grassland. They assumed that land use history and management inputs (fertiliser, herbicides and irrigation), may create a unique soil environment that would bring about the support and selection of specific groups of organisms. They proved this by using phospholipids fatty acids analysis (PLFA) profiles that showed distinct microbial communities between the cultivated land-use types.

4.2.2 Determining specific functional groups in the rhizosphere

Though the establishment of AM fungi observed in the study did not increase or decrease the total bacteria isolated in the rhizosphere of *Cynodon dactylon* and *Pelargonium graveolens* plants, the isolation of specific functional groups in the mycorrhizal treated and control plots indicated a change in species composition (Fig.3.8). This was evident from the Gram stain results (Table 3.3) as fluctuations in the number of Gram-positives and Gram-negative bacteria in the rhizosphere of the MBT Site were observed. This relationship, however, could be misleading since the random sampling of colonies for Gram staining may have influenced the results. Furthermore, spatial heterogeneity of microbes in the soil, which is a well-known problem in microbial diversity studies (Kirk *et al*., 2004), could account for the Gram result fluctuations. Soil is heterogeneous and contains microhabitats that support microbial growth. As a result, microorganisms can form aggregates in soil in the form of clumps, hot spots or microsites as influenced by plant species (Kirk *et al*., 2004). This fact can be related to the study by Kuske *et al*., (2002) who determined the existence of bacterial heterogeneity in the rhizospheres of the grasses *Stipa*, *Hilaria* and *Bromus*. Using terminal-RFLP they observed that differences in the bacterial composition were influenced by the plant species used, which indicated that plants were capable of influencing the spatial heterogeneity of soil microorganisms. However, due to limited information on the spatial distribution of microorganisms in soil, random soil sampling in a traditional manner would underestimate microbial populations or species composition (Kirk *et al*., 2004; Trevors, 1998). In spite of the fluctuations in the Gram result, it could be deduced that Gram-positive rods were the major phenotypic bacterial types at the MBT and LHF Sites. This is agreement with

the study of da Silva and Nahas (2002) who observed the predominance of Gram-positive rods in the rhizosphere of brachiaria grass or chicken pea. On the contrary, the study conducted by Secilia and Bagyaraj (1987) observed more Gram-negative bacteria in pot cultures planted with Guinea grass. Trevors (1998) stated that the rhizosphere generally contains more Gram-negative bacteria than Gram-positive bacteria. However, this generalisation can also be questioned given the fact that soil-influencing factors such as soil depth, plant species, root exudates, soil texture and pH can also influence microbial types in the rhizosphere. Alternatively, this could mean that the more Gram-positive bacteria obtained in this study could have been influenced by random sampling.

The bacterial functional groups and fungi were determined in this study by ease of isolation using selective media, the ecological significance and the potential role they play in soil fertility or biocontrol along with AM fungi (Bianciotto *et al*., 1996a). The two land use types differed in the amount of culturable pseudomonads and phosphate solubilising microorganisms (Fig. 3.8), with the LHF Site having the greater bacterial species number. This result confirms reports of PSB and pseudomonads being predominantly found in agricultural soils and the ability of land use to influence species composition (Steenwerth *et al*., 2005; Salles *et al*., 2004). Large reserves of P in complexed form are contained in agricultural soils due to accumulated application of P fertilisers. Hence, the prevalence of these organisms in such soil lies in their ability to hydrolyse or solubilise certain complexed form of P with the aid of the enzyme acid phosphatase, which subsequently leads to improvement of plant P uptake (Rodriguez and Fraga, 1999).

Bacterial isolates belonging to the genera *Pseudomonas*, *Burkholderia* and *Bacillus* have been found to belong to the group of bacteria called PGPR and are known for their ecological use as biofertilisers (Gentili and Jupponen, 2006). Various bacterial species such as *Pseudomonas flavus*, *Bacillus megaterium* and *Burkholderia glathei* belonging to the above-mentioned genera were isolated in this study and were capable of solubilising $CaHPO_4$, thus indicating their potential exploitation for use as plant growth promoters. Pseudomonads are Gram-negative spore-forming rods that are common soil inhabitants and are known for their metabolic diversity (Madigan *et al*., 1998). Some species belonging to this genus are either saprotrophic and are involved

in suppression of plant diseases that promote plant growth and yield (Kloepper *et al*., 1980). The possible mechanism by which this group achieves such benefits to plants is through the production of plant growth regulators such as auxins, gibberellin, siderophores and indole acetic acid (Ahmad *et al*., 2005). Isolate PSA A1 (Table 3.4), which was isolated from the MBT Site, was identified as *Pseudomonas fulva* and is not regularly isolated from soils. The 96% identity obtained (Table 3.4) was below the cut off set for a significant species level in this study. Therefore, at species level, molecular identification of *Pseudomonas fulva* remains inconclusive. However, this organism belongs to the *Pseudomonas putida* (Trevisan) group that has been found to stimulate the development of AM fungal mycelium or increase the susceptibility of plants and mycorrhizal fungi colonisation (Gryndler and Vosátka, 1996; Meyer and Linderman, 1986b). This group of organisms has also been reported to mineralise inositol phosphate through the production of the enzyme phytase, thereby increasing P availability to plants to increase plant growth (Rodriguez and Fraga, 1999). Gryndler and Vosátka (1996) determined the response of *Gl. fistulosum* to treatments with *Pseudomonas putida* using maize. They observed that a high AM fungal colonisation of the plants roots was obtained when the fungus was inoculated together with *Pseudomonas putida* than when inoculated alone. Therefore, there is a possibility that *Pseudomonas fulva* could have promoted mycorrhizal establishment at the study Site were they were isolated. However, further studies to support the interaction between *Pseudomonas fulva* and AM fungi in pot or field trials are required.

Isolate N2A A53 and isolate NBP C31 (Table 3.4) were identified as *Bacillus megaterium*, which are spore forming mesophiles that have been found as the predominant populations in bulk soils and in the rhizosphere of plants such as potato, strawberry and oilseed rape (Smalla *et al*., 2001). In this study, a 98% similarlity to the Genbank nucleotide sequence is an indication that the isolate was well identified at the species level. *B. megaterium* was isolated from the rhizosphere soil of the LHF Site on N_2 free medium indicating their capability to solubilise insoluble P as well as to fix nitrogen. Ding *et al*., (2005) confirmed this by using PCR degenerate primers for the *nifH* genes. This gene indicates whether a bacterium has nitrogenase activity and possesses the *nifH* gene sequence, which is responsible for its nitrogen fixing capability (Ding *et al*., 2005). Furthermore, *B. megaterium* has been reported to produce a range of enzymes such as protease that degrades proteins and others that

degrades pectin and starch (Carrim *et al*., 2006), thereby improving nitrogen and carbon cycling as well as soil fertility. Studies by Alagawadi and Gaur (1988) reported the P solubilising ability of the species *B. polymyxa* in conjunction with *Rhizobium* to improve the nutrient content and uptake of P and N in chiken pea plants when fertilised with rock phosphate. On the other hand, plant colonisation studies have revealed that members of this genus have different colonisation patterns on plants as endophytes (i.e organisms that grow in plant structures) (McInroy and Kloepper, 1995). Some species such as *B. pumilus* and *B. mojavensis* have been found colonising the root, stem, leaves and twigs of tomato and coffee plants (Yan *et al*., 2003; Nair *et al*., 2002). However, the colonisation pattern of *B. megaterium* is yet to be fully ascertained since they have been found to colonise different areas of the plant depending on plant species (Liu *et al*., 2006). Hence, the isolation of *B. megaterium* in the farming Site, which have been reported as predominant species in such soils, portrays their roles in nutrient cycling or as plant growth promoters.

Similarly, species of *Burkholderia* were found to be bifunctional in this study because they were isolated as free-living N_2 fixers and as PSB. Isolate NBP C33 (Table 3.4) was identified as a bacterium in the Burkholdericeae family, while isolate N2A A53 (Table 3.4) was identified as *Burkholderia glathei*. The percentage identities of these two organisms were observed to be below the cut off mark for genus and species levels set for this study. Therefore, their identification as belonging to the genus *Burkholderia* was not conclusive. This genus was formed in 1992 after the proposed reclassification of *Pseudomonas* RNA homology group II by Yabuuchi *et al*., (1992). Members of this genus are Gram-negative aerobic, non-sporing straight rods that are known for their functional roles in the environment, agriculture, bioremediation and plant growth stimulation (Salles *et al*., 2004; Estrada-De Los *et al*., 2001). Most species use poly-β- hydroxybutyrate as a carbon source and are reported to live in close associations with AM fungi (Prescott *et al*., 2005; Bianciotto and Bonafante, 2002). The isolation of *B. glathei* from the rhizosphere of cucumber plants was reported by Mansfeld-Giese *et al*., (2002) who determined the bacterial populations associated with the mycelium of *Gl. intraradices* using fatty acid methyl ester analysis and culture dependent techniques. Furthermore, studies by Salles *et al*., (2004) reported the occurrence of this genus in soils cultivated with barley, grass (*Lolium perenne*), oats, or maize. They also observed that different species of this

genus were influenced by plant species and land use history of the Sites examined. This could mean that the presence of members of this genus isolated in this study could vary with land use and cultivated plant species. In addition, the ability of the isolated *Burkholderia* organisms to solubilise phosphate and fix nitrogen was confirmed by the analysis of the phenotypic and physiological characteristics of this genus in a study conducted by Pandey *et al*., (2005). Similarly, the species *B. cepacia*, *B. kururiensis* and *B. vietnamiensis* were isolated by Estrada-De Los *et al*., (2001) on N_2 free medium. These organisms were reported to have *nifH*DK genes that are responsible for nitrogen fixing capability. Therefore, the presence of this genus in the rhizosphere could play functional roles in nitrogen fixation and solubilisation of inorganic P.

Isolate Act C29 and Act C31 (Table 3.4) were identified as an actinomycetales bacterium and *Streptomyces* sp., respectively. Actinomycetes are natural soil inhabiting microbes that are found to be dominant in most soils. In this study, actinomycete representatives were observed to be predominant compared to other bacterial groups. This is in accordance with the findings of Heur *et al*., (1997) who reported the predominance of actinomycetes when the rhizosphere of transgenic and wild type potato plants was analysed. Smalla *et al*., (2001) also obtained a high proportion of members of this genus in the rhizosphere of oilseed rape and strawberry. Members of this group have been reported to play active roles in the decomposition of organic material (Heur *et al*., 1997) due to the production of various catalytic enzymes as well as the metabolic compounds. Gomes *et al*., (2000) reported the ability of five *Streptomyces* species to produce the enzyme endochitinase, which was observed to inhibit the growth of *Fusarium solani*, *Aspergillus parasiticus* and *Colletotrichium gloesporiodes* fungal pathogens, thus indicating their biocontrol activity. The mechanism of biocontrol activity is probably exerted through the action of 1, 3 ßglucanase (Hong *et al*., 2002) and chitinase on the glucan or chitin of the fungal cell wall. In addition, *Actinomyces pyogenes*, an endophytic bacterium, obtained from the leaves and stem of *Jacaranda decurrens* was reported to have amylolytic and proteolytic capability (Carrim *et al*., 2006). The ability of this organism and *Streptomyces cyaneus* (Petinate *et al*., 1999) to produce the enzyme amylase or protease indicates their potential role in biotechnology and agriculture to degrade amylose and proteins. Other degradative properties reported by members of

this group include, lignin degradative ability (Pasti *et al.*, 1990) and bacteriolytic activity (Vinogradova *et al.*, 1988). The biochemical properties of this group indicate that their presence at the two study Sites would be beneficial in rhizosphere interactions because of these varying metabolic and or degradation activities.

The restriction digest of 16S rDNA of these "standards" (identified functional bacterial species) and cultured bacterial samples obtained from the two study Sites were successfully conducted (Fig. 3.11). A visual analysis of the digest patterns indicated that there were differences and similarities in the banding patterns. The similarities in the banding patterns were related to the undigested samples, which when compared to the standards could probably mean that they belonged to the *Pseudomonas* group. However, these differences and/or similarities are not conclusive as this method experiences loss of smaller fragments and banding patterns can be too complex for analysis (Kirk *et al.*, 2004). In addition, it is presumed that the restriction enzymes used in this study may have been quite general and would not have extracted variances in banding patterns. A recommendation would be to use restriction enzymes for specific groups of organisms as well as to explore the use of T-RFLP, which is highly sensitive and can be used for comparative analysis (Marsh, 1999). This method is less complex than the banding patterns of RFLP, in that the restriction of nucleotide sequences will only occur at the terminal end fragment as a result of the fluorescently tagged primer (Marsh, 1999).

Fungi, which were not the main focus of this study, were found present in similar amounts in the rhizosphere of *C. datylon* and *Pelargonium graveolens* at the MBT and LHF Site, respectively (Fig. 3.8). From Table 3.5, the percentage identities of all fungal isolates were above the significant percentage identity set for this study for genus and species, with the exception of isolate D31. Therefore, the fungal species were identified with a high level of certainty. Qualitatively most isolated and identified genera were found to be common saprotrophs or pathogens (Table 3.5 and Table 3.6) that are involved in plant-fungal interactions. Saprotrophic fungi are one of the major contributors to the ecosystem as they release compounds through the degradation of both dead and living organic matter in soils; thus promoting or inhibiting growth of microorganisms in the rhizosphere (Garcia-Romera *et al.*, 1998). The most frequently isolated genera in this study from both Sites included

Penicillium, *Fusarium*, *Aspergillus and Trichoderma*. Studies by Malik and Sandhu (1973) revealed the cellulolytic ability of 10 fungal isolates among which were *Aspergillus* spp., *Fusarium solani*, *Alternaria humicola* and *Nigaspora* spp. They observed that these organisms increased the decomposition of organic matter in the form of fresh kallar grass and farmyard manure within 2 weeks of incubation in a saline soil. This indicates that the presence of these fungal saprotrophs in the soil would be beneficial in degradation of litter and plant materials (Gray and Williams, 1971). Furthermore, species like *Trichoderma harzianium*, which was isolated in this study (Table 3.5), have been used as biocontrol agents for other fungal pathogens, a process referred to as mycoparasitism (Brimmer and Boland, 2003). This fungus attacks target organisms such as *Pythium* and *Rhizoctonia solani* first, by penetrating the cell wall using chitinolytic enzymes. This process is followed by the production of antimicrobial compounds that permeates the hyphae thereby preventing resynthesis of the host cell wall. Finally, the fungus grows in the empty hyphae of the target organism after dissolution of the cytoplasm, an event that leads to cell rupture (Brimmer and Boland, 2003*). Exserohilium rostratum*, which was identified in this study (Table 3.5), is a known human pathogen that causes phaeohyphomycoses. However, it has been reported to occur in soils and is a common plant pathogen of grasses (Pratt, 2000; Guiraud *et al*., 1997). Pratt and Brink (2007), reported the response of seven *C. dactylon* cultivars to the pathogens *Exserohilium rostratum* and *Bipolaris spicifera* in a field experiment. They observed that all cultivars of the grass were susceptible to these two pathogens and the degree of susceptibility determined the persistence the grasses in the field. Though, some of the cultivars showed varying persistence with the fungus *B. spicifera*, no cultivar showed less susceptibility to *Exserohilum rostratum*, thus indicating their strong pathogenicity. This could also explain the isolation of this fungus in the rhizosphere of the MBT Site which was cultivated with *C. dactylon*. In addition, Guiraud *et al*., (1997) isolated the species *Exserohilum sodomii* from soil samples collected around the Dead Sea. They observed that this species was able to produce extra cellular polysaccharides and enzymes such as phenoloxidases, lipases, amylases and proteases. They also reported the ability of this fungus to have antimicrobial activity against pathogenic organisms such as *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. This could mean that members of this group could also play positive functional roles in the rhizosphere through its activity in spite of its pathogenicity.

The following genera, *Aspergillus*, *Penicillum* and *Cephalosporium* were isolated in this study to be capable of solubilising P as observed on the NBRIP medium. This is in accordance with the findings of Banik and Dey (1982) who observed the capability of *Aspergillus fumigatus* and *A. candidus* to solubilise rock phosphate as well as possessing a cellulose degrading capability. Similarly, Omar (1998) studied 36 fungal isolates for their ability to solubilise rock phosphate. *Aspergillus niger* and *Penicillium citrinum* were reported to solubilise rock phosphate in higher amounts, which is in agreement with this study, as large clearing zones of about 9 mm was evident on NBRIP medium. As such, members of these genera when present in soils could facilitate in the mineralisation of P present in complex forms.

The isolation of readily culturable microorganisms has been the widely used method in microbial diversity studies (Kirk *et al*., 2004; Meyer and Linderman, 1986b). This method is preferably used in determining the physiological and biochemical activities of culturable microorganisms in the ecosystem (Ellis *et al*., 2003). However, the use of this method has its limitations as different growth factors such as media concentration; pH and nutrient constituents may affect the observation and deductions drawn from this method (Basu *et al*., 2005). Another culture dependent method that is used in microbial diversity studies is CLPP technique. This method involves the use of BIOLOG plates to access the usage of 95 different carbon sources by microorganisms (Garland and Mill, 1991). This allows for the culturable microbial community to be determined based on substrate utilisation and the average metabolic respiration of C by these organisms. Hence, it is reliable, less time consuming, sensitive and could be used in further studies. However, CLPP has its disadvantages as it takes into account only organisms that can utilise the carbon source present (Garland and Mill, 1991).

4.2.3 Determination of the unculturable microbial populations in the rhizosphere

DGGE allows a large number of samples to be analysed concurrently and is a good tool for screening similarities or differences between communities (Kirk *et al*., 2004). In the present study, the method was attempted to investigate the bacterial community at the MBT Site. Visual observation revealed similarities in the patterns that resulted in a total of 12 bands which reflected dominant species present (Fig. 3.9). Similarities were observed in the number of bands but not in the intensity of bands. Sharma (2003) determined the structural and functional characterisation of bacteria in the rhizosphere of three legume plants *Vicia faba*, *Pisum sativum* and *Lupinus albus*. In one of the experiments were DGGE analysis was conducted on 10 samples collected from different pots of the same plant, visual similarities in DGGE profiles were observed. But when analysed using Gelcompar II software it was found that the samples were only 90% similar and not 100% as expected. Thus analysis of DGGE band patterns requires more than just visual analysis and the PCR bias of this method (i.e. the need to obtain high purity PCR product before adequate separation is observed) requires optimisation. However, this method would have been further applied if not for the experimental problems in extraction efficiency and period of the study.

PCR amplification from environmental samples has been reported to be highly problematic due to the presence of humic and fulvic acids that co-purify with the DNA thereby inhibiting *Taq* polymerase during amplification (Wechter *et al*., 2003). Other problems such as DNA polymerase error (inability of *Taq* polymerase to proofread inserted bases), primer selectivity and interference from DNA flanking the template region, decreases the accuracy of this method (Hanssen *et al*., 1998; Wang and Wang, 1997). In spite of these PCR pitfalls, this method has been extensively used to study microbial diversity. The ease of analysing many samples and the use of universal or group specific primers to target organisms or taxa of interest have led to its prevalent application (Kirk *et al*., 2004). The method of DNA extraction determines a successful PCR amplification (Niemi *et al*., 2001). As a result, the inability to successfully reproduce the combined PCR protocol of Kauffaman *et al*., (2004), Yeates *et al*., (1998) and Zhou *et al*., (1996) in this study, was subsequently

optimised using the Qiagen DNeasy plant mini kit. Components in the kit such as the QIAshredder and DNeasy spin column were used to remove cell debris and salt precipitates to obtain a purified DNA product. This kit, which was actually designed for plant DNA extraction, was also used for bacterial DNA and yielded products that were amplifiable. This indicated that the efficacy of developed kits for soil DNA extractions would be much more reliable and reproducible than the use of chemical and enzymatic methods of extraction (Niemi *et al*., 2001) if quantity and quality of DNA extracted is not to be compromised. Therefore, the use of soil DNA kits, although not used in this study, is highly recommended.

The purpose of cloning the bacterial DNA was to overcome the limitations of DGGE analysis as well as to identify organisms present in the PCR product resulting from total soil DNA extraction. One of the benefits of this method was the ability to optimise cloning efficiency by adjusting the ligation volumes based on the purified PCR product yield. Also the use of a positive and negative ligation controls gave an indication of suboptimal or unsuccessful reactions. It can be said that the cloning was suboptimal as <50 white colonies was observed in the positive control reaction. While in the negative control <30 blue colonies were observed which is an indication that compared to the standard reaction, more of the white colonies contained inserts. Out of a total of 28 clones, only 10 clones had the correct insert size (600-500bp) (Fig. 3.16), while the others had smaller inserts. The incorrect insert size could be due to factors such as presence of inhibitors in the PCR product, multiple PCR products being generated and cloned into the pGEM-T vector or the presence of dimmers formed from UV exposure during gel purification (Promega, 2005). Clones, upon sequencing, belonged to the genera of bacteria *Acidobacterium*, *Actinobacterium*, alpha *Proteobacterium*, *Propionibacterium*, *Bradyrhizobium* and *Sporichthya* (Table 3.7).

Based on the percentage identity chosen for this study, clones 1MBT7, 2MBT4 and 4LHF1 (Table 3.7) obtained from the MBT and LHF Sites were successfully identified as *Propionibacterium sp*. *Propionibacterium* are Gram-positive non-sporulating, non-motile anaerobic organisms that are known to ferment lactic acid, carbohydrates and polyhydoxyl alcohols by producing propionic acid, acetic acid and CO_2 (Madigan *et al*., 1998). These organisms have no defined source of origin

(Madigan *et al*., 1998) but activities of *Propionibacterium freudenreichii* have been reported in soils. This organism oxidises propionate and ferrous hydrous in the presence of humic acids when incubated under anaerobic conditions into acetate and ferric iron. This oxidation reaction increases acetate in the soil which favours fermenting bacteria that in turn transfer electrons via humic acids to ferric iron (Benz *et al*., 1998). Furthermore, *Propioniobacterium shermanii* was reported to have the capability to produce the enzyme polyphosphate (polyP)-glucokinase that was suggested to increase the amount of short chains polyP encountered in the intraradical hyphae of the AM fungus *Gigaspora margarita*. This was because the enzymes produced by this organism breaks down long chain polyP into short chain polyP that is involved in the metabolism and transport of P by AM fungi (Capaccio and Callow, 1982). Additionally, *Propionibacterium pentosaceum* was reported to reduce nitrate to nitrite in an anaerobic medium (Van Gent-Ruijters *et al.,* 1975), which could have an ecological importance in nitrogen fixation. The culture-dependent method used in this study was aerobic and therefore would have prevented the isolation of *Propionibacterium* sp. as well as any other anaerobic microorganism on media.

Clone 3LHF was obtained from inoculated plots at the LHF Site and was identified as *Bradyrhizobium* sp. with an accepted percentage identity of 99% (Table 3.7). This group are common soil organisms closely related to *Rhizobium* and are involved in root nodule formation in legumes and non-leguminous plants (Antoun *et al*., 1998). These groups of organisms fix nitrogen mainly in the nodules of legumes, however, in non-leguminous plants such as rice, *Brasicca napus* and *Arabidopsis thaliana*, nodule like structures have been reported to be formed which may be responsible for nitrogen fixation in such plants (Antoun *et al*., 1998; Trinick and Hadobas, 1995; Price *et al*., 1984). They have also been reported to form tripartite associations with AM fungi (Prescott *et al*., 2005). In a pot experiment conducted by Ramadan and Attia (2006), they observed that the dual inoculation of *Bradyrhizobium japonicum* strain and a mixture of different *Glomus* species increased nodulation of soybeans than the separate inoculation using these microorganisms. *Bradyrhizobium* are well known for their nitrogen fixing capability, however, they have also been reported to possess characteristics of PGPR, which includes P solubilisation capability, production of siderophores, production of IAA and the ability to be antagonistic towards plant pathogens (Antoun *et al*., 1998). In addition these organisms have been reported to

possess a hydrogenase gene that recycles hydrogen thereby increasing nitrogenase activity with their symbiont. This was observed by Baginsky *et al*., (2002), who showed using molecular techniques, that the inactivation of the hydrogenase gene in *Bradyrhizobium japonicum* affected the nitrogen content of black eyed pea.

Spirochthya polymorpha (Table 3.7) that was obtained from the LHF Site (clone 3MBT5) is a soil-inhabiting microbe that belongs within the order actinomycetales (Tamura *et al*., 1999). Due to the 96% identity of the organism (Table 3.7) obtained in this study, the identification to the species level remains inconclusive. This genus is characterised by a Gram-positive cell wall and is a facultative anaerobe. Their cell wall contains large amounts of L-diaminopimelic acid that acts as a precursor in lysine synthesis in bacteria (Suzuki *et al*., 1999; Gilvarg, 1959). In addition, this genus is differentiated from the actinomycetales group by their ability to release motile flagellate conidia in the presence of water (Prescott *et al*., 2005). This group of actinomycetes has not been studied extensively perhaps due to their rare nature but has been reported, however, to be isolated from soils using selective agar such as humic acid vitamin gellan-gum medium (Suzuki *et al*., 1999). This means that inability to culture this group of organisms in this study was due to media used. According to Tamura *et al*., (1999) *Sporichthya brevicatena* isolated from a soil sample had biochemical properties such as the ability to utilise simple and complex sugars (glucose, inositol, D-xylose, glycerol, lactose and melibose) as carbon sources and the ability to convert nitrite to nitrate. Hence, this could mean that directly or indirectly, this group of organisms through its biochemical activities may be of ecological importance in relation to nitrogen fixation and carbon degradation. However, further investigations into this area are required.

Actinobacteria (clones 3LHF3, 4LHF2) and alpha Proteobacteria (clone 3MBT6) (Table 3.7) are large divisions in the bacterial classification that consist of a wide variety of soil organisms and that were obtained from the MBT and the LHF Sites. Actinobacteria are a class that consists of ecologically significant genera that have been found present in the rhizosphere. Genera belonging to this class include *Actinomyces*, *Arthrobacter*, *Corynebacterium*, *Mycobacterium*, *Micrococcus Streptomyces* and *Propionibacterium* (Prescott *et al*., 2005). Gremion *et al*., (2003) who employed cloning techniques, reported the prevalence of actinobateria in the bulk

and rhizosphere soil contaminated with heavy metals. This was attributed to the fact that members in this group are metabolically active either by increasing plant growth due to production of phytohormones or through nitrogen fixation. A member of this group, *Streptomyces* sp. was identified to have a high affinity nickel transporter gene that enable it to survive on high nickel concentrations of up to 10mM $NiCl_2$ (Amoroso *et al*., 2000). Such characteristics could possibly indicate their participation in the removal of heavy metals from the environment. The alpha *Proteobacteria* class includes organisms that are capable of growing at low-level nutrients, commonly referred to as oligotrophs. Genera belonging to this class include *Rhodospirillum*, *Methylobacterium*, *Rhizobium*, *Nitrobacter and Beijerinkia*. Some of the mentioned genera are known for their metabolic activities in the rhizosphere such as the ability to fix nitrogen or solubilise insoluble phosphates (Rodriguez and Fraga, 1999). In addition, this group of bacteria have been proposed to protect plants or bacteria from toxic effects of heavy metals or enhance hyper-accumulating properties in plants due to their presence in metal contaminated soils (Gremion *et al*., 2003).

Furthermore, the identification to species level of clones 3HLF3, 3LHF, 4LHF2, 4LHF3 and 4LHF4 (Table 3.7) in spite of some of their percentage identity being above the significant value set for this study, remain inconclusive. These clones were observed to either belong to the class Proteobacteria, Actinobacteria or Acidobacteria (Table3.7) that consist of other genera. However, based on the phylogenetic diversity of isolated clones, similar groups of bacteria were obtained from the studies carried out by An *et al*., (2004). Their study analysed from rice field soil, the degrading characteristics of nitrate-reducing bacteria and the microbial communities involved in toluene degradation using DGGE analysis. Majority of the isolated clones belonged to these classes. Therefore, it could be said that these groups of bacteria have limit-less roles in the environment where they are obtained.

Many soil bacteria have been referred to as culturable or “unculturable” organisms. There have been arguments that certain groups of organisms are viable but not culturable on media and can only be identified molecularly (Dobrovol’Skaya *et al*., 2001). However, analysis of the 16S rDNA gene sequences could possibly provide information as to the formulation of media required to culture them when compared to other similar cultured species or bacterial class. According to Janssen *et al*., (2002)

improving media composition would increase the chances of obtaining these "unculturable" organisms. In this study, a cladogram analysis was conducted to illustrate similarities between nucleotide sequences obtained using culture dependent and culture independent technique (Fig. 3.17). Clones indicated in groups D, E and F were clearly differentiated from the nucleotide sequences in group A, B, C (Fig. 3.17). This indicates that there is little or no similarity between the clones and bacterial cultures (standards). Therefore, both methods are complimentary as they provide an insight into the diversity of soil bacterial community (Dobrovol'skaya *et al*., 2001). This is in accordance to the findings of Ellis *et al*., (2003) who reported differences in phylogenetic groups of bacteria based on the culture dependent and culture independent approach that they used, which they expected to have theoretically yielded similar results. This study also expected such similarities between methods but then other influential factors such as soil spatial heterogeneity, bacterial dominance both on media or molecularly and possible degrading of DNA in soil were considered (Marschner *et al*., 2001a; Trevors, 1996).

4.3 Arbuscular mycorrhizal fungal population assessment in soil

AM fungi are naturally occurring in most soils and are non- host specific (Smith and Read, 1997). The presence of this fungus is determined by accessing the infectivity and rate of colonisation of propagules such as spores, hyphae and infected root fragments (Brundrett *et al*., 1996). AM fungal populations studies are usually limited to determination of spore density in a given soil (Smith and Dickson, 1997).

Spore separation using a wet sieving method is one of the easiest and most frequently used procedures to examine the presence of AM fungi in soil. The limitations of this method as recorded in this study, was the failure to differentiate dead from viable spores, the loss of spores smaller than 45µm sieve mesh size or spores adhering to organic particles which may be discarded in extraction process. Therefore, it should be noted that the use of spore density method though reliable gives an underestimation of the total AM fungal spore population (Schenck, 1982). This is so said in that small endophytic spores like *Gl. tenue* may be lost during extraction process as well as spores parasitised by soil mammals or insects and the presence of

non-sporulating species that may not be detected through the standard technique (Liu and Luo, 1994). Hence the population referred to is one that is underestimated due to the above factors (Dames, 1991). Other techniques such as the use of specific antibodies, DNA probes, specific PCR primers, DGGE and isozyme banding patterns have been developed to overcome this problem (Kwong ma, 2004; Stukenbrock and Rosendahl, 2005; Tisserant *et al*., 1998), but were not used in this study.

Results from the spore enumeration analysis (Table 3.8) revealed variations in spore density at the MBT Site. From the pilot study, an average of 71 spores/100g of soil was enumerated but at 3 months spore density was 80 spores/100g of soil. The difference between the pilot result and that obtained after 3 months was very much anticipated due to inoculation of plots with the mycorrhizal inoculum. But then, other factors such as seasonal variations or spatial heterogeneity of spores probably influenced results (Uhlmann *et al*., 2004; Kabir *et al*., 1997; Anderson *et al*., 1983). Such influence was detected by Kwong ma (2004) who reported that species of AM fungi (*Acaulospora colossica*) sporulate profusely at the beginning of summer, remain viable as spores throughout the summer period and is only physiologically active in the cool season plant (e.g wild garlic) community. In this regard, collection of samples that is, from pilot sampling (August, 2005) to the last sampling at 9 months (June, 2006) can be seasonally categorised as spring, summer and winter seasons in South Africa with the highest spore density recorded during the summer period. There is a tendency that indigenous species of AM fungi though not active at one seasonal period, may become effective pending favourable environmental conditions that aids colonisation and subsequent release of spores into the soil. Due to this reason and other ecological factors the population of AM fungi through spore density is somewhat underrated.

High spore density variability, which was evident between the control plots at 3 months and other spore density values (Table 3.8), may be accounted for by vectors of dispersal such as wind. The MBT Site is open to windy conditions due to lack of windbreaks; therefore, AM fungal spores can be blown along with soil particles from one plot to another. The effect of wind dispersal was indicated in the study conducted by Allen *et al*., (1989). They observed that AM fungal spores were dispersed by wind up to 2 km from the disturbed Site of interest by monitoring the wind dispersal

patterns and subsequent spore enumeration-identification analysis. In addition, there is possibility that the spores enumerated in the control plots at the 3 months were not viable as indicated from the initial MPN assay. However, this can be ascertained by conducting a viability test (tetrazolium test) which is reliable (Meier and Charvat, 1993) though not conducted in this study. This invariably means that viable spores would have germinated to initiate colonisation, thereby resulting in low numbers. Non-viable spores would remain in soil, extracted numbers high but will not germinate.

