

https://dspace.org/

Arbuscular mycorrhiza and soil microbial interactions in sugarcane agriculture in KwaZuluNatal, South Africa

Jamal-Ally, Sumaiya Faizal

2013-07-30
http://hdl.handle.net/10539/12930

Downloaded from a DSpace repository. https://dspace.org/



ARBUSCULAR MYCORRHIZA AND SOIL MICROBIAL INTERACTIONS IN SUGARCANE AGRICULTURE IN KWAZULU–NATAL, SOUTH AFRICA

SUMAIYA FAIZAL JAMAL–ALLY

2013

PHD THESIS

FACULTY OF SCIENCE

ARBUSCULAR MYCORRHIZA AND SOIL MICROBIAL INTERACTIONS IN SUGARCANE AGRICULTURE IN KWAZULU–NATAL, SOUTH AFRICA

SUMAIYA FAIZAL JAMAL-ALLY

A Thesis, submitted to the Faculty of Science, University of the Witwatersrand, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, 2013

In the Name of God Most Gracious, Most Beneficent:

I declare that this Thesis is my own, unaided work.

The information used in my Thesis, has been obtained whilst working as a PhD student under the aegis of the South African Sugarcane Research Institute, Mount Edgecombe, and was subsequently completed (p/t) whilst employed as a lecturer in Microbiology, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, South Africa.

This material was financially supported by the National Research Foundation, Thuthuka (RiT) - any opinion, findings and conclusions or recommendations expressed in this material are those of the author(s) and therefore the NRF does not accept any liability in regard thereto.

My Thesis is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



SUMAIYA FAIZAL JAMAL-ALLY

Signed on this 30 day of April 2013 in Pietermaritzburg, KwaZulu-Natal.

ABSTRACT

A novel holistic approach was used to study the mycotrophic nature of commercial sugarcane varieties grown in KwaZulu-Natal, South Africa. All five varieties were mycotrophic, but N12 had the highest overall mycorrhization and was selected for a pot study to assess the growth response of sugarcane to inoculation with indigenous arbuscular mycorrhizal (AM) fungi and microflora. The pot study suggested that sugarcane will respond positively to inoculation with AM fungi, but the effects are most clear in the early phase of growth and less obvious in later elongation phases. This observation, taken together with the ability of sugarcane to grow well in sterile soil without microflora additions suggests that the plant may be facultatively mycotrophic. A multivariate analysis determined the nutrient relationships between soil and corresponding leaf nutrient levels on 72 sugarcane field plants, categorised according to either high or low percentage colonisation. Highly colonised plants were found to have more positive nutrient correlations compared to lower percentage colonised plants. AM fungi were identified from spore morphology and associated mycorrhizal bacteria (AMB) were identified by 16s rDNA analysis. Partial molecular identification was conducted using a universal eukaryotic forward NS31 primer and general fungal AM1 primers confirming the spores to be of AM fungi origin. A nested PCR was performed, using the universal fungal primers, NS5 and ITS4, followed by primer combinations to target sequences of specific Glomalean groups. Only partial molecular identification was conducted, as RFLPs were not successfully optimised. DNA from the *Acaulospora gerdemannii/Acaulospora trappei* group, *Glomus occultum/Glomus brasilianum* group, *Glomus mosseae/Glomus intraradices* group, *Glomus etunicatum/ Glomus clariodeum* group and *Acaulosporaceae sensu stricto* were detected, indicating AM fungi diversity. Bacteria, *Brevibacillus reuszeri* isolated from *Scutellospora nigra*, *Bacillus megaterium* and *Stenotrophomonas maltophilia* isolated from *Glomus geosporum*, *Paenibacillus chitinolyticus* and *Bacillus cereus* isolated from *Acaulospora mellea* and *Gigaspora margarita* spores respectively, were tested

for biocontrol capability against pathogenic nematodes of *Paratrichodorus, Meloidogyne* and *Pratylenchus* genera. *Meloidogyne* was the least susceptible to associated mycorrhizal bacteria biocontrol and *Paratrichodorus* the most susceptible. These studies have contributed to understanding the role of AM in sugarcane agriculture in South Africa.

Keywords: arbuscular mycorrhiza, sugarcane varieties, multivariate analysis, nutrient relationships, associated mycorrhizal bacteria, biocontrol, nematodes

I LOVINGLY DEDICATE MY THESIS TO

My Creator, for making all things possible;

My wonderful husband Hassen Ally, for his eternal love and encouragement;

My beautiful son Muhammad-Suhayl, for his little fingers “helping mom type”,

My amazing parents Rasheeda and Faizal Jamal, for your continuous love and dedication to our family;

My sister Naasiha Jamal, my brother Yacoob Jamal and sister-in-law Nazeefa Jamal, for your continuous love and support.

ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisor Prof CJ Straker (Wits) for patience and guidance throughout this study and especially encouraging me to continue with my PhD even after my paralysis.

I sincerely thank my co-supervisor Dr RS Rutherford (SASRI) for support and advice throughout this study.

I sincerely thank Mufti Ismail Ibn Musa Menk, and Qaris Haroon Taylor, for spiritual guidance, wisdom and oceans of knowledge.

Sincere thanks to the National Research Foundation (Thuthuka – RiT) for financially supporting this material.

Special thanks to:

- Dr Sackey Yobo (UKZN), for help and advice with using the SAS programmes.
- Dr Patrice Cadet (SASRI/CIRAD), for advice during the field trials and the ADE-4 statistical analysis.
- Dr Shaun Berry (SASRI), for providing the nematodes and technical staff during sample collection and processing.
- Prof Mark Laing, for being an inspiration and a wonderful mentor.
- Dr Unathi Kolanisi, for her moral support.
- Jayson Kuppusamy from Merck, for invaluable advice.
- My colleagues and students, for support and encouragement.
- SASRI and the University of KwaZulu-Natal, for granting me the opportunity and facilities to complete my PhD.

RESEARCH OUTPUTS

1. Original publication submitted to Journal of Plant Nutrition

Title: Multivariate Analysis of Plant-Soil Nutrient Relationships in Arbuscular Mycorrhizal Sugarcane Grown in KwaZulu-Natal, South Africa

Authors: Sumaiya F. Jamal-Ally[ab*#], Stuart Rutherford[b], Colin J. Straker[a]

[a] Mycology Research Laboratory, School of Molecular and Cell Biology, University of the Witwatersrand, Private Bag 3, Wits 2050, Johannesburg, South Africa

[b] Crop Protection, South African Sugar Research Institute, Private Bag X02, Mount Edgecombe 4300, South Africa

*Corresponding author: Sumaiya F Jamal-Ally

[#]Present address: Microbiology, School of Life Sciences, University of KwaZulu-Natal, Private Bag X01, Scottville, Pietermaritzburg 3209, South Africa

Email: jamal@ukzn.ac.za ; Telephone: +27 829737496;
Facsimile: +27 33 2606209

2. Conference poster presentation

Sumaiya F. Jamal-Ally; Stuart Rutherford; Colin J. Straker. "Arbuscular Mycorrhizas and Soil Microbial Interactions in Sugarcane Agriculture in KwaZulu-Natal, South Africa". The 7th International Conference on Mycorrhiza (ICOM 7), The Energy and Resources Institute; India Habitat Centre, New Delhi, 6 to 11 January 2013.

3. Conference oral presentation

Jamal, S. F.; Cadet, P.; Rutherford, R. S.; Straker, C. J. "Effect of mycorrhiza on the nutrient uptake of sugarcane". Proceedings of the 78th Annual Congress of South African Sugar Technologists' Association, held at Kwa-Shukela, Mount Edgecombe, South Africa, 27-30 July 2004. pp 343-347. (paper)

4. Seminars

- Sumaiya F. Jamal-Ally. "Arbuscular Mycorrhizas: An Under Earth Revolution." School of Life Sciences Seminar Series, held at the University of KwaZulu-Natal, Agricultural Campus, Pietermaritzburg, South Africa, 24 August 2012.

- Sumaiya F. Jamal- Ally. "Enhanced Uptake of Nutrients In Arbuscular Mycorrhizal Sugarcane Grown in the North Coast Region of Kwazulu-Natal". School of Biochemistry, Genetics and Microbiology Research Day, held at the University of KwaZulu-Natal, Agricultural Campus, Pietermaritzburg, South Africa, 14 June 2011.

TABLE OF CONTENTS

DECLARATION.....ii

ABSTRACT III

ACKNOWLEDGEMENTS VI

RESEARCH OUTPUTS.....vii

LIST OF FIGURES XIII

LIST OF TABLES XVII

LIST OF SYMBOLS.....XX

CHAPTER 1

GENERAL INTRODUCTION.....1

1.1 RATIONALE AND AIMS 2

LITERATURE REVIEW

1.2 THE IMPORTANCE OF SUGARCANE 4

1.3 SUGARCANE DISEASES AND PESTS 6

1.3.1 Sugarcane Fungal Pathogens 7

1.3.2 Sugarcane Bacterial Pathogens.....8

1.3.3 Nematodes 9

1.4. MYCORRHIZAL FUNGI 12

1.4.1 Arbuscular Mycorrhizal (AM) Fungi 14

1.4.2 Arbuscular mycorrhizal sugarcane 16

1.4.3 Nutrient uptake in arbuscular mycorrhizal fungal symbiosis 17

1.5 BACTERIAL SYMBIONTS 23

1.5.1 Bacteria associated with nitrogen nutrition 23

1.5.2 Mycorrhiza associated bacteria 24

CHAPTER 2

ARBUSCULAR MYCORRHIZA STATUS IN COMMERCIAL VARIETIES OF SUGARCANE IN P-FIXING AND NON P-FIXING SOIL REGIONS OF KWAZULU-NATAL AND THE GROWTH RESPONSE OF SUGARCANE IN THE GLASSHOUSE TO INOCULATION WITH INDIGENOUS ARBUSCULAR MYCORRHIZAL FUNGI

2.1 INTRODUCTION 26

2.2 MATERIALS AND METHODS 30

2.2.1 Sampling commercial varieties from field trials to determine percentage mycorrhization. 30

2.2.2 AM fungi root assessment - Mycorrhization 30

2.2.3 AM fungi spore extraction 31

2.2.4 Experimental design of the glasshouse pot trial 31

2.2.5 Statistical Analysis 33

2.3 RESULTS 33

2.3.1 Field variety trials 33

2.3.2 Glasshouse pot trial 35

2.4 DISCUSSION 38

2.5 CONCLUSION 41

CHAPTER 3

MULTIVARIATE ANALYSIS OF PLANT-SOIL NUTRIENT RELATIONSHIPS IN ARBUSCULAR MYCORRHIZAL SUGARCANE GROWN IN THE NORTH COAST REGION OF KWAZULU-NATAL, SOUTH AFRICA

3.1 INTRODUCTION 42

3.2 MATERIALS AND METHODS 45

3.2.1 Sampling 45

3.2.2 Soil Analysis 45

3.3.3 Leaf Analysis 45

3.3.4 Mycorrhization 45

3.2.6 Multivariate Statistical Analysis 46

3.3 RESULTS 47

3.4 DISCUSSION 56

3.5 CONCLUSION 63

CHAPTER 4

MORPHOLOGICAL AND PARTIAL MOLECULAR IDENTIFICATION OF ARBUSCULAR MYCORRHIZAL FUNGI ISOLATED FROM SUGARCANE PLANTATIONS IN KWAZULU-NATAL, SOUTH AFRICA

4.1 INTRODUCTION 65

4.2 MATERIALS AND METHODS 68

4.2.1 Soil types 68

4.2.2 Crude spore extraction from soil 69

4.2.3 Spore treatment and morphotyping 69

4.2.4 Morphological identification of spores 69

4.2.5 Molecular analysis 70

4.3 RESULTS 72

4.3.1 Morphological identification 72

4.3.2 Partial molecular identification 78

4.4 DISCUSSION 83

4.5 CONCLUSION 88

CHAPTER 5

MOLECULAR IDENTIFICATION OF BACTERIA ISOLATED FROM ARBUSCULAR MYCORRHIZAL FUNGAL SPORES ASSOCIATED WITH SUGARCANE AND PRELIMINARY TESTING OF ASSOCIATED MYCORRHIZAL BACTERIA AS BIOCONTROL AGENTS FOR NEMATODES *IN-VITRO*

5.1 INTRODUCTION 89

5.2 MATERIALS AND METHODS 91

5.2.1 Soil sites 91

5.2.2 Spore extraction and identification 92

5.2.3 Arbuscular Mycorrhizal Bacteria isolation and pure cultures 92

5.2.4 DNA Extraction Protocol 92

5.2.5 Agarose Gel Electrophoresis 93

5.2.6 PCR Amplification 93

5.2.7 PCR cycling parameters 93

5.2.8 DNA Purification 94

5.2.9 Cloning and Sequencing 94

5.2.10 Sequence analysis 96

5.2.11 Preliminary study to determine the effect of AMB on nematodes *in-vitro* 96

5.2.12 Statistical Analysis 97

5.3 RESULTS 97

5.3.1. Identification of bacterial isolates 97

5.3.2 Preliminary study to determine the effect of AMB on nematodes *in-vitro* 99

5.4 DISCUSSION 102

5.5 CONCLUSION 106

CHAPTER 6

SUMMARY AND CONCLUSION

SUMMARY 108

6.1 Arbuscular mycorrhiza (AM) status in commercial varieties of sugarcane in P-fixing and non P-fixing soil regions of KwaZulu-Natal and the growth response of sugarcane in the glasshouse to inoculation with indigenous AM fungi 109

6.2 Multivariate analysis of plant-soil nutrient relationships in arbuscular mycorrhizal sugarcane grown in the North Coast region of KwaZulu-Natal, South Africa 111

6.3 Morphological and partial molecular identification of arbuscular mycorrhizal fungi isolated from sugarcane plantations in KwaZulu-Natal, South Africa 113

6.4 Molecular identification of bacteria isolated from AM Ffunglspores associated with sugarcane and preliminary testing of Associated Mycorrhiza Bacteria (AMB) as biocontrol agents for nematodes *in-vitro* 115

CONCLUSION 117

REFERENCES 118

APPENDICES 155

LIST OF FIGURES

Figure 3.1: Convex hulls indicating homogeneity of soil factors for La Mercy 2-2 sampled area. Variables were categorized according to (1) low and (2) high mycorrhizal status. 50

Figure 3.2a-d: Correlation circles (F1xF2) of the PCA for low (L) and high (H) mycorrhization status soil (s) and leaf (l) nutrient variables. Arrows in the same direction indicate positively correlated variables, while arrows in the opposite direction to each other indicate negatively correlated variables. 52

Figure 3.3a-d: Factor maps (F1XF2) for low (L) and high (H) percentage mycorrhization status soil (s) and leaf (l) factors transformed by the co-inertia analysis. 53

Figure 3.4: Co-inertia crossed tables indicating the sum of the cross products between soil and leaf factors for (a) low percentage mycorrhization status and (b) high mycorrhization status. Circles indicate positive or strong associations and squares indicate negative or weak associations. The size of the circles and squares are proportional to the absolute values. 55

Figure 4.1: *Acaulospora mellea,* (a) thin outer layer of spore wall (swl1); laminated inner layers (swl2 and swl3); (b) outer hyaline layer of inner wall one (gwl1); inner hyaline layer of inner wall one (gwl2); (c) second hyaline inner wall (gw2); (d) inner layer of inner wall staining red-purple in Melzer's solution. 73

Figure 4.2: *Gigaspora margarita,* (a) globose white spores; spore is terminal on septate hyphae (sh), suspensor or sporogenous cell (sc); spore walls (sw); (b) outer spore wall (swl1); inner laminate layer of spore wall (swl2) and inner papillate layer (swl3); (c) Suspensor cell wall's outer layer (scwl1) and inner layer (scwl2). 74

Figure 4.3: *Glomus geosporum,* (a) whole spore with subtending hypha (sh); spore wall (sw); (b) hyaline outer spore wall (swl1); red-brown laminated middle wall (swl2); yellow-brown inner spore wall (swl3).....75

Figure 4.4: *Scutellospora nigra*, (a) whole spore with subtending hyphae (10X); (b) crushed spores (20X) and (c) spore wall (40X) in Meltzer's solution indicating double porous outer spore wall (swl1) and inner spore wall (swl2).....76

Figure 4.5: *Scutellospora verrucosa*, (a) spore (s); germination shield (gs); suspensor cell (cs); (b-c) plug (p); subtending hypha (sh); spore wall (sw) constituting an outer wall (swl1) and an inner wall (swl2).....77

Figure 4.6: Agarose gel confirming all spores to be arbuscular mycorrhizal. Primers AM1 and NS31 amplified 550 bp fragments of genomic DNA. Spores were typed according to colours: Orange (O), Black (B), Brown (Br) and Red (R).....78

Figure 4.7: Agarose gel representing amplified ±270 bp DNA fragment from second round PCR of Brown spore DNA using primers ARCH 1311 and ITS4.....79

Figure 4.8: Agarose gel representing amplified 800 bp and 1000 bp DNA fragments from second round PCR of Orange, Black, Brown and Red

spore DNA using primers GLOM1310 and ITS4. Bands above these were 1200 bp from the first round PCR. 80

Figure 4.9: Agarose gel representing amplified ±400 bp DNA fragment from second round PCR of Orange, Black, Brown and Red spore DNA using Primers LECT1670 and ITS4. Additionally Red spores indicated the presence of 100 bp and 800 bp bands. 81

Figure 4.10: Agarose gel representing amplified ±400 bp DNA fragment from second round PCR of Orange, Black, Brown and Red spore DNA using Primers ACAU1660 and ITS4. 82

Figure 5.1: Tryptone soy agar plates indicating (a) contaminating fungi around spores washed only with Millipore sterilized water (b) no fungal contamination around surface sterilized spores treated with EDTA after a 48 h incubation at 30 °C. 98

Figure 5.2: Examples of pure bacterial colonies sub-cultured from plates containing surface sterilized AM fungal spores. 98

LIST OF TABLES

Table 2.1: Mean percentage mycorrhization of individual commercial sugarcane varieties compared between Zinkwazi II and Klipp III soil types. Means indicated with the same letters were not significantly (ns) different (P> 0.05) and s = significantly different ($P \leq 0.05$).....34

Table 2.2: Mean percentage mycorrhization compared (n=6) across five varieties grown at Zinkwazi II and Klipp III respectively. Means indicated with the same letter were not significantly (ns) different ($P > 0.05$) and s = significantly different ($P \leq 0.05$).....35

Table 2.3: LSD analysis of mean stalk length (cm) per treatment (n=10) per month for N12 sugarcane grown under three different treatments in P-fixing soil (Klipp III) and non P-fixing soil (La Mercy 2-2). LSD analysis of mean biomass (g) of stems and leaves per treatment (n=10) harvested after 7 months. Treatments indicated with the same letter were not significantly different (ns) ($P > 0.05$) and s = significantly different ($P \leq 0.05$). 37

Table 3.1: Optimum nutrient levels required for good sucrose yields (adapted from Myer *et al.*, 2011).....48

Table 3.2: Physico-chemical soil and leaf mean nutrient levels for sugarcane N12 variety grown at La-Mercy 2-2, North coast region. 49

Table 3.3: Highlighted nutrient relationships for (a) low and (b) high percentage mycorrhization status crossed tables. 56

Table 4.1: Reclassification of AM fungi, based on taxonomy and molecular phylogeny (Schüßler and Walker, 2010).....66

Table 4.2: AM species diversity found in sugarcane fields of South India and Iran. 67

Table 4.3: PCR Primer sequences and annealing temperatures. 71

Table 5.1: BLAST description of sequences and accession numbers for Associated Mycorrhizal Bacteria. 99

Table 5.2: Three-way ANOVA for nematode mortality after AMB treatments. Values represent transfomed (arcsine) percentage mortality. Nematodes were monitored at 1, 2 and 3 hour intervals to determine effectivity of the treatments. Treatments indicated with the same letter were not significantly different ($P \geq 0.05$). 101

Table A - 1: Recipe for Melzer's solution (adapted from INVAM, 2012)… 155

Table A - 2: Recipe for Polyvinyl-Lacto-Glycerol (PVLG) (adapted from: INVAM, 2012).....155

Table A - 3: Guideline for PCR amplification of DNA for partial molecular analysis of AM fungal spores associated with sugarcane (adapted from: http://www.promega.com/~/media/files/resources/protocols) 156

Table A - 4: Guideline for PCR amplification of AMB DNA isolated from AM fungal spores associated with sugarcane (adapted from: http://www.promega.com/~/media/files/resources/protocols).. 156

Table B - 1: Isolate 4 forward sequences producing significant alignments. 157

Table B - 2: Isolate 4 reverse sequences producing significant alignments. 161

Table B - 3: Isolate 5 forward sequences producing significant alignments. 164

Table B - 4: Isolate 5 reverse sequences producing significant alignments. 166

Table B - 5: Isolate 6 forward sequences producing significant alignments. 168

Table B - 6: Isolate 6 reverse sequences producing significant alignments. 171

Table B - 7: Isolate 7 forward sequences producing significant alignments. 174

Table B - 8: Isolate 7 reverse sequences producing significant alignments. 176

Table B - 9: Isolate 8 forward sequences producing significant alignments. 178

Table B - 10: Isolate 8 reverse sequences producing significant alignments. 180

LIST OF SYMBOLS

ADE-4	Analysis of Environmental Data software
AM	arbuscular mycorrhiza
AMB	associated mycorrhizal bacteria
ANOVA	analysis of variance
BLAST	basic local alignment search tool
bp	base pairs
C	carbon
°C	degrees centigrade
cm	centimetre
cm^3	cubic centimetres
CTAB	cetyl trimethyl ammonium bromide
$dS.m^{-1}$	deciSiemens per metre
dH_2O	distilled and deionized water
DNA	deoxyribose nucleic acid
dNTP	deoxyribose nucleotide triphosphate
°E	degree East
EC_e	Electrical conductivity if the extract
e.g.	for example
EDTA	ethylene diamine tetra acetic acid
et al.	and others
FAS	fertiliser advisory service
g	gram
h	hour
i.e.	that is

ITS	internal transcribed spacer
kg	kilograms
kPa	kilopascal
L	litre
LB	Luria Bertani
m	metre
m^2	square metre
mg	milligram
$mg.m^{-3}$	milligrams per cubic meter
$mg.kg^{-1}$	milligrams per kilogram = ppm (parts per million)
$mg.mL^{-1}$	milligram per millilitre
mM	millimolar
$mm.m^{-1}$	millimetre per meter
Min(s)	minute(s)
N	nitrogen
nm	nanometer
NCBI	National Centre for Biotechnology Information
P	phosphorus
PCA	principal component analysis
PCR	polymerase chain reaction
PGPR	plant growth promoting bacteria
RFLP	restriction fragment length polymorphism
Pi	inorganic P
PVLG	polyvinylalcohol lacto glycerol
RNA	ribose nucleic acid

rDNA	ribosomal deoxyribonucleic acid
rpm	rotation per min
rRNA	ribosomal ribonucleic acid
°S	degree South
SD	standard deviation
SE	standard error
sec	seconds
sp.	species (singular)
spp.	species (plural)
SSU	small subunit
TBE	tris-boric-EDTA
TSA	tryptone soy agar
µ	micron (10^{-6})
µL	microlitre
$\mu L.mL^{-1}$	microlitre per millilitre
$U.\mu L^{-1}$	units per microliter
v.i.z.	namely
V	volts
w/v	weight per volume

CHAPTER 1

GENERAL INTRODUCTION

"The South African sugar industry is one of the world's leading cost competitive producers of high quality sugar and makes an important contribution to employment, particularly in rural areas, to sustainable development and to the national economy. The industry produces an estimated average of 2.2 million tons of sugar per season. About 60% of this sugar is marketed in the Southern African Customs Union (SACU). The remainder is exported to markets in Africa, Asia and the Middle East" (SASA, 2012).

South African sugarcane land currently constitutes 428,000 hectares extending between the latitudes of 25°S - 31°S, mostly within the eastern province of KwaZulu-Natal (Moore and Hudson, 2007). Sixty eight percent of the cane is grown within 30 km of the coast, 17% in the high rainfall area of the KwaZulu-Natal midlands and 15% is grown in the northern irrigated areas of the country (O'Reilly, 1998). On average 2.5 million tons of sugar per season is produced from sugarcane harvested from these lands (Moore and Hudson, 2007).

Approximately 45,300 registered cane growers exist in South Africa, of which 43,500 are small-scale growers with plots of no more than 10 ha and account for only 11% of the total sugarcane (Snyman *et al.,* 2008) and of these 29 130 registered cane growers are based in KwaZulu-Natal province (SASA, 2012). Their success or failure determines whether they have enough to eat. These growers face many challenges: their soil is often degraded from overuse; they lack quality seed, fertilizer and irrigation while diseases, pests and drought threaten their crops. Women do up to eighty percent of the work on farms. They are responsible for both producing the food and preparing it for their families. When small-scale growers obtain more out of their land and labour, their families eat better, earn more money, and lead healthier lives. It is possible to reduce hunger and poverty on a large scale by focusing on the environment and including the needs of small-scale growers.

1.1 RATIONALE AND AIMS

Sugarcane ecosystems are no different from other agroecosystems world-wide. Sugarcane varieties are ravaged by insect pests and diseases that lead to severe crop losses because of the continuous impairment to plant physiology. However, these pests and disease may be subject to a degree of biocontrol (Boland and Kuykendall, 1998).

Sustainable farming systems rely in part on recycling nutrients through materials such as crop residues, manure and biological inputs. Root systems range enormously in their morphology, longevity, activity and composition, because of both environmental and species differences but their function is generally to supply the plant with water and mineral nutrients and to anchor it to the soil. The systems are usually associated with a mycorrhizosphere that contains a dense distribution of microbes compared to soil without roots present. The microbial biomass in the soil is the driving force of most terrestrial ecosystems because it controls the rates of turnover and mineralisation of organic substrates. Bacteria, fungi and nematodes are important microbes to consider when studying plant disease and prevention, as they are adapted to life in the soil (Killham, 1994).

Developing and breeding improved varieties for South African sugarcane industry, is one of the core functions of the South African Sugarcane Research Institute (SASRI) (Parfitt, 2005). Like other global breeding programmes, focus is placed on improved sugarcane yields, sugar content, ratooning ability, maintaining or improving disease resistance, and maintaining acceptable fibre levels for milling. While global crises in agricultural production, resulted in the development of new high yield cultivars, which showed a good response to fertilizer application, more uniform productivity and shorter maturation times; high energy costs of fertilizer production, diminishing availability of rock phosphate and natural resources as well as environmental issues increased contamination of aquatic systems by agrochemicals and greenhouse gas

emissions has prompted SASRI to move toward more sustainable farming systems at the same time taking into account the needs of small-scale growers.

Therefore the long term goals at SASRI for sugarcane crop protection and sustainability includes the reduction in the use of agrochemicals and increased use of beneficial microbiological systems. Research contributing to the use of beneficial microbiological systems in enhancing availability of nutrients already present in the soil and the effects of this synergism between these beneficial microorganisms and sugarcane is therefore important. Since the use of arbuscular mycorrhizal (AM) fungi together with other soil microbes in sugarcane agriculture in South Africa is not fully understood this research was aimed at:

- Assessing the natural mycorrhization status of field-grown AM sugarcane varieties grown in both P-fixing and non P-fixing soils as well as assessing the most mycotrophic of these varieties for growth (stalk elongation) enhancement/suppression by indigenous AM fungi with and without soil microbiota under glasshouse conditions (see Chapter 2).

- Investigating the soil-plant nutrient interactions in mycotrophic sugarcane under field conditions (see Chapter 3).

- Identifying indigenous AM fungi associated with sugarcane in KwaZulu-Natal, South Africa (see Chapter 4).

- Molecularly identifying associated mycorrhizal bacteria (AMB) and testing their "biocontrol" capability against sugarcane parasitic nematodes in a preliminary study (see Chapter 5).

In this introductory chapter the background knowledge of the sugarcane agrosystem including soil-borne pests and diseases as well as beneficial microorganisms - arbuscular mycorrhizal fungi, and associated mycorrhizal bacteria are reviewed. Subsequently the four experimental chapters are presented as papers, followed by a concluding chapter.

1.2 THE IMPORTANCE OF SUGARCANE

Sugarcane is the world's most efficient collector of solar energy, storing this energy in a huge quantity of biomass in the form of sugar and fibre. The sugarcane crop is of great importance to the agricultural sector of South Africa and in the general economy of many other tropical developing countries. While being a very important source of foreign exchange for our economy, it also provides employment for our people in all parts of the sugar industry from the fields to processing in the mills and factories. The earliest accounts of sugarcane cultivation in South Africa were in 1635, when Portuguese explorers, shipwrecked near the mouth of the Umzimkulu River, KwaZulu-Natal province, recorded sugarcane to be one of the crops grown by the local inhabitants (Moore and Hudson, 2007).

Sugarcane plants are tall tropical perennial grasses, classified in the division Magnoliophyta, class: Liliopsida, order: Poales, family: Poaceae, sub-family: Andropogoneae and genus: *Saccharum* (Barnes, 1974). Six species occur in two different forms, wild species being *Saccharum spontaneum* and *Saccharum robustum* and cultivated species being *S. barberi, S. sinense, S. edule* and *S. officinarum* (Sreenivasan, 1987; Irvine, 1999). Cane breeding by plant breeders was originally restricted to *S. officinarum. Saccharum officinarum* L. the so-called 'noble' sweet canes, were believed to be derived from Sac*charum robustum* Brandes & Jeswiet (ex Grassl) (2n=80) through chance mutations which were preserved by the island natives of The New Guinea and associated islands of the Malayan archipelago (Warner, 1962). *S. robustum* was proposed to be a natural hybrid species between *Saccharum spontaneum* L. and *Miscanthus floridulus* (Labill) (Daniels, 1973). The history of *Saccharum spp*. hybrids started about 100 years ago in Java in response to the need for resistance to sereh disease. *S. spontaneum* clone, Glagah (2n=112), was used in an interspecific hybridisation with a noble cane. A selected hybrid was backcrossed to noble types and the

process was repeated until in 1921 and the first 'supercane', POJ2878, was produced (Simmonds, 1976).

Interspecific hybridisation was later repeated in India where a local *S. spontaneum* clone Coimbatore (2n=64), was hybridised with *S. officinarum* clone Vellai (= Bourbon/Lahaina/ (Otaheite) producing Co205, a hybrid more suited to the drier subtropical conditions of India. The initial breeding programs of Java and India utilized only thirteen to fifteen clones of *S. officinarum, Saccharum barberi* Jesw. and S*accharum sinense* Roxb. *(S. sinense* and *S. barberi* clones being natural hybrids of *S. officinarum* and *S. spontaneum* originating in north-east India and China) (Naidu and Sreenivasan, 1987).

The propagation of sugarcane is usually vegetative and initiated by planting setts or seedcane (pieces of stalk planted as single to three-budded setts) in a furrow. Seedlings produced by specialist nurseries and tissue culture (plantlets) are also used as these provide disease free seedcane to new projects or for quick multiplication of elite cultivars with specific advantages. Sett germination varies with seasons, cultivars and treatments. Some cultivars develop roots first while in others shoot development occurs first (Meyer *et al.,* 2011).

The root system is fibrous and shallow, with buttress roots developing to anchor the plant (Moore and Nuss, 1987). Below-ground primary shoots and roots emerge from each node developing into the stool (conglomeration of secondary roots and shoots). This build-up in the shoot population over time is referred to as tillering. The germinating buds produce primary tillers (oldest shoots). Secondary tillers grow from the primary shoots and the tertiary tillers from the secondary shoots. At full canopy, when full leaf ground cover is achieved, shading out of the smaller tillers occurs and they start dying. Stalk elongation and maturity is reached with an increase in sugar production and storage (Meyer *et al.,* 2011). Stalks form stools maturing at 2-3 metres in length and 2-3 cm in width. Each leaf comprises a leaf sheath and lamina. Flowering indicates the change from vegetative to the reproductive stage, with no further production of

leaves or internodes. Once the young developing internodes below the flower have fully expanded, growth of the stalk ceases (Meyer *et al.,* 2011). Harvesting occurs from 12-24 months and is dependent on temperature, water and maturation of the cane. Sugarcane is a perennial and ratooning (repeated harvesting), usually annually, for up to five to ten years or more is practiced, for economic reasons (van Dillewijn, 1952; Spaull and Cadet, 1990; van Antwerpen, 1999).

The two most important purposes for producing and harvesting sugarcane apart from employment and providing a sustainable living for farmers are: 1) production of sucrose, and 2) production of setts, used as seeds for planting (Barnes, 1974). Economically important by-products are bagasse and molasses. Bagasse is the residual woody fibre of cane and can be used as fuel for the boilers (in the generation of process steam) and lime kilns, production of paper, agricultural mulch and so on (Chen *et al.,* 1993). Molasses is the remaining liquor in the manufacturing process after separation of the crystallised sucrose and is produced in two forms: blackstrap (inedible for humans) or edible syrup. Blackstrap is used industrially to produce ethanol, compressed yeast, citric acid and rum. It is also used as animal feed additive. Edible molasses syrup is often blended with maple syrup and invert sugars (Barnes, 1974).

Since 2000, increased fuel prices and fuel shortages have caused the price of fertilizers and pesticides to spiral upwards. Nitrogen fertilizer prices have risen by forty percent and potassium fertilizers by sixty five percent. We are now looking toward soil sustainability without using huge amounts for fertilizers and pesticides, contributing to developing more eco-friendly farming systems thereby enabling sustainability.

1.3 SUGARCANE DISEASES AND PESTS

A large number of diseases affect sugarcane productivity significantly (Rott *et al.,* 2000). This is partly due to its nature, propagation, cultivation and management

strategies. Systemic pathogens (occurring within the plant tissues) can be readily introduced into a new crop by the planting of infected seedcane or ratooning which permits these pathogens to survive and multiply from one ratoon crop to the next. Ratooning also favours infection by non-systemic diseases, such as those caused by foliar pathogens (Meyer *et al*., 2011).

1.3.1 Sugarcane Fungal Pathogens

Fungal pathogens cause huge losses to the agricultural industry in South Africa and all over the world (Hardar, *et al.,* 1979). Sugarcane fungal pathogens are known to spread to different continents and different sugarcane varieties.

Sugarcane rust is caused by two species of fungi, *Puccinia melanocephala* and *Puccinia kuenhnii* basiodiomycetes, belonging to the Uredinales order (Ryan & Egan, 1989; Raid and Comstock, 2006; Bittencourt *et al.,* 2010). Brown rust caused by *Puccinia melanocephala*, is found anywhere sugarcane is grown (Comstock *et al.,* 1992, Raid & Comstock, 2000). Orange rust caused by *Puccinia kuenhnii* is limited to Hawaii, Fiji, Sumatra, Papua New Guinea and Australia (Magarey, 2000; Magarey *et al.,* 2001). Severe rust has caused reductions in both stalk mass and stalk numbers, thereby reducing cane tonnage (Raid and Comstock, 2006; Bittencourt *et al.,* 2010).

Smut, is caused by the fungus *Ustilago scitaminea* (Lee-Lovick, 1978) a basidiomycete belonging to the Ustilaginales order (Martinez-Espinoza *et al.,* 2002; McFarlane and McFarlane, 2002). Whip-like sori are produced from lateral bud meristems of infected stalks. Whips are composed of core host tissue surrounded by a thin layer of black spores (Comstock, 2000).

In South Africa the root fungal pathogen *Pythium arrhenomanes,* a heterokont, belonging to the Pythiales order, causes root rot in sugarcane. *Pythium* usually infects the immature roots such as the fine roots, nodal, primary root tips, with most rotting occurring in the lateral, secondary and tertiary roots, consequently reducing root growth and production of tillers: this ultimately results in reddening and lesion formation on roots and lower stalk weights. *Pachymetra*

root rot symptoms caused by *Pachymetra chaunorhiza* differs from *Pythium* root rot, in that the larger roots are infected resulting in the retarded root development, plant growth and a susceptibility to water stress (Rott *et al.,* 2000; Rutherford *et al.,* 2002).

1.3.2 Sugarcane Bacterial Pathogens

Pathogenic bacteria are also present and affect different plant communities in different ways. Often, they elicit symptoms in plants similar to those produced by other pathogenic organisms. The number of major diseases caused by bacteria is relatively small compared with those caused by fungi. Most bacterial pathogens produce one major symptom type. However some bacterial diseases may result in a syndrome comprising two or more different symptoms. Bacteria enter host tissue through wounds or natural openings such as hydathodes in leaves, lenticels in corky tissue and nectaries in blossoms (Palm *et al.,* 1968).

Proteobacteria belonging to the Enterobacteriales order, *Erwinia* genus, cause soft rot in sugarcane, fleshy fruits, vegetable and ornamental crops throughout the world. The disease starts as lesions on the leaves, stems and roots of host plants. This results in slime masses of bacteria, which eventually leads to rot of the infected part of host plant. Decaying tissue turns white, cream-coloured, grey, brown or black. It gives off putrid odour, which is caused by secondary invading bacteria that are growing in the decomposing tissues (Bradbury, 1977). Soft rot bacteria produce enzymes that hydrolyse both cell wall components and the pectic substances between walls, resulting in host cell separation and tissue softening by the enzymes. These enzymes also cause tissue degradation, causing subsequent exudation of water into the intercellular spaces, eventually resulting in tissue degradation and necrosis (Palm *et al.,* 1968).

Ratoon Stunting Disease (RSD) caused by *Leifsonia xyli* subsp *xyli* previously *Clavibater xyli* subsp *xyli,* of the Actinomycetales order, is transmitted through the soil. External symptoms include stunting, poor growth, which might not be noticed if most plants in the field are affected, or it might be attributed to other

causes. Yield losses become more pronounced with the age of ratoons. In some varieties, there is reddening of the internal tissue of the stalk, near the node where leaf tissue exists. Cane yield loss is variety dependent. The disease is established within a field by the use of infected plant cane. The pathogen is further spread via cutting implements such as cane knives and harvesters that contaminate the cut surfaces of the healthy plants (Davis and Bailey, 2000; Hoy & Flynn, 2001; Texas Plant Disease Handbook, 2012).

Leaf Scald is caused by *Xanthomonas albilineans* of the Xanthomonadales order. The initial characteristic symptom is a white streak (pencil line) 1-2 mm wide on the leaf, which follows the direction of the main veins. The streaks may later become enlarged and the affected leaf becomes wilted and necrotic. The white tissue streak may also be visible on the leaf sheaths. Symptoms may disappear, although plants remain infected or plants maybe infected and grow without showing any symptoms. Mature stalks may suddenly wilt and die, sometimes without the previous appearance of other symptoms. The bacteria are transmitted by infected cutting and by implements used to cut stalks. There is evidence for soil and water transmission as well (Saumtally, *et al.,* 1996; Texas Plant Disease Handbook, 2012).

1.3.3 Nematodes

Nematodes are non-segmented microscopic worms. Species such as *Helicotylenchus*, *Xiphinema*, *Meloidogyne* and *Pratylenchus* have received a lot of attention. A wide variety of nematodes function at different trophic levels in the soil food web. Pathogenic or more specifically plant parasitic nematodes attack different parts of the plants. These nematodes are different from the free living nematodes, as that prey on either bacteria, fungi or other nematodes (Spaull and Cadet, 1990).

The role of nematodes in agriculture and their impact on sugarcane yields

Nematodes may be useful indicators of soil quality because of their diversity and different functions at different trophic levels. Blair *et al.* (1996) have proposed approaches to assessing the status of soil quality by counting the number of nematodes in different trophic levels, as they are microscopic and live in water films, the changes in the nematode populations, therefore, will reflect changes in soil microenvironments. Nematodes are found in every soil-type throughout the world. They can cause serious damage to sugarcane roots, particularly in sandy soils. When prominent in large numbers they restrict root growth affecting nutrient and water uptake, which in turn limit shoot growth. Many species of plant parasitic nematodes feed on the roots of sugarcane (SASRI, 2006). Root-feeders, for example, are plant parasites that concentrate around roots of stressed or susceptible plants (Ingham, 2000). They suck out liquid nutrients and inject damaging materials into the root cells, causing both structural and physiological damage. Symptoms of attack by these root feeders are swelling of stems or roots, irregular branching, deformed leaves, lack of blossoming and galls on roots (Kerrigan *et al.,* 1998). Furthermore, roots damaged by nematodes are more prone to invasion by pathogenic bacteria and fungi (SASRI, 2012) resulting in reduction in the yield and number of ratoons from one planting, and subsequently weed problems increase due to slower canopy formation of the affected cane sugarcane (SASRI, 2006).

Eighty species of the thirty-two genera of plant parasitic nematodes have been found associated with sugarcane in SA, but only species of *Paratrichodorus, Xiphinema, Meloidogyne*, and *Pratylenchus* are regarded as important pests. Results of a survey in KwaZulu-Natal (KZN) where my research was conducted show these genera to be widespread in sandy soils and occurred in 90% or more of surveyed fields (SASRI, 2012).

Symptoms of nematode damage

Above ground symptoms are similar to those of moisture and nutritional stress. They include stunted and patchy growth with the plants bearing narrow, longitudinally rolled leaves. Tillering may also be reduced so that fewer stalks are produced and the stalks themselves are thin and stunted (SASEX, 1970).

Below ground, symptoms are more diagnostic and include sparse root systems, short and stubby lateral roots. Galls may be visible on the roots if *Meloidogyne* is present. If *Pratylenchus* is abundant, small reddish-brown lesions are present. Where *Paratrichodorus* is present in large numbers, stubby lateral roots are seen on the root system (SASEX, 1970).

Nematode - sugarcane interactions

Roots of sugarcane are normally attacked simultaneously by a number of nematode species, some or all of which may cause serious damage. The sugarcane plant itself is also responsible for the dynamics of the nematode populations and this is due to the replacements of one root system by another (Spaull and Cadet, 1990). In order to understand the importance of nematodes and to explain their mechanisms of damage, it is important to consider the different components of the nematode community in relation to both the development of the roots and to the evolution of growth parameters that contribute to the yield of the crop (Spaull and Cadet, 1990).

Sugarcane yield is the function of the number, length and diameter of the stalks, while sucrose yield is a function of the sucrose content of the stalks. Root damage by nematodes results in a reduction in the number and length of stalks, and occasionally has a significant influence on the diameter and sucrose content (Spaull and Cadet, 1990). In plant cane the reduction in the number of stalks takes place primarily during the period of maximum tiller development i.e. while the plant is largely dependent on the sett root system. A reduction in stalk length may also be apparent during this period, and in the presence of certain

nematodes, increasing in magnitude through to harvest. Therefore, stalk length may be affected by damage to both sett roots and shoot roots (Cadet *et al.,* 2005).

In South Africa, ratoon cane is almost as badly affected by nematodes as the plant-cane. Crop loss is due primarily to a reduction in stalk length. This is attributed to the considerable damage to the shoot roots caused by ectoparasitic *Xiphinema* and *Pratylenchus* spp. During the initial period of shoot development, large numbers of ectoparasitic nematodes are present in the soil and very few endoparasites are present in the roots (Spaull and Cadet, 1990). Evidence from previous field trials indicate that the control of nematodes in sandy soils by nematicide use in SA, increases the yield by several hundred tons per annum, but besides being expensive, nematicides can be deadly to humans as well as the nematodes (Spaull, 1991; SASEX, 2012).

1.4. MYCORRHIZAL FUNGI

As researchers, we are required to exploit environmental conditions or manipulate the prevailing planting environment to increase agricultural crop yields, while simultaneously reducing stresses caused by diseases and pests, as well as drought. Plant adaptation mechanisms lie in the fact that each community in the agroecosystem influences the other.

Since 1885, extensive research revealed that some plant roots, though extensively invaded by fungi, were not diseased. Frank (1885) found a symbiotic association between the plant and fungus and termed this mycorrhiza i.e. fungus root associations formed between plants and fungi that colonise the cortical tissue of roots. The term “mycorrhiza” does not denote the fungal symbiont (Smith and Smith, 2012). Research indicated that some plants could not grow without their fungal symbionts or mycobionts known as mycorrhizal fungi. These fungi are ubiquitous in most of the higher plants, with arbuscular mycorrhizal fungi being the most common. Non-mycorrhizal plants therefore are considered

abnormal (Bryce and Kendrick, 2000; Smith and Smith, 2012) as mycorrhizal plants influence the structure of plant communities (Santos, 2006) by bridging the soil-root environment i.e. symbiotic root fungi provide the plants with nutrients from the soil to roots in exchange for photosynthates from the leaves. This critical linkage between plant roots and the soil in nutrient deficient soils, by mycorrhizal fungi, can lead to improved plant growth and reproduction; aiding plant communities under environmental stress and mycorrhizal plants require reduced amounts of fertiliser and can withstand heavy metal, as well as acid rain pollution better than non-mycorrhizal plants. These plants grow better in nutrient deficient soils of marginal lands, on mine spoils and at high altitudes. They also survive transplant shock better, they are more resistant to soil-borne diseases, withstand higher soil temperatures and higher soil salinity and wider extremes of soil pH (Clark *et al.,* 1999; Bryce *et al.,* 2000, Evelin *et al.,* 2009).

