

ECOLOGICAL STUDIES OF INDIGENOUS BAMBARA GROUNDNUT RHIZOSPHERE BACTERIA AND THEIR METABOLIC ACTIVITIES

LIBRARY	
MAFIKENG CAMPUS	
CALL NO:	2021 -02- 0 1
ACC.NO.:	
NORTH-WEST UNIVERSITY	

C. F. AJILOGBA

0000-0003-0706-4210

B. Tech. (Hons) Microbiology

MSc Biology

Thesis submitted for the degree *Philosophiae Doctor* in
Biology at the
Mafikeng Campus of the North-West University

Promoter: Prof. Olubukola Oluranti Babalola

Graduation: October 2017

Student number: 23703563

<http://dspace.nwu.ac.za/>



DECLARATION

I, the undersigned, Caroline Fadeke Ajilogba, declare that this thesis submitted to the North-West University for the degree of Doctor of Philosophy in Biology in the Faculty of Science, Agriculture and Technology, School of Environmental and Health Sciences, and the work contained herein is my original work with exemption to the citations and that this work has not been submitted at this or any other university in partial or entirely for the award of any degree.

STUDENT NAME

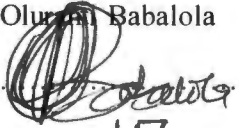
Caroline Fadeke Ajilogba

SIGNATURE.....

DATE.....13-10-2017

SUPERVISOR NAME

Professor Olubukola Olurukan Babalola

SIGNATURE.....

DATE.....13-10-17

DEDICATION

This work is dedicated to;

My lovely husband, Mark Makinde Ajilogba, (who believes so much in me and supported me),
our great children Victoria, GodsDelight, Demmy and Dammy, and

My father, Pst. Daniel Olushola Akinpelumi and my mother of blessed memory, Mummy Stella
Tunlabi Akinpelumi, (who, as a lover of plants, taught me hard work).

ACKNOWLEDGEMENTS

I want to thank my maker and the One who formed me from my mother's womb and made me who I am today. I thank My Lord and Saviour Jesus Christ, for loving me all the way, and the Holy Spirit, who taught me and has made me to excel.

I wish to express my profound gratitude and appreciation to my advisor and promoter, Prof. Olubukola O. Babalola, for her guidance in the course of carrying out the experiments, encouragement to write articles which have helped to boost my work, patience in reading and indispensable criticism of my manuscript. I appreciate you Ma.

I acknowledge the North-West University for bursary award to pursue the PhD degree. My profound appreciation goes to the Head of Department (HOD) of Biological Sciences, Prof. E. Mukwevho and all academic and support staff of the Department of Biological Sciences for their love and assistance during this work, as well as all Faculty and staff members of the School of Environmental and Health Sciences, North-West University, for their valuable co-operation.

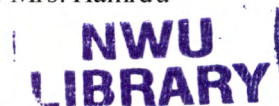
I want to appreciate ARC-PPRI for my training on the use of BIOLOG and its extraordinary staff (Mr. Johan Habig, Ms. Selina and Ms. Johannes) for their extreme dedication that made all the BIOLOG analyses possible. My profound gratitude also goes to Dr. Patrick Adebola and Dr. Rasheed Adeleke of ARC-VOPI and ARC-ISCW respectively for their technical support in carrying out some of the chemical analyses. Mr. Sizwe of the Chemistry Department of the NWU, Mafikeng campus, was helpful with the GC-MS analysis. I will not forget Dr. Chris Udomboso, Dr. Seun Alawode and Mr. Emmanuel Okon, who, inspite of their busy schedules helped with my statistical analysis and Dr. Andres Gomez of the J. Craig Venter Institute, California, USA, who taught me how to interpret my analysed NGS data.

Many thanks to Dr. M. F. Adegboye, for her kind assistance and support both technically and otherwise in the course of this study. I want to appreciate Prof. E. Ebenso and Prof. O. Useh and family, thank you for your guidance, counsels, encouragement and always looking out for me. God bless you all. My parents, Pastor D. O. Akinpelumi, thank you daddy for your support and my mother of blessed memory Mrs. S. T. Akinpelumi, I put in so much effort trusting that I would take better care of you than this, but you are in a better place now, I thank you for inspiring me to work with plants. You are the best farmer I ever knew! My husband's parents, Rtd. Captain and

Mrs. Ajilogba Arowolo, thank you for your support. To my siblings Dr. B. F. Taiwo and Mrs. R. A. Omotayo, their husbands, my brothers and sisters-in law and my entire family, I say thank you for your overwhelming love, spiritual, financial, and emotional support.