AM fungal spores are taken to be the precursor to the presymbiotic stage of the AM fungal life cycle (Giovannetti, 2000) despite the fact that they are not the only colonisation propagules (Smith and Read, 1997). However, based on this generalisation, spores once relieved of spore dormancy or triggered by environmental factors will germinate and go through the complete fungal life cycle only to be produced by the extraradical hyphae (Fig. 1.4). Thus the decrease in spore numbers after 3 months at the MBT Site can be attributed to this phenomenon, time, or seasonal factors. However, no study as at yet has related reductions in AM fungal spore density to its life cycle, which can possibly be the case if spores were viable. On the other hand, Troeh and Loynachan (2003) observed a similar decreasing trend in spore density in soil planted with soybean when AM fungal survival in a continuous maize, soya bean and fallow cropping was determined over a period of 3 years in three different areas.

Studies of AM fungal populations in various Sites categorised by land-use type have become a common practice in urban ecology (Grimm *et al*., 2000). The study Sites, which can be referred to as a disturbed community, differed in AM fungal spore density. The spore density found at the LHF Site (Table 3.9) were above the range of spore density values (0-68 spores/100g) reported by Cousin *et al*., (2003) for all the disturbed Sites evaluated. Agricultural practices are known to affect AM fungal community structure and as such low spore densities and mycorrhization have been reported (Douds and Millner, 1999). However, this study cannot ascertain this fact since comparisons were with a mining Site rather than a similar management practice. The high variability in spore density between the two Sites (Table 3.8 and Table 3.9) may be as a result of different ecological factors, which include soil fertility and plant

species (De Oliveira and De Oliveira, 2005; Miller and Jackson, 1998; Smith and Read, 1997). Dames (1991) confirmed the impact of soil fertility when the effect of factors such as available P, pH, major cations, organic C, total N and moisture on spore density of AM fungal species in Nylsvley Nature Reserve, South Africa were determined. It was observed that available P and moisture were positively correlated with spore density indicating that the sporulation and infectivity of AM fungi are sensitive to these factors. However, other soil factors were found either to be negatively or positively correlated depending on the AM fungal species. Similarly, De Oliveira and De Oliveira (2005) concluded that AM fungi sporulation is seasonal, dependent on soil moisture and other soil factors. This possibly implied that the irrigation problem encountered in this study could have affected spore density values at the MBT Site, since moisture can affect spore germination phases which include hydration, activation, germ tube emergence and hyphal growth processes (Sinegani *et al*., 2005; Dames, 1991). Similarly, effects of seasonal variation was reported by Uhlmann *et al*., (2004) who compared the species diversity and spore numbers of AM fungi in winter-rainfall areas of South Africa and summer-rainfall areas of Namibia. They reported a low spore density of less than 75 spores/100g soil during the winter-rainfall season in South Africa than in the summer-rainfall season in Namibia (greater than 105 spores/100g soil). However, their findings were not solely attributed to seasonal variations in the two areas but also to host suitability and geographical distance, which may be a factor of variation in spore density between the Sites in this study.

Spore production varies with respect to species composition, dormancy and variability (Smith and Read, 1997). Because AM fungal spores are not the only colonisation propagules, difficulties have been experienced in ascertaining the contributions of different AM fungal propagules in the colonisation of root systems particularly in the field (Smith and Read, 1997). Determination of the infectivity potential of AM fungal propagules through the use of MPN technique has become widely used and is frequently employed in the production of inoculum (Brundrett *et al*., 1997; Douds and Thomson, 1994). This technique considers AM fungal life cycles and measures the collective infectivity of AM fungal propagules including non-sporulating species that may be present (Troeh and Loynachan, 2003). But this method is limited by the failure to detect propagules like hyphae that are destroyed due to disturbance.

Therefore, other methods such as whole cores and the use of nylon mesh bags with varying pore sizes have been employed to determine the contribution of AM hyphal networks to colonisation (Requena *et al*., 1996), though not conducted in the present study. In this study, it was observed that the MBT Site had a zero infectivity potential during the preliminary analysis. The increase from zero to 850 propagules /100g of soil is an indication that the inoculum used has the potential to improve the AM fungal infectivity when native propagules are low or futile under field conditions. From the observed spore density and infectivity at the LHF Site (5500 propagules /100g soil) which was higher than the MBT Site, it was expected that the percentage colonisation of the LHF Site should be at least 2 times greater than the percentage root colonisation at the MBT Site. This was not the case as close percentage colonisation values was recorded at 9 months and 5 months at the MBT and LHF Site, respectively (age factor). However, at 10 months at the LHF Site the percentage colonisation had increased to 19.4% in the inoculated plots and 8.2% in the control plots (Dames, 2006, Mycoroot unpublished report). This is more comparable to the MBT Site at 9 months thereby, confirming the resultant expectation.

Percentage root colonisation has been reported to be one of the major parameters used to measure biological productivity and nutrient use efficiency of plants inoculated with AM fungi (Kurle and Pfleger, 1994). This parameter also reflects the viability of AM fungal propagules in soil. The viability of AM fungal spores has always been questioned in terms of root colonisation because spores are not the only propagules capable of colonising host plant roots. A significant difference was evident in the root colonisation at 9 months for the MBT Site and the LHF Site at 5 months (Table 3.8 and Table 3.9). This result can be related to the findings of Eriksson (2001), who recorded a percentage colonisation rate of 39.7-42.1% at Sites with continuous management regimes when cultivated with *Ranunculus acris*, *Achillea millefolium* and *Anthriscus sylvestris*. Even though the percentage root colonisation obtained from in this study were lower, it is still possible that the continuous management practice at the LHF Site may be responsible for the higher percentage root colonisation recorded. This is because the land history of the Site has been routinely used for farm purpose with varying plant species which can bring about AM species diversity or richness. However, was not determined in this study may influence AM effectivity and infectivity. Alternatively, soil factors may also be responsible for the different

colonisation rate between Sites since it was reported to be a likely factor that affects root colonisation (Nehl *et al*., 1998). Generally, this study recorded a low percentage root colonisation in the field Sites (Table 3.8 and Table 3.9) compared to other studies that used similar land use management regimes in their analysis (Nehl *et al*., 1998; Thompson, 1994b). For example, studies by Nehl *et al*., (1998) recorded a percentage root colonisation of at least 38% after 48 days of sown cotton plants in three different fields. Since root colonisation by AM fungi is aided by spores or other infective propagules, relating the spore density and the MPN values could help in understanding why a low percentage root colonisation of 9.4% and 11.4% was recorded at the two study Sites (Table 3.8 and Table 3.9). From the spore enumeration analysis it can be said that majority of the “spores” were not viable given the level of MPN values. If the MPN reflects the colonisation potential of propagules in the field and was conducted successfully in a controlled environment; it probably means that there were certain processes that affected mycorrhizal fungi colonisation in the field (Abbott and Gazey, 1994). On the other hand, there is a possibility that the low percentage root colonisation obtained in the study Sites could be as a result of sampling procedure. This conclusion is based on the fact that the prevalence of mycorrhizal colonisation occurs at the root tips (Smith and Walker, 1981). Hence, sampling with a core borer 15 cm in depth may not be adequate to obtain samples containing the highly colonised section of the plant. Therefore, to obtain a good representation sample for percentage root colonisation, sampling at various soil depths should be considered. Additionally, fine roots occur at soil depths 1-60 cm, while the thickest roots occur at depths less than 20 cm (Fisher and Jayachandran, 1999). In this study, the root samples obtained from the MBT and LHF Site will rather be referred to as thin roots because of the root structure of the plant species used which means that a greater percentage colonisation was expected. However, according to the study of Skinner (2006), it was reported that AM fungi can occur in thick and fine roots but to a higher degree, maturation of roots are likely to affect location and colonisation of the root. This she concluded based on the majority of percentage colonisation found in young roots of lovegrass that were 3 weeks old conducted in a pot experiment. This is contradictory and may not be applicable to this study, as no root colonisation was recorded at 3 months from the MBT Site.

Glomalin is a recalcitrant glycoprotein produced by the hyphae of AM fungi but sloughs off and adheres to soil particles (Wright, 2000; Wright *et al*., 1998). Most information concerning this protein has been studied in relation to its role in soil aggregation and water stability (Rillig *et al*., 2002; Franzluebber *et al*., 2000). But its use as a bio-indicator for AM fungal presence is advantageous because it is AM fungal specific and could be easily assayed using Bradford's or immunoreactive antibodies (Lovelock *et al*., 2004b; Wright and Upadhyaya, 1998). The use of the AM fungal glycoprotein (glomalin) in this study was employed to confirm the presence and activity of the AM extraradical hyphal growth in the soil. The preliminary analysis of glomalin concentration at the MBT Site was 0.121 mg/ml and later at 3 months increased to 0.211mg/ml. Subsequently a decrease in glomalin concentration after 3 months was observed (Fig. 3.18) which perhaps was as a result of glomalin decomposition or rate of production in the soil (Steinberg and Rillig, 2003). Lutgen *et al*., (2003) raised an issue of seasonality in glomalin concentrations which could also be a factor responsible for the decrease in glomalin concentrations over time at the MBT Site (Fig. 3.18). They argued that since AM fungal spores and percentage root colonisation are subjected to seasonal changes, the density of AM fungal extraradical hyphae and its exuded product, glomalin, might also be influenced by seasonal variations. Hence, they determined the glomalin concentrations over time which revealed a rise and fall trend with a 24.5% change between highest and lowest mean concentrations (Lutgen *et al*., 2003). Similarly, the insignificant differences between treatment plots over time are in agreement with the suggestion that glomalin is relatively stable in soils and that several sampling events may not capture response variables (Lutgen *et al*., 2003). However, this assumption is tentative and has not been proven using a wider range of ecosystems. The total glomalin concentrations between plots at the MBT Site were not significantly different from each other with the exception of the concentration at the 9th month sampling. This could be related to two possible scenarios, which are glomalin stability or the spread of AM fungal extraradical hyphae from inoculated plots into control plots. Glomalin has been reported to remain stable in soil long after the decomposition of the hyphae (Rillig *et al*., 2003). Studies by Wright *et al*., (1998), revealed the possibility of obtaining a significant difference in glomalin change over a period of 2 or 3 years that will go undetected in a single year. Therefore, it is possible that plots already contained glomalin, irrespective of inoculation and can remain stable for a long period of time.

Little is known on how far the extraradical hyphae of AM fungi can spread in soils. However, according to Sanders *et al*., (1977), the spread of AM fungal extraradical hyphae is dependent on the fungal species and has been studied based on the different growth promoting effects of AM fungal species. Further studies would therefore be required to ascertain the extent of extraradical hyphal growth. In this regard, the spread of the extraradical hyphae can influence glomalin concentrations between plots when released, given that field trial plots are not compartmentalised, thus the difficulty in ascertaining this fact in the present study.

The method of quantifying glomalin is of great importance as sensitivity and precision of techniques may be required to verify obtained values of glomalin concentration. Bradford's protein assay is rapid and cheaper than the enzyme linked immunosorbent assay (ELISA) method which makes use of monoclonal antibodies that are not commercially available. These two methods measure different fractions of glomalin, which includes total glomalin (TG), easily extractable glomalin (EEG), immunoreactive easily extractable glomalin (IREEG) and immunoreactive total glomalin (IRTG) (Wright and Upadhyaya, 1998). These fraction types differ from the concentration of citrate buffer used and the period of incubation during the extraction process as well as in the detection method (Lutgen *et al*., 2003). The ELISA assay is very specific and sensitive as it captures glomalin proteins that have likely undergone microbiological mediated changes (Wright and Upadhyaya, 1998). However, the Brafords assay, which measures TG and EEG, is widely used due to its advantage of being more rapid and cost effective. It has also been shown by SDS-page analysis that crude glomalin extracts determined by this method had similar banding patterns to glomalin extracts from single AM fungal species. Thus, indicating its reliability (Rillig *et al*., 2001).

The rate of glomalin production and decomposition of AM hyphae in soil has not been well studied and the biochemical nature of this glycoprotein makes it a possible source of carbon for soil organisms. Klironomos and Ursic (1998) studied the effect of microathropods (collembola) densities on the extraradical hyphae of AM fungi as a food source. They reported that these microathropods reduced the efficacy of AM symbioses as they distorted the hypal network through their mode of feeding which could influence glomalin production. Steinberg and Rillig (2003) evaluated the

possible decomposition of AM fugal hyphae and glomalin by exploiting the non-saprotrophic nature of AM fungal hyphae. They found a decline in the total glomalin extracted, which they proposed to be attributed to the partial activity of soil organisms that contribute to the solubilisation of glomalin or decrease its sorption to soil particles. However, the mechanism of how these organisms decompose glomalin and extraradical AM fungal hyphae is still not certain and requires further investigation.

The impact of different crops and management practices on soil quality could be assessed by measuring soil aggregate stability and glomalin production by AM fungi (Rillig *et al*., 2003; Borie *et al*., 2000; Wright and Anderson, 2000). Glomalin is linked to soil carbon storage and has been reported to account for 30% of total carbon found in soils (Haddad and Sarkar, 2003). In this study, a clear distinction between the amount of glomalin found at the LHF Site at 5 months and MBT Site at 9 months was evident (Fig. 3.19). The LHF Site was found to have an average total glomalin concentration of 0.282 mg/ml while the MBT Site had a concentration of 0.102 mg/ml. Rillig *et al*., (2003), studied the distribution and decomposition of glomalin in an agricultural, native forest and afforested soils. Unlike the increase in glomalin concentration in the farm Site that was observed in this study, Rillig *et al*., (2003) observed a concentration of glomalin 3.06 mg/ml in their agricultural soil which was lower than the concentration of glomalin in the native forest and afforested soils. This indicates that glomalin concentrations vary with land disturbance that affect intact hyphal network. In addition, the lower concentration of glomalin at the MBT Site can be attributed to the previous non-existence of host plants thereby resulting in reduced presence of extraradical hyphae in soil. The glomalin concentrations obtained in this study and that of Rillig *et al*., (2003), perhaps could be as a result of the different land use management comparison or the effect of soil fertility. Rillig *et al*., (2003) also determined total glomalin concentrations in relation to soil chemical characteristics. They observed that soil characteristics such as Ca, Mg, K and pH did not correlate with total glomalin concentrations but reported a positive correlation with C and N content. However, in spite of this being a possible factor in glomalin variations, it may not be applicable in this study since the MBT Site had a higher value of %C and low NH_4 content than the LHF Site and vice versa (Table 3.1). Similarly, studies by Wright and Upadhyaya (1998) highlighted the possible abundance of total glomalin in clay soils as they obtained a total glomalin concentration of 14 mg/ml in soil rich in

clay than in other soil types they compared. This could be attributed to the ability of glomalin to bind more effectively with clay particles as clay soils are rich in minerals and will have enough binding sites for the glomalin compound. Therefore, the soil from the LHF Site will have a high tendency to accumulate and retain glomalin when sloughed off from AM fungal hyphae.

4.4 Alternative host plants from around the mine area

AM fungi are ubiquitous in soil and are reported to have evolved with terrestrial plants (Brundrett, 2002). Apart from the nutritional and soil functioning role of AM fungi, these organisms have been reported to influence plant community structure through the varying host response to AM fungal species (Van der Heijden, 1998).

This study discovered that the plant species examined were mainly endomycorrhizal with the majority being the arbuscular mycorrhizal type. Root colonisation type, when referred to as endomycorrhizal, had hyphal coils that penetrated the root cortical cells but with the absence of vesicles and arbuscules (Smith and Read, 1997). Ericoid mycorrhizal fungi though are known to form hyphal coils are restricted to the epidermal cells and have very specific host plants, which can be easily differentiated from AM fungal colonisation (Molina *et al*., 1992). The plant species were classified into families and were found to be members of Hyacinthaceae, Fabaceae, Asteraceae, Eurphorbiaceae and Scrophulariaceae (Table 3.9). Asteraceae and Fabaceae are the most common families in the Cape flora based on the number of species (Goldblatt and Manning, 2000).

The plant species *Selago corymbosa* (Scrophulariaceae) was found to be endomycorrhizal, similar to the studies by Hawley and Dames (2004) and Skinner (2001) who observed the root of species *Halleria lucida* to be AM. The fact that they were observed to be endomycorrhizal in this study does not mean that they do not form an AM type of colonisation. To confirm the relationship, sampling of the same species throughout the year may clarify the type of colonisation formed. According to Wang and Qui (2006) species belonging to this family form AM type colonisation only. This was based on their findings that out of 58 species, 40 were observed to

form AM type colonisation, while the rest were either non-mycorrhizal or facultatively AM mycorrhizal plants. However, this family has about 418 species (Goldblatt and Manning, 2000). Therefore, generalisations of the family being AM should be investigated, as there are still possibilities that some species such as *Selago corymbosa*, which was not among the 58 plant species examined by Wang and Qui (2006), could form other endomycorrhizal type colonisation.

According to Molina *et al*., (1992) some host plants from the family Asteraceae have been reported to form ectomycorrhizal, ericoid mycorrhizal and AM colonisation. Three species in this family were identified as *Pentzia incana*, *Elytropappus rhinocerotis* and *Helichrysum rosum*, which were found to be AM colonised with *Paris* type hyphal coiling (Table. 310). *Elytropappus rhinocerotis* is a plant known for its nutritive value for black rhino and has been found to possess medicinal chemical constituents (Bergh, 2006; Levyns, 1935). Studies by Skinner (2001), Allsopp and Stock (1993) confirmed the findings in this study that the mycorrhizal status of *Elytropappus rhinocerotis* and *Helichrysum rosum* is AM. *Pentzia incana* is an unpalatable woody species that thrives in diverse areas (Todd and Hoffman, 1999). Turnau and Mesjasz-Przybylowicz (2003) collected plant species from different areas including from the Agnes mine in Mpumalanga Province, South Africa. They found that the roots of four Ni-hyperaccumulating species (*Berkheya coddii, B. zeyherii, Senecio anomalochorus and S. coronatus*) belonging to the family Asteraceae formed AM symbioses. The plant species collected around the mine area (MBT) that belong to this family, is an indication that these plants may not just be a source of food for browsers but could be capable of exhibiting accumulatory properties like the *B. coddii*, thereby serving as potential host plants for rehabilitation of mine spoils. However, this property was not determined in the present study.

Albuca canadensis belonged to the Hyacinthaceae family and formed AM type of colonisation. In the survey carried out by Wang and Qui (2006) to determine the mycorrrhizal status of 3,617 species from 263 families, it was observed that all selected species of the Hyacinthaceae family were AM colonised. Similarly findings of other species in this family were found colonised by AM fungi (Berndt *et al*., 2003; Skinner, 2001).

Fabaceae, which is the second largest plant family, has also been reported to have genera that can form ectomycorrhizal, ericoid mycorrhizal and AM type of colonisation (Brundrett *et al.*, 1996; Molina *et al.*, 1992). However, the genus *Indigofera* was not among the listed genera. In this study a species in the genus *Indigofera* was found to be AM colonised. This is in agreement with Skinner (2001), Allsopp, and Stock (1993) who found other species such as *Indigofera stricta*, *Indigofera stipularis* and *Indigofera poliotes* to be colonised by AM fungi. Kohno and Marumoto (2004) in their study on the revegetation of a volcanic devastated slope using AM fungi showed that the species *Indigofera pseudotinctoria* grew well with approximately 80% of the roots being colonised by AM fungi, indicating the pioneering nature of some *Indigofera* species. This could be a possible good choice for revegetation of the MBT Site.

All the plant species examined for mycorrhizal status were found to have varying morphological features. The AM colonised roots possessed vesicles, which varied in their morphology and position in the roots (Fig. 3.21). The plant species *Pentzia incana* and *Elytropappus rhinocerotis* (Table 3.10) were found to form *Paris* type AM colonisation. *Paris* type colonisation strategies have been reported to be more frequent than the *Arum* type (Smith and Smith, 1997) probably because of the short life span of the *Arum* type structure (Smith and Read, 1997). Due to the argument as to which structural type of AM fungi were found in various plant species, Yamato (2004) examined weed species and herbaceous plants growing in a vacant area. It was observed that the *Arum* type of colonisation was found in the majority of the fast growing species, especially the weed plants, due to the wide spread of their roots in soil, while the *Paris* type were predominant in the slow growing herbaceous plants. In relation to this, the plant species selected in this study are mainly herbaceous plants and slow growing, which may account for the *Paris* type being mainly observed. The majority of studies that determined the mycorrhizal status or the morphological types of AM found in plants from diverse families reported a greater occurrence of the *Paris* type than the *Arum* type (Hawley and Dames, 2004, Yamato and Iwasaki, 2002; Smith and Smith, 1997). In this study the rare occurrence of *Arum* type mycorrhiza could be attributed to the presence either of different fungal species, host root structure or under sampling. For example, in the root structure of *C. dactylon*, vesicles were found to conform to the structural boundary of the cell (Fig. 3.4) with helical

hyphal coils, while in the *P. graveolens*, vesicles were found scattered around cortical cells. Cavagnaro *et al*., (2001) evaluated the influence of fungal identity on the morphological structure of arbuscular mycorrhizal fungi using a wild type tomato crop and six different species of AM fungi. They concluded that plant host control was not solely a determining factor since three species (*Gl. intraradices*, *Gl. mosseae* and *Gl. versiforme*) formed the *Arum* type and the remaining (*G. margarita*, *Gl. coronatum* and *Scutellospora calospora*) formed the *Paris* type. It is known that the formation of vesicles is common in certain groups of AM fungal species (Glomineae) (Morton and Benny, 1990; Smith and Read, 1997). Therefore, it could be extrapolated from the results (Table 3.9) that majority of the plant species that formed vesicles must be colonised by members of the order. On the other hand, the *Indigofera* sp. was the only plant species that had arbuscules in the root cortical cells. Arbuscles are responsible for nutrient transfer but its formation in terms of the arbuscle cycle is said to vary with plant species or is absent due to seasonal effects or environmental stress (Smith and Read, 1997; Smith and Smith, 1997). To confirm the seasonal effects on the presence or absence of arbuscules, it would be worthwhile to sample the same species that form AM symbiosis over extended seasonal periods.

The examined plants species were found to have similar habitual patterns (Table 3.9). They thrive in areas that reflect their ability to survive in harsh environmental conditions. Thus, the determined mycorrhizal status of the plants should be considered alongside their capability to thrive in harsh conditions and these could be an indication of their potential use in revegetation or phytoremediation. Amongst the examined host plants, this study recommends the use of *Indigofera* sp. in rehabilitation as they have a high level of performance in degraded areas and are N_2 fixing legumes that can promote nitrogen content in soils.

CHAPTER 5

SUMMARY AND CONCLUSIONS

5 Summary and Conclusions

5.1 The potential of arbuscular mycorrhizal fungi in rehabilitation of mine spoils

The benefits and wide host range of AM fungi has led to it being used as a bio-inoculant to improve plant nutrition and growth. This study focussed on the use of AM fungi, in conjunction with fertilisers, to rehabilitate an overburdened soil resulting from kaolin clay mining. In spite of the problems associated with the field trial, such as inadequate irrigation and resultant poor plant growth in most plots, the pot trial confirmed the use of AM fungal inoculum as an efficient environmentally friendly product that can be used in rehabilitation. Since AM fungi have been reported to be affected by fertilisers (Kurle and Pfleger, 1994), this study deemed it necessary to access the compatibility of Organic Tea and 3:1:5 NPK fertilisers with AM fungi.

Both fertilisers were capable of improving plant growth but in terms of compatibility with AM fungi, this study showed that the Organic Tea was not compatible as it reduced fungal colonisation relative to the 3:1:5 NPK. The significance of this is that fertilisers low in P can be applied moderately in plant systems combined with the use of AM fungal inoculum to attain maximum plant growth and yield. However, further analysis into the compatibility of other fertilisers with AM fungi is recommended.

What may seem as an omission during the pot trial set-up in this study, was not having another control that included pots treated with only AM fungal inoculum. However, it should be noted that this was deliberate as the effect of fertilisers on AM fungi was the determinant and not AM fungal effect on plant growth. This study opposes the replacement of chemical fertilisers with AM fungi, because it is irresponsible in an environment that is disturbed and no longer supports natural nutrient cycling process to recommend the use of only AM fungal inoculum. These fungi also require access to nutrients and do not make nutrients rather they will be more effective using lower concentrations of nutrients.

In addition, it would be worthwhile to carry out controlled experiments to determine the developmental stage at which these fertilisers hinder AM fungal colonisation. For example since the asymbiotic and presymbiotic stages of AM fungi are non-host dependent, an attempt could be made to germinate spores *in vitro* before inoculating them into pot cultures. This could aid in determining whether the fertilisers should be applied before the planting, since native AM populations could be inactive or during plant growth when AM fungal colonisation is already established.

5.2 Effect of introduced AM fungal inoculum on soil microbial populations

It well known that AM fungi interact with a wide range of soil microorganisms, but this interaction differs from one geographical region to another. In South Africa, the use of AM fungi as an inoculum in agriculture and rehabilitation is gaining recognition and therefore it is necessary to understand its interaction with rhizospheric organisms from a field perspective. Most studies have used pot trials to determine interaction effects with few field trials, which are indeed necessary if AM fungi are to be exploited successfully. This study, though not without limitations in the field trial, encourages more field analysis to be carried out and for longer periods of time. Also, proper irrigation systems should be set-up to avoid limitations in plant growth or other rhizospheric processes. Studies using field trials provide the formulation of guidelines as to the application and management of AM fungal populations in different land use types.

This study aimed at improving the understanding of AM fungal interactions in the rhizosphere using a field trial which was to an extent successful. The use of culture independent technique was employed to achieve this aim bearing in mind that some organisms “cannot” be cultured, even when selective media are used. However, the culture dependent technique, which was successfully applied in this study, was important as it enabled the physiological and cellular state of organisms to be determined. With the use of culture dependent techniques, it was evident that AM fungi had no effect on the culturable microbial numbers but had the potential to change species composition as indicated by the functional group composition in study Site plots. For example the presence of P solubilising microorganisms in the

agricultural AM fungal treated plots. On the other hand, because many molecular methods are dependent on PCR due to its flexibility and rapid nature, users must be aware of the shortcomings of this method. The culture independent technique, though not without setbacks was able to give an insight into the genetic variations in the rhizosphere. The identified clones belonging to the genera *Propionibacterium*, *Bradyrhizobium*, *Sporichthya*, *Acidobacterium and Actinobacterium* indicated the possible co-existence of these organisms that have varying physiology and function in the rhizosphere. However, a recommendation would be to study the individual or combination effects of some species in these genera with AM fungi.

Land-use management, which is frequently applied by industries and land owners, was found to have an effect on the culturable microbial population. This effect was suggested to vary due to the Site that was used, the geographical location and cultivation manner. Hence, confirming the hypothesis of this study that microbial interaction with AM fungi varies according to land use management. However, the use of statistical analysis to confirm this is far from sufficient as other factors such as soil nutrients and plant species influence such interaction studies. Hence, it would be necessary to take into account each affecting factor and involving the use of a more complex system, which will take into account biotic and abiotic factors affecting field trial analysis. This will then enable the use of multivariate analysis or principal component analysis to study factor effects.

5.3 Functional groups of soil microbial populations and their interaction with arbuscular mycorrhizal fungi

Functional groups, which are known to play key roles in the rhizosphere, were determined using selective media. Nitrogen fixers and actinomycete*s* were found to be the predominant functional groups in the rhizosphere of the two study Sites used. However, P solubilisers and *Pseudomonas* were found to be predominant at the farming Site. In addition, the identified species from the genera *Pseudomonas*, *Bacillus and Burkholderia* indicated the potential to be used as PGPR in managed systems, though this is not the first time that they have been reported to interact with AM fungi as PGPR or biofertilisers (Rodriguez and Fraga, 1999). Other functional

groups such as dentrifying bacteria, chitinase producers and hydrogenase producers play significant roles in the rhizosphere and as such could be further determined. The fungi identified in this study highlighted the presence of some pathogenic organisms for which control measures can be taken through the use of other fungal biocontrol agents when they become a problem or indirectly by the use of AM fungal inoculum to increase plants tolerance to diseases. This is because disease would only develop in the presence of a virulent pathogen, a susceptible host and favourable environmental conditions (Agrios, 1997).

Extraction and analysis of 16S rDNA sequences of selected functional groups and clones obtained directly from soil extractions enabled a judgement on the comparison of culture dependent and culture independent techniques. From the sequence similarity tree, it is likely that if more sequence data were obtained from both techniques a validated comparison using phylogenetics would be more appropriate. However, this study acknowledges the limitations of culture dependent technique and supports the fact that both methods be employed to study microbial interactions (Dobrovol'skaya *et al*., 2001). Furthermore, optimisation strategies in PCR amplication is highly recommended due to the desirable use of PCR-DGGE compared to cloning and the possible use of other methods such as fluorescent *in situ* hybridisation.

5.4 Effect of land use management on arbuscular mycorrhizal fungal population and infectivity

The AM fungal population is a key factor to improve plant sustainability and soil fertility due to its symbiotic benefits (Smith and Read, 1997). The ability to optimise and manage AM fungi in a field situation would bring about more information on how management practices such as mining and agriculture influence the AM fungal community and function. This study confirmed that management practices and most likely soil characteristics had an impact on the AM fungal population. The reliance on spore density to determine AM fungal population is subjective and provides an underrated result due to the factors that influence sporulation. Therefore, intensive sampling at different seasons with increased sample size is recommended. Also it will

be important to identify members of the AM fungal community since AM fungal species differs in physiology and geographic distribution. In addition, AM fungal species are known to differ in their effectivity (Dodd and Thomson, 1994), hence it would be worth focusing on other species to determine what factor, or land management practice affects its efficacy in both field and pot trials. Furthermore, in natural environments generally, the presence of more than one species of AM fungi in soil will brings about combined effects in terms of infectivity and effectivity with the host plants.

5.5 Mycorrhizal status of selected plants growing around the mine area

The mycorrhizal status of selected plant species were successfully determined with the majority being AM colonised. This study supports reports of the prevalence of AM associations in plant species examined in the Eastern Cape region (Hawley and Dames, 2004; Skinner, 2001). Despite, the low number of plant species examined in this study, the result confirms the mycorrhizal status of species that have initially been determined. The collection of these plant species in this study was based on the surrounding area, which could also be a determinant in selecting plant species for rehabilitation potential. By doing so, the ecological importance and the potential use of the plants selected could easily be exploited.

5.6 Recommendations

Although this study provided an insight into the different rhizospheric interactions that can occur under different land use management, further studies would be required to take into account main biotic and abiotic factors that affect field trial analysis. In addition, the time duration of this study limited any possible changes that may have occurred in the interaction effects. It would therefore, be pertinent to conduct field trials over periods of years with at least two growing seasons to enable differences in bacterial rhizospheric numbers and structure to be observed (Yang and Crowley, 2000). Furthermore, the use of other culture dependent techniques such as CLPP together with plate counts would provide more information as to the active cultural

populations in the rhizosphere. Similarly, the use of other culture independent techniques such as DGGE and T-RFLP would enable the functional but "unculturable" bacterial populations to be monitored.

This study has so far confirmed previous observations that land use management will affect AM fungal population in soils, which is involved in several interactive associations in the rhizosphere. The use of AM fungi as inoculants in agriculture and environmental rehabilitation is becoming more widely accepted as being the key to maintaining soil health and vitality because of the intimate link they form between the host plant and the soil environment. Analyses into the interaction of AM fungi and rhizospheric organisms using culture dependent and independent technique has provided an insight into the more complex associations that may exist; thereby, leading to enhancement of nutrient cycling processes and development of sustainable ecosystems.

References

Abbott, L.K. and Gazey, C. 1994. An ecological view of the formation of VA mycorrhizas. Plant Soil. **159**: 69-78.

Abd-All, M.H., Omar, S.A. and Karanxha, S. 2000. The impact of pesticides on AM and nitrogen-fixing symbioses in legumes. Applied Soil Ecology. **14**: 191-200.

Ahmad, F., Ahmad, I. and Khan, M.S. 2005. Indole acetic acid production by the indigenous isolates of *Azotobacter* and fluorescent *Pseudomonas* in the presence and absence of tryptophan. Turkish Journal of Biology. **29**: 29-34.

Agrios, G.N. 1997. Plant Pathology. Academic Press, Fourth Edition. USA.

Ahn, P.M. 1970. West African Soils. Oxford University Press, London.

Alagawadi, A.R. and Gaur, A.C. 1988. Associative effect of *Rhizobium* and phosphate-solubilizing bacteria on the yield and nutrient uptake of chickpea. Plant and Soil. **105**: 241-246.

Al-Karaki, G., McMichael, B. and Zak, J. 2004. Field response of wheat to arbuscular mycorrhizal fungi and drought stress. Mycorrhiza. **14**: 263-269.