Mycorrhizas have been characterised as follows: Ectomycorrhizas, Monotropoid mycorrhizas, Arbutoid mycorrhizas, Ectendomycorrhizas, Ericoid mycorrhizas, Orchid mycorrhizas, and finally Arbuscular mycorrhizas (Smith and Read, 2008).

Ectomycorrhizas (ECM) are formed mostly by basidiomycetes. An ectomycorrhizal root is characterised by a sheath or mantle made up of compact fungal tissue surrounding the root, a labyrinthine inward growth of hyphae between epidermal and cortical cells called the Hartig net and an external mycelium, which forms connections with the soil and sporocarps of the fungi. ECM fungi can be found on about ninety percent of the trees in temperate forests and each tree can be associated with many fungal species concurrently (Smith and Read, 2008).

Monotropoid mycorrhizal fungi such as those infecting the Monotropaceae within the Ericales form a thick compact mycelial sheath and a Hartig net one cell deep in the root cortex. The penetration of the epidermal and outer cortical cells is limited and achieved by the development of extensively invaginated fungal

pegs, resulting in specialized transfer cells. These fungi can also be ectomycorrhizal when associated with trees (Smith and Read, 2008).

Arbutoid mycorrhizal fungi are of the Ectendo type and among the Ericaceae (order Ericales) the fungal sheath is well developed while the Hartig net is restricted to the outer layers of cells. They exhibit hyphae coiled within the epidermal cells of the root, which differentiates them from the ectomycorrhizas (Harley and Harley, 1987; Smith and Read, 2008).

Although members of the Ericales exhibit three types of mycorrhizas - ericoid, arbutoid and monotropoid, the ericoid type is the most important with the fungal hyphae growing loosely over the lateral "hair" roots of the host plant, penetrating the single layer of cortical cells and filling them with intracellular hyphal coils. The apex of the "hair" root is not colonised and the stele is never penetrated. The endophyte can occupy as much as eighty percent of the total volume of heavily colonised roots (Schmid *et al.,* 1995).

Orchid mycorrhizas have no sheath but hyphae penetrate intracellularly. For all or part of their life cycle, orchids are obligately dependent on their mycorrhizal partner. Under natural conditions, orchid seeds will not successfully germinate or develop without the mycorrhizal fungal infection as the fungus provides the carbon compounds to the orchid during the non-photosynthetic portion of its life cycle, ceasing during the photosynthetic phase. In green orchids there is some evidence that adult plants have the capability of supplying the fungus with recent photosynthate (Laiho, 1986; Sieverding *et al.,* 1991).

1.4.1 Arbuscular Mycorrhizal Fungi

Fungal endophytes thought to represent early endomycorrhiza have been found in the root-like organs of Asteroxylon and Rhynia from the Devonian period (360-410 million years ago) and other possible fossil records date back to the Cambrian period (± 550 million years ago) (Pirozynski and Dalpe, 1989). Endomycorrhizas (now an obsolete name for arbuscular mycorrhizas (AM), according to Brundrett, (2004) may have been instrumental in colonisation of

land by primitive vascular plants in the late Silurian, ± 420 million years ago (Pirozynski, 1981). AM fungi are classified in the phylum Glomeromycota, class Glomeromycetes containing four orders, eleven families and seventeen genera (Schüßler and Walker, 2010). Species are soil-borne, obligate biotrophic symbionts (Douds *et al.,* 1999; Franken and Gianinazzi-Pearson, 1996) that play an essential role in plant growth enhancement in natural ecosystems and sustainable agroecosystems (Johnson *et al.,* 1997) that have low inputs of chemical fertilizers and biocides (Bethlenfalvay and Schüepp, 1994; Jeffries and Barea, 1994; Hooker *et al.,* 1994). Within the ecosystems, improved plant establishment, enhanced plant nutrient uptake, plant protection against cultural and environmental stresses and improved soil structure are some of the functions that the AM symbiosis promotes (Smith and Read, 2008).

Arbuscular mycorrhiza (AM) is a term used to denote the symbiotic structure formed between the arbuscular mycorrhizal fungi and the host root (Smith and Smith, 2012). AM fungi form symbiotic associations with most roots of Angiosperms, Gymnosperms, Pteridophytes and Bryophytes. AM comprises the host root, AM fungi internal mycelia and AM fungi external mycelia. The morphology of host roots is not extensively changed by colonization by AM fungi and hyphae are restricted to the cortical region of the roots (Smith and Smith, 1989, 2012).The fungus initially grows between the cortical cells, but soon penetrates the host cell wall and grows within the cell. Neither the fungal cell wall nor the host cell membrane is breached. As the fungus grows the host cell membrane invaginates, enveloping the fungus and creating a new compartment where solubilised material is stored. This apoplastic space prevents direct contact between the symbionts (Read, 1984).

Arbuscules - small tree-like, highly-branched, modified haustoria - persist in the active form for about 1-15 days (Harrison, 1999; Bryce and Kendrick, 2000, Smith and Read, 2008) providing the symbiotic interface (comprising the plasma membranes of the host and roots separated by the apolastic compartment

between them) for nutrient exchange between the fungus and host. This interfacial apoplast contains the interfacial matrix (varying amounts of plant and fungal wall material). Conditions within the interfacial matrix, remain relatively constant, with mostly the pH and solute concentrations being modified by the symbiotic activities of the host and fungus, (Smith and Smith, 2012). Branched AM external mycelium containing infection units (± 2 or more per cm), expand beyond the rhizospheric and nutrient depletion zones as well as colonizing many different parts of the same or neighbouring root systems. This ensures nutrients are derived from the soil and organic C may be obtained from many plants. These root/s systems may also be colonized by more than one AM fungi (Harley and Smith, 1983; Olsson *et al.,* 2002; Öpik *et al.,* 2006). Unlike the interfacial matrix, the external mycelium may be subjected to extensive variations in soil conditions, such as soil type, pH, nutrient availability, seasonal temperature and water availability (Smith and Smith, 2012).

Reproductive spores can be formed in the root or the soil (Sylvia, 1990; Sylvia, 1994). AM fungi except species of *Gigaspora* and *Scutellospora* also form thin-walled, lipid filled, intercellular vesicles i.e. terminal swellings of hyphae with a storage function within the roots (Paszkowski, 2006; Sanders, 2008). AM fungi generally have very large and thick walled resting spores, which are stimulated to germinate by the proximity of the plant roots under optimal seed germination and root growth conditions. When the fungus encounters a receptive root or root hair, an appressorium is formed and penetration occurs at the root tip, in the elongation region. Symbiosis is thus initiated in the juvenile tissues (Bryce and Kendrick, 2000).

1.4.2 Arbuscular mycorrhizal sugarcane

Although sugarcane agroecosystems have been maintained for thousands of years globally, research on the diversity and impact of AM fungi on sugarcane agroecosystems has only in recent years accelerated. Morphological studies on diversity of AM fungi in Iran and South India indicated approximately thirty AM

fungi species to be associated with varieties of sugarcane. *Glomus aggregatum, G. fasciculatum, G. geosporum* and *G. mosseae* were found to be commonly associated with sugarcane grown in both Iran and South India (Kariman *et al.,* 2005; Srikumar *et al.,* 2009; Rokni *et al.,* 2010, Rokni and Goltepeh, 2011). Greenhouse experiments as well as laboratory experiments, using *Glomus mosseae* as an inoculant, showed enhanced plant root development and growth. Spore inoculation of micropropagated and callus derived seedlings also produced profuse rooting, early establishment of tissue culture plantlets in field conditions and improved yields (Wang *et al.,* 1995; Muniyamma *et al.,* 2000, Naik, 2001).

1.4.3 Nutrient uptake in arbuscular mycorrhizal fungal symbiosis

After decades of dedicated research, it is now well established that "mycorrhizas", not roots, are the chief organs of nutrient uptake by land plants" (Smith and Read, 2008, Smith and Smith, 2012). As indicated, AM fungi are important symbiotic constituents of agroecosystems, including that of sugarcane (Kelly *et al.,* 2001; Naik, 2001; Rokni *et al.,* 2010, Rokni and Goltepeh, 2011). AM stimulate uptake of macro-nutrients particularly P, N and micro-nutrients such as Zn, Cu and Fe from beyond the rhizosphere in nutrient-deficient soils (Chen and Zhao, 2009; Kothari *et al.,* 1991; Liu *et al.,* 2000, Cardoso, 2006). Studies also indicate the increased resistance of higher plants to heavy metals in highly colonised AM plants (Bradley *et al.,* 1982; Dupré de Boulois *et al.,* 2005; Chen and Zhao, 2009; Subramanian *et al.,* 2008).

Other studies have indicated that the plant host nutritional status is significant for establishment and functioning of a good mutualistic symbiotic relationship. AM fungi supply their host plant with nutrients in return for carbohydrates and are therefore more efficient in acquiring nutrients per unit length than non-mycorrhizal roots, even in the absence of increased nutrient uptake; the largest effect is on P acquisition, followed by N acquisition (Smith and Read 2008, Smith and Smith 2012). Nutrient uptake in AM plants occurs via two potential uptake pathways; either, directly from the soil or via the AM root (the major uptake

route). The AM symbiotic pathway is dependent on three essential processes: 1) soil fungal mycelium nutrient uptake; 2) translocation of the nutrients over a distance to the hyphae, arbuscules and coils within the plant root; and 3) transfer of the nutrients from the fungal cells to the plant cells. Of significant importance is P and N. Compared to nutrient "sufficient" plants, hosts with P and N-deficiencies release more exudates into the soil, which in turn stimulates root colonization by AM fungi (Blanke *et al.,* 2011; Harrison 2005; Schwab *et al.,* 1991; Yoneyama *et al.,* 2007a,b). It has already been well proven that nutrient deficiencies in plants positively affects carbon allocation to the fungus within the root (Blanke *et al.,* 2011; Olsson *et al.,* 2002, 2005) firmly establishing the symbiosis (Bücking and Shachar-Hill 2005).

Nutrient exchange in AM occurs via the interfacial apoplast created by the invaginated host plasma membranes in colonised cortical cells or across fungal-host membrane interfaces, intercellularly. Carbon in the form of hexoses, are transferred to the fungi, while nutrients are transferred to the plant tissues. Due to the lack of cytoplasmic continuity, bidirectional transfer of nutrients relies on the efflux of nutrients from the AM fungi and uptake by root tissue and vice versa (Smith and Read, 2008). These transport processes are driven by the electrochemical potential gradients and mechanisms that govern solute exchange, charge balance and the respective cytoplasmic pH (Shachar-Hill *et al.,* 1995).

Part of this research entails the nutrient uptake in sugarcane, it is therefore important to understand the significant mechanisms that govern nutrient exchange between AM fungi and the host plants involving C, P and N metabolism in AM symbiosis as indicated below.

Carbon (C)

Evidence of the AM fungi life cycles being completely dependent on obtaining organic C from host plants is both direct and indirect. Indirect evidence occurs in

the in the form of increased growth of extraradical mycelium and spore production upon mycorrhization (Smith and Read, 2008). Direct evidence obtained using nuclear magnetic resonance (NMR) studies, resulted in the proposition of the following scheme describing the metabolism of organic carbon in exchange for P and N in AM symbiosis: (1) the uptake of hexoses by the intraradical mycelium (IRM), (2) glycogen and trehalose formation from hexose within the host roots, particularly before sporulation, (3) triacylglycerides (TAGs) stored in the IRM is then exported to the extraradical mycelium (ERM) where, (4) transformation of the TAGs into gluconeogenic precursors for hexose occurs via the glyoxylate cycle, (5) active sysnthesis of Arginine (Arg) via the usual AM fungi metabolic pathway, (6) association of Arg with polyphosphates (polyP) within mycorrhizal vacuoles and (7) active dark fixation of carbon dioxide occurs in the AM fungi (Pfeffer *et al.,* 2001; Smith and Read, 2008).

Further studies of AM intraradical structures indicate the functioning of glycolysis, the tricarboxylic acid (TCA) cycle as well as the phosphate pentose pathway (PPP) (Harrier *et al.,* 1998). AM fungi are oleogenic fungi and store large amounts of TAGs in oleosomes, which circulate throughout the AM fungal colony. TAGs play an important role in the morphogenic and reproductive events such as sporulation (Bago *et al.,* 2002). Host derived hexoses are metabolised by the fungi via glycolysis to triose and acetyl-CoA. Fatty acids are synthesised normally via the acetyl-CoA carboxylase and fatty acid synthase complex. These are then subsequently elongated and/or desaturated and esterified with a glycerol moiety via acetyltransferases to constitute TAGs (Bago *et al.,* 2000). At this stage is important to note that studies provide evidence for cycling of C between the IRM and ERM; however to date studies also indicate no evidence for C transfer from the IRM to the host plant. To date considerable differences in C expended by AM fungi in exchange for P have already been established. It is unclear as to whether these differences occur due to variations in patterns or colonisation rates, hyphal extension in the soil, the subtle differences in

membrane transport capacity or differences in respiratory efficiency (Smith and Read, 2008).

Phosphorus (P)

AM fungal mycelium in the soil can penetrate the smaller pores in the soil particles compared to the plant root hairs, giving the AM symbiosis a great advantage compared to reduced nutrient uptake directly from the soil. P, which is required in large amounts is poorly mobile and occurs in the soil solution in low concentrations, is mobilised by AM. P depletion in the rhizosphere therefore gives AM plants a distinct advantage compared to non-mycorrhizal plants, which eventually become P deficient, ultimately resulting in poor plant growth (Smith and Read, 2008).

A variety of evidence indicates the presence of different P transporters and H^+-ATPases at uptake sites. Organic phosphate (Po) is present in the soil as phospholipids, nucleic acids and phytate and does not provide a major pool to non-mycorrhizal plants. Some AM fungi do appear to hydrolyse some Po and influence the phosphate production in plants (Smith and Read, 2008). The uptake of orthophosphates usually in the form of $H_2PO_4^-$ require large amounts of energy due to the large electrochemical potential gradient present between the soil solution and the AM fungi cytoplasm (Schachtman *et al.,* 1998). It is used for synthesis of the key molecules for cellular functioning, before being stored in vacuoles where pH~6.5 is maintained (Rasmussen, *et al.,* 2000, Smith and Smith, 2012).

Inorganic P (Pi), on the other hand is usually crystalized in insoluble forms as Ca, Fe and Al phosphates depending on soil pH, hence its low solubility in soils. Further contributing to the low soil solubility is the fact that the crystals may be chemically bound to soil clay particles (Smith and Smith, 2012). Less tightly bound or labile Pi is more available to plants when the soil pH is 6.5. However

root exudates such as citrate and oxalate increases the availability of P, as these compounds are chelating agents (Marschner, 1995, Comerford, 1998).

A Pi concentration gradient exists between the soil solution and plant/fungal cytoplasm; soil solution [Pi] = $\leq$ 10 μm and plant [Pi] = 10 mM (Mimura, 1999). Pi absorption is dependent on low and high affinity energy systems. The physiological role of transporters can be interpreted from their different pH optima; the H^+/Pi symporter system works optimally at a neutral pH, while the Na^+/Pi system works optimally at an alkaline pH. This suggests that Pi uptake is efficient with two systems over a broad pH range (Martinez and Persson, 1998).

Following uptake, Pi is first incorporated into the cytosolic Pi pool, which has a constant Pi concentration gradient to maintain cell functioning viz. energy generation and biosynthesis of phospholipid, nucleic acid and precursors of carbohydrate polymers such as UDP-glucose. When fungi take up more Pi than is required, the excess is stored in vacuoles, thereby achieving effective buffering of the cytosolic [Pi] (Kilonsky *et al.,* 1990). Pi transport in the vacuole is maintained by an electrical potential gradient not a chemical gradient created by vacuolar ATPase (Massonneau *et al.,* 2000).

Key mechanisms governing the bidirectional transfer of nutrients on which the symbiosis is based is still unknown. Very little is known of the mechanisms of P loss from fungus to the interfacial apoplast surrounding intracellular arbuscules or hyphal coils. Abnormally rapid efflux of P from the intracellular fungal structures may be disadvantageous to the fungus. Charge balances must be maintained when transfers of negatively charged P forms occur during uptake into hyphae, long distance translocation and transfer across the symbiotic interface occurs. Since reverse transfers of negatively charged ions seem unlikely, the simultaneous transfer of positively charged ions (e.g. K^+ or arginine) with Pi (Smith and Smith 2011, 2012) is also required.

Arbuscule formation is P sensitive. Very high P concentrations in the soil impede root colonization and arbuscule formation (Bolan *et al.,* 1984; Goerge, 2000).

Research by Abbott and Robson (1979) concluded that levels of soil phosphorus greater than that required for plant growth eliminated the development of the arbuscules. Schubert and Hayman (1986) found AM to be ineffective when 100 mg or more of P isadded per kilogram of soil (100 $mg.kg^{-1}$). Other researchers have reported that mycorrhizal infections tend to die out in soils containing or given high P (Dalpè *et al.,* 2006; Santos, 2006). The developments of mycorrhizal relationships were the greatest when soil phosphorus levels were at 50 $mg.kg^{-1}$ or less (Schubert and Hayman, 1986). Prior to inoculating soil with AM fungi, a soil test should be conducted as phosphorus levels greater than 50 $mg.kg^{-1}$ the addition of AM fungi will probably be ineffective. The level of phosphorus in the plant also has been shown to influence the establishment of AM with high levels inhibiting colonisation by AM fungi (Menge *et al.,* 1978). This is of particular interest to us as sugarcane is cultivated in both P-fixing and non P-fixing soils.

Nitrogen (N)

Mineralisation and mobilisation of nitrogen in the soil is dependent on rhizospheric and free-living microorganisms. These microorganisms play an important role in nitrification, denitrification and the conversion of N_2 gas to ammonium. AM fungi absorb both nitrates and ammonium (less mobile) from the soil solution. Usually these inorganic forms are relatively mobile in the soil and transported to roots by mass flow in the soil solution. However, when N depletion zones are experienced, the external hyphae of AM plants are able to transport the N to the roots. AM plants thus exhibit increased N concentrations compared to non-mycorrhizal plants. AM plants may also be symbiotic with nitrogen fixing bacteria (see section 1.5 below), contributing to the enhanced nutrient uptake mobilised by this tri-partite symbiosis (Smith and Read, 2008).

Studies have indicated that the extraradical hyphae of AM take up the inorganic nitrogen and assimilate it into organic nitrogen in the form of arginine (Arg). Arginine is then transferred to the intraradical mycelium and metabolised to the respective C-skeleton and inorganic nitrate forms, before being translocated

once again in its inorganic forms to the interfacial apoplast and then the plant root. Once released in the intraradical mycelium the carbon skeleton rejoins the C intraradical pool and used for metabolic translocation or structural purposes. It is clear that plant enzymes and pH regulation mechanisms play an important role during N assimilation. The mechanisms of AM assimilation including enzymatic activities of glutamine synthetase (GS has a higher affinity for ammonium), glutamate synthase and ammonium transporters is unclear (Bago *et al.,* 2002; Govindarajulu, *et al.,* 2005; Jin *et al.,* 2005).

1.5 BACTERIAL SYMBIONTS

1.5.1 Bacteria associated with nitrogen nutrition

Nitrogen fixing bacteria such as *Rhizobacterium* form symbiotic associations with legume roots. Visible nodules are created where bacteria infect a growing root hair. The plant supplies carbon compounds to the bacteria, which in turn convert atmospheric nitrogen (N_2) into a form utilised by the plant. In general nitrifying bacteria change ammonium (NH_4^+) to nitrite (NO_2^-), which in turn is converted to nitrate (NO_3^-). Nitrate, which is highly mobile in the soil, is the preferred form of nitrogen for most crops. Denitrifying bacteria are anaerobic and convert nitrate to nitrogen (N_2) or nitrous oxide (N_2O) gas (Ingham, 2000 and Smith and Read, 2008). Plants require ten times more N than P, therefore compared to non-mycorrhizal plants, when AM plants also share a symbiosis with plant symbiotic bacteria, N supply to plants increases. However, improved nodulation, N_2 fixation improved plant growth and general health in AM plants only occur in the absence of P-stress. Therefore, even when non-mycorrhizal plants are steadily supplied with P the differences between AM and non-mycorrhizal plant growth and general plant health are negated. Under agricultural field and natural conditions, since a steady P supply is not always feasible as nutrients are in short supply, AM fungi-plant symbiosis is important for survival (Smith and Read, 2008).

1.5.2 Associated Mycorrhizal Bacteria

Symbiotic fungi, free-living parasitic bacteria, microfungi and protozoa thrive in the same ecological niche, using root exudates as carbon substrata. Garbaye and Bowen (1989) showed that bacterial strains isolated from the ectomycorhizosphere stimulated mycorrhizal formation. They were named Mycorrhiza Helper Bacteria (MHB). Further research indicated that most MHBs are fluorescent pseudomonads and sporulating bacilli. These species are common in all rhizospheres, but a small portion is MHB. These MHBs are found embedded in the ectomycorrhizal mantle sheath or the inner tissues of sporocarps, suggesting a close association with the fungus. Fungus specific MHBs suggest that the competitive ability of the ECM fungi to infect receptive sites on the root is partly controlled by surrounding bacteria (Garbaye, 1991).

Strains of *Burkholderia cepacia* and *B. gladioli* have been reported to be antagonistic towards nematodes (Kloepper *et al.,* 1992). A *B. cepacia* strain has been shown to inhibit egg hatch and mobility and second stage juveniles of *M. incognita in-vitro* (Meyer, 2000) and *in-vivo* (Meyer *et al.,* 2002). The occurrence of various bacteria on the surface or within the cytoplasm of AM fungal spores has been reported (Mosse, 1962; Varma *et al.,* 1981). Mycorrhizal associated bacteria found embedded AM fungi spore walls were termed Associated Mycorrhizal Bacteria (AMB) (Roesti *et al.,* 2005).

Tilak *et al.,* (1989) were able to isolate nitrogen fixing *Azospirillum* spp. from the surface-sterilized spores of AM fungal species, and suggested that these diazotrophs may contribute to the N nutrition of the mycorrhiza. Sward (1981a-c) observed binary fission of 'bacteria like organisms' within spores of *Gigaspora margarita*, which spread into the growing germ tube, where the organisms became concentrated in apical and subapical sites. These bacteria are now identified as *Burkholderia* endosymbionts (Bianciotto *et al.,* 2000).

Some *Pseudomonas* and *Xanthomonas* spp. enhance plant growth by producing compounds that inhibits the growth of pathogens or reduce invasion by plant

pathogens including nematodes and indirectly promote plant growth by preventing or minimising the degradative effects of pathogens by producing antibiotics, toxins and growth enzymes. For example, certain strains of *Pseudomonas fluorescens* have antifungal activity that inhibits some plant pathogens (Ingham, 2000).

Many papers have described nitrogen fixing bacteria in AM fungi and suggested that *Azospirillum* and *Acetobacter* species may play an important role in fertilization (Isopi *et al.,* 1995 and Tilak *et al.,* 1989). According to Bianciotto *et al.,* (1996), however these investigations did not demonstrate the presence of intercellular bacteria. The bacteria could just be present over the thickened and layered cell walls of the AM fungal spores.

CHAPTER 2

ARBUSCULAR MYCORRHIZA (AM) STATUS IN COMMERCIAL VARIETIES OF SUGARCANE IN P-FIXING AND NON P-FIXING SOIL REGIONS OF KWAZULU-NATAL AND THE GROWTH RESPONSE OF SUGARCANE IN THE GLASSHOUSE TO INOCULATION WITH INDIGENOUS AM FUNGI

2.1 INTRODUCTION

Developing and breeding improved varieties for South African sugarcane industry, is one of the core functions of the South African Sugarcane Research Institute (SASRI) (Parfitt, 2005). Like other global breeding programmes, focus is placed on improved sugarcane yields, sugar content, ratooning ability, maintaining or improving disease resistance, and maintaining acceptable fibre levels for milling (Parfitt, 2005; Rokni, 2011).

Breeding commercial sugarcane varieties relied on the Co or NCo varieties (Coimbatore heritage) resulting in a narrow genealogy. Three *Saccharum spontaneum* clones, two or three *S. barberi* clones, between nine and eleven *S. officinarum* clones and two unknowns were involved in the ancestry of South African varieties (N varieties were named for the province they were bred in viz. Natal (now KwaZulu-Natal). Breeders faced formidable obstacles such as the narrow genetic base, the high ploidy of *Saccharum* spp. hybrids, the regular occurrence of aneuploids and an inability to control the outcome of crosses. Conditions in temperature controlled glasshouses were manipulated ensuring synchronised flowering and production of viable pollen. Once seed production occurred, a screening and selection programme was initiated, where many clones from single crosses were reduced to a selected few and material was bulked up over the years. Selection in the first few years proved difficult due to lack of clonal material to work with and the effect of environment and genome interactions. The breeding process spans fifteen years before new varieties are released to growers (RS. Rutherford* personal communication).

Sugarcane life cycle includes the seed (stalk setts or cuttings containing nodal primordia are used) and vegetative, ripening and reproductive stages. Seed cane may be dormant or ripe for germination. The vegetative stages comprises: i) Germination and emergence - buds containing embryonic shoots, sprouts under favourable conditions and gives rise to a primary stalk; ii) Seedling - lives at the expense of the reserves present in the seed cane piece, and partially uses water and nutrients provided for by the first roots; iii) Tillering or formative stage starts around 40 days after planting and may last up to 120 days - although 6-8 tillers are produced from a bud, ultimately only 1.5 to 2 tillers per bud remains to form canes; iv) Stem elongation or the grand growth phase involving frequent and rapid leaf production - under favourable conditions stalks grow rapidly almost 4-5 internodes per month and the grand growth phase starts from 120 days after planting and lasts up to 270 days in a 12-month crop; v) Ripening and maturation phase in a twelve-month crop lasts for about three months starting from 270-360 days where sugar synthesis and rapid accumulation of sugar takes place during this phase. The reproductive stage or occurrence of flowering is influenced by variety as well as by environmental conditions; flower initiation causes the apical meristem to switch from vegetative to floral development, causing stalk elongation to cease (Netafim, 2012).

Characteristics of varieties involved in this study are as follows: N12 is a very reliable, low risk cane with good disease resistance i.e. intermediately resistant to smut and mosaic, nematodes and eldana to a certain extent; it is resistant to ratoon stunting disease (RSD), rust, leaf scald and red rot diseases; it is also frost tolerant, and grows well in poorer, water-logged and weak sands; it grows well on the coast and in the midlands. N31 grows particularly well in the midlands. It is not as resilient to disease as N12 as it is susceptible to smut and red rot and intermediately susceptible to mosaic, eldana and nematodes; but grows well under water-stressed conditions. N35 grows well in the coastal regions but does not grow well under water stressed or water logged conditions especially in the midlands; it is resistant to smut, mosaic and leaf scald, while showing

intermediate resistance to RSD, rust and red rot; its resistance to nematodes is unknown. N37 grows better under high potential humic soils of the midlands; it shows resistance to mosaic, but is susceptible to smut and intermediately susceptible to rust, red rot and Eldana. N39 initial trial results indicated better growth on low potential soil on the coast where it out performs N12; it is resistant to eldana, and rust, but shows intermediate resistance to mosaic (SASRI, 2002).

Although, researchers have verified the colonization of sugarcane by AM fungi in countries such as Australia, Brazil, Iran and India (Andreloa, 1982; Kelly *et al.*, 2001; Naik, 2001; Rokni *et al.*, 2010), the status of indigenous AM fungi in South African sugarcane commercial varieties is yet to be determined. AM fungi are integrated into agricultural plant production in two ways; either by inoculation of plants with known AM fungi inoculants or managing the indigenous AM fungi populations with transplants being pre-inoculated for green houses (Lovato *et al*., 1996). Plant responses to AM fungal colonisation can range from highly positive to highly negative (Klironomos, 2003) therefore the use of known, commercial AM fungi inocula (isolated from "foreign" soils stored in culture collections) may have little relevance to indigenous AM fungi-host interactions (Klironomos, 2003).

Commercial sugarcane varieties are grown under P-fixing and non P-fixing soil conditions at various locations in the KwaZulu-Natal province. Fields are either unfertilized or minimally fertilized by small-scale farmers, for economic reasons. AM fungi influences P uptake and the level of P also determine AM fungi colonization (Kling and Jacobson, 1998; Barea *et al*., 2002). Most of the sugarcane is therefore grown under nutrient stressed natural conditions, in both P-fixing and non P-fixing soils, making AM fungi important in the sugarcane agricultural sector. P-fixation is a term that is used to describe both P-sorption and P precipitation in the soil (http://www.ctahr.hawaii.edu/oc/freepubs/pdf/

pnm9.pdf). Since both P-sorption and P precipitation reduce phosphorus availability, P-fixing soils are considered to have less available phosphorus compared to non P-fixing soils.

Whilst some studies have indicated that soil microorganisms enhance AM fungi germination, AM fungi root colonisation and mycorrhizal plant growth (Azcon-Aguilar and Barea, 1985; Azcon-Aguilar *et al*., 1986; Meyer and Linderman, 1986), other studies have reported the suppressive effects of soil microorganisms on plant growth (Hetrick *et al*., 1986). The effects of microflora and AM fungi on sugarcane varieties grown in SA has never been established and there was also great concern that the introduction of commercial inocula into our sugarcane agroecosystems may have undesirable effects on indigenous AM fungi populations and through competition reduce the diversity in sugarcane soils as well as other neighbouring natural soils. These potential effects have never been explored in local sugarcane P-fixing and non P-fixing agrosystems.

The aim of this study was therefore two-fold: a) to assess, the mycorrhization status of five field AM sugarcane varieties (N12, N31, N35, N37, N39) grown in both P-fixing and non P-fixing fields/plantations and b) to design a glasshouse study to assess the effects of AM fungi on sugarcane plant growth levels in P-fixing and non P-fixing soil types under the following three treatments:

- *Sterilized soil (S)* was used to assess the effects of site specific "indigenous" AM fungi inoculum on sugarcane growth levels.

- *Non sterile soil (NS)* was used to assess growth levels under natural soil field conditions in which site specific AM fungal spores as well as other site indigenous microflora were present. The effects of non-site "non-indigenous" AM fungi (isolated from the other field) on sugarcane growth levels were also assessed.

- *Sterilized washed soil plus soil bacterial microflora (SW)* was used to assess the effects of indigenous microflora on sugarcane growth levels. The soil was

washed to remove the excess nutrients that may have resulted from sterilization. Growth levels of uninoculated plants and plants inoculated with both site “indigenous” and non-site “non-indigenous” AM fungi were also assessed.

2.2 MATERIALS AND METHODS

2.2.1 Sampling commercial varieties from field trials to determine percentage mycorrhization.

Every year SASRI carries out variety trials to determine the most suitable variety for growing in particular locations (Barker and Davis, 2005). For logistical and economic reasons these variety trials located at P-fixing soil (Klipp III) in the KwaZulu-Natal Midlands (29°21`S - 30°43`E) and non P-fixing soil (Zinkwazi II), on the KwaZulu-Natal north coast (29°16`S – 31°25`E) were used for this investigation. Five sugarcane varieties viz. N12, N31, N35, N37 and N39 were analysed for percentage mycorrhization in early spring (August), as part of their routine sampling for disease and FAS analysis. Six plants per variety were randomly chosen within the variety plot. The soil and roots were collected on the stool where the leaf was sampled. Roots were extracted from the soil via sieving using the 750 µm sieve. Percentage mycorrhization was determined (see section 2.2.2) and analysed (see section 2.2.5).

2.2.2 AM fungi root assessment - Mycorrhization

Mycorrhization, using percentage colonisation of sugarcane roots by AM fungi, was determined using the gridline intersect method (modification according to Brundrett *et al*., 1996). Roots were initially cleared by heating in 10% w/v KOH (potassium hydroxide) for 5 min to prevent background staining, followed by acidification in 2% HCl for 15 minutes after rinsing. Fungal structures in plant tissues were observed by staining with 0.03% w/v Trypan blue or 0.03% w/v Chlorazol black E (CBE) in lactoglycerol (1:1:1 lactic acid, glycerol and water). After staining, the roots were destained in 50% glycerol. The roots were dispersed randomly in a nine centimetre diameter petri-dish with grid lines

(14/11 cm). Intersections between the grid lines and roots were quantified and roots designated as either colonised or non-mycorrhizal. The portion of root length that was mycorrhizal and the total root length was calculated from the conversion factor derived from the total length of grid lines (14/11 cm) and the area of the dish. Assessment of a minimum of 100 intersections was conducted and samples were randomised and counted several times to increase the accuracy.

2.2.3 AM fungi spore extraction

AM fungi spore inocula was extracted from field soils using a modified wet sieving and centrifugation isolation and extraction method (Brundrett *et al*., 1996). Soil (300 cm^3) was soaked with water then washed (maximum of six times) through a series of sieves (750 µm, 500 µm, 200 µm, 150 µm, 90 µm, 75 µm, 53 µm and 45 µm). Spore suspensions were centrifuged using the Avanti J-26 XPI High Performance centrifuge for five minutes at 2000 *g* at 4 °C to remove minor floating debris in solution. The spore pellets were re-suspended in 60% w/v sucrose, balanced, vortexed and centrifuged for two minutes at 10 000 *g* at 4 °C. The sucrose was carefully washed-off the spores with distilled water in a 45 µm sieve. Spore cocktails (± 500 mixed spores) were collected on glass fibre filter-paper (Advantec GA-55) by means of vacuum-filtration using the Buchner Vacuum Filtration System. In order to ensure adequate representation of all species present inocula of approximately 500 spores per pot was mixed into the top 5 cm of soil. Differences in field spore densities were disregarded. Indigenous AM fungal spores were site specific while non-indigenous AM fungal spores were non-site specific.

2.2.4 Experimental design of the glasshouse pot trial

Substrate soils were collected from two different sugarcane fields: non P-fixing sandy soil (Fernwood series, FAO: Dystric and Eutric Rhegosols, USDA: Entisols) from La Mercy (L) on the KwaZulu-Natal north coast (29°37`S - 31°7`E) and P-fixing soil (Cartref series, FAO: Gleyic luvisol, USDA: Inceptisol) from Klipp III (K)

(29°21`S - 30°43`E). Soils were sieved through a 2 mm mesh screen to remove gravel and debris before being decanted into 5 L pots and subjected to the following three treatments in a completely randomised fashion.

Sterilized soil (S) – soils were autoclaved (121 kPa, 120 °C for 40 min followed by a second sterilization 24 h later). This treatment was used to assess the effects of indigenous AM fungi on sugarcane growth levels (n=10). Indigenous AM fungi refers to AM fungi isolated from the matching soil type i.e. AM fungi from P-fixing/non P-fixing soils were inoculated into pots containing P-fixing/non P-fixing soils respectively. Controls for this treatment were not inoculated with AM fungi (n=10).

Non sterile soil (NS) - were used to mimic natural field conditions in which indigenous microflora including AM fungi were present (not identified – but detected through AM fungi spore extraction (n=10). The effects of "non-indigenous" AM fungi on sugarcane growth levels were also assessed (n=10). Non-indigenous AM fungi refers to AM fungi isolated from the unmatched soil type i.e. AM fungi from P-fixing/non P-fixing soils were inoculated into pots containing non P-fixing/P-fixing soils respectively.

Sterilized washed soil plus soil bacterial microflora(SW) – soils were autoclaved (121 kPa, 120 °C for 40 min followed by a second sterilization 24 h later), washed to remove excess nutrients from denatured organic material and dead matter and supplemented with an indigenous bacterial solution. This treatment was set up to assess the effects of indigenous microflora on sugarcane growth levels (control, n=10). Additionally the interactive effects of microflora and both "indigenous" and "non-indigenous" AM fungi on sugarcane plants were also assessed (n=10).

Bacterial inocula were prepared by blending 200 cm^3 of the matched soil with 500 mL water and passing the slurry through 500 µm, 90 µm, 50 µm and 38 µm sieves, which trapped the debris and AM fungal spores and nematodes (discarded) while smaller microflora were collected in a beaker.

To stimulate plant germination, single-budded sugarcane setts (N12) were placed in hot water baths at 50 °C for 20 min before planting. AM fungi inocula consisting of approximately 500 AM spores were mixed into the top 5 cm potted soil before the setts were planted.

Pots (5 L) were placed in the glasshouse, temperatures were regulated within the 28 °C - 30 °C range and relative humidity between 35-35% range. Plants were irrigated once a day and rotated every four weeks. Sugarcane height (stalk length - cm) was measured every four weeks, for a period of twenty-eight weeks, before harvesting i.e. heights were measured during the vegetative phases of sugarcane growth: emergence and seedling (month 1), tillering (months 2-5) and elongation (months 6-7). At harvest the above ground biomass (g) was determined.

2.2.5 Statistical Analysis

One way Analysis of variance (ANOVA) was performed on all data collected using SAS-General Linear Model (GLM) procedure (SAS, 2008); differences among treatments and the means were separated using the Fischer's Least Significant Difference (LSD) test at 5% level.

2.3 RESULTS

2.3.1 Field variety trials

The mycorrhizal status of the five varieties, N12, N31, N35, N37 and N39, grown under P-fixing and non P-fixing field conditions were determined. Table 2.1 shows mean percentage AM fungi colonisation of sugarcane roots between the non P-fixing and P-fixing soil types for the same variety. All mycorrhization means were less than fifty percent but only N35 variety showed significantly lower mycorrhization (P = 0.008) in P-fixing soil whereas no differences between soils were found for the other varieties. N12 variety had the highest overall percentage AM fungi colonization followed by N39, N35, N31 and N37. Thereafter, percentage mycorrhization was compared between the respective

varieties grown in the same field (Table 2.2). No significant differences in mycorrhization between varieties grown in the non P-fixing fields were observed. However, N12 and N39 showed significantly higher growth than N37 in the P-fixing soil (Table 2.2). Overall, N12 appeared to be the most mycotrophic, thus most suitable for the glasshouse pot trial.

Table 2.1: Mean percentage mycorrhization of individual commercial sugarcane varieties compared between Zinkwazi II and Klipp III soil types. Means indicated with the same letters were not significantly (ns) different ($P > 0.05$) and s = significantly different ($P \leq 0.05$).

Soil types	**Mean percentage mycorrhization (± SE)**
N12 Zinkwazi II	37.33 ± 6.26 [a]
N12 Klipp III	41.83 ± 3.41 [a]
Significance level	$P = 0.542$ (ns)
Fishers LSD (5%)	15.892
N31 Zinkwazi II	30.83 ± 6.10 [a]
N31 Klipp III	30.83 ± 3.79 [a]
Significance level	$P = 1.000$ (ns)
Fishers LSD (5%)	15.993
N35 Zinkwazi II	38.33 ± 3.06 [a]
N35 Klipp III	26.17 ± 2.09 [b]
Significance level	$P = 0.008$ (s)
Fishers LSD (5%)	8.259
N37 Zinkwazi II	32.50 ± 4.12 [a]
N37 Klipp III	27.50 ± 5.68 [a]
Significance level	$P = 0.492$ (ns)
Fishers LSD (5%)	15.634
N39 Zinkwazi II	41.00 ± 3.88 [a]
N39 Klipp III	31.50 ± 6.18 [a]
Significance level	$P = 0.222$ (ns)
Fishers LSD (5%)	16.259

Table 2.2: Mean percentage mycorrhization compared (n=6) across five varieties grown at Zinkwazi II and Klipp III respectively. Means indicated with the same letter were not significantly (ns) different ($P > 0.05$) and s = significantly different ($P \leq 0.05$).

Varieties	Mean percentage mycorrhization (±SE)
Zinkwazi II N12	37.33 ± 6.26 [a]
Zinkwazi II N31	30.83 ± 6.10 [a]
Zinkwazi II N35	38.33 ± 3.06 [a]
Zinkwazi II N37	32.50 ± 4.12 [a]
Zinkwazi II N39	41.00 ± 3.88 [a]
Significance level	*P* = 0.564 (ns)
Fishers LSD (5%)	14.139
Klipp III N12	41.83 ± 3.41 [a]
Klipp III N31	30.83 ± 3.79 [ab]
Klipp III N35	26.17 ± 2.09 [ab]
Klipp III N37	27.50 ± 5.68 [b]
Klipp III N39	31.50 ± 6.18 [a]
Significance level	*P* = 0.145 (ns)
Fishers LSD (5%)	13.077

2.3.2 Glasshouse pot trial

Statistically analysed results comparing sugarcane growth levels and above ground biomass for three treatments: *Sterilized soil (S); Non-sterile soil (NS) and Sterilized washed soil plus soil bacterial microflora (SW)* either inoculated with AM fungi or uninoculated grown in P-fixing (K) and non P-fixing (L) soils, are presented in Table 2.3.

During the emergence and seedling phase (month 1), in P-fixing (K) soils: a) stalk heights were significantly higher in plants grown in sterilized soil and inoculated with AM fungi (KSA) compared to uninoculated sterilized soil (KS), indicating the enhanced effect of indigenous AM fungi; b) stalk heights were significantly

higher in plants grown in sterilized washed soil plus soil microflora and inoculated with AM fungi from both K and L (KSWA) compared to all other treatments besides KSA; c) growth levels of plants grown in non-sterile soils (KNS) already containing AM fungi and microflora, were not significantly different from plants already containing indigenous AM fungi and microflora and inoculated with non-indigenous AM fungi from L (KNSA); d) AM fungi inoculated plants grown in sterilized washed soil plus soil microflora (KSWA) were also significantly taller compared to plants grown in uninoculated sterile soil plus microflora (KSW) and non-sterilized (natural) soils with non-indigenous AM fungi form L (KNSA); further no significant differences were found in growth levels between KSW and KNSA (Table 2.3).

In non P-fixing (L) soils: e) stalk heights were also significantly higher in plants grown in sterilized soil and inoculated with AM fungi (LSA) than uninoculated sterilized soil (LS), also indicating the advanced effects of indigenous AM fungi; f) growth levels of plants grown in non-sterile soils (LNS) already containing AM fungi and microflora were significantly lower from plants already containing indigenous AM fungi and microflora and inoculated with non-indigenous AM fungi from K (LNSA), indicating AM fungi from P-fixing soils influenced plant growth in non-sterile soils; g) stalk elongation were significantly higher plants inoculated with AM fungi and grown in sterilized (LSA) compared to non-sterile soil (LNSA); h) stalk lengths appeared higher, but not significantly so in the AM fungi inoculated plants grown in sterilized washed soil plus soil microflora (LSWA) compared to the uninoculated sterilized washed soil plus soil microflora (LSW) (Table 2.3).

Table 2.3: LSD analysis of mean stalk length (cm) per treatment (n=10) per month for N12 sugarcane grown under three different treatments in P-fixing soil (Klipp III) and non P-fixing soil (La Mercy 2-2). LSD analysis of mean biomass (g) of stems and leaves per treatment (n=10) harvested after 7 months. Treatments indicated with the same letter were not significantly different (ns) ($P > 0.05$) and s = significantly different ($P \leq 0.05$).