I will want to appreciate my lovely husband, Mark M. Ajilogba, for believing in me and supporting me, Sweetie, you are the best! To our great and godly seeds, Victoria, GodsDelight, Demmy and Dammy, you are such a great force to be associated with, your understanding and encouragement is second to none, you are well appreciated. To my household family, Mary, Simon and Seyi, thanks for your love and support.

My gratitude also goes to the Olubambis who God used to connect me to my supervisor, you are more than family to me and the Olorundares, for their love, support, encouragement and believe in me that I can do all things through Christ who strengthens me. Thank you Mr. and Mrs. Hamidu and family for support on every side.



Rev. and Pastor (Mrs) Olowodola and members of the Four Square Gospel Church Asokoro, Rev. and Rev. (Mrs.) Ajetomobi and members of Men of Issachar Vision, Pastor and Pastor (Mrs.) Gbolahan Akinola and members of Kingdom Impact Christian Centre, Accra Ghana, Pastor and Mrs. Bulus Alim and members of the RCCG Tree of Righteousness Parish, Jos Nigeria, Pastor and Mrs. Ochigbo, of the RCCG Life sanctuary, Jos, Prof. and Prof. (Mrs.) Oduaran, Chief Justice and Mrs. Mogoeng and the members of the RCCG, Mmabatho, for their spiritual, prayer and financial support. To members of RCCG, Dibate Assembly for their love, kind support and encouragement, God has just began with us. Mr. and Mrs. Mustvangwa, your support has been so great, God bless you.

Thanks to all my friends, the Adejayans, the Fasholas, the Aremus, sister Ruth Adebayo, sister Olobatoke, sister Mamsi, Dns C. Famodimu, the Fayemis, the Omotayos, the Olorunfemis, brother Dan Medoye, my fellow students in the Microbial Biotechnology group, God bless you all. Together with a host of others that have not been able to mention but have affected my life and academics one way or the other, I say thank you all. Re a leboga.

Caroline Fadeke Ajilogba (07/06/2017)

GENERAL ABSTRACT

With the rise in world population and decrease in food supply due to global climate change, food security becomes very pertinent. Malnutrition, food scarcity and poverty have consistently affected population growth. This issue has driven scientists to seek out other plants that have been understudied but have potentials for food security. Metabolites from rhizobacteria have also been found to be important in improving crop yield and most especially rhizobacteria from legumes like Bambara groundnut. This study was designed to harness the interaction between Bambara groundnut, various rhizobacteria in the soil, their metabolites and their roles in biocontrol and biofertilization. This, in turn, will help to increase crop yield by resisting pests and diseases. This will also improve plant growth and productivity.

In this study, soil from the rhizosphere of Bambara groundnut was screened for Plant growth-promoting properties (PGP) of isolated rhizobacteria. They included indole acetic acid (IAA), hydrogen cyanide (HCN), phosphate solubilization (PS) and ammonia production (NH_3P) activities using standard methods. In addition, antifungal assay using a dual culture method was used to analyze the biocontrol properties of the isolates and their phylogenetic identification were carried out using the 16S gene. Forty three (43) bacterial isolates from the Bambara groundnut rhizosphere were screened for their plant growth-promoting and biocontrol potentials. Out of this, 41.87% showed positive actions in one or some of the PGP tests, 27.91% were all able to produce these enzymes: catalase, oxidase and protease. All the isolates were able to produce ammonia while 4.65%, 12.28% and 27.91% produce HCN, IAA and solubilize phosphate, respectively making them important target as biocontrol and biofertilizer agents. Among the isolates, the species identified included *Bacillus*, *Kocuria*, *Arthrobacter* and *Enterobacter*. Growth of *Fusarium graminearum* was suppressed *in vitro* by 9.3% of the isolates while 16.3% were

antagonistic against *Bacillus cereus* and *Enterobacter faecalis*. This study reveals the PGP and biocontrol potentials of rhizobacteria from Bambara groundnut rhizosphere.