Allen, M. F., Hipps, L. E. and Wooldridge, G. L. 1989. Wind dispersal and subsequent establishment of VA mycorrhizal fungi across a successional arid landscape. Landscape Ecology. **2**: 165-171.

Allen, M.F., Smith, W.K., Moore, T.S. and Christensen, M. 1981. Comparative water relations and photosynthesis of mycorrhizal and non-mycorrhizal *Bouteloua gracilis* H.B.K. lag ex steud. New Phytologist. **88**: 683-693.

Allsopp, N. and Stock, W.D. 1993. Mycorrhizal status of plants growing in the Cape floristic region, South Africa. Bothalia. **23**: 91-104.

Amaranthus, M.P. 1999. Mycorrhizal management. A look beneath the surface at plant establishment and growth. The Spring Florida Landscape Architecture Quarterly. USA.

Amerian, M.R. and Stewart, W.S. (2001). Effect of 2 species of arbuscular mycorrhizal fungi on growth assimilation and leaf water relations in maize (*Zea mays*). Aspects of Applied Biology. **63**: 1-6.

Ames, R.N., Reid, P.P., Porter, L.K. and Cambardella, C. 1983. Hyphal uptake and transport of nitrogen from two ^{15}N-labelled sources by *Glomus mosseae*, a vesicular-arbuscular mycorrhizal fungus. New Phytologist. **95**: 381-396.

Amoroso, M.J., Schubert, D., Mitscherlich, P., Schumann, P. and Kothe, E. 2000. Evidence for high affinity nickel transporter genes in heavy metal resistant *Streptomyces* species. Journal of Basic Microbiology. **40**: 295-301.

An, Y.J., Joo, Y.H., Hong, I.Y., Ryu, H.W. and Cho, K.S. 2004. Microbial characterization of toluene-degrading denitrifying consortia obtained from terrestrial and marine ecosystems. Applied Microbiology and Biotechnology **65**: 611-619.

Anderson, R.C., Liberta, A.E., Dickman, L.A. and Katz, A.J. 1983. Spatial variation in vesicular-arbuscular mycorrhiza spore density. Bulletin of the Torrey Botanical Club. **10**: 519-525.

Andrade, G., Mihara, K.L., Linderman, R.G. and Bethlenfalvay, G.J. 1997. Bacteria from the rhizosphere and hyposphere soils of different arbuscular-mycorrhizal fungi. Plant and Soil. **192**: 71-79.

Antoun, H., Beauchamp, C.J., Goussard, N., Chabot, R. and Lalande, R. 1998. Potential of *Rhizobium* and *Bradyhizobium* species as plant growth promoting rhizobacteria on non-legumes: Effect on radishes (*Raphanus sativus* L.). Plant Soil. **204**: 57-67.

Arden-Clarke, C. and Hodges, R.D. 1988. The environmental effect of conventional and organic/biological farming systems. II soil ecology, soil fertility and nutrient cycles. Biology Agriculture and Horticulture. **5**: 223-287.

Ashman, M.R. and Puri, G. 2002. Essential Soil Science: A clear and concise introduction to soil science. Blackwell Science Limited, United Kingdom.

Aspray, T.J., Eirian Jones, E., Whipps, J.M. and Bending, G.D. 2006. Importance of mycorrhization helper bacteria cell density and metabolite localization for the *Pinus sylvestris-lactarius rufus* symbiosis. FEMS Microbiological Research. **56**: 25-33.

Atkins, I.S. 2005. Microbial Environmental Surveillance: A molecular biology manual. IACR Rothamsted.

Atkinson, D., B. Addeley, J.A., Goicoechea, N., Green, J., Sánchez-díaz, M. and Watson, C.A. 2002. Arbuscular mycorrhizal fungi in low input agriculture. *In* Mycorrhizal Technology in Agriculture: From Genes to Bioproducts *Edited by* S. Gianinazzi, H. Scüepp, J.M. Barea and K. Haselwandter. Birkhäuser Verlag., Basel. pp. 211-222.

Atlas, R.M. 1984. Diversity of microbial communities. Advances in Microbial Ecology. **7**: 1-47.

Augé, R.M. 2001. Water relations, drought and vesicular arbuscular mycorrhizal symbiosis. Mycorrhiza. **11**: 3-42.

Augé, R.M. 2000. Stomatal behaviour of arbuscular mycorrhizal plants. *In* Arbuscular Mycorrhizas: Physiology and Function. *Edited by* Y. Kapulnik and J.D.D. Douds. Kluwer Academic Publishers, Netherlands. pp. 201-237.

Azcón-Aguilar, C. and Barea, J.M. 1996. Arbuscular mycorrhizas and biological control of soil-borne plant pathogens-an overview of the mechanisms involved. Mycorrhiza. **6**: 457-464.

Azcón-Aguilar, C. and Barea, J. 1992. Interactions between mycorrhizal fungi and other rhizosphere micororganisms. *In* Mycorrhizal Functioning. *Edited by* M.F. Allen. Chapman and Hall, Inc., New York, USA. pp. 163-198.

Azcón, R., Ambrosano, E. and Charest, C. 2003. Nutrient acquisition in mycorrhizal lettuce plants under different phosphorus and nitrogen concentration. Plant Science. **165**: 1137-1145.

Azcón-Aguilar, C., Jaizme- Vega, M.C. and Calvet, C. 2002. The contribution of arbuscular mycorrhizal fungi to the control of soil-borne plant pathogens. *In* Mycorrhizal Technology in Agriculture: From Genes to Bioproducts *Edited by* S. Gianinazzi, H. Scüepp, J.M. Barea and K. Haselwandter. Birkhäuser Verlag, Basel. pp. 187-197.

Azcón-Aguilar, C., Bago, B. and Barea, J.M. 1999. Saprophytic growth of AMF. *In* Mycorrhiza: Structure, function, molecular biology and biotechnology. *Edited by* A. Varma and B. Hock. Springer-Verlag, Berlin. pp. 391-407.

Bâ, M.A., Plenchette, C., Danthu, p., Duponnois, R. and Guissou, T. 2000. Functional compatibility of two arbuscular mycorrhizae with thirteen fruit trees in senegal. Agroforestry Systems. **50**: 95-105.

Baginsky, C., Brito, B., Imperial, J., Palacios, J. and Ruiz-Argüeso, T. 2002. Diversity and evolution of hydrogenase systems in rhizobia . Applied and Environmental Microbiology. **68**: 4915-4924.

Bago, B. and Bécard, G. 2002. Bases of biotrophy of arbuscular mycorrhizal fungi. *In* Mycorrhizal Technology in Agriculture: From Genes to Bioproducts. *Edited by* S. Gianinazzi, H. Scüepp, J.M. Barea and K. Haselwandter. Birkhäuser Verlag, Basel. pp. 33-48.

Bago, B., Shachar-Hill, Y. and Pfeffer, P. 2000. Dissecting carbon pathways in arbuscualr mycorrhizas with NMR spectroscopy. *In* Current advances in mycorrhizae research. *Edited by* Gopi K, Podila and Douds D.D. The American Phytopathological Society, Minnesota, pp. 111-126.

Bagyaraj, J.D. 1984. Biological interaction with VA mycorhizal fungi *In* VA mycorrhiza. *Edited by* L.L.C. Powel and J.D. Bagyaraj. CRC press, Boca Raton. pp. 131-153.

Balestra, G.M. and Misaghi, I.J. 1997. Increasing the efficiency of the plate counting method for estimating bacterial diversity. Journal of Microbiological Methods. **30**: 111-117.

Banik, S. and Dey, B.K. 1982. Available phosphate content of an alluvial soil as influenced by inoculation of some isolated phosphate-solubilizing micro-organisms. Plant Soil. **69**: 353-364.

Barea, J., Azcón, R. and Azcón -Aguilar, C. 2002b. Mycorrhizosphere interactions to improve plant fitness and soil quality. Antonie Van Leeuwenhoek. **81**: 343-351.

Barea, J., Pozo, M.J. and Azcón-Aguilar, C. (2005).Microbial co-operation in the rhizosphere. Journal of Experimental Botany. **56**: (417)1761-1778.

Barea, J.M., Gryndler, M., Lemanceau, P., Schüepp, H. and Azcón, R. 2002a. The rhizosphere of mycorrhizal plants. *In* Mycorrhizal Technology in Agriculture: From Genes to Bioproducts. *Edited by* S. Gianinazzi, H. Schüepp, J.M. Barea and K. Haselwandter. Birkhäuser Verlag, Basel. pp. 1-18.

Barker, J.S., Tagu, D. and Delp, G. 1998. Regulation of root and fungal morphogenesis in mycorrhizal symbioses. Plant Physiology. **116**: 1201-1207.

Barnett, H.L. 1962. Illustrated genera of imperfect fungi. Burgess Publishing Company, USA.

Basu, S., Pal, A. and Desai, P.K. 2005. Quality control of culture media in a microbiology laboratory. Indian Journal of Medical Microbiology. **23**: 159-163.

Belimov, A.A., Kojemiakov, A.P. and Chuvarliyeva, C.V. 1995. Interaction between barley and mixed cultures of nitrogen fixing and phosphate-solubilizing bacteria. Plant and Soil. **173**: 29-37.

Benabdellah, K., Azcon-Aguilar, C. and Ferrol, N. 1998. Soluble and membrane symbiosis-related polypeptides associated with the development of arbuscular mcyorrhizas in tomato (*Lycopersicum esculentum*). New Phytologist. **140**: 135-143.

Bentivenga, S.P. and Morton, J.B. 1994. Systemics of glomalean endomycorrhizal fungi: Current views and future directions. *In* Mycorrhizae and Plant Health. *Edited by* F.L. Pfleger and R.G. Linderman. APS press, Minnesota. pp. 283-308.

Benz, M., Schink, B. and Brune, A. 1998. Humic acid reduction by *Propionibacterium freudenreichii* and other fermenting bacteria. Applied and Environmental Microbiology. **64**: 4507-4512.

Bergh, N. 2006. *Elytropappus rhinocerotis* [online]. Available from http://www.plantzafrica.com/plantefg/elytrorhino.htm [Accessed Februrary 2007].

Berndt, R., Görke, C., Langer, E., Mennicken, M., Uhlmann, E. and Oberwinkler, F. 2003. Biodiversity of Southwestern African fungi: Interactions with higher plants, functional diversity and DNA-taxonomy. Biota Africa. http://www.biota-africa.org

Bianciotto, V. and Bonfante, P. 2002. Arbuscular mycorrhizal fungi: A specialised niche for rhizospheric and endocellular bacteria. Antonie Van Leeuwenhoek. **81**: 365-371.

Bianciotto, V., Lumini, E., Bonfante, P. and Vandamme, P. 2003. 'Candidatus glomeribacter gigasporarum' gen. nov., sp. nov., an endosymbiont of arbuscular mycorrhizal fungi. International Journal of Systemic Evolutionary Microbiology. **53**: 121-124.

Bianciotto, V., Minerdi, D., Perotto, S. and Bonfante, P. 1996. Cellular interactions between arbuscular mycorrhizal fungi and rhizosphere bacteria. Protoplasma. **193**: 123-131.

Bianciotto, V., Lumini, E., Lanfranco, L., Minerdi, D., Bonfante, P. and Perotto, S. 2000. Detection and identification of bacterial endosymbionts in arbuscular mycorrhizal fungi belonging to the family Gigasporaceae. Applied Environmental Microbiology. **66**: 4503-4509.

Bianciotto, V., Bandi, C., Minerdi, D., Sironi, M., Tichy, H.V. and Bonfante, P. 1996. An obligately endosymbiotic mycorrhizal fungus itself harbors obligately intracellular bacteria. Applied Environmental Microbiology **62**(8): 3005-3010.

Biró, B., Köves-Péchy, K., Vörös, I., Takács, T., Eggenberger, P. and Strasser, R.J. 2000. Interrelations between *Azospirillum* and *Rhizobium* nitrogen-fixers and arbuscular mycorrhizal fungi in the rhizosphere of alfalfa in sterile, AMF-free or normal soil conditions. Applied Soil Ecology. **15**: 159-168.

Bonfante, P. and Perotto, S. (2000). Outside and inside the roots: cell to cell interactions among arbuscular mycorrhizal fungi, bacteria and host plant: In Current Advances in Mycorrhizae Research, Edited by Gopi K, Podila and Douds D.D. The American Phytopathological Society, Minnesota.141-155.

Borie, F.R., Rubio, R., Morales, A. and Castillo, C. 2000. Relationship between arbuscular mycorrhizal hyphal density and glomalin production with physical and chemical characteristics of soils under no-tillage. Revista Chilena De Historia Natural. **73**: 749-756.

Borneman, J., Skroch, P.W., O'Sullivan, K.M., Palus, J.A., Rumjanek, N.G., Janssen, J.L., Nienhuis, J. and Triplett, E.W. 1996. Molecular microbial diversity in agricultural soils in wisconsin. Applied and Environmental Microbiology. **62:** 1935-1943.

Bossio, D.A., Scow, K.M., Gunapala, N. and Graham, K.J. 1998. Determinants of soil microbial communities: Effects of agricultural management, season and soil type on phospholipid fatty acid profiles. Microbial Ecology. **36**: 1-12.

Bothe, H. and Hildebrandt, U. 2002. Arbuscular mycorrhizal fungi nitrate assimilation: Genes and ecophysiological aspects. *In* Mycorrhizal Technology in Agriculture: From Genes to Bioproducts *Edited by* S. Gianinazzi, H. Scüepp, J.M. Barea and K. Haselwandter. Birkhäuser Verlag, Basel. pp. 123-128.

Bouamri, R., Dalpé, Y., Serrhini, M.N. and Bennani, A. 2006. Arbuscular mycorrhizal fungi species associated with rhizosphere of *Phoenix dactylifera* L. in Morocco. African Journal of Biotechnology. **5**(6): 510-516.

Bradford, M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. **72**: 248-254.

Braunberger, P.G., Miller, M.H. and Peterson, R.L. 1991. Effect of phosphorus nutrition on morphological characteristics of vesicular-arbuscular mycorrhizal colonization of maize. New Phytologist. **119**: 107-113.

Brimner, T.A. and Boland, G.J. 2003. A review of the non-target effects of fungi used to biologically control plant diseases. Agriculture, Ecosystems and Environment. **100**: 3-16.

Brundrett, M. 2004. Diversity and classification of mycorrhizal associations. Biological Review. **79:** 473-495.

Brundrett, M., Bougher, N., Dell, B., Grove, T. and Malajczuk, N. 1996. Working with mycorrhizas in forestry and agriculture. ACISR monograph. Pirie Printers, Canberra.

Brundrett, M.C. 2002. Co-evolution of roots and mycorrhizas of land plants. New Phytologist. **154**: 275-304.

Bücking, H. and Shachar-Hill, Y. 2005. Phosphate uptake, transport and transfer by the arbuscular mycorrhizal fungus *Glomus intraradices* is stimulated by increased carbohydrate availability. New Phytologist. **165**: 899-912.

Buckley, D.H. and Schmidt, T.M. 2001. The structure of microbial communities in soil and the lasting impact of cultivation. Microbial Ecology **42**: 11-21.

Busman, L., Lamb, J., Randall, G., Rehm, G. and Schmitt, M. 2002. The nature of phosphorus in soils [online]. Available from http://awqa.org/pubs/waterqual/phosphate.pdf [Accessed November 2006].

Busse, M.D. and Ellis, J.R. 1984. Vesicular-arbuscular mycorrhizal (*Glomus fasciculatum*) influence on soybean drought in high phosphorus soil. Canadian Journal of Botany. **63**: 2290-2294.

Calvet, C., Barea, J.M. and Pera, J. 1992. *In vitro* interactions between the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae* and some saprophytic fungi isolated from organic substrates. Soil Biology and Biochemistry. **24**: 775-780.

Capaccio, L.C.M. and Callow, J.A. 1982. The enzymes of polyphosphate metabolism in vesicular-arbuscular mycorrhizas. New Phytologist **91**: 81-91.

Caravaca, F., Tortosa, G., Carrasco, L., Cegarra, J. and Roldã¡n, A. 2006. Interaction between AM fungi and a liquid organic amendment with respect to enhancement of the performance of the leguminous shrub *Retama sphaerocarpa*. Biological and Fertility of Soils. **43**: 30-38.

Cardoso, I.M. and Kuyper, T.W. 2006. Mycorrhizas and tropical soil fertility. Agriculture, Ecosystems & Environment. **116**: 72-84.

Caris, C. and Hordt, W. 1998. Studies of iron transport by arbuscular mycorrhizal hyphae from soils to peanut and sorghum plants. Mycorrhiza. **8**: 35-39.

Carlile, M.J., Watkinson, S.C. and Gooday, G.M. 2001. The fungi. Elsevier Academic Press, San Diego.

Carrim, A. J. I., Barbosa, E.C. and Vieira, J. D. G. 2006. Enzymatic activity of endophytic bacterial isolates of *Jacaranda decurrens* cham. (carobinha-do-campo). Brazilian Archives of Biology and Technology. **49**: 353-359.

Cavagnaro, T.R., Gao, L., Smith, F.A. and Smith, S.E. 2001. Morphology of arbuscular mycorrhizas is influenced by fungal identity. New Phytologist **151**: 469-475.

Chabot, R., Antoun, H. and Cescas, M.C. 1996. Growth promotion of maize and lettuce by phosphate-solubilising *Rhizobium leguminosarum biovar phaseoli*. Plant and Soil. **184**: 311-321.

Chhabra, M.L., Bhatnagar, M.K. and Sharma, M.P. 1992. Influence of vesicular-arbuscular (VA) mycorrhizal fungus on important diseases of maize. Indian Phytopathology. **45**: 235-236.

Chiarini, L., Bevivino, A., Dalmastri, C., Nacamulli, C. and Tabacchioni, S. 1998. Influence of plant development, cultivar and soil type on microbial colonization of maize roots. Applied Soil Ecology. **8**: 11-18.

Christensen, H. and Jakobsen, I. 1993. Reduction of bacterial growth by vesicular-arbuscular mycorrhizal fungus in the rhizosphere of cucumber (*Cucumis sativus* L.). Biology and Fertility of Soils. **15**: 253-258.

Coetzee, J.C. 1982. A note on the E*ndogone* and *Acaulospora* in South Africa. South African Journal of Botany. **1**: 38-39.

Compton, J.E. and Cole, D.W. 2001. Fate and effects of phosphorus additions in soils under N2-fixing red alder. Biogeochemistry. **53**: 225-247.

Cordier, C., Gianinazzi, S. and Gianinazzi-Pearson, V. 1996. Colonisation patterns of root tissues by *Phytophthora nicotianae* var. *parasitica* related to reduced disease in mycorrhizal tomato. Plant Soil. **185**: 223-232.

Courtright, E.M., Wall, I.H. and Virginia, R.A. 2001. Determining habitat suitability for soil invertebrates in an extreme environment: The McMurdo dry valleys, antarctica. Antarctic Science. **13**: 9-77.

Cousins, J.R., Hope, D., Gries, C. and Stutz, J.C. 2003. Preliminary assessment of arbuscular mycorrhizal fungal diversity and community structure in an urban ecosystem. Mycorrhiza. **13**: 319-326.

Cuenca, G., De Andrade, Z. and Escalante, G. 1998. Diversity of Glomalean spores from natural, disturbed and revegetated communities growing on nutrient-poor tropical soils. Soil Biology and Biochemistry. **30**: 711-719.

Cullen, D.W. and Hirsch, P.R. 1998. Simple and rapid method for direct extraction of microbial DNA from soil for PCR. Soil Biology and Biochemistry. **30**: 983-993.

Da Silva, P. and Nahas, E. 2002. Bacterial diversity in soil in response to different plants, fertilisers and liming. Brazillian Journal of Microbiology. **33**: 304-310.

da Silva, K.R., Salles, J.F., Seldin, L. and van Elsas, J.D. 2003. Application of a novel *Paenibacillus*-specific PCR-DGGE method and sequence analysis to assess the diversity of *Paenibacillus* spp. in the maize rhizosphere. Journal of Microbiological Methods. **54**: 213-231.

Dalpé, Y., Diop, T.A., Plenchette, C. and Gueye, M. 2000. Glomales species associated with surface and deep rhizosphere of *Faidherbia albida* in Senegal. Mycorrhiza. **10**: 125-129.

Dames, J.F. 1991. The distribution of vesicular arbuscular mycorrhizal fungi in the savanna region of Nylsvley nature reserve in relation to soil fertility factors. Masters thesis. University of the Witswaterand, Johannesburg.

Davies, F.T., Potter, J.R. and Linderman, R.G. 1993. Drought resistance of mycorrhizal pepper plants independent of leaf P concentration- response in gas exchange and water relations. Physologia Plantarum. **87**: 45-53.

De Freitas, J.R., Banerjee, M.R. and Germida, J.J. 1997. Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (B*rassica napus* L.). Biology and Fertility of Soils. **24**: 358-364.

De Oliveira, A. N. and De Oliveira, L. A. 2005. Seasonal dynamics of arbuscular mycorrhizal fungi in plants of *Theobroma grandiflorum* Schum and *Paullinia cupana Mart.* of an agroforestry system in central Amazonia, Amazonas State, Brazil. Brazilian Journal of Microbiology. **36**: 262-270.

Dell, B. 2002. Role of mycorrhizal fungi in ecosystems. CMU Journal. **1**: 47-60.

Dhillon, K.S., Yagodeen, B.A. and Pleshkov, A.C. 1983**.** Micronutrients and nitrogen metabolism **I**. Effects of different levels of micronutrients in maize plants. Plant and Soil. **73**: 355-363.

Di Pietro, A., Lorito, M., Hayes, C.K., Broadway, R.M. and Harman, G.E. 1993. Endochitinase from *Gliocladium virens*: Isolation, characterisation and synergistic antifungal activity in combination with gliotoxin. Phytopathology. **83**: 308-313.

Dimroth, P. 1994. Bacterial sodium ion-coupled energetics. Antonie Van Leeuwenhoek. **65**: 381-95.

Ding, Y., Wang, J., Liu, Y. and Chen, S. 2005. Isolation and identification of nitrogen-fixing bacilli from plant rhizosphere in Beijing region. Journal of Applied Microbiology. **99**: 1271-1281.

Diop, T.A., Gueye, M., Dreyfus, B.L., Plenchette, C. and Strullu, D.G. 1994. Indigenious arbuscular mycorrhizal fungi associated with *Acacia albida* del. in different areas of senegal. Appied Enviromental Microbiology. **60**: 3433-3436.

Dobrovol'skaya, T.G., Lysak, L.V., Zenova, G.M. and Zvyagintsev, D.G. 2001. Analysis of soil bacterial diversity: Methods, potentiality and prospects. Microbiology. **70**: 149-167.

Dodd, J.C. and Thompson, B.D. 1994. The screening and selection of inoculant arbuscular-mycorrhizal and ectomycorrhizal fungi. *In* Management of Mycorrhizas in Agriculture, Horticulture and Forestry. *Edited by* A.D. Robson, L.L. Abbot and N. Malajckzuk. Kluwer Academic Publishers, Netherlands. pp. 149-158.

Dodd, J. C., Dougall, T. A., Clapp, J. P., Jeffries, P. 2002. The role of arbuscular mycorrhizal fungi in plant community establishment at Samphire Hoe, Kent, UK. Biodiversity Conservation. **11**:39–58.

Douds Jr, D.D., Galvez, L., Franke-Snyder, M., Reider, C. and Drinkwater, L.E. 1997. Effect of compost addition and crop rotation point upon VAM fungi. Agriculture, Ecosystems and Environment. **65**: 257-266.

Douds, D.D. and Millner, P.D. 1999. Biodiversity of arbuscular mycorrhizal fungi in agroecosystems. Agriculture, Ecosystems & Environment. **74**: 77-93.

Douds, D.D., Pfeffer, P.E. and Scachar-Hill, Y. 2000. Carbon partitioning, cost and metabolism of arbuscular mycorrhizas. *In* Arbuscular Mycorrhizas: Physiology and Function. *Edited by* Y. Kapulnik and J.D.D. Douds. Kluwer publishers, Netherlands. pp. 107-129.

Drijber, R.A., Doran, J.W., Parkhurst, A.M. and Lyon, D.J. 2000. Changes in soil microbial community structure with tillage under long-term wheat fallow management. Soil Biology and Biochemistry. **32**: 1419-1430.

Dumas-Gaudot, E., Armelle, G., Cordier, C., Gianinazzi, S. and Gianinazzi-pearson, V. 2000. Modulation of host defence systems *In* Arbuscular Mycorrhizas: Physiology and Function. *Edited by* Y. Kapulnik and J.D.D. Douds. Kluwer Academic Publishers, Netherlands. pp. 173-200.

Elgaali, H., Hamilton-Kemp, T.R., Newman, M.C., Collins, R.W., Yu, K. and Archbold, D.D. 2002. Comparison of long-chain alcohols and other volatile compounds emitted from food-borne and related Gram-positive and Gram-negative bacteria. Journal of Basic Microbiology. **42**: 373-380.

Elliott, M.L. and Des Jardin, E.A. 1999. Comparison of media and diluents for enumeration of aerobic bacteria from *Bermuda* grass golf course putting greens. Journal of Microbiological Methods. **34**: 193-202.

Ellis, R.J., Morgan, P., Weightman, A.J. and Fry, J.C. 2003. Cultivation-dependent and independent approaches for determining bacterial diversity in heavy metal contaminated soil. Applied and Environmental Microbiology. **69**: 3223-3230.

Entry, J.A., Rygiewicz, P.T., Watrud, L.S. and Donnelly, P.K. 2002. Arbuscular mycorrhizal response to adverse soil conditions. *In* Arbuscular Mycorrhizae: Interactions in Plants, Rhizosphere and Soils *Edited by* A.K. Sharma and B.N. Johri. Science Publishers, Inc., Hampshire. pp. 135-158.

Eriksson, Å. 2001. Arbuscular mycorrhiza in relation to management history, soil nutrients and plant species diversity. Plant Ecology. **155**: 129-137.

Estrada-De Los, S., Bustillos-Cristales, P., Caballero-Mellado, R. and Caballero-Mellado, J. 2001. *Burkholderia*, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. Applied Environmental Microbiology **67**: 2790-2798.

Fagbola, O., Osonubi, O., Mulongoy, K. and Odunfa, S.A. 2001. Effects of drought stress and arbuscular mycorrhiza on the growth of *Gliricidia sepium* (jacq). walp and *Leucaena leucocephala* (lam.) de wit. in stimulated eroded soil conditions. Mycorrhiza. **11**: 215-223.

Farrar, J., Hawes, M., Jones, D. and Lindow, S. 2003. How roots control the flux of carbon to the rhizosphere. Ecology. **84**: 827-837.

Fillion, M., St-Arnaud, M. and Fortin, J.A. 1999. Direct interaction between the arbuscular mycorrhizal fungus *Glomus intraradices* and different rhizosphere microorganisms. New Phytologist. **141**: 525-533.

Fitter, A.H. 1985. Functioning of vesicular-arbuscular mycorrhiza on growth and water relations of plants. New Phytologist. **89**: 599-608.

Fitter, A.H. and Garbaye, J. 1994. Interaction between mycorrhizal fungi and other soil organisms. *In* Management of Mycorrhizas in Agriculture, Horticulture and Forestry. *Edited by* A.D. Robson, L.L. Abbot and N. Malajckzuk. Kluwer Academic Publishers, Netherlands. pp. 47-68.

Fisher, J.B. and Jayachandran, K. 1999. Root structure and arbuscular mycorrhizal colonization of the palm *Serenoa repens* under field conditions. Plant and Soil. **217:** 229–241.

Fletcher, A., Moot, D.J. and Stone, P. 2004. Fertiliser P effects on biomass partitioning and quality of sweet corn in a cool temperate environment. *In* New directions for a diverse planet: Proceedings of the 4th international crop science congress. Brisbane, Australia.

Fontanet, X., Estaun, V., Camprubi, A. and Calvet, C. 1998. Fungicides added to potting substrate affect mycorrhizal symbiosis between a peach- almond rootstock and *Glomus* sp. Horticultural Science. **33**: 1217-1219.

Franzluebber, A.J., Wright, S.F. and Stuedemann, J.A. 2000. Soil aggregation and glomalin under pastures in the Southern Piedmont USA. Soil Science Society of America. **64**: 1018-1026.

Frey, B. and Schüepp, H. 1993. Acquisition of nitrogen by external hyphae of arbuscular mycorrhizal fungi associated with *Zea mays* L. New Phytologist. **124**: 221-230.

Friberg, S. 2001. Distribution and diversity of arbuscular mycorrhizal fungi in traditional agriculture on the Niger inland Delta, Mali West Africa. CBM: Skriftserie. **3**: 53-80.

Fromin, N., Hamelin, J., Tarnawski, S., Roesti, D., Jourdain-Miserez, K., Forestier, N., Teyssier-Cuvelle, S., Gillet, F., Arangno, M. and Rossi, P. 2002. Statistical analysis of denaturing gel electrophoresis (DGE) finger-printing patterns. Environmental Microbiology. **4**: 634-643.

Galal, Y.G.M., El-ghandour, I.A., Osman, M.E. and Abdel-Raouf, A.M.N. 2003. The effect of inoculation by mycorrhizae and *Rhizobium* on the growth and yield of wheat in relation to nitrogen and phosphorus fertilization as assessed by ^{15}N techniques. Symbiosis. **34**: 171-183.

Garbaye, J. 1994. Helper bateria: A new dimension to the mycorrhizal symbiosis. New Phytologist. **128**: 197-210.

Garbeva, P., van Veen, J.A. and van Elsas, J.D. 2004. Microbial diversity in soil: Selection microbial populations by plant and soil type and implications for disease suppressiveness. Annual Review of Phytopathology. **42**: 243-270.

Garbeva, P., Overbeek, L.S., Vuurde, J.W. and Elsas, J.D. 2001. Analysis of endophytic bacterial communities of potato by plating and denaturing gradient gel electrophoresis (DGGE) of 16S rDNA based PCR fragments. Microbial Ecology **41**: 369-383.

Garcia-Romera, I., Garcia-Garrido, J.M., Martin, J., Fracchia, S., Mujica, M.T., Godeas, A. and Ocampo, J. 1998. Interactions between saprophytic *Fusarium* starins and arbuscular mycorrhizas of soybean plants. Symbiosis. **24**: 235-246.

Gardes, M. and Bruns, T.D. 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Molecular Ecology. **2**: 113-118.

Garland, J.L. and Mills, A.L. 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilisation. Applied Environmental Microbiology **57**: 2351-2359.

Gaur, A. and Adholeya, A. 2004. Review article: Prospects of arbuscualr mycorrhizal fungi in phytoremediation of heavy metal contaminated soils. Current Science. **86**: 528-534.

Gentili, F. and Jumpponen, A. 2006. Potential and possible uses of bacterial and fungal biofertilisers. *In* Handbook of microbial biofertilizers. *Edited by* M.K. Rai. Food Products Press, London. pp. 1-18.

Gildon, A. and Tinker, P.B. 1983. Interactions of vesicular-arbuscular mycorrhizal infection and heavy metal in plants. New Phytologist. **95**: 247-261.

Gilvarg, C. 1959. N-succinyl-L-diaminopimelic acid. The Journal of Biological Chemistry. **234**: 2955-2959.

Giovannetti, M. 2000. *In* Arbuscular Mycorrhizas: Physiology and Function. *Edited by* Y. Kapulnik and J.D.D. Douds. Kluwer Academic publishers, Netherlands. pp. 47-68.

Giovannetti, M., Azzolini, D. and Citernesi, A.S. 1999. Anastomosis formation and nuclear and protoplasmic exchange in arbuscular mycorrhizal fungi. Applied and Environmental Microbiology. **65**: 5571-5575.

Giovannetti, M.,Fortuna, Citernesi, A.S., Morini, S. and Nuti, M.P. 2001. The occurrence of anastomosis formation and nuclear exchange in intact arbuscular mycorrhizal networks. New Phytologist. **151**: 717-724.

Giovannetti, M. and Sbrana, C. 1998. Meeting a non-host: The behaviour of AM fungi. Mycorrhiza. **8**: 123-130.

Giri, B. and Mukerji, K.G. 2004. Mycorrhizal inoculant alleviates salt stress in *Sesbania aegyptiaca* and *Sesbania grandiflora* under field conditions: Evidence for reduced sodium and improved magnesium uptake. Mycorrhiza. **14**: 307-312.

Girvan, M.S., Bullimore, J., Pretty, J.N., Osborn, A.M. and Ball, A.S. 2003. Soil type is the primary determinant of the composition of the total and active bacterial communities in arable soils. Applied Environmental Microbiology **69**: 1800-1809.

Gobat, J.M., Aragno, M. and Matthey, W. 2004. The living soil. fundamentals of soil science and soil biology. Science Publishers, USA.