		Mean stalk length (cm) ± SE per month														Mean Biomass (g) ± SE	
Soil type	**Treatments**	**1**		**2**		**3**		**4**		**5**		**6**		**7**			
P-fixing	KS	9.85 ± 1.16	e	20.6 ± 1.28	a	26.65 ± 0.92	a	29.30 ± 0.83	a	38.45 ± 1.85	b	61.80 ± 2.99	def	80.45 ± 3.71	d	117.92 ± 20.96	c
	KSA	13.70 ± 1.15	ba	22.7 ± 1.25	a	27.15 ± 1.38	a	31.10 ± 1.78	a	39.90 ± 3.23	b	64.40 ± 4.39	cde	84.60 ± 6.06	cd	138.056 ± 31.84	bc
	KNS	9.70 ± 0.70	de	14.85 ± 0.98	b	16.85 ± 1.75	b	20.20 ± 1.19	b	29.55 ± 2.30	c	58.90 ± 7.90	ef	92.30 ± 12.62	bcd	179.334 ± 36.26	abc
	KNSA	10.80 ± 0.89	bcde	13.65 ± 0.72	b	17.10 ± 0.87	b	19.70 ± 1.10	b	26.70 ± 1.99	c	50.60 ± 4.40	f	78.40 ± 5.01	d	103.335 ± 24.10	c
	KSW	10.75 ± 1.10	bcde	21.25 ± 1.62	a	27.75 ± 1.53	a	31.20 ± 1.46	a	44.50 ± 4.31	ab	85.55 ± 5.21	a	110.75 ± 7.01	a	223.96 ± 26.11	abc
	KSWA	14.35 ± 0.86	a	21.6 ± 1.32	a	26.05 ± 1.71	a	28.70 ± 1.80	a	41.45 ± 3.37	ab	74.50 ± 4.82	abcd	102.00 ± 5.90	abc	163.13 ± 31.33	abc
non P-fixing	LS	10.05 ± 1.26	de	22.3 ± 1.25	a	28.75 ± 1.52	a	31.55 ± 1.61	a	48.35 ± 2.06	a	82.35 ± 4.95	ab	108.65 ± 5.42	ab	206.94 ± 27.06	abc
	LSA	13.10 ± 1.28	abc	23.10 ± 1.53	a	26.70 ± 1.72	a	29.35 ± 1.61	a	41.35 ± 1.62	ab	77.45 ± 4.31	abc	102.10 ± 6.57	abc	177.66 ± 28.30	abc
	LNS	9.00 ± 0.91	e	15.40 ± 1.08	b	18.95 ± 1.00	b	20.95 ± 1.04	b	28.25 ± 2.75	c	61.50 ± 3.78	def	93.45 ± 5.94	abcd	163.91 ± 29.46	abc
	LNSA	12.30 ± 1.14	abcd	16.55 ± 1.02	b	20.00 ± 1.15	b	22.05 ± 1.20	b	26.85 ± 1.76	c	61.35 ± 5.05	def	90.10 ± 5.24	cd	142.55 ± 23.21	bc
	LSW	10.50 ± 1.19	de	21.45 ± 0.85	a	27.60 ± 1.03	a	28.55 ± 0.60	a	41.15 ± 2.10	ab	68.85 ± 3.54	bcde	89.10 ± 4.93	cd	145.98 ± 23.75	bc
	LSWA	12.30 ± 0.90	abcd	22.90 ± 1.41	a	27.20 ± 1.71	a	31.20 ± 2.06	a	44.00 ± 3.29	ab	74.05 ± 5.40	abcd	91.70 ± 5.48	bcd	151.78 ± 24.45	abc
P value		0.0046	(s)	< 0.0001	(s)	< 0.0001	(s)	< 0.0001	(s)	< 0.0001	(s)	< 0.0001	(s)	0.0067	(s)	0.1211	(ns)
LSD		2.9700		3.4219		3.9184		3.9803		7.5002		13.6570		18.2490		77.256	
Alpha = 0.05																	

Key

K	Klipp III	L	La Mercy 2-2
S	sterile	S	sterile
SA	sterile + indigenous AM fungi from Klipp III	SA	sterile + indigenous AM fungi from La Mercy 2-2
NS	non -sterile	NS	non -sterile
NSA	non sterile + AM fungi from La Mercy 2-2 (non-indigenous)	NSA	non sterile + AM fungi from Klipp III (non-indigenous AM fungi)
SW	sterile + washed + bacterial solution from Klipp III	SW	sterile + washed + bacterial solution from La Mercy 2-2
SWA	sterile + washed + bacterial solution + AM fungi from Klipp III (indigenous) and La Mercy 2-2 (non-indigenous)	SWA	sterile + washed + bacterial solution from La Mercy 2-2 + AM fungi from La Mercy 2-2 (indigenous) and Klipp III (non-indigenous)

During the tillering phase (months 2-5) in both P-fixing (K) and non P-fixing (L) soils: a) significantly taller plants occurred in sterile soils compared to non-sterile soil with or without AM fungi; b) no significant differences occurred between sterile treatments irrespective of AM fungi inoculation; c) plants grown in sterilized soil, washed to remove dead matter and inoculated with indigenous bacterial microflora (SWA) were significantly taller than plants grown in natural soils (Table 2.3) .

During the elongation phase (months 6-7): a) In both soil types longer stalks were generally significantly higher in inoculated sterile soils (SA) than in inoculated natural soils (NSA); b) in both soil types there were no differences between any of the treatments irrespective of AM fungi inoculation. In P-fixing soils, stalks were significantly higher in sterile washed and AM fungi inoculated soil (SWA) compared to AM fungi-inoculated natural soils (NSA) (Table 2.3).

There were no significant differences between treatments and controls in above ground biomass (Table 2.3).

2.4 DISCUSSION

The AM status of several South African commercial cane varieties growing in KwaZulu-Natal province in P-fixing soil in the midlands and non P-fixing soil on the coast was confirmed in the field study i.e. all varieties of sugarcane grown in KwaZulu-Natal are naturally mycotrophic. AM fungi root analysis of field varieties indicated mycorrhization to be generally less than fifty percent. N12 showed overall higher mycorrhization but only to N37 and N39 and only in P-fixing soil. Only the N35 variety showed different levels of colonization in different soils types at the time of sampling. Overall, levels of mycorrhization in respective varieties were very similar, suggesting that breeding had not interfered with the genetic pre-disposition of the plant to form AM and that edaphic characteristics are not strong deterministic factors in the plant's dependence on AM.

The interrelationships between AM fungi and other soil microorganisms is dynamic: some studies have indicated soil microorganisms to enhance spore germination, host root colonisation and mycorrhizal plant growth (Azcon-Aguilar and Barea, 1985, Azcon-Aguilar *et al.,* 1986, Meyer and Linderman, 1986), while other studies indicated suppression of AM fungi effects by soil microorganisms (Hetrick *et al*., 1986; Ross, 1980; Hetrick *et al*., 1987; Wilson *et al.,* 1988. Furthermore, in sugarcane agroecosystems, plants are not grown under sterilized soil conditions yet most scientific experiments compare mycorrhizal plants with non-mycorrhizal plants growing in sterilized soils only and the effects of microflora in non-sterile soils were not taken into consideration in these studies.

For these reasons, the glasshouse experiment was designed to further assess if AM fungi and microflora levels enhanced or suppressed growth levels of N12 sugarcane grown in both P-fixing and non P-fixing soil under three treatments: Sterilised soil (S); Non-sterile soil (NS), and Sterilised washed soil plus soil bacterial microflora (SW) with or without combinations of AM fungi inocula. Unfortunately, root analysis of AM fungi colonisation for this pot trial could not be fully established as some softer roots disintegrated during the clearing process using 10% potassium hydroxide and so correlations between growth and levels of colonisation in AM fungi-inoculated and non-sterile treatments could not be made due to root fragility. Although the use of cocktails of isolated native spore inocula did not provide information on the individual AM fungi taxa-plant interactions, the limited time-frame did not allow the establishment of trap cultures to produce multiple pure indigenous AM fungi cultures and spore viability assays were not conducted during the usage of the inocula. Since multiple species of AM fungi co-exists in agroecosystems, using “indigenous” site specific mixed whole spore inoculum for this study was preferred.

The overall picture clearly indicates that patterns in differences between treatments are affected by the growth phase and the soil type. For example, during the seedling stage the pattern of differences in both soils are similar, with

highest growth in all treatments receiving AM fungi inoculum (Table 2.3). However, by the elongation phase in the P-fixing soil, growth tended to be higher in sterile treatments with microbial additions with or without AM fungi inoculum; whereas in the non P-fixing soil, growth also tended to be higher in sterile treatments with or without AM fungi inoculum, but without a microbial addition (Table 2.3). There are a number of variables, which would determine these patterns, which in a preliminary study of this nature were not monitored.

Sterilization alters the structure and physico-chemical properties of the soil (Smith and Smith, 1980) where beneficial and toxic nutrients may be released into the soil (Warcup, 1957; Wallace *et al*., 1973) and data on the physico-chemical characteristics of the Klipp III and La Mercy 2-2 soils or the effects of autoclaving on those were not available. Non-sterile soils also contain microorganisms such as the native AM fungi, pathogenic fungi, beneficial and pathogenic bacteria, nematodes that may have been removed (Linderman, 1988), and there was no data available on the biotic components apart from confirmation that both soil types did contain AM fungi.

The correlation of growth response to AM fungi colonisation levels could not be analysed because of the lack of colonisation data. However, the ability of sugarcane to grow comparatively well in sterile soils without any additions suggests it is not obligately dependent on AM fungi, unlike the peanut (*Arachis hypogaea*) which appears to be obligately mycotrophic (Middleton *et al*., 1989; Al-Khaliel, 2010). Middleton *et al*. (1989) observed that effective soil sterilisation caused significant reductions in peanut plant growth and these could be reversed by the addition of AM fungi inoculum, and were able to measure a high degree of correlation between root colonisation levels and plant growth parameters. Al-Khaliel *et al.* (2010) also observed the positive effect of added AM fungi inoculum and soil microbiota in sterilised soils on growth of peanut, using up to 12 different growth indices, but because they used species-specific inocula of *Glomus mosseae* and *Glomus fasciculatum* could conclude that soil type could

influence the effectiveness of specific AM fungi inocula. These authors also suggested that in their study, the suppressive effects of other soil microorganisms in non-sterile soils on growth were largely by way of their competitive effects in general or due to presence of mycoparasites, and that the positive effects of the addition of a microbial solution as a treatment could be concentration-dependant (Al-Khaliel *et al*., 2010).

In terms of the interactions between different treatments and growth stage, Al-Khaliel *et al*. (2010) found no effect of AM fungi inoculation on growth of peanut in the first month and Middleton *et al*. (1989) observed the positive influence of AM fungi on growth only after flowering. In this study, positive effects of AM fungi were observed in the first month of growth, but by the elongation phase the positive effects of AM fungi were not clear-cut and the study did not continue into the maturation and flowering stages of growth.

2.5 CONCLUSION

The results presented in this preliminary study, illustrated that South African varieties of sugarcane growing in the field are naturally mycotrophic and that sugarcane growth is enhanced rather than suppressed by AM fungi and bacterial microflora. It may be that the positive effects of AM fungi in sugarcane occur during the early establishment phase but AM fungi are less important later on and that sugarcane is not obligately mycotrophic. Although these are important features, they require more rigorous research involving assessment of a greater range of growth parameters (including spore viability assays) longer into the growth cycle of the plant as well as AM colonisation data to better understand the interactions between favourable AM fungi and microflora for AM sugarcane.

CHAPTER 3

MULTIVARIATE ANALYSIS OF PLANT-SOIL NUTRIENT RELATIONSHIPS IN ARBUSCULAR MYCORRHIZAL SUGARCANE GROWN IN THE NORTH COAST REGION OF KWAZULU-NATAL, SOUTH AFRICA

3.1 INTRODUCTION

Arbuscular mycorrhizal fungi are obligate biotrophic symbionts (Smith and Smith, 2012) that colonise sugarcane roots (Kariman *et al*., 2005; Srikumar *et al*., 2009; Rokni *et al*., 2010, Rokni and Goltepeh, 2011,) and improve the functioning of the root system within the soil. This symbiotic association allows AM fungi to produce extraradical hyphae that extend several centimetres into the soil, binding the soil aggregates, which in turn helps maintain the soil structure and microbial diversity, establishing a hyphal network. The extraradical hyphae create a surface area greater than the roots alone, enabling the plant better nutrient and water access by exploring larger volumes of soil and overcoming nutrient and water depletion zones (Rhodes *et al*., 1975, Hetrick *et al*., 1988; Clark and Zeto, 1996a-c).

As obligate biotrophs AM fungi are dependent on their host for their C supply, as they cannot absorb sugar through their extraradical mycelium. Once absorbed the C is incorporated into lipids, which are metabolized through the glyoxylate cycle, and transported to the rest of the mycelium. This extraradical mycelium in turn, enables an extended “root system”, which allows the absorption of nutrients such as phosphorus, zinc and copper to the intraradical hyphae, and subsequent transfer to the host (Bolan, 1991, Pfeffer *et al*., 1999; Hammer *et al.*, 2011).

AM fungi therefore may play an essential role in accommodating the mechanism of sucrose formation in sugarcane, as many nutrients are required for the structural, functional, electrochemical and metabolic functions, as well as for growth, maintaining the plant lifecycle and ratooning in sugarcane.

Macronutrients include nitrogen (N) phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulphur (S). Micronutrients include iron (Fe), copper (Cu), manganese (Mn), zinc (Zn), boron (Bo) molybdenum (Mo) and chlorine (Cl) (Sinha, 2004). Beneficial but non-essential nutrients include sodium (Na), cobalt (Co), vanadium (Va), nickel (Ni), selenium (Se), aluminium (Al) and silicon (Si) (Sinha, 2004).

Furthermore, elements are classified according to their functionality. Structural elements such as carbon (C), hydrogen (H) and oxygen (O) are important for carbohydrate synthesis in the cell wall and for formation of the protoplasm framework. S, P and N are important protoplasmic elements required for protein synthesis (Sinha, 2004). Mg and N are constituents of chlorophyll. Ca forms calcium pectate that contributes to the cell wall, while P is the constituent of the nucleo-protein. Several micronutrient elements such as Fe, Cu, Mo, Zn, Mn and Cl have catalytic roles as cofactors of apoenzymes. Monovalent cations increase membrane permeability, while di- and trivalent cation decrease permeability. Cell sap pH is variable and fluctuations are maintained by the phosphate system that acts as the mineral element buffer (Sinha, 2004).

Osmotic potentials are maintained by mineral sap containing water soluble elements and K in the guard cells maintain the osmotic balance. Ca, K and Mg neutralize the toxic effects of other elements by maintaining ion balance. Mn, for example, is toxic to barley plants when present in the 300-400 $mg.kg^{-1}$ range. Si however, neutralizes this toxic effect. Other potentially toxic elements such as arsenic (As), Cu and mercury (Hg) are similarly neutralized (Sinha, 2004).

Mechanisms of sucrose formation, accumulation and storage include: i) energy capture from solar radiation involving photosystem I and II, ii) non-/cyclic photophosphorylation, iii) ferrodoxin and photoreduction of $NADP^{+}$, iv) CO2 assimilation via the C4 pathway (Alexander, 1973). According to Glasziou and Gayler (1972), movement of sugars occur between phloem and free space (which includes cell walls). In this free space, sucrose is hydrolysed to glucose and

fructose by acid invertase, before carrier-mediated transfer into metabolic compartments of the parenchyma cells occur. Here phosphorylation, interconversion, synthesis of uridine diphosphate-glucose and synthesis again to a sucrose phosphate occur. The sucrose phosphate molecule is then transferred to the storage compartment, where it is hydrolysed by acid invertase (immature tissue only) to sucrose once again (Alexander, 1973).

Further, sugarcane growth requires suitable soil, ideally: fertile, deep (up to 1.5 m), well drained and aerated soil (air-filled porosity: 10 – 12%) which is loamy to clayey in texture with a bulk density of 1.1 to 1.4 $mg.m^{-3}$. Topography should be moderate (1 to 3°), soil pH optima: 6.5 (range: 5 to 8.5), available water holding capacity: 150 $mm.m^{-1}$ depth of soil, groundwater table: below 1.5 to 2 m, critical soil salinity level (EC_e – Electrical conductivity if the extract) : <1.7 $dS.m^{-1}$ above which yields decrease (Netafim, 2012). For good sucrose yields the recommended sugarcane nutrient uptake per ton of cane yield is, 0.7 – 1.2 kg N, 0.4 – 0.8 kg P_2O_5 (phosphorus oxide), and 1.8 – 3.5 kg K_2O (potassium oxide). Optimum leaf nutrient levels required for good sucrose yields are also required (Table 3.1) and peak water requirements are 6 – 7 mm per day in South Africa (Netafim, 2012).

In chapter 2, the mycotrophic nature of sugarcane in South Africa was confirmed and some light was thrown on the influence of soil microbiota on growth in two soil types, without reference to actual soil nutrient status. Given the paucity of studies on the functioning of AM in sugarcane, this study was undertaken to assess the relationship between soil nutrients and uptake (nutrients in the leaves) in the presence of AM fungi, to ascertain the symbiotic role of AM in the nutrient uptake of N12 sugarcane. Since N12 was the most mycotrophic of all the commercial cane varieties it was chosen for this investigation. The design of the study involved using multivariate statistics to analyse the relationships between soil and plant nutrients and degree of mycorrhization of a single variety of sugarcane growing in a non P-fixing sandy soil.

3.2 MATERIALS AND METHODS

3.2.1 Sampling

An existing 4000 m^2 sugarcane plantation consisting 71 plots of 14 month-old N12 variety sugarcane was used for this investigation. The trial was located at La Mercy 2-2 on the KwaZulu-Natal north coast (29°36`S - 31°06`E). The field, which was unfertilized and known to be affected by salinity, was divided into 72 plots. The numbers of plants per plot varied as ratoons do not yield exact numbers. Soil and leaf sampling was done as part of a routine for FAS (fertiliser advisory service) analysis, in early summer, by the field team. A plant was randomly chosen within the plot and the third leaf sampled. The rhizospheric soil and roots were collected on the stool where the leaf was sampled.

3.2.2 Soil Analysis

Seventy-two soil samples (300 cm^3) were analysed for nutrients by the fertiliser advisory service (FAS) laboratory of the South African Sugarcane Research Institute (Barnard *et al.*, 1990; Meyer *et al*., 1997). Soil physico-chemical analysis included pH, P ($mg.kg^{-1}$), K ($mg.kg^{-1}$), S ($mg.kg^{-1}$), Ca ($mg.kg^{-1}$), Mg ($mg.kg^{-1}$), Na ($mg.kg^{-1}$), Zn ($mg.kg^{-1}$), Fe ($mg.kg^{-1}$), Mn ($mg.kg^{-1}$) and Al ($mg.kg^{-1}$) and % C (Table 3.2). N could not be analysed due to a technical problem with the FAS analyser at the time of the study. Soil was categorised as non P-fixing sandy soil, Fernwood series, FAO: Dystric and Eutric Rhegosols, USDA: Entisols.

3.3.3 Leaf Analysis

In the leaves N (%), P (%), K (%), S (%), Ca (%), Mg (%), Zn ($mg.kg^{-1}$), Mn ($mg.kg^{-1}$), Cu ($mg.kg^{-1}$), Fe ($mg.kg^{-1}$), N/S ($mg.kg^{-1}$) and Si (%) contents were analysed by FAS (Barnard *et al.*, 1990; Meyer *et al.*, 1997). Only 71 samples could be analysed.

3.3.4 Mycorrhization

Root samples (n=72) were analysed for AM fungi colonisation i.e. as a percentage of root length using the gridline-intersect method described in section 2.2.2.

3.2.6 Multivariate Statistical Analysis

The multivariate analysis software and graphical display: Analysis of Environmental Data software (ADE-4) (Thioulouse *et al*., 1997) was used to analyse the data viz. leaf and soil nutrient levels (see sections 3.2.2 and 3.2.3 above) measured on N12 sugarcane. The following methods were used in this analysis:

Convex hulls were used to determine if any measure of dissimilarity within the two mycorrhization categories (1) and (2), were influenced by soil factors. Graphically convex hulls joined the most extreme point of each cloud of points for each category (Thioulouse *et al*., 1997).

The principal component analysis (PCA) was then computed to calculate new synthetic variables or principal components, which are the linear combinations of the original variables accounting for as much of the variance in the original data as possible (Hotelling, 1933; Rammet, 2007). This was adapted as variables were expressed in different units ($mg.kg^{-1}$ and percentage), therefore data sets viz. objects (rows) and variables (columns) had to be represented in a new system of coordinates where the maximum amount of variation from the original data set was depicted on two axes (F1 and F2) (Ramette, 2007).

Permutation tests were used to determine if relationships between the leaf and soil tables were statistically significant, the random permutation test of the co-structure between two tables was performed using the ADE-4 co-inertia fixed-D test. One thousand permutations were used for an alpha level of 0.05 (Thioulouse *et al*., 1997).

Co-inertia analysis (COAI) which had been implemented only in the ADE-4 software (Thioulouse *et al*., 1997) was suitable for quantitative and/or qualitative environmental variables (Dray, 2003). Co-inertia analysis is a general coupling method that maximizes the co-inertia between the variables of the two tables' viz. leaf and soil tables for each category. In this study the PCA-PCA coupling method (Cadet and Thioulouse, 1998) was employed to check for co-structures

or relationships within the leaf-soil covariance matrix. The flexibility of the co-inertia analysis, allowed for a maximized covariance when factors were computed for each table (Thioulouse *et al*., 1997).

The co-inertia analysis was also used to compute and display a crossed table containing the sum of the cross products between the leaf and soil variables for each category, represented by either circles or squares. Circles corresponded to positive factorial values and squares corresponded to negative factorial values. The size of each symbol was proportional to the absolute value. Circles indicated a strong/stronger association/relationship between the leaf and soil variables. Squares indicated a weak/weaker association/relationship between the leaf and soil variables (Thioulouse *et al*., 1997).

3.3 RESULTS

Assuming AM colonisation to be caused by background or indigenous AM populations; to determine if AM fungi played a role in buffering effects of nutrient uptake between the soil and leaves, two mycorrhization categories were determined: (1) low percentage mycorrhization (range 9-26%) and (2) high percentage mycorrhization (range: 32-53%). Corresponding soil and leaf nutrient levels for the respective samples were matched and categorized according to these categories. Only 71 samples could be statistically analysed. One leaf sample was not fully analysed due to insufficient material.

The optimum nutrient levels required for good sucrose yields are indicated in Table 3.1. The average soil and leaf nutrient levels were also determined (Table 3.2). Results of the leaf analysis indicate that most of the nutrients except for K fell below the optima for good sucrose yields. In the high percentage mycorrhization, status category Mn and Fe seemed to be slightly elevated compared to the low percentage status plant values. However soil Na in highly mycorrhizal plants were lower (55.74 $mg.kg^{-1} \pm 6.04$) compared to low mycorrhization status soils (60.96 $mg.kg^{-1} \pm 7.50$) (Table 3.2).

Table 3.1: Optimum nutrient levels required for good sucrose yields (adapted from Myer *et al.*, 2011).

Leaf nutrients	**Optimum range**
N (%)	1.80-2.40
P (%)	0.19-0.25
K (%)	1.05-1.45
S (%)	0.12-0.20
Si (%)	0.75-1.50
Ca (%)	0.15-0.25
Mg (%)	0.08-0.18
Zn ($mg.kg^{-1}$)	13.0-20.0
Mn ($mg.kg^{-1}$)	15.0-75.0
Cu ($mg.kg^{-1}$)	3.0-7.0
Fe ($mg.kg^{-1}$)	75.0-200.0
B ($mg.kg^{-1}$)	2.0-7.0
Mo ($mg.kg^{-1}$)	0.05-0.15

Table 3.2: Physico-chemical soil and leaf mean nutrient levels for sugarcane N12 variety grown at La-Mercy 2-2, North coast region.

	Mean nutrient levels (±SE)			
Nutrient	**(1) Low % mycorrhization**		**(2) High % mycorrhization**	
Soil				
pH	5.23	± 0.11	5.18	± 0.12
P ($mg.kg^{-1}$)	38.59	± 2.88	39.21	± 3.38
K ($mg.kg^{-1}$)	66.30	± 5.53	63.13	± 5.73
S ($mg.kg^{-1}$)	11.11	± 0.33	11.38	± 0.65
Ca ($mg.kg^{-1}$)	113.44	± 13.21	110.29	± 14.49
Mg ($mg.kg^{-1}$)	22.93	± 2.02	22.50	± 2.50
Na ($mg.kg^{-1}$)	55.74	± 6.04	60.96	± 7.50
Zn ($mg.kg^{-1}$)	1.17	± 0.10	1.15	± 0.10
Fe ($mg.kg^{-1}$)	62.96	± 2.31	64.04	± 4.93
Al ($mg.kg^{-1}$)	7.07	± 0.30	7.58	± 0.26
Mn ($mg.kg^{-1}$)	7.22	± 0.57	7.92	± 0.95
C (%)	0.25	± 0.01	0.23	± 0.02
Leaf				
N (%)	1.40	± 0.03	1.38	± 0.03
P (%)	0.15	± 0.00	0.15	± 0.00
K (%)	1.39	± 0.03	1.43	± 0.03
S (%)	0.12	± 0.00	0.12	± 0.00
Si (%)	0.50	± 0.04	0.49	± 0.03
Ca (%)	0.22	± 0.01	0.21	± 0.00
Mg (%)	0.12	± 0.00	0.12	± 0.00
Zn ($mg.kg^{-1}$)	19.22	± 0.44	18.25	± 0.37
Mn ($mg.kg^{-1}$)	47.52	± 1.76	45.58	± 1.33
Cu ($mg.kg^{-1}$)	4.67	± 0.09	4.46	± 0.10
Fe ($mg.kg^{-1}$)	106.07	± 5.33	95.38	± 3.58
N/S ($mg.kg^{-1}$)	11.77	± 0.30	11.73	± 0.21

The ADE-4 multivariate analysis required the categorisation of the leaf and soil nutrient levels according to the mycorrhization to establish the coding within the ADE-4 programme i.e. 1 = low percentage mycorrhization and 2 = high percentage mycorrhization. Each category consisted of two tables i.e. leaf and soil nutrient level variables represented by twelve and eleven columns respectively.

Soil homogeneity of the entire soil sampling area, was established using convex hulls. Forty-two percent of the variability was kept to create the F1 and F2 axes of the factorial plan (Figure 3.1). Overlapping convex hulls and both gravity centres (category 1 and 2) located within the vicinity of each other indicated that the dissimilarities were influenced by a factor other than soil i.e. arbuscular mycorrhizas.

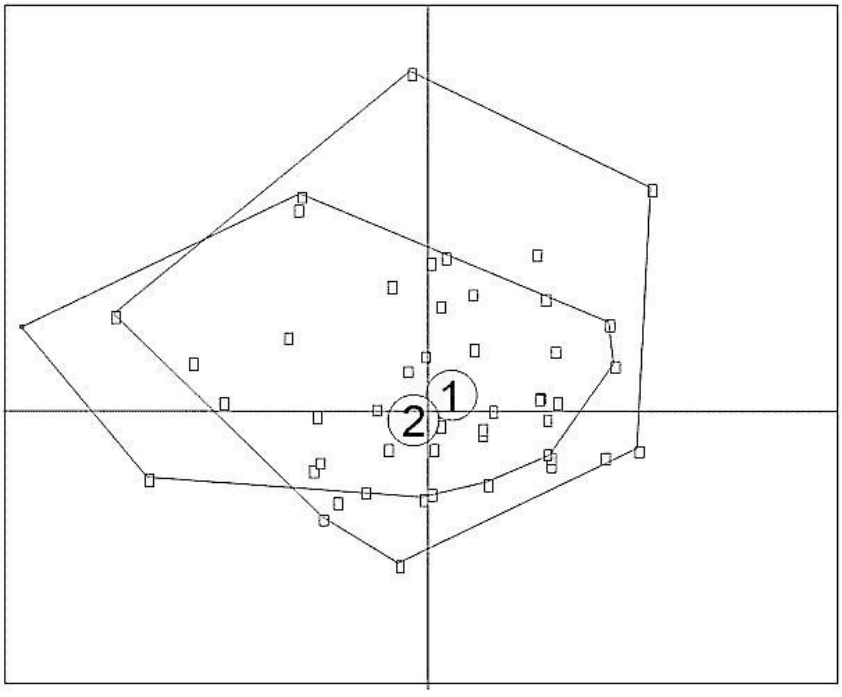


Figure 3.1: Convex hulls indicating homogeneity of soil factors for La Mercy 2-2 sampled area. Variables were categorized according to (1) low and (2) high mycorrhizal status.

For each soil and leaf table in the respective category a PCA was computed on correlation matrices to transform the data creating “dummy variables” or dimensionless variables removing the influence of magnitude differences between the units ($mg.kg^{-1}$) and percentages. A z-score transformation to the values of each variable was applied i.e. computing the difference between the original value and the mean variable (centering), subsequently dividing the difference by the standard deviation (SD) variable (standardization). PCA allowed the data to be represented in a new system of coordinates where the maximum amount of variation from the original data set was depicted on two axes (F1 and F2).

PCA correlation circles were used to graphically display the correlations amongst the standardized descriptor variables. Each arrow corresponded to one variable. Long arrows had a high correlation with factors. Arrows in the same direction corresponded to positively correlated variables, while arrows in opposing directions corresponded to negatively correlated variables. For low percentage mycorrhization status soil (Figure 3.2a) Ca, Mg and pH were found to be positively correlated with the first factor (F1), while Al was negatively correlated to the variables. In the low mycorrhization status leaf correlation circle (Figure 3.2b); K was well correlated with the positive F1. For high percentage mycorrhization status soil (Figure 3.2c) Ca and Mg, Zn and K were positively correlated. Al was negatively correlated to the pH and C. In the high mycorrhization status leaf (Figure 3.2d) correlation circle Ca is well correlated with the F1, Si and Zn were positively correlated and K was negatively correlated to Mg. PCA files were more importantly created to run the co-inertia analysis.

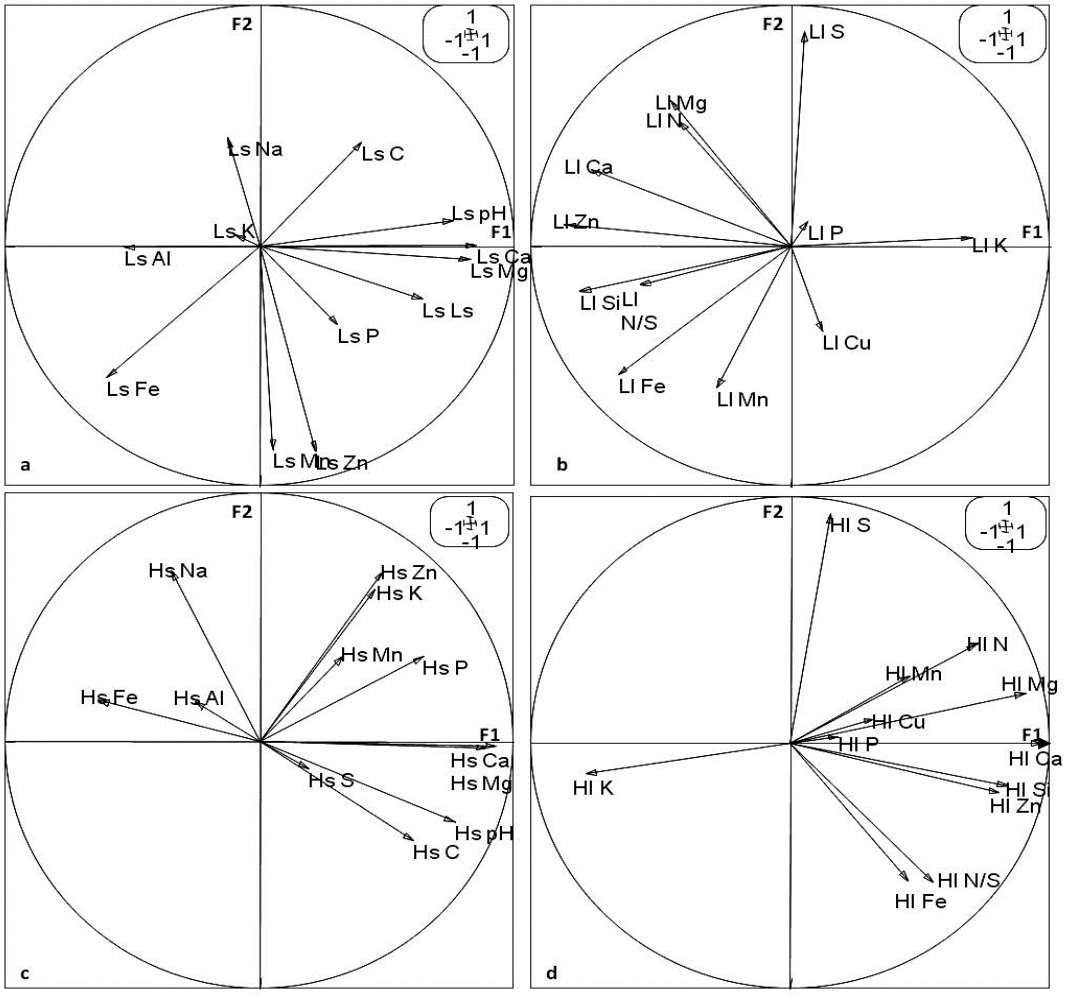


Figure 3.2a-d: Correlation circles (F1xF2) of the PCA for low (L) and high (H) mycorrhization status soil (s) and leaf (l) nutrient variables. Arrows in the same direction indicate positively correlated variables, while arrows in the opposite direction to each other indicate negatively correlated variables.

To determine if relationships between the leaf and soil tables were statistically significant, the random permutation test of the co-structure between two tables was performed (ADE-4 co-inertia fixed-D test).One thousand permutations were used for an alpha level of 0.05 ($P = 0.05$). Soil and leaf relationships were statistically significant: $P<0.001$ for the low percentage mycorrhizal status tables and $P<0.020$ for the high percentage mycorrhizal status tables, thus allowing the simultaneous reading of both soil and leaf tables in each category.

In the co-inertia analysis, for the rows of the two tables (soil and leaf) to be simultaneously permutated, the soil and leaf PCAs were normalized to correct the distribution shapes for variables that departed from the normality, to obtain a homogenous variance for variables known as factorial values. These factorial values were projected on leaf and soil factorial maps (Figure 3.3a-d) within each category. These normalised PCAs were important to create the files to run the ADE-4 co-inertia two tabled analysis. In the high percentage mycorrhization status category, 75% of variability was kept for the leaf tables and 68% of variability for the soil tables to create the F1 and F2 axes.

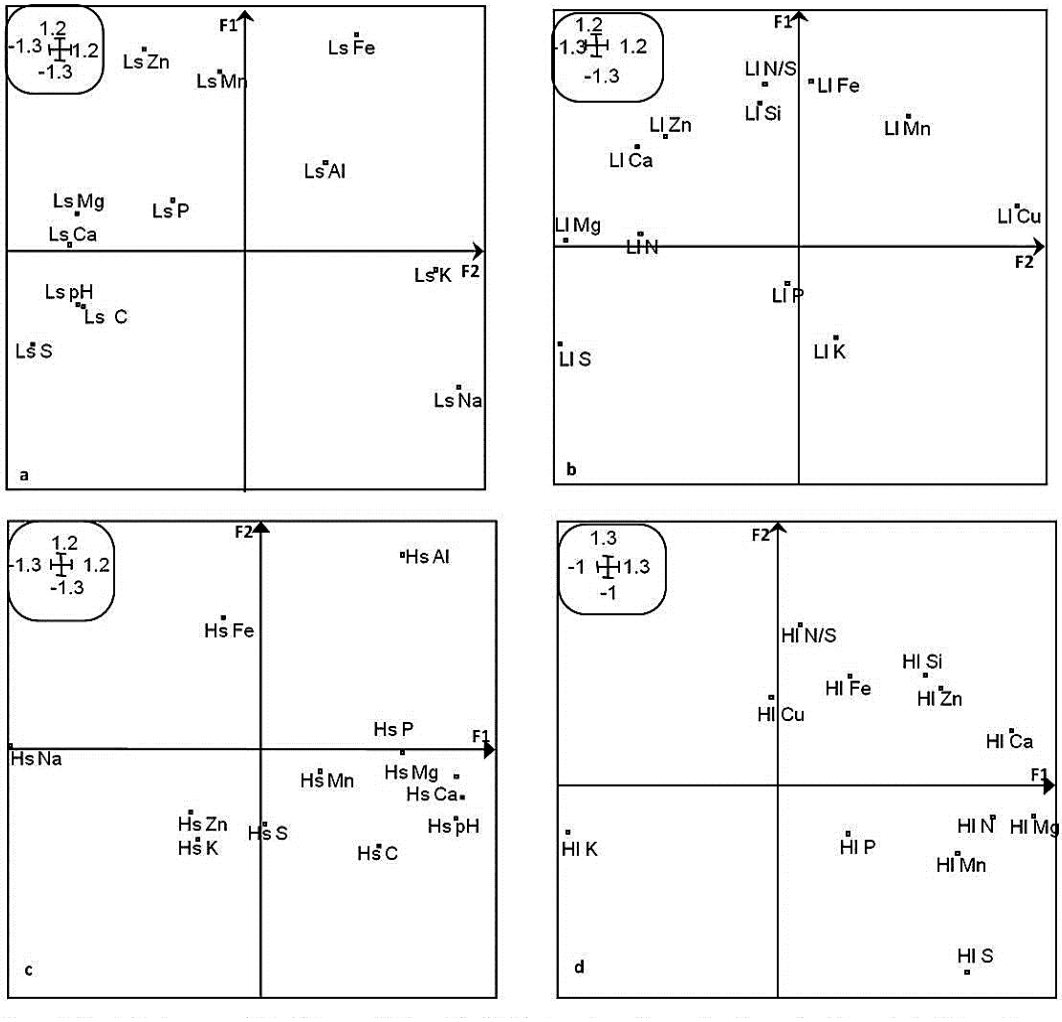


Figure 3.3a-d: Factor maps (F1XF2) for low (L) and high (H) percentage mycorrhization status soil (s) and leaf (l) factors transformed by the co-inertia analysis**.**

A summary of the two tabled analysis containing the sum of the cross products between the soil and leaf variables for each category was projected onto factor maps (Figure 3.4a-b). These describe all the relationships between soil and leaf nutrient levels and their relative importance. Each variable was located according to the first factorial values of both tables for each category i.e. factorial values indicated in Figure 3.3 a-d. For the leaf, the co-inertia analysis rotated the factorial plan of the PCA around F1. Circles and squares, corresponding to positive and negative associations respectively within each category, revealed relationships between the soil and leaf factors to be evidently similar or different in plants with high and low levels of mycorrhization.

More positive nutrient relationships were evident in highly colonized plants compared to lower percentage colonized plants (Table 3.3a). Positive relationships in high mycorrhizal plants appeared to be between: soil pH - leaf N, P, S, Mg and Mn; soil P - leaf Zn and Fe; soil Ca – leaf N, Ca and Mn, soil Mg – leaf Ca and Mn; soil Na – leaf K; soil Al – leaf A, Zn, Si, Ca, Mg and Fe, soil C – leaf P and Mn. Different positive relationships appeared in low percentage mycorrhizal plants viz. soil K – leaf Cu; soil S – leaf S and Mg; soil Zn – leaf Ca, Zn and Si; soil Fe – leaf Cu and Si; soil Mn- leaf Fe. Weak relationships in highly mycorrhizal plants occurred between: soil Mn, Zn – leaf Cu; soil pH, Ca, Mg, Al – leaf K, while in low percentage mycorrhizal plants: soil pH, Mg, C – leaf Cu; soil K – leaf N; soil Na – leaf Ca appeared to have weak relationships. Some relationships were not considered important either due to both categories having similar sized circles or squares i.e. nutrient values projected appeared were similar in size e.g. soil C – leaf K Table 3.3b); or were products of the crossed table indicated by a tiny circle/square in one category and a corresponding small square/circle in the other category e.g. soil P – leaf Cu (Table 3.3b).

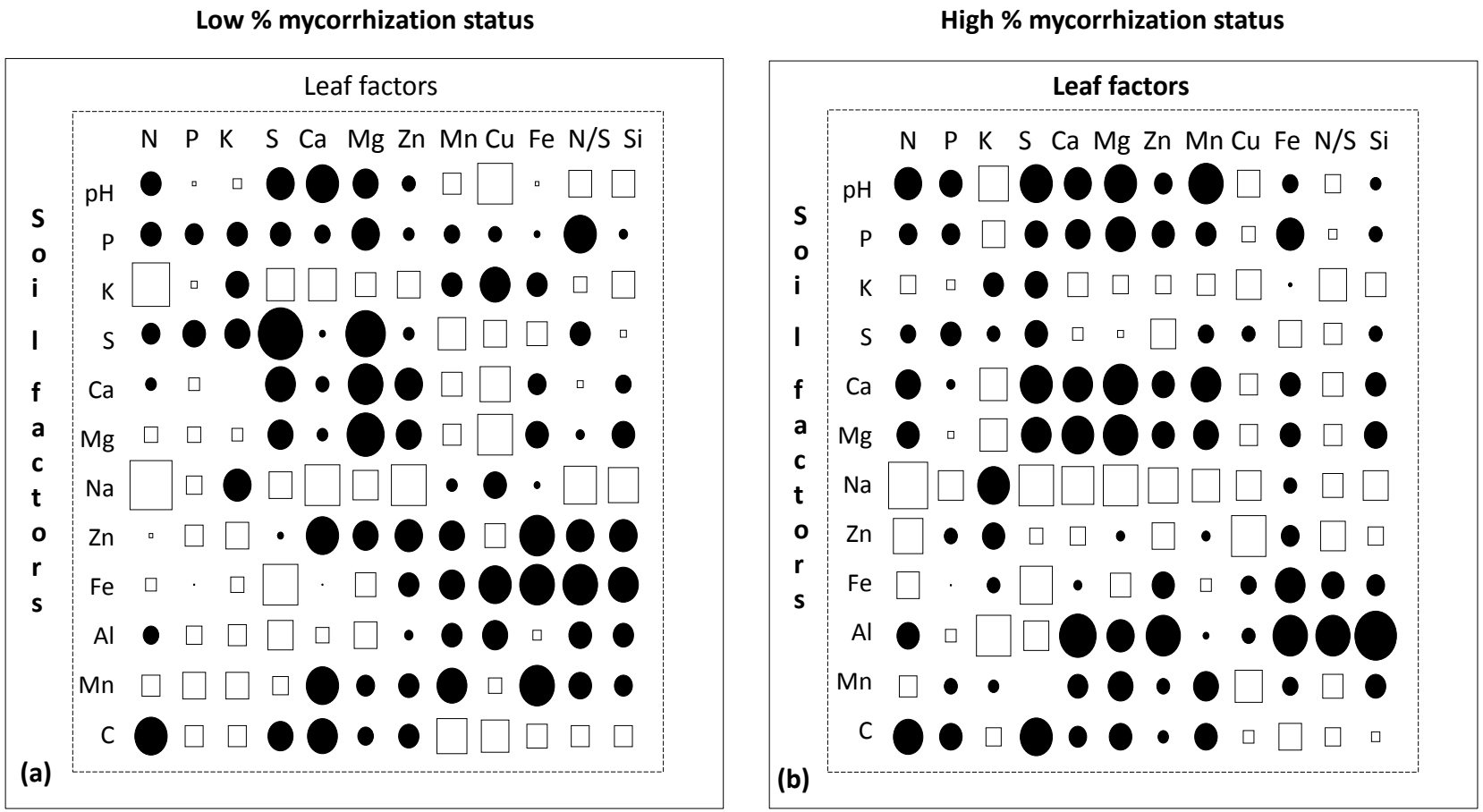


Figure 3.4: Co-inertia crossed tables indicating the sum of the cross products between soil and leaf factors for (a) low percentage mycorrhization status and (b) high mycorrhization status. Circles indicate positive or strong associations and squares indicate negative or weak associations. The size of the circles and squares are proportional to the absolute values.

Table 3.3: Highlighted nutrient relationships for (a) low and (b) high percentage mycorrhization status crossed tables.

Low percentage mycorrhization status			
Positive/Strong relationships (●)		**Negative/Weak relationships (□)**	
Soil	Leaf	Soil	Leaf
K	Cu	pH; Mg; C	Cu
S	S; Mg	K	N
Zn	Ca; Zn; Si	Na	Ca
Fe	Cu; Si		
Mn	Fe		
(a)			
High percentage mycorrhization status			
Positive/Strong relationships (●)		**Negative/Weak relationships (□)**	
Soil	Leaf	Soil	Leaf
pH	N; P; S; Mg; Mn	Mn; Zn	Cu
P	Zn; Fe	pH; Ca; Mg; Al	K
Ca	N; Ca; Mn		
Mg	Ca; Mn		
Na	K		
Al	N; Zn; Si; Ca; Mg; Fe		
C	P; Mn		
(b)			

3.4 DISCUSSION

Small-scale sugarcane farming is usually low-input and/or organic by default, due to the rising costs of fertilizers and agrochemicals (Cardoso *et al*., 2006). The use therefore of indigenous AM fungi is beneficial in terms of site-specific sustainability for these small-scale farmers i.e. enhanced acquisition of water, phosphorous and nitrogen (Evelin *et al*., 2009, Garg *et al*., 2010), translocation of other essential nutrients such as Zn and Cu to the host plant, increased overall absorption capacity of roots, protection against soil-borne pathogens, improved tolerance to abiotic stresses, increased production of growth hormones, maintaining soil aggregation stability, increased resistance to diseases, secretion of antibiotics, increased plant biodiversity of restored ecosystems (Evelin *et al*., 2009; Garg *et al*., 2010) contribution to weathering, dissolution and cycling of nutrients within the soil ecosystem (Finlay and Rosling, 2006; Wallander, 2006; Garg and Chandel, 2009; Helgason and Fitter, 2009). In sugarcane agriculture the

nutrient uptake and nutrient use efficiency of varieties play an important role in nutrient management (Schumann *et al.,* 1998 and Rakkiyappan *et al*., 2007).