Due to the underutilized nature of Bambara groundnut and its resistant qualities to drought and harsh environmental conditions, some of the bacterial isolates were analyzed for their production of volatile organic compounds (VOCs). Volatile organic compounds are secondary metabolites produced by living organisms including bacteria in response to metabolic activities in the environment. In order to assess the production of these bioactive compounds, isolates were screened for the production of new compound or known compounds from Bambara groundnut rhizosphere. The antibacterial activities of crude extracts from three selected isolates were determined against *E. faecalis*, *Pseudomonas aeruginosa*, *Microbacterium cryophilus* and *B. cereus*. The butanol, hexane, ethyl acetate and petroleum ether extract of BAMr, BAMhi and BAMli were active when subjected to gas chromatography-mass spectrometry (GC-MS) analysis to ascertain the chemical components and structure of the bioactive compounds. Some of the VOCs released are Phthalan, p-xylene, tropeolin, tropone, fumaronitrile, tridecane and isocarboxazid. It was observed that not much work has been carried out to extract these compounds from Bambara groundnut rhizosphere. So far this is the first time that these bioactive compounds are extracted from Bambara groundnut rhizobacteria and are very potent antibacterial compounds.

The functional diversity of the rhizospheric bacterial in growth stages of Bambara groundnut can be used to assess its impact on crop production. The rhizosphere of Bambara groundnut at different growth stages was also assessed for catabolic diversity and the pattern of metabolism by the bacterial community as a function of the carbon source utilization profile (CSUP) of the rhizosphere using BIOLOGTM. Soil samples were analyzed using a 96-well carbon source plate to determine the mostly utilized source of carbon by microbes in the soil samples. Cluster analysis

revealed a shift in soil microbial community diversity and activity over the plant growth stages. Bacterial abundance and diversity were higher at 4 and 8 WAP and lowest in the bulk soil before planting. The highest utilization of alcohols, amides, amines, aromatic chemicals, brominated chemicals and phosphorylated chemicals was found in the control treatments. The highest utilization of carboxylic acids, ester, amino acids and polymers and carbohydrates were found in the treatments across growth stages. This implies that the soil samples between 4 WAP and 12 WAP were richer in diversity of microbial species and their abundance making the soil important in crop production. With the structure of carbon source utilization in the rhizosphere of Bambara groundnut, the diversity of bacterial that enhanced the richness and diversity in the soil was determined and planting strategies can be formulated.

The bacterial communities at the different growth stages of Bambara groundnut and the bulk soil was also determined. Paired end illumina Miseq sequencing of 16S rRNA was carried on the soil samples of the bacterial community. The phyla operational taxonomic units (OTU) were dominated by actinobacter (30.1%), proteobacter (22%), acidobacter (20.9%), bacteroides (8.4%), chloroflex (4.5%) and firmicutes (4.4%) in all soil samples. Samples from bulk soil had the least average percent phyla (O1 and O4) while samples at 16 WAP (F1 and F4) had the highest average percent phyla. *Rubrobacter* was the most predominant genera, after which is *Acidobacterium* and *Skermanella*. It was observed from the analysis of OTU that there was significant change in the bacterial structure of the rhizosphere with a higher abundance of potential plant growth promoting rhizobacteria, at the different growth stages which included genera such as *Bacillus* and *Acidobacterium*. These results demonstrated that the bacterial communities of Bambara groundnut rhizosphere in the field are dynamic and changes with abundance at growth stages of the plant.