Goicoechea, N., Antolín, M.C. and Sánchez-Díaz, M. 1997. Influence of arbuscular mycorrhizae and *Rhizobium* on nutrient content and water relations in drought stressed alfalfa. Plant and Soil. **192**: 261-268.

Goldblatt, P.B.A. and Manning, J. 2000. Cape plants: A conspectus of the Cape flora of South Africa. ABC press, Epping.

Gomes, R.C., Semedo, L.T.A.S., Soares, R.M.A., Alviano, C.S., Linhares, L.F. and Coelho, R.R.R. 2000. Chitinolytic activity of actinomycetes from a cerrado soil and their potential in biocontrol. Letters in Applied Microbiology. **30**: 146-150.

Gonzalez-Chavez, C., D'Haen, J., Vangronsveld, J. and 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.

Gosling, P., Hodge, A., Goodlass, G. and Bending, G.D. 2006. Arbuscular mycorrhizal fungi and organic farming. Agriculture, Ecosystems and Environment. **113**: 17-35.

Gray, T.R.G. and Williams, S.T. 1971. Soil micro-organisms. Oliver and Boyd, Edinburgh.

Grayston, S.J., Wang, S., Campbell, C.D. and Edwards, C.D. 1998. Selective influence of plant species on microbial diversity in the rhizosphere. Soil Biology and Biochemistry. **30**: 369-378.

Gremion, F., Chatzinotas, A. and Harms, H. 2003. Comparative 16S rDNA and 16S rRNA sequence analysis indicates that actinobacteria might be a dominant part of the metabolically active bacteria in heavy metal-contaminated bulk and rhizosphere soil. Environmental Microbiology. **5**: 896-907.

Griffioen, W.A.J., Ietswaart, J.H. and Ernst, W.H.O. 1994. Mycorrhizal infection of an *Agrostis capillaris* population on a copper contaminated soil. Plant and Soil. **158**: 83-89.

Grimm, N.B., Grove, J.M., Pickett, S.T.A. and Redman, C.L. 2000. Integrated approaches to long-term studies of urban ecological systems. Bioscience. **50**: 571-584.

Gryndler, M. 2000. Interactions of arbuscular mycorrhizal fungi with other soil organism. *In* Arbuscular Mycorrhizas: Physiology and Function. *Edited by* Y. Kapulnik and J.D.D. Douds. Kluwer Academic Publishers, Netherlands. pp. 239-262.

Gryndler, M. and Vosátka, M. 1996. The response of *Glomus fistulosum*-maize mycorrhiza to treatments with culture fractions from *Pseudomonas putida*. Mycorrhiza. **6**: 207-211.

Gryndler, M., Hrselova, H., Chvaytalova, I. and Jansa, J. 1998. The effect of selected plant hormones on *in vitro* proliferation of hyphae of *Glomus fistulosum*. Biologia Plantarum. **41**: 255-263.

Guiraud, P., Steiman, R., Seigle-Murandi, F. and Sage, L. 1997. *Exserohilum sodomii*, a new species isolated from soil near the dead sea (israel). Antonie Van Leeuwenhoek. **72**: 317-325.

Haddad, M.J. and Sarkar, D. 2003. Glomalin, a newly discovered component of soil organic matter: Part I-- environmental significance. Environmental Geosciences. **10**: 91-98.

Hall, T.A. 1999. Bioedit: A user friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acids Symposium Series. **41:** 95-98.

Hallberg, K. 2001. Restriction enzyme fragment length polymorphism (RFLP): A short overview.

Hamel, C., Morin, F., Granger, R.L. and Smith, D.L. 1994. Mycorrhizal colonisaton increases herbicide toxicity in apple. Journal of American Society and Horticultural Science. **119**: 1255-1260.

Hanssen, M.C., Tolker-Nielsen, T., Givskov, M. and Molin, S. 1998. Biased 16S rDNA PCR amplification caused by interference from DNA flanking the template region. FEM Microbiology and Ecology. **26**: 141-149.

Harinikumar, K.M. and Bagyaraj, D.J. 1989. Effect of cropping sequence, fertilizers and farmyard manure on vesicular-arbuscular mycorrhizal fungi in different crops over three consecutive seasons. Biology and Fertility of Soils. **7**: 173-175.

Harris, J.L. 2000. Safe, low-distortion tape touch method for fungal slide mounts. Journal of Clinical Microbiology. **38**: 4683-4684.

Hattingh, M.J. 1972. A note on the fungus endogone. Journal of South African Botany. **38**: 29-31.

Häussling, M. and Marschner, H. 1989. Organic and inorganic soil phosphates and acid phosphatase activity in the rhizosphere of 80-year-old Norway spruce (*Picea abies* (L.) karst) trees. Biology and Fertility of Soils. **8**: 128-133.

Hawkes, C.V. 2003. Nitrogen cycling mediated by biological soil crust and arbuscular mycorrhizal fungi. Ecology. **84**: 1553-1562.

Hawkins, H., Johansen, A. and George, E. 2000. Uptake and transport of organic and inorganic nitrogen by arbuscular mycorrhizal fungi. Plant and Soil. **226**: 275-285.

Hawley, G.L. and Dames, J.F. 2004. Mycorrhizal status of indigenous tree species in forest biome of the Eastern Cape, South Africa. South African Journal of Science. **100**: 633-637.

Helgason, T., Fitter, A.H. and Young, J. P. W. 1999. Molecular diversity of arbuscular mycorrhizal fungi colonising Hyacinthoides non-scripta (bluebell) in a semi natural woodland. Molecular Ecology. **8:** 659-666.

Hepper, C.M., Sen, R., Azcon-Aguilar, C. and Grace, C. 1988. Variation in certain isozymes amongst different geographical isolates of the vesicular-arbuscular mycorrhizal fungi *Glomus clarum*, *Glomus monosporum* and *Glomus mosseae*. Soil Biology and Biochemistry. **20**: 51-59.

Heuer, H., Krsek, M., Baker, P., Smalla, K. and Wellington, E. 1997. Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Applied Environmental Microbiology **63**: 3233-3241.

Hijri, M. and Sanders, I.R. 2005. Low gene copy number shows that arbuscular mycorrhizal fungi inherit genetically different nuclei. Nature. **433**: 160-163.

Hoffman, M.T. and Mitchell, D.T. 1986. The root morphology of some legume spp. in the Western Cape and the relationship of vesicular arbuscular mycorrhizas with dry mass and phosphorus content of *Acacia saligna* seedlings. South African Journal of Botany. **52**: 316-320.

Ibekwe, A.M., Kennedy, A.C., Frohne, P.S., Papiernik, S.K., Yang, C.H. and Crowley, D.E. 2002. Microbial diversity along transect of agronomic zones. FEMS Microbiology Ecology. **39:** 183-191.

Ibekwe, A.M. and Kennedy, A.C. 1998. Fatty acid methyl ester (FAME) profiles as a tool to investigate community structure of two agricultural soils. Plant and Soil. **206**: 151-161.

Ieswart, J., Griffioen, A. and Ernst, W. 1992. Seasonality of VAM infection in three populations of *Agrostis capillaris* (Graminea) on soil with or without heavy metal enrichment. Plant and Soil. **139**: 67-73.

Illmer, P. and Schinner, F. 1992. Solubilization of inorganic phosphates by microorganisms isolated from forest soils. Soil Biology and Biochemistry. **24**: 389-395.

Internet 1: Open-cast mining. 2007. In *Wikipedia, The Free Encyclopedia* [online]. Available from http://en.wikipedia.org/w/index.php?title=Open-pit_mining&oldid=111538987 [Accessed March, 2007].

Internet 2: National Technical Information Service. Stream corridor restoration- principles, processes and practices [online]. Available from http://www.ntis.gov/products/bestsellers/stream-corridor.asp?loc=4-2-0 [Accessed October, 2006].

Isaac, S. 1992. Fungal-plant interactions. Chapman and Hall, 2-6 Boundary Row, London SE1 8HN.

Jamal, A., Ayub, N., Usman, M. and Khan, A.G. 2002. Arbuscular mycorrhizal fungi enhance zinc and nickel uptake from contaminated soils by soyabean and lentil. International Journal of Phytoremediation. **4**: 205-221.

Jansa, J., Mozafar, A., Anken, T., Ruh, R., Sanders, I.R. and Frossard, E. 2002. Diversity and structure of AMF communities as affected by tillage in a temperate soil. Mycorrhiza. **12**: 225-234.

Janssen, P.H., Yates, P.S., Grinton, B.E., Taylor, P.M. and Sait, M. 2002. Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions acidobacteria, actinobacteria, proteobacteria and verrucomicrobia. Applied Environmental Microbiology **68**: 2391-2396.

Jasper, D.A. 1994. Management of mycorrhizas in revegetation. *In* Management of Mycorrhizas in Agriculture, Horticulture and Forestry. *Edited by* A.D. Robson, L.L. Abbot and N. Malajckzuk. Kluwer Academic Publishers, Netherlands.

Johansen, A., Finlay, R.D. and Olsson, P.A. 1996. Nitrogen metabolism of external hyphae of the arbuscular mycorrhizal fungus *Glomus intraradices*. New Phytologist. **133**: 705-712.

Johansson, J., Leslie, R.P. and Finlay, R.D. 2004. Microbial interactions in the mycorrhizosphere and their significance for sustainable agriculture. FEM Microbiology Ecology. **48**: 1-13.

Johnson, R.T. and Case, C.L. 2007. Laboratory experiments in microbiology. Benjamin and Cummings Publishing Co. San Francisco.

Johnson, S.E., Lauren, J.G.C., Welch, R.M. and Duxbury, J.M. 2005. A comparison of the effects of micronutrient seed priming and soil fertilization on the mineral nutrition of chickpea (*Cicer arietinum*), lentil (*lens culinaris*), rice (O*ryza sativa*) and wheat (*Triticum aestivum*) in Nepal. Experimental Agriculture. **41**: 427-448.

Jones, D.L., Hodge, A. and Kuzyakov, Y. 2004. Plant and mycorrhizal regulation of rhizodeposition. New Phytologist. **163**: 459-480.

Juang, T.C. 2007. The manufacturing and application of organic compound fertilizers. Food and Fertiliser Technology center. http://www.agnet.org/library/eb/431/

Kabir, Z. 2005. Tillage or no-tillage: Impact on mycorrhizae. Canadian Journal of Plant Science. **85**: 23-29.

Kabir, Z., Halloran, I.P., Fyles, J.W. and Hamel, C. 1997. Seasonal changes of arbuscular mycorrhizal funi as affected by tillage practices and fertilization: Hyphal density and mycorrhizal root colonisation. Plant and Soil. **192**: 285-293.

Kabir, Z., O'Halloran, I.P., Fyles, J.W. and Hamel, C. 1998. Dynamics of the mycorrhizal symbiosis of corn : Effects of host physiology, tillage practice and fertilization on spatial distribution of extra-radical mycorrhizal hyphae in the field. Agriculture, Ecosystems & Environment. **68**: 151-163.

Kauffmann, I.M., Schmitt, J. and Schmid, R.D. 2004. DNA isolation from soil samples for cloning in different hosts Applied Microbiology and Biotechnology. **64**: 665-670.

Kendrick, B. 1992. The fifth kingdom. Mycologue publications, Waterloo.

Kennedy, N., Brodie, E., Connolly, J. and Clipson, N. 2004. Impact of lime, nitrogen and plant species on bacterial community structure in grassland microcosms. Environmental Microbiology. **6**: 1070-1080.

Kent, A.D. and Triplett, E.W. 2002. Microbial communities and their interactions in soil and rhizosphere ecosystems. Annual Review of Microbiology **56**: 211-236.

Khan, A.G. 2006. Mycorrhizoremediation-an enhanced form of phytoremediation. Journal of Zhejiang University Science B. **7**: 503-514.

Khan, A.G. 2003. Vetiver grass as an ideal phytosymbiont for glomalian fungi for ecological restoration of heavy metal contaminated declict land. *In* Proceedings of the third international conference on Vetiver and exhibition, China, *Edited by* Vetiver grass as an ideal phytosymbiont for glomalian fungi for ecologicalrestoration of heavy metal contaminated declict land. pp. 466.-474.

Kiers, E.T., Lovelock, C.E., Krueger, E.L. and Herre, E.A. 2000. Differential effects of tropical arbuscular mycorrhizal fungal inocula on root colonization and tree seedling growth: Implications for tropical forest diversity. Ecology Letters. **3**: 106-113.

Kirk, J.L., Beaudette, L.A., Hart, M., Moutoglis, P., Klironomos, J.N., Lee, H. and Trevors, J.T. 2004. Methods of studying soil microbial diversity. Journal of Microbiological Methods. **58**: 169-188.

Kling, M. and Jakoben, I. 1998. Arbuscular mycorrhiza in soil quality assessment. Ambio. **27**: 29-34.

Klironomos, J.N. and Ursic, M. 1998. Density-dependent grazing on the extraradical hyphal network of the arbuscular mycorrhizal fungus, *Glomus intraradices*, by the collembolan, *Folsomia candida*. Biological and Fertility of Soils. **26**: 250-253.

Klironomos, J.N., McCune, J., Hart, M. and Neville, J. 2000. The influence of arbuscular mycorrhizae on the relationship between plant diversity and productivity. Ecology Letters. **3**: 137-141.

Kloepper, J .W., Leong, J., Teintze, M. and Schroth, M. N. 1980. *Pseudomonas* siderophores: A mechanism explaining disease suppressive soils. Current Microbiology. **4**: 317–320.

Kohno, N. and Marumoto, T. 2004. Revegetation of volcanic devastated slope using the symbiosis with arbuscular mycorrhizal fungi in mt.unzen-fugen, nagasaki prefecture. (Unpublished).

Koide, R.T. and Mosse, B. 2004. A history of research on arbuscular mycorrhizal fungi. Mycorrhiza. **14**: 145-163.

Koide, R.T. and Kabir, Z. 2000. Extraradical hyphae of the mycorrhizal fungus *Glomus intraradices* can hydrolyse organic phosphate. New Phytologist. **148**: 511--517.

Koide, R.T. and Schreiner, R.P. 1992. Regulation of the vesicular-arbuscular mycorrhizal symbiosis. Annual Review of Plant Physiology and Plant Molecular Biology. **43**: 557-581.

Kothari, S.K., Marschner, H. and Römheld, V. 1991. Contribution of the VA mycorrhizal hyphae in acquisition of phosphorus and zinc by maize grown in a calcareous soil. Plant and Soil. **131**: 177-185.

Kozdroj, J. and van Elsas, J.D. 2000. Application of polymerase chain reaction- denaturing gradient gel electrophoresis for comparison of direct and indirect extraction methods of soil DNA used for microbial community finger-printing. Biology Fertility of Soils. **31**: 372-378.

Krishna, K.R., Balakrishna, A.N. and Bagyaraj, D.J. 1982. Interaction between a vesicular-arbuscular mycorrhizal fungus and *Streptomyces cinnamomeous* and their effects on finger millet. New Phytologist. **92**: 401-405.

Kucey, R.M.N. 1987. Increased phophorus uptake by wheat and field beans inoculated with a phosphorus-solubilizing *Penicillum bilaj* strain and with vesicular-arbuscular mycorrhizal fungi. Applied Environmental Microbiology. **53**: 2699-2703.

Kuhn, G., Hijri, M. and Sanders, I.R. 2001. Evidence for the evolution of multiple genomes in arbuscular mycorrhizal fungi. Nature. **414**: 745-748.

Kung'u, J.B. 2004. Effect of vesicular-arbuscular mycorrhiza (VAM) inoculation on growth performance of *Senna spectabillis*. *In* Managing Nutient Cycles to Sustain Soil Fertility in Sub-Saharan Africa. *Edited by* A. Bationo. Academic Science Publishers. Nairobi. pp. 432-446.

Kurle, J.E. and Pfleger, F.L. 1994. The effect of cultural practices and pesticides on VAM fungi. *In* Mycorrhizae and Plant Health. *Edited by* F.L. Pfleger and R.G. Linderman. APS press, Minnesota. pp. 101-131.

Kuske, C.R., Ticknor, L.O., Miller, M.E., Dunbar, J.M., Davis, J.A., Barns, S.M. and Belnap, J. 2002. Comparison of soil bacterial communities in rhizospheres of three plant species and the interspaces in an arid grassland. Applied and Environmental Microbiology. **68**: 1854-1863.

Kwong ma, W. 2004. A polymerase chain reaction and denaturing gradient gel electrophoresis procedure for analysis of arbuscular mycorrhizal fungi in soil. Masters thesis. University of Saskatchewan, Saskatoon.

Lanfranco, L., Bianciotto, V., Lumini, E., Souza, M. and Morton, J. B. Bonfante, P. 2001. A combined morphological and molecular approach to characterize isolates of arbuscular mycorrhizal fungi in *Gigaspora* (Glomales). New Phytologist. **152**: 169-179.

Larkin, R.P. 2003. Characterization of soil microbial communities under different potato cropping systems by microbial population dynamics, substrate utilization and fatty acid profiles. Soil Biology and Biochemistry. **35**: 1451-1466.

Lee, S.B., Milgroom, M.G. and Taylor, J.W. 1988. A rapid high yield mini-prep method for isolation of total genomic DNA from fungi. Fungal Genetics Newsletters. **35**: 23-24.

Levyns, M.R. 1935. A revision of *Elytropappus* Cass. Journal of South African Botany. **1**: 89.

Leyval, C., Joner, E.J., de Val, C. and Haselwandter, K. 2002. Potential of AM fungi for bioremediation. *In* Mycorrhizal Technology in Agriculture: From Genes to Bioproducts. *Edited by* S. Gianinazzi, H. Scüepp, J.M. Barea and K. Haselwandter. Birkhäuser Verlag, Basel. pp. 175-186.

Li, X., George, E. and Marschner, H. 1991. Phosphorus depletion and ph decrease at the root-soil and hyphae-soil interfaces of VA mycorrhizal white clover fertilized with ammonium. New Phytologist. **119**: 397-404.

Linderman, R.G. 1994. Role of VAM fungi in biocontrol. *In* Mycorrhizae and Plant Health. *Edited by* F.L. Pfleger and R.G. Linderman. APS press, Minnesota. pp. 1-25.

Linderman, R.G. 1992. Vesicular-arbuscular mycorrhizae and soil microbial interactions. . *In* Mycorrhizae in Sustainable Agriculture. *Edited by* G.J. Bethlenfalvay and R.G. Linderman. ASA Special Publication, Madison. pp. 65-77.

Linderman, R.G. 1988. Mycorrhizal interaction with the rhizosphere microflora: The mycorrhizosphere effect. Phytopathology. **78**: 366-371.

Lipson, D.A., Raab, T.K., Schmidt, S.K. and Monson, R.K. 1999. Variation in competitive abilities of plants and microbes for specific amino acids. Biology and Fertility of Soils. **29**: 257-261.

Liu, A., Hamel, C., Hamilton, R.I. and Ma, B.L. 2000. Acquisition of Cu, Zn, Mn and Fe by mycorrhizal maize (*Zea mays* L.) grown in soil at different P and micronutrient levels. Mycorrhiza. **9**: 331-336.

Liu, W.T., Marsh, T.L., Cheng, H. and Forney, L.J. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Applied and Environmental Microbiology. **63**: 4516-4522.

Liu, R. and Luo, X. 1994. A new method to quantify the inoculum potential of arbuscular mycorrhizal fungi. New Phytologist **128**: 89-92.

Liu, X., Zhao, H. and Chen, S. 2006. Colonization of maize and rice plants by strain *Bacillus megaterium* C4. Current Microbiology. **52**: 186-190.

Lovelock, C.E., Wright, S.F. and Nichols, K.A. 2004a. Using glomalin as an indicator for arbuscular mycorrhizal hyphal growth: An example from the tropical rain forest soil. Soil Biology and Biochemistry. **36:** 1009-1012.

Lovelock, C.E., Wright, S.F., Clark, D.A. and Ruess, R.W. 2004b. Soil stocks of glomalin produced by arbuscular mycorrhizal fungi across a tropical rain forest landscape. Journal of Ecology. **92**: 278-287.

Ludwig-Müller, J.L. 2000. Hormonal balance in plants during colonization by mycorrhizal fungi: In Arbuscular Mycorrhizas: Physiology and Function. *In Edited by* Kapulnik and J. Douds. Kluwer Publishers, Netherlands. pp. 263-285.

Lutgen, E.R., Muir-Clairmont, D., Graham, J. and Rillig, M.C. 2003. Seasonality of arbuscular mycorrhizal hyphae and glomalin in Western Montana grassland. Plant Soil. **257**: 71-83.

Lynch, J.M. and Whipps, J.M. 1990. Substrate flow in the rhizosphere. Plant and Soil. **129**: 1-10.

Madigan, M.T., Martinko, J.M. and Parker, J. 1998. Brock Biology of Microorganisms. Prentice-Hall International, USA.

Malik,K. A. Sandhu,G. R. 1973. Decomposition of organic matter by fungi in saline soils. Mycopathologia. **50**: 339-347.

Mansfeld-Giese, K., Larsen, J. and Bødker, L. 2002. Bacterial populations associated with mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices*. FEM Microbiology and Ecology. **41**: 133-140.

María-Laura, G. 2002. Cellular and molecular aspect. *In* Arbuscular Mycorrhizae: Interactions in Plants, Rhizosphere and Soils. *Edited by* A.K. Sharma and B.N. Johri. Science Publishers, USA. pp. 231-246.

Maropola, M.A. 2006. Assessment of the compatibility between organic fertiliser and arbuscular mycorrhizal fungi. BSc Honours report. Rhodes University, Grahamstown.

Marschner, P. and Timonen, S. 2005. Interactions between plant species and mycorrhizal colonisation on the bacterial community composition in the rhizosphere. Applied Soil Ecology. **28**: 23-36.

Marschner, P., Crowley, D.E. and Lieberei, R. 2001a. Arbuscular mycorrhizal infection changes the bacterial 16S rDNA community composition in the rhizosphere of maize. Mycorrhiza. **11**: 297-302.

Marschner, P., Crowley, D. and Yang, C.H. 2004. Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type. Plant and Soil. **261**: 199-208.

Marschner, P., Yang, C.H., Lieberei, R. and Crowley, D.E. 2001b. Soil and plant specific effects on bacterial composition in the rhizosphere. Soil Biology and Biochemistry. **33**: 1437-1445.

Marsh, T.L. 1999. Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. Current Opinion in Microbiology. **2**: 323-327.

Martensson, A.M. and Carlgren, K. 1994. Impact of phosphorus fertilization on VAM diaspores in 2 Swedish long-term field experiment. Agriculture, Ecosystems & Environment. **47**: 327-334.

Martin, A.R.H. and Noel, A.R.A. 1960. The Flora of Albany and Bathurst. Replika Process, United Kingdom.

McAllister, C.B., Garciaromera, I., Godeas, A. and Ocampo, J.A. 1994. Interactions between *Trichoderma koningii, Fusarium-solani* and *Glomus mosseae* - effects on plant-growth, arbuscular mycorrhizas and the saprophyte inoculants. Soil Biology and Biochemistry. **26**: 1363-1367.

McGonigle, T.P., Miller, M.H., Evans, D.G., Fairchild, G.L. and 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.

McInroy, J.A. and Kloepper, J.W. 1995. Survey of indigenous bacterial endophytes from cotton and sweet corn. Plant Soil. **173**: 337-342.

McLaughlin, A. and Mineau, P. 1995. The impact of agricultural practices on biodiversity. Agriculture, Ecosystems and Environment. **55**: 201-212.

Mehta, S. and Nautiyal, C.S. 2001. An efficient method for qualitative screening of phosphate-solubilizing bacteria. Current Microbiology. **43**: 51-56.

Meier, R. and Charvat, I. 1993. Reassessment of tetrazolium bromide as a viability stain for spores of vesicular-arbuscular mycorrhizal fungi. American Journal of Botany. **80**: 1007-1015.

Mena-Violante, H.G., Ocampo-Jimenez, O., Dendooven, L., Martinez-Soto, G., Gonzalez-Castaneda, J., Davies, F.T. and Olalde-Portugal, V. 2006. Arbuscular mycorrhizal fungi enhance fruit growth and quality of chile ancho (*Capsicum annuum* L. cv san luis) plants exposed to drought. Mycorrhiza. **16**: 261-267.

Meyer, J.R. and Linderman, R.G. 1986a. Response of subterranean clover to dual inoculation with vesicular-arbuscular mycorrhizal fungi and a plant growth-promoting bacterium, *Pseudomonas putida*. Soil Biology and Biochemistry. **18**: 185-190.

Meyer, J.R. and Linderman, R.G. 1986b. Selective influence on populations of rhizosphere or rhizoplane bacteria and *Actinomycetes* by mycorrhizad formed by *Glomus fasiculatum* . Soil Biology and Biochemistry. **18**: 191-196.

Miller, R.L. and Jackson, L.E. 1998. Survey of vesicular-arbuscular mycorrhizae in lettuce production in relation to management and soil factors. Journal of Agricultural Science. **130**: 173-182.

Minerdi, D., Bianciotto, V. and Bonfante, P. 2002. Endosymbiotic bacteria in mycorrhizal fungi: From their morphology to genomic sequence. Plant and Soil. **244**: 211-219.

Minerdi, D., Fani, R., Gallo, R., Boarino, A. and Bonfante, P. 2001. Nitrogen fixation genes in an endosymbiotic *Burkholderia* strain. Applied and Environmental Microbiology. **67**: 725-732.

Molina, R., Massicotte, H. and Trappe, J.M. 1992. Specificity phenomena in mycorrhizal symbiosis: Community-ecological consequences and practical implications. *In* Mycorrhizal Functioning. *Edited by* M.F. Allen. Chapman and Hall, Inc., New York, USA. pp. 357-423.

Moore, D.D., Ausubel, F.M., Kingston, R., Smith, J.A. and Brent, R. 1987. Current Protocols in Molecular Biology. Wiley, John and Sons, Incorporated, USA.

Morton, J.B. and Redecker, D. 2001. Two new families of Glomales, Archaeosporaceae and Paraglomaceae, with two new genera, *Archaeospora* and *Paraglomus*, based on concordant molecular and morphological characters. Mycologia. **93**: 181-195.

Morton, J.B. and Benny, G.L. 1990. Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): A new order, Glomales, two new sub-orders, Glomineae and Gigasporineae and two new families, Acaulosporaceae and Gigasporaceae, with emendation of Glomaceae. Mycotaxon. **37**: 471-491.

Muchovej, R.M. 2004. Importance of mycorrhizae for agricultural crops. Gainesville, FL 32611. Institute of Food and Agricultural Sciences (IFAS), University of Florida. SS-AGR-170.

Mujica, M.T., Fracchia, S., Ocampo, J.A. and Godeas, A. 1999. Influence of the herbicides chlorsulfuron and glyphosate on mycorrhizal soybean intercropped with the weeds *Brassica campestris* or *Sorghum halepensis*. Symbiosis. **27**: 73-81.

Mukasa-Mugerwa, T.M. 2005. Biocontrol potential of arbuscular mycorrhizal fungi against *Fusarium*. BSc Honours report. Rhodes University, Grahamstown.

Muthukumar, T. and Udaiyan, K. 2002. Arbuscular mycorrhizal fungal composition in semi-arid soils of Western ghats, Southern India. Current Science. **82**: 624-628.

Muthukumar, T., Udaiyan, K. and Rajeshkannan, V. 2001. Response of neem (*Azadirachta indica* A. juss) to indigenous arbuscular mycorrhizal fungi, phosphate-solubilizing and asymbiotic nitrogen-fixing bacteria under tropical nursery conditions. Biology and Fertility of Soils. **34**: 417-426.

Muyzer, G., Teske, A., Wirsen, C.O. and Jannasch, H.W. 1995. Phylogenetic relationships of thiomicrospira species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Archives of Microbiology. **164**: 165-172.

Naeem, S., Chapin III, C.F.S., Costanza, R., Ehrlich, P.R., Golley, F.B., Hooper, D.U., Lawton, J.H., OíNeill, R.V., Mooney, H.A., Sala, O.E., Symstad, A.J. and Tilman, D. 1999. Biodiversity and ecosystem functioning: Maintaining natural life support processes. Issues in Ecology. **4**: 1-12.

Nagahashi, G. 2000. *In vitro* and *in situ* techniques to examine the role of root exudates during AM fungus-host interactions. *In* Arbuscular Mycorrhizas: Physiology and Function. *Edited by* Y. Kapulnik and J.D.D. Douds. Kluwer Academic Publishers, Netherlands. pp. 287-305.

Nagahashi, G. and Douds Jr, D.D. 1997. Appressorium formation by AM fungi on isolated cell walls of carrot roots. New Phytologist. **136**: 299-304.

Nair, J.R., Singh, G. and Sekar, V. 2002. Isolation and characterization of a novel *Bacillus* strain from coffee phyllosphere showing antifungal activity. Journal of Applied Microbiology. **93**: 772-780.

Nehl, D.B., Allen, S.J. and Brown, J.F. 1998. Slow arbuscular mycorrhizal colonisation of field-grown cotton caused by environmental conditions in the soil. Mycorrhiza. **8**: 159-167.

Nicholson, P.S. and Hirsch, P.R. 1998. The effect of pesticides on the diversity of culturable soil bacteria. Journal of Applied Microbiology. **84**: 551-558.

Niemi, R.M., Heiskanen, I., Wallenius, K. and Lindström, K. 2001. Extraction and purification of DNA in rhizosphere soil samples for PCR-DGGE analysis of bacterial consortia. Journal of Microbiological Methods. **45**: 155-165.

Norris, V., Grant, S., Freestone, P., Canvin, J., Sheikh, F.N., Toth, I., Trinei, M., Modha, K. and Norman, R.I. 1996. Calcium signalling in bacteria. Journal of Bacteriology. **178**: 3677-3682.

Ocampo, J.A., Martin, J. and Hayman, D.S. 1980. Influence of plant interactions on vesicular-arbuscular mycorrhizal infections. **I**. Host and non-host plants grown together. New Phytologist **84**: 27-35.

Oehl, F. and Sieverding, E. 2004. *Pacispora,* a new vesicular arbuscular mycorrhizal fungal genus in the glomeromycetes. Journal of Applied Botany. **78**: 72-82.

Oehl, F., Sieverding, E., Ineichen, K., Mader, P., Boller, T. and Wiemken, A. 2003. Impact of land use intensity on the species diversity of arbuscular mycorrhizal fungi in agroecosystems of central Europe. Applied Environmental Microbiology **69**(5): 2816-2824.

Okalebo, J.R., Gathua, K.W. and Woomer, P.L. 1993. Laboratory methods of soil and plant analysis: A working manual. Marvel EPZ, Nairobi.

Omar, S.A. 1998. The role of rock-phosphate-solubilizing fungi and vesicularâ€"arbusular-mycorrhiza (VAM) in growth of wheat plants fertilized with rock phosphate. World Journal of Microbiology and Biotechnology. **14**: 211-218.

Onguene, N.A. and Habte, M. 1995. Nitrogen and phosphorus requirements for raising mycorrhizal seedlings of *Leucaena leucocephala* in containers. Mycorrhiza. **5**: 347-356.

Øvreås, L. 2000. Population and community level approaches for analyzing microbial diversity in natural environments. Ecology Letters. **3**: 236-251.

Pandey, P., Kang, S.C. and Maheshwari, D.K. 2005. Isolation of endophytic plant growth promoting *Burkholderia* sp. MSSP from root nodules of *Mimosa pudica*. Current Science. **89**: 177-180.

Parvathi, K., Venkateswarlu, K. and Rao, A.S. 1984. Toxicity of soil-applied fungicides and gypsum to the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae* in groundnut. Canadian Journal of Botany. **63**: 1673-1675.

Pasti, M.B., Pometto, A.L., Nuti, M.P. and Crawford, D.L. 1990. Lignin-solubilizing ability of actinomycetes isolated from termite (Termitidae) gutt. Applied and Environmental Microbiology. **56**: 2213-2218.

Pattinson, G.S., Warton, D.I., Misman, R. and McGee, P.A. 1997. The fungicides terrazole and terraclor and the nematicide fenamiphos have little effect on the root colonisation by *Glomus mosseae* and growth of cotton seedlings. Mycorrhiza. **7**: 155-159.

Paulitz, T.C. and Linderman, R.G. 1991. Lack of antagonism between the biocontrol agent *Gliocladium virens* and vesicular arbuscular mycorrhizal fungi. New Phytologist **117**: 303-308.

Paulitz, T.C. and Linderman, R.G. 1989. Interaction between fluorescent pseudomonads and VA mycorrhizal fungi. New Phytologist. **113**: 37-45.

Paustian, T. 2006. Microbiology and Bacteriology: The world of microbes [online]. Available from http://www.bact.wisc.edu/Microtextbook/index.php?name=Sections&req=viewarticle&artid=5 [Accessed 2006].

Pawlowska, T.E. 2005. Genetic processes in arbuscular mycorrhizal fungi. FEMS Microbiology Letters. **251**: 185-192.