The nutrient relationships formed in highly mycorrhizal and minimally mycorrhizal plants are of importance to sugarcane agriculture as nutrient requirements of sugarcane are determined based on the level of nutrients in select index tissues such as the leaves at specific ages of the crop (Lakshikantham, 1983). Although the exact regulation mechanisms of nutrient exchange is still in its research phase (Hammer, 2011; Smith and Smith, 2012), the further understanding of the nutrient relationships between the soil and low-input sugarcane under mycorrhizal conditions was required. An existing low-input field of fourteen month N12 sugarcane located at La-Mercy, KwaZulu-Natal was therefore ideal for this study.

Statistical analysis required both rhizospheric soil and corresponding leaves to be collected on the stool where the leaf was sampled. Soil and leaf samples were sent to the FAS for nutrient analysis, which was successfully carried out on all but one of the leaf samples, thus the respective soil and mycorrhization results could not be considered. Analysis of nutrient levels indicated the average soil pH to be acidic and below the optima for sugarcane. This soil was also non P-fixing. Plants grown on acidic soil often suffer mineral stresses and deficiencies (Marschner, 1991; Foy, 1992). We observed most leaf nutrient levels except for K to be below optima (Tables 3.1 and 3.2) indicating that these sugarcane plants should be deficient in P, Ca, Mg and K (Marschner, 1991; Foy, 1992). However, symptoms of deficiency usually observed were not visible, indicating that “something” was “buffering” the nutrient levels between the soil and sugarcane. Since results in the previous study already indicated N12 to be most mycotrophic (see section 2.3.1), it was then hypothesized that the “something” that buffered the nutrient levels in low-input sugarcane farming was the AM fungi.

Uptake of nutrients by sugarcane depends on the availability and the effectiveness of root systems for absorption. It is clear that the uptake by AM

fungi is influenced by low levels of micronutrients in the soil, with its effectiveness increased by the external hyphae (Liu *et al*., 2000). In this study most nutrient levels in the soil tested appeared to be low, in keeping with the general fact that acidic soils have low fertility and that the mechanism of tolerance of sugarcane to grow in acidic soil is influenced by AM fungi (Haynes and Hamilton, 1999).

To investigate this further, two categories in this field of sugarcane were established: (1) low percentage mycorrhization status and (2) high percentage mycorrhization status - no plants were non-mycorrhizal, further confirming the mycotrophic nature of sugarcane. The mycotrophic nature was also observed in Iran (Kariman *et al*., 2005; Rokni *et al*., 2010 and Rokni and Goltepeh, 2011) and South India (Srikumar *et al*., 2009) in many cane varieties. To understand the complexity of the nutrient relationships the ADE-4 multivariate statistical analysis package was used to analyse 71 roots, soil and respective leaf samples (as indicated one leaf sample could not be analysed by FAS).

Methods used in the multivariate analysis included permutations, convex hulls, principal component analysis and PCA-PCA coinertia analysis. Using convex hulls, soil homogeneity of the overall sampling unfertilized area was established, indicating relationships to be influenced by another factor viz. AM fungi colonisation, validating the hypothesis. Homogeneity allowed further analysis using the PCA-PCA co-inertia analysis, which determined the relationships between the nutrients in the soil sample and respective leaf to be either positive or negative in highly colonised plants compared plants having a low mycorrhizal status.

Statistical significance was established via permutation tests ($P = 0.05$) where $P < 0.001$ for the low percentage mycorrhizal status tables and $P < 0.020$ for the high percentage mycorrhizal status tables, thus enabling the simultaneous reading of both tables in each category. The ADE-4 programme effectively displayed the crossed tables containing the sum of the crossed products between the leaf and

soil variables for each category. Circles and squares were proportional to the leaf – soil cross products indicated either a strong/positive relationship or weak/negative relationships. Some relationships were not considered either due to both categories having similar nutrient values and were products of the crossed table indicated by a tiny circle/square in one category and/or a corresponding small square/circle in the other category. These crossed tables (Figure 3.4a-b) were used verify associations between soil nutrients and nutrients in the leaf in AM sugarcane.

Crossed tables indicated, positive relationships in highly mycorrhizal plants between soil pH and leaf N, P, S, Mg and Mn. Rhizosphere pH has been implicated in nutrient cycling within the soil, metabolic activities of microorganisms, AM fungi spore germination, AM fungi spore production, AM fungi colonisation and plant growth (Johnson *et al*., 1984; Wang *et al.,* 1993; Marschner, 1995 and Comerford, 1998, Clark *et al*., 1999b). In general phosphorus fertilizers (H_3PO_4) will inhibit AM fungi spore germination and spore production, as they reduce the pH of the zone around the fertilizer band to as low as 1.5, which then filters into the rhizosphere. This acidity of rhizospheric soil pH tends to reduce the uptake of soil nutrients such as P, Ca, Mg, K and Zn (Foy 1992; Clark *et al*., 1999b) by plants while increasing the solubility of Al and Mn making them toxic to plants at low concentrations (Clark *et al*., 1999b; Harter, 2007).

Mycorrhizal relationships were found to be the greatest when soil phosphorus levels were at 50 $mg.kg^{-1}$ or less (Schubert and Hayman, 1986). In this study, FAS soil analysis (Table 3.2) indicated P levels to be 39.21 $mg.kg^{-1}$ ± 3.38 (low mycorrhizal status) and 38.59 $mg.kg^{-1}$ ± 2.88 (high mycorrhizal status) which would be linked to the likelihood of AM fungi colonisation of sugarcane. The relationship between soil P and leaf Zn, Fe in highly mycorrhizal plants were stronger compared to minimally mycorrhizal plants, implicating AM fungi in micronutrient uptake, which correlates with results from studies done on maize

by Liu *et al*., (2000) i.e. mycorrhizal contribution to Zn, Cu, Mn and Fe uptake by maize was significantly influenced by soil P and micronutrient levels.

Using a pot experiment to demonstrate the action mechanisms of arbuscular mycorrhizal (AM) fungi in phosphorus (P) uptake, Sharif and Claassen (2011), reported increased P uptake of AM inoculated *Capsicum annuum L.,* plants to be caused by increased P concentration in the soil solution and by the increased P absorbing surface area coming from the external hyphae of AM fungi. According to Marschner (1986) the availability of Mn and Fe is dependent on both soil pH and redox potential. In highly mycorrhizal sugarcane the relationship between soil pH and leaf Mn, Fe were stronger compared to low mycorrhizal status plants, whereas in maize Mn acquisition in AM plants were found to be decreased (Kothari, 1991), while Fe uptake was increased in maize grown in Fe deficient soils (Clark and Zeto, 1996c).

Under sugarcane production acidification of soil occurs (Haynes and Hamilton, 1999). Since pH influences the P levels in the soil, which in turn influences the mycorrhizal development and root colonisation (Abbot and Robson, 1984), pH therefore influences the mycorrhizal development and colonisation. Acidity caused through the carbonation of soil water (pH 5.6) via respiration by plants, and the conversion of ammonium ions and phosphates also contribute to the pH value of the soil (Harter, 2007). The pH for low mycorrhizal plants (pH 5.18 ± 0.12) and that of highly mycorrhizal plants (pH 5.23 ± 0.11) was not drastically different, and fell within the pH range that promotes AM fungi germination, spore production and root colonisation (Clark *et al*., 1999b). Many studies have shown AM fungi to thrive in acidic soils compared to alkaline or neutral soils (Clark *et al*., 1999b, Clark, 1997). Another study conducted on the coast and midlands of KwaZulu-Natal indicated a decrease in soil pH, Ca and Mg while Al increased as the period under sugarcane cultivation increased (Qongqo and van Antwerpen, 2000).In this study sugarcane was shown to have AM fungi colonised

roots at pH ≥ 5.18, which correlates with results from studies done on switchgrass (Clark *et al*., 1999a-b).

Aluminium oxides begin to dissolve at about pH 5.5, but at pH below 4, free Al ions increase in the soil solution, making it toxic to plants (Harter, 2007). In this study Al toxicity in the soil was not a problem. Al oxides and Fe oxides absorb P, limiting its uptake by the plant (Clark *et al*., 1999b). This correlates to the crossed tabled products indicating the relationship between Al and P to be extremely weak in this soil type. This relationship was even weaker in highly colonised roots. Inorganic P is usually crystalized in insoluble forms as Ca, Fe and Al phosphates depending on soil pH. To compensate for this root exudates such as citrate and oxalate increases the availability of P, as Ca, Fe and Al are chelating agents (Marschner, 1995 and Comerford, 1998, Smith and Read, 2008).

The positive relationship between soil Al and leaf Si was more apparent in highly colonised roots. Sugarcane is a silicon accumulator, which strongly responds to Si supplies. Under field conditions cane yields in the leaf dry matter was found to increase when Si was increased (Anderson, 1991). In sugarcane, the absence of Si decreases the incorporation of inorganic phosphates into ADP, ATP and sugar phosphates (Cheong and Chan, 1973) in turn affecting photosynthesis and sucrose formation. Si prevents nutrient imbalance, metal toxicity and enhances plant tolerance to environmental stresses such as cold, heat, drought pests and disease (Vlamis and Williams, 1967; Meyer and Keeping, 2002). Above average Si uptake hence contributes to the many reasons why N12 sugarcane variety yields optimum sucrose and is most resistant to the stalk borer *Eldana saccharina*. Several reports in the literature suggest that Si nutrition has a definite agronomic role in sugarcane crop cultivation, especially on weathered tropical organic soils such as Entisols (Savant *et al*., 1999.)

In highly mycorrhizal plants the co-inertia analysis revealed the relationship between soil Ca, Mg and leaf Ca and showed Mg to be positively stronger compared to minimally colonised plants, further establishing the buffering

effects of mycorrhization. Mg and Ca are co-factors of chlorophyll and are enzyme activators and promote photosynthesis, plant growth and symbiosis (Marschner, 1995). Results indicated that the relationship between soil Ca, Mg and leaf K were weaker and that between soil Na and leaf K to be stronger in highly mycorrhizal plants, corresponding with the majority of other investigations that indicated lower K concentrations in mycorrhizal plants compared to non-mycorrhizal plants (Smith and Read, 2008). Potassium plays an important role in the photosynthetic processes in sugarcane (Hartt and Burr, 1965).

Ca stimulates net uptake of K at low pH (Viets, 1944) due to its stabilizing effect on plasma membranes in non-mycorrhizal plants. Our results indicated the association between the soil Ca, Mg and the leaf K to be weaker in highly mycorrhizal plants. This can be due to K forming weak complexes and therefore being readily exchangeable (Wyn *et al*., 1979) with Na and not Mg or Ca cations. This may also be the reason for the weak relationship between Na and Ca compared to the stronger relationship between soil Na and leaf K in for both plant types. These ions have specific chemical properties that are sufficiently similar to compete for absorption and transport sites on the plant root surfaces (Fageria *et al*., 2002). The translocation of one of these ions will reduce the translocation of the other ions (Schimansky, 1981).

During sugar storage, K cations and organic-acid anions are reduced while there is an increase in the concentration of reducing sugars. This holds true for storage in sugar beet i.e. the concentrations of sucrose increase during storage, while the concentrations of K cations decrease (Beringer *et al*., 1986). Therefore, plants with increased sugar storage and decreased K cations will experience an increased translocation of Na to the leaves to regulate osmotic functions (Willmer and Mansfield, 1969).

Salinity is another factor besides pH that influences host plant growth and AM fungi symbiosis is soil salinity. Its effects on plant growth includes: i) nutrient

imbalances in the plant; ii) the reduction of soil solution osmotic potential, which may lead to physiological drought in the plant and iii) the toxicity of excessive Na and Cl ions, which disrupts enzymes, proteins, organelles, the plasma membrane, photosynthesis, and respiration (Evelin *et al.,* 2009). The effect of salinity on AM fungi includes inhibition of AM fungi hyphal growth and root colonisation (Estaun, 1989; McMillen *et al*., 1998; Jahromi *et al*., 2008).

Results from this study on AM fungi occurrence under saline conditions, concur with studies indicating the negative impact of salinity on AM fungi colonisation (Evelin *et al*., 2009) i.e. low percentage colonisation occurred were soil Na levels were higher (60.96 mg.kg $^{-1}$ ± 7.50) compared to increased root colonisation occurring when Na levels were lower (55.74 mg.kg $^{-1}$ ± 6.04). Further, crossed tables (Figure 3.4) indicated very negative associations between soil Na and most leaf nutrients except for leaf K for all mycorrhizal plants. The soil Na and leaf K association was however stronger in high status mycorrhizal plants compared to minimally mycorrhizal plants. Many studies have indicated AM fungi colonisation to enhance K absorption under saline conditions while preventing Na translocation to the shoots, thereby, reversing the effect of salinity on K and Na nutrition (Olrovich and Ashford, 1993; Alguacil *et al*., 2003; Giri *et al*., 2007; Sharifi *et al*., 2007; Zuccarini and Okurowska, 2008).

3.5 CONCLUSION

While not all crossed table relationships could be explained, this study provides a global perspective on soil-plant nutrient relationships of a genetically homogeneous plant like sugarcane. While pH and salinity play an important role in nutrient plant uptake, it is clear that AM fungi “buffers” nutrient uptake mechanisms in sugarcane grown in infertile, acidic and saline soils. The AM fungi buffering effects are governed by multi-complex processes and factors involving the biological, physiological, molecular and signalling mechanisms. As these multi-process mechanisms are difficult to measure directly, perhaps mechanistic simulation models (Claassen and Steingrobe, 1999) should also be used for a

better understanding of root and hyphae properties in relation to the “buffering” mechanisms of the symbiosis.

CHAPTER 4

MORPHOLOGICAL AND PARTIAL MOLECULAR IDENTIFICATION OF ARBUSCULAR MYCORRHIZAL FUNGI ISOLATED FROM SUGARCANE PLANTATIONS IN KWAZULU-NATAL, SOUTH AFRICA.

4.1 INTRODUCTION

Arbuscular mycorrhizas (AM) belonging to the phylum Glomeromycota form multidimensional associations with plants integrating diverse morphological, functional and evolutionary categories. Similarities exist in plant lineages, anatomical features and evolutionary traces (Brundrett, 2004; Redecker and Raab, 2006).

Until recently identification and taxonomy of the AM was traditionally based on spore morphology. This together with the use of molecular technology, based on a comprehensive SSU rRNA analysis, has now allowed for the reclassification of the phylum Glomeromycota, class Glomeromycetes into four orders, eleven families and seventeen genera (Table 4.1 adapted from Schüßler and Walker, 2010) with taxonomic changes in the Glomerales based on the characterisation of the *Glomus* type species, *Glomus macrocarpum*; Diversporales - creation of the new genus *Redeckera* and several former *Glomus* species transferred to Diversispora; and Archaeosporales - synonymisation of Intraspora with Archaeospora. Currently ± 228 AM fungal species are described (Schüßler and Walker, 2010).

Table 4.1: Reclassification of AM fungi, based on taxonomy and molecular phylogeny (Schüßler and Walker, 2010).

Orders (4)	Families (11)	Genera (17)
Glomerales	Glomeraceae	*Glomus*
		Funneliformis (former *Glomus* Group Aa*, 'Glomus mosseae clade'*)
		Rhizophagus (former *Glomus* Group Ab, *'Glomus intraradices clade'*)
		Sclerocystis (basal in former *Glomus* Group Ab)
	Claroideoglomeraceae	*Claroideoglomus* (former *Glomus* Group B*, 'Glomus claroideum clade'*)
Diversisporales	Gigasporaceae	*Gigaspora*
		Scutellospora
		Racocetra (including *Racocetra weresubiae*)
	Acaulosporaceae	*Acaulospora* (including the former *Kuklospora*)
	Entrophosporaceae	*Entrophospora* (with unclear phylogenetic affiliation)
	Pacisporaceae	*Pacispora*
	Diversisporaceae	*Diversispora* (including several *Glomus* species, e.g. BEG47, recently transferred)
		*Otospora (*unclear phylogenetic affiliation)
Paraglomerales	Paraglomeraceae	*Paraglomus*
Archaeosporales	Geosiphonaceae	*Geosiphon*
	Ambisporaceae	*Ambispora*
	Archaeosporaceae	*Archaeospora* (including the former *Intraspora*)

Morphological studies on diversity of AM fungi in Iran and South India indicated approximately thirty AM fungi species to be associated with different varieties of sugarcane (Kariman *et al*., 2005; Srikumar *et al*., 2009; Rokni *et al*., 2010, Rokni and Goltepeh, 2011). *Glomus aggregatum, G. fasciculatum and G. geosporum* were found to be associated with sugarcane grown in both Iran and South India (Table 4.2). In South Africa, many species of AM fungi associated with other plants have been isolated and identified, *Acaulospora scrobiculata, Glomus rubiforme, Acaulospora scrobiculata, Acaulospora mellea, Acaulospora tuberculata, Glomus etunicatum, Glomus rubiforme, Gigaspora spp.* and *Scutellospora sp*. were isolated from rhizospheres of cassava (*Manihot esculenta*) plants growing in Limpopo and Mpumalanga provinces (Straker *et al*., 2010). *Glomus etunicatum, G. intraradices, G. occultum and Gigaspora albida* were

identified to be associated with *Vangueria infausta*, an indigenous fruit tree to South Africa (Gaur *et al*., 1999).

Table 4.2: AM species diversity found in sugarcane fields of South India and Iran.

South India		**Iran**	
(Srikumar *et al*., 2009)	(Kariman *et al*., 2005)	(Rokni *et al*., 2010)	(Rokni and Goltepeh, 2011)
Acaulospora scrobiculata	*Acaulospora rugosa*	*Entrophospora colombiana*	***Glomus aggregatum***
Gigaspora margarita	*Glomus ambisporum*	***Glomus aggregatum***	*G. ambisporum*
Glomus aggregatum	*G. claroideum*	*G. caledonium*	*G. caledonium*
G. deserticola	*G. coremioides*	*G. coronatum*	*G. coronatum*
G. fasciculatum	*G. etunicatum*	*G. diaphanum*	*G. diaphanum*
G. geosporum	***G. fasciculatum***	*G. eburneum*	*G. eburneum*
G. mosseae	***G. geosporum***	*G. manihotis*	*G. etunicatum*
Sclerocystis pachycaulis	*G. intraradices*	*G. microcarpum*	***G. geosporum***
S pakistanica	*G. lamellosum*	*Pacispora scintillans*	*G. intraradices*
S. sinuosa	*G. liquidambaris*	*Paraglomus occultum*	*G. manihotis*
Scutellospora heterogama	*G. luteum*		*G. microcarpum*
	G. microcarpum		***G. mosseae***
	G. rubiformis		*G. lamellosum*
	G. sinousum		*Kuklospora colombiana*
	G. versiforme		*Pacispora scintillans*
	G. viscosum		*Paraglomus occultum*

The detection and discrimination of species is difficult and many are inadequately defined (Gamper *et al*., 2009) and natural communities usually harbour AM fungi from several phylogenetic clades. Molecular characterization and the availability of pure reference cultures help in establishing traits of a particular species, or phylotypes, (Hart and Reader, 2002; Munkvold *et al*., 2004; Maherali and Klironomos, 2007; Gamper, 2009).

Amplification of the internal transcribed spacers (ITS) and associated 18S, 5.8S or 28S rDNA regions has been used extensively in molecular fungal taxonomy. Redecker (2000) designed primers based on the sequence data of 18S, 5.8S rRNA genes including the ITS regions to identify major phylogenetic subgroups of

AM fungi to genus or specific level from DNA extracted from colonized roots using a nested PCR technique.

The major phylogenetic groups amplified by specific primers ARCH1311 and ITS4 were *Acaulospora gerdemannii/Acaulospora trappei* group, *Glomus occultum/Glomus brasilianum* group. Primers GLOM1310 and GLOMS 5.8R targeted the *Glomus mosseae/Glomus intraradices*, *Glomus versiforme, Acaulospora rugosa, Acaulospora spinosa* and *Scutellospora dipapillsoa/ Scutellospora pellucida* group. ACAU1660 targeted the *Acaulosporaceae sensu stricto* group. The LETC1670 and ITS4 targeted the *Glomus etunicatum /Glomus clariodeum* group. Lastly, primers GIGA 5.8R and ITS1F were used to target the *Gigasporaceae* group (Redecker, 2000). Since then the small subunit rRNA (SSU rRNA) gene has been used for AM fungi-specific primers because it is less variable than ITS but allows for sufficient resolution to the species level (Lee *et al*., 2008).

The aim of this study was to identity AM fungi isolated from two sugarcane plantations in South Africa using morphological approaches. A partial molecular analysis was carried out to determine if AM fungal spores isolated from sugarcane soils belonged to any of the phylogenetic groups described by Redecker (2000).

4.2 MATERIALS AND METHODS

4.2.1 Soil types

Spores were isolated from two different soil types: non-P-fixing sandy soil (Fernwood series, FAO: Dystric and Eutric Rhegosols, USDA: Entisols) at La Mercy 2-2 on the KwaZulu-Natal north coast (29°37`S - 31°7`E) and P-fixing, soil (Cartref series, FAO: Gleyic luvisol; USDA: Inceptisol) from Klipp III (29°21`S - 30°43`E). Soil types were the same as that used to investigate the response of sugarcane in the glasshouse to inoculation with indigenous AM fungi (see section 2.2.4).

4.2.2 Crude spore extraction from soil

Spores were extracted using modified wet sieving and centrifugation method (Brundrett *et al*., 1996) (see section 2.2.3).

4.2.3 Spore treatment and morphotyping

After extraction, spores were washed with water and EDTA (ethylene diamine tetra acetic acid) due to its antimicrobial properties. After rinsing, the spores were further disinfected with Chloramine-T (2%) and "two drops" Tween 20 before being washed three times with distilled water. The spores were then aseptically transferred into a microtube containing 50 mM EDTA. Spores were initially morphotyped according to their colour types and coded
O = orange (± 250 spores); B = black (± 250 spores); BR = brown (± 250 spores); R = red (± 250 spores) and further morphotyped according to their size, shape and appearance, using a Zeiss Stemi DVH stereo microscope.

4.2.4 Morphological identification of spores

Diagnostic slides were made with and without Melzer's solution (Appendix A, Table A - 1), and PVLG (polyvinylalcohol lacto glycerol) (Appendix A, Table A - 2). Melzer's solution reacts with certain spore walls, staining different wall structures. PVLG serves as an adhesive and allow slides to become semi-permanent. Ultra-fine forceps were used to place spores from the same group (identical with respect to colour, size and shape) into the mount mixture and allowed to set for 2 min before adding the cover-slip. Pressure was gently applied to the cover-slip to crush the spores (INVAM, 2012).

Slides were then viewed under the light microscope (Olympus AX 70 Provis) and images were captured using Soft Imaging System Analysis 3.0 software. Identification was based on diagnostic feature such as cell wall (in terms of colour, thickness, wall numbers for each spore and width of each layer). The spores were identified to genus or species according to the synoptic keys from the INVAM website (http://invam.caf.wvu.edu); Koske and Walker (1985); Trappe (1977 and 1982); Walker (1982); Schenck and Perez (1990); Schüßler and

Walker (2010) and arbuscular mycorrhizal fungi (Glomeromycota) *Endogone and Complexipes* species deposited in the Department of Plant Pathology, University of Agriculture in Szczecin, Poland website: http://www.zor.zut.edu.pl/Glomeromycota/index.html).

4.2.5 Molecular analysis

DNA extraction and purification

Spores that were initially morphotyped according to their colour types and coded O = orange (± 250 spores); B = black (± 250 spores); BR = brown (± 250 spores); R = red (± 250 spores) were physically ruptured a using a sterile pipette tip. These spores had not been morphologically identified at this stage. DNA from each respective tube, containing ± 250 spores was subsequently extracted using the NucleoSpin® Plant II Kit protocol (Macherey-Nagel). DNA was purified and concentrated using the Zymo DNA Clean & Concentrator (Zymo Research) with modifications: DNA Binding Buffer (100 µL) was added to 50 µL and the DNA sample, vortexed and loaded into a Zymo-Spin Column was placed into a 2 ml collection tube and centrifuged at full speed (10 000 *g*) for 10 seconds. The pellet was washed (200 µL wash buffer) and centrifuged at full speed for 10 seconds, then washed a second time before centrifuging for 30 seconds. After placing the spin column into a new microtube, the DNA was eluted with autoclaved MilliQ water. Purified DNA was stored at 4 °C until further use.

Polymerase chain reaction

Initially DNA was amplified using universal fungal primers, followed by PCR using genus specific primers. Combinations of the primers of Redecker (2000) were used (Table 4.3).

Table 4.3: PCR Primer sequences and annealing temperatures**.**

Primer 1	Sequence	Primer 2	Sequence	PCR annealing T°
Universal				
ITS1F	5'CTTGGTCATTTAG AGGAAGTAA3'	ITS4 (R)	3'TCCTCCGCTTATTG ATATGC5'	61 °C for 5 cycles 60 °C for 25 cycles
NS5	5'AACTTAAAGGAA TTGACGGAAG 3'	ITS4 (R)	3'TCCTCCGCTTATTG ATATGC5'	51 °C
AM1	5'TTGGAGGGCAAG TCTGGTGCC 3'	NS31	5'TTGGAGGAGGGCA AGTCTGGTGCC3'	56 °C
Genus specific				
GLOM1310	5'AGCTAGGYCTAAC ATTGTTA3'	ITS4 (R)	3'TCCTCCGCTTATTGA TATGC	57 °C
ARCH1311	5'TGCTAAATAGCCA GGCTGY3'	ITS4 (R)	3'TCCTCCGCTTATTGA TATGC5'	57.7 °C
ACAU1660	5'TGAGACTCTCGGA TCGG3'	ITS4 (R)	3'TCCTCCGCTTATTGA TATGC5'	61 °C
LETC1670	5'GATCGGCATCGGT GAGT3'	ITS4 (R)	3'TCCTCCGCTTATTGA TATGC5'	60 °C
GIGA5.8	3'ACTGACCCTCAAG CAKGTG5'	ITS1F	5'CTTGGTCATTTAGA GGAAGTAA3'	52.5 °C
GLOM 5.8	3' TCCGTTGTTGAAAG TGATC5'	ITS1F	5'CTTGGTCATTTAGA GGAAGTAA3'	52.5 °C

Each PCR reaction contained GoTaq Colourless Master Mix (1X) (Promega); forward and reverse primers (0.5 mM, 1.5 µL) each; Genomic DNA (obtained by crushing spores with a yellow tip in a PCR microtube) and autoclaved MilliQ water was used to make up a total reaction volume of 25 µL (Appendix A, Table A - 3). For the negative control 2 µL of autoclaved MilliQ water was used to make up a total reaction volume of 25 µL (Appendix A, Table A - 3). PCR cycling parameters included initial denaturation step of 94 °C for 5 min, followed by 35 amplification cycles of denaturation (94 °C for 1 min), respective annealing temperatures (1 min) and extension (72 °C for 1 min). Thereafter, there was an additional 7 min at 72 °C for final extension and 4 °C holding temperature. PCR products were then subjected to a second round amplification using a similar

protocol, with genus specific primers and respective annealing temperatures (Table 4.1) (Appendix A, Table A - 3). Products were analysed and photographed using Versadoc Quantity One Image Analyzer.

Agarose Gel Electrophoresis

This standard technique was used to separate, and identify purified DNA fragments via electrophoreses through an ethidium bromide (0.5 $\mu L.mL^{-1)}$ stained agarose gel (1%) at 100 volts for 5 min followed by 80 volts for 90 min in Tris-Boric-EDTA (1 M) buffer. The gel was analysed and photographed using Versadoc Quantity One Image Analyzer.

4.3 RESULTS

4.3.1 Morphological identification

Spore examinations indicated the abundance of five spore types indicated below.

***Acaulospora mellea* Spain & Schenck**

(INVAM reference accession BR983A, Schenk *et al.*, 1984.)

Family: Acaulosporaceae Order: Diversisporales

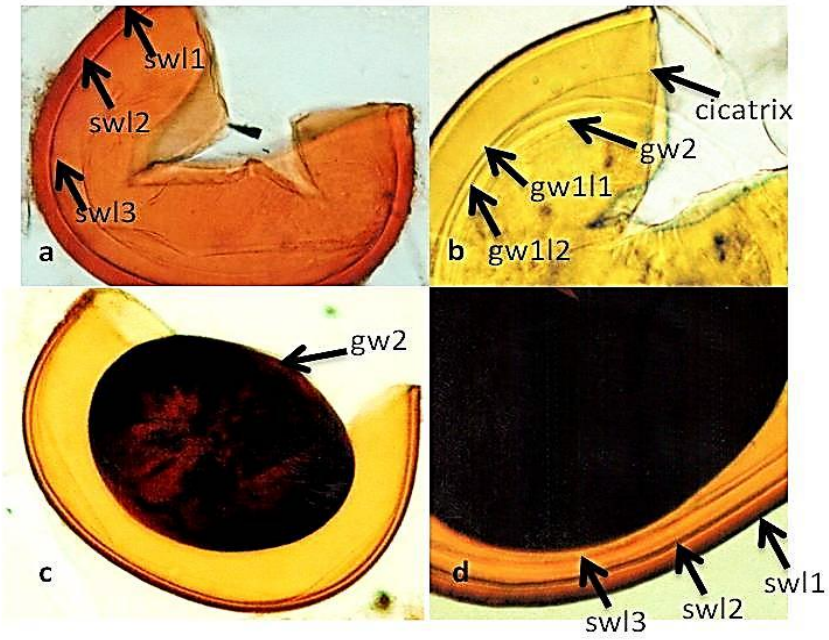


Figure 4.1: *Acaulospora mellea,* (a) thin outer layer of spore wall (swl1); laminated inner layers (swl2 and swl3); (b) outer hyaline layer of inner wall one (gwl1); inner hyaline layer of inner wall one (gwl2); (c) second hyaline inner wall (gw2); (d) inner layer of inner wall staining red-purple in Melzer's solution.

Spores appeared globose to subglobose honey-brown in colour. Spore walls consisted of an outer layer with two inner laminated layers (Figure 4.1a and d). Two layers of the germinal inner walls were also visible (Figure 4.1b) as was the cicatrix (scar indicating contact region between the spore and saccule). The inner layer of the inner wall stained red-purple in Melzer's solution (Figure 4.1c). These spores were present only in the La Mercy 2-2 sandy soil.

***Gigaspora margarita* Becker & Hall**

(INVAM reference accession WV205A; Becker and Hall, 1976)

Family: Gigasporaceae Order: Diversisporales

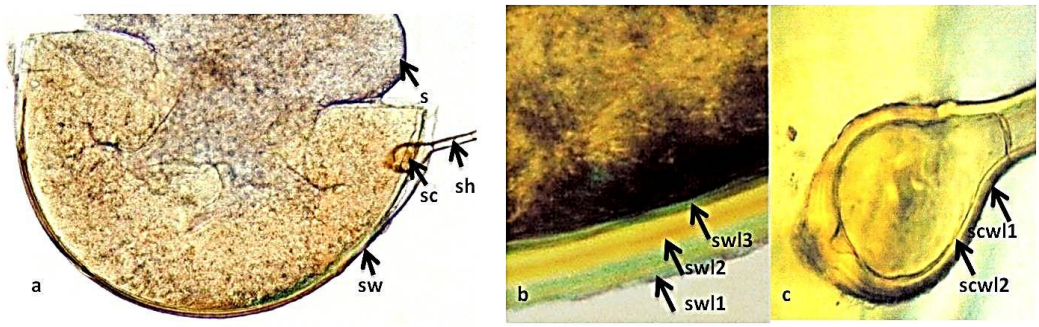


Figure 4.2: *Gigaspora margarita,* (a) globose white spores; spore is terminal on septate hyphae (sh), suspensor or sporogenous cell (sc); spore walls (sw); (b) outer spore wall (swl1); inner laminate layer of spore wall (swl2) and inner papillate layer (swl3); (c) Suspensor cell wall's outer layer (scwl1) and inner layer (scwl2).

Spores appeared globose, pearly-white in colour with laminated walls (Figure 4.2a). Three cell wall layers were evident (Figure 4.2b). Suspensor or sporogenous cells consisting two layers were also visible with septate hyphae (Figure 4.2c) (http://www.zor.zut.edu.pl/*Glomeromycota*/Gigaspora%20 margarita.html).

***Glomus geosporum* (Nicolson & Gerdemann) Walker**

(INVAM reference accession CA112; Walker, 1982).

Family: Glomaceae Order: Glomerales

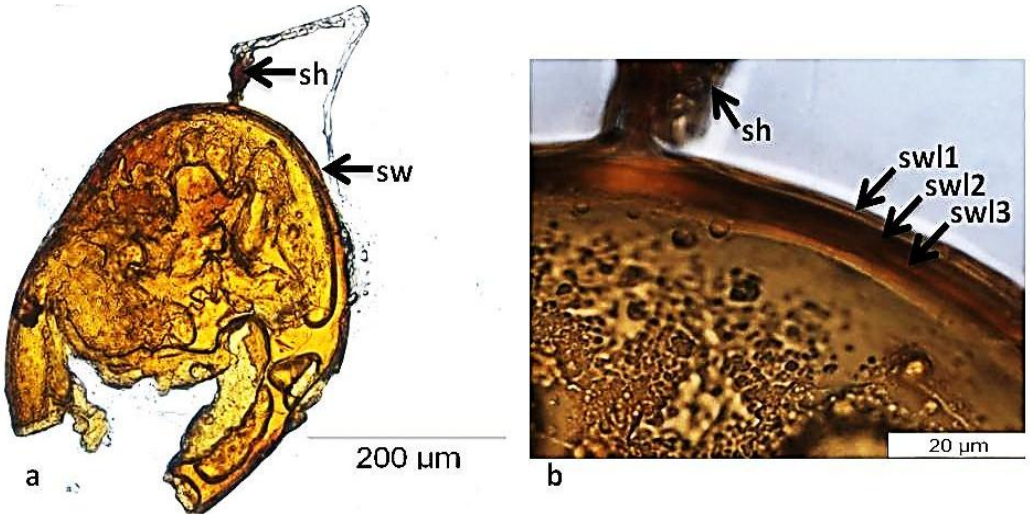


Figure 4.3: *Glomus geosporum,* (a) whole spore with subtending hypha (sh); spore wall (sw); (b) hyaline outer spore wall (swl1); red-brown laminated middle wall (swl2); yellow-brown inner spore wall (swl3).

Spores were yellow-brown to dark-orange in colour depending on maturity (the older the spore the darker the colour). Spores appeared subglobose to globose (Figure 4.3). Three spore wall layers were observed: hyaline outer wall, red-brown laminated middle wall and a yellow-brown inner wall. The subtending hyphae had a width of 18.5 µm. Depending on maturity, older spores had larger and more curved hyphae. Average spore size was 148.1 µm (n=10). Spore wall contents appeared granular indicating older spores.

***Scutellospora nigra* (J.F. Redhead) C. Walker & F.E. Sanders**

Family: Gigasporaceae Order: Diversisporales

(No INVAM reference accession; Nicolson and Schenck, 1979; Walker and Sanders, 1986; Schenk and Perez, 1990; Schüßler and Walker, 2010).

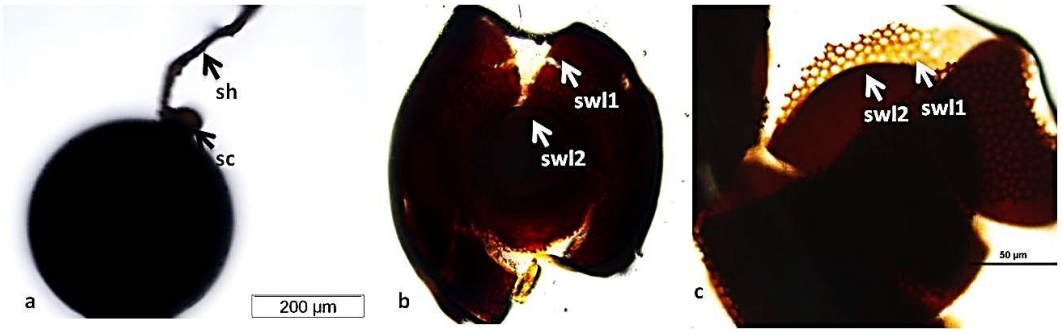


Figure 4.4: *Scutellospora nigra*, (a) whole spore with subtending hyphae (10X); (b) crushed spores (20X) and (c) spore wall (40X) in Meltzer's solution indicating double porous outer spore wall (swl1) and inner spore wall (swl2).

Spores black in colour and were globose in shape with a suspensor cell (diameter of 55 µm and subtending hyphae (Figure 4.4a). When subjected to Melzer's solution spores appeared brown in colour. Spore sizes ranged from 100-300 µm depending on maturity (n=10). The outer wall appeared double-pored compared to the inner spore wall (Figure 4.4b-c).

***Scutellospora verrucosa* (Koske & C. Walker) C. Walker & F.E. Sanders**

(INVAM reference accession VA105B; Koske and Walker, 1985, Walker and Sanders, 1986)

Family: Gigasporaceae Order: Diversisporales

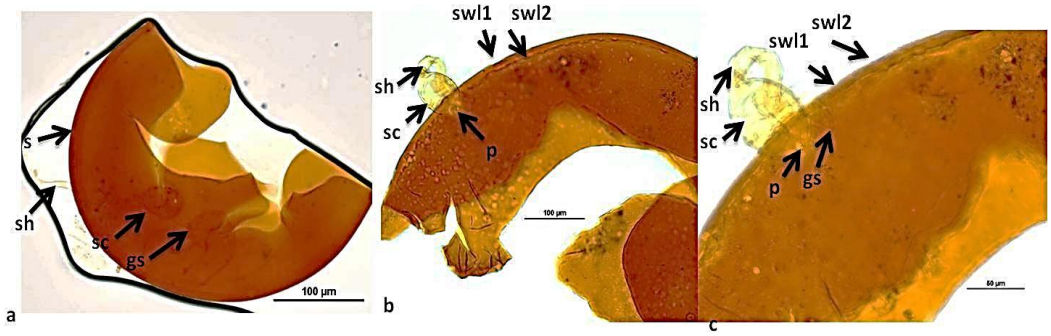


Figure 4.5: *Scutellospora verrucosa*, (a) spore (s); germination shield (gs); suspensor cell (cs); (b-c) plug (p); subtending hypha (sh); spore wall (sw) constituting an outer wall (swl1) and an inner wall (swl2).

Spores appeared orange-brown in colour (Figure 4.5) and were sub-globose to globose in shape. Bulbous suspensor cells with plugs were observed as well as a germination shield. The hyphae appeared aseptate. An outer spore wall adhering to the inner laminated spore wall was also evident. Cell walls consisted of two layers adhering to each other and appearing dark-orange to brown in colour upon reaction with Melzer's solution. This spore type was present in La Mercy 2-2 soil only.

4.3.2 Partial molecular identification

Spores morphotyped according to their colour types and coded O = orange (± 250 spores); B = black (± 250 spores); BR = brown (± 250 spores); R = red (± 250 spores), but not yet morphologically identified were subjected to partial molecular identification. PCR using primers AM1 and NS31 resulted in the amplification of expected 550 bp DNA fragments from all four morphotypes (Figure 4.6). Upon verification the morphologically different spores were further analysed using a nested PCR. The fungal universal primers NS5 and ITS4 were used to amplify 1200 bp fragments that were subsequently used as templates for the second round amplification step using group specific primer combinations.

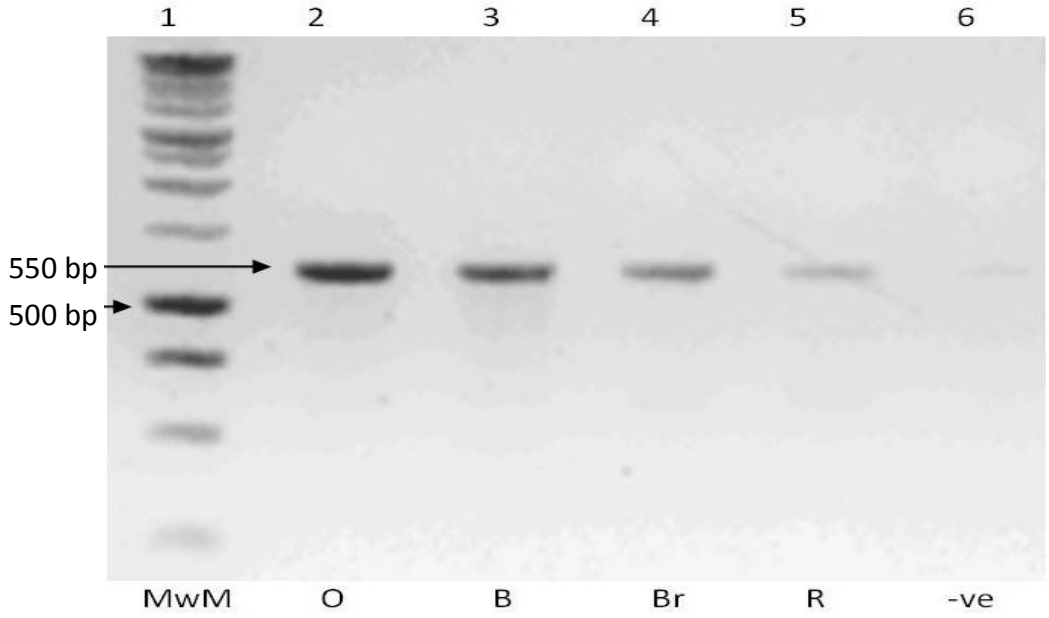


Figure 4.6: Agarose gel confirming all spores to be arbuscular mycorrhizal. Primers AM1 and NS31 amplified 550 bp fragments of genomic DNA. Spores were morphotyped according to colours: Orange (O), Black (B), Brown (Br) and Red (R).

Pairing the ITS4 reverse primer with group specific forward primers produced the following results. ARCH1311 only amplified the DNA from the brown morphotype spores producing a 270 bp fragment (Figure 4.7).

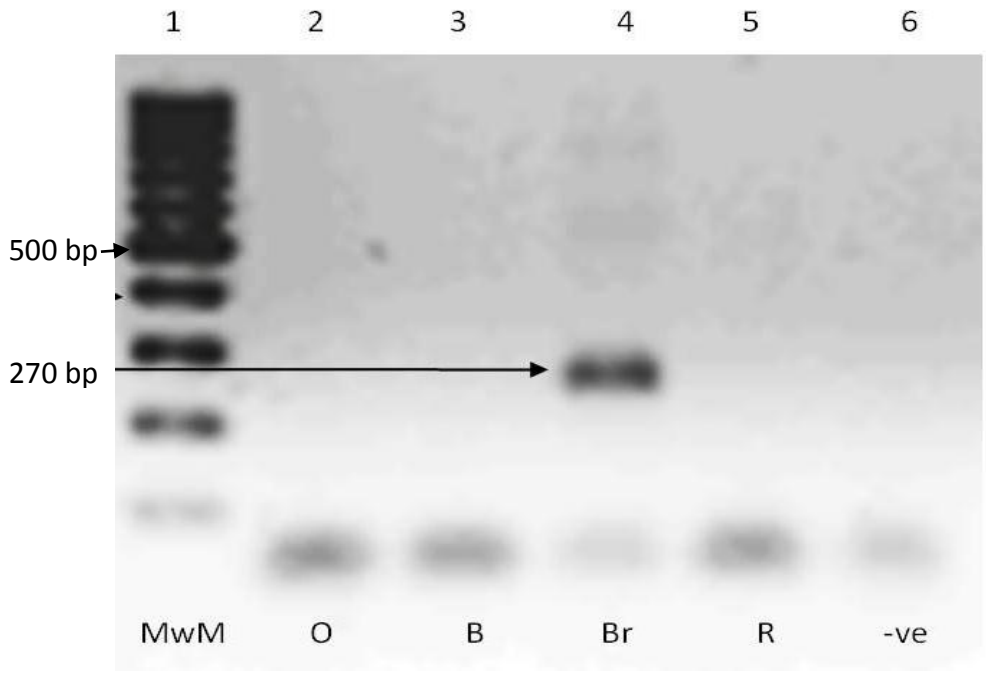


Figure 4.7: Agarose gel representing an amplified ±270 bp DNA fragment from the second round PCR of Brown spores DNA using primers ARCH 1311 and ITS4.