Given the knowledge above of the bacterial community, isolated bacteria, their metabolites and activities *in vitro*, BAMr, BAMhi and BAMli were applied as biofertilizers to Bambara groundnut grown on the field. Bambara groundnut seeds were coated with bacteria and the isolates left for 48 hours before planting them in the field using complete randomized block experimental design with three replications. The Null hypothesis stated that there is no significant difference between the treatments BAMli, BAMhi, BAMr and control while the alternative hypothesis emphasized that there exists a significant difference between the treatments. Growth parameters such as length of plant, number of leaves, number of branches and number of seeds were measured. The results were significant for both the treatment/bacterial effect and the block/week effect at a 5% level of significance (treatment: $F = 12.028$, $p = 0.001$; block: $F = 105.350$, $p = 0.000$). Since p-value is less than 0.05, the Null hypothesis is rejected in favour of the alternative hypothesis. However, the overall model used for this data was significant ($F = 196.068$, $p = 0.000$) signifying that this model fits the data. Characterization of BAMhi, BAMli and BAMr using the 16S rRNA gene reveals their identity as *B. amyloliquefaciens*, *B. thuringiensis* and *Bacillus* spp respectively. These *Bacillus* strains have proved to be plant growth-promoting agents that can be used as biofertilizers to enhance the growth of crops in order to improve yield. This is the first time that rhizobacteria from Bambara groundnut rhizosphere is applied as biofertilizer.

TABLE OF CONTENTS

DECLARATION	i
DEDICATION	ii
ACKNOWLEDGEMENTS	iii
GENERAL ABSTRACT	v
TABLE OF CONTENTS	ix
LIST OF ABBREVIATIONS	xvi
LIST OF FIGURES	xviii
LIST OF TABLES	xx
LIST OF PUBLICATIONS	xxi
CHAPTER ONE	1
1.1 General Introduction.....	1
1.2 Problem Identification	2
1.3 Research Questions	2
1.4 Research Aims and Objectives.....	3
CHAPTER TWO	4
BAMBARA GROUNDNUT-BACTERIA INTERACTION; SOURCE OF FOOD SECURITY	4
Abstract	4
2.1 Introduction	5
2.2 History of Bambara groundnut.....	6
2.3 World Bambara production	7
2.4 Agronomy and morphology	8
2.5 Importance of bambara groundnut cultivation worldwide	9
2.5.1 Agronomic advantages	9
2.5.2 Nutritional advantages.....	10
2.5.2.1 Consumption	11
2.5.2.2 Medicinal.....	11
2.6 Bambara groundnut-bacterial interactions	12
2.6.1 Symbiotic interaction	15
2.6.2 Non symbiotic interaction.....	17
2.6.3 Nitrogen fixation and food security	18

2.7 Cultural cultivation of Bambara groundnut and food security	20
2.7.1 Mixed cropping	21
2.7.2 Intercropping.....	23
2.7.3 Crop rotation	24
2.8 Conclusion.....	24
CHAPTER THREE.....	25
METABOLIC DIVERSITIES OF RHIZOSPHERIC BACTERIA FOR BIOTECHNOLOGICAL PROCESSES.....	25
Abstract	25
3.1 Introduction	26
3.2 Metabolism and volatile organic compounds (VOCs)	27
3.2.1 Microbial volatile organic compounds	28
3.2.1.1 Volatile organic compounds (VOCs).....	28
3.2.1.2 Rhizobacteria as sources of volatile organic compounds	29
3.2.1.3 Ability and adaptation of rhizobacteria to produce volatile organic compounds.....	30
3.2.1.4 Contribution of rhizobacteria from legumes to production of volatile organic compounds	31
3.3 Functions of metabolites in plant-bacteria interactions.....	31
3.3.1 Phenazines	31
3.3.2 Toxin production	32
3.3.3 Hydrogen cyanide	32
3.4 Mechanism of detection of metabolites from rhizobacteria	33
3.5 Conclusion.....	34
CHAPTER FOUR.....	35
EVALUATION OF PGPR AND BIOCONTROL ACTIVITIES OF BACTERIA ISOLATED FROM BAMBARA GROUNDNUT RHIZOSPHERE.....	35
Abstract	35
4.1 Introduction	36
4.2 Materials and methods.....	38
4.2.1 Planting of Bambara groundnut.....	38
4.2.2 Soil sampling and collection	39
4.2.3 Soil Analysis	40
4.2.3.1 Physical and chemical analysis of samples	40