Pawlowska, T.E. and Charvat, I. 2004. Heavy metal stress and developmental patterns in arbuscular mycorrhizal fungi. Applied and Environmental Microbiology. **70**: 6643-6649.

Pawlowska, T.E. and Charvat, I. 2002. Influence of edaphic and environmental factors on arbuscular mycorrhizae. *In* Arbuscular Mycorrhizae: Interactions in Plants, Rhizosphere and Soils. *Edited by* A.K. Sharma and B.N. Johri. Science Publishers, Inc., Hampshire. pp. 105-134.

Pawlowska, T.E. and Taylor, J.W. 2004. Organization of genetic variation in individuals of arbuscular mycorrhizal fungi. Nature. **427**: 733-737.

Petinate, S.D., Martins, R.M., Coelho, R.R., Meirelles, M.N., Branquinha, M.H. and Vermelho, A.B. 1999. Influence of growth medium in proteinase and pigment production by *Streptomyces cyaneus*. Memórias do Instituto Oswaldo Cruz. **94**: 173-177.

Pfleger, F.L., Stewart, E.L. and Noyd, R.K. 1994. Role of VAM fungi in mine land revegetation. *In* Mycorrhizae and Plant Health. *Edited by* F.L. Pfleger and R.G. Linderman. APS press, Minnesota.

Picini, D. and Azcon, R. 1987. Effect of phosphate-solubilising bacteria and vesicular-arbuscular mycorrhizal fungi on the utilization of Bayovar rockphosphate by alfalfa plants using a sand vermiculite medium. Plant and Soil. **50**: 45-50.

Podeszfinski, C., Dalpé, Y. and Charest, C. 2002. *In situ* turfgrass establishment: **I**. Responses to arbuscular mycorrhizae and fertilization. Journal of Sustainable Agriculture. **20**: 44-46.

Porteous, L.A., Seidler, R.J. and Watrud, L.S. 1997. An improved method for purifying DNA from soil for polymerase chain reaction amplification and molecular ecology applications. Molecular Ecology. **6**: 787-791.

Porter, J.N., Wilhelm, J.J. and Tresner, H.D. 1960. Method for the preferential isolation of *Actinomycetes* from soils. Applied Microbiology. **8**: 174-178.

Pozo, M.J., Slezack-Deschaumes, S. and Dumas-Gaudot, E. 2002a. Plant defense responses induced by arbuscular mycorrhizal fungi. *In* Mycorrhizal Technology in Agriculture: From Genes to Bioproducts *Edited by* S. Gianinazzi, H. Scüepp, J.M. Barea and K. Haselwandter. Birkhäuser Verlag, Basel. pp. 103-111.

Pozo, M.J., Cordier, C., Dumas-Gaudot, E., Gianinazzi, S., Barea, J.M. and Azcon-Aguilar, C. 2002b. Localized versus systemic effect of arbuscular mycorrhizal fungi on defence responses to *Phytophthora* infection in tomato plants. Journal of Experimental Botany. **53**: 525-534.

Pratt, R.G. 2000. Diseases caused by dematiaceous fungal pathogens as potential limiting factors for production of *Bermuda* grass on swine effluent application sites. Agronomy Journal. **92**: 512-517.

Pratt, R.G. and Brink, G.E. 2007. Forage Bermudagrass cultivar responses to inoculations with *Exserohilum rostratum* and *Bipolaris spicifera* and relationships to field persistence. Crop Science. **47**: 239-244.

Prescott, L.M., Harley, J.P. and Klein, D.A. 2005. Microbiology. McGraw-Hill, New-York.

Price, G. D., Mohapatra, S. S., Gresshoff, P. M. 1984. Structure of nodules formed by *Rhizobium* Strain ANU289 in the nonlegume *Parasponia* and the Legume Siratro (*Macroptilium atropurpureum*). Botanical Gazette. **145**: 444-451.

Promega, C. 2005. Technical Manual, pGEM-T and pGEM-T Easy Vectors. Promega Corporation, USA.

Quilambo, O.A. 2003. The vesicular-arbuscular mycorrhizal symbioses. African Journal of Biotechnology. **2**: 539-546.

Ramadan,M. A. E. H. and Attia, M. 2006. The use of arbuscular mycorrhizal fungi to protect plants and soil using contaminated water. Geophysical Research Abstracts 03129. SRef-ID: 1607-7962/gra/EGU06-A-03129.

Ravnskov, S., Nybroe, O. and Jakobsen, I. 1999. Influence of an arbuscular mycorrhizal fungus on *Pseudomonas fluorescens* DF57 in rhizosphere and hyphosphere soil. New Phytologist **142**: 113-122.

Redecker, D. 2005. Glomeromycota. arbuscular mycorrhizal fungi and their relative(s) [online]. Available from http://tolweb.org/Glomeromycota/28715/2005.07.01 [Accessed August 2006].

Redecker, D. 2002. Molecular identification and phylogeny of arbuscular mycorrhhizal fungi. Plant and Soil. **244**: 67-73.

Redecker, D., Kodner, R. and Graham, L.E. 2000. Glomalean fungi from the ordovician. Science. **289**: 1920-1921.

Renker, C., Blanke, V. and Buscot, F. 2005. Diversity of arbuscular mycorrhizal fungi in grassland spontaneously developed on area polluted by a fertilizer plant. Environmental Pollution. **135**: 255-266.

Requena, N., Jimenez, I., Toro, M. and Barea, J.M. 1997. Interactions between plant-growth-promoting rhizosbacteria (PGPR), arbuscular mycorrhizal fungi and *Rhizobium* spp. in the rhizosphere of *Anthyllis cytisoides*, a model legume for revegetation in mediterranean semi-arid ecosystems. New Phytologist. **136**: 667-677.

Requena, N., Jeffries, P. and Barea, J. 1996. Assessment of natural mycorrhizal potential in a desertified semiarid ecosystem. Applied Environmental Microbiology **62**: 842-847.

Rillig, M.C., Wright, S.F. and Eviner, V.T. 2002. The role of arbuscular mycorrhizal fungi and glomalin in soil aggregation: Comparing effects of five plant species. Plant and Soil. **238**: 325-333.

Rillig, M.C., Wright, S.F., Nichols, K.A., Schmidt, W.F. and Torn, M.S. 2001. Large contribution of arbuscular mycorrhizal fungi to soil carbon pools in tropical forests soils. Plant and Soil. **233**: 167-177.

Rillig, M.C. 2004. Arbuscular mycorrhizae and terrestrial ecosystem processes. Ecology Letters. **7**: 740-754.

Rillig, M.C., Ramsey, P.W., Morris, S. and Paul, E.A. 2003. Glomalin, an arbuscular-mycorrhizal fungal soil protein, responds to land-use change. Plant and Soil. **253**: 293-299.

Rodriguez, H. and Fraga, R. 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnology Advances. **17**: 319-339.

Rosen, C.J. and Bierman, P.M. 2005. Managing Soil Fertility in an Organic System [online]. Available from http://www.extension.umn.edu/distribution/horticulture/components/M1191.pdf [Accessed Februrary 2007].

Rowell, D.L. 1994. Methods and Application. Longman Group, UK.

Rufyikiri, G., Thiry, Y., Wang, L., Delvaux, B. and Declerck, S. 2002. Uranium uptake and translocation by arbuscular mycorrhizal fungus, *Glomus intraradices*, under root-oragn culture conditions. New Phytologist. **156**: 275-281.

Rufyikiri, G.S., Declerck, S., Dufey, J.E. and Delvaux, B. 2000. Arbuscular mycorrhizal fungi might alleviate aluminium toxicity in banana plants. New Phytologist. **148**: 343-352.

Ryan, M.H. and Graham, J.H. 2002. Is there a role for arbuscular mycorrhizal fungi in production agriculture? Plant Soil. **244**: 263-271.

Safir, G.R. 1994. Invovlement of cropping systems, plant produced compounds and inoculum production in the functioning of VAM fungi. *In* Mycorrhizae and Plant Health. *Edited by* F.L. Pfleger and R.G. Linderman. APS press, Minnesota. pp. 239-259.

Saito, M. 2000. Sysmbiotic exchange of nutrients in arbuscular mycorrhizas: Transport and transfer of phosphorus. *In* Arbuscular mycorrhizas: Physiology and function *Edited by* Y. Kapulnik and J.D.D. Douds. Kluwer Academic publishers, Netherlands. pp. 85-106.

Salles, J.F., van Veen, J.A. and van Elsas, J. D. 2004. Multivariate analyses of *Burkholderia* Species in soil: Effect of crop and land use history. Applied and Environmental Microbiology. **70**: 4012-4020.

Sanders, I.R. 2002. Ecology and evolution of multigenomic arbuscular mycorrhizal fungi. American Naturalist. **160**: 128-141.

Sanders, F.E., Tinker, P.B., Black, R.L.B. and Palmerley, S.M. 1977. The development of endomycorrhizal root systems: **I**. Spread of infection and growth-promoting effects with four species of vesicular-arbuscular endophyte. New Phytologist **78**: 257-268.

Sbrana, C., Agnolucci, M., Bedini, S., Lepera, A., Toffanin, A., Giovannetti, M. and Nuti, M.P. 2002. Diversity of culturable bacterial populations associated to *Tuber borchii* ectomycorrhizas and their activity on *T. borchii* mycelial growth. FEMS Microbiology Letters. **211**: 195-201.

Scervino, J.M., Ponce, M., Erra-bassells, R., Vierheilig, H., Ocampo, J. and Godeas, A. 2005. Flavonoids exclusively present in mycorrhizal roots of white cloverexhibit a different effect on arbuscular mycorrhizal fungi than flavonoids exclusively present in non-mycorrhizal roots of white clover. Journal of Plant Interactions. **1**: 15-22.

Schachtman, D.P., Reid, R.J. and Ayling, S.M. 1998. Phosphorus uptake by plants: From soil to cell. Plant Physiology. **116**: 447-453.

Schenck, N.C. 1982. Methods and principles of mycorrhizal research. American Phytopathological Society, St. Pauls Minnesota.

Schreiner, R.P. and Bethlenfalvay, G.J. 1997. Mycorrhizae, biocides and biocontrol 3. effects of three different fungicides on the developmental stages of three AM fungi. Biology and Fertility of Soils. **24**: 18-26.

Schrey, S.D., Schellhammer, M., Ecke, M., Hampp, R. and Tarkka, M.T. 2005. Mycorrhizal helper bacterium *Streptomyces* AcH 505 induces differential gene expression in the ectomycorrhizal fungus *Amanita muscaria*. New Phytologist. **168**: 205-216.

Schüßler, A., Schwarzott, D. and Walker, C. 2001. A new phylum, the glomeromycota: Phylogeny and evolution. Mycological Research. **105**: 1413-1421.

Schwarzott, D., Walker, C. and Schüßler, A. 2001. *Glomus,* the largest genus of the arbuscular mycorrhizal fungi (Glomales), is nonmonophyletic. Molecular Phylogenetics and Evolution. **21**: 190-197.

Secilia, J. and Bagyaraj, D.J. 1987. Bacteria and actinomycetes associated with pot cultures of vesicular-arbuscular mycorrhizas. Canadian Journal of Botany. **33**: 1069-1073.

Sharma, K.D., Kumar, S. and Gough, L.P. 2000. Rehabilitation of land mined for limestone in the Indian desert. Land Degradation and Development. **11**: 563-573.

Sharma, S. 2003. Structural and functional characterization of bacterial diversity in the rhizosphere of three grain legumes. Doctorate thesis. Institute of Soil Ecology, National Research Centre for Environmental Health. Neuherberg, Germany.

Sinegani, A.S., Mahboobi, A.A. and Nazarizadeh, F. 2005. The effect of agricultural practices on the spatial variability of arbuscular mycorrhiza spores. Turkish Journal of Biology. **29**: 149-153.

Singh, A.N., Raghubanshi, A.S. and Singh, R. 2002. Review article: Plantation as a tool for mine spoils restoration. Current Science. **82**: 1432-1441.

Singh, R. and Adholeya, A. 2002. Plant and fungal responses to colonisation. *In* Arbuscular Mycorrhizae: Interactions in Plants, Rhizosphere and Soils *Edited by* A.K. Sharma and B.N. Johri. Science Publishers, Inc., Hampshire. pp. 213-230.

Singh, S. and Kapoor, K.K. 1998. Effects of inoculation of phosphate-solubilizing microorganisms and an arbuscular mycorrhizal fungus on mungbean grown under natural soil conditions. Mycorrhiza. **7**: 249-253.

Skinner, A. 2001. A mycorrhizal survey of indigenous plant species within Featherstone kloof and DassieKrantz area on the grahamstown commonage, Eastern Cape, South Africa. BSc Honours report. Rhodes University, Grahamstown.

Skinner, A. 2006. Investigating the effect of *Glomus etunicatum* colonization on the structure and phloem transport in roots of *Eragrostis curvula* (Umgeni). Masters thesis. Rhodes University, Grahamstown.

Sliwinski, M.K. and Goodman, R.M. 2004. Comparison of crenarchaeal consortia inhabiting the rhizosphere of diverse terrestrial plants with those in bulk soil in native environments. Applied Environmental Microbiology **70**: 1821-1826.

Smalla, K., Wieland, G., Buchner, A., Zock, A., Parzy, J., Kaiser, S., Roskot, N., Heuer, H. and Berg, G. 2001. Bulk rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: Plant-dependent enrichment and seasonal shifts revealed. Applied and Environmental Microbiology. **67**: 4742-4751.

Smith, F.A. and Smith, S.E. 1997. Tansley review no. 96. Structural diversity in (vesicular)-arbuscular mycorrhizal symbioses. New Phytologist **137**: 373-388.

Smith, G.S. 1988. The role of phosphorus nutrition in interactions of vesicular- arbuscular mycorrhiza fungi with soil borne nematodes and fungi. Phytopathology. **78**: 371-374.

Smith, S. and Dickson, S. 1997. VA mycorrhizas: Basic research techniques. Cooperative Research Centre for Soil and Land Management, Glen Osmond.

Smith, S.E. and Read, D.J. 1997. Mycorrhizal Symbiosis. Academic Press, UK.

Smith, S.E. and Gianinazzi-Pearson, V. 1990. Phosphate uptake and arbuscular activity in mycorrhizal *Allium cepa* L.: Effects of photon irradiance and phosphate nutrition. Australian Journal of Plant Physiology. **17**: 177-188.

Smith, S.E. and Walker, N.A. 1981. A quantitative study of mycorrhizal infection in *Trifolium*: Separate determination of the rates of infection and of mycelial growth. New Phytologist **89**: 225-240.

Söderberg, K.H., Olsson, P.A. and Baath, E. 2002. Structure and activity of the bacterial community in the rhizosphere of different plant species and the effect of arbuscular mycorrhizal colonisation. FEM Microbiology Ecology. **40**: 223-231.

Souchie, E.L., Saggin-Júnior, O.J., Silva, E.M.R., Campello, E.F.C., Azcón, R. and Barea, J.M. 2006. Communities of P-solubilising bacteria, fungi and arbuscular mycorrhizal fungi in grass pasture and secondary forest of Paraty, RJ– Brazil. Annals of the Brazilian Academy of Sciences. **78**: 183-193.

StatSoft, I. 2005. STATISTICA (data analysis software system). version 7.1.

Steenwerth, K.L., Jackson, L.E., Calderón, F.J., Stromberg, M.R. and Scow, K.M. 2003. Soil microbial community composition and land use history in cultivated and grassland ecosystems of coastal California. Soil Biology and Biochemistry. **35**: 489-500.

Steinberg, P.D. and Rillig, M.C. 2003. Differential decomposition of arbuscular mycorrhiza fungal hyphae and glomalin. Soil Biology and Biochemistry. **35**: 191-194.

Stukenbrock, E.H. and Rosendahl, S. 2005. Clonal diversity and population genetic structure of arbuscular mycorrhizal fungi (*Glomus* spp.) studied by multilocus genotyping of single spores. Molecular Ecology. **14**: 743-752.

Stutz, J.C., Copeman, R., Martin, C.A. and Morton, J.B. 2000. Patterns of species composition and distribution of arbuscular mycorrhizal fungi in arid regions of Southwestern North America, Namibia and Africa. Canadian Journal of Botany. **28**: 237-245.

Sukarno, N.S., Smith, S.E. and Scott, E.S. 1993. The effect of fungicides on vesicular-arbuscular mycorrhizal symbiosis. I.the effect on vesicular-arbuscular mycorrhizal fungi and plant growth. New Phytologist. **25**: 139-147.

Suresh, C.K. and Bagyaraj, D.J. 2002. Mycorrhiza-microbe interaction: Effect on rhizosphere. *In* Arbuscular Mycorrhizae: Interactions in Plants, Rhizosphere and Soils *Edited by* A.K. Sharma and B.N. Johri. Science Publishers Inc., Hampshire. pp. 7-28.

Suzuki, S., Okuda, T. and Komatsubara, S. 1999. Selective isolation and distribution of *Sporichthya* strains in soil. Applied and Environmental Microbiology. **65**: 1930-1935.

Sward, R.J. 1981. The structure of the spores of *Gigaspora magarita* **I**. The dormant spore. New Phytologist. **87**: 761-768.

Swift, E.C. 2006. Mycorrhiza and soil phosphorus levels [online]. Available from http://www.coopext.colostate.edu/TRA/PLANTS/index.html#http://www.coopext.colostate.edu/TRA/PLANTS/mycorrhiza.html [Accessed January 2007].

Sylvia, D., Alagely, A., Chellemi, D. and Demchenko, L. 2001. Arbuscular mycorrhizal fungi influence tomato competition with Bahiagrass. Biology and Fertility of Soils. **34**: 448-452.

Sylvia, D.M. and Jarstfer, A.G. 1994. Production of inoculum and inoculation with AMF. *In* Management of Mycorrhizas in Agriculture, Horticulture and Forestry *Edited by* A.D. Robson, L.L. Abbot and N. Malajckzuk. Kluwer Academic Publishers, Netherlands.

Sylvia, D.M., Fuhrmann, J.J., Hartel, P.G. and Zuberer, D. 1998. Principles and Applications of Soil Microbiology. Prentice Publishers, New Jersey.

Tamura, T., Hayakawa, M. and Hatano, K. 1999. *Sporichthya brevicatena* sp. nov. International Journal of Systematic Bacteriology. **49**: 1779-1784.

Thompson, J.P. 1994a. What is the potential for management of mycorrhizas in agriculture? *In* Management of Mycorrhizas in Agriculture, Horticulture and Forestry. *Edited by* A.D. Robson, L.L. Abbot and N. Malajckzuk. Kluwer Academic Publishers, Netherlands. pp. 191-200.

Thompson, J.P. 1994b. Inoculation with vesicular-arbuscular mycorrhizal fungi from cropped soil overcomes long-fallow disorder of linseed (linum usitatissimum L.) by improving P and Zn uptake. Soil Biology and Biochemistry. **26**: 1133-1143.

Tian, C., He, X., Zhong, Y. and Chen, J. 2002. Effects of VA mycorrhizae and frankia dual inoculation on growth and nitrogen fixation of *Hippophae tibetana*. Forest Ecology and Management. **170**: 307-312.

Timonen, S. and Marschner, P. 2005. Mycorrhizosphere concept. *In* Microbial activity in the rhizosphere. *Edited by* K.G. Mukerji, C. Manoharachary and J. Singh. Springer Verlag, Berlin.

Tisserant, B., Brenac, V., Requena, N., Jeffries, P. and Dodd, J.C. 1998. The detection of *Glomus* spp. (arbuscular mycorrhizal fungi) forming mycorrhizas in three plants, at different stages of seedling development, using mycorrhiza-specific isozymes. New Phytologist. **138**: 225-239.

Tobar, R., Azcón, R. and Barea, J.M. 1994. Improved nitrogen uptake and transport from ^{15}N-labelled nitrate by external hyphae of arbuscular mycorrhiza under water-stressed conditions. New Phytologist. **126**: 119-122.

Todd, S.W. and Hoffman, M.T. 1999. A fence-line contrast reveals effects of heavy grazing on plant diversity and community composition in Namaqualand, South Africa. Plant Ecology. **142**: 169-178.

Toljander, J.F. 2006. Interaction between soil bacteria and arbuscular mycorrhizal fungi. Doctoral thesis, Swedish University of Agricultural Sciences, Uppsala.

Toro, M., Azcon, R. and Barea, J. 1997. Improvement of arbuscular mycorrhiza development by inoculation of soil with phosphate-solubilizing rhizobacteria to improve rock phosphate bioavailability ((sup32)P) and nutrient cycling. Applied Environmental Microbiology **63**: 4408-4412.

Trappe, J. M., Molina, R. and Castellano, M. 1984. Reaction of mycorrhizal fungi and mycorrhiza formation to pesticides. Annual Review Phytopathology. **22**: 331-359.

Treseder, K.K. 2004. A meta-analysis of mycorrhizal responses to nitrogen, phosphorus and atmosperic CO_2 in field studies. New Phytologist. **164**: 347-355.

Trevors, J.T. 1998. Bacterial biodiversity in soil with an emphasis on chemically-contaminated soils. Water, Air, & Soil Pollution. **101**: 45-67.

Trevors, J. T. 1996. DNA in soil: adsorption, genetic transformation, molecular evolution and genetic microchip. Antonie van Leeuwenhoek. **70**: 1–10.

Trinick, M. J. and Hadobas, P. A. 1995. Formation of nodular structures on the non-legumes *Brassica napus*, *B.campestris*, *B. juncea* and *Arabidopsis thaliana* with *Bradyrhizobium* and *Rhizobium* isolated from *Parasponia* spp. or legumes grown in tropical soils. Plant Soil. **172**: 207–219.

Troeh, Z. I. and Loynachan, T.E. 2003. Endomycorrhizal fungal survival in continuous corn, soybean and fallow. Agronomy Journal. **95**: 224-230.

Turnau, K. and Mesjasz-przybylowicz, J. 2003. Arbuscular mycorrhiza of *Berkheya coddii* and other Ni-hyperaccumulating members of Asteraceae from ultramafic soils in South Africa. Mycorrhiza. **13**: 185-190.

Turnau, K., Ryszyka, P. and Gianinazzi-Pearson, V. 2001. Identification of arbuscular mycorrhizal fungi in soils and roots of plants colonizing zinc wastes in Southern Poland Mycorrhiza. **10**: 169-174.

Turnau, K., Kottke, I. and Oberwinkler, F. 1993. Element localization in mycorrhizal roots of *Pteridium aquilinum* (L.) kuhn collected from experimental plots treated with cadium dust. New Phytologist. **123**: 313-324.

Tylka, G.L., Hussey, R.S. and Roncadori, R.W. 1991. Axenic germination of vesicular-arbuscular mycorrhizal fungi: Effects of selected *Streptomyces* species. Phytopathology. **81**: 754-759.

Uhlmann, E., Görke, C., Petersen, A. and Oberwinkler, F. 2006. Arbuscular mycorrhizae from arid parts of Namibia. Journal of Arid Environments. **64**: 221-237.

Uhlmann, E., Görke, C., Petersen, A. and Oberwinkler, F. 2004. Comparison of species diversity of arbuscular mycorrhizal fungi in winter-rainfall areas of South Africa and summer-rainfall areas of Namibia. Mycological Progress. **3**: 267-274.

Ulrike, M. 2003. Conservation and divergence of signaling pathways between roots and soil microbes - the *Rhizobium*-legume symbiosis compared to the development of lateral roots, mycorrhizal interactions and nematode-induced galls. Plant and Soil. **255**: 105-119.

Uphoff, N., Ramamurthy, P. and Steiner, R. 1991. Managing irrigation. Sage Publications, New Delhi.

Van Aarle, I.M., Cavagnaro, T.R., Smith, S.E., Smith, F.A. and Dickson, S. 2005. Metabolic activity of *Glomus intraradices* in *Arum*- and *Paris*-type arbuscular mycorrhizal colonization. New Phytologist. **166**(2): 611-618.

van der Heijden, M.G., Boller, T., Wiemken, A. and Sander, I.R. 1998a. Different arbuscular mycorrhizal fungal species are potential determinants of plant community structure. Ecology. **79**: 2082-2091.

van der Heijden, M. G. A., Klironomos, J.N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., Wiemken, A. and Sander, I.R. 1998b. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature. **396**: 69-72.

van der Heijden, M. G. and Cornelissen, J..H. 2002. The critical role of plant-microbe interactions on biodiversity and ecosystem functioning: Arbuscular mycorrhizal associations as an example. *In* Biodiversity and Ecosystem Functioning: Synthesis and Perspectives *Edited by* M. Loreau, S. Naeem and P. Inchausti. Academic Press, New York. pp. 181-192.

Van Gent-Ruijters, M.L., DeVries, W. and Southamer, A.H. 1975. Influence of nitrate on fermentation pattern, molar growth yields and synthesis of cytochrome b in *Propionibacterium pentosaceum*. Journal General Microbiology. **88**: 36-48.

Van Oudtshoorn, F. 1992. Guide to Grasses of South Africa. National Book Printers. Cape Town.

Van Zwieten, L. 2006. Impact of agricultural inputs on soil organisms [online]. Available from http://www.wormdigest.org/index2.php?option=com_content&do_pdf=1&id=299-[Accessed November 2006].

Vassilev, N., Vassileva, M. and Nikolaeva, I. 2006. Simultaneous P-solubilizing and biocontrol activity of microorganisms: Potentials and future trends. Applied Microbiology and Biotechnology. **71**: 137-144.

Vázquez, M. M., César, S., Azcón, R. and Barea, J. M. 2000. Interaction between arbuscular mycorrhizal fungi and other microbial inoculants (*Azospirillum, Pseudomonas, Trichoderma*) and their effect on microbial population and enzyme activities in the rhizosphere of maize plants. Applied Soil Ecology. **15**: 261-272.

Vessey, J.K. 2003. Plant growth promoting rhizobacteria as biofertilizers. Plant Soil. **255**(2): 571-586.

Vierheilig, H., Scheweiger, P. and Brundrett, M. 2005. An overview of methods for the detection and observation of arbuscular mycorrhizal fungi in roots. Physiologia Plantarum. **125**: 393-404.

Vinogradova, K.A., Kirillova, N.P. and Polin, A.N. 1988. Bacteriolytic enzymes produced by actinomycetes. **I**. The physicochemical properties of the enzymes and the spectrum of their lytic action. Nauchnye Doki Vyss Shkoly Biol Nauki. **8**: 12-26.

Wali, M.K. 1999. Ecological succession and the rehabilitation of disturbed terrestrial ecosystems. Plant and Soil. **213**: 195-220.

Walker, C. and Schüßler, A. 2004. Nomenclatural clarifications and new taxa in the glomeromycota. Mycological Research. **108**: 981-982.

Wall, D.H. and Virginia, R.A. 1999. Controls on soil biodiversity: Insights from extreme environments. Applied Soil Ecology. **13**: 137-150.

Wall, L.G. 2000. The actinorhizal symbiosis. Journal of Plant Growth Regulation. **19**: 167-182.

Walley, F.L. and Germida, J.J. 1997. Response of spring wheat (*Triticum aestivum*) to interactions between *Pseudomonas* species and *Glomus clarum* NT 4. Biology and Fertility of Soils. **24**: 365-371.

Wamberg, C., Christensen, S., Jakobsen, I., Müller, A.K. and Sørensen, S.J. 2003. The mycorrhizal fungus (*Glomus intraradices*) affects microbial activity in the rhizosphere of pea plants (*Pisum sativum*). Soil Biology and Biochemistry. **35**: 1349-1357.

Wan, M.T., Rahe, J.E. and Watts, R.G. 1998. A new technique for determining the sub lethal toxicity of pesticides to the vesicular-arbuscular mycorrhizal fungus *Glomus intraradices*. Environmental Toxicology and Chemistry. **17**: 1421-1428.

Wang, B. and Qiu, Y.L. 2006. Phylogenetic distribution and evolution of mycorrhizas in land plants. Mycorrhiza. **16**: 299-363.

Wang, G.C.Y. and Wang, Y. 1997. Frequency of formation of chimeric molecules as a consequence of PCR co-amplification of 16S rRNA genes from mixed bacterial genomes. Applied Environmental Microbiology. **62**: 4645-4650.

Wardle, D.A and Van der Putten, W.H. 2002. Biodiversity, ecosystem functioning and above-ground-below-ground linkages. *In* Biodiversity and Ecosystem Functioning: Synthesis and perspectives *Edited by* M. Loreau, S. Naeem and P. Inchausti. Academic Press, New York. pp. 155-168.

Wechter, P., Williamson, J., Robertson, A. and Kluepfel, D. 2003. A rapid, cost-effective procedure for the extraction of microbial DNA from soil. World Journal of Microbiology and Biotechnology. **19**: 85-91.

Weissenhorn, I., Leyval, C., Belgy, G. and Berthelin, J. 1995. Arbuscular mycorrhizal contribution to heavy metal uptake by maize (*zea mays L.*) in pot culture with contaminated soil. Mycorrhiza. **5**: 245-251.

Wheeler, D.L., Barrett, T., Benson, D.A., Bryant, S.H., Canese, K., Chetvernin, V., Church, D.M., Dicuccio, M., Edgar, R., Federhen, S., Geer, L.Y., Kapustin, Y., Khovayko, O., Landsman, D., Lipman, D.J., Madden, T.L., Maglott, D.R., Ostell, J., Miller, V., Pruitt, K.D., Schuler, G.D., Sequeira, E., Sherry, S.T., Sirotkin, K., Souvorov, A., Starchenko, G., Tatusov, R.L., Tatusova, T.A., Wagner, L. and Yaschenko, E. 2006. Database resources of the national center for biotechnology information. Nucleic Acids Research.[online]. Available from www.ncbi.nlm.nih.gov [Accessed January 2007].

Wicklow-Howard, M. 1994. Vesicular arbuscular mycorrhizae from sagebrush steppe habitat in Western Idaho and parts of Eastern ecosystem and central Oregon. Eastside Ecosytem Management Project. Boise State University Boise, Idaho. http://www.icbemp.gov/science/wicklow1.pdf

Will, M.E. and Sylvia, D.M. 1990. Interaction of rhizosphere bacteria, fertilizer and vesicular-arbuscular mycorrhizal fungi with sea oats. Applied Environmental Microbiology **56**: 2073-2079.

Withers, P.J., lay, S.D. and Breeze, V.G. 2001. Phosphorus transfer in runoff following application of fertilizer, manure and sewage sludge Journal of Environmental Quality. **30**: 180-188.

Wright, S.F. 2000. A fluorescent antibody assay for hyphae and glomain from arbuscular mycorrhizal fungi. Plant and Soil. **226**(2): 171-177.

Wright, S.F., and Anderson, R.L. 2000. Aggregate stability and glomalin in alternative crop rotations for the central Great Plains. Biological and Fertility of Soils. **31**: 249-253.

Wright, S.F. and Upadhyaya, A. 1998. A survey for aggregate stability and glomalin, a glycoprotein produced by hyphae of arbuscular mycorrhizal fungi Plant and Soil. **198:** 97-107.

Wright, S.F., Upadhyaya, A. and Buyer, J.S. 1998. Comparison of N-linked oligosaccharides of glomalin from arbuscular mycorrhizal fungi and soils by capillary electrophoresis. Soil Biology and Biochemistry. **30**: 1853-1857.

Xavier, L.J.C. and Germida, J.J. 2003. Bacteria associated with *Glomus clarum* spores influence mycorrhizal activity. Soil Biology and Biochemistry. **35**: 471-478.

Xu, H., Wang, R. and Mridha, A.U. 2000. Effects of organic and chemical fertilisers and arbuscular mycorrhizal inoculation on growth and quality of cucumber and lettuce. Journal of Crop Production. **3**: 313-324.

Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T. and Arakawa, M. 1992. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes, 1981) comb. nov. Microbiology and Immunology. **36**: 1251-1275.

Yamato, M. 2004. Morphological types of arbuscular mycorrhizal fungi in roots of weeds on vacant land. Mycorrhiza. **14**: 127-131.

Yamato, M. and Iwasaki, M. 2002. Morphological types of arbuscular mycorrhizal fungi in roots of understory plants in Japanese deciduous broadleaved forests. Mycorrhiza. **12**: 291-296.

Yan, Z., Reddy, M.S. and Kloepper, J.W. 2003. Survival and colonization of rhizobacteria in a tomato transplant system. Canadian Journal of Microbiology. **49**: 383-389.

Yang, C.H. and Crowley, D.E. 2000. Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. Applied Environmental Microbiology. **66**: 345-351.

Yeates, C., Gillings, M.R., Davison, A.D., Altavilla, N. and Veal, D.A. 1998. Methods for microbial DNA extraction from soil for PCR amplification. Biological Procedures Online. **1**: 40-47.

Zar, J.H. 1999. Biostatistical Analysis. Prentice Hall, New Jersey.