GLOM1310 amplified DNA 800 bp and 1000 bp fragments from the Orange, Black and Brown spores (Figure 4.8) (the bands larger than 1000 bp were first round excess DNA).

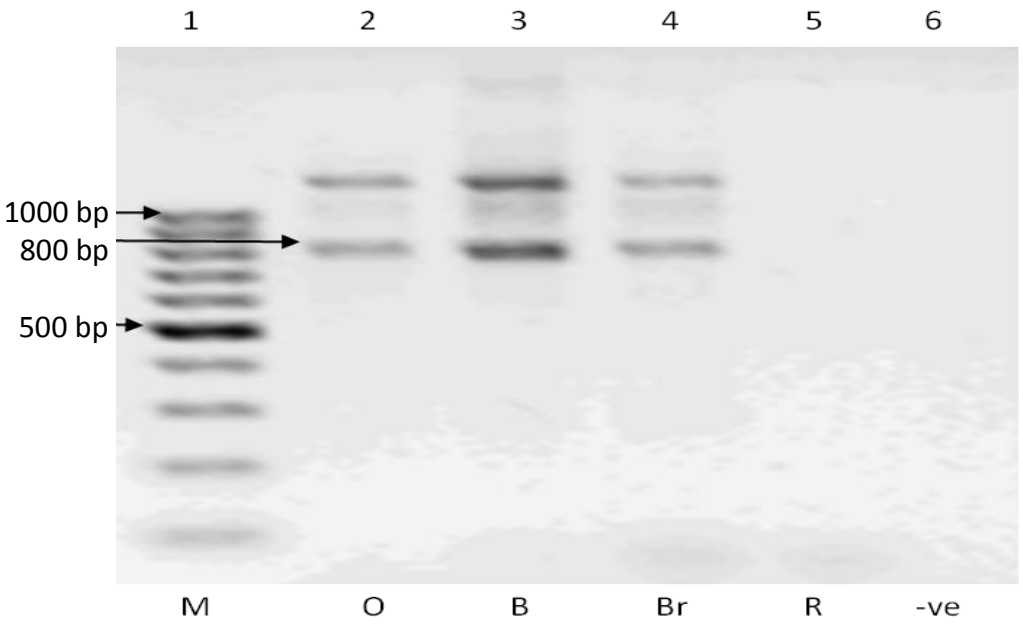


Figure 4.8: Agarose gel representing amplified 800 bp and 1000 bp DNA fragments from second round PCR of Orange, Black and Brown spores DNA using primers GLOM1310 and ITS4. Bands above these were 1200 bp from the first round PCR.

LETC1670 primer amplified 400 bp DNA fragments from all spores, as well as 100 bp and 800 bp fragments from the red spore (Figure 4.9).

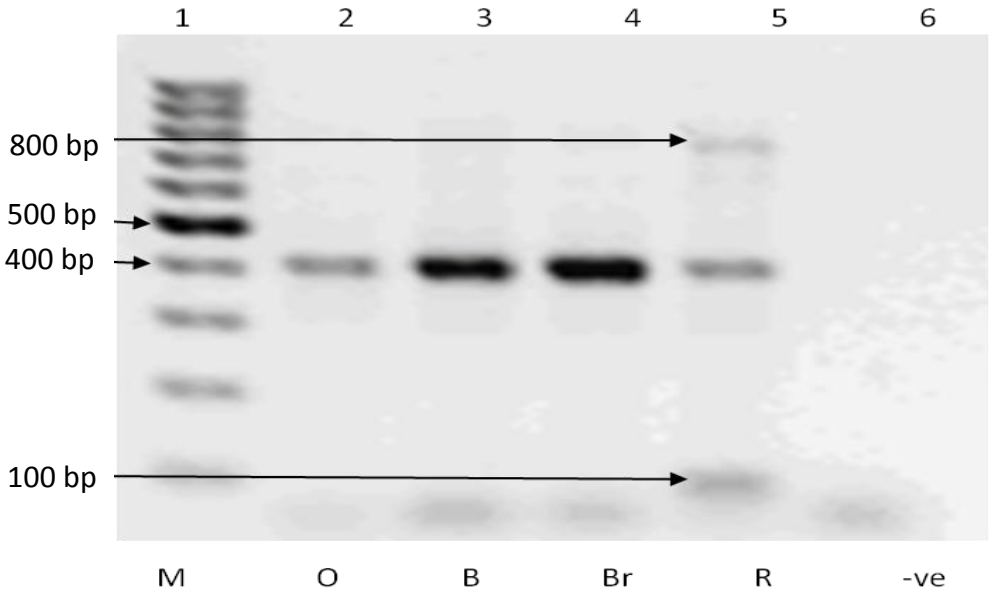


Figure 4.9: Agarose gel representing amplified ±400 bp DNA fragment from second round PCR of Orange, Black, Brown and Red spore DNA using Primers LECT1670 and ITS4. Additionally Red spores indicated the presence of 100 bp and 800 bp bands.

ACAU1660 also amplified 400 bp fragments for all spores (Figure 4.10) PCR using reverse primers GLOM5.8R and GIGA5.8R together with forward ITS1F primer did not reveal any DNA amplification.

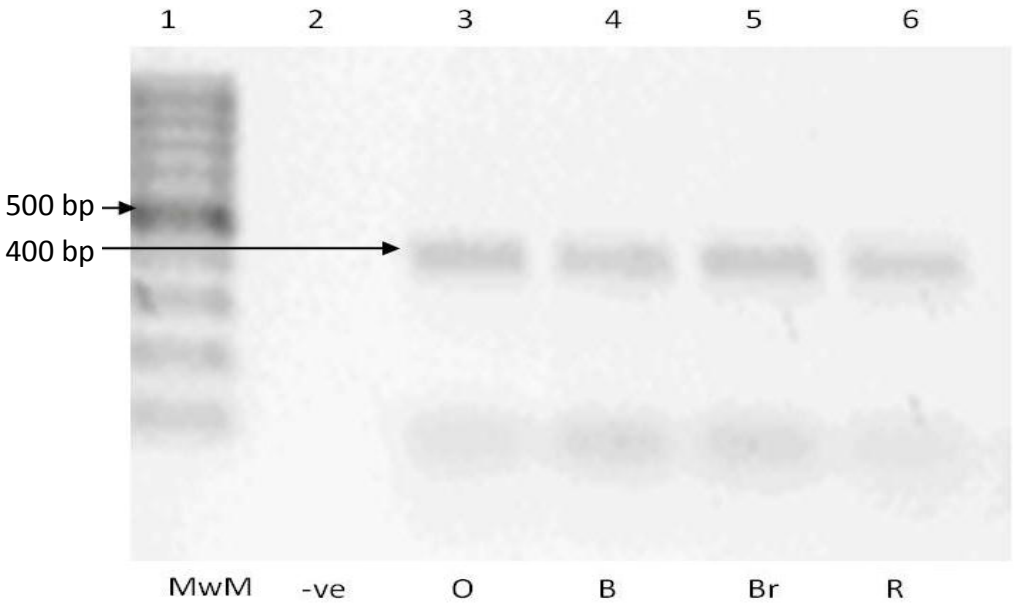


Figure 4.10: Agarose gel representing amplified ±400 bp DNA fragment from second round PCR of Orange, Black, Brown and Red spore DNA using Primers ACAU1660 and ITS4.

4.4 DISCUSSION

Although prevalent in most ecosystems much remains unknown about AM patterns of diversity at local and global scales (Chaudhary *et al*., 2008). Identification using spore morphology depends on spore production, which is highly dependent on environmental conditions resulting in many spores been produced or none at all (Redecker *et al*., 2003). In natural communities, different AM fungi species can colonise the same root system, but limited variation in hyphal morphology in both plant and soil makes identification very difficult (Lee *et al.*, 2008).

In this study, five spore types were identified morphologically as *Acaulospora mellea, Gigaspora margarita, Glomus geosporum, Scutellospora nigra* and *Scutellospora verrucosa.* Four spores belonged to the Diversisporales order and one to the Glomerales order, three spore types belonged to the Gigasporaceae family, one spore type to the Acaulosporaceae family, and one to the Glomaceae family. No trap cultures were established, as spores were isolated directly from the fields before identification.

Acaulospora mellea spores were found mainly in La Mercy 2-2 sandy soils. This spore type was not reported to be associated with sugarcane varieties in Iran and South India (Table 4.2). Schenck and Perez (1990) reported these spores to be found in sandy soils. This AM fungi was also recorded among roots of wild and cultivated plants growing in forest nurseries, uncultivated and cultivated soils of Poland (Blaszkowski, 1993a), as well as in maritime (Blaszkowski, 1993b, 1994) and inland (Blaszkowski *et al*., 2002) sand dunes of Poland; in cultivated and uncultivated soils of Florida, Massachusetts, North Carolina, Rhode Island, U. S. A. (Schenck *et al*. 1984; Sylvia 1986; Bever *et al*., 1996; Douds and Schenck, 1990; Koske and Gemma, 1997), Brazil (Schenck *et al*., 1984; Grandi and Trufem, 1991), Mexico (Estrada-Torres *et al*., 1992), Colombia (Sieverding *et al*. 1986; Saif, 1987; Sieverding and Toro, 1988; Sieverding, 1989; Dodd *et al*., 1990), Cameroon (Musoko *et al*., 1994), China (Mei-ging and You-shan, 1992) and in

cassava soils in South Africa (Straker *et al*., 2010). In other parts of Africa, *Acaulospora mellea* have been identified from forest and grassland soils in Western Kenya (Shepherd *et al*., 1996) and in *Terminalia* plantations and undisturbed forests of Cameroon (Mason *et al*., 1992; Musoko *et al*., 1994).

Gigaspora margarita appeared abundantly upon extraction (spore counts were not recorded) in both soil types of South African sugarcane fields, but not reported for Iran and South Indian sugarcane. These spores were also reported in cultivated and uncultivated soil in the U.S. (Nicolson and Schenck, 1979; Schenck and Kinloch, 1980; Schenck and Smith, 1981; Koske and Gemma, 1997), cultivated and uncultivated plants of Japan (Saito and Vargas, 1991), New Zealand (Hall, 1977), China (Wu *et al*., 2002), Canada (Marcel *et al*., 1988; Hamel *et al*., 1994), Mexico (Estrada-Torres *et al*., 1992), Cuba (Ferrer and Herrera, 1980), and South America (Moreira-Souza *et al*., 2003; Sieverding, 1989).

Glomus geosporum identification was uncomplicated due to its wall thickness and number, no reaction to Melzer's solution and the presence of constricted hyphae. The variation in size and colour may be due to the maturity, change in climate and soil types. This spore species is found in soils throughout the year and has a wide host range (Walker, 1982). They seemed to occur in moderately in both South African soil types, and were evident in sugarcane fields of both Iran and South India. Spores were also reported from cultivated and natural sites of Canada (Hamel *et al*., 1994), U. S. A (Miller *et al*., 1985; Schenck and Smith, 1981; Walker *et al*., 1982), Brazil (Moreira-Souza *et al*., 2003), Italy (Puppi and Riess, 1982), The Netherlands and Germany (Hildebrandt *et al*., 2001; Oehl *et al*., 2003), France and Switzerland (Oehl *et al*., 2003), Israel (Blaszkowski *et al*., 2001; Haas and Menge, 1990), China (Wu *et al*., 2002), and New Zealand (Hall, 1977).

Scutellospora nigra (synonyms: *Gigaspora nigra* J.F. Redhead, 1979; *Dentiscutata nigra* (J.F. Redhead) Sieverd., F.A. Souza & Oehl, 2008) (Schüßler and Walker, 2010) spores were classified as mature spores seemingly more abundant in Klipp III loamy soils on extraction compared to La Mercy 2-2 sandy soils (spore counts

were not recorded). These spores were not recorded in sugarcane soils of Iran and South India. According to Schenck and Perez (1990), loamy soils tend to contain more *S. nigra* spores and were reported to be associated with a diverse flora in Nigeria and the U.S.A.

Scutellospora verrucosa was identified from South African La Mercy 2-2, sandy soils only. These were not reported in Iranian and South Indian sugarcane soils. These spores were reported to be distributed in the coastal dunes in the U.S.A., the Bahamas and Brazil (Schenck and Perez, 1990).

In South Africa, besides sugarcane, species of AM fungal spores were previously identified from rhizospheres of cassava which included: *Glomus etunicatum, Glomus rubiforme, Acaulospora mellea, Acaulospora scrobiculata, Acaulospora tuberculata, Gigaspora sp. 1, Gigaspora sp. 2, Scutellospora sp.* (Straker *et al*., 2010); while *Glomus etunicatum*, *Glomus intraradices, Glomus occultum and Gigaspora albida* species were found to be associated with an indigenous fruit tree, *Vangueria infausta* (Gaur *et al*,. 1999). Only *Acaulospora mellea* was found to be common in sugarcane and cassava rhizospheres. While, morphological studies on diversity of AM fungi in Iran and South India indicated approximately thirty AM fungi species to be associated with different varieties of sugarcane with *Glomus aggregatum* and *G. fasciculatum* were found to be commonly associated with sugarcane grown in both Iran and South India(Kariman *et al*., 2005; Srikumar *et al*., 2009; Rokni *et al*., 2010, Rokni and Goltepeh, 2011); only *G. geosporum* appeared to be commonly associated with sugarcane from South Africa, Iran and South India.

Spores for this study were isolated directly from soil as crude spore inocula and were not necessarily indicative of those that infected the sugarcane roots, as spore viability was also not determined. Spores were not propagated in trap cultures indicating that spores may not have been healthy, however evidence of isolated spores being parasitized or degraded were absent, making them identifiable. Identification of healthy spores still does create difficulties with

respect to spore size or other morphological features that are too similar or scarce in some AM fungi (Redecker *et al*., 2003). Using whole-soil crude spore inocula did not provide information on the individual AM fungi taxa-sugarcane interactions and the use of directly isolated crude spore inocula for identification is not necessarily indicative of those that infect the sugarcane roots (Habte and Osorio, 2001).

Molecular DNA isolation of AM fungi from sugarcane roots was not successful. Therefore partial molecular identification of spores morphotyped according to their colour types and coded O= orange (± 250 spores); B = black (± 250 spores); BR = brown (± 250 spores); R = red (± 250 spores), but not yet morphologically identified) was conducted using a universal eukaryotic forward NS31 primer and a general fungal AM1 primer. These primers excluded plant DNA sequences, and although these primers were not absolutely specific for AM fungi they successfully amplified partial SSU DNA of AM fungi (Helgason *et al*., 1998; Helgason *et al.,* 1999). The expected fragment size (550 bp) was obtained from the DNA of all five morphotypes, confirming the spores to be of AM fungi origin (Figure 4.1). Thereafter, the nested PCR procedure of Redecker (2000) was used which involved a first amplification using the universal fungal primers, NS5 and ITS4, followed by primer combinations to target sequences of specific Glomalean groups (Table 4.3).

This partial molecular analysis indicated the presence of DNA from the *Acaulospora gerdemannii/ Acaulospora trappei* group, *Glomus occultum/Glomus brasilianum* group (brown spores), *Glomus mosseae/Glomus intraradices* group (orange, black and brown spores), *Glomus etunicatum/Glomus clariodeum* group (all spores) and *Acaulosporaceae sensu stricto* (all spores) i.e. five of eight phylogenetic groups described by Redecker (2000). Only partial molecular identification was conducted, as RFLPs were not successfully optimised. The grouping of spores according to size and colour-types as well as the limited spore numbers limited further analysis i.e. partial molecular identification could not be

matched to the morphologically identified spores. Redecker (2000) used colonised plant roots instead of AM fungal spores for molecular analysis. Genetic variation is caused mainly by nuclei been passed onto the next generation at every generation. For genetic exchange, there are assumptions brought forward, firstly genetic drift could have occurred by random assortment of genetically different nuclei during spore formation; however during spore formation, if this drift occurred there would be a probability of AM fungi loosing genetic information, not gaining of internal genetic variation (Sanders *et al*., 2008).

Genetic exchange via anastomoses has been shown to occur widely between hyphae belonging to the same and different germlines of the same isolates in *Glomus mosseae, Glomus caledonium* and *Glomus intraradices* (Giovannetti *et al*., 2003) i.e. nuclei exchange between individuals from different isolates do occur (Redecker, 2003; Giovannetti *et al*., 1999). This exchange may result in information flow, as well as physiological and genetic integration, between mycelia belonging to different AM fungi species (Giovannetti *et al*., 1999), resulting in a single spore containing nuclei form different species, hence the additional banding produced for primers LETC1670 and GLOM1310.

Selective PCR amplification depends on primer specificity, but some studies revealed that these primers do not amplify sequences from some recently described AM fungi groups and sometimes they amplify the DNA of other organisms (Clapp *et al*., 1995, 1999; Helgason *et al*., 1999). Although, internal transcribed spacer (ITS) sequences were used extensively for molecular taxonomy (Redecker, 2000; Renker *et al*., 2006), they exhibited high levels of variation within AM fungi species and even within single spores (Sanders *et al*., 1995; Lloyd-Macgilp *et al*., 1996).

Lee *et al*. (2008) designed new Glomeromycota-specific PCR primers AML1 (5’ ATC AAC TTT CGA TGG TAG GAT AGA 3’) and AML2 (GAA CCC AAA CAC TTT GGT TTC C-3’) based on representative SSU rRNA gene sequences of AM fungi from each phylogenetic group (Schüßler, 2007). These primers amplify all subgroups of

arbuscular mycorrhizal fungi (AM fungi, Glomeromycota), and exclude sequences from other organisms. Since these primer sequences were unpublished at the time of this study, it is recommended that these primers designed to facilitate rapid detection and identification directly from field-grown plant roots, be used in future molecular analysis of AM fungi.

4.5 CONCLUSION

It is now firmly established that sugarcane soils in South Africa are colonised by the *Acaulospora, Gigaspora, Glomus* and *Scutellospora* genera. AM fungi genetic diversity was evident in spores isolated from both plantations. Conflicting spore morphologies were evident in some cases as spores were directly extracted from the field, before analysis or in some cases spores were immature. Identified spores require further confirmation by crop inoculation, re-isolation and re-identification as well as the establishment of trap and single spore cultures. Future work on identification of indigenous AM fungi in sugarcane fields in to South Africa would also include the bulking of spores using *in-vitro* techniques and complete DNA sequence analysis as well as the use of recently designed Glomeromycota AML1 and AML2 primers.

CHAPTER 5

MOLECULAR IDENTIFICATION OF BACTERIA ISOLATED FROM ARBUSCULAR MYCORRHIZAL FUNGAL SPORES ASSOCIATED WITH SUGARCANE AND PRELIMINARY TESTING OF ASSOCIATED MYCORRHIZAL BACTERIA (AMB) AS BIOCONTROL AGENTS FOR NEMATODES *IN-VITRO*

5.1 INTRODUCTION

The soil system constitutes the rhizosphere (Hiltner, 1904), the mycorrhizosphere (Oswald and Ferchau, 1968) and the hyphosphere (Marschner, 1995). Arbuscular mycorrhiza (AM), rhizosphere bacteria, nematodes and plants are ecologically linked to nutrient cycles (Sanders *et al.,* 1995; Smith and Read, 2008) within the mycorrhizosphere niche. Nematode host range is rivalled by AM fungi colonising about 90% of higher plants (Vos *et al.,* 2011) and these AM fungi play a role in reducing the development of nematodes (Zhang *et al*., 2008, 2009; Liu *et al.*, 2011) as well as inducing systemic resistance against plant-parasitic nematodes in root systems (Elsen *et al.*, 2008; Hao *et al*., 2012).

Plant parasitic nematodes have been recorded in 24 countries and approximately 275 nematodes species in 48 genera were isolated from sugarcane roots and its rhizosphere (Spaull and Cadet, 1991). Lesion nematodes, *Pratylenchus zea* and *P. brachyurus*, are common and highly pathogenic: they are migratory endoparasites that attack new roots and destroy fine roots, causing lesions which become necrotic ultimately reducing the root mass. Common root-knot nematodes include *Meloidogyne javanica* and *M. incognita*, which are highly pathogenic and cause swelling and galls on sett roots and young shoot roots, resulting in root growth stunting. *Paratrichodorus minor* is a common, highly pathogenic ectoparasitic nematode. *Xiphinema spp.* and *Paralogodorous spp.* are common highly pathogenic dagger and needle nematodes respectively (Stirling and Blair, 2000). Sugarcane roots are usually attacked by many species at once, overwhelming the root system causing serious damage and drastically reducing the growth and sustainability of sugarcane production (Cadet and Spaull, 2003).

Eighty species within the 32 genera of plant parasitic nematodes in South Africa have been found associated with sugarcane, but only *Paratrichodorus, Xiphinema, Meloidogyne* and *Pratylenchus* are regarded as severely pathogenic pests (Cadet and Spaull, 2005). *Meloidogyne* and *Pratylenchus* damage large numbers of setts roots, causing delay and retardation of primary root emergence resulting in fewer tillers, while, *Trichodorus* and *Paratrichodorus* cause extensive sett root damage resulting in the inability for water uptake by the plants thus inhibiting stalk elongation. Root damage results in reduction of the number and length of stalks while diameter and sucrose content are sometimes severely affected (Cadet and Spaull*,* 2005). Severe infestations reduce cane yields by 20-50% due to reduction in stalk length (Stirling and Blair, 2000; Cadet and Spaull, 2005). *M. javanica* can reduce yields by 15 tons cane per hectare per annum, equivalent to approximately 25% of the production capacity under rainfed conditions (Cadet and Spaull, 2003). Since control options of nematodes are limited due to the reduction of nematicides and soil fumigants as a result of environmental and health concerns (Sikora *et al.,* 2008), interest now lies in the use of AM fungi as biocontrol options for these parasitic nematodes (Vos *et al.,* 2011).

However, the biocontrol ability of associated mycorrhizal bacteria (AMB) found embedded in AM fungi spore walls (Roesti *et al.,* 2005) was of interest for this study. These bacteria were also found within the cytoplasm of AM fungal spores (Mosse, 1962; Varma *et al.,* 1981). AMB play a role in mycorrhizal colonisation (Garbaye, 1994), plant nutrient uptake, plant growth promotion as well as protection against pathogens (Schelke and Peterson, 1996) and they influence microbial predators (Siddiqui and Mahmood, 1995). Tilak *et al.* (1989) were able to isolate nitrogen fixing *Azospirillum* spp. from the surface-sterilized spores of AM fungi species, and suggested that these diazotrophs may contribute to the nitrogen nutrition of the mycorrhiza. Sward (1981a-c) observed binary fission of 'bacteria like organisms' within spores of *Gigaspora margarita*, which spread into the growing germ tube, where the organisms became concentrated in apical and

subapical sites. These bacteria are now identified as *Burkholderia* endosymbionts (Bianciotto *et al.,* 2000).

Some *Pseudomonas* and *Xanthomonas spp*. can enhance plant growth by producing a compound that inhibits the growth of pathogens or reduces invasion by plant pathogens including nematodes. Strains of *Burkholderia cepacia* and *B. gladioli* have been reported to be antagonistic towards nematodes (Kloepper *et al.,* 1992). A *B. cepacia* strain has been shown to inhibit *Meloidogyne incognita* egg hatching, mobility and second stage juveniles both *in-vitro* and *in-vivo* (Meyer, 2000; Meyer *et al.,* 2002).

Results of a survey in KwaZulu-Natal where this research was conducted showed *Paratrichodorus, Xiphinema, Meloidogyne* and *Pratylenchus* to be widespread in sandy soils and were present in 90% or more of surveyed fields (SASRI, 2001). Due to this extensive problem, a preliminary investigation was conducted to test the effectiveness of AMB as natural control agents of soil pathogens. Since AMB may be the contributing factor for the effectiveness of AM fungi as biocontrol agents against nematodes, AMB isolated from AM fungal spores associated with sugarcane soils of KwaZulu-Natal were identified and tested for possible biocontrol effects on just three nematode genera viz. *Meloidogyne, Pratylenchus and Paratrichodorus.*

5.2 MATERIALS AND METHODS

5.2.1 Soil sites

Two different soil types: non P-fixing sandy soil (Fernwood series, FAO: Dystric and Eutric Rhegosols, USDA: Entisols) at La Mercy (LM) on the KwaZulu-Natal north coast (29°37`S - 31°7`E) and P-fixing, soil (Cartref series, FAO: Gleyic luvisol; USDA: Inceptisol) from Klipp III (K) (29°21`S - 30°43`E), were collected from the rhizosphere for this investigation.

5.2.2 Spore extraction and identification

Spores were extracted using a modified wet sieving and centrifugation isolation and extraction method (Brundrett *et al.*, 1996) (see section 2.2.3). Spores were morphologically identified (see section 4.2.3) as *Scutellospora nigra, Glomus geosporum*, *Gigaspora margarita* and *Acaulospora mellea.*

5.2.3 Arbuscular Mycorrhizal Bacteria isolation and pure cultures

After extraction and identification, five spores per respective species were treated with 50 mM EDTA (ethylenediaminetetraacetic acid) while the other five were washed in sterile distilled water (controls). EDTA is an antifungal agent that was used to fungal proliferation after spores were placed on Tryptone Soy agar (0.8% TSA, Biorad) plates. Plates incubated for 48 h in a 30 °C incubator before growth was evident. Bacteria occurring around treated spore plates were sub-cultured on TSA plates and subsequently Gram-stained to determine if the cultures were pure.

5.2.4 DNA Extraction Protocol

(Modification of Protocol for DNAs, Quiagen Corp. http://www.qiagen.com.)

The pure isolates were then grown in Tryptone Soy broth (10 $g.L^{-1}$ pH 7.5) for 48 h before being used for DNA extraction. To a 2 mL microtube, 750 µL CTAB based Carlson lysis buffer, 100 mM Tris-HCl pH 9.5, 1.4 M NaCl, 1 % polyethylenglycol 8000, 20 mM EDTA, 10 mM β-mercaptoethanol, Sigma) (Carlson *et al.,* 1991) was added to bacterial suspension (500 µL). Samples were incubated for 40 min and inverted every 10 min, then centrifuged at 10 000 *g* for 10 min at room temperature. The supernatant was decanted into a new microtube and 750 µl of chloroform: isoamyl alcohol (24:1) added to the supernatant. Samples were then centrifuged for 10 min at 10 000 *g*. The upper phase was transferred to clean microtubes to a measured volume. An equal volume of isopropanol was added and tubes were inverted several times to mix and precipitate the DNA. The tubes were again centrifuged for 10 min at 10 000 *g* to sediment DNA. The pellets were washed by rinsing the sides with 250 µL ice

cold absolute ethanol. The samples were spun at 4 °C for 10 min. The ethanol was then decanted and the pellet left to dry. The pellet was resuspended in 50 µL Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). RNAse plus was diluted 1:9, and 2 µL was added to the suspension and incubated overnight in the refrigerator.

5.2.5 Agarose Gel Electrophoresis

Genomic DNA was electrophoresed through an ethidium bromide (0.5 $\mu L.mL^{-1}$) stained agarose gel (1%) at 100 volts for 5 min followed by 80 volts for 90 min in Tris-Boric-EDTA (1 M) buffer. The gel was analysed and photographed using Versadoc Quantity One Image Analyzer.

5.2.6 PCR Amplification

Once confirmation of successful extraction of genomic DNA was obtained, DNA was used to amplify the 16S region of the bacterial genome using universal primers. The 16S rDNA region of approximately 1500 bp was amplified using the fungal general primers: FGP 281 (UnivBactF, Inqaba) 5'ATGGARAAGYTTGATC CTGGCTCA3' and FGP 153 (UnivBactR, Inqaba) 5'AAGGAGGGGATCCAGCCGCA3'.

Each PCR reaction mix contained GoTaq Colourless Master (1X) (Promega). Forward and reverse Primers (0.5 mM, 1.5 µL each), genomic DNA (2 µL) and autoclaved MilliQ water. For the negative control 2 µL of autoclaved MilliQ water was used to make up a total reaction volume of 50 µL (Appendix A, Table A - 4).

5.2.7 PCR cycling parameters

PCR was performed using a Gradient Thermal Cycler (Merck). PCR cycling parameters included an initial denaturation step of 94 °C for 5 min, 40 amplification cycles consisting of the following segments: denaturation (94 °C for 1 min), annealing (56 °C for 1 min) and extension (72 °C). The final extension step was programmed for 10 min at 72 °C. Samples were then stored at 4 °C until electrophoresis.

5.2.8 DNA Purification

Following gel electrophoresis, the DNA 1500 bp bands were viewed under the excised UV transilluminator and the DNA bands of interest were excised using a clean scalpel. The gel slices were weighed and put into microtubes. The Promega Wizard SV Gel and PCR Clean–Up System (Promega) was used to elute the DNA: Membrane Binding Solution was added at a 1:1 ratio i.e. 10 µL of solution per 10 mg of agarose gel slice. The mixture was vortexed and incubated at 65 °C to dissolve the gel, then transferred to the SV minicolumn assembly and incubated at room temperature for 1 min to allow the DNA to bind to the silica. After centrifuging the mixture at 14 000 *g* for 1 min, the flow-through was discarded and the minicolumn inserted into the collection Tube. The DNA was washed with 700 µL of Membrane Wash Solution and again centrifuged at 14 000 *g* for 5 min. After transferring to a clean microtube, 50 µL of Nuclease-Free water was added before incubation at room temperature. The DNA was stored at 4 °C until cloning was performed.

5.2.9 Cloning and Sequencing

Ligation using the pGRM®-T Easy vector system (PROMEGA)

Purified DNA fragments (1500 bp) were then inserted into the pGC blue cloning vector (GC Cloning and Amplification Kit, Lucigen Corporation) by GC cloning and transformed into mutated *Escherichia coli* competent cells. The GC cloning technology is based on the unique combination of a G-tailed insert DNA and a C-tailed vector. For this purpose, following PCR amplification, the DNA fragments were subjected to a G-tailing reaction. The pGC cloning vector is also supplied with de-phosphorylated 5’ ends; hence the need for 5’ phosphorylated PCR products. In addition, the pGC Blue Vector contains the kanamycin resistance gene which eliminates the growth of non-transformed “satellite” colonies in the screening process. The vector also included opposing SP6 and T7 promoters for expression studies.

Ligation to the pGC Blue Cloning Vector

In the ligation reaction the G-tailed, phosphorylated insert is ligated with pre-processed pGC Blue Vector. The ligation was performed as follows: the GC Cloning Vector Premix was briefly centrifuged before use and gently mixed by pipetting up and down several times. The following components were combined in a 1.5 mL microtube, adding the ligase lastly: 2.0 µL DNA insert, 2.5 µL 4X pGC Blue Vector Premix, 1.0 µL CloneSmart DNA Ligase (2 $U.\mu L^{-1}$), 4.5 µL dH_20, to give a 10.0 µL total reaction volume. The reaction mixture was then mixed by gently pipetting up and down and thereafter incubated at 25 °C for 60 min. The ligation reaction was incubated at 70 °C for 15 min.

Preparation for Transformation

After heat denaturation of the ligation mixture, the sample was cooled to room temperature for 15 sec followed by 0-4 °C for 15 sec to condense water vapour inside the tube. The sample was centrifuged at 14 000 *g* for 1 min to collect condensation and collect precipitated material. The samples were then ready for transformation into the *E. coli* 10G Competent Cells. These *E. coli* cells were heat shocked at 42 °C followed by incubation on ice.

Transformation Protocol for Chemically Competent cells

The heat-denatured GC Cloning ligation reaction (3.5 µL) was added to 40 µL of the *E. coli* competent cell suspension. This cell/ligation mixture was incubated on ice for 30 min. Room temperature recovery medium (260 µL) was added to the cells in the culture tube. Tubes were then placed in a shaking water bath at 250 rpm for 1 h at 37 °C. Transformed cells (100 µL) were plated onto Tryptone Yeast Agar (1.5%) plates containing 30 $\mu g.mL^{-1}$ kanamycin plus XGAL (50 $mg.mL^{-1}$ in N, N-dimethylformamide) and IPTG (0.5 mM in 20% ethanol). Plates were incubated overnight at 37 °C. The pGC Blue Vector uses the standard blue/white colony screening based on *lac*Z alpha complementation. Recombinant colonies

were white and non-recombinant colonies were blue. White colonies where then transferred to LB broth and incubated at 37 °C at 150 rpm.

Plasmid purification and DNA Sequencing

After incubation the broth suspensions were centrifuged at 10 000 *g,* and the supernatant removed. The QIAprep Spin Miniprep Kit Protocol was used to isolate and purified the recombinant DNA. These templates were then were sent to the Department of Biochemistry (SASRI) and sequenced using Applied Biosystems ABI 310 Genetic Sequencer.

5.2.10 Sequence analysis

Raw sequences were edited using the Staden software package and were aligned using the CLUSTAL X 2.0 programme. Homology searches of the 16S rDNA for the respective isolates were performed using the Basic Local Alignment Search tool (BLAST, 2011 - http://blast.ncbi.nlm.nih. gov /Blast.cgi); algorithm (Zhang *et al.,* 2000). Ambiguities, vector and primer sequences were removed before nucleotide sequences were submitted to search entries in the National Centre for Biotechnology Information (NCBI) nucleotide databases. The significance of the homology was based on the Score and E-value. Significant homology was indicated by setting a minimum score of 500 (Appendix B, Tables B1- B10).

5.2.11 Preliminary study to determine the effect of AMB on nematodes *in-vitro*

Twenty-four hour cultures of the respective bacterial isolates (AMB) were grown in 30 mL of Tryptone Soy Broth (3 $g.L^{-1}$) (incubated at 30 °C). The cultures were centrifuged at 10 000 *g* for 5 min and the pellets re-suspended in Ringer's solution. Centrifugation and re-suspension was repeated twice. Twelve live nematodes of the *Paratrichodorus*, *Pratylenchus* and *Meloidogyne* genera isolated by the Nematology Department (SASRI), from sugarcane fields mentioned above (see section 5.2.2) were placed into separate watch glasses containing 500 µL of the bacterial suspension (bacterial concentrations were not

considered at this early stage of the study). Four replicates per isolate and one control consisting of the respective nematodes in pure Ringers solution were sampled. The nematodes were analysed hourly over a 3 h period. Analysis were done under the Zeiss Stemi DVH stereo dissecting microscope at 25 °C. Nematode mortality was indicated by non-motile stick-like bodies that floated to the top of the suspension.

5.2.12 Statistical Analysis

Analysis of variance (ANOVA) was performed on all data collected using SAS-General Linear Model (GLM) procedure (SAS, 2008) after arcsine transformation and the means for nematode mortality were separated using the Least Significant Difference (LSD) test at 5% level.

5.3 RESULTS

5.3.1. Identification of bacterial isolates

Acaulospora mellea, Glomus geosporum, Gigaspora margarita and *Scutellospora nigra* spores (identified in chapter 4) were used to isolate associated bacteria. Untreated spores washed only with Millipore sterilized water, supported the growth of contaminating fungi as well as bacteria (Figure 5.1a), while EDTA surface-sterilized spores supported only bacterial growth (Figure 5.1b).

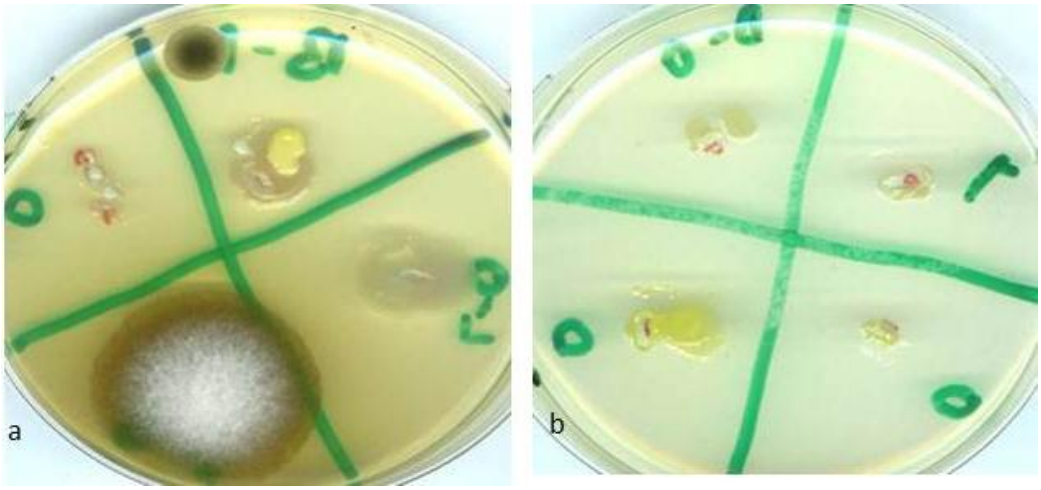


Figure 5.1: Tryptone soy agar plates indicating (a) contaminating fungi around spores washed only with Millipore sterilized water (b) no fungal contamination around surface sterilized spores treated with EDTA after a 48 h incubation at 30 °C.

Bacterial colonies established from the surface-sterilized spores and streaked onto TSA plates to obtain pure cultures were predominantly cream and yellow in colour (Figure 5.2), but differing in colony texture and shape. Gram stains indicated pure cultures after multiple subcultures.



Figure 5.2: Examples of pure bacterial colonies sub-cultured from plates containing surface sterilized AM fungal spores.

Genomic DNA from eleven bacterial isolates was successfully extracted and agarose gel electrophoresis showed clear bands the intensity of which was indicative of the amount of DNA extracted. Bacterial growth from spore surfaces indicated that these bacteria are closely associated with AM fungal spores. Partial 16S rDNA sequences were subjected to nucleotide-nucleotide BLAST and compared with GenBank reference sequences (Appendix B, Tables B1 - B10) for species identification. *Brevibacillus reuszeri* was isolated from *Scutellospora nigra*, while *Bacillus megaterium* and *Stenotrophomonas maltophilia* were isolated from *Glomus geosporum* spores. *Paenibacillus chitinolyticu*s and *Bacillus cereus* were isolated from *Acaulospora mellea* and *Gigaspora margarita* spores respectively (Table 5.1).

Table 5.1: BLAST description of sequences and accession numbers for Associated Mycorrhizal Bacteria.

Bacterial Isolate #	BLAST Description	Accession	Total score	E-value
4	*Brevibacillus reuszeri strain* DSM 9887 16S ribosomal RNA, partial sequence.	NR040982.1	754	0
5	*Bacillus megaterium* strain SW1:2 16S ribosomal RNA gene, partial sequence.	HQ124332.1	769	0
6	*Bacillus chitinolyticus* gene for 16S ribosomal RNA	AB021183.1	767	0
7	*Stenotrophomonas maltophilia* partial 16S rRNA gene, strain c6.	AJ293468.1	811	0
8	*Bacillus cereus* strain WQ9-2 16S ribosomal RNA gene, partial sequence.	HM067839.1	771	0

5.3.2 Preliminary study to determine the effect of AMB on nematodes *in-vitro*

These AMBs were then used in a preliminary *in-vitro* study to determine their effect on survival of three plant parasitic nematode genera associated with sugarcane: *Meloidogyne*, *Pratylenchus* and *Paratrichodorus*. Nematodes were monitored at 1, 2 and 3 hour intervals to determine effectivity of the treatments. Percentage mortality was transformed (arcsine) before three-way ANOVA

analysis. The nematodes differed in their susceptibility to the AMB treatments and all interactive effects were significant (Table 5.2).

Paratrichodorus was the most susceptible to AMB effects with *Brevibacillus reuszeri* causing the highest mortality and *Stenotrophomonas maltophilia* the lowest, and similar mortality caused by *Bacillus megaterium, Paenibacillus chitinolyticus* and *Bacillus cereus* by three hours (Table 5.2). *Pratylenchus* fell into an intermediate group with respect to susceptibility to AMB, with *Bacillus cereus* causing the highest mortality and *Stenotrophomonas maltophilia* having no inhibitory effect at all on this nematode. *Meloidogyn*e proved to be the least susceptible to AMB biocontrol with the highest mortality caused by *Brevibacillus reuszeri* (significantly less than the highest mortality caused in *Pratylenchus*) and the other bacteria all exerting low or negligible effects on the nematode's mortality, especially in the early stages of incubation (Table 5.2).

Table 5.2: Three-way ANOVA for nematode mortality after AMB treatments. Values represent transformed (arcsine) percentage mortality. Nematodes were monitored at 1, 2 and 3 hour intervals to determine effectivity of the treatments. Treatments indicated with the same letter were not significantly different ($P \geq 0.05$).

AMB treatments	**Host nematode**	**Arcsine % Mortality per hour**			
		1 h	2 h	3 h	
Brevibacillus reuszeri	*Paratrichodorus*	0.94	ghi	1.57	k	1.57	k
Bacillus megaterium	*Paratrichodorus*	0.44	bcde	1.15	ij	1.36	jk
Paenibacillus chitinolyticus	*Paratrichodorus*	0.45	bcde	0.45	bcde	1.36	jk
Bacillus cereus	*Paratrichodorus*	0.84	fghi	1.15	ij	1.36	jk
Stenotrophomonas maltophilia	*Paratrichodorus*	0.17	abc	0.35	abcd	1.15	ij
Bacillus cereus	*Pratylenchus*	0.18	abc	0.66	defg	1.05	hij
Brevibacillus reuszeri	*Meloidogyne*	0.00	a	0.45	bcde	0.75	efgh
Brevibacillus reuszeri	*Pratylenchus*	0.08	ab	0.34	abcd	0.53	cdef
Paenibacillus chitinolyticus	*Pratylenchus*	0.35	abcd	0.53	cdef	0.53	cdef
Paenibacillus chitinolyticus	*Meloidogyne*	0.00	a	0.35	abcd	0.35	abcd
Bacillus megaterium	*Pratylenchus*	0.00	a	0.08	ab	0.27	abc
Stenotrophomonas maltophilia	*Meloidogyne*	0.00	a	0.00	a	0.17	abc
Bacillus cereus	*Meloidogyne*	0.00	a	0.00	a	0.08	ab
Bacillus megaterium	*Meloidogyne*	0.00	a	0.00	a	0.00	a
Stenotrophomonas maltophilia	*Pratylenchus*	0.00	a	0.00	a	0.00	a
Controls - no bacteria	*Meloidogyne*	0.00	a	0.00	a	0.00	a
Controls - no bacteria	*Pratylenchus*	0.00	a	0.00	a	0.00	a
Controls - no bacteria	*Paratrichodorus*	0.00	a	0.00	a	0.00	a

Effects	**F-value**	**P-value**	**Significance**	**Description**
Bacteria	30.51	<0.001	***	Highly significant
Nematode	118.13	<0.001	***	Highly significant
Time	35.90	<0.001	***	Highly significant
Bacteria x Nematode	9.83	<0.001	***	Highly significant
Bacteria x Time	2.16	0.023	*	Significant
Nematode x Time	4.29	0.003	**	Highly significant
Bacteria x Nematode x Time	1.93	0.014	*	Significant
	%CV =71.30			S.E = 0.279

5.4 DISCUSSION

Bacteria in this study were isolated directly from AM fungal spores treated with EDTA to reduce contaminating fungi due to its antifungal capacity. EDTA also acts as a chelating agent with divalent metals within the cell itself i.e. metals such as Mg^{2+} and Ca^{2+} required by essential enzymes (Kubo *et al.*, 2005); this promoted the growth of dominant bacteria found on the AM fungi spore surfaces.

Spore walls of most AM fungal spores are composed of three layers: an outer hyaline layer that decays until it sloughs off leaving a granular surface, a laminated middle layer and a more rigid inner layer that often adheres to the middle layer. The thin hyaline layer is composed mainly of chitin and has often been found to be colonized by microorganisms that become enmeshed in this wall layer in several *Glomus* species (Roesti *et al.*, 2005). The isolation of bacteria directly from the AM fungi indicated them to be AMBs. Sequence analysis indicated the AMBs to be *Brevibacillus reuszeri* (isolated from *Scutellospora nigra*), *Bacillus megaterium* and *Stenotrophomonas maltophilia* (isolated from *Glomus geosporum* spores) and *Paenibacillus chitinolyticu*s and *Bacillus cereus* (isolated from *Acaulospora mellea* and *Gigaspora margarita* spores respectively) (Table 5.1).

Further, it was established that most AMB treatments were effective biocontrol agents against nematodes *Paratrichodorus* and *Pratylenchus*, but less so against *Meloidogyne in-vitro*. However, there were differences in susceptibility between the nematodes to the bacteria, with *Paratrichodorus* being the most susceptible and *Meloidogyne* the least. Moreover, variability in bacterial virulence was host dependent (Table 5.2). Nematophagous bacteria affect nematodes by parasitizing them; producing toxins, antibiotics or enzymes; interfering with nematode-plant-host recognition; competing for nutrients; inducing systemic resistance of plants; and promoting plant health (Siddiqui and Mahmood, 1999). Some nematophagous bacteria such *Brevibacillus laterosporus* strain G4 and

Bacillus sp. B16 are able to penetrate the cuticle barrier to infect and kill a nematode host under some conditions.