4.2.3.1.1 Determination of CEC and extractable cations.....	40
4.2.3.1.2 Nitrogen analysis of soil samples.....	41
4.2.3.1.3 Determination of nitrate composition.....	41
4.2.3.1.4 Determination of pH and redox potential.....	41
4.2.3.1.5 Determination of organic matter	41
4.2.4 Preparation of soil samples for bacterial isolation	42
4.2.5 Culturing and isolation of bacteria from soil samples.....	42
4.2.6 PGPR and biochemical analysis of bacteria isolates.....	43
4.2.6.1 Detection of hydrogen cyanide (HCN) production	43
4.2.6.2 Determination of indole acetic acid (IAA) production	43
4.2.6.3 Determination of Phosphate solubilisation (PS)	44
4.2.6.4 Detection of ammonia (NH ₃) production	44
4.2.6.5 Determination of 1-aminocyclopropane-1-carboxylate (ACC)	44
4.2.6.6 Catalase activity	44
4.2.6.7 Assay for protease production.....	45
4.2.6.8 Oxidase activity.....	45
4.2.7 Antifungal effect assay	45
4.2.8 Antibacterial effect assay	45
4.2.9 Isolation of genomic DNA.....	46
4.2.10 PCR Amplification targeting the 16S rRNA	47
4.2.11 Agarose gel electrophoresis procedure.....	47
4.2.12 Sequencing and phylogenetic analysis	48
4.2.13 Supporting data.....	49
4.3 Results	49
4.3.1 Physical and chemical characterization of samples	49
4.3.1.1 Cation Exchange Capacity (CEC), Nitrate and Nitrogen analysis of soil samples	49
4.3.1.2 pH and Redox tolerance of soil samples	53
4.3.2 Organic matter content of soil samples	54
4.3.3 Culturing and isolation of bacteria from soil samples.....	54
4.3.4 PGPR activities.....	56
4.3.5 Biocontrol activities	61
4.3.5.1 Antifungal activities	61

4.3.5.2 Antibacterial activities	61
4.3.6 Molecular identification of selected isolates	62
4.3.7 Phylogenetic analysis and diversity	63
4.4 Discussion	66
4.5 Conclusion.....	72
CHAPTER FIVE	73
THE INFLUENCE OF BAMBARA GROUNDNUT GROWTH STAGES AND LANDRACES ON SOIL BACTERIAL COMMUNITIES	73
Abstract	73
5.1 Introduction	74
5.2 Materials and Methods	76
5.2.1 Preparation of Bambara groundnut landraces for sample collection	76
5.2.2 Sample collection.....	76
5.2.3 Analysis of bacterial community in the soil samples /microbial activities	77
5.2.3.1 Soil Chemical Analysis	77
5.2.4 Soil Microbial Functional Diversity.....	77
5.2.4.1 Sample preparation	77
5.2.4.2 Inoculation of soil samples in BIOLOG GN2 Plates	77
5.2.4.3 Determination of functional diversity	78
5.2.4.4 Analysis of Carbon source utilization profiles (CSUP)	78
5.2.5 Statistical Analyses	79
5.3 Results	79
5.3.1 Soil chemical analysis	79
5.3.2 AWCD analysis	80
5.3.3 Diversity Indices	83
5.3.4 Dendrogram constructed to compare Microbial richness and evenness over time	85
5.3.5 Cluster analysis to compare Microbial functional diversity	88
5.4 Discussion	93
5.5 Conclusion.....	96
CHAPTER SIX	98
GC-MS ANALYSIS OF VOLATILE ORGANIC COMPOUNDS FROM BAMBARA GROUNDNUT RHIZOBACTERIA AND THEIR ANTIBACTERIAL PROPERTIES	98

Abstract	98
6.1 Introduction	98
6.2 Materials and Methods	100
6.2.1 Cultivation of the isolates	100
6.2.2 Extraction and partial purification of the crude extracts from the rhizobacteria isolates	100
6.2.3 Biological assay	101
6.2.4 Antagonism assay against phytopathogenic fungi and bacteria	101
6.2.5 Gas chromatography-mass spectrometry (GC-MS) analysis	102
6.2.6 Mass spectrometer	103
6.3 Results	103
6.3.1 Cultivation of Rhizobacteria isolates	103
6.3.2 Antibacterial assay of Volatile Organic Compounds (VOCs)	103
6.3.3 GC-MS analysis carried out on the rhizobacteria isolates	105
6.3.4 Analysis of GC-MS chromatograms for <i>B. amyloliquefaciens</i>	107
6.3.4.1 Benzene and Butanol extract	107
6.3.4.2 Chloroform and ethyl acetate extract	109
6.3.4.3 Hexane