Zelles, L., Rackwitz, R., Bai, Q.Y., Beck, T. and Besse, F. 1995. Discrimination of microbial diversity by fatty acid profiles of phospholipids and lipopolysaccharides in differently cultivated soils. Plant and Soil. **170**: 115-122.

Zhou, J., Bruns, M.A. and Tiedje, J.M. 1996. DNA recovery from soils of diverse composition. Applied Environmental Microbiology. **62**: 316-322.

Zhu, Y. G. and Miller, R. M. 2003. "Carbon cycling by arbuscular mycorrhizal fungi in soil-plant systems." Trends in Plant Science. **8**:407-409.

Zhu, H.H. and Yao, Q. 2004. Localized and systemic increase of phenols in tomato roots induced by *Glomus versiforme* inhibits *Ralstonia solanacearum*. Journal of Phytopathology. **152**: 537-542.

Appendices

APPENDIX A

A1. Organic Tea (Product of Guano Organic fertiliser)

Constituents

N – 1.42%

P – 0.5%

K – 0.3%

Ca – 0.54%

Mg – 0.16%

Na – 1.30%

Fe – 0.5%

Cu – 0.82mg

Zn – 1.54mg

B – 1.82mg

APPENDIX B

Selective media

B1. Nitrogen-free medium (N_2A) (Paustian, 2006)

Component	Gram / Litre
Glucose	10.0
$CaCO_3$	1.0
KH_2PO_4	1.0
$MgSO_4.7H_2O$	0.2
$FeSO_4.7H_2O$	0.3
NaCl	0.2
$NaMo_4$.2H2O	0.05
Agar	15.0

Autoclave at 121°C for 15 mins and allow to cool before dispensing into Petri dishes.

B2. National Botanical Research Institute's Phosphate growth medium (NBRIP) (Mehta and Nautiyal, 2001).

Component	**Gram / Litre**
Glucose	10.0
$Ca_3(PO_4)_2$	5.0
$MgCl_2$. $6H_2O$	5.0
$MgSO_4.7H_2O$	0.25
KCl	0.2
$(NH_4)_2SO_4$	0.1
Agar	15.0

Autoclave at 121°C for 15 mins and allow to cool before dispensing into Petri dishes.

B3. Benette's Modification of Lindenbein's Medium (BLM) (Porter *et al.*, 1960)

Component	**Gram / Litre**
Glycerol	10.0
L- arginine	
NaCl	0.2
$CaCO_3$	1.0
$FeSO_4.7H_2O$	0.3
$MgSO_4.7H_2O$	0.2
Agar	15.0

Autoclave at 121°C for 15 mins and allow to cool before dispensing into Petri dishes.

B4. **Nutrient Agar (Biolab, laboratories)**

Component	Gram / Litre
Meat extract	1.0
Peptone	5.0
Yeast extract	2.0
Sodium Chloride	8.0
Agar	15.0

Full strength: 31g in 1000ml distilled water. Autoclave at 121°C for 15 mins and allow to cool before dispensing into Petri dishes. pH 7.1.

B5. Tryptone Soy Agar (Biolab, Laboratories)

Component	Gram / Litre
Tryptone	15.0
Soy peptone	5.0
Sodium Chloride	5.0
Agar	15.0

Full strength: 38g in 1000ml distilled water. Autoclave at 121°C for 15 mins and allow to cool before dispensing into Petri dishes. pH 7.3.

APPENDIX C

Recipes for Gram staining

C1. Gram crystal violet solution

To make a crystal violet stock solution, 20g of crystal violet was dissolved in 100ml ethanol. Similarly, 1g of ammonium oxalate was dissolved in 100ml of water to make a

stock solution. A working solution of crystal violet was the prepared by mixing 1ml of the crystal violet stock with 10ml of water and 40ml of the oxalate stock solution.

C2. Gram iodine solution

1g iodine

2g potassium iodide

3g sodium biocarbonate

Make up to 300ml with sterile water.

C3. Gram decoloriser solution

95% ethanol

95% acetone

Mix equal volumes of each to make up the solution.

C4. Gram safranin solution

To make a stock solution, 2.5g of Safranin O was dissolved in 95% ethanol. A working solution was obtained by diluting one part of the stock with five parts of water.

C5. Gram staining procedure

Bacterial colony to be stained was picked with a sterile wire loop and smeared thinly on a microscope slide. Smear was heat fixed by passing over flame. Slide was stained with crystal violet for 60 sec and washed with water for 1 min. After washing, iodine solution which acts as a mordant was added and left to stand for 60 sec; this was also washed for 1 min. To decolorise the crystal violet and allow cells to be stained with the subsequent dye, acetone solution was dropped on smear until no trace of crystal violet dye was seen. This was further rinsed with water and then stained with safranin solution for 60 secs; which was then rinsed with water for 1 min and allowed to air dry before examining under the microscope. Gram-positive cells retained the crystal violet dye (purple) while Gram-negative cells stained pink or red (retained safranin dye).

APPENDIX D

Competent cell preparation

A colony of DH5α bacteria was inoculated into a test tube containing 5ml Luria Broth. The culture was grown overnight with shaking (200rpm) at 37ºC. Overnight cultures (500µl) were inoculated into 50ml Luria Broth which was placed at 37ºC with shaking until the OD_{600nm} was between 0.6-0.8. Cultures were chilled on ice and centrifuged at 5000g for 5mins at 4ºC. The supernatant was removed and the pellet re-suspended with 50ml 0.1M $MgCl_2$ which was further incubated on ice for 2 mins. Cells were centrifuged (5000 rpm) for 5 mins at 4ºC and the resultant pellet re-suspended in 25ml 0.1M $CaCl_2$. Tubes were incubated on ice for 1hr. After incubation, cells were centrifuged at 5000 rpm and the pellet re-suspended in 2.5ml 0.1M CaCl2 and 2.5ml 30% glycerol on ice. Competent cells were then stored in 100µl aliquots at -70ºC.

APPENDIX E

Cloning recipes (Promega, 2005)

E1. Isopropyl-beta-D-thiogalactopyranoside solution (0.1M)

1.2g IPTG

Make up with water to a 50ml final volume. Filter-sterilise and store at 4ºC.

E2. 5-bromo-4-chloro-3-indolyl-b-D-galactoside (2ml)

100mg X-Gal

Dissolved in 2ml N, N'-dimethylformamide. Protect from sunlight and store at -20ºC.

E3. Luria Bertani Broth

10g Tryptone

5g Yeast Extract

5g NaCl

Adjust pH to 7.0 with NaOH.

To solidify, add 15g Agar powder.

E4. LB plates with ampicillin/IPTG/X-Gal

Agar (15g) was added to LB medium. This was autoclaved and allowed to cool to 50ºC before adding filter sterilised ampicillin to a final concentration of 100µg/ml. Subsequently, 0.5mM IPTG and 80µg/ml X-Gal was added. Medium was stirred on a magnetic stirrer using sterile magnetic bars and poured into sterile Petri-dishes. Upon solidification agar was stored at 4ºC for less than a month.

E5. SOC medium

2g Tryptone

0.5g Yeast Extract

1ml, 1MNaCl

0.25ml, 1M KCl

1ml, 2M Mg^{2+} stock (filter-sterilised)

1ml, 2M glucose (filter-sterilised).

The Tryptone, Yeast Extract, NaCl and KCl were dissolved in 97ml distilled water by stirring. The solution was autoclaved and cooled to room temperature. The Mg^{2+} stock and 2M glucose was added to a final concentration of 20mM. The final volume was made up to 100ml with sterile distilled water.

E6. 2M Mg^{2+} stock

20.33g $MgCl_2.6H_2O$

24.65g $MgSO_4.7H_2O$

Make up to 100ml with distilled water. Filter sterilise

APPENDIX F

F1. Bacterial aligned nucleotide sequences

```
CLUSTAL W (1.83) multiple sequence alignment


DQ1442127_Actinomycetales_      ----------------CTCAG-ACGAACGCTGGCGGCGTGCTTAACACATG 34
DQ663172_Streptomyces           AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATG 50
Actc29                          --------------------------------------------------
ActC31                          --------------------------------------------------
AY154378_Burkholderia           --------------------ATTGAACGCTGGCGGCATGCCTTACACATG 30
DQ490307_Burkholdericeae        AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCATGCCTTACACATG 50
DQ904608_Bacillus               ---------------------------CTGGGCGGCGTGCCTAATACATG 23
DQ464386_Pseudomonas            --------------------------------------------------
AM161143_Pseudomonas            --------------------------------------------------
PSAC41                          --------------------------------------------------
PSAA1                           --------------------------------------------------
3LHF6                           --------------------------------------------------
1MBT7                           --------------------------------------------------
3LHF5                           --------------------------------------------------
3LHF3                           --------------------------------------------------
4LHF2                           --------------------------------------------------
2MBT4                           --------------------------------------------------
4LHF1                           --------------------------------------------------
3LHF                            --------------------------------------------------
4LHF4                           --------------------------------------------------
4LHF3                           --------------------------------------------------
N2AA52                          --------------------------------------------------
```

```
NBPC31                          --------------------------------------------------
N2AA53                          --------------------------------------------------
NBPC33                          --------------------------------------------------

DQ1442127_Actinomycetales_      CAAGTCGAACGATGAACCTCCTTCG------GGAGGGGATTAGTGGCGAA 78
DQ663172_Streptomyces           CAAGTCGAACGATGAACCACTTC--------GGTGGGGATTAGTGGCGAA 92
Actc29                          --------------------------------------------------
ActC31                          --------------------------------------------------
AY154378_Burkholderia           CAAGTCGAACGG-CAGCACGGGAGT---AATCCTGGTGGCGAGTGGCGAA 76
DQ490307_Burkholdericeae        CAAGTCGAACGG-CAGCACGGGGGC---AACCCTGGTGGCGAGTGGCGAA 96
DQ904608_Bacillus               CAAGTCGANCGAACTGATTAGAAGCTTGNTTCTNTGANGTTAGCGGCGGA 73
DQ464386_Pseudomonas            --------------------------------------------------
AM161143_Pseudomonas            --------------------------------------------------
PSAC41                          --------------------------------------------------
PSAA1                           --------------------------------------------------
3LHF6                           --------------------------------------------------
1MBT7                           --------------------------------------------------
3LHF5                           --------------------------------------------------
3LHF3                           --------------------------------------------------
4LHF2                           --------------------------------------------------
2MBT4                           --------------------------------------------------
4LHF1                           --------------------------------------------------
3LHF                            --------------------------------------------------
4LHF4                           --------------------------------------------------
4LHF3                           --------------------------------------------------
N2AA52                          --------------------------------------------------
NBPC31                          --------------------------------------------------
N2AA53                          --------------------------------------------------
NBPC33                          --------------------------------------------------

DQ1442127_Actinomycetales_      CGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTG 128
DQ663172_Streptomyces           CGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTG 142
Actc29                          --------------------------------------------------
ActC31                          --------------------------------------------------
AY154378_Burkholderia           CGGGTGAGTAATACATCGG-AACGTGTCCTGTAGTGGGGGATAGCCCGGC 125
DQ490307_Burkholdericeae        CGGGTGAGTAATACATCGG-AACGTGTCCTGGAGTGGGGGATAGCCCGGC 145
DQ904608_Bacillus               CGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGG 123
DQ464386_Pseudomonas            --------------------------------------------CGTTCG 6
AM161143_Pseudomonas            --------------------------------------------------
PSAC41                          --------------------------------------------------
PSAA1                           --------------------------------------------------
3LHF6                           --------------------------------------------------
1MBT7                           --------------------------------------------------
3LHF5                           --------------------------------------------------
3LHF3                           --------------------------------------------------
4LHF2                           --------------------------------------------------
2MBT4                           --------------------------------------------------
4LHF1                           --------------------------------------------------
3LHF                            --------------------------------------------------
4LHF4                           --------------------------------------------------
4LHF3                           --------------------------------------------------
N2AA52                          --------------------------------------------------
NBPC31                          --------------------------------------------------
N2AA53                          --------------------------------------------------
NBPC33                          --------------------------------------------------

DQ1442127_Actinomycetales_      GAAACGGGGTCTAATACCGGATACTGACC-------CGCTTGGGCATCCA 171
DQ663172_Streptomyces           GAAACGGGGTCTAATACCGGATACTGATC-------ATCTTGGGCATCCT 185
Actc29                          --------------------------------------------------
ActC31                          --------------------------------------------------
AY154378_Burkholderia           GAAAGCCGGATTAATACCGCATACGATCT--------ACGGAAGA-AAGC 166
DQ490307_Burkholdericeae        GAAAGCCGGATTAATACCGCATACGCTCG--------AGAGAGGA-AAGC 186
DQ904608_Bacillus               GAAACCGAAGCTAATACCGGATAGGATCTTCTCCTTCATGGGAGATGATT 173
DQ464386_Pseudomonas            GAAACGAACGCTAATACCGCATACGTCCT--------ACGGGAGA-AAGC 47
AM161143_Pseudomonas            --------------------------------------------------
PSAC41                          --------------------------------------------------
PSAA1                           --------------------------------------------------
3LHF6                           --------------------------------------------------
```

```
1MBT7                           --------------------------------------------------
3LHF5                           --------------------------------------------------
3LHF3                           --------------------------------------------------
4LHF2                           --------------------------------------------------
2MBT4                           --------------------------------------------------
4LHF1                           --------------------------------------------------
3LHF                            --------------------------------------------------
4LHF4                           --------------------------------------------------
4LHF3                           --------------------------------------------------
N2AA52                          --------------------------------------------------
NBPC31                          --------------------------------------------------
N2AA53                          --------------------------------------------------
NBPC33                          --------------------------------------------------

DQ1442127_Actinomycetales_      AGCGGTTCGAAAGCTCCGGCGGTGCAGGATGANCCCGCGGCCTATCAGCT 221
DQ663172_Streptomyces           TGGTGATCGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCT 235
Actc29                          --------------------------------------------------
ActC31                          --------------------------------------------------
AY154378_Burkholderia           GGGGGATCGCAAGACCTCGCGCTATAGGGGCGGCCGATGGCAGATTAGCT 216
DQ490307_Burkholdericeae        GGGGGATCTTCGGACCTCGCGCTCAAGGGGCGGCCGATGGCGGATTAGCT 236
DQ904608_Bacillus               GAAAGATGGTTTCGGCTATCACTTACAGATGGGCCCGCGGTGCATTAGCT 223
DQ464386_Pseudomonas            AGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCT 97
AM161143_Pseudomonas            --------------------------------------------------
PSAC41                          --------------------------------------------------
PSAA1                           --------------------------------------------------
3LHF6                           --------------------------------------------------
1MBT7                           --------------------------------------------------
3LHF5                           --------------------------------------------------
3LHF3                           --------------------------------------------------
4LHF2                           --------------------------------------------------
2MBT4                           --------------------------------------------------
4LHF1                           --------------------------------------------------
3LHF                            --------------------------------------------------
4LHF4                           --------------------------------------------------
4LHF3                           --------------------------------------------------
N2AA52                          --------------------------------------------------
NBPC31                          --------------------------------------------------
N2AA53                          --------------------------------------------------
NBPC33                          --------------------------------------------------

DQ1442127_Actinomycetales_      TGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAG 271
DQ663172_Streptomyces           TGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAG 285
Actc29                          --------------------------------------------------
ActC31                          --------------------------------------------------
AY154378_Burkholderia           AGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCTGGTCTGAG 266
DQ490307_Burkholdericeae        AGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAGCTGGTCTGAG 286
DQ904608_Bacillus               AGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCATAGCCGACCTGAG 273
DQ464386_Pseudomonas            AGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAG 147
AM161143_Pseudomonas            --------------------------------------------------
PSAC41                          --------------------------------------------------
PSAA1                           --------------------------------------------------
3LHF6                           --------------------------------------------------
1MBT7                           --------------------------------------------------
3LHF5                           --------------------------------------------------
3LHF3                           --------------------------------------------------
4LHF2                           --------------------------------------------------
2MBT4                           --------------------------------------------------
4LHF1                           --------------------------------------------------
3LHF                            --------------------------------------------------
4LHF4                           --------------------------------------------------
4LHF3                           --------------------------------------------------
N2AA52                          --------------------------------------------------
NBPC31                          --------------------------------------------------
N2AA53                          --------------------------------------------------
NBPC33                          --------------------------------------------------

DQ1442127_Actinomycetales_      AGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA 321
DQ663172_Streptomyces           AGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA 335
```

```
Actc29                        --------------------------------------------------
ActC31                        --------------------------------------------ACGGGA 6
AY154378_Burkholderia         AGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA 316
DQ490307_Burkholdericeae      AGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA 336
DQ904608_Bacillus             AGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA 323
DQ464386_Pseudomonas          AGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGA 197
AM161143_Pseudomonas          --------------------------------------------------
PSAC41                        ----------------------CNCCTCCTNCGGGAGGCAGCAGTGGGGA 28
PSAA1                         --------------------------------------------------
3LHF6                         --------------------------------------------------
1MBT7                         --------------------------------------------------
3LHF5                         --------------------------------------------------
3LHF3                         --------------------------------------------------
4LHF2                         --------------------------------------------------
2MBT4                         --------------------------------------------------
4LHF1                         --------------------------------------------------
3LHF                          --------------------------------------------------
4LHF4                         --------------------------------------------------
4LHF3                         --------------------------------------------------
N2AA52                        --------------------------------------------------
NBPC31                        --------------------------------------------------
N2AA53                        --------------------------------------------------
NBPC33                        --------------------------------------------------

DQ1442127_Actinomycetales_    GGCAGCAGTGGGGAATATTGCAC--AATGGGCGAAAGCCTGA-TGCAGCG 368
DQ663172_Streptomyces         GGCAGCAGTGGGGAATATTGCAC--AATGGGCGAAAGCCTGA-TGCAGCG 382
Actc29                        -----------------------G--CATGGGCGAA-GCCTGA-TGCAGCG 24
ActC31                        GGCAGCAGTGGGGAATATTGCACAGNATGGGCGAAAGCCTGA-TGCAGCG 55
AY154378_Burkholderia         GGCAGCAGTGGGGAATTTTGGAC--AATGGGGGAAACCCTGA-TCCAGCA 363
DQ490307_Burkholdericeae      GGCAGCAGTGGGGAATTTTGGAC--AATGGGGGCAACCCTGA-TCCAGCA 383
DQ904608_Bacillus             GGCAGCAGTAGGGAATCTTCCGC--AATGGACGAAAGTCTGA-CGGAGCA 370
DQ464386_Pseudomonas          GGCAGCAGTGGGGAATATTGGAC--AATGGGCGAAAGCCTGA-TCCAGCC 244
AM161143_Pseudomonas          ---------------------------TGGGCGAA-GCCTGA-TCCAGCC 21
PSAC41                        ATATNNNNNNNNNNNNGTAGGNC--AATGGGCGAAAGCCTGA-TCCAGCC 75
PSAA1                         ------------------GAAGG--NATGGGCGAA-GCCTGA-TCCAGCC 28
3LHF6                         -------------------CNGG--NATGGGCGCA-GCCTGA-TCCAGCC 27
1MBT7                         ----------------------TAGCATGGGCGGAAGCCTGA-TGCAGCA 27
3LHF5                         ---------------------TNCGCATGGGCGAAAGCCTGAACGCAGCG 29
3LHF3                         -----------------------TACCAGGGCGAA-GCCTGA-CGCANC- 24
4LHF2                         ---------------------GGGTGNATGGGCGGA-GCCTGA-TCCAGC- 27
2MBT4                         -----------------------TGCATGGGCGGAAGCCTGA-TGCAGC- 25
4LHF1                         -----------------------TNCATGGGCGGA-GCCTGA-TGCANC- 24
3LHF                          -----------------------GGCATGGGCGCA-GCCTGA-TCCAGCC 25
4LHF4                         --------------------------------------------------
4LHF3                         -------------------CTANCATNTTGGNNAA-CCCTGCATGCAGCG 30
N2AA52                        -------------------TCGCCCGCCGGCGCCCCGCGCCCGTCCCGCC 31
NBPC31                        --------------------------------------------------
N2AA53                        --------------------------------------------------
NBPC33                        --------------------------------------------------

DQ1442127_Actinomycetales_    ACGCC-GCGTG-AGGGATGAC-GGCCTTCGGGTT---GTAAACCTCTTTC 412
DQ663172_Streptomyces         ACGCC-GCGTG-AGGGATGAC-GGCCTTCGGGTT---GTAAACCTCTTTC 426
Actc29                        ACGCC-GCGTG-AGGGATGAC-GGCCTTCGNNTTT--GTAAACCTCTTTC 69
ActC31                        ACACAAGCGTG-AGGGATGAC-GGCCTTCGGGTCTNCGTAAACCTCGTAA 103
AY154378_Burkholderia         ATGCC-GCGTG-TGTGAAGAA-GGCCTTCGGG-TT--GTAAAGCACTTTT 407
DQ490307_Burkholdericeae      ATGCC-GCGTG-TGTGAAGAA-GGCCTTCGGG-TT--GTAAAGCACTTTT 427
DQ904608_Bacillus             ACGCC-GCGTG-AGTGATGAA-GGCTTTCGGG-TC--GTAAAACTCTGTT 414
DQ464386_Pseudomonas          ATGCC-GCGTG-TGTGAAGAA-GGTCTTCGGA-TT--GTAAAGCACTTTA 288
AM161143_Pseudomonas          ATGCC-GCGTG-TGTGAAGAA-GGTCTTCGGA-TT--GTAAAGCACTTTA 65
PSAC41                        ATGCC-GCGTG-TGTGAAGAA-GGTCTTCGGACTT--GTAAAGCACTTTA 120
PSAA1                         ATGCC-GCGTG-TGTGAAGAA-GGTCTTCGNCNTT--GTAAAGCACGAAA 73
3LHF6                         ATGCC-GCGTG-AGTGATGAA-GGCCTTCGGG-TT--GTAAAACTCTTTT 71
1MBT7                         ACGCC-GCGTG-TGGGATGAACGGCCTTCGGGTTT--GTAAACCGCTTTC 73
3LHF5                         ACGCC-GCGTG-AGGGACGAA-GGCCTTCGGGTC---GTAAACCTCTTTC 73
3LHF3                         ACGCC-GCGTG-CGGGA-GAA-GGTCTTCGGGTTG---TAAACCGCTTTC 67
4LHF2                         ACGCC-GCGTG-AGGGATGAC-GGCCTTCGGGNNC---NAAACCTCTTTC 71
2MBT4                         ACGCC-GCGTG-CGGGATGAC-GGCCTTCGGGTTN---TAAACCGCTTTC 69
4LHF1                         ACGCC-GCGTG-CGGGATGAC-GGCCTTCGGGTTG---TAAACCGCTTTC 68
3LHF                          ATGCC-GCGTG-AGTGATGAA-GGCCCTAGGGTTC---TAAAGCTCTTTT 69
```

```
4LHF4                             ----------------------------------------GACCTCNAAA 10
4LHF3                             ACGCCGCCGTGGAGCGATNAA-CCCCTTCNCGGGNTCTAAANGCTCTTTC 79
N2AA52                            GCCCCCGCCCGCCGTCNAGNATAACTTTGAGGATT--ACCAGTCTTGCGA 79
NBPC31                            -----------GNATCNAGNNNAANTT-GAGNANN--ANCAGTCTTGCGA 36
N2AA53                            --------------GNATGNNGGACCCTGATNNNN--NATAATCTTGCGA 34
NBPC33                            --------------GNANGNNNGACCCTGATNANN--AATAATCTTGCGA 34
                                                                             *

DQ1442127_Actinomycetales_        A-GCAGGGAAGAAGCG---------------------AAAGTGACGGTA- 439
DQ663172_Streptomyces             A-GCAGGGAAGAAGCG---------------------AAAGTGACGGTA- 453
Actc29                            A-GCAGGGAAGAAGCG---------------------AAAGTGACGGTTT 97
ActC31                            A-AAAGGGAAGAAGCG---------------------AAAGTGACGGTAC 131
AY154378_Burkholderia             G-TCCGGAAAGAAAACTTCTGCCCTAATAC-GGTGGGAGGATGACGGTA- 454
DQ490307_Burkholdericeae          G-TCCGGAAAGAAATCCTCTGGGTTAATAC-CTCGGGGGGATGACGGTA- 474
DQ904608_Bacillus                 G-TTAGGGAAGAACAAGTACGAGAGTAACT-GCTCGTACCTTGACGGTA- 461
DQ464386_Pseudomonas              A-GTTGGGAGGAAGGGTTGTAGATTAATAC-TCTGCAATTTTGACGTTA- 335
AM161143_Pseudomonas              A-GTTGGGAGGAAGGGTTGTAGATTAATAC-TCTGCAATTTTGACGTTA- 112
PSAC41                            A-GTTGGGAGGAAGGGTTGTAGATTAATTCCTCTGCAATTTTGACGTTA- 168
PSAA1                             AAGTTGGGAGGAAGGGTTGTAGATTA-TTCNCCTGCAATTTTGAAGAAA- 121
3LHF6                             G-GCGGGGACGATAA--------------------------TGACGGTA- 93
1MBT7                             -GCCTGTGACGAAGCG---------------------TGAGTGACGGTA- 100
3LHF5                             AGCAGGGNAAGAAGCG---------------------AAAGTGACGGTA- 101
3LHF3                             ACCAGGGNACGAAGGCAACTGGGTTAATAG--CCCAGAAGTTGACGGTA- 114
4LHF2                             AN-AACAGACGAAGCTGACTCCACGTGTGN--TTCCGGG--TGACGGTA- 115
2MBT4                             G-CCTGTGACGAAGCGT---------------------GAGTGACGGTAA 97
4LHF1                             G-CCTGTGACGAAGCGT---------------------GAGTGACGGTAA 96
3LHF                              G-TGCGGGAAGA--------------------------TAATGACGGTAC 92
4LHF4                             A--AAAGGACGAAGNGC----------------------AGTGACGGTT- 35
4LHF3                             TNCAGGGCNNGAATNA-------------------------NGACGAGTN 104
N2AA52                            CCGTACTCCCCAGGTNCGGAGTGCT-------------TAATGCAGTTAG 116
NBPC31                            CCGTACTCCCCAGG-CNNTAGTGCT-------------TAATGC-GTTAG 71
N2AA53                            CCGTACTCCCCAGGTCNTTCAACTT-------------CACGGAGAATAG 71
NBPC33                            CCGTACTCCCCAGG-CTCNCAACTT-------------CACGC-GAATAG 69
                                             *

DQ1442127_Actinomycetales_        CCTGCAGAAGAAGCGCCG----GCTAACTAC-GTG-CCAGCAG-CCGCGG 482
DQ663172_Streptomyces             CCTGCAGAAGAAGCGCCG----GCTAACTAC-GTG-CCAGCAG-CCGCGG 496
Actc29                            CCTGCAGAAGAAGCGCCG----GCTAACTAC-GTG-CCAGCAG-CCGCGG 140
ActC31                            CCTGCAGAAGAAGCGCCGAAAAGCTAACTAC-GTG-CCAGCAG-CCGCGG 178
AY154378_Burkholderia             CCGGAAGAATAAGCACCG----GCTAACTAC-GTG-CCAGCAG-CCGCGG 497
DQ490307_Burkholdericeae          CCGGAAGAATAAGCACCG----GCTAACTAC-GTG-CCAGCAG-CCGCGG 517
DQ904608_Bacillus                 CCTAACCAGAAAGCCACG----GCTAACTAC-GTG-CCAGCAG-CCGCGG 504
DQ464386_Pseudomonas              CCGACAGAATAAGCACCG----GCTAACTCT-GTG-CCAGCAG-CCGCGG 378
AM161143_Pseudomonas              CCGACAGAATAAGCACCG----GCTAACTCT-GTG-CCAGCAG-CCGCGG 155
PSAC41                            CCGACAGAATAAGCACCG----CCTAACTCT-GTG-CCAGCAG-CCGCGG 211
PSAA1                             CCGACAGAATAAGCACCG----GCTAACTCT-GTG-CCAGCAG-CCGCGG 164
3LHF6                             CCCGCAGAATAAGCTCCG----GCTAACTTC-GTG-CCAGCAG-CCGCGG 136
1MBT7                             ATGGGTAAAGAAGCACCG----GCTAACTAC-GTG-CCAGCAG-CCGCGG 143
3LHF5                             CCTGCAGAAGAAGCACCG----GCTAACTAC-GTG-CCAGCAG-CCGCGG 144
3LHF3                             CTTGCAGAAGAAGGTCCG----GCTAACTAC-GTG-CCAGCAG-CCGCGG 157
4LHF2                             TGTGNAGAAGAAGCACCG----GCCAACTAC-GTG-CCAGCAG-CCGCGG 158
2MBT4                             TGGGTA-AAGAAGCACCG----GCTAACTAC-GTG-CCAGCAG-CCGCGG 139
4LHF1                             TGGGTA-AAGAAGCACCG----GCTAACTAC-GTG-CCAGCAG-CCGCGG 138
3LHF                              CGCAAGTAATAAGCCCCG----GCTAACTTC-GTG-CCAGCAG-CCGCGG 135
4LHF4                             TTTCCCNAACANGNGTCA----GAAAANTACCATG-CCACCNG-CCNCGG 79
4LHF3                             CCTGTNNTAACCAGTCTGCGC-NCTAACTACGGTGACCNGCAGACCGCGA 153
N2AA52                            CTGCAGCACTAAAGGGCGG---AAACCCTCTAACACTTAGCACTCATCGT 163
NBPC31                            CTGCAGCACTAAAGGGCGG---AAANTCNCTAACACTTAGCACTCATCGT 118
N2AA53                            CTACGTTACTAAGGAAATG---AA--TNTCCAACAACTAGTTGACATCGT 116
NBPC33                            CTTCGTTACTAAGGAAATG---AA--TNNTCCNCAACTAGNTGACANCGN 114
                                                                              *  **

DQ1442127_Actinomycetales_        --TAATACG-TAG---GGCGCGAGCGT-TGTCC-GGAATTATTGGGCGTA 524
DQ663172_Streptomyces             --TAATACG-TAG---GGCGCGAGCGT-TGTCC-GGAATTATTGGGCGTA 538
Actc29                            --TAATACG-TAG---GGCGCGAGCGT-TGTCC-GGAATTATTGGGCGTA 182
ActC31                            --TAATACG-TAG---GGCGCGAGCGT-TGTCC-GGAATTATTGGGCGTA 220
AY154378_Burkholderia             --TAATACG-TAG---GGTGCGAGCGT-TAATC-GGAATTACTGGGCGTA 539
DQ490307_Burkholdericeae          --TAATACG-TAG---GGTGCGAGCGT-TAATC-GGAATTACTGGGCGTA 559
DQ904608_Bacillus                 --TAATACG-TAG---GTGGCAAGCGT-TATCC-GGAATTATTGGGCGTA 546
DQ464386_Pseudomonas              --TAATACA-GAG---GGTGCAAGCGT-TAATC-GGAATTACTGGGCGTA 420
AM161143_Pseudomonas              --TAATACA-GAG---GGTGCAAGCGT-TAATC-GGAATTACTGGGCGTA 197
```

```
PSAC41                            --TAATACA-GAG---GGTGCAAGCGT-TNATCGCCAATTACTGGGCGTA 254
PSAA1                             --TAATACA-GAG---GGTGCAAGCGT-TTNTC-CCAATTACTGGGCGNA 206
3LHF6                             --TAATACG-AAG---GGGGCTAGCGT-TGTTC-GGAATTACTGGGCGTA 178
1MBT7                             --TGATACG-TAG---GGTGCGAGCGC-TGTCC-GGATTTATTGGGCGTA 185
3LHF5                             --TAATACG-TAG---GGTGCAAGCGT-TGTCC-GGAATTATTGGGCGTA 186
3LHF3                             --TAATACG-TAG---GGACCGAGCGT-TGTCC-GGAATCATTGGGCGTA 199
4LHF2                             --TAATACG-TAG---GGTGCGAGCGT-TGTCC-GGAATTATTGGGCGTA 200
2MBT4                             --TGATACG-TAG---GGTGCGAGCGT-TGTCC-GGATTTATTGGGCGTA 181
4LHF1                             --TGATACG-TAG---GGTGCGAGCGT-TGTCC-GGATTTATTGGGCGTA 180
3LHF                              --TAATACG-AAG---GGGGCTAGCGT-TGCTC-GGAATCACTGGGCGTA 177
4LHF4                             A-TNAGANG-TAGC--GGCGCGAGCGTCTGTACCNGATNTATTGGGNGNA 125
4LHF3                             GATNNTACGGTAGGACNGCGTAGACGATNNTTCGNNAGTTACTGNGANTA 203
N2AA52                            T-TACGGCG-TGG---ACTACCAGGGT--ATCTAATCCTGTTTGCTCCCC 206
NBPC31                            T-TACGGCG-TGG---ACTACCAGGGT--ATCTAATCCTGTTTGCTCCCC 161
N2AA53                            T-TAGGGCG-TGG---ACTACCAGGGT--ATCTAATCCTGTTTGCTCCCC 159
NBPC33                            T-TAGGGCG-TGG---ACTACCAGGGT--ATCTAATCCTGTTTGCTCCCC 157
                                     *         *            *                **

DQ1442127_Actinomycetales_        AAGAGCTC-GTAGGCGGCTT-GTCACGTCGGTTGTGAAAGCCCGGGGCTT 572
DQ663172_Streptomyces             AAGAGCTC-GTAGGCGGCTT-GTCACGTCGGTTGTGAAAGCCCGGGGCTT 586
Actc29                            AAGAGCTC-GTAGGCGGCTT-GTCACGTCGGTTGTGAAAGCCCGGGGCTT 230
ActC31                            AAGAGCTC-GTAGGCGGCTT-GTCACGTCGGTTGTGAAAGCCCGGGGCTT 268
AY154378_Burkholderia             AAGCGTGC-GCAGGCGGTTC-GTTAAGACAGATGTGAAATCCCCGGGCTT 587
DQ490307_Burkholdericeae          AAGCGTGC-GCAGGCGGTTC-GCTAAGACCGATGTGAAATCCCCGGGCTT 607
DQ904608_Bacillus                 AAGCGCGC-GCAGGCGGTTT-CTTAAGTCTGATGTGAAAGCCCACGGCTC 594
DQ464386_Pseudomonas              AAGCGCGC-GTAGGTGGTTC-GTTAAGTTGGATGTGAAAGCCCCGGGCTC 468
AM161143_Pseudomonas              AAGCGCGC-GTAGGTGGTTT-GTTAAGTTGGATGTGAAAGCCCCGGGCTC 245
PSAC41                            AAGCGCGCCGTAGGTGGTTCCGTTAAGTTGGATGTGAAAGCCCCGGGCTC 304
PSAA1                             AAGAGCGC-GTAGGTGGTTT-GTTAAGTTGGATGTGAAAGCCCCGGGCTC 254
3LHF6                             AAGCGTGC-GCAGGCGGCTT-CTCAAGTCAGGGGTGAAAGCCCAGAGCTT 226
1MBT7                             AAGGGCTC-GTAGGTGGTTG-ATCGCGTCGGAAGTGTAATCTTGGGGCTT 233
3LHF5                             AAGAGCTC-GTAGGCGGCTT-GTCACGTCTGCTGTGAAAACTCGGGGCCC 234
3LHF3                             AAGAGCGT-GTAGGCGGCTC-GCTAAGTCTTGCGTGAAATCTCGGGGCTC 247
4LHF2                             AAGGGCTC-GTAGGCGGTTT-GTCGCGTCGGGAGTGAAATCCACTGGCTT 248
2MBT4                             AAGGGCTC-GTAGGTGGTTG-ATCGCGTCGGAAGTGTAATCTTGGGGCTT 229
4LHF1                             AAGGGCTC-GTAGGTGGTTG-ATCGCGTCGGAAGTGTAATCTTGGGGCTT 228
3LHF                              AAGGGTGC-GTAGGCGGGTC-TTTAAGTCAGGGGTGAAATCCTGGAGCTC 225
4LHF4                             AANAGCTC-GGATGCCGNTT-GTCNCGTNTACCGTGNNNACTTGNGGCTC 173
4LHF3                             AACGGTGT-GNAGGCGGNTG-TTTANGTNTGGNGTGAAATCTCCCCGCCC 251
N2AA52                            ACGCTTTC-----GCGCCTCAGCGTCAGTTACAGACCAAAAAGCCGCCTT 251
NBPC31                            ACGCTTTC-----GCGCCTCAGCNTCAGTTACAGACCAAAAAGCCGCCTT 206
N2AA53                            ACGCTTTC-----GNGCNTGAGCGTCAGTATTGGCC-AGGGGGNTGCCTT 203
NBPC33                            ACGCTTTC-----GNGCATGAGCGTTNNTNCNAGCCCAGGGGGCTGCCTT 202
                                  *             *    *               *             *

DQ1442127_Actinomycetales_        A-ACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAG 621
DQ663172_Streptomyces             A-ACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAG 635
Actc29                            A-ACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAG 279
ActC31                            A-ACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTGCGGTAGGGGAG 317
AY154378_Burkholderia             A-ACCTGGGAACTGCATTTGTGACTGGCGAGCTAGAGTATGGCAGAGGGG 636
DQ490307_Burkholdericeae          A-ACCTGGGAACTGCATTGGTGACTGGCGAGCTAGAGTGTGGCAGAGGGG 656
DQ904608_Bacillus                 A-ACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGAAA 643
DQ464386_Pseudomonas              A-ACCTGGGAACTGCATTCAAAACTGTCGAGCTAGAGTATGGTAGAGGGT 517
AM161143_Pseudomonas              A-ACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTACGGTAGAGGGT 294
PSAC41                            ACACCTGGGAACTGCATTCAAAACTGTCGAGCTAGAGTATGGTAGAGGGT 354
PSAA1                             A-ACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTACGGTAGAGGGT 303
3LHF6                             A-ACTCTGGAATTGCCTTTGAGACTGGGTGGCTTGAGTACGGGAGAGGTG 275
1MBT7                             A-ACCCTGAGCGTGCTTTCGATACGGGTTGACTTGAGGAAGGTATGGGAG 282
3LHF5                             A-ACCCCGAGCCTGCAGTGGATACGGGCTAGCTAGAGTGCGGTAGGGGAG 283
3LHF3                             A-ACCCCGAGCGGTCGTGAGAAACTGGCGGGCTANAGTGCGGGTGAGGAG 296
4LHF2                             A-ACTGGTGGCTTGCTTTCNATACGGGCAGACTGGAGGTATGCAGGGNAA 297
2MBT4                             A-ACCCTGAGCGTGCTTTCGATACGGGTTGACTTGAGGAAGGTATGGGAG 278
4LHF1                             A-ACCCTGAGCGTGCTTTCGATACGGGTTGACTTGAGGAAGGTATGGGAG 277
3LHF                              A-ACTCCAGAACTGCCTTTGATACTGAGGATCTTGAGTTCGGGAGAGGTG 274
4LHF4                             A-ACNCCNNGCCTGCNNNCGATACGNNCAGGCTNGAGTNCAGTANGGGAG 222
4LHF3                             A-ACTGGGAGGGTGCGCCNAATACTGANTGACTCGAGTGNNNCANAGGTG 300
N2AA52                            CGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGA 301
NBPC31                            CGCCACTGGNGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGA 256
N2AA53                            CGNCATCGGTATTCCTCCACATCTCTACGCATTTCACTGNTACACGTGGA 253
NBPC33                            CGNCATCGGTATTCCTCCACATCTCTACGCATTTCACTGNTACACGTGGA 252
                                      *          *                  *  *
```

```
DQ1442127_Actinomycetales_      ATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACAC 671
DQ663172_Streptomyces           ATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACAC 685
Actc29                          ATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACAC 329
ActC31                          ATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACAC 367
AY154378_Burkholderia           GGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAATAC 686
DQ490307_Burkholdericeae        GGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAATAC 706
DQ904608_Bacillus               AGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACAC 693
DQ464386_Pseudomonas            GGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACAC 567
AM161143_Pseudomonas            GGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACAC 344
PSAC41                          GGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACAC 404
PSAA1                           GGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACAC 353
3LHF6                           AGTGGAATTCCCAGTGTAGAAGTGAAATTCGTAGATATTGGGAAGAACAC 325
1MBT7                           AATGGAATTCCTGGTGGAGCGGTGGAATGCGCAGATATCAGGAGGAACAC 332
3LHF5                           ACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACAC 333
3LHF3                           AGTGGAATTCCTGGTGTAGCGGTGAAATGCGCANATATCAGGAAGAACAC 346
4LHF2                           AACGGAATTCCTGGNGTAGCGGNGAAATGCGCAAATTTCNGGGGGAAACC 347
2MBT4                           AATGGAATTCCTGGTGGAGCGGTGGAATGCGCAGATATCAGGAGGAACAC 328
4LHF1                           AATGGAATTCCTGGTGGAGCGGTGGAATGCGCAGATATCAGGAGGAACAC 327
3LHF                            AGTGGAACTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCAAGAACAC 324
4LHF4                           GCTGGAATTCCTGGTGTAGCGGTGAAACGCGCAGATATCAAGANGAACAC 272
4LHF3                           ANTNNAATTCCTGGNGNAGCGGNGANATGCCTNCATNTCATGAGGAACAC 350
N2AA52                          ATTCCGCTTTTCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTC 351
NBPC31                          ATTCCGCTTTTCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTC 306
N2AA53                          ATTCTACCCCCCTCTGACANACTCTANNCTGCCAGNCACAAATGNAGNTN 303
NBPC33                          ATTCTACCCCCCTCTGNCANACTCTANNTCGCNAGTCACNAATGCAGTTC 302


DQ1442127_Actinomycetales_      CGG-TGGCGAAGGCG-GATCTCTGGGCC--GATACTGACGCTGAGGAGCG 717
DQ663172_Streptomyces           CGG-TGGCGAAGGCG-GATCTCTGGGCC--GATACTGACGCTGAGGAGCG 731
Actc29                          CGG-TGGCGAAGGCG-GATCTCTGGGCC--GATACTGACGCTGAGGAGCG 375
ActC31                          CGG-TGGCGAAGGCG-GATCTCTGGGCC--GATACTGACGCTGAGGAGCG 413
AY154378_Burkholderia           CGA-TGGCGAAGGCA-GCCCCCTGGGCC--AATACTGACGCTCATGCACG 732
DQ490307_Burkholdericeae        CGA-TGGCGAAGGCA-GCCCCCTGGGCT--AACACTGACGCTCATGCACG 752
DQ904608_Bacillus               CAG-TGGCGAAGGCG-GCTTTTTGGTCT--GTAACTGACGCTGAGGCGCG 739
DQ464386_Pseudomonas            CAG-TGGCGAAGGCG-ACCACCTGGACT--GATACTGACACTGAGGTGCG 613
AM161143_Pseudomonas            CAG-TGGCGAAGGCG-ACCACCTGGACT--GATACTGACACTGAGGTGCG 390
PSAC41                          CAG-TGGCGAAGGCG-ACCACCTGGACT--GATACTGACACTGAGGTGCG 450
PSAA1                           CAG-TGGCGAAGGCG-ACCACCTGGACT--GATACTGACACTGAGGTGCG 399
3LHF6                           CGG-TGGCGAAGGCG-GCTCACTGGCCC--GTAACTGACGCTCATGCACG 371
1MBT7                           CAG-TGGCGAAGGCG-GTTCTCTGGGCC--TTTCCTGACGCTGAGGAGCG 378
3LHF5                           CGG-TGGCGAAGGCG-GGTCTCTGGACC--GATACTGACGCTGAAGAGCG 379
3LHF3                           CCG-TGGCGAAGGCG-GCTCTCTGGAAC---GCACTGACNCTGAAACGCG 391
4LHF2                           CGGGNGGNAAAGGGG-GTTTNCTNGGGNATGNCCNNGACCCTAAAAAAAA 396
2MBT4                           CAG-TGGCGAAGGCG-GTTCTCTGGGCC--TTTCCTGACGCTGAGGAGCG 374
4LHF1                           CAG-TGGCGAAGGCG-GTTCTCTGGGCC--TTTCCTGACGCTGAGGAGCG 373
3LHF                            CAG-TGGCGAAGGCG-GCTCACTGGCCC--GATACTGACGCTGAGGCACG 370
4LHF4                           CGG-TGGCGAANGCTCGGTCTCTGGTCC--GATACTGACGCTNAAGAGCG 319
4LHF3                           CNCGNGGTGTATACC-NCTCNCNGGANC--ATNACTGACGCTGATACACC 397
N2AA52                          CAC--GGTTGAGCCGTGGGCTTTCACAT--CAGACTTAAGAAACCGCCTG 397
NBPC31                          CAC--GGTTGAGCCGTGGGCTTTCACAT--CAGACTTAAGAAACCGCCTG 352
N2AA53                          CCA--GGTTAAGCCCGGGGATTTCACAT--CGNTCTTAGCAAAANGCCTG 349
NBPC33                          CCA--GGTTAAGCCCGGGGATTTCACAT--CGNNCTTAGCNAANCGNCTG 348
                                *    **   *                          *

DQ1442127_Actinomycetales_      AAAGCGTGGGGAGCGAA-CAGG--ATTAGATACCCTGGTAGTCCACGCCG 764
DQ663172_Streptomyces           AAAGCGTGGGGAGCGAA-CAGG--ATTAGATACCCTGGTAGTCCACGCCG 778
Actc29                          AAAGCGTGGGGAGCGAA-CAGG--ATTAGATACCCTGGTAGTCCACGCCG 422
ActC31                          AAAGCGTGGGGAGCGAA-CAGG--ATTAGATACCCTGGTAGTCCACGCCG 460
AY154378_Burkholderia           AAAGCGTGGGGAGCAAA-CAGG--ATTAGATACCCTGGTAGTCCACGCCC 779
DQ490307_Burkholdericeae        AAAGCGTGGGGAGCAAA-CAGG--ATTAGATACCCTGGTAGTCCACGCCC 799
DQ904608_Bacillus               AAAGCGTGGGGAGCAAA-CAGG--ATTAGATACCCTGGTAGTCCACGCCG 786
DQ464386_Pseudomonas            AAAGCGTGGGGAGCAAA-CAGG--ATTAGATACCCTGGTAGTCCACGCCG 660
AM161143_Pseudomonas            AAAGCGTGGGGAGCAAA-CAGG--ATTAGATACCCTGGTAGTCCACGCCG 437
PSAC41                          AAAGCGTGGGGAGCAAA-CAGG--ATTAGATACCCTGGTAGTCCACGCCG 497
PSAA1                           AAAGCGTGGGGAGCAAA-CAGG--ATTAGATACCCTGGTAGTCCACGCCG 446
3LHF6                           ACAGCGTGGGGATCAAA-CAGG--ATTAGATACCCTGGTAGTCCACGCCG 418
1MBT7                           AAAGCGTGGGGAGCGAA-CAGG--CTTAGATACCCTGGTAGTCCACGCTG 425
3LHF5                           AAAGCGTGGGGAGCGAAACAGG--ATTAGATACCCTGGTAGTCCACGCCG 427
3LHF3                           AAAGCGTGGGGAGCAAACAGG---ATTANATACCCTGGTAGTCCCCCCCN 438
4LHF2                           AAAANTNGGGGGANANAANAGGATNTTAAAACCCCGGGGNNNCCCCCCCC 446
```

```
2MBT4                           AAAGCGTGGGGAGCGAA-CAGG--CTTAGATACCCTGGTAGTCCACGCTG 421
4LHF1                           AAAGCGTGGGGAGCGAA-CAGG--CTTAGATACCCTGGTAGTCCACGCTG 420
3LHF                            AAAGCGTGGGGAGCAAA-CAGG--ATTAGATACCCTGGTAGTCCACGCCG 417
4LHF4                           AAAGCGNGGGGAGCGAA-CAGG--ATTACANACCCTGGTAGTCCACNCTG 366
4LHF3                           NNAGCGNGNTTNNCANA-CAGG--ATTAGATACCCTGACAGTCCNCGCCC 444
N2AA52                          CGCGCGCTTTACGCCCAATAATTCGCGGAAAACGCTTGCCACCTACGTAT 447
NBPC31                          CGCGCGCTTTACGCCCAATAATTC-CGGATAACGCTTGCCACCTACGTAT 401
N2AA53                          CGCACGNTTTACGCCCAGTGAATTCCGATTAACGCTCGCACCCTACGTAT 399
NBPC33                          CGCACGCTTTACGCCCAGT-AATTCCNATTAANGCTCGCACCCTACGTAT 397
                                                 *                  *       *  *

DQ1442127_Actinomycetales_      TAAACGGT--GGGCACTAGGTGTGGGC-GACATTCCACGTCG----TCCG 807
DQ663172_Streptomyces           TAAACGGT--GGGCACTAGGTGTGGGC-GACATTCCACGTCG----TCCG 821
Actc29                          TAAACGGT--GGGCACTAGGTGTGGGC-GACATTCCACGTCG----TCCG 465
ActC31                          TAAACGGG--GGGAACTAGGTGTGGGCAGACATTCCACGTTG----TCCG 504
AY154378_Burkholderia           TAAACGAT--GTCAACTAGTTGTTGGG--GA--TTCATTTCC----TTAG 819
DQ490307_Burkholdericeae        TAAACGAT--GTCAACTAGTTGTTGGG--GA--TTCATTTCC----TTAG 839
DQ904608_Bacillus               TAAACGAT--GAGTGCTAAGTGTTAGA--GGGTTTCCGCCCT----TTAG 828
DQ464386_Pseudomonas            TAAACGAT--GTCAACTAGCCGTTGGG--AGCCTTGAGCTC-----TTAG 701
AM161143_Pseudomonas            TAAACGAT--GTCAACTAGCCGTTGGA--ATCCTTGAGATT-----TTAG 478
PSAC41                          TAAACGAT--GTCAACTAGCCGTTGGA--AGCCTTGAGCTC-----TTAG 538
PSAA1                           TAAACGAT--GTCAACTAGCCGTTGGA--ATCCTTGAGATT-----TTAG 487
3LHF6                           TAAACTAT--GAACGCTAGCCGTTGGG--TAGCTT--GCTAT----TCAG 458
1MBT7                           TAAACGGT--GGGTACTAGGTGTGGGG-TCCATTCCACGGGT----TCCG 468
3LHF5                           TAAACGTT--GGGCGCTCGGTGTGGGA-TCCCTTCCACGGGT----TCCG 470
3LHF3                           TAAACAAT--GGACCCTAGGNGNGGGNGGGGNCNANCCCNNCCGNGCNAA 486
4LHF2                           CAAAAANTTNGGGGNCCGGNGGGGGGGGAAAAATTCNCCCCGGGGGNTCN 496
2MBT4                           TAAACGGT--GGGTACTAGGTGTGGGG-TCCATTCCACGGGT----TCCG 464
4LHF1                           TAAACGGT--GGGTACTAGGTGTGGGG-TCCATTCCACGGGT----TCCG 463
3LHF                            TAAACGAT--GAATGCCAGCCGTTAGT-GGGTTTACTCA-------CTAG 457
4LHF4                           TAAACGTT--GGGCGCTAGGTGTGGCNGGACCTCTCCGTGTTC---TCTG 411
4LHF3                           TNNACGAT--GCANATTTNGTGTGNNT----AGNTCANTCCN----NCCG 484
N2AA52                          T--ACCGC--GGCTGCTGGCACGTAGTTAGCCGTGGCTTTCT----GGTT 489
NBPC31                          T--ACCGC--GGCTGCTGGCACGTAGTTAGCCGTGGCTTTCT----GGTT 443
N2AA53                          T--ACCGC--GGCTGCTGGAACNTATTTAGCNGGNGCTTATT----CTTC 441
NBPC33                          T--ACCGC--GGCTGCTGGCACGTANTTAGCNGGTGCTTATT----CTTC 439
                                   *       *

DQ1442127_Actinomycetales_      TGCCGCAGCTAA-CGCATTAAGTGCCC-CGCCTGGGG-AGTACGGCCGCA 854
DQ663172_Streptomyces           TGCCGCAGCTAA-CGCATTAAGTGCCC--GCCTGGGG-AGTACGGCCGCA 867
Actc29                          TGCCGCAGCTAA-CGCATTAAGTGCCC-CGCCTGGGG-AGTACGGCCGCA 512
ActC31                          TGCCGCAGCTAA-CGCATTAAGTGCCAGCGCCTGGGG-AGTACGGCCGCA 552
AY154378_Burkholderia           TAACGTAGCTAA-CGCGTGAAGTTGACCG-CCTGGGG-AGTACGGTCGCA 866
DQ490307_Burkholdericeae        TAACGTAGCTAA-CGCGTGAAGTTGACCG-CCTGGGG-AGTACGGTCGCA 886
DQ904608_Bacillus               TGCTGCAGCTAA-C-CATTAAGCACTCCG-CCTGGGG-AGTACGGTCGCA 874
DQ464386_Pseudomonas            TGGCGCAGCTAA-CGCATTAAGTTGACCG-CCTGGGG-AGTACGGCCGCA 748
AM161143_Pseudomonas            TGGCGCAGCTAA-CGCATTAAGTTGACCG-CCTGGGG-AGTACGGCCGCA 525
PSAC41                          TGGCGCAGCTAATCGCATTAAGTTGACCGACCTGGGG-AGTACGGCCGCA 587
PSAA1                           TGGCGCAGCTAA-CGCATTAAGTTGACCG-CCTGGGG-AGTACGGCCGCA 534
3LHF6                           TGGCGCAGCTAA-CGCATTAAGCGTTCCN-CCTGGGG-AGTACNGCCNCA 505
1MBT7                           TGCCGTAGCTAA-CGCTTTAAGTACCCCG-CCTGGGG-AGTACGGCCGCG 515
3LHF5                           TGCCGCANCTAA-CGCGTTAAGCGCCCNC-CCTGGGG-AGTACGGCCNC- 516
3LHF3                           ANTAACNATTAAC-------------------------------------- 499
4LHF2                           NGGGGNGNCAAAAAAAAAAAANNATANACCCCCCCCC-CCCGGGGGNAAA 545
2MBT4                           TGCCGTAGCTAA-CGCTTTAAGTACCCCG-CCTGGGA-AGTACGGCCGCG 511
4LHF1                           TGCCGTAGCTAA-CGCTTTAAGTACCCCG-CCTGGGG-AGTACGGCCGCG 510
3LHF                            TGGCGCAGCTAA-CGCTTTAAGCATTCCG-CCTGGGG-AGTACGGTCGCA 504
4LHF4                           TGNCGCAGCTAA-CGCATTAAGCTGCCCCNCCTGGGGCAGTACGGCCGNA 460
4LHF3                           NNCCNGAGCTAA-CGNGNTNNNNNTGCCG-CCTGGATGAGTACANTCGCA 532
N2AA52                          AGGTACCGTCCAAGGTACGAAGCAAATACTCTCGTACTTGTTCTTCCCTA 539
NBPC31                          AGGTACCGTC-AAGGTACGA-GCAGTTACTCTCGTACTTGTTCTTCCCTA 491
N2AA53                          CGGTACCGTCATCCACACNNCGNATTANGNCNNGNGTTT--TCTTTCCGG 489
NBPC33                          CGGTACCGTCATCCCCCCGAGGNATTAACCCAGAGGATT--TCTTTCCGG 487

DQ1442127_Actinomycetales_      AGGC-----------------TAAAAC-TCAAAGGAATTG--ACGGGGGC 884
DQ663172_Streptomyces           AGGC-----------------TAAAAC-TCAAAGGAATTG--ACGGGGGC 897
Actc29                          AGGC-----------------TAAAAC-TCAAAGGAATTG--ACGGCGG- 541
ActC31                          AGGCANTTTT----------TTNTTTN-TCAAAGGAATTG--ACGGCGG- 588
AY154378_Burkholderia           AGA-----------------TTAAAAC-TCAAAGGAATTG--ACGGGGAC 896
DQ490307_Burkholdericeae        AGA-----------------TTAAAAC-TCAAAGGAATTG--ACGGGGAC 916
```

```
DQ904608_Bacillus               AGA-----------------CTGAAAC-TCAAAGGAATTG--ACGGGG-- 902
DQ464386_Pseudomonas            AGG-----------------TTAAAAC-TCAAATGAATTG--ACGGGGGC 778
AM161143_Pseudomonas            AGG-----------------TTAAAAC-TCAAATGAATTG--ACGGGGAA 555
PSAC41                          AGGGTTNTNNNTNATCAAACTTNNNNCATCCAAGGAATTG--ACGGCGGG 635
PSAA1                           AGG-----------------TTAAAAC-TCAAAGGAATTG--ACGGCGGG 564
3LHF6                           AGG-----------------TTGAAAC-TCAAAGGAATTT--ACCGCNGN 535
1MBT7                           AGG-----------------CTAAAAC-TCAAAGGAATTG--ACGGCGGG 545
3LHF5                           ANG-----------------NTNAAAC-TCAANG-AATTN--ACCGCCGG 545
3LHF3                           --------------------------------------------------
4LHF2                           AAANCCCCCCCNNTTTTTTTTTTTTTTTTAAAAAAAAAAA--AAANNNNG 593
2MBT4                           AGG-----------------CTAAAAC-TCAAAGGAATTG--ACGGCGGG 541
4LHF1                           AGG-----------------CTAAAAC-TCAAAGGAATTG--ACGGCGGG 540
3LHF                            AGA-----------------TTAAAAC-TCAAAGGAATTG--ACGGCGGG 534
4LHF4                           NGG-----------------CTAAAAC-TCAAANGANTTNT-NNNGCGNG 491
4LHF3                           NNGT----------------CTGANACTCACACCTAATTGACTNTGGCGC 566
N2AA52                          ACAACAGAGT---------TTTACGAC-CCGAAAGCCTTCATCACTCACG 579
NBPC31                          ACAACAGAGT---------TTTACGAC-CCGAAAGCCTTCATCACTCACG 531
N2AA53                          ACAAAAGTGN---------TTTACANC-CCGANGGGCTTCTTCACACACG 529
NBPC33                          ACAAAAGTGC---------TTTACAAC-CCGAAGGCCTTCTTCACACACG 527


DQ1442127_Actinomycetales_      CCGCACAAGCGGCGGAGC-ATGTGGCTTAATTCGACGCAACGCGAAGAAC 933
DQ663172_Streptomyces           CCGCACAAGCGGCGGAGC-ATGTGGCTTAATTCGACGCAACGCGAAGAAC 946
Actc29                          --GCGGGGGCGGCGGGAC-G---GGCGCGGGGCGCCGGCGGGCGA----- 580
ActC31                          --GCGGGGGCGGNGGGAC-G---GGCGCGGGGNGCGGCGG-GCGA----- 626
AY154378_Burkholderia           CCGC-CAAGCGGTGGATG-ATGTGGATTAATTCGATGCAACGCGAAAAAC 944
DQ490307_Burkholdericeae        CCGCACAAGCGGTGGATG-ATGTGGATTAATTCGATGCAACGCGAAAAAC 965
DQ904608_Bacillus               --------------------------------------------------
DQ464386_Pseudomonas            CCGCACAAGCGGTGGAGC-ATGTGGTTTAATTCGAAGCAACGCGAGAA-- 825
AM161143_Pseudomonas            TCGAATTCCCGCGGCCGCCATGGCGGCCGGGAGCATGCGACGTCGGGCCC 605
PSAC41                          CGGGGGCGGCGGGACGGGCGCGGGGCNCCGGCGGGCGA------------ 673
PSAA1                           CGGGGGCGGCGGGACGGGCGCGGGGCGC-GGNGGGCG------------- 600
3LHF6                           CNGGGNCCGCCGNACNGNCCCGGGNCCC--GCCGGNNAATCNAATTCCCC 583
1MBT7                           CGGGGGCGGCGGGACGGGCGCGGGGCGC-GGCGGGCGAATCGAATTCCCG 594
3LHF5                           CCGGGGCCGCNGNACNGGCCCNGGGCCC-CGCNGGCNANTCNANTTCCC- 593
3LHF3                           --------------------------------------------------
4LHF2                           GGGNGGG------------------------------------------- 600
2MBT4                           CGGGGGCGGCGGGACGGGCGCGG--CGC-GGCGGGCGAATCACTAGTGAA 588
4LHF1                           CGGGGGCGGCGGGACGGGCGCGGGGCGC-GGCGGGCGAATCACTAGTGAA 589
3LHF                            CGGGGGCGGCGGGACNGGCGCGGGGCGC-GGCGGGCGAATCACTAGTGAA 583
4LHF4                           CCGGGACGGCCGCACGGNCCCGCGGCGCANTCACTNGAATCACTAGTGAA 541
4LHF3                           NAGTCNCGGCCNTACNNGCTCCCTGNGC-GACATTGCNATCACTACTGAA 615
N2AA52                          CGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGC- 628
NBPC31                          CGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGTG 581
N2AA53                          AGGNATTGCTGGATCAGGGTTGCCCCCATTGTCCAAAATTCCCCACTGC- 578
NBPC33                          CAGCATTGCTGGATCAGGGTTGCCCCCATTGTCCAAAATTCCCCACTGC- 576


DQ1442127_Actinomycetales_      CTTACCAAGGCTTGACATACACCGGAAAGCATCAGA----GATGGTGCCC 979
DQ663172_Streptomyces           CTTACCAAGGCTTGACATACACCGGAAAACCCTGGA----GACAGGGTCC 992
Actc29                          --------------------------------------------------
ActC31                          --------------------------------------------------
AY154378_Burkholderia           CTTACCTACCCTTGACATGGTCGGAACCCTGCTGAAAGGTGGGGGTGCTC 994
DQ490307_Burkholdericeae        CTTACCTACCCTTGACATGGACGGAATCCCGCTGAGAGGTGGGAGTGCTC 1015
DQ904608_Bacillus               --------------------------------------------------
DQ464386_Pseudomonas            --------------------------------------------------
AM161143_Pseudomonas            AATTCGCCCTWGGGGNNCCG------------------------------ 625
PSAC41                          --------------------------------------------------
PSAA1                           --------------------------------------------------
3LHF6                           NGNCCCCCTNGGNGNC---------------------------------- 599
1MBT7                           CGGCCGCCATGGCGGCCGGGAGCATGCGACGTCNGGCCCAATTCGCCCTA 644
3LHF5                           CGGNC-CCCTNGGGGNCGGGAAAATGNAANN-CNGGCCCANTCC-CCCNA 640
3LHF3                           --------------------------------------------------
4LHF2                           --------------------------------------------------
2MBT4                           TTCGCGGCCGCCTG---CAGGTCGACCATATGGGAGAGCTCCCAACGCGT 635
4LHF1                           TTCGCGGCCGCCTG---CAGGTCGACCATATGGGAGAGCTCCCAACGCGT 636
3LHF                            TTCGCGGCCGCCTG---CAGGTCGACCATATGGGAGAGCTCCCAACGCGT 630
4LHF4                           TTCNNGGNCGCCTG-CATGTCGACNATATGGNAGAGCTCCCAANNCGTTN 590
4LHF3                           TTCGCGNCCNNCTG---CAGGNCGACCNTATGGGAGAGCTCCCTNCGCGT 662
N2AA52                          TGCCTCCCGTAGGAGA---------------------------------- 644
NBPC31                          CCCCNCCNGTAGGA------------------------------------ 595
```

```
N2AA53                          TGCCTCCCGTAGGAGGA--------------------------------- 595
NBPC33                          TGCCTCCCGNAGGA------------------------------------ 590


DQ1442127_Actinomycetales_      CCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCG 1029
DQ663172_Streptomyces           CCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCG 1042
Actc29                          --------------------------------------------------
ActC31                          --------------------------------------------------
AY154378_Burkholderia           GAAAGAGAACCGATACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCG 1044
DQ490307_Burkholdericeae        GAAAGAGAACCGTTGCACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCG 1065
DQ904608_Bacillus               --------------------------------------------------
DQ464386_Pseudomonas            --------------------------------------------------
AM161143_Pseudomonas            --------------------------------------------------
PSAC41                          --------------------------------------------------
PSAA1                           --------------------------------------------------
3LHF6                           --------------------------------------------------
1MBT7                           TAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACT 694
3LHF5                           NNGGGGGCG----------------------------------------- 649
3LHF3                           --------------------------------------------------
4LHF2                           --------------------------------------------------
2MBT4                           TGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTGGGCGTA 685
4LHF1                           TGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTA 686
3LHF                            TGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTA 680
4LHF4                           GCATGATANCTTGANTATNCNANNAGANGTCACCTAGATAGNTTGGCGTA 640
4LHF3                           TNGATGCATAGCTTGANNATTCTATANTGTCACCTANNNACCTTGGCCGT 712
N2AA52                          --------------------------------------------------
NBPC31                          --------------------------------------------------
N2AA53                          --------------------------------------------------
NBPC33                          --------------------------------------------------


DQ1442127_Actinomycetales_      TGAGATGTTGGGTTAAGTCCCGCAA-CGAGCGCAACCCTTGTCCCGTGTT 1078
DQ663172_Streptomyces           TGAGATGTTGGGTTAAGTCCCGCAA-CGAGCGCAACCCTTGTCCCGTGTT 1091
Actc29                          --------------------------------------------------
ActC31                          --------------------------------------------------
AY154378_Burkholderia           TGAGATGTTGGGTTAAGTCCCGCAA-CGAGCGCAACCCTTGTCCTTAGTT 1093
DQ490307_Burkholdericeae        TGAGATGTTGGGTTAAGTCCCGCAA-CGAGCGCAACCCTTGTCTCTAGTT 1114
DQ904608_Bacillus               --------------------------------------------------
DQ464386_Pseudomonas            --------------------------------------------------
AM161143_Pseudomonas            --------------------------------------------------
PSAC41                          --------------------------------------------------
PSAA1                           --------------------------------------------------
3LHF6                           --------------------------------------------------
1MBT7                           GGGAAAACCCTGGCGTTACCCAACT-TAATCGCCTTGCAGCACATCCCCC 743
3LHF5                           --------------------------------------------------
3LHF3                           --------------------------------------------------
4LHF2                           --------------------------------------------------
2MBT4                           ATCATGGTCATAGCTGTTTCCTGNG-TGAAANTGTTATCCGCTCANANTT 734
4LHF1                           ATCATGGTCATAGCTGTTTCCTGTG-TGAAATTGTTATCCGCTCACAATT 735
3LHF                            ATCATGGTCATAGCTGTTTCCTGTG-TGAAATTGTTATCCGCTCACAATT 729
4LHF4                           ATCATGGGCATANCTGTTTCNTGTGGTGAAATTGTNATCCGCTCACAATT 690
4LHF3                           ATTCNTGGTCATAAATGTNTCCTGNGCGAANNTGTTATCNACTCACCNNT 762
N2AA52                          --------------------------------------------------
NBPC31                          --------------------------------------------------
N2AA53                          --------------------------------------------------
NBPC33                          --------------------------------------------------


DQ1442127_Actinomycetales_      GCCAGCAGGCCCTTGTGGTGC-TGGGGACTCACGGGAGACCGCCGGGGTC 1127
DQ663172_Streptomyces           GCCAGCAGGCCCTTGTGGTGC-TGGGGACTCACGGGAGACCGCCGGGGTC 1140
Actc29                          --------------------------------------------------
ActC31                          --------------------------------------------------
AY154378_Burkholderia           GCTACGCAA--------------GAGCACTCTAAGGAGACTGCCGGTGAC 1129
DQ490307_Burkholdericeae        GCTACGAAA--------------GGGCACTCTAGAGAGACTGCCGGTGAC 1150
DQ904608_Bacillus               --------------------------------------------------
DQ464386_Pseudomonas            --------------------------------------------------
AM161143_Pseudomonas            --------------------------------------------------
PSAC41                          --------------------------------------------------
PSAA1                           --------------------------------------------------
3LHF6                           --------------------------------------------------
1MBT7                           TTTCGCCAGCTGGCGTAATAG-CGAAAAGNCCGCACCGATCGCCCTTCCN 792
```

```
3LHF5                           --------------------------------------------------
3LHF3                           --------------------------------------------------
4LHF2                           --------------------------------------------------
2MBT4                           CCCC-CAACATACNANCCNGA-AGC-TNAANTGTAAANCCTGGGGNGCCN 781
4LHF1                           CCNCACAACATACGAGCCGGA-AGCATAAAGTGTAAAGCCTGGGGGGCCT 784
3LHF                            CCCCACAACATACGAGCCNGA-AGCATAAAGTGTAAAGCCTGGGGTGCCT 778
4LHF4                           CCACACAACNTACAANCCCGAANGCATAACCTGNAANGCCTGAGGTGCCT 740
4LHF3                           CCCCCGACATANGAGCCNNANGCATAAGGGNNANGTCNGNTTCGNCTANT 812
N2AA52                          --------------------------------------------------
NBPC31                          --------------------------------------------------
N2AA53                          --------------------------------------------------
NBPC33                          --------------------------------------------------

DQ1442127_Actinomycetales_      AACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCT 1177
DQ663172_Streptomyces           AACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCT 1190
Actc29                          --------------------------------------------------
ActC31                          --------------------------------------------------
AY154378_Burkholderia           AAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGT 1179
DQ490307_Burkholdericeae        AAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGT 1200
DQ904608_Bacillus               --------------------------------------------------
DQ464386_Pseudomonas            --------------------------------------------------
AM161143_Pseudomonas            --------------------------------------------------
PSAC41                          --------------------------------------------------
PSAA1                           --------------------------------------------------
3LHF6                           --------------------------------------------------
1MBT7                           ACAGTTGCNCANCCTGAATTGGCGAATGGACCCNCCCTGTANCGCCCATT 842
3LHF5                           --------------------------------------------------
3LHF3                           --------------------------------------------------
4LHF2                           --------------------------------------------------
2MBT4                           AATGAGTGANCTA-CTCNN-TTANTTGNGTTG-GCTCCCTGNCC-NNTTC 827
4LHF1                           AATGAGTGAGCTA-CTCACATTAATTGCGTTGCGCTCACTGCCC-CTTTC 832
3LHF                            AATGAGTGAGCTAACTCCCATTAATTGCGTTGCGCTCACTGCCCGCTTTC 828
4LHF4                           AATGAANGACCTAACTCACATTAATTGCGTTGCCCNCNTGCCCGCTTTTC 790
4LHF3                           GATGGAGCTANNCNCATAATTGCGNTNGCTCNAGCCCNNTTCNC-NNCGA 861
N2AA52                          --------------------------------------------------
NBPC31                          --------------------------------------------------
N2AA53                          --------------------------------------------------
NBPC33                          --------------------------------------------------

DQ1442127_Actinomycetales_      TGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCG 1227
DQ663172_Streptomyces           TGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCG 1240
Actc29                          --------------------------------------------------
ActC31                          --------------------------------------------------
AY154378_Burkholderia           AGGGCTTCACACGTCATACAATGGTCGGAACAGAGGGCTGCCAACCCGTG 1229
DQ490307_Burkholdericeae        AGGGCT-CACACGTCATACAATGGTCGGAACAGAGGGTTGCCAACCCGCG 1249
DQ904608_Bacillus               --------------------------------------------------
DQ464386_Pseudomonas            --------------------------------------------------
AM161143_Pseudomonas            --------------------------------------------------
PSAC41                          --------------------------------------------------
PSAA1                           --------------------------------------------------
3LHF6                           --------------------------------------------------
1MBT7                           AACCCGGCGGGTG------------------------------------- 855
3LHF5                           --------------------------------------------------
3LHF3                           --------------------------------------------------
4LHF2                           --------------------------------------------------
2MBT4                           CNNTCGGNAAACTT------------------------------------ 841
4LHF1                           CAGTCGGGAACCTGTCNTGCC----------------------------- 853
3LHF                            CAGTCGGGAAACCTGTCNNGCCANCTGCATTAATGAATCNGCCACCCNNG 878
4LHF4                           AANCNGGNAACCTGNCTGGCTAATGAATTAATAAACCGCCAAANAACGGG 840
4LHF3                           AACCTGTCTNGCCTCTNNCTAATNATCGGANACCCCCGAAAAAANNGGNT 911
N2AA52                          --------------------------------------------------
NBPC31                          --------------------------------------------------
N2AA53                          --------------------------------------------------
NBPC33                          --------------------------------------------------

DQ1442127_Actinomycetales_      AGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCA 1277
DQ663172_Streptomyces           AGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCA 1290
Actc29                          --------------------------------------------------
```

```
ActC31                          --------------------------------------------------
AY154378_Burkholderia           AGGGGGAGCTAATCCCAGAAAACCGATCGTAGTCCGGATCGTAGTCTGCA 1279
DQ490307_Burkholdericeae        AGGGGGAGCTAATCCCAGAAAACCGATCGTAGTCCGGATTGCACTCTGCA 1299
DQ904608_Bacillus               --------------------------------------------------
DQ464386_Pseudomonas            --------------------------------------------------
AM161143_Pseudomonas            --------------------------------------------------
PSAC41                          --------------------------------------------------
PSAA1                           --------------------------------------------------
3LHF6                           --------------------------------------------------
1MBT7                           --------------------------------------------------
3LHF5                           --------------------------------------------------
3LHF3                           --------------------------------------------------
4LHF2                           --------------------------------------------------
2MBT4                           --------------------------------------------------
4LHF1                           --------------------------------------------------
3LHF                            GGAAAAGGCGGNTTGGNNNATTGGGGGCTNTTCCGNTTCCTCGNTCATGA 928
4LHF4                           AAAGNCNNTTNGNTTTTTGGGGTTNTTCCGGTTCCTANTTNATGAAATNC 890
4LHF3                           NNAAATTGGCNANTTCCNNTCCCNAAAAANGACCNTTGCGGNCGTNTTCN 961
N2AA52                          --------------------------------------------------
NBPC31                          --------------------------------------------------
N2AA53                          --------------------------------------------------
NBPC33                          --------------------------------------------------


DQ1442127_Actinomycetales_      ACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCT 1327
DQ663172_Streptomyces           ACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCT 1340
Actc29                          --------------------------------------------------
ActC31                          --------------------------------------------------
AY154378_Burkholderia           ACTCGACTACGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCAT-GCC 1328
DQ490307_Burkholdericeae        ACTCGAGTGCATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCAT-GCC 1348
DQ904608_Bacillus               --------------------------------------------------
DQ464386_Pseudomonas            --------------------------------------------------
AM161143_Pseudomonas            --------------------------------------------------
PSAC41                          --------------------------------------------------
PSAA1                           --------------------------------------------------
3LHF6                           --------------------------------------------------
1MBT7                           --------------------------------------------------
3LHF5                           --------------------------------------------------
3LHF3                           --------------------------------------------------
4LHF2                           --------------------------------------------------
2MBT4                           --------------------------------------------------
4LHF1                           --------------------------------------------------
3LHF                            ATCCCTGNNCTCGGNCNTTCGG---------------------------- 950
4LHF4                           NTGNNTTCGGNNTTCNGATGGGGNAAAANTTATCCAATNCCNCAAAGNGG 940
4LHF3                           NANGCCNNACCNTTACANNNTCNCAAAGGCGNAATCCGGTTTCACGGATC 1011
N2AA52                          --------------------------------------------------
NBPC31                          --------------------------------------------------
N2AA53                          --------------------------------------------------
NBPC33                          --------------------------------------------------


DQ1442127_Actinomycetales_      GCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAA 1377
DQ663172_Streptomyces           GCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAA 1390
Actc29                          --------------------------------------------------
ActC31                          --------------------------------------------------
AY154378_Burkholderia           GCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGG 1378
DQ490307_Burkholdericeae        GCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGG 1398
DQ904608_Bacillus               --------------------------------------------------
DQ464386_Pseudomonas            --------------------------------------------------
AM161143_Pseudomonas            --------------------------------------------------
PSAC41                          --------------------------------------------------
PSAA1                           --------------------------------------------------
3LHF6                           --------------------------------------------------
1MBT7                           --------------------------------------------------
3LHF5                           --------------------------------------------------
3LHF3                           --------------------------------------------------
4LHF2                           --------------------------------------------------
2MBT4                           --------------------------------------------------
4LHF1                           --------------------------------------------------
3LHF                            --------------------------------------------------
4LHF4                           TNTCCCGNTNTCCCNGGAAACNGGGGNAACCCNNGAAAANANTGGNCCNA 990
```

```
4LHF3                           ANGNNAAACGCNNNAAAAAATNTGGCTTNCCCCNGAAANGCCNNAACCNT 1061
N2AA52                          --------------------------------------------------
NBPC31                          --------------------------------------------------
N2AA53                          --------------------------------------------------
NBPC33                          --------------------------------------------------


DQ1442127_Actinomycetales_      AGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGCTG 1427
DQ663172_Streptomyces           AGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGCTG 1440
Actc29                          --------------------------------------------------
ActC31                          --------------------------------------------------
AY154378_Burkholderia           AGTGGGTTTTACCAGAAGTGGCTAGTCTAACCGCAAGGAGGACGGTCACC 1428
DQ490307_Burkholdericeae        AGTGGGTTTTACCAGAAGTGGCTAGTCTAACCGCAAGGAGGACGGTCACC 1448
DQ904608_Bacillus               --------------------------------------------------
DQ464386_Pseudomonas            --------------------------------------------------
AM161143_Pseudomonas            --------------------------------------------------
PSAC41                          --------------------------------------------------
PSAA1                           --------------------------------------------------
3LHF6                           --------------------------------------------------
1MBT7                           --------------------------------------------------
3LHF5                           --------------------------------------------------
3LHF3                           --------------------------------------------------
4LHF2                           --------------------------------------------------
2MBT4                           --------------------------------------------------
4LHF1                           --------------------------------------------------
3LHF                            --------------------------------------------------
4LHF4                           AAGGCCCCAAAANNCCAAAACCCCNAANGNCNGNTTTTTNGGTTTTTTCC 1040
4LHF3                           AAAGGNCCNATCNTGNCNTTTTCAAAANTTNCCCCCTTTACATTTNAAAA 1111
N2AA52                          --------------------------------------------------
NBPC31                          --------------------------------------------------
N2AA53                          --------------------------------------------------
NBPC33                          --------------------------------------------------


DQ1442127_Actinomycetales_      TCGAAGGTGGGACTGGCGATTGGGACGAAGTCTAACCAAGGTAGCCGA-- 1475
DQ663172_Streptomyces           TCGAAGGTGGGACTGGCGATTGGGACGAAGTCGTAACAAGGTAGCCGTA- 1489
Actc29                          --------------------------------------------------
ActC31                          --------------------------------------------------
AY154378_Burkholderia           ACGGTAGGATTCATGACTGGGGTG-------------------------- 1452
DQ490307_Burkholdericeae        ACGGTAGGATTCATGACTGGGGTGAAGTCGTAACAAGGTAACCGT----- 1493
DQ904608_Bacillus               --------------------------------------------------
DQ464386_Pseudomonas            --------------------------------------------------
AM161143_Pseudomonas            --------------------------------------------------
PSAC41                          --------------------------------------------------
PSAA1                           --------------------------------------------------
3LHF6                           --------------------------------------------------
1MBT7                           --------------------------------------------------
3LHF5                           --------------------------------------------------
3LHF3                           --------------------------------------------------
4LHF2                           --------------------------------------------------
2MBT4                           --------------------------------------------------
4LHF1                           --------------------------------------------------
3LHF                            --------------------------------------------------
4LHF4                           NNNCCCNCNCCCCCTNAAAAAATCCAAAATNNNCCNNANNNNGAAAGGNN 1090
4LHF3                           ATNNNCTTCNNTNAAANGGGGGAAACCCCCNNNTTAAAAA---------- 1151
N2AA52                          --------------------------------------------------
NBPC31                          --------------------------------------------------
N2AA53                          --------------------------------------------------
NBPC33                          --------------------------------------------------


DQ1442127_Actinomycetales_      --------------------------------------------------
DQ663172_Streptomyces           --------------------------------------------------
Actc29                          --------------------------------------------------
ActC31                          --------------------------------------------------
AY154378_Burkholderia           --------------------------------------------------
DQ490307_Burkholdericeae        --------------------------------------------------
DQ904608_Bacillus               --------------------------------------------------
DQ464386_Pseudomonas            --------------------------------------------------
AM161143_Pseudomonas            --------------------------------------------------
PSAC41                          --------------------------------------------------
```

```
PSAA1                             --------------------------------------------------
3LHF6                             --------------------------------------------------
1MBT7                             --------------------------------------------------
3LHF5                             --------------------------------------------------
3LHF3                             --------------------------------------------------
4LHF2                             --------------------------------------------------
2MBT4                             --------------------------------------------------
4LHF1                             --------------------------------------------------
3LHF                              --------------------------------------------------
4LHF4                             GNAAACCNAAGAATTAAAATTTCCCNGGTTCCCCCGANNGCCCNNGNGGT 1140
4LHF3                             --------------------------------------------------
N2AA52                            --------------------------------------------------
NBPC31                            --------------------------------------------------
N2AA53                            --------------------------------------------------
NBPC33                            --------------------------------------------------

DQ1442127_Actinomycetales_        --------------------------------------------------
DQ663172_Streptomyces             --------------------------------------------------
Actc29                            --------------------------------------------------
ActC31                            --------------------------------------------------
AY154378_Burkholderia             --------------------------------------------------
DQ490307_Burkholdericeae          --------------------------------------------------
DQ904608_Bacillus                 --------------------------------------------------
DQ464386_Pseudomonas              --------------------------------------------------
AM161143_Pseudomonas              --------------------------------------------------
PSAC41                            --------------------------------------------------
PSAA1                             --------------------------------------------------
3LHF6                             --------------------------------------------------
1MBT7                             --------------------------------------------------
3LHF5                             --------------------------------------------------
3LHF3                             --------------------------------------------------
4LHF2                             --------------------------------------------------
2MBT4                             --------------------------------------------------
4LHF1                             --------------------------------------------------
3LHF                              --------------------------------------------------
4LHF4                             NCCCTTTNCANNNGGGAAAANGAACTTTNCCCTTTTNCNNNGGAAAGNGG 1190
4LHF3                             --------------------------------------------------
N2AA52                            --------------------------------------------------
NBPC31                            --------------------------------------------------
N2AA53                            --------------------------------------------------
NBPC33                            --------------------------------------------------

DQ1442127_Actinomycetales_        --------------------------------------------------
DQ663172_Streptomyces             --------------------------------------------------
Actc29                            --------------------------------------------------
ActC31                            --------------------------------------------------
AY154378_Burkholderia             --------------------------------------------------
DQ490307_Burkholdericeae          --------------------------------------------------
DQ904608_Bacillus                 --------------------------------------------------
DQ464386_Pseudomonas              --------------------------------------------------
AM161143_Pseudomonas              --------------------------------------------------
PSAC41                            --------------------------------------------------
PSAA1                             --------------------------------------------------
3LHF6                             --------------------------------------------------
1MBT7                             --------------------------------------------------
3LHF5                             --------------------------------------------------
3LHF3                             --------------------------------------------------
4LHF2                             --------------------------------------------------
2MBT4                             --------------------------------------------------
4LHF1                             --------------------------------------------------
3LHF                              --------------------------------------------------
4LHF4                             NTTCCNAANANNNNTNAAANCNNANGGGGNNCCCCCCCAGGGGGNGNNNN 1240
4LHF3                             --------------------------------------------------
N2AA52                            --------------------------------------------------
NBPC31                            --------------------------------------------------
N2AA53                            --------------------------------------------------
NBPC33                            --------------------------------------------------
```

```
DQ1442127_Actinomycetales_      --------------------------------------------------
DQ663172_Streptomyces           --------------------------------------------------
Actc29                          --------------------------------------------------
ActC31                          --------------------------------------------------
AY154378_Burkholderia           --------------------------------------------------
DQ490307_Burkholdericeae        --------------------------------------------------
DQ904608_Bacillus               --------------------------------------------------
DQ464386_Pseudomonas            --------------------------------------------------
AM161143_Pseudomonas            --------------------------------------------------
PSAC41                          --------------------------------------------------
PSAA1                           --------------------------------------------------
3LHF6                           --------------------------------------------------
1MBT7                           --------------------------------------------------
3LHF5                           --------------------------------------------------
3LHF3                           --------------------------------------------------
4LHF2                           --------------------------------------------------
2MBT4                           --------------------------------------------------
4LHF1                           --------------------------------------------------
3LHF                            --------------------------------------------------
4LHF4                           AANNCCCNNNCCCNTTNNTTTTTNTNTTTTNTTTGAAANAAAAAAANTTT 1290
4LHF3                           --------------------------------------------------
N2AA52                          --------------------------------------------------
NBPC31                          --------------------------------------------------
N2AA53                          --------------------------------------------------
NBPC33                          --------------------------------------------------
```

```
DQ1442127_Actinomycetales_      --------------------------
DQ663172_Streptomyces           --------------------------
Actc29                          --------------------------
ActC31                          --------------------------
AY154378_Burkholderia           --------------------------
DQ490307_Burkholdericeae        --------------------------
DQ904608_Bacillus               --------------------------
DQ464386_Pseudomonas            --------------------------
AM161143_Pseudomonas            --------------------------
PSAC41                          --------------------------
PSAA1                           --------------------------
3LHF6                           --------------------------
1MBT7                           --------------------------
3LHF5                           --------------------------
3LHF3                           --------------------------
4LHF2                           --------------------------
2MBT4                           --------------------------
4LHF1                           --------------------------
3LHF                            --------------------------
4LHF4                           CCCCNAAAAAAAAAAAAAAAAANGGG 1316
4LHF3                           --------------------------
N2AA52                          --------------------------
NBPC31                          --------------------------
N2AA53                          --------------------------
NBPC33                          --------------------------
```

F2. Fungal aligned nucleotide sequences

>A1 *Ampelomyces* sp.

AGNNANAGGTTTCAGTAGGNGANCTGCGGAAGGATCATTACCTAGGAGTT
GTAGGCNTTGCCTGCTATCTCTTACCCATGGTCTTTTGAGTACCTTACGT
TTCCTCGGTGGGTTCGCCCACCGATTGGACAAACTTAAACCCTTTGCAGT
TGAAATCAGCGTCTGAAAAAACTTAATANTTACNACTTTCAACAACGGAT
CTCTTGGNTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTG
TGAATTGCAGAATTCAGTGAATCATCNAATCTTTGANCGCACATTGCGCC

CCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAA
GCTCTGCTTGGTGTTGGGTGTNTGTCTCCTGTANACTCNCCTTAAAACAA
TTGGCAGCCGGCGTATNGATTTCGGAGCGCAGTACATCTCGCGCTTTGCA
CTCATAACGACGACATCCAANAGTACATTTTTACACTCTTGACCTCNGAT
CAGGTAGGGATACCCGCTGANCTTAGNCATACAAGNAAAANCGGAANGAA

>A4 *Exserohilium rostratum*

GNGNCGGNTCTCAGCNAGGAGNACTGCGGAGGGATCATTACACAACAAAA
ATGTNNAGGGGTGTGGTNNGCTGGCNACAGCCGNCCGCCCCAAGTATTTN
TCACCCGTGTCNTTTGCGCACTTTGTGTTTACCTGGGCNAGTTCNCTCGC
CNCCANGACCNANCCATAAANCTTTTTTNATGCNGNNGCNATCAGCGTNA
NTANAATAATTNAANTTATNAAAACTTTNNACNACGGATCTCTTGGCTCT
GGCANCNATGAAGAACNNAGNNAAATGCGATACATAGNGTNAATTGCAGA
ATNCANTGAANCATCAAATCTTTGANCGCNCATTGCGCCCTTTGNTATTC
CNAAGGGCATGCCTGNTCTAGCNCCNTNTGTACCCNCAAGCTGTGNNTGG
TGTTGGGCGTCNTNNNNTCNCTCCCCTAGANGNGGAGACTCGCCTTNNA
ACNATNGNNACCNGANCTACTGGTTTTCGGAGCNNATNACACATTTGCGC
CTTCCANTCCACGGGGCGNNATCNANNATGNCTNTGTTNTCTACANCNTA
TCCACATTTNGACCNCAGATCAAGCAGGNATACCCGCTGANCTNAGGCAT
ATAGGAAAACCGAAGAAA

>A52 *Trichoderma harzianum*

NGGGANGGTCTCCGGAGGAGAACAGCGGAGGGATCATTACCGAGTTTACA
ACNCCCAAACCCAATGATGAACGTTACCAAACTGTTGCCTCGGTTGTCCN
TCTCTGCCCCGGGTGCGTCGCAGCCCCGGACCAAGGCGACCCGCCGGAGG
ACCAACCAAAACTCTTTTTGTATACCCCCTCGCGGGTTTTTTTATAATCT
GAGCCTTCTCGGCGCCTCTCGTAGGCGTTTCGAAAATGAATCAAAACTTT
CAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
NATAAGTANTGTGAATTGCAGAATTCAGTGAATCATCNAATCTTTGAACG
CACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATT
TCAACCCTCGAACCCCTCCGGGGGGTCGGCGTTGGGGATCGGCCCTCCCT
TAGCGGGTGGNCGTCTCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTC
NTGCGNAGTAGTTTGCACACTCGCATCGGGAGCGCGGCGCGTCCACAGNC
GTTAAACACCCAACTTCTGAAATGTTGACCTCGGATCAGGTAGGAATACC
CGCTGANCTTAAGCATATCATNAAAGCGGAAGAAA

>B1 *Fusarium oxysporum*

GTTGGNGAANGGCGGNGGGANCATTACCGAGTTTACAACNCCGANACCCC
TGNGAACATACCACTTGTTGCCTCGGCGGATCNNCNCGCTCCCGGTAAAA
CGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTATATGTAACTTC
TGAGTAAAACCATAAATANATCAAAACTTTCAACAACGGATCTCTTGGTT
CTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTANTGTGANTTGCA
GAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCNCCAGTAT
TCTGGCGGGCATGCCTGTTCNAGCGTCATTTCAACCCTCNAGCACAGCTT
GGTGTTGGGACTCGCGTTAATTCGCGTTCCTCNAATTGATTGGCGGTCAC
NTCGAGCTTCCATAGCGTAGNANTAAAACCCTCGTTACTGGTAATCGTCG
CGGCCACNCCGTTAAACCCCANCTTCTGAATGTTGACCTCNGATCAAGTA
GGAATACCCGCTGANCTTAAGCATATCAGNAAAGCGGGAGGAAA

>C31 *Ampelomyces* sp.

ANNNNNNNNNNNNNNNNNGNGACCNGCGGAAGGATCATTACCTAGAGTTG

TANNCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTACGTTT
CCTCGGTGGGTTCGCCCACCGATTGGACAAACTTAAACCCTTTGCAGTTG
AAATCAGCGTCTGAAAAAACTTAATAGTTACNACTTTCAACAACGGATCT
CTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTG
AATTGCAGAATTCAGTGAATCATCNAATCTTTGAACGCACATTGCGCCCC
TTGGTATTCCNTGGGGCATGCCTGNTCGAGCGTCATTTGTACCTTCAAGC
TCTGCTTGGTGTTGGGTGTTTGTCTCCTGTAGACTCGCCTTAAAACAATT
GGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACT
CATAACGACGACATCCAAAAGTACATTTTTACACTCTTGACCTCNGATCA
GGTAGGGATACCCGCTGANCTTAGNCATACANNAAAAGGGGNAGAAAA

>C51 *Fusarium* sp.

CGGGGGGAGCTATCANAGGNGGAGGAACNGCGGAGGGATCATTACCGAGT
TTACAANNNCGAAACCCCNGAAGAAACATACCTATACGTTGCCTCGGCGG
TTCAGCNCGCGCCCCGNAAAAAGGGACGGCCCGCCCGAGGACCCCTAAAC
TCTGTTTTTAGTGGAACTTCTGAGTAAAACAAACANATAANTCAAAACTT
TCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATG
CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCNAATCTTTGAAC
GCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCAT
TTCAACCCTCAAGCTCAGCTTGGTGTTGGGACTCGCGGTAACCCGCGTTC
CCCAAATCGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAATCATAC
ACCTCGTTACTGGTAATCGTCGCGGNCACGCCGTAAAACCCCAACTTCTG
AATGTTGACCTCNGATCATTGTANGAATACCCGCTGANCTTAAGCATATC
AAGAAGCGGAGGAA

>D11 *Ampelomyces* sp.

ANNGTAAGGTTTCNGTAGGNGAACTGCGGAAGGATCATTACCTAGAGTTG
TACNGCTTTGCCTGCTATCTCTTACCCATGGTCTTTTGAGTACCTTACGT
TTCCTCGGTGGGTTCGCCCACCGATTGGACAAACTTAAACCCTTTGCAGT
TGAAATCAGCGTCTGAAAAAACTTAATANTTACNACTTTCAACAACGGAT
CTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTG
TGAATTGCAGAATTCAGTGAATCATCNAATCTTTGAACGCACATTGCGCC
CCTTGGTATTCCNTGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAA
GCTCTGCTTGGTGTTGGGTGTNTGTCTCCTGTAGACTCGCCTTAAAACAA
TTGGCAGCCGGNGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCA
CTCATAACGACGACATCCAAAAGTACATTTTTACACTCTTGACCTCNGAT
CAGGTAGGGATACCCGCTGANCTTAAGCCATATCANNAAAAGCGGAAGNA
AA

>D31 *Fusarium* sp.

GGGGGAGCTATCAGTAGGAGGGAANGAACNGCGGNGGGATCATTACCGAG
TTTACAANCNCCNAAACCCNGGAAAAACATACCTATACGTTGCCTCGGCG
GTTCNGCNCGCGCCNNGAAAAAAGGGACGGCCCGCCCGAGGACCCCTAAA
CTCTGTTTTTAGTGGAACTTCTGAGTAAAACAAACANNTAANTCAAAACT
TTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAAT
GCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATNGAATCTTTGAA
CGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCA
TTTCAACCCTCAAGCTCAGCTTGGTGTTGGGACTCGCGGTAACCCGCGTT
CCCCAAATCGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAATCATA
CACCTCGTTACTGGTAATCGTCGCGGCCACGCCGTAAAACCCCAACTTCT

```
GAATGTTGACCTCGGATCNTGTNTNNTTACCCGCTGANCTTAAGCATATC
AATAAGCGGAGGAA
```

>D53 *Fusarium* sp.

```
AGGGNAGGGTCTCGTTGGAGNACAGCGGAGGGATCATTACCGAGTTTACA
ACTCCCAAACCCCTGTGAACATACCACTTGNTTGCCTCGGCGGATCNNCC
CGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTT
CTATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAA
CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAG
TAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATT
GCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACC
CTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTCGCGTTCCTCAAAT
TGATTGGCGGTCACNTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGTT
ACTGGTAATCGTCGCGGCCACGCCGTTAAACCCCAACTTCTGAATGTTGA
CCTCNGATCAGGTAGGAATACCCGCTGANCTTAAGCATATCATNAAAGCG
NAAAGAA
```

APPENDIX G

Roots clearing and staining solutions (Smith and Dickson, 1997).

G1. 50% ethanol

1000ml ethanol

1000ml distilled water

G2. 5% KOH

100g KOH

2L distilled water

G3. Alkaline Peroxide H_2O_2

3ml NH_4OH (Ammonia)

30ml 10% H_2O_2

567ml distilled water

G4. 0.1M HCl (32% MW36.46)

22.79ml HCl

2L Distilld water

G.5 Lactoglycerol trypan blue stain

Lactic acid: Glycerol: Water (13:12:16)

520ml lactic acid

480ml Glycerol

640ml distilled water

0.82g Trypan blue

G6. Lactoglycerol Destain

Lactic acid: Glycerol: Water (13:12:16)

520ml lactic acid

480ml Glycerol

640ml distilled water

APPENDIX H

Bradford standards and Standard curve (Bradford, 1979).

H1. BSA stock solution/standards

Step 1: Protein stock solution was prepared by dissolving 16mg of Bovine Serum Albumin (BSA) in 10ml double distilled water.

Standards were prepared to a final volume of 160µl in micro-centrifuge tubes as follows:

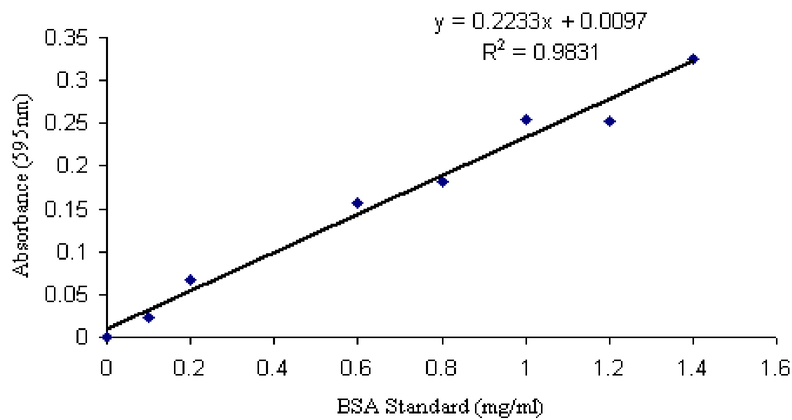
Standard (mg/ml)	Vol. BSA stock	Vol. ddH_2O
0.1	10	150
0.2	20	140
0.4	40	120
0.6	60	100
0.8	80	80
1.0	100	60

1.2	120	40
1.4	140	20
1.6	160	0

Step 2: Using a 96 well titre plates, 5µl of standards, blanks and samples were aliquoted into the wells with replicates because of pipetting errors.

Step 3: Bradford's reagent (250µl) were added to each well and left at room temperature for 15 mins.

H2. Standard curve



APPENDIX I

Solutions and Buffers

I1. TE (Tris/EDTA) buffer pH 8.0

Tris/HCl pH 8.0 10mM

EDTA pH 8.0 10mM

I2. 30% PEG/ 1.6M NaCl
30g PEG
9.369 NaCl
100ml distilled water

I3. 10% SDS (sodium dodecyl sulphate)
10g SDS
100ml distilled water
Warm to 65°C to allow SDS to dissolve.