The *Brevibacillus* genus contains aerobic, endospore-forming bacteria and was separated from the *Bacillus brevis* group based on molecular data (Shida *et al.,* 1996). Some strains of *Brevibacillus* such as *Brevibacillus laterosporus* have been found to have nematicidal activities i.e. they contain the hydrolytic enzymes involved in the stages of penetration of the surface (cuticle) and digestion of the host nematode (Huang *et al.,* 2005). This preliminary study indicates *B. reuszeri* to be the most effective biocontrol activity against *Paratrichodorus*, i.e. it killed 75% of the nematodes after just 1 h and 100% after 3 h but further research into the functions of *Brevibacillus reuszeri* in the soil is still required.

*Bacillus species h*ave been found to have nematotoxic activity against *Panagrellus redivivus* nematodes. These bacteria produced extracellular proteins that killed about 80% of the tested nematodes within 24 h (Qiuhong *et al.,* 2006). *Bacillus megaterium* a Gram-positive, aerobic, endospore forming, rod shaped bacterium (Vary *et al.,* 2007), was used in the biocontrol of *Meloidogyne graminicola* i.e. these bacteria were found to reduce the severity of root-knot galling and nematode penetration in rice roots over a period of 20 days (Padgham and Sikora, 2007). *Bacillus cereus,* a facultative, anaerobic, endospore-forming rod was used as a biocontrol agent to protect alfalfa from seedling disease caused by *Phytophthora megasperma* f. sp. *medicaginis*. (Handelsman *et al.,* 1990) and promoted mycorrhiza-assisted germination (Wilkinson *et al.,* 1994).

However, only certain isolates of *Bacillus* may exhibit nematotoxic effects over a longer period of time (Padgham and Sikora, 2006) than tested in this study, i.e. *B. megaterium* was most effective against *Paratrichodorus* with no effect on *Meloidogyne* nematodes after 3 h and no significant effect on *Pratylenchus*. *B. cereus* was found to be the most effective against *Paratrichodorus* followed

closely by *Pratylenchus* after just 3 h and was minimally effective against *Meloidogyne*.

*Paenibacillus chitinolyticus (*Kuroshima *et al.,* 1996), is a Gram-positive, facultatively anaerobic, endospore-forming bacterium, previously known as *Bacillus chitinolyticus (*Lee *et al.,* 2004) and produces chitinase (Jami al Ahmadi *et al.*, 2008). This genus was originally included within the genus *Bacillus* and then reclassified as a separate genus in 1993 (Ash *et al.,* 1993). Chitinase is a glycosyl hydrolase, which catalyses the degradation of chitin by hydrolysing chitin to its oligomers and monomer, N-acetyl-β-D-glucosamine (Jami al Ahmadi *et al.*, 2008). Chitin, an insoluble linear β-1, 4-linked polymer of N-acetyl glucosamine (acetamido-2-deoxy-D-glucose) is the structural component of fungal cell wall, exoskeleton of insects and crustaceans (Flach *et al.,* 1992). In this study *Paenibacillus chitinolyticus* was most effective as a biocontrol against *Paratrichodorus*, but indicated limited affectivity against *Pratylenchus* and *Meloidogyne.*

Stenotrophomonas maltophilia - previously classified as *Pseudomonas maltophilia*, then *Xanthomonas maltophilia* (Palleroni and Bradbury, 1993; Denton and Kerry, 1998), a Gram-negative, non-fermentative bacillus (Bollet *et al.,* 1995; Berg *et al.,* 1998, Gilligan *et al.,* 2003) also produces chitinase (Chen and Caswell-Chen, 2001). *S. maltophilia* C3 strain was also reported to be nematotoxic against *Caenorhabditis elegans* and *Heterodera schachitii* nematodes (Chen and Caswell-Chen, 2001) and *S. maltophilia* G2 had a high nematotoxic activity against *Panagrellus redivivus* and *Bursaphelenchus xylophilus*, which are free-living and plant-parasitic nematodes respectively (Huang *et al.,* 2009). Insunza *et al.* (2002) reported *S. maltophilia* to suppress the *Trichodorid* nematode population and Cronin *et al.* (1997) reported that *S. maltophilia* reduced egg hatch of the potato cyst nematode, *Globodera rostochiensis*. In this study S*. maltophilia* had the best biocontrol on

Paratrichodorus nematodes and no biocontrol on *Pratylenchus*. There was no significant effect on *Meloidogyne* either.

Soil bacteria are associated with various groups of AM fungi (Paulitz and Linderman, 1991) and changes in the communities of microorganisms occur in the mycorrhizosphere (Andrade *et al.,* 1997; Wamberg *et al.,* 2003). Dual and multimicrobial inoculations with AM fungi and plant growth promoting rhizobacteria (PGPR) have been shown to improve plant nutrition, growth and reduce plant diseases more effectively compared to single inoculations (Akhtar and Siddiqui 2008; Siddiqui and Akhtar 2009). Liu *et al.* (2011), reported that dual inoculations with *G. mosseae* and *Bacillus sp.*, improved tomato plant growth and inhibited the development of *M. incognita*. This dual combination was found to be most effective in the reduction of penetration of the tomato roots by nematodes, nematode reproduction, and nematode-incited disease (Liu *et al.*, 2011).

Colonisation of plants by AM fungi therefore provides a bioprotectional effect against a broad range of nematodes (Pinochet *et al.,* 1996; Elsen *et al.,* 2003a-b; Hol and Cook, 2005, Zhang *et al*., 2008, 2009, Vos *et al*., 2011). Zhang *et al*. (2008) reported that pre-inoculation of cucumber plants with AM fungi *Glomus intraradices*, *Glomus mosseae*, and *Glomus versiforme* significantly reduced the reproduction of the root knot nematode *Meloidogyne incognita* in a pot experiment i.e. the number of eggs per root system was significantly decreased by AM fungus inoculation, with no significant difference among the three AM fungal isolates. In a later study conducted to investigate the tolerance of cucumber plants (*Cucumissativus* L.) to *M. incognita* after inoculation with *Glomus intraradices,* Zhang *et al.* (2009) reported that mycorrhizal colonization increased shoot dry weight, shoot length, leaf numbers, root fresh weight and shoot P concentration, whereas nematode penetration and reproduction were significantly suppressed during the early stages of plant growth and that P fertilizer conferred tolerance of cucumber plants to *M. incognita* (Zhang *et al*.,

2009). In tomato roots (*Solanum lycopersicum* cv. Marmande) *G. mosseae* was found to continuously suppress root-knot nematode *M. incognita* throughout the early infection phase of root penetration and subsequent life stage development (Vos *et al*., 2011).

In a most recent study Hao *et al*. (2012) indicated that colonisation by AM fungi reduced both gall formation on roots of the grapevine and nematode number in the surrounding soils. The suppressive effects of *Glomus* (*syn. Rhizophagus*) *intraradices* BEG141 against *Xiphinema index* nematodes on roots of grapevine rootstock SO4 (*Vitis berlandieri3V. riparia*) were shown to increase with time and these effects were greater when the nematode was post-inoculated rather than co-inoculated with the arbuscular mycorrhizal (AM) fungus (Hao *et al*., 2012).

Soil bacteria associated with various groups of AM fungi may complement mycorrhizal activities, particularly in biological control in agricultural systems (Budi *et al.,* 1999). The above mentioned studies confirm that AM fungi and various PGPR have bioprotection capabilities against pathogenic nematodes, while this preliminary study indicates the biocontrol capabilities of AMB. The synergistic effects of particularly AM fungi-AMB in activity and function to suppress the effects of nematodes in South African sugarcane varieties therefore demands further experimentation.

5.5 CONCLUSION

This study confirms the presence of bacteria on AM fungal spores isolated from sugarcane agroecosystems. The nature of the interaction between these two groups of organisms is still unclear but the discussion above indicates that some of these AMB are implicated as natural biocontrol agents of some pathogenic nematodes. The fact that some AMB can act as a biological control agent while improving AM fungi formation opens the possibility for future studies using an the multimicrobial AM fungi-AMB inoculation approach for ensuring healthy sugarcane ecosystems. The preliminary study to determine the nematoxic effect

of AMB on nematodes *in-vitro* now requires further consideration as the recommended dosage and microbial inhibitory concentrations are yet to be determined. Further experimentation *in-vitro* will help reveal mechanisms of interactions.

CHAPTER 6

SUMMARY

South African sugarcane varieties are ravaged by insect pests and diseases that lead to severe crop losses because of the continuous impairment to plant physiology. Research at SASRI is focused on improving sugarcane yields, sugar content, ratooning ability, maintaining or improving disease resistance, and maintaining acceptable fibre levels for milling. This led to the development of new high yield cultivars, which showed a good response to fertilizer application, more uniform productivity and shorter maturation times. However high energy costs of fertilizer production, diminishing availability of rock phosphate and natural resources as well as increased contamination of aquatic systems by agrochemicals and greenhouse gas emissions has also prompted SASRI to now move toward more sustainable sugarcane farming systems, by reducing the use of agrochemicals and increasing use of beneficial microbiological systems, at the same time taking into account the needs of small-scale growers.

To date the use of arbuscular mycorrhizal (AM) fungi together with other soil microbes in sugarcane agriculture in South Africa is not fully understood. Studies globally have thus far indicated approximately thirty AM fungi species to be associated with varieties of sugarcane, while glasshouse experiments as well as laboratory experiments using individual AM fungi species as inocula showed enhanced plant root development and growth. Given the paucity and fragmented nature of these studies, on the functioning of AM in sugarcane, a holistic and unique approach was required to study the mycotrophic nature of commercial sugarcane varieties grown in the KwaZulu-Natal region. The outcomes of my studies are summarized below.

6.1 Arbuscular mycorrhiza (AM) status in commercial varieties of sugarcane in P-fixing and non P-fixing soil regions of KwaZulu-Natal and the growth response of sugarcane in the glasshouse to inoculation with indigenous AM fungi

This first study was a preliminary study done to assess the natural mycorrhization status of field-grown AM sugarcane varieties grown in both P-fixing and non P-fixing soils as well as assessing the most mycotrophic of these varieties for growth (stalk elongation) enhancement/suppression by indigenous AM fungi with and without soil microbiota under glasshouse conditions.

The naturally mycotrophic nature of commercial sugarcane varieties N12, N31, N35, N37 and N39, indigenous to P-fixing and non P-fixing regions of KwaZulu-Natal was confirmed. Root percentage colonisation was determined via the gridline intersect method and all varieties were mycorrhizal with values below 50% and the N12 variety proving to be the most mycotrophic at the time of sampling. Overall levels of mycorrhization in varieties were similar, suggesting that breeding had not interfered with the genetic pre-disposition of the plant to form AM and that edaphic characteristics are not strong deterministic factors in the plant's dependence on AM.

To assess the effect of AM fungi and microflora inoculation on growth of N12 sugarcane in a glasshouse study, three treatments were created: sterilized soil; non-sterile soil, and sterilized washed soil plus soil bacterial microflora, which were then inoculated with AM fungi or uninoculated. Plants inoculated with AM fungi were generally taller than uninoculated controls, and plants grown in sterilized soil, washed and supplemented with microflora also indicated higher growth levels, indicating that microflora and AM fungi enhanced rather than suppressed sugarcane growth. These positive effects of AM fungi and microflora in sugarcane were evident mostly during the early establishment phase, compared with the maturing stages. This part of the study could have been made

stronger had the colonisation data not been compromised due to the fragility of the roots.

Results presented in the preliminary study illustrated that South African varieties of sugarcane growing in the field are naturally mycotrophic and that sugarcane growth is enhanced rather than suppressed by AM fungi and bacterial microflora. It may be that the positive effects of AM fungi in sugarcane occur during the early establishment phase but AM fungi are less important later on and that sugarcane is not obligately mycotrophic. This observation, taken together with the ability of sugarcane to grow well in sterile soil without microflora additions, suggests that the plant may be facultatively mycotrophic. This hypothesis would have to be explored in a more rigorous study, which measures a greater range of growth and metabolic parameters continuing into the maturation and flowering phases, and confirmed in the field, but would aid in decisions around when to apply AM fungi inoculum if it were to become a routine part of sugarcane agriculture.

In this regard, the type of inoculum would need to be further investigated i.e. a crude cocktail of spores of unidentified indigenous species, as used in this study, versus pure single species inoculum. An avenue to explore would be the bulking of AM fungi sugarcane spores using *in-vitro* cultures methods and transformed sugarcane roots. A focus of future research could be the *in-vitro* modification of sugarcane roots to hairy root status which has not been established before. These could then be used as starter cultures for bulking up host specific AM fungi inocula for sugarcane agriculture.

6.2 Multivariate analysis of plant-soil nutrient relationships in arbuscular mycorrhizal sugarcane grown in the North Coast region of KwaZulu-Natal, South Africa

Given the paucity of field trial studies on the functioning of AM in sugarcane, this study successfully indicated the potential buffering effects of AM under nutrient deficient and stressed conditions and further provided a novel holistic perspective on soil-plant nutrient relationships of the genetically homogeneous sugarcane plants under AM fungi colonisation. N12 variety was chosen, as it was the most mycotrophic in variety studies and had already been used in the glasshouse trial. An existing 4000 m^2 sugarcane plantation consisting of 71 plots of 14 month old N12 variety sugarcane was used for this investigation. The plantation was located at La Mercy 2-2 on the KwaZulu-Natal north coast (29°36`S - 31°06`E). This non P-fixing sandy soil plantation was deliberately chosen due to soil acidity and salinity factors and a history of lack of fertilizer application which would, in all likelihood, result in the plant experiencing mineral stresses and deficiencies (confirmed by leaf analysis). This scenario was ideal to study the effects of mycorrhization on plant nutrient uptake.

Soil and leaf sampling was done in early summer, percentage AM fungi colonisation was determined and soil and leaf nutrient levels were analysed. To determine if AM fungi played a role in buffering effects of nutrient uptake between the soil and leaves, two mycorrhization categories were determined: (1) low percentage mycorrhization (range 9-26%) and (2) high percentage mycorrhization (range: 32-53%). Corresponding soil and leaf nutrient levels for the respective samples were matched and categorized, to allow statistical analysis via the ADE-4 multivariate analysis programme. This programme used by SASRI at the time of this study was used to generate complicated graphical maps and crossed tables for each category. This allowed us to see the differences in relationships between nutrients in the soil and those found in the leaves, from a

holistic level. To my knowledge, this approach to study AM fungi-sugarcane interactions is novel.

The ADE-4 programme also allowed the viewing of convex hulls, which in this study indicated soil homogeneity, allowing the assumption that dissimilarities were influenced by AM fungi. PCA-PCA coupling allowed co-structures or relationships within the leaf-soil covariance matrix to be determined. The flexibility of the co-inertia analysis allowed for a maximized covariance when factors were computed for each table. To determine if relationships between the leaf and soil tables were statistically significant, the random permutation test of the co-structure between two tables was performed (co-inertia fixed-D test). One thousand permutations were used for an alpha level of 0.05 ($P = 0.05$). Soil and leaf relationships were statistically significant: $P < 0.001$ for the low percentage mycorrhizal status tables and $P < 0.020$ for the high percentage mycorrhizal status tables, thus allowing the simultaneous reading of both soil and leaf tables in each category.

The co-inertia analysis was also used to compute and display a crossed table containing the sum of the cross products between the leaf and soil variables for each category. More positive nutrient relationships were evident in highly colonized plants compared to lower percentage colonized plants. Positive relationships in high mycorrhizal plants appeared to be between: soil pH - leaf N, P, S, Mg and Mn; soil P - leaf Zn and Fe; soil Ca – leaf N, Ca and Mn, soil Mg – leaf Ca and Mn; soil Na – leaf K; soil Al – leaf A, Zn, Si, Ca, Mg and Fe, soil C – leaf P and Mn. Different positive relationships appeared in low percentage mycorrhizal plants viz. soil K – leaf Cu; soil S – leaf S and Mg; soil Zn – leaf Ca, Zn and Si; soil Fe – leaf Cu and Si; soil Mn- leaf Fe. Weak relationships in highly mycorrhizal plants occurred between: soil Mn, Zn – leaf Cu; soil pH, Ca, Mg, Al – leaf K, while in low percentage mycorrhizal plants: soil pH, Mg, C – leaf Cu; soil K – leaf N; soil Na – leaf Ca appeared to have weak relationships.

While not all crossed tabled relationships could be explained, this study clearly indicated that AM fungi “buffers” nutrient uptake mechanisms in sugarcane grown in infertile, acidic and saline soils. Since AM fungi buffering effects are governed by multi-complex processes it is recommended mechanistic simulation models should be taken into consideration for a better understanding of root and hyphal properties in relation to the “buffering” mechanisms of symbiosis. At this point it is important to note that nutrient deficiencies were expected to be evident in the plant, however, symptoms of deficiency usually observed were not visible on plant leaves. In future work, I recommend that root samples also be analysed for nutrient levels, as this will provide an even better idea into AM fungi-plant nutrient dynamics. Roots were not analysed for nutrient levels, as FAS analysis required a larger sample size than used for percentage mycorrhization. As this trial was still on-going at the time, further disruption of root structures was not allowed, as this would have interfered with several plant dynamics for that trial. Furthermore, the N of the soil could not be measured due to technical problems with the FAS analyser.

6.3 Morphological and partial molecular identification of arbuscular mycorrhizal fungi isolated from sugarcane plantations in KwaZulu-Natal, South Africa

After establishing the “buffering” effects of mycorrhizas in sugarcane nutrient uptake, it was important to identify the AM fungi associated with the sugarcane soils used in this study. This third study firmly established that sugarcane soils in South Africa are colonised by the *Acaulospora*, *Gigaspora, Glomus* and *Scutellospora* genera. Five spore types were identified morphologically as *Acaulospora mellea, Gigaspora margarita, Glomus geosporum, Scutellospora nigra* and *Scutellospora verrucosa.* Four spores belonged to the Diversisporales order and one to the Glomerales order. Three spore types belonged to the Gigasporaceae family, one spore type to the Acaulosporaceae family, and one to the Glomaceae family.

Spores directly extracted from the field, included both mature and immature, making morphological identification somewhat challenging, with respect to spore size or other morphological features that are too similar or scarce in some AM fungi. These spores were isolated directly from the mycorrhizospheres of sugarcane from P-fixing and non P-fixing soils, at the same time the crude spore inocula were isolated for the pot study of chapter 2, as this saved time as trap cultures could not be established.

While morphological studies on diversity of AM fungi in Iran and South India indicated approximately thirty AM fungi species to be associated with different varieties of sugarcane with *Glomus aggregatum* and *G. fasciculatum* were found to be commonly associated with sugarcane grown in both Iran and South India; only *G. geosporum* appeared to be commonly associated with sugarcane from South Africa, Iran and South India. In South Africa, species of AM fungal spores were also identified by Straker *et al*. (2010); from rhizospheres of cassava which included: *Glomus etunicatum, Glomus rubiforme, Acaulospora mellea, Acaulospora scrobiculata, Acaulospora tuberculata, Gigaspora sp. 1, Gigaspora sp. 2, Scutellospora sp.* while (Gaur *et al*,(1999) identified *Glomus etunicatum*, *G. intraradices, G. occultum and Gigaspora albida* species to be associated with an indigenous fruit tree, *Vangueria infausta*. Only *Acaulospora mellea* was found to be common in sugarcane and cassava rhizospheres.

Identified spores require further confirmation by crop inoculation, re-isolation and re-identification. Molecular analysis was attempted using already established primer sequences (Redecker, 2000). However, DNA isolation of AM from sugarcane roots as conducted by Redecker, (2000) was not successful. Therefore partial molecular identification of spores morphotyped according to their colour types and coded (O= orange ($\pm$ 250); B = black ($\pm$ 250); BR = brown ($\pm$ 250); R = red ($\pm$ 250), but not yet morphologically identified). Molecular analysis indicated the presence of DNA from the *A. gerdemannii/A. trappei* group, *G. occultum/G. brasilianum* group (brown spores), *Glomus mosseae/*

intraradices group (orange, black and brown spores), *Glomus etunicatum/ clariodeum* group (all spores) and Acaulosporaceae *sensu stricto* (all spores) i.e. five of eight phylogenetic groups described by Redecker (2000). Only partial molecular identification was conducted, as RFLPs could not be carried out. Further optimisation was required but sample volume limited this process and partial molecular identification could not be matched to the morphologically identified spores.

Future work on identification of indigenous AM fungi in sugarcane fields in South Africa would include the bulking of spores in trap and single culture *in-vitro* culture techniques, and complete DNA sequence analysis using recently designed Glomeromycota AML1 and AML2 primers by Lee *et al*. (2008). These primers are recommended as they were based on representative SSU rRNA gene sequences of AM fungi from each phylogenetic group (Schüßler, 2007) are meant to amplify all subgroups of AM fungi, they exclude sequences from other organisms and they were also designed to facilitate rapid detection and identification directly from field-grown plant roots.

6.4 Molecular identification of bacteria isolated from AM fungal spores associated with sugarcane and preliminary testing of Associated Mycorrhiza Bacteria (AMB) as biocontrol agents for nematodes *in-vitro*

In this study, arbuscular mycorrhiza bacteria (AMB) from indigenous AM fungi were successfully isolated and identified. Bacteria were isolated directly from spores of *Acaulospora mellea*, *Gigaspora margarita, Glomus geosporum* and *Scutellospora nigra* after treatment to remove any fungal contaminants. After establishing pure cultures of the dominant bacteria, DNA sequence analysis indicated these AMB to be *Brevibacillus reuszeri* isolated from *Scutellospora nigra*, *Bacillus megaterium* and *Stenotrophomonas maltophilia* isolated from

Glomus geosporum. *Paenibacillus chitinolyticu*s and *Bacillus cereus* were isolated from *Acaulospora mellea* and *Gigaspora margarita* spores respectively.

Thereafter the AMB were tested for biocontrol capability against pathogenic plant nematodes, *Paratrichodorus*, *Pratylenchus* and *Meloidogyne* in a preliminary study. *Brevibacillus reuszeri* caused the highest mortality against *Paratrichodorus,* followed by *Bacillus megaterium, Paenibacillus chitinolyticus, Bacillus cereus and Stenotrophomonas maltophilia. Brevibacillus reuszeri was* moderately effective against *Meloidogyne* but less effective against *Pratylenchus. Stenotrophomonas maltophilia, Bacillus megaterium, Paenibacillus chitinolyticus, Bacillus cereus* indicated low or no biocontrol activity against *Meloidogyne and Pratylenchus* respectively*. Meloidogyn*e proved to be the least susceptible to AMB biocontrol and *Paratrichodorus* the most susceptible. All interactive effects were significant, demonstrating an indirect benefit of AM fungi to act as a biocontrol agent against plant parasitic nematodes by way of their spore-associated helper bacteria.

Since variability in bacterial virulence was host dependent, I recommend that this be further studied in pot trials since the nature of the interaction between these two groups of organisms is still unclear. The fact that some AMB can act as a biological control agent while improving AM fungi formation opens the possibility for future studies using an the multimicrobial AM fungi-AMB inoculation approach for ensuring healthy sugarcane ecosystems. The preliminary study to determine the nematoxic effect of AMB on nematodes *in-vitro* requires further consideration as the recommended dosage and microbial inhibitory concentrations are yet to be determined. Further experimentation *in-vitro* will help reveal mechanisms of interactions.

CONCLUSION

The use of indigenous arbuscular mycorrhizal fungi as inocula in sugarcane agriculture is indeed vital to the sugarcane industry of South Africa with respect to long-term crop protection and sustainability. Internationally, there has been little research done on the AM symbiosis with sugarcane and no studies have emanated from South Africa. This project provided novel holistic insights into AM sugarcane in South Africa, not only in relation to plant growth and nutrient relationships, but also in relation to some of the associated microflora and microfauna, both beneficial and non-beneficial. This novel approach confirmed the importance of arbuscular mycorrhiza and other soil microorganisms in sugarcane crop protection and sustainability. Future work commands further analyses into the interaction of AM fungi and rhizospheric organisms using *in-vitro* culture methods and optimised molecular strategies to provide more insight into the more complex associations within the sugarcane agroecosystems.

REFERENCES

Abbott, L.K. and Robson A.D. (1984), The effect of mycorrhizae on plant growth, In: Powell, C.L. and Bagyaraj D.J. eds. *VA mycorrhiza*. Florida, USA: CRC Press Inc., pp 113-130.

Akhtar, M.S. and Siddiqui, ZA. (2008), Biocontrol of a root-rot disease complex of chickpea by *Glomus intraradices, Rhizobium sp*. and *Pseudomonas striata*. *Crop Protection*, 27: 410–417.

Alexander, A.G. (1973), *Sugarcane Physiology: A comprehensive study of the Saccharum source-to-sink system*. Amsterdam, Netherlands: Elsevier Scientific Publishing Company, pp 69-269.

Alguacil, M.M. Hernandez, J.A. Caravaca, F. Portillo, B. and Roldan, A. (2003), Antioxidant enzyme activities in shoots from three mycorrhizal shrub species afforested in a degraded semi-arid soil. *Physiologia Plantarum,* 118: 562–570.

Anderson, D.L. (1991), Soil and leaf nutrient interactions following the application of calcium silicate slag to sugarcane. *Fertilizer Research,* 30: 9-18.

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

Andreloa, F. (1982), Vesicular arbuscular mycorrhiza in sugarcane (*Saccharum officinarum*). *Piracicaba,* 83: 74.

Ash, C. Priest, F.G. and Collin, M.D. (1993), Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Proposal for the creation of a new genus *Paenibacillus*. *Antonie Van Leeuwenhoek International Journal of General Molecular Microbiology*, 64: 253-260.

Azcon-Aguilar, C. and Barea, J. M. (1985), Effect of soil microorganisms on formation of vesicular-arbuscular mycorrhizas. *Transactions of the British Mycological Society,* 83: 536–537.

Azcon-Aguilar, C. Diaz-Rodriguez, R. M. and Barea, J. M. (1986), Effect of soil microorganisms on spore germination and growth of vesicular-arbuscular mycorrhizal fungus *Glomus mosseae*. *Transactions of the British Mycological Society,* 86: 337–340.

Bago, B. Pfeffer, P.E. and Sachar-Hill, Y. (2000), Carbon Metabolism and transport in arbuscular mycorrhiza. *Plant Physiology,* 124: 949-957.

Bago, B. Pfeffer, P.E. Zipfel, W. Lammers, P. and Shachar-Hill, Y. (2002), Tracking metabolism and imaging transport in arbuscular mycorrhizal fungi, metabolism and transport in fungi. *Plant and soil,* 244: 189-197.

Barea, J. M. Azcon, R. and Azcon-Aguilar, C. (2002), Mycorrhizosphere interactions to improve plant fitness and soil quality. *Antonie Van Leeuwenhoek International Journal of General Molecular Microbiology*, 81: 343–351.

Barker, B. and Davis, S.B. (2005), Characteristics of cane varieties based on sugar processing parameters. *South African Sugarcane Technologist's Association. [Online]* http://www.sasta.co.za/wpcontent/uploads/Proceedings/ 2000s/2005_barker_characterisation %20of%20cane%20varieties.pdf).

Barnard, R.O. Buys, A.J. Coetzee, J.G.K. Du Prees, C.C. Meyer, J.H. van Vuuren, J.A.J. Volschen, J.E. Bessinger, F. and Lambrechts, J.J.N. (1990), *Handbook of standard soil testing methods for advisory purposes*. South African Sugar Technologists' Association, Mount Edgecombe, South Africa, pp 118-162.

Barnes, A. C. (1974), *The Sugarcane*. Aylesbury, UK: Leonard Hill books, pp 570-573.

Becker, W.N. and Hall, I.R. (1976), *Gigaspora margarita*, a new species in the Endegonaceae. *Mycotaxon,* 4: 155-160.

Berg, G. Marten, P. and Bahl, H. (1998), Population dynamics of bacteria including antifungal species in the rhizosphere of oilseed rape during its life cycle. *Archives of Phytopathology and Plant Protection,* 31: 215-224.

Beringer, H. Koch, K. and Lindhauder, M.G. (1986), Sucrose accumulation and osmotic potentials in sugar beet at increasing levels of potassium nutrition. *Journal Science of Food Agriculture,* 37: 211-218.

Bethlenfalvay, G.J. and Schüepp, H. (1994), Arbuscular mycorrhizas and agrosystem stability, In: Gianinazzi, S. and Schüepp, H. eds. *Impact of Arbuscular Mycorrhizas on Sustainable Agriculture and Natural Ecosystems*, Switzerland, Basel: Birkhauser Verlag, pp 117–131.

Bever, J. D. Morton, J. B. Antonovics, J. and Schultz, P. A. (1996), Host-dependent sporulation and species diversity of arbuscular mycorrhizal fungi in a mown grassland. *Journal of Ecology*, 84: 71-82.

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

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 and Environmental Microbiology*, 66: 4503-4509.

Bittencourt, M.F. Griffin, J.L. Bourreaux, J.M. and Blouin, D.C. (2010), Seasonal growth, shading potential and yield of six sugarcane cultivars. *Journal of American Society of Sugarcane Techniques,* 30: 21-36.

Blair, J.M. Bohlen, P.J. and Freckman, D.W. (1996), Soil invertebrates as indicators of soil quality, In: Doran, J.W. and Jones, A.J. eds., *Methods for*

Assessing Soil Quality. Madison, USA: Soil Science Society of America Special Publication 49, SSSA, pp 273-291.

Blanke, V. Wagner, M. Renker, C. Lippert, H. Michulitz, M. Kuhn, A. J. and Buscot, F. (2011), Arbuscular mycorrhizas in phosphate-polluted soil: interrelations between root colonization and nitrogen. *Plant and Soil*, 343(1-2): 379-392 (doi:10.1007/s11104-011-0727-9).

BLAST (Basic Local Alignment Search Tool) (2011), [Online] http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Blaszkowski, J. (1993a), Comparative studies of the occurrence of arbuscular fungi and mycorrhizae (*Glomales*) in cultivated and uncultivated soils of Poland. *Acta Mycologica*, 28: 93-140.

Blaszkowski, J. (1993b), The occurrence of arbuscular fungi and mycorrhizae (Glomales) in plant communities of maritime dunes and shores of Poland. *Bulletin of the Polish Academy of Sciences - Biological Sciences*, 41: 377-392.

Blaszkowski, J. (1994), Arbuscular fungi and mycorrhizae (Glomales) of the Hel Peninsula, Poland. *Mycorrhiza*, 5: 71-88.

Blaszkowski, J. Tadych, M. Madej, T. Adamska, I. Iwaniuk, A. (2001), Arbuscular mycorrhizal fungi (Glomales, Zygomycota) of Israeli soils. Mat. II Polsko-Izraelskiej Konf. Nauk. nt. "Gospodarowanie zasobami wodnymi i nawadnianie roslin uprawnych". *Przeglad Naukowy Inzynieria I Ksztaltowanie Srodowiska*, 22: 8-27.

Blaszkowski, J. Tadych, M. and Madej, T. (2002), Arbuscular mycorrhizal fungi (Glomales, Zygomycota) of the Bledowska Desert, Poland. *Acta Societatis Botanicorum Poloniae*, 71: 71-85.

Bolan, N.S. Robson, A.D. and Barrow, N.J. (1984), Increasing phosphorus supply can increase the infection of plant roots by vesicular-arbuscular mycorrhizal fungi. *Soil Biology and Biochemistry,* 16: 419-420.

Bolan, N.S. (1991), A critical review on the role of mycorrhizal fungi in the uptake of phosphorus by plants. *Plant Soil,* 134: 189-207.

Bolan, G.J. and Kuykendall, L.D. (1998), *Plant-microbe interaction and biological control*. New York, USA: Marcel Dekker, Inc., pp 111-126.

Bollet, C. Davin, R.A. and De Micco, P. (1995), A simple method for selective isolation of Stenotrophomonas maltophilia from environmental samples. *Applied Environmental Microbiology*, 61: 1653-1654.

Bradbury, J.F. (1977), *Erwinia carotovora* var.carotovora. *CMI descriptions of pathogenic fungi and bacteria*, 552:2.

Bradley, R. Burt, A.J. Read, D.J. (1982), The biology of mycorrhiza in the Ericaceae VIII. The role of mycorrhizal infection in heavy metal resistance. *New Phytologist*, 91:197–209.

Brundrett, M.C. Bougher, N., Dell, B. Grove, T.S. and Malajczuk, N. (1996), Examining Mycorrhizal Associations. *Working with Mycorrhizas in Forestry and Agriculture*. Canberra, Australia: Pirie Printers, pp 173-186.

Brundrett, M.C. (2004), Diversity and classification of mycorrhizal associations. *Biological Reviews,* 79: 473–495 (doi: 10.1017/S1464793103006316).

Bryce, B. and Kendrick, P. (2000), Mycorrhizas: Mutualistic Plant-Fungus symbiosis, In: The *Fifth Kingdom*. 3rd Edition. Massachusetts, USA: Focus Publishing Co., pp 32-277.

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: 89-912.

Budi, S.W. Van Tuinen, D. Martinotti, G. and Gianinazzi, S. (1999), Isolation from the *sorghum bicolour* mycorrhizosphere of a bacterium compatible with

arbuscular mycorrhiza development and antagonistic towards soilborne fungal pathogens. *Applied and Environmental Microbiology*, 65: 5148-5150.

Cadet, P. and Spaull, V.W. (2003), Effect of nematodes on the sustained production of sugarcane in South Africa. *Field Crops Research*, 83: 91–100.

Cadet, P. and Spaull V.W. (2005), Nematode parasites of sugarcane, In: Luc, M. Sikora, R.A. and Bridge, J. eds. *Plant parasitic Nematodes in subtropical agriculture*. Cambridge, USA: CAB International, pp 645-671.

Cadet, P. and Thioulouse, J. (1998), Identification of soil factors that relate to plant parasitic nematode communities on tomato and yam in the French West Indies. *Applied Soil Ecology,* 8: 35-49.

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

Carlson, J.E. Tulsieram, L.K. Glaubitz, J.C. Luk, V.W.K. Kauffeldt, C and Rutledge, R. (1991), Segregation of random amplified DNA markers in F1 progeny of conifers. *Theory of Applied Genetics*, 83:194–200.

Chaudhary, V.B. Lau, M.K. and Johnson, N.C. (2008), Macroecology of microbes – biogeography of the Glomeromycota, In: Varma, A. ed. *Mycorrhiza* 3rd Edition, Berlin, Germany: Springer-Verlag, pp 529-620.

Cheong, Y.W.Y. and Chan, P.Y. (1973), Incorporation of P^{32} in phosphate esters of the sugar cane plant and the effect of Si and Al on the distribution of these esters. *Plant and Soil*, 38 (1):113-123 (doi: 10.1007/BF00011221).

Chen, J. and Caswell-Chen, E. (2001), Effects of *Stenotrophomonas maltophilia* on *Caenorhabditis elegans* and *Heterodera schachitii*. *International C. elegans Meeting,* Online Key: citeulike: 3305162.

Chen, X.-H. and Zhao, B. (2009), Arbuscular mycorrhizal fungi mediated uptake of nutrient elements by Chinese milk vetch (*Astragalus sinicus L*.) grown in lanthanum spiked soil. *Biology and Fertility of Soils,* 45: 675–678.

Claassen, N. and Steingrobe, B. (1999), Mechanistic simulation models for a better understanding of nutrient uptake from soil, In: Rengel, Z. ed. *Mineral Nutrition of Crops: Fundamentals Mechanisms and Implications.* New York, USA: The Haworth Press, Inc., pp 327–367.

Clapp, J.P. Young, J.P.W. Merryweather, J.W. and Fitter, A.H. (1995), Diversity of fungal symbionts in arbuscular mycorrhizas from a natural community. *New Phytologist,* 130: 259–265.

Clapp, J.P., Fitter, A.H. and Young, J.P.W. (1999), Ribosomal small subunit sequence variation within spores of an arbuscular mycorrhizal fungus, *Scutellospora sp*. *Molecular Ecology*, 8: 915–921.

Clark, R. B. (1997), Arbuscular mycorrhizal adaptation, spore germination, root colonization and host plant growth and mineral acquisition at low pH. *Plant and Soil,* 192: 15-22.

Clark, R.B. and Zeto, S.K. (1996a), Growth and root colonization of mycorrhizal maize grown on acid and alkaline soil. *Soil Biology and Biochemistry,* 28: 1505–1511.

Clark, R.B. and Zeto, S.K. (1996b), Mineral acquisition by mycorrhizal maize grown on acid and alkaline soil. *Soil Biology and Biochemistry,* 28: 1495-1530.

Clark, R.B. and Zeto, S.K. (1996c), Iron acquisition by mycorrhizal maize grown on alkaline soil. *Journal of Plant Nutrition,* 19: 247-264.

Clark, R.B. Zobel, R.W. and Zeto, S.K. (1999a), Effects of mycorrhizal fungus isolates on mineral acquisition by *Panicum virgatum* in acidic soil. *Mycorrhiza*, 9: 167–176.

Clark, R.B., Zeto, S.K. and Zobel, R.W. (1999b), Arbuscular mycorrhizal fungal isolate effectiveness on growth and root colonization of *Panicum virgatum* in acidic soil. *Soil Biology and Biochemistry,* 3 (13): 1757-1763.

Colla, G. Rouphael, Y. Cardarelli, M. Tullio, M. Rivera, C.M. and Rea, E. (2008), Alleviation of salt stress by arbuscular mycorrhizal in zucchini plants grown at low and high phosphorus concentration. *Biology and Fertility of Soils,* 44: 417–424.

Comerford, N.B. (1998), Soil Phosphorus bioavailability, In: Lynch, J.P. and Diekerman, J. eds. *Phosphorus in plant biology*. Maryland, USA: American Society of Plant Physiologists, pp 136-147.

Comstock, J.C. (2000), Smut, In: Rott, P., Bailey, R.A., Comstock, J.C., Croft, B.J. and Salem Saumtally, A. eds. *A Guide to Sugarcane Diseases*, CIRAD and ISSCT, pp 181–185.

Comstock, J.C. Shine, J.J. and Raid, R.N. (1992), Effect of rust on sugarcane growth and biomass. *Plant Disease*, 76: 175-177.

Cronin, D. Moënne-Loccoz, Y. Dunne, C. and O'Gara, F. (1997), Inhibition of egg hatch of the potato cyst nematode Globodera rostochiensis by chitinase-producing bacteria. *European Journal of Plant Pathology*, 103: 433–440.

Dalpé, Y. Adriano de Souza, F. and Declerck, S. (2005), Host and Non-Host Impact on the Physiology of the AM Symbiosis, In: Declerck, S, Strullu, D.G. and Fortin J.A. eds. *Soil biology vol 4: In-vitro Culture of Mycorrhizas*, London, UK: Springer, pp 49-71.

Davies, M.I. and Bailey, R.A. (2000), Ratoon stunting, In: Rott, P., Bailey, R.A., Comstock, J.C., Croft, BJ. and Saumtally, A.S. eds., *A Guide to Sugarcane Diseases.* CIRAD-ISSCT, pp 49-54.

Denton, M. and Kerr, K. (1998), Microbiological and clinical aspects of infection associated with Stenotrophomonas maltophilia. *Clinical Microbiology Reviews*, 11(1): 57–80.

Dodd, J. C. Arias, I. Koomen, I. and Hayman, D. S. (1990), The management of populations of vesicular-arbuscular mycorrhizal fungi in acid-infertile soils of a savannah ecosystem. *Plant and Soil*, 122: 241-247.

Douds, D. D. and Schenck, N. C. (1990), Relationship of colonization and sporulation by VA mycorrhizal fungi to plant nutrient and carbohydrate contents. *New Phytologist,* 116: 621-627.

Douds, D. D. and Millner, P. D. (1999), Biodiversity of arbuscular mycorrhizal fungi in agroecosystems. *Agriculture, Ecosystems & Environment*, 74(1-3): 77-93 (doi:10.1016/S0167-8809(99)00031-6).

Dupré de Boulois, H. Delvaux, B. and Declerck, S. (2005), Effects of arbuscular mycorrhizal fungi on the root uptake and translocation of radio caesium. *Environmental Pollution,* 134: 515–524.

Elsen, A. Baimey, H. Swennen, R. and De Waele, D. (2003a), Relative mycorrhizal dependency and mycorrhiza-nematode interaction in banana cultivars (*Musa spp.*) differing in nematode susceptibility. *Plant Soil*, 256: 303–313.

Elsen, A. Beeterens, R. Swennen, R. and De Waele, D. (2003b), Effects of an arbuscular mycorrhizal fungus and two plant-parasitic nematodes in Musa genotypes differing in root morphology. *Biology of Fertile Soils*, 38: 367–376.

Elsen, A. Gervacio, D. Swennen, R. and Waele, D.D. (2008), AM fungi-induced biocontrol against plant parasitic nematodes in *Musa sp.*: a systemic effect. *Mycorrhiza*, 18: 251–256.

Estrada-Torres, A. Varela, L. Hernandez-Cuevas, L. and Cavito, M. (1992), Some arbuscular mycorrhizal fungi from the state of Tlaxa. Mexico. *Revista Mexicana de Micología*, 8: 85-110.

Evelin, H. Kapoor, R. and Giri, B. (2009), Arbuscular mycorrhizal fungi in alleviation of salt stress: a review. *Annals of Botany*, 104 (7): 1263-80 (doi:10.1093/aob/mcp251).

Fageria, N.K. and Zimmermann, F. J. P. (1998), Influence of pH on growth and nutrient uptake by crop species in an Oxisol. *Communications in Soil Science and Plant Analysis,* 29: 17-18, 2675-2682.

Fageria, N. K. Baligar, V. C. and Clark, R. B. (2002), Micronutrients in Crop Production. *Advances in Agronomy*, 77: 189-272.

Ferrer, R. L. and Herrera, R. A. (1980), El genero *Gigaspora* Gerdemann *et* Trappe (Endogonaceae) en Cuba. *Revista del Jardín Botánico Nacional*, 1: 43-66.

Finlay, R.D. and Rosling, A. (2006), Integrated nutrient cycles in forest ecosystems, the role of ectomycorrhizal fungi, In: Gadd, G.M. ed. *Fungi in biogeochemical cycles*, Cambridge, UK: Cambridge University Press, pp 28–50.

Flach, J.P. Pilot, E. and Jollies, P. (1992), What's new in chitinase research? *Experientia*, 48: 701-716.

Foy, C.D. (1992), Soil chemical factors limiting plant root growth. *Advances in Soil Science,* 19: 87-149.

Frank, A. B. (1885), Ueber die auf Wurzelsymbiose beruhende Ernährung gewisser Bäume durch unterirdische pilze.*Berichte der Deutschen Botanischen Gesellschaft,* 3: 128-145.

Franken, P. and Gianinazzi-Pearson, V. (1996), Construction of genomic phage libraries of the arbuscular mycorrhizal fungi *Glomus mosseae* and *Scutellospora castanea* and isolation of ribosomal RNA genes. *Mycorrhiza,* 6: 167–173.

Gamper, H.A. Walker, C. and Schüßler, A. (2009), *Diversispora celata* sp. nov: molecular ecology and phylotaxonomy of an inconspicuous arbuscular mycorrhizal fungus. *New Phytologist, 182*: 495–506.

Garbaye, J. (1991), Costs and benefits of mycorrhizas: Implications for functioning under natural conditions. *Experientia*, 47: 350-354.

Garbaye, J. (1994), Helper bacteria: a new dimension to the mycorrhizal symbiosis. *New Phytologist,* 128: 197-210.

Garbaye, J. and Bowen, G.D. (1989), Stimulation of ectomycorrhizal infection of Pinus radiate by some microorganism associated with the mantle of ectomycorrhizas*. New Phytologist*, 112: 383-388.

Garg, N. and Chandel, S. (2010), Arbuscular mycorrhizal networks: process and functions- a review. *Agronomy for Sustainable Development,* 30: 581–599.

Gaur, A. Van Greuning, J.V. Sinclair, R.C. and Eicker, A. (1999), Arbuscular mycorrhizas of *Vangueria infausta* Burch. Subsp. *Infausta (Rubiaceae)* from South Africa. *South African Journal of Botany,* 65: 434–436.

Gerdemann, J.W. and Trappe, J.M. (1974), The Endogonaceae in the Pacific Northwest. *Mycologia Memoir,* 5: 76.

Gilligan, P.H. Lum, G. Van Damme, P. A. R. and Whittier, S. (2003), *Burkholderia, Stenotrophomonas, Ralstonia, Brevundimonas, Comamonas, Delftia, Pandoraea, and Acidivorax*, In: Murray PR, Baron EJ, Jorgensen JH *et al.* eds. *Manual of Clinical Microbiology* 8th edition. Washington DC, USA: ASM Press, pp 729–748.

Giovannetti, M. Azzolini, D. and Citernesi, A.S. (1999), Anastomoses formation and nuclear and protoplasmic exchange in arbuscular mycorrhizal fungi. *Applied and Environmental Microbiology*, 65(12): 5571–5575.

Giovannetti, M. Sbrana, C. Strani, P, Agnolucci, M. Rinaudo, V. and Avio, L. (2003), Genetic diversity of isolates of *Glomus mosseae* from different geographic areas detected by vegetative compatibility testing and biochemical and molecular analysis. *Applied and Environmental Microbiology*, 69: 616–624 (doi: 10.1128/AEM.69.1.616-624).

Giri, B. Kapoor, R. and Mukerji, K.G. (2007), Improved tolerance of *Acacia nilotica* to salt stress by arbuscular mycorrhiza, *Glomus fasciculatum*, maybe partly

related to elevated K^+ /Na^+ ratios in root and shoot tissues. *Microbial Ecology*, 54: 753–760.

Glasziou, K. T. and Gayler, K. R. (1972), Sucrose accumulation in sugarcane. Role of cell walls in sucrose transport. *Plant Physiology,* 49: 912-913.

Goerge, E. (2000), Nutrient Uptake, In: Kapulink, Y. and Douds. Jr. D.D. eds. *Arbuscular Mycorrhizas: Physiology and Function*. Dordrecht, Netherlands: Kluwer Academic Publishers, pp 307-344.

Govindarajulu, M., Pfeffer, P. and Jin, H. (2005), Nitrogen transfer in arbuscular mycorrhizal symbiosis. *Nature,* 345: 819-823.

Grandi, R. A. P. and Trufem, S. F. B. (1991), Fungos micorrízocos vesículo-arbusculares em *Marantaceae cultivadas* no Instituto de Botânica, São Paulo, SP. Revista Brasil. *Botany,* 14: 89-95.

Hadar, Y. Chet, I. and Henis. Y. (1979), Biological control of *Rhizoctonia solani* damping-off with wheat bran culture of *Trichoderma harziamum*. *Phytopathology*, 69: 64-68.

Haas, J. H., Menge, J. A. (1990), VA-mycorrhizal fungi and soil characteristics in avocado (*Persea americana* Mill.) orchard soils. *Plant and Soil,* 127: 207-212.

Habte, M. and Osorio, N.W. (2001), Arbuscular mycorrhizas: producing and applying arbuscular mycorrhizal inoculum. Honolulu, Hawaii: College of Tropical Agriculture and Human Resources (CTAHR), University of Hawaii, pp 2-47.

Hall, I. R. (1977), Species and mycorrhizal infections of New Zealand Endogonaceae. *Transactions of the British Mycological Society*, 68: 341-356.

Hamel, C. Dalpé, Y. Lapierre, C. Simard, R. R. and Smith D. I. (1994), Composition of the vesicular-arbuscular mycorrhizal fungi population in an old meadow as affected by pH, phosphorus and soil disturbance. *Agriculture, Ecosystems & Environment,* 49: 223-231.

Hammer, E. C. Nasr, H. Pallon J. Olsson P.A. and Wallander H. (2011), Elemental composition of arbuscular mycorrhizal fungi at high salinity. *Mycorrhiza* 21: 117–129. (doi 10.1007/s00572-010-0316-4).

Handelsman, J. Raffel, S. Mester, E. H. Wunderlich, L. and Grau, G.R. (1990), Biological Control of Damping-Off of Alfalfa Seedlings with *Bacillus cereus* UW85. *Applied and Environmental Microbiology,* 56(3): 713-718.

Hao, Z. Fayolle, L. van Tuinen, D. Chatagnier, O. Li, X. Gianinazzi, S. and Gianinazzi-Pearson, V., (2012), Local and systemic mycorrhiza-induced protection against the ectoparasitic nematode *Xiphinema index* involves priming of defence gene responses in grapevine. *Journal of Experimental Botany*, 63(10): 3657-3672. (doi:10.1093/jxb/ers046).

Harley, J.L. and Harley, E.L. (1987), A check-list of mycorrhiza in the British flora. *New Phytologist,* 105: 1-102.

Harley, J.L. and Smith, S.E. (1983), *Mycorrhizal symbiosis*. London: Academic Press, pp 20-483.

Harrison, M.J. (1999), Biotrophic interfaces and nutrient transport in plant/fungal symbiosis. *Journal of Experimental Biology*, 50: 1013-1022.

Harrison, M.J. (2005), Signalling in the arbuscular mycorrhizal symbiosis. *Annual Reviews in Microbiology,* 59: 19–42.

Hart, M.M. and Reader, R.J. (2002), Taxonomic basis for variation in the colonization strategy of arbuscular mycorrhizal fungi. *New Phytologist,* 153: 335–344.

Harter, R.D. (2007), Acid soil of the tropics. Echo technical note, pp 1-11. [Online] http://www.echonet.org.

Hartt, C.E. and Burr, G.O. (1965), Factors affecting photosynthesis in sugarcane. *Proceedings of the International Society of Sugar Cane Technology,* 12: 590-609.

Haynes, R.J. and Hamilton, C.S. (1999), Effects of sugarcane production on soil quality: a synthesis of world literature. *Proceedings of the South African Sugar Technologists' Association,* 73: 46-52.

Helgason, T. and Fitter, A. (2009), Natural selection and the evolutionary ecology of the arbuscular mycorrhizal fungi (*Phylum Glomeromycota*). *Journal of Experimental Botany*, 60: 2465–2480.

Helgason, T. Daniell, T.J. Husband, R. Fitter, A.H. and Young, J.P.W. (1998), Ploughing up the wood-wide web. *Nature,* 394: 431.

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

Hetrick, B. A. D. Wilson, G. W. T. and Kitt D. G. (1987), Suppression of mycorrhizae by soil microorganisms, In: Sylvia, D. M. Hung L. L and. Graham J. H eds. *Proceeding of the 7th North American Conference on Mycorrhizae*. Florida, USA: IFAS, University of Florida, pp 15.

Hetrick, B.A.D. Laslie, J.F. Wilson, G.T. and Kitt, D.G. (1988), Physical and topological assessment of effects of vesicular-arbuscular mycorrhizal fungus on root architecture of big bluestem. *New Phytologist,* 110: 85-96.

Hildebrandt, U. Janetta, K. Ouziad, F. Renne, B. Nawrath, K. and Bothe, H. (2001), Arbuscular mycorrhizal colonization of halophytes in Central European salt marshes. *Mycorrhiza,* 10: 175-183.

Hiltner, L. (1904), Über neuere Erfahrungen und Probleme auf dem Gebiet der Bodenbakteriologie und unter besonderer erüksichtigung der Gründüngung und Brache - Recent insights and problems in the area of soil bacteriology under special consideration of the use of green manure and fallowing. *Arbeiten der Deutschen Landwirtschaftlichen*, 98: 59–78.

Hol, G.W.H. and Cook, R. (2005), An overview of arbuscular mycorrhizal fungi-nematode interactions. *Basic Applied Ecology,* 6: 489–503.

Hooker, J.E. Jaizme-Vega, M. Atkinson, D. (1994), Biocontrol of plant pathogens using arbuscular mycorrhizal fungi, In: Gianinazzi, S. and Schüepp, H. eds., *Impact of Arbuscular Mycorrhizas on Sustainable Agriculture and Natural Ecosystems*. Basel, Switzerland: Birkhäuser Verlag, pp 191–200.

Hotelling, H. (1933), Analysis of a complex of statistical variables into principal components. *British Journal of Educational Psychology,* 24: 417–441.

Hoy, l. W. and Flynn, l.L. (2001), Control of ratoon stunting disease of sugarcane in Louisiana with seedcane produced through micropropagation and resistant cultivars. *Proceedings of the International Society of Sugar Cane Technologists Congress*, 24: 417-421.

Huang, X. Liu, J. Ding, J. He, Q. Xiong, R. and Zhang, K. (2009), The investigation of nematicidal activity in Stenotrophomonas maltophilia G2 and characterization of a novel virulence serine protease. *Canadian Journal of Microbiology*, 55(8): 934-942.

Huang, X. Tian, B. Niu, Q. Yang, J. Zhang, L. and Zhang, K. (2005), An extracellular protease from Brevibacillus laterosporus G4 without parasporal crystals can serve as a pathogenic factor in infection of nematodes. *Research in Microbiology,* 156: 719–727.

Hussey, R.S. and Roncadori, R.W. (1982), Vesicular-Arbuscular mycorrhiza may inhibit nematode activity and improve plant growth. *Plant Disease*, 66: 9-14.

http://www.ctahr.hawaii.edu/oc/freepubs/pdf/pnm9.pdf

Ingham, E.R. (2000), Bacteria, In: Tugel, A.J. Lewandowski, A.M. and Happe-von, D. eds. *Soil Biology Primer.* The Soil and water conservation Society. Chapter three. [Online] http://soils.usda.gov/sqi/concepts/soil_biology.

Insunza, V. Alström, S. and Eriksson, K. B. (2002), Root bacteria from nematicidal plants and their biocontrol potential against trichodorid nematodes in potato. *Plant and Soil,* 241(2): 271-278.

INVAM, (2012), International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi. [Online] http://invam.caf.wvu.edul. 2012.

Irvine, J.E. (1999), *Saccharum sp.* as horticultural classes. *Theoretical and Applied genetics,* 98(2): 186-194.

Isopi, R. Fabbri, P. Del Gallo, M. and Puppi, G. (1995), Dual inoculation of *Sorghum bicolour* (L.) and *Moench sp bicolor* with vesicular-arbuscular mycorrhizae and *Acetobacter diazotrophicus*. *Symbiosis,* 18: 43–55.

Jahromi, F. Aroca, R. Porcel, R. and Ruiz-Lozano, J.M. (2008), Influence of salinity on the *in-vitro* development of *Glomus intraradices* and on the *in-vivo* physiological and molecular responses of mycorrhizal lettuce plants. *Microbial Ecology,* 55: 45–53.

Jami al- Ahmadi, K. Yazdi, M.T. Najafi, M.F. Shahverdi, A.R. Faramarzi, M.A. Zarrini, G.H. and Behravan, J. (2008), Isolation and characterisation of chitinolytic enzyme producing microorganism, *Paenibacillus chitinolytic* JK2 from Iran. *Research Journal of Microbiology,* 3(6): 395-404. doi: 10.3923/jm.2008.395.404.

Jeffries, P. and Barea, J.M. (1994), Biogeochemical cycling and arbuscular mycorrhizas in the sustainability of plant–soil systems. In: Gianinazzi, S. and Schüepp, H. eds., *Impact of Arbuscular Mycorrhizas on Sustainable Agriculture and Natural Ecosystems*. Basel, Switzerland: Birkhäuser Verlag, pp 101–115.

Jin, H. Pfeffer, P. Douds, D. Piotrowski, E. Lammers, P. and Shachar-Hill, Y. (2005), The uptake, metabolism, transport and transfer of nitrogen in arbuscular mycorrhizal symbiosis. *New Phytologist*, 168: 119-127.

Johnson, N.C. Graham, J.H. and Smith, F.A. (1997), Functioning of mycorrhizal associations along the mutualism–parasitism continuum. *New Phytologist,* 135: 575–585.

Kariman, K.H. Goltapeh, E.M. and Minassian, V. (2005), Arbuscular mycorrhizal fungi from Iran. *Journal of Agricultural Technology,* 1(2): 301-313.

Kelly, R. M. Edwards, D. G. Thompson, J. P. and Magarey, R. C. (2001), Responses of sugarcane, maize, and soybean to phosphorus and vesicular-arbuscular mycorrhizal fungi. *Australian Journal of Agricultural Research*, 52(7): 731-743.

Kerrigan, J. and Nagel, M. (1998), Ohio Master Gardener Online Manual. Ohio State University Extension. http://www.hcs.ohio-state.edu/mg/manual/index.

Killham, K. (1994), *Soil Ecology*. Cambridge, UK: Cambridge University Press, pp 150-203.

Kilonsky, D.J. Herman, P.K. and Emr, S.D. (1990), The fungal vacuole composition, function and biogenesis. *Microbiological Review*, 54: 266-292.

Kling, M. and Jakobsen, I. (1998), Arbuscular mycorrhiza in soil quality assessment. *Ambio*, 27: 29–34.

Klironomos, J.N. (2003), Variation in plant response to native and exotic arbuscular mycorrhizal fungi. *Ecology*, 84(9): 2292–2301

Kloepper, J.W. and Beauchamp, C.J. (1992), A review of issues related to measuring of plant roots by bacteria. *Canadian Journal of Microbiology*, 38: 1219-1232.

Koske, R. E. and Gemma, J. N. (1997), Mycorrhizae and succession in plantings of beach grass in sand dunes. *American Journal of Botany*, 84: 118-130.

Koske, R.E. and Walker, C. (1985), Species of *Gigaspora* with roughened outer walls. *Mycologia,* 77(5): 702-720.

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

Kubo, I. Sang, H. L. and Tae, J. H. (2005), Effect of EDTA alone and in combination with polygodial on growth of Saccharomyces cerevesiae. *Journal of Agricultural and Food Chemistry,* 53: 1818-1822.

Kuroshima, K. Sakane, T. Tawita, R. and Yokota, A. (1996), *Bacillus ehimensis sp.* nov. and *Bacillus chitinolyticus sp*. nov., new chitinolytic members of the genus *Bacillus*. *International Journal of Systematic Bacteriology*, 46: 76-80.

Laiho, O. (1986), Practical forestry needs more research on ectomycorrhiza physiology in Finland, In: Gianinazzi-Pearson, V. and Gianinazzi, S. eds. *Physiological and Genetical Aspects of Mycorrhizae*, Paris, France: INRA, pp 489-492.

Lakshmikantham, M. (1983), *Technology in Sugarcane Growing*. 2nd Edition. Andhra Pradesh, India: Agricultural University Hyderabad, pp 259.

Lee, J. Lee, S. and Young, J.P.W. (2008), Improved PCR primers for the detection and identification of arbuscular mycorrhizal fungi. *FEMS Microbiology Ecology*, 65: 339-349.

Lee, J-S, Pyun, Y- R. and Bae, K. S. (2004), Transfer of *Bacillus ehimensis* and *Bacillus chitinolyticus* to the genus *Paenibacillus* with emended descriptions of *Paenibacillus ehimensis* comb. nov. and *Paenibacillus chitinolyticus* comb. nov. *International Journal of Systematic and Evolutionary Microbiology*, 54: 929-933.

Lee-Lovick, G. (1978), Smut of sugarcane-*Ustilago scitaminea*. Review of Plant *Pathology Commonwealth Mycological Institute*, 57(5): 181–187.

Linderman, R. G. (1988), Mycorrhizal Interactions with the Rhizosphere Microflora: The mycorrhizosphere effect. *Phytopathology*, 78: 366-371.

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, R. Mei, D. Xia, W. Min, L. and Xingzhong, L. (2011), Suppression of the root-knot nematode [*Meloidogyne incognita*(Kofoid & White) Chitwood] on tomato by dual inoculation with arbuscular mycorrhizal fungi and plant growth-promoting rhizobacteria. *Mycorrhiza,* 22: 289–296.

Lloyd-Macgilp, S.A. Chambers, S.M. Dodd. J.C. Fitter. A.H. Walker, C. and Young J.P.W. (1996), Diversity of the ribosomal internal transcribed spacers within and among isolates of *Glomus mosseae* and related mycorrhizal fungi. *New Phytologist*, 133: 103–111.

Lovato, P.E. Gianinazzi-Pearson, V. Trouvelot, A. and Gianinazzi, S. (1996), The state of art of mycorrhizas and micropropagation. *Advances in Horticultural Science,* 10: 46–52.

Magarey, R.C. (2000), Orange rust, In: Rott, P., Bailey, R.A., Comstock, J.C., Croft, B.J. and Saumtally, A.S. eds. *A Guide to Sugarcane Diseases*. CIRAD ISSCT, pp 121-125.

Magarey, K.C. Croft, B.J. and Willcox, T.G. (2001), An epidemic of orange rust on sugarcane in Australia. *Proceedings of the International Society of Sugar Technologists' Association,* 24: 410-416.

Maherali, H. and Klironomos, J.N. (2007), Influence of phylogeny on fungal community assembly and ecosystem functioning. *Science* 316: 1746–1748.

Marcel, G. A. van der Heiden, M. G. A., Klironomos, J. N. Ursic, M. Moutoglis, P. Streitwolf-Engel, R. Boller, T., Wiemken, A. and Sanders I. R. (1988), Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. *Nature,* 396: 69-72.

Marschner, H. (1986), Relationship between mineral nutrition and plant diseases and pests. *Mineral Nutrition of Higher Plants*. London, UK: Academic Press, pp 340-390.

Marschner, H. (1991), Mechanisms of adaptation of plants to acid soils. *Plant & Soil,* 134: 1-20.

Marschner, H. (1995), Ion uptake mechanisms of individual cells and roots. *Mineral Nutrition of Higher Plants*. London, UK: Academic Press, pp 30-72.

Martinez, P. and Persson, B.L. (1998), Identification, cloning and characterization of a depressible Na^+ coupled phosphate transporter in *Saccharomyces cerevisiae*. *Molecular Genetics and Genomics,* 258: 628-638.

Martinez-Espinoza, A.D. Garcia-Pedrajas, M.D. and Gold, S.E. (2002), The Ustilaginale as plant pests and model system. *Fungal Genetics and Biology,* 35: 1–20.

Mason, P.A. Musoko, M.O. Last, F.T. (1992), Short-term changes in vesicular arbuscular mycorrhizal spore population in *Terminalia* plantations in Cameroon. In: Read, D.J. Lewis, D.H. Fitter, A.H. and Alexander, I.J. eds. Mycorrhizas in ecosystems. Oxon, UK: C.A.B International, pp. 261–267.

Massonneau, A. Martinoia, E. Dietz, K.J. and Mimura, T. (2000), Phosphate uptake across the tonoplast of intact vacuoles isolated from suspension-cultured cells of *Catharanthus roseaus* (l.) G. Don. *Planta*, 211: 390-395.

McMillen, B. Juniper, S. and Abbott, L.K. (1998), Inhibition of hyphal growth of a vesicular-arbuscular mycorrhizal fungus in soil containing sodium chloride limits the spread of infection from spores. *Soil Biology and Biochemistry,* 30: 1639–1646.

Mei-ging, Z. and You-shan, W. (1992), Eight species of VA mycorrhizal fungi from northern China. *Acta Mycologica Sinica*, 11: 258-267.

Menge, J.A. Steirle, D. Bagyaraj, D.J. Johnson, E.L.V. and Leonard, R.T. (1978), Phosphorus concentrations in plants responsible for inhibition of mycorrhizal infection. *New Phytologist*, 80: 575- 578.

Meyer, H.W.J. (2000), *The transfer of agricultural information to rural communities.* Unpublished doctoral Thesis, University of Pretoria, South Africa.

Meyer, J. and Keeping M.G. (2002), Calcium silicate enhances resistance of sugarcane to African stalk borer *Eldana saccharina* Walker (*Lepidoptera: Pyralidae*), *Agricultural and Forest Entomology*, 4: 265-274.

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

Meyer, J. Tucker, A.B. and Wood R.A. (1997), The SASEX (now SASRI) Fertiliser Advisory Service: over 40 years' service to the South African Sugar Industry. *Proceedings of the South African Sugar Technologists' Association,* 71: 42-49.

Meyer, S. L. F. Massoud, D. J. Chitwood, D .J. and Roberts, D.P. (2000), Evaluation of *Trichoderma virens* and *Burkholderia cepacia* for antagonistic activity against root-knot nematode, *Meloidogyne incognita*. *Nematology*, 2: 871-879.

Meyer, S. L. F. Roberts, D. P. Chitwood, D. J. Carta, L. K. Lumsden, R.D. and Mao, W. (2001), Application of *Burkholderia cepacia* and *Trichoderma virens*, alone and in combinations, against *Meloidogyne incognita* on bell pepper. *Nematropica*, 31: 75-86.

Meyer, J. Rein, P. Turner, P. and Mathias, K. (2011), Good management practices for the cane sugar industry, In: Meyer J ed. *I.F.C. Cane Management Practices*. PGBI Sugar & Bio-Energy (Pty) Ltd. *Online Manual Produced For the International Finance Corporation*. pp 1-696. [Online] http://www.bonsucro.com/assets /ifc _webinar.pdf

Middleton, K. J. Bell, M. J. and Thompson, J. P. (1989), Effects of soil sterilization, inoculation with vesicular-arbuscular mycorrhizal fungi and cropping history on peanut (*Arachis hypogaea* L.) growth in an oxisol from subtropical Australia. *Plant and Soil,* 117(1): 41–48.

Mimura, T. (1999), Regulation of phosphate transport and homeostasis in plant cells. *International Review of Cytology*, 191: 149-200.

Moore, B. and Hudson, J.R. (2007), The South African sugar industry: shaped by history. *Sugar Industry Zuckerindustrie*, 132(6): 425-432.

Moore, P.H. Nuss, K.J. (1987), Flowering and flower synchronization, In: Heinz D.J, ed. *Sugarcane improvement through breeding*. Amsterdam, Netherlands: Elsevier, pp 273-311.

Moreira-Souza, M. Truem, S. F. B. Gomes-da-Costa, S. M. and Cardoso, E. J. B. N. (2003), Arbuscular mycorrhizal fungi associated with *Araucaria angustifolia* (Bert.) O. Ktze. *Mycorrhiza,* 13: 211-215.

Mosse, B. (1962), The establishment of vesicular-arbuscular mycorrhizae under aseptic conditions. *Journal of General Microbiology*, 27: 509-520.

Muniyamma, M. Bharati, B. K. and Reddy, C. N. (2000), Effect of VAM on root induction in in-vitro sugarcane (*Saccharum officinarum* L.) seedlings - a new technique. *Mycorrhiza News,* 12: 13-16.

Munkvold, L. Kjoller, R. Vestberg, M. Rosendahl, S. and Jakobsen, I. (2004), High functional diversity within species of arbuscular mycorrhizal fungi. *New Phytologist,* 164: 357–364.

Musoko, M. Last, F. T. and Mason, P. A. (1994), Populations of spores of vesicular-arbuscular mycorrhizal fungi in undisturbed soils of secondary semideciduous moist tropical forest in Cameroon. *Forest Ecology and Management*, 63: 359-377.

Naidu, M.K. and Sreenivasan, T.V. (1987), Conservation of sugarcane germoplasm. *Copersucar International Sugarcane Breeding Workshop*, São Paulo: Copersucar, pp 33–53.

Naik, G.R. (2001), Vesicular arbuscular mycorrhiza. *Sugarcane Biotechnology*. New Hampshire, USA: Science Publishers Inc., pp 87-91.

Netafim (2012), [Online] http://www.netafimindia.com/agriculture-sugarcane.html.; http://www.sugarcanecrops.com/crop_growth_phases/germination_establishment_phase

Nicolson, T.H. and Schenck, N.C. (1979), Endogonaceous mycorrhizal endophytes in Florida. *Mycologia,* 71: 178-198.

O'Reilly, G. (1998), The South African sugar industry. *International Sugar Journal*, 100: 266-268.

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

Olrovich, D.A. and Ashford, A.E. (1993), Polyphosphate granules are artifact of specimen preparation in the ectomycorrhizal fungus *Pisolithus tinctorius*. *Protoplasma,* 173: 91–102.

Olsson, P.A. van Aarle, I.M. Allaway, W.G. Ashford, A.E. Rouhier, H. (2002), Phosphorus effects on metabolic processes in monoxenic arbuscular mycorrhiza cultures. *Plant Physiology,* 130: 1162–1171.

Olsson, P.A. Burleigh, S.H. and van Aarle, I. (2005), The influence of external nitrogen on carbon allocation to *Glomus intraradices* in monoxenic arbuscular mycorrhiza. *New Phytologist*, 168: 677–686.

Olsson, P.A. Rahm, J. and Aliasgharzad, N. (2010), Carbon dynamics in mycorrhizal symbioses is linked to carbon costs and phosphorus benefits. *FEMS Microbiology Ecology,* 72: 123–131.

Öpik, M. Moora, M. Liira, J., Zobel, M. (2006), Composition of root-colonizing arbuscular mycorrhizal fungal communities in different ecosystems around the globe. *Journal of Ecology,* 94: 778–790 (doi:10.1111/j.1365-2745.2006.01136.x).

Oswald, E. T. and Ferchau, H. A. (1968), Bacterial associations of coniferous mycorrhizae*. Plant Soil*, 28: 187–192.

Padgham, J.L. and Sikora, R.A. (2007), Biological control potential and modes of action of *Bacillus megaterium* against *Meloidogyne graminicola* on rice. *Crop Protection*, 26: 971–977.

Palleroni, N. and Bradbury, J. (1993), *Stenotrophomonas*, a new bacterial genus for *Xanthomonas maltophilia* (Hugh 1980) Swings *et al.* 1983*. International Journal of Systematic Bacteriology*, 43(3): 606–9 (doi:10.1099/00207713-43-3-606).

Palm, C.E. Dykstra, W.W. Ferguson, G.R. Hansberry, R. Hayes, W.J. Jr. Hazleton, L.W. Horsfall, J.G. Knipling, E.F. Leach, L.D. Louvorn, R.L. and Swanson, G.A. (1968), Plant Disease Development and control. *Principles of plant and animal pest control.* National Academy of Sciences, Washington DC, USA. Volume 1, pp 13-15.

Parfitt, R.C. (2005), Release of sugarcane varieties in South Africa. *Proceedings of the South African Sugarcane Technologists' Association*. [Online] www.sasta.co.za.

Paszkowski, U. (2006), A journey through signalling in arbuscular mycorrhizal symbiosis. *New Phytologist* 72: 35-46.

Paulitz T. C. and Linderman R. G. (1991), Mycorrhizal interactions with soil organisms, In: Arora, D.K., Rai, B., Mukerji, K.G., Knudsen, G.R. eds. *Handbook of*

Applied Mycology, vol. 1: Soil and Plants. New York, USA: Marcel Dekker, pp 77–128.

Pfeffer, P.E. Douds, D.D. Be´ card, G. and Shachar-Hill, Y. (1999), Carbon uptake and the metabolism and transport of lipids in and arbuscular mycorrhiza. *Plant Physiology*, 120: 587–598.

Pinochet, J. Calvet, C. Camprubi, A. and Fernandez, C. (1996), Interactions between migratory endoparasitic nematodes and arbuscular mycorrhizal fungi in perennial crops. A review. *Plant Soil*, 185: 183–190.

Pirozynski, K.A. (1981), Interactions between fungi and plants through the ages. *Canadian Journal of Botany*, 59: 1824-1827.

Pirozynski, K.A. and Dalpe, Y. (1989), Geological history of the Glomaceae with particular reference to mycorrhizal symbiosis. *Symbiosis,* 7: 1–36.

Puppi, G. and Riess, S. (1982), Notes on the occurrence of endogonaceous spores and vesicular-arbuscular mycorrhizal associations in woodland sites in the middle valley of the Tiber (Italy). Acc. *Lincei-Rend. Sc. fis. mat. e nat,* 22: 279-286.

Qiuhong, N. Xiaowei, H. Baoyu, T. Jinkui, Y. Jiang, L. Lin, Z. and Keqin, Z. (2006), Bacillus sp. B16 kills nematodes with a serine protease identified as a pathogenic factor. *Applied Microbiology Biotechnology*, 69: 722–730 (doi 10.1007/s00253-005-0019-5).

Qongqo, L.L. and van Antwerpen, R. (2000), Effect of long-term sugarcane production on physical and chemical properties of soils in KwaZulu-Natal. *Proceedings of the South African Sugar Technologists' Association,* 74: 114-121.

Raid, R.N. and Comstock, J.C. (2006), *Sugarcane Rust Disease*. Agronomy Department document SS-AGR-207. University of Florida/IFAS, Gainesville, FL 32611.

Rakkiyappan, P. Thnagavelu, S. Bhagyalakshmi, K.V. and Radhamani, R. (2007), Uptake of Nitrogen, Phosphorus and Potassium by some promising mid late maturing sugarcane clones. *Sugar Technology*, 9(1): 23-27.

Ramette, A. (2007), Multivariate analyses in microbial ecology. *FEMS Microbiology Ecology,* 62: 142-60.

Rasmussen, N. Lloyd, D.C. Ratcliffe, R.G. Hansen, P.E. and Jakobson, I. (2000), ^{31}P NMR for the study of P metabolism and translocation in arbuscular mycorrhizal fungi. *Plant and Soil,* 226: 245-253.

Read, D.J. (1984), The structure and function of vegetative mycelium of mycorrhizal roots, In: Jennings, D.H. and Rayner, A.D.M. eds. *The Ecology and Physiology of the Fungal Mycelium*. New York, USA: Cambridge University Press, pp 215-240.

Redecker, D. (2000), Specific Primers to identify arbuscular mycorrhizal fungi within colonized roots. *Mycorrhiza,* 10: 73-80.

Redecker, D. Hijri, I. and Wiekem, A. (2003), Molecular Identification of Arbuscular Mycorrhizal Fungi in Roots: Perspectives and Problems. *Folia Geobotanica,* 38: 113-124

Redecker, D. Raab, P. (2006), Phylogeny of the Glomeromycota (arbuscular mycorrhizal fungi): recent developments and new gene markers. *Mycologia,* 98: 885-895.

Rhodes, L.H. and Gerdemann, J.W. (1975), Phosphate uptake zones of mycorrhizal and non-mycorrhizal onions. *New Phytologist*, 75: 555-561.

Roesti, D. Ineichen, K. Braissant, O. Redecker, D. Wiemken, A. and Aragno, M. (2005), Bacteria Associated with Spores of the Arbuscular Mycorrhizal Fungi *Glomus geosporum* and *Glomus constrictum*. *Applied and Environmental Microbiology*, 71: 6673- 6679.

Rokni, N. Goltapeh, M.E. and Alizadeh, A. (2010), Some new recorded species of arbuscular mycorrhizal fungi associated with sugarcane crop in Iran. *Journal of Agricultural Technology,* 6: 67-78.

Rokni, N. and Goltapeh, M.E. (2011), Diversity of arbuscular mycorrhizal fungi associated with common sugarcane varieties in Iran. *Journal of Agricultural Technology,* 7(4): 1017-1022.

Ross, J.P. (1980), Effect of nontreated field soil on sporulation for vesicular-arbuscular mycorrhizal fungi associated with soybean. *Phytopathology*, 70: 1200–1205.

Rott, P. Bailey, R.A. Comstock, C. Croft, B.J. and Saumtally, S.A. (2000), Diseases cause by fungi. *A Guide to Sugarcane Diseases*. CIRAD and ISSCT, pp 126 and 141.

Rutherford, S.R. (2011), Senior Pathologist, Crop Biology Resource Centre, Crop Protection Programme, South African Sugarcane Research Institute (SASRI). **personal communication*.

Rutherford, R.S. van Antwerpen, T. Conlong, D.E. Keeping, M.G. McFarlane, S.A. and Vogel, J.L. (2002), Promoting plant health: Potential for the use of plant-associated micro-organisms in the biological control of pathogens and pests in sugarcane. *Proceedings of the South African Sugar Technologists' Association*, 76: 289-300.

Saif, S. R. (1987), Growth responses of tropical forage plant species to vesicular-arbuscular mycorrhizae. *Plant and Soil,* 97: 25-35.

Saito, M. and Vargas, R. (1991), Vesicular-arbuscular mycorrhizal fungi in some humus-rich Ando soils of Japan. *Soil Microorganisms*, 38: 3-15.

Sanders, I.R. Alt, M. Groppe, K. Boller, T. and Wiemken, A. (1995), Identification of ribosomal DNA polymorphisms among and within spores of the Glomales. *New Phytologist,* 130: 419-427.

Sanders, I. R. Clapp, J.P. and Wiemken, A. (1996), The genetic diversity of arbuscular mycorrhizal fungi in natural ecosystems-a key to understanding the ecology and functioning of the mycorrhizal symbiosis. *New Phytologist*, 133(1): 123-134.

Sanders, J.R. (2008), Ecology and Evolution of symbiotic Organisms. [Online] http://www.unil.ch/dee/page7238_en.html.

Santos, C.J., Finlay, D.R. and Tehler, A. (2006), Molecular analysis of Arbuscular mycorrhizal fungi colonizing semi-natural grassland along a fertilization gradient. *New Phytologist,* 172(1): 159-168.

SAS - Statistical Analysis System (2008), *SAS/STAT® 9.2. User Guide*. North Carolina, USA: [Online] http://sas.com.]

SASA- South African Sugar Association (2012), Mount Edgecombe, South Africa. [Online] http://www.sasa.org.za/Libraries; http://www.sasa.org.za/HomePage1.aspx

SASEX-South African Sugar Experiment Station (1970), Pest of sugarcane in South Africa. South African Sugar Association Experiment Station, Bulletin number 8.

SASEX -South African Sugar Experiment Station (1999), Identification and management of the soils of the South African Sugar Industry. 3rd Edition, Mount Edgecombe, South Africa: South African Sugar Association Experiment Station, pp 112 - 118. [Online] http://www.sasa.org.za/divisions/SASugarCaneResearch Institute/Publications.aspx

SASRI-South African Research Institute (2006), variety information sheets 8.2, 2012. Mount Edgecombe, South Africa. [Online] http://www.sugar.org.za/sasri/variety/index.html; http://www.sasa.org.za/ divisions/SASugarCaneResearchInstitute/Publications.aspx.

SASRI- South African Sugarcane Research Institute (2012), Senior certificate course notes in sugarcane agriculture. Nematodes and their control. *Diseases and Pests*. Mount Edgecombe, South Africa.

Savant, N. K. Korndörfer, G. H., Datnoff, L.E. and Snyder, G. H. (1999), Silicon nutrition and sugarcane production: A Review, *Journal of Plant Nutrition*, 22(12): 1853-1903.

Schachtman, D.P. Reid, R.J. and Ayling, S.M. (1998), Phosphorus uptake by plants: from soil to cell. *Plant Physiology,* 116: 447-453.

Schelke, M. and Peterson, R.L. (1996). Supression of common pathogens by helper bacteria and ectomycorrhizal fungi *in-vitro*. *Mycorrhiza*, 6: 481-485.

Schenck, N. C. and Kinloch, R. A. (1980), Incidence of mycorrhizal fungi on six field crops in monoculture on a newly cleared woodland site. *Mycologia,* 72: 445-456.

Schenck N. C. and Smith G. S. (1981), Distribution and occurrence of vesicular-arbuscular mycorrhizal fungi on Florida agricultural crops. Soil and Crop Science Society of Florida, 40: 171-175.

Schenck, N. C. Spain J. L, Sieverding E. and Howeler, R. H. (1984), Several new and unreported vesicular-arbuscular mycorrhizal fungi (*Endogonaceae*) from Colombia. *Mycologia*, 76: 685-699.

Schenck, N.C. and Perez, Y. (1990), *Manual for identification of VA mycorrhizal fungi*, Florida, USA: Synergistic Publications, University of Florida pp 4-39.

Schimansky, C. (1981). The influence of certain experimental parameters on the flux characteristics of Mg in the case of barley seedlings grown in hydroculture. *Landwirtschaftliche Forschung*, 34: 154-165.

Schmid, E. Oberwinkler, F. and Gomez, L.D. (1995), Light and electron microscopy of a host-fungus interaction in the roots of some epiphytic ferns from Costa Rica. *Canadian Journal of Botany*, 73: 991-996.

Schubert, A. and Hayman, D.S. (1986), Plant growth responses to vesicular-arbuscular mycorrhizae. XVI. Effectiveness of different endophytes at different levels of soil phosphate. *New Phytologist,* 103: 79-80.

Schüßler, A. Schwarzott, D. Walker, C. (2001), A new fungal phylum, the Glomeromycota: phylogeny and evolution. *Mycological Research*, 105: 1413-1421.

Schüßler, A. Martin, H., Cohen, D. Fitz, M., Wipf, D. (2006), Characterization of a carbohydrate transporter from symbiotic glomeromycotan fungi. *Nature,* 444: 933–936 (doi:10.1038/nature05364).

Schüßler, A. and Walker, C. (2010), The Glomeromycota. A species list with new families and new genera. Arthur Schüßler & Christopher Walker, Gloucester. Published in libraries at The Royal Botanic Garden Edinburgh, The Royal Botanic Garden Kew, Botanische Staatssammlung Munich, and Oregon State University, pp 1-56.

Schwab, S.M., Menge, J.A. and Tinker, P.B. (1991), Regulation of nutrient transfer between host and fungus in vesicular arbuscular mycorrhizas. *New Phytologist,* 177: 387–398.

Shachar-Hill, Y. Pfeffer, P.E. Douds, D. Osman, S.F. Doner, L.W. and Ratcliffe, R.G. (1995), Partitioning of intermediate carbon metabolism in VAM colonised leek. *Plant Physiology,* 108: 7-15.

Sharif, M. and Claassen, N. (2011), Action mechanisms of arbuscular mycorrhizal fungi in phosphorus uptake by *Capsicum annuum* L*. Pedosphere*, 21(4): 502–511.

Sharifi, M. Ghorbanli, M. and Ebrahimzadeh H. (2007), Improved growth of salinity-stressed soybean after inoculation with pre-treated mycorrhizal fungi. *Journal of Plant Physiology,* 164: 1144–1151.

Shepherd, K.D. Jefwa, J. Wilson, J. Ndufa, J.K. Ingleby, K. and Mbuthu, K.W. (1996), Infection potential of farm soils as mycorrhizal inocula for *Leucaena leucocephela*. *Biology and Fertility of Soils,* 22: 16–21.

Shida, O. Takagi, H. Kadowaki, K. and Komagata, K. (1996), Proposal for Two New Genera, *Brevibacillus gen*. nov. and *Aneurinibacillus gen*. nov. *International Journal of Systematic Bacteriology*, 46(4): 939-946.

Siddiqui, Z.A. and Akhtar, M.S. (2009), Effects of antagonistic fungi, plant growth-promoting rhizobacteria, and arbuscular mycorrhizal fungi alone and in combination on the reproduction of *Meloidogyne incognita* and growth of tomato. *Journal of General Plant Pathology*, 75: 144–153.

Siddiqui, Z.A. and Mahmood, I. (1999), Role of bacteria in the management of plant parasitic nematodes: a review. *Bioresource Technology*, 69: 167–179.

Sieverding, E. El-Sharkawy, M. Hermandez, A. P. and Toro, T. (1986), Influence of water stress on growth and formation of VA mycorrhiza of 20 cassava cultivars. Physiological and genetical aspects of mycorrhizae. *Proceedings of the European symposium*, Dijon, France: INRA, 1-5 July, pp 717-720.

Sieverding, E. (1989), Ecology of VAM fungi in tropical agrosystems. *Agriculture, Ecosystems, and Environment,* 29: 369–390.

Sieverding, E. and Toro, S. T. (1988), Influence of soil water regimes on VA mycorrhiza. . *Journal of Agronomy and Crop Science*, 161: 322-332.

Sieverding, E. (1991), Vesicular-arbuscular mycorrhiza management in tropical agrosystems, Schriftenreihe *der GTZ*, 224: 339-365.

Sikora, R.A. Pocasangre, L. zum Felde, A. Niere, B. Vu, T.T. and Dababat, A.A.(2008), Mutualistic endophytic fungi and in-planta suppressiveness to plant parasitic nematodes. *Biological Control,* 46: 15–23.

Sinha, R.K. (2004), Mineral nutrition and absorption. *Modern Plant Physiology*. Pangbourne, UK: Alpha Science International Ltd, pp 101-110.

Smith S.E. and Read D.J. (2008), *Mycorrhizal Symbiosis*. 3rd Edition. London, UK: Academic Press, pp 13-638.

Smith, F.A. and Smith, S.E. (1980), Mycorrhizal Infection and growth of *Trifolium subterraneum*: Use of sterilized soil as a control treatment. *New Phytologist*, 88: 299-309.

Smith, S.E. and Smith, F.A. (2011), Roles of arbuscular mycorrhizas in plant nutrition and growth: new paradigms from cellular to ecosystems scales. *Annual Review of Plant Biology*, 62: 227–250. doi:10.1146/annurev-arplant-042110-103846

Smith, S.E. and Smith, F.A. (2012), Fresh perspectives on the roles of arbuscular mycorrhizal fungi in plant nutrition and growth. *Mycologia,* 104(1): 1-13.

Snyman, S.J. Baker, C. Huckett, B.I. McFarlane, S.A. van Antwerpen T. Berry, S. Omarjee, J. Rutherford, R.S. and Watt, D.A. (2008), South African Sugarcane Research Institute: Embracing biotechnology for crop improvement research. *Sugar Technology*, 10(1): 1-13.

Spaull, V. (1991), Nematodes, *Pests of sugarcane in South Africa*. Mount Edgecombe, South Africa: SASRI Publishers, Bulletin 8: 19-23.

Spaull, V.W. and Cadet, P. (1990), Nematode parasites of sugarcane, In: Luc, M., Sikora, R.A. and Bridge, J. eds. *Plant Parasitic Nematodes in Subtropical and Tropical Agriculture*. Wallingford, UK: CAB International, pp 461–491.

Sreenivasan, T.V. Ahloowalia, B.S. and Heinz, D.J. (1987), Cytogenetics, In: Heinz, D.J. ed. *Developments in crop sciences II. Sugarcane improvement through breeding*. Amsterdam, Netherlands: Elsevier, pp 211-253.

Srikumar, R. Murugaian, P. and Thangaraj, R. (2009), Survey of arbuscular mycorrhizal fungi-associated with sugarcane in of South India, *Agricultural Science Digest,* 29(2): 19-22.

Stirling, G. and Blair, B. (2000), Nematodes, In: Rott. P., Bailey. A., Comstock. J.C., Croft. B.J. and Saumtally. A.S. eds. *A Guide to Sugarcane Diseases.* CIRAD and ISSCT, pp 299-305.

Straker, C.J. Hilditch, A.J. and Rey, M.E.C. (2010), Arbuscular mycorrhizal fungi associated with cassava (*Manihot esculenta Crantz*) in South Africa. *South African Journal of Botany,* 76: 102–111.

Subramanian, K.S. Bharathi, C. and Jegan, A. (2008), Response of maize to mycorrhizal colonization at varying levels of zinc and phosphorus. *Biology and Fertility of Soils,* 45: 133–144.

Sward, R.J. (1981a), The structure of the spores of *Gigaspora margaritar* I. The dormant spore. *New Phytologist*, 87: 761-768.

Sward, R.J. (1981b), The structure of the spores of Gigaspora margaritar II. Changes accompanying germination. *New Phytologist*, 88: 661-666.

Sward, R.J. (1981c), The structure of the spores of Gigaspora margaritar III. Germ-tube emergence and growth. *New Phytologist*, 88: 667-673.

Sylvia, D. M. (1986), Spatial and temporal distribution of vesicular-arbuscular mycorrhizal fungi associated with *Uniola paniculata* in Florida foredunes. *Mycologia,* 78: 728-734.

Texas Plant Disease Handbook. (2012), Sugar crops. Texas Agricultural and Mechanical University (TAMU), Department of Plant Pathology and Microbiology. [Online] http://plantdiseasehandbook.tamu.edu/industry-specialty/sugar-crops/

Thioulouse, J. Chessel, D. Doledec, S. and Olivier, J.M. (1997), ADE-4: A multivariate analysis and graphical display software. *Statistics and Computing*, 7: 75-83.

Tilak, K.V.B.R., Lil, C.Y. and Ho, I. (1989), Occurrence of nitrogen-fixing Azospirillum in vesicular- arbuscular mycorrhizal fungi. *Plant and Soil,* 11: 286-288.

Trappe, J.M. (1977), Three New Endogonaceae: *Glomus constrictus, Sclerocystis clavispora* and *Acaulospora scrobiculata*. *Mycotaxon,* 6: 359-366.

Trappe, J.M. (1982), Synoptic key to the genera and species of Zygomycetous mycorrhizal fungi. *Phytopathology,* 72(8): 1102-1108.

Trappe, J.M. Bloss, H.E. and Menge, J.A. (1984), *Glomus deseticola* sp nov. *Mycotaxon,* 20: 123-127.

van Antwerpen, R. (1999), Sugarcane root growth and relationships to aboveground biomass. *Proceedings of the South African Sugar Technologists' Association*, 73: 89-95.

van Dillewijn, C. (1952), *Botany of sugarcane*, Massachusetts, USA: Waltham, pp 1- 370.

Varma, A.K. Singh, K. Lall, V.K. (1981), Lumen bacteria from endomycorrhizal spores. *Current Microbiology,* 6: 207-211.

Vary, P.S. Biedendieck, R. Fuerch, T. Meinhardt, F. Rohde, M. Deckwer, W.D. and Jahn, D. (2007), *Bacillus megaterium* - from simple soil bacterium to industrial protein production host. A Minireview. *Applied Microbiology and Biotechnology*, 76: 957–967 (doi 10.1007/s00253-007-1089-3).

Viets, Jnr F.G. (1944), Calcium and other polyvalent cations as accelerators of ion accumulation by excised barley roots. *Plant Physiologist,* 19: 466-480.

Vos, C. Geerinckx, K. Mkandawire, R. Panis, B. De Waele, D. Elsen, A. (2011), Arbuscular mycorrhizal fungi affect both penetration and further life stage development of root-knot nematodes in tomato. *Mycorrhiza*, 22(2):157-63.

Vlamis, J. and Williams, D.E. (1967), Manganese and silicon interaction in the *Gramineae*. *Plant Soil,* 27: 131-140.

Walker, C. (1982), Species in the Endogonaceae, a new species and new combination. *Mycotaxon,* 15: 49-61.

Walker, C. and Sanders , F.E. 1986. Taxonomic concepts in the Endogonaceae: III. The separation of *Scutellospora* gen. nov. from *Gigaspora* Gerd. & Trappe. *Mycotaxon 27:* 169-182.

Wallace, A. Frolich, E. F. and Alexander, G.V. (1973), Effect of steam sterilization of soil on two desert plant species. *Plant and Soil*, 39: 453-456.

Wallander, H. (2006), Mineral dissolution by ectomycorrhizal fungi, In: Gadd, G.M. ed., *Fungi in Biogeochemical Cycles*. Cambridge, UK: Cambridge University Press, pp 28–50.

Wamberg, C. Christensen, S. Jakobsen, I. Muller, A.K. and Sorensen, 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.

Wang, G.M. Stribley, D.P., Tinker P.B. and Walker, C. (1993), Effects of pH on Arbuscular mycorrhiza I. Field observation on long term liming experiments at Rothhamsted and Woburn. *New Phytologist*, 124: 465-472.

Wang, Y.Z. Zhang, M.Q. Ke, Y.Q. Pym, T.G. and Zheng, S.K. (1995), Effect of mycorrhiza pre inoculation on root development of sugarcane. *Fuji Agricultural University,* 24: 318-322.

Warcup, J. H. (1957), Chemical and biological aspects of soil sterilisation. *Soils and Fertilisers*, 20: 1-5.

Wilkinson, K.G. Dixon K.W. Sivasithamparam, K. and Ghisalberti, E.L. (1994), Effect of IAA on symbiotic germination of an Australian orchid and its production by orchid-associated bacteria. *Plant and Soil*, 159(2): 291-295.

Willmer, C.M. and Mansfield, T.A. (1969), Active cation transport and stomatal opening: a possible physiological role of sodium ions. *Planzenphysiol,* 61: 398-400.

Wilson, G. W. T. Daniels, B. A. H. and Kitt, D. G. (1988), Suppression of mycorrhizal growth response of big bluestem by non-sterile soil. *Mycologia,* 80: 338–343.

Wu, T. Hao. W. Lin, X. Shi, Y. (2002), Screening of arbuscular mycorrhizal fungi for the revegetation of eroded red soils in subtropical China. *Plant and Soil,* 239: 225-235.

Wyn, J.R.G. Brady, C.J. and Spiers, J. (1979), Ionic and osmotic relations in plant cells, In: Laidman D. and Wyn J.R.G eds. *Recent advances in Biochemistry of Cereals*. London, UK: Academic press, pp 63-103.

Yoneyama, K. Yoneyama, K. Takeuchi, Y. Sekimoto, H.(2007a), Phosphorus deficiency in red clover promotes exudation of orobanchol, the signal for mycorrhizal symbionts and germination stimulant for root parasites. *Planta,* 225: 1031–1038.

Yoneyama, K. Xie, X. Kusumoto, D. Sekimoto, H. Sugimoto, Y. and Takeuchi, Y. (2007b), Nitrogen deficiency as well as phosphorus deficiency in sorghum

promotes the production and exudation of 5-deoxystrigol, the host recognition signal for arbuscular mycorrhizal fungi and root parasites. *Planta,* 227: 125–132.

Zhang, Z. Schwartz, S. Wagner, L. and Miller, W. (2000), "A greedy algorithm for aligning DNA sequences". *Journal of Computational Biology*, 7(1-2): 203-14.

Zhang, L. Zhang, J. Christie, P. and Li, X. (2008), Pre-inoculation with arbuscular mycorrhizal fungi suppresses root knot nematode (*Meloidogyne incognita*) on cucumber (*Cucumis sativus*). *Biology and Fertility of Soils*, 45: 205-211 (doi: 10.1007/s00374-008-0329-8).

Zhang, L. Zhang, J. Christie, P. and Li, X. (2009), Effect of inoculation with the arbuscular mycorrhizal fungus G*lomus intraradices* on the root-knot nematode *Meloidogyne incognita* in cucumber. *Journal of Plant Nutrition*, 32(6): 967–979 (doi: 10.1080/01904160902870739).

Zuccarini, P. and Okurowska, P. (2008), Effects of mycorrhizal colonization and fertilization on growth and photosynthesis of sweet basil under salt stress. *Journal of Plant Nutrition*, 31: 497–513.

APPENDICES

APPENDIX A

Recipes for Melzer's solution and Polyvinyl Lacto Glycerol (PVLG)

PVLG was used to permanently mount whole or broken spores on glass slides. More permanent mounts were made by mixing Melzer's solution with PVLG in a volume ratio of 1:1 and storing the mixture in a dark bottle. (INVAM, 2012).

Table A - 1: Recipe for Melzer's solution (adapted from INVAM, 2012).

Reagents	Quantity
Chloral hydrate	100 g
Distilled water	100 mL
Iodine	1.5 g
Potassium iodide	5.0 g

Table A - 2: Recipe for Polyvinyl-Lacto-Glycerol (PVLG) (adapted from INVAM, 2012).

PVLG Reagents	Quantity
Distilled water	100 mL
Lactic acid	100 mL
Glycerol	10 mL
Polyvinyl alcohol (PVA)	16.6 g

PCR Guidelines

Table A - 3: Guideline for PCR amplification of DNA for partial molecular analysis of AM fungal spores associated with sugarcane (adapted from: http://www.promega.com/~/media/files/resources/protocols).

Reagents	Total reaction volume (25 µL)	Final Concentration
GoTaq Colourless Master Mix (2X)	12.5 µl	1X
forward primer, 10 µM	0.5 - 2.5 µl	0.1–1.0 µM
reverse primer, 10 µM	0.5 - 2.5 µl	0.1–1.0 µM
DNA template	1 – 5 µl	< 250 ng
Nuclease-free water	as required	

Table A - 4: Guideline for PCR amplification of AMB DNA isolated from AM fungal spores associated with sugarcane (adapted from: http://www.promega.com/~/media /files/resources/protocols).

Reagents	Total reaction volume (50 µL)	Final Concentration
GoTaq Colourless Master Mix, 2X	25 µL	1X
forward primer, 10 µM	0.5 – 5.0 µL	0.1–1.0 µM
reverse primer, 10 µM	0.5 – 5.0 µL	0.1–1.0 µM
DNA template	1 – 5 µL	< 250 ng
Nuclease-free water	as required	

PARTIAL SEQUENCE ALIGNMENT IDENTITIES USING BLAST FOR BACTERIA ISOLATED AND CULTURED FROM AM FUNGI SPORES.

BLAST Reference: http://blast.ncbi.nlm.nih.gov/Blast.cgi

Zhang, Z; Schwartz, S.; Wagner, L and Miller, W. (2000), "A greedy algorithm for aligning DNA sequences", J Comput Biol 2000; 7(1-2):203-14.

Bacterial isolate 4 (Forward Primer T7)

5'GGGCCGacGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTcTGGAG
AGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACtTGCAAGTCGAGC
GAGTCTCTTCGGAGGCTAGCGGCGGACGGGTGAGTAACACGTAGGCAACCTGCCTCTC
AgACTGGGATAACATAGGGAAACTTATGCTAATACCGGATAGGTTTTTGGACCGCATG
GTCCGAAAAGAAAAGATGGCTTCGGCTATCACTGGGAGATGGGCCTGCGGCGCATTG
GCTAGTTGGTGGGGTAACGGCCTACCAAGGCGACgATGCGTAGCCGACCTGAGAGGG
TGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAG
GGAATTTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAACGATGAAGGT
CTTCGGATTGTAAAGTTCTGTTGTTAGGGAC 3'

Table B - 1: Isolate 4 forward sequences producing significant alignments.

Accession	Description	Max score	Total score	Query coverage	E-value	Max ident
AP008955.1	Brevibacillus brevis NBRC 100599 DNA, complete genome	789	1.160e+04	100%	0.0	99%
NR_029132.1	Brevibacillus reuszeri strain 39 16S ribosomal RNA,	789	789	99%	0.0	99%

	partial sequence >dbj|D78464.1| Brevibacillus reuszeri DNA for 16S ribosomal RNA					
HQ703912.1	Brevibacillus reuszeri strain NBM83 16S ribosomal RNA gene, partial sequence	785	785	99%	0.0	98%
DQ444285.1	Brevibacillus brevis strain YJ011 16S ribosomal RNA gene, partial sequence	778	778	99%	0.0	98%
JF628155.1	Uncultured bacterium clone GDIC2IK01D77HL 16S ribosomal RNA gene, partial sequence	771	771	97%	0.0	99%
HM590699.1	Brevibacillus reuszeri strain MHB1B 16S ribosomal RNA gene, partial sequence	771	771	98%	0.0	98%
GQ284343.1	Brevibacillus sp. PCWCS5 16S ribosomal RNA gene, partial sequence	771	771	99%	0.0	98%

HM453885.1	Brevibacillus sp. SUT47 16S ribosomal RNA gene, partial sequence	767	767	99%	0.0	98%
GU321101.1	Brevibacillus sp. B59 16S ribosomal RNA gene, partial sequence	767	767	99%	0.0	98%
GU321098.1	Brevibacillus sp. B34 16S ribosomal RNA gene, partial sequence	767	767	99%	0.0	98%
GQ284518.1	Brevibacillus sp. TDSAS2-32 16S ribosomal RNA gene, partial sequence	767	767	99%	0.0	98%
EU605978.1	Brevibacillus brevis strain B12 16S ribosomal RNA gene, partial sequence	767	767	99%	0.0	98%
AJ457160.1	Brevibacillus sp. AV-Pb partial 16S rRNA gene	763	763	99%	0.0	98%
D78460.1	Brevibacillus formosus DNA for 16S ribosomal RNA	763	763	99%	0.0	97%
JF772473.1	Brevibacillus sp. bE28(2011) 16S ribosomal RNA gene, partial	761	761	99%	0.0	98%

	sequence					
FJ481959.1	Brevibacillus agri isolate KZ17 16S ribosomal RNA gene, partial sequence	761	761	99%	0.0	98%
EU534619.1	Uncultured bacterium clone nbt181h04 16S ribosomal RNA gene, partial sequence	761	761	99%	0.0	98%

Bacterial Isolate 4 (Reverse Primer SP6)

5'TTATCTTTAGTTGCCAGCATTCAGTTGGGCACTCTAGAGAGACTGCCGTCGACAAGAC
GGAGGAAGGCGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACG
TGCTACAATGGTTGGTACAACGGGACGCTAGCCCGCGAGGGTATGCCAATCTCTTAAA
ACCAATCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGT
AATCGCGGATCAGCATGCCGCGGTGAGTACGTTCCCGGGCCTTGTACACACCGCCCGTC
ACACCACGGGAGTTTGCAACACCCGAAGTCGGTGAGGTAACCGCAAGGAGCCAGCCG
CCGAAGGTGGGGTAGATGACTGGGGTGAAGTCGTAACAAGGTATCCGTACCGGAAGG
TGCGGCTGGATCCCCTCCTTAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCAT
ATGGGAGAGCTCCCAACGCGTGGA 3'

Table B - 2: Isolate 4 reverse sequences producing significant alignments.

Accession	Description	Max score	Total score	Query coverage	E-value	Max ident
GU321101.1	Brevibacillus sp. B59 16S ribosomal RNA gene, partial sequence	632	632	85%	5e-178	98%
NR_040982.1	Brevibacillus reuszeri strain DSM 9887 16S ribosomal RNA, partial sequence >dbj|AB112715.1| Brevibacillus reuszeri gene for 16S rRNA, partial sequence, strain:DSM 9887T	628	628	80%	7e-177	99%
FJ937913.1	Brevibacillus agri strain LS97 16S ribosomal RNA gene, partial sequence	627	627	85%	2e-176	97%
FJ481959.1	Brevibacillus agri isolate KZ17 16S ribosomal RNA gene, partial sequence	627	627	85%	2e-176	97%
EU231618.1	Brevibacillus sp. TCCC17020 16S ribosomal RNA gene, partial sequence	627	627	85%	2e-176	97%
EF640968.1	Bacillus sp. NAP2-2 16S ribosomal RNA gene, partial sequence	627	627	85%	2e-176	97%

AY319301.1	Brevibacillus agri strain NCHU1002 16S ribosomal RNA gene, complete sequence	627	627	85%	2e-176	97%
AB039334.1	Brevibacillus agri DNA for 16S rRNA, strain:M1-5	625	625	85%	8e-176	97%
GU321098.1	Brevibacillus sp. B34 16S ribosomal RNA gene, partial sequence		621	85%	1e-174	97%
AP008955.1	Brevibacillus brevis NBRC 100599 DNA, complete genome	621	9312	85%	1e-174	97%
EU816695.1	Brevibacillus borstelensis clone DQ-1 16S ribosomal RNA gene, partial sequence	621	621	85%	1e-174	97%
DQ350830.1	Brevibacillus borstelensis strain MH301 16S ribosomal RNA gene, complete sequence	621	621	85%	1e-174	97%
DQ350828.1	Brevibacillus borstelensis strain IPH801 16S ribosomal RNA gene, complete sequence	621	621	85%	1e-174	97%
M10111.1	B.brevis 16S rRNA	621	621	85%	1e-174	97%
EU816694.1	Brevibacillus borstelensis clone K11 16S ribosomal RNA gene, partial sequence	619	619	86%	4e-174	97%
DQ350837.1	Brevibacillus borstelensis strain U404 16S ribosomal RNA gene, complete sequence	619	619	85%	4e-174	97%
DQ350827.1	Brevibacillus borstelensis strain IPH701 16S ribosomal RNA gene, complete sequence >gb|JN098704.1| Uncultured Brevibacillus sp. clone KMC9 16S ribosomal RNA gene, partial sequence	619	619	85%	4e-174	97%

AJ438305.2	Brevibacillus sp. R-12868 partial 16S rRNA gene	619	619	84%	4e-174	97%
AM910315.1	Brevibacillus sp. R-31301 partial 16S rRNA gene, strain R-31301	617	617	83%	1e-173	98%

Bacterial Isolate 5 (Forward Primer T7)

5’CAATTGGGCCGaGTCGCATGCTCCCGGcCGCCATGGCGGCCGCGGGAATTCGATTAA
GGAGGGATCCAGCCGCACCTTCCGATACGGCTACCTTGTTACGACTTCACCCCAATCAT
CTGTCCCACCTTAGGCGGCTAGCTCCTTACGGTTACTCCACCGACTTCGGGTGTTACAA
GCTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCAT
GCTGATCCGCGATTACTAGCGATTCCAGCTTCATGTAGGCGAGTTGCAGCCTACAATCC
GAACTGAGAATGGTTTTATGGGATTGGCTTGACCTCGCGGTCTTGCAGCCCTTTGTACC
ATCCATTGTAGCACGTGTGTAGCCCAGGCCATAAGGGGCATGATGATTTGACGTCATCC
CCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTAAATGCTGGCA
ACTAAGATCAA 3’

Table B - 3: Isolate 5 forward sequences producing significant alignments.

Accession	Description	Max score	Total score	Query coverage	E-value	Max ident
JN082514.1	Uncultured Bacillus sp. clone 405 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
JN082388.1	Uncultured Bacillus sp. clone 206 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
HQ242770.1	Bacillus aryabhattai isolate PSB57 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
GU726179.1	Bacillus sp. KZ_AaeF_Ma2 16S ribosomal RNA gene, complete sequence	769	769	100%	0.0	99%
HQ124332.1	Bacillus megaterium strain SW1:2 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
HM104232.1	Bacillus megaterium strain LAMA 262 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
CP001982.1	Bacillus megaterium DSM319, complete genome	769	8451	100%	0.0	99%
CP001983.1	Bacillus megaterium QM	769	8438	100%	0.0	99%

	B1551, complete genome					
GU191918.1	Bacillus megaterium strain SB 3112 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
DQ660362.1	Bacillus megaterium strain MPF-906 16S ribosomal RNA gene, complete sequence	769	769	100%	0.0	99%
AJ717381.1	Bacillus megaterium 16S rRNA gene, isolate AC46b1	769	769	100%	0.0	99%
DQ267829.1	Bacillus megaterium strain 2-37-4-1 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
AB066347.1	Bacillus sp. No.49 gene for 16S rRNA, partial sequence	769	769	100%	0.0	99%
AB066345.1	Bacillus sp. No.54 gene for 16S rRNA, partial sequence	769	769	100%	0.0	99%
JN178876.1	Uncultured bacterium clone TX2_8P11 16S ribosomal RNA gene, partial sequence	767	767	99%	0.0	99%
JF309239.1	Bacillus sp. 3548BRRJ 16S ribosomal RNA gene, partial sequence	767	767	99%	0.0	99%
GU726176.1	Bacillus sp. KZ_AalM_Mm1 16S ribosomal RNA gene, complete sequence	767	767	99%	0.0	99%
HQ285770.1	Bacillus aryabhattai strain 33D 16S ribosomal RNA gene, partial sequence	767	767	99%	0.0	99%

Bacteria Isolate 5 (reverse Primer SP6)

5’AAAGCCTTCATCACTCACGCGGCGTTGCTCCGTCGGACTTTCGTCCATTGCGGAAGAT
TCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATC
ACCCTCTCAGGTCGGCTATGCATCGTTGCCTTGGTGAGCCGTTACCTCACCAACTAGCTA
ATGCACCGCGGGCCCATCTGTAAGTGATAGCCGAAACCATCTTTCAATCATCTCCCATG
AAGGAGAAGATCCTATCCGGTATTAGCTTCGGTTTCCCGAAGTTATCCCAGTCTTACAG
GCAGGTTGCCCACGTGTTACTCACCCGTCCGCCGCTAACGTCATAGAAGCAAGCTTCTA
ATCAGTTCGCTCGACTTGCATGTATTAGGCACGCCGCCAGCGTTCATCCTGAGCCAGGA
TCAAACTCTCCATAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAG
AGCTCCCAACGCGTGGATGAA 3’

Table B - 4: Isolate 5 reverse sequences producing significant alignments.

Accession	Description	Max score	Total score	Query coverage	E-value	Max ident
gi|341831776|JN082514.1	Uncultured Bacillus sp. clone 405 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
gi|341831650|JN082388.1	Uncultured Bacillus sp. clone 206 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
gi|325660530|HQ242770.1	Bacillus aryabhattai isolate PSB57 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
gi|312164965|GU726179.1	Bacillus sp. KZ_AaeF_Ma2 16S ribosomal RNA gene, complete sequence	769	769	100%	0.0	99%
gi|306029937|HQ124332.1	Bacillus megaterium strain SW1:2 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
gi|295790204|HM104232.1	Bacillus megaterium strain LAMA 262 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
gi|294799901|CP001982.1	Bacillus megaterium DSM319, complete	769	8451	100%	0.0	99%

	genome					
gi|294346812|CP001983.1	Bacillus megaterium QM B1551, complete genome	769	8438	100%	0.0	99%
gi|281192853|GU191918.1	Bacillus megaterium strain SB 3112 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
gi|110006621|DQ660362.1	Bacillus megaterium strain MPF-906 16S ribosomal RNA gene, complete sequence	769	769	100%	0.0	99%
gi|56542030|AJ717381.1	Bacillus megaterium 16S rRNA gene, isolate AC46b1	769	769	100%	0.0	99%
gi|82468524|DQ267829.1	Bacillus megaterium strain 2-37-4-1 16S ribosomal RNA gene, partial sequence	769	769	100%	0.0	99%
gi|15076638|AB066347.1	Bacillus sp. No.49 gene for 16S rRNA, partial sequence	769	769	100%	0.0	99%
gi|15076636|AB066345.1	Bacillus sp. No.54 gene for 16S rRNA, partial sequence	769	769	100%	0.0	99%
gi|340805152|JN178876.1	Uncultured bacterium clone TX2_8P11 16S ribosomal RNA gene, partial sequence	767	767	99%	0.0	99%
gi|326580989|JF309239.1	Bacillus sp. 3548BRRJ 16S ribosomal RNA gene, partial sequence	767	767	99%	0.0	99%
gi|312164962|GU726176.1	Bacillus sp. KZ_AalM_Mm1 16S ribosomal RNA gene, complete sequence	767	767	99%	0.0	99%
gi|310006709|HQ285770.1	Bacillus aryabhattai strain 33D 16S ribosomal RNA gene, partial sequence	767	767	99%	0.0	99%
gi|291062039|GU566318.1	Bacillus sp. KL2(2010) 16S ribosomal RNA gene, partial sequence	767	767	99%	0.0	99%

Bacterial Isolate 6 (Forward Primer Sp6)

5’TCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGATTCGATTAAGGAGGGGATCCAG
CCGCACCTTCCGATACGGCTACCTTGTTACGACTTCACCCCAATCATCTACCCCACCTTCG
GCGGCTGGCCCCTTGCGGTTACCTCACCGACTTCGGGTGTTGTAAACTCTCGTGGTGTG
ACGGGCGGTGTGTACAAgACCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATT
ACTAGCAATTCCGACTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGAGACCGG
CTTTTGTAGGATTGGCTCCACCTCGCGGCTTCGCTTCCCGTTGTACCGGCCATTGTAGTA
CGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCGCCTTCCTCCG
GTTTGTCACCGGCAGTCAACTTAGAGTGCCCAGCCTTACCTGCTGGCAACTAAGTTCAA
3’

Table B - 5: Isolate 6 forward sequences producing significant alignments.

Accession	Description	Max score	Total score	Query coverage	E-value	Max ident
AB021183.1	Bacillus chitinolyticus gene for 16S ribosomal RNA	767	767	89%	0.0	99%
HE577054.1	Paenibacillus polymyxa M1 main chromosome, complete genome	734	1.021e+04	90%	0.0	97%
CP002213.1	Paenibacillus polymyxa SC2, complete genome	734	1.022e+04	90%	0.0	97%
AB428571.1	Paenibacillus graminis gene for 16S ribosomal RNA, complete sequence	728	728	90%	0.0	97%
AB480884.1	Paenibacillus fujiensis genes for 16S rRNA, 16S-23S ribosomal intergenic spacer, 23S rRNA, complete and partial sequence	726	726	90%	0.0	97%
AB092351.1	Paenibacillus fujiensis gene for 16S ribosomal RNA, partial sequence	726	726	90%	0.0	97%
AY237109.1	Paenibacillus sp.	725	725	91%	0.0	97%

	A11 16S ribosomal RNA gene, partial sequence					
HQ005270.1	Paenibacillus vortex strain V453 16S ribosomal RNA gene, complete sequence	723	723	90%	0.0	97%
AJ299573.1	Paenibacillus sp. CZ89 partial 16S rRNA gene, isolate CZ89	723	723	90%	0.0	97%
FR727737.1	Paenibacillus polymyxa partial 16S rRNA gene, strain M1	721	721	89%	0.0	97%
GU321104.1	Paenibacillus sp. B69 16S ribosomal RNA gene, partial sequence	719	719	91%	0.0	96%
DQ232773.1	Paenibacillus campinasensis strain BL11 16S ribosomal RNA gene, partial sequence	719	719	91%	0.0	96%
JF309265.1	Paenibacillus timonensis strain 3584BRRJ 16S ribosomal RNA gene, partial sequence	717	717	87%	0.0	98%
AB482199.1	Paenibacillus abekawaensis gene for 16S rRNA, complete sequence, strain: MG1	715	715	90%	0.0	96%
AJ316314.1	Paenibacillus sp. LMG 20244 partial 16S rRNA gene, strain LMG 20244	715	715	88%	0.0	97%
FJ719492.1	Paenibacillus sp. B27 clone 3 16S ribosomal RNA gene, partial sequence	713	713	91%	0.0	96%
AB480709.1	Paenibacillus sp. PE0C gene for 16S rRNA, partial sequence	713	713	90%	0.0	96%

EF452233.1	Paenibacillus polymyxa strain HKA-15 16S ribosomal RNA gene, partial sequence	713	713	91%	0.0	96%
NR_040854.1	Paenibacillus chitinolyticus strain HSCC 596T (IFO 15660T) 16S ribosomal RNA, partial sequence >dbj|AB045100.1| Bacillus chitinolyticus gene for 16S rRNA, partial sequence	713	713	83%	0.0	99%
AB045099.1	Bacillus chitinolyticus gene for 16S rRNA, partial sequence	713	713	83%	0.0	99%

Bacterial Isolate 6 (reverse Primer T7)

5’CATCACTCACGCGGCGTTGCTCCGTCAGACTTGCGTCCATTGCGGAAGATTCCCTACT
GCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGTTCACCCTTTC
AGGTCGGCTACGCATCGTCGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCC
GCAGGCCCATCCGTAAGCCACAGATTACTCCGTGTTTCACGATCTCTCCATGCGGAGAA
ACCGATTATCCGGTCTTAGCTACCGTTTCCGGTAGTTATCCCGATCTTACAGGCAGGTTG
CCTACGTGTTACTCACCCGTCCGCCGCTAACCATCAGAGAAGCAAGCTTCTCATCAAGTC
CGCTCGACTTGCATGTATTAGGCACGCCGCCAGCGTTCGTCCTGAGCCAGGATCAAACT
TTCCATAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCC
AACGCGTGGATGAA 3’

Table B - 6: Isolate 6 reverse sequences producing significant alignments.

Accession	**Description**	**Max score**	**Total score**	**Query coverage**	**E-value**	**Max ident**
AB021183.1	Bacillus chitinolyticus gene for 16S ribosomal RNA	732	732	83%	0.0	98%
AF156314.1	Marine bacillus NRRLB-14909 16S ribosomal RNA gene, partial sequence	726	726	85%	0.0	98%
AY556413.1	Bacillus sp. SSL3 16S ribosomal RNA gene, partial sequence	726	726	85%	0.0	98%
AY556409.1	Bacillus sp. SGE2 16S ribosomal RNA gene, partial sequence	726	726	85%	0.0	98%
AY556411.1	Bacillus sp. SGE4 16S ribosomal RNA gene, partial sequence	715	715	85%	0.0	97%
AM162308.1	Paenibacillus sp. 4GH03-07 partial 16S rRNA gene	706	706	83%	0.0	98%
NR_040854.1	Paenibacillus chitinolyticus strain HSCC 596T (IFO 15660T) 16S ribosomal RNA, partial sequence >dbj|AB045100.1| Bacillus chitinolyticus gene for 16S rRNA, partial sequence	702	702	80%	0.0	98%

AM162338.1	Paenibacillus sp. AS40 partial 16S rRNA gene	695	695	82%	0.0	98%
EU839149.1	Soil bacterium 02G-07 16S ribosomal RNA gene, partial sequence	691	691	82%	0.0	97%
AB045099.1	Bacillus chitinolyticus gene for 16S rRNA, partial sequence	688	688	80%	0.0	98%
FJ174604.1	Paenibacillus chitinolyticus strain 146XG36 16S ribosomal RNA gene, partial sequence	684	684	79%	0.0	98%
AB461735.1	Paenibacillus sp. M325 gene for 16S rRNA, partial sequence, strain: M325	682	682	81%	0.0	97%
FJ174602.1	Paenibacillus chitinolyticus strain 137XG33 16S ribosomal RNA gene, partial sequence	680	680	79%	0.0	98%
FJ174585.1	Paenibacillus chitinolyticus strain 34XG60 16S ribosomal RNA gene, partial sequence	676	676	79%	0.0	98%
EF123224.1	Paenibacillus sp. RIZO1 16S ribosomal RNA gene, partial sequence	673	673	80%	0.0	97%
AY359637.1	Paenibacillus polymyxa strain KCTC3717 16S ribosomal RNA gene, partial sequence	669	669	79%	0.0	97%
FR727704.1	Paenibacillus sp. IMM02 partial 16S rRNA gene, strain IMM02	667	667	79%	0.0	97%
AM900790.1	Paenibacillus sp. PA227 partial 16S rRNA gene, isolate PA227	654	654	85%	0.0	95%
AF181573.1	Paenibacillus cf. polymyxa 16S ribosomal RNA gene, partial sequence	654	654	80%	0.0	96%
GU599102.1	Uncultured soil bacterium clone HB_Ca_M_178 16S ribosomal RNA gene, partial sequence	643	643	85%	0.0	94%
EU637621.1	Bacterium C-TJ14 16S ribosomal RNA gene,	643	643	73%	0.0	98%

	partial sequence					
AF378695.1	Paenibacillus turicensis clone A2 16S ribosomal RNA gene, complete sequence	643	643	85%	0.0	94%
GQ383913.1	Paenibacillus sp. 2CJ4 16S ribosomal RNA gene, partial sequence	640	640	76%	4e-180	97%
AB190129.1	Paenibacillus sp. D-39-25-6 gene for 16S rRNA, partial sequence	638	638	85%	1e-179	94%

Bacterial Isolate 7 (Forward Primer T7)

5’GCTCCCGGCCGCCATGGCGGCCGCGGGATTCGATTAAGGAGGGGATCCAGCCGCAC
CTTCCGATACGGCTACCTTGTTACGACTTCACCCCaGTCATCGGCCACACCGTGGCAAGC
GCCCTCCCGAAGGTTAAGCTACCTGCTTCTGGTGCAACAAACTCCCATGGTGTGACGGG
CGGTGTGTACAAGGCCCGGGAACGTATTCACCGCAGCAATGCTGATCTGCGATTACTA
GCGATTCCGACTTCATGGAGTCgAGTTGCAGACTCCAATCCGGACTGAGATAGGGTTTC
TGGGATTGGCTTACCGTCGCCGGCTTGCAGCCCTCTGtCCCTACCATTGTAGTACGTGTG
TAGCCCTGGCCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTC
ACCGGCGGTCTCCTTAGAGTTCCCACCATTACGTGCTGGCAACTAAGGACAAGGGCTGC
GCTCGTTGC3’

Table B - 7: Isolate 7 forward sequences producing significant alignments.

Accession	Description	Max score	Total score	Query coverage	E-value	Max ident
gi|306032257|GQ476505.1	Uncultured bacterium clone 721-M13F 16S ribosomal RNA gene, partial sequence	817	817	92%	0.0	99%
gi|193245054|EU816936.1	Uncultured Stenotrophomonas sp. clone l-35-2 16S ribosomal RNA gene, partial sequence	815	815	93%	0.0	99%
gi|193245051|EU816933.1	Uncultured Stenotrophomonas sp. clone l-43 16S ribosomal RNA gene, partial sequence	815	815	93%	0.0	99%
gi|326698745|JF346011.1	Uncultured Stenotrophomonas sp. clone Al6h-57 16S ribosomal RNA gene, partial sequence	813	813	92%	0.0	99%
gi|317160942|GU459217.1	Stenotrophomonas maltophilia strain QS3 16S ribosomal RNA gene, partial sequence	813	813	92%	0.0	99%
gi|256861409|GQ360071.1	Stenotrophomonas maltophilia strain	813	813	92%	0.0	99%

	pp5c 16S ribosomal RNA gene, partial sequence					
gi|209423310|FJ193661.1	Uncultured Stenotrophomonas sp. clone GI2-SSB-cs-C11 16S ribosomal RNA gene, partial sequence	813	813	92%	0.0	99%
gi|126149287|AB294556.1	Stenotrophomonas maltophilia gene for 16S ribosomal RNA, partial sequence, strain: IAM 12672	813	813	92%	0.0	99%
gi|343200890|NR_041577.1	Stenotrophomonas maltophilia strain IAM 12423 16S ribosomal RNA, partial sequence	813	813	92%	0.0	99%
gi|343776783|CP002986.1	Burkholderia sp. JV3, complete genome	811	3247	92%	0.0	99%
gi|298107802|HM447936.1	Uncultured Stenotrophomonas sp. clone T222G7 16S ribosomal RNA gene, partial sequence	811	811	92%	0.0	99%
gi|334847550|JF357614.1	Uncultured Stenotrophomonas sp. clone ASC7 16S ribosomal RNA gene, partial sequence	811	811	92%	0.0	99%
gi|321146008|HQ912766.1	Uncultured Stenotrophomonas sp. clone ASC45 16S ribosomal RNA gene, partial sequence	811	811	92%	0.0	99%
gi|317184316|HQ652605.1	Stenotrophomonas sp. p22(2011) 16S ribosomal RNA gene, partial sequence	811	811	92%	0.0	99%
gi|307713794|HM438644.1	Uncultured Stenotrophomonas sp. clone T311H8 16S ribosomal RNA gene, partial sequence	811	811	92%	0.0	99%
gi|306410506|HQ200414.1	Stenotrophomonas maltophilia strain Dh 16S ribosomal RNA gene, partial sequence	811	811	92%	0.0	99%

Bacterial Isolate 7 (Reverse Primer Sp6)

5’ACGCGGTATGGCTGGATCAGGCTTGCGCCCATTGTCCAATATTCCCCACTGCTGCCTC
CCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTGATCATCCTCTCAGACCAGC
TACGGATCGTCGCCTTGGTGGGCCTTTACCCCGCCAACTAGCTAATCCGACATCGGCTC
ATTCAATCGCGCAAGGTCCGAAGATCCCCTGCTTTCACCCGTAGGTCGTATGCGGTATT
AGCGTAAGTTTCCCTACGTTATCCCCCACGACAGAGTAGATTCCGATGTATTCCTCACCC
GTCCGCCACTCGCCACCCAAGGAGCAAGCTCCTCTGTGCTGCCGTTCGACTTGCATGTG
TTAGGCCTACCGCCAGCGTTCACTCTGAGCCAGGATCAAACTTTCCATAATCACTAGTG
AATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTGGATG3’

Table B - 8: Isolate 7 reverse sequences producing significant alignments.

Accession	Description	Max score	Total score	Query coverage	E-value	Max ident
EU373409.1	Stenotrophomonas maltophilia strain YRL04 16S ribosomal RNA gene, partial sequence	734	734	85%	0.0	99%
AY770720.1	Uncultured gamma proteobacterium from sea squirt Microcosmus sp. clone MspS83 16S ribosomal RNA gene, partial sequence	734	734	85%	0.0	100%
JF290248.1	Uncultured bacterium clone PE7-30M 16S ribosomal RNA gene, partial sequence	732	732	84%	0.0	100%
JN200221.1	Uncultured bacterium clone 109109E08 16S ribosomal RNA gene, partial sequence	732	732	84%	0.0	100%
JN199944.1	Uncultured bacterium clone 1081080470775 16S ribosomal RNA gene, partial sequence	732	732	84%	0.0	100%
JN199936.1	Uncultured bacterium clone 1081080420744 16S ribosomal RNA gene, partial sequence	732	732	84%	0.0	100%
HQ905871.1	Uncultured bacterium clone VA0916S297 16S ribosomal RNA gene,	732	732	84%	0.0	100%

	partial sequence					
GU013678.1	Uncultured Stenotrophomonas sp. clone PGM-6 16S ribosomal RNA gene, partial sequence	732	732	84%	0.0	100%
HQ445836.1	Uncultured bacterium clone Luq_GN490_051 16S ribosomal RNA gene, partial sequence	732	732	84%	0.0	100%
HQ445729.1	Uncultured bacterium clone Luq_GN470_018 16S ribosomal RNA gene, partial sequence	732	732	84%	0.0	100%
FN826236.1	Uncultured bacterium partial 16S rRNA gene, clone UK14.39_V02193E_035	732	732	84%	0.0	100%
FN826222.1	Uncultured bacterium partial 16S rRNA gene, clone UK14.32_V02193E_018	732	732	84%	0.0	100%
HM557604.1	Uncultured bacterium clone TIFI1100 16S ribosomal RNA gene, partial sequence	732	732	84%	0.0	100%
GQ206319.1	Uncultured Stenotrophomonas sp. clone A6H6M9 16S ribosomal RNA gene, partial sequence	732	732	84%	0.0	100%
GU563764.1	Uncultured alpha proteobacterium clone Bmc13 16S ribosomal RNA gene, partial sequence	732	732	84%	0.0	100%
GU451206.1	Uncultured bacterium clone A1H1M9 16S ribosomal RNA gene, partial sequence	732	732	84%	0.0	100%
FJ675503.1	Uncultured bacterium clone LL141-8H2 16S ribosomal RNA gene, partial sequence	732	732	84%	0.0	100%
FN401250.1	Uncultured bacterium partial 16S rRNA gene, clone HH3_f5	732	732	84%	0.0	100%
FN401248.1	Uncultured bacterium partial 16S rRNA gene, clone HH3_e6	732	732	84%	0.0	100%

Bacterial Isolate 8 (Forward Primer T7)

5'CgCATGCTCCCGGCCGCCATGGCGGCCGCGGGATTCGATTATGGAGAGTTTGATCCT
GGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAA
GAGCTTGCTCTTATGAAGGTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCC
ATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGC
ATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGCCGCATT
AGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTACCCGACCTGAGAGG
GTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTA
GGGAATCTTCCGCAATGGACgAAAGTCTGACGGACACCGCCGCGTGAGTGATGAAGGC
TTTCGGGTCGT3'

Table B - 9: Isolate 8 forward sequences producing significant alignments.

Accession	Description	Max score	Total score	Query coverage	E-value	Max ident
CP002508.1	Bacillus thuringiensis serovar finitimus YBT-020, complete genome	771	1.077e+04	99%	0.0	98%
HM588153.1	Bacillus anthracis strain RSNPB13 16S ribosomal RNA gene, partial sequence	771	771	99%	0.0	98%
CP001746.1	Bacillus cereus biovar anthracis str. CI, complete genome	771	8433	99%	0.0	98%
HM067839.1	Bacillus cereus strain WQ9-2 16S ribosomal RNA gene, partial sequence	771	771	99%	0.0	98%
CP001903.1	Bacillus thuringiensis BMB171, complete genome	771	1.075e+04	99%	0.0	98%
CP001598.1	Bacillus anthracis str. A0248, complete genome	771	8451	99%	0.0	98%
CP001215.1	Bacillus anthracis str. CDC 684, complete genome	771	8440	99%	0.0	98%
CP001407.1	Bacillus cereus 03BB102, complete genome	771	1.079e+04	99%	0.0	98%
CP000227.1	Bacillus cereus Q1,	771	9980	99%	0.0	98%

	complete genome					
AM293345.1	Bacillus thuringiensis 16S rRNA gene, strain BAB-Bt2	771	771	99%	0.0	98%
AM293344.1	Bacillus thuringiensis 16S rRNA gene, strain DAB-Bt6	771	771	99%	0.0	98%
AM293343.1	Bacillus thuringiensis 16S rRNA gene, strain DAB-Bt4	771	771	99%	0.0	98%
CP001283.1	Bacillus cereus AH820, complete genome	771	9209	99%	0.0	98%
AM292033.1	Bacillus thuringiensis 16S rRNA gene and 16S-23S IGS, strain SBSBT-2	771	771	99%	0.0	98%
CP001176.1	Bacillus cereus B4264, complete genome	771	1.077e+04	99%	0.0	98%
AM779002.1	Bacillus thuringiensis 16S rRNA gene and 16S-23S IGS, strain SBS-BT6	771	771	99%	0.0	98%
AM778999.1	Bacillus thuringiensis 16S rRNA gene and 16S-23S IGS, strain SBS-BT3	771	771	99%	0.0	98%
CP001177.1	Bacillus cereus AH187, complete genome	771	1.072e+04	99%	0.0	98%
AM292032.1	Bacillus thuringiensis 16S rRNA gene and 16S-23S IGS, strain CMBLBT-5	771	771	99%	0.0	98%
AM292029.1	Bacillus thuringiensis 16S rRNA gene and 16S-23S IGS, strain CMBLBT-1	771	771	99%	0.0	98%
EU586795.1	Bacillus cereus strain MZ-30 16S ribosomal RNA gene, partial sequence	771	771	99%	0.0	98%
EF028102.1	Bacillus thuringiensis 16S ribosomal RNA gene, partial sequence	771	771	99%	0.0	98%

Bacterial Isolate 8 (Reverse Primer Sp6)

5'TTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACC
GGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACG
TGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAA
ACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGT
AATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTC
ACACCACGAGAGTTTGTAACACCCGAAGTCGGTGGGGTAACCTTTTTGGAGCCAGCCG
CCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTaGCCGTATCGGAAGG
TGCGGCTGGATCCCCTCCTTAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCAT
ATGGGAGAGTCCC3'

Table B - 10: Isolate 8 reverse sequences producing significant alignments.

Accession	Description	Max score	Total score	Query coverage	E-value	Max ident
AY853168.1	Bacillus sp. 4 16S ribosomal RNA gene, partial sequence	671	671	87%	0.0	99%
GQ360073.1	Bacillus cereus strain pp9k 16S ribosomal RNA gene, partial sequence	669	669	87%	0.0	99%
AJ539175.1	Bacillus cereus partial 16S rRNA gene	669	669	87%	0.0	99%
AJ563290.1	Bacillus cereus partial 16S rRNA gene, strain VA1	669	669	87%	0.0	99%
AJ539225.1	Bacillus cereus partial 16S rRNA gene, strain A78	669	669	87%	0.0	99%
FR851254.1	Bacillus sp. PR1.7 partial 16S rRNA gene, strain PR1.7	667	667	87%	0.0	99%
CP001907.1	Bacillus thuringiensis serovar chinensis CT-43, complete genome	667	1.160e+04	87%	0.0	99%
CP002508.1	Bacillus thuringiensis serovar finitimus YBT-020, complete genome	667	9322	87%	0.0	99%
HM771661.1	Bacillus cereus strain PPB6 16S ribosomal RNA gene, partial	667	667	87%	0.0	99%

	sequence >gb|JN215517.1| Bacillus cereus strain VB21 16S ribosomal RNA gene, partial sequence					
HQ200405.1	Bacillus anthracis strain B 16S ribosomal RNA gene, partial sequence	667	667	87%	0.0	99%
HQ156459.1	Bacillus cereus strain HMT6 16S ribosomal RNA gene, partial sequence	667	667	87%	0.0	99%
HM348811.1	Bacillus cereus strain BZ-001H 16S ribosomal RNA gene, partial sequence	667	667	87%	0.0	99%
CP001746.1	Bacillus cereus biovar anthracis str. CI, complete genome	667	7323	87%	0.0	99%
HM067839.1	Bacillus cereus strain WQ9-2 16S ribosomal RNA gene, partial sequence	667	667	87%	0.0	99%
CP001903.1	Bacillus thuringiensis BMB171, complete genome	667	1.267e+04	87%	0.0	99%
GU939625.1	Bacillus anthracis strain TC-3 16S ribosomal RNA gene, partial sequence	667	667	87%	0.0	99%
GU566355.1	Bacillus sp. D12(2010) 16S ribosomal RNA gene, partial sequence	667	667	87%	0.0	99%
GU566345.1	Bacillus sp. R5(2010) 16S ribosomal RNA gene, partial sequence	667	667	87%	0.0	99%
GU014292.1	Bacillus sp. VITABR9 16S ribosomal RNA gene, partial sequence	667	667	88%	0.0	99%
FJ932761.1	Bacillus thuringiensis strain 61436 16S ribosomal RNA gene, partial sequence	667	667	87%	0.0	99%
CP001598.1	Bacillus anthracis str. A0248, complete genome	667	7337	87%	0.0	99%
CP001215.1	Bacillus anthracis str.	667	7326	87%	0.0	99%

	CDC 684, complete genome					
CP001407.1	Bacillus cereus 03BB102, complete genome	667	9289	87%	0.0	99%
EU429672.1	Bacillus thuringiensis serovar israelensis 16S ribosomal RNA gene, partial sequence	667	667	87%	0.0	99%
EU429671.1	Bacillus thuringiensis serovar tenebrionis 16S ribosomal RNA gene, partial sequence	667	667	87%	0.0	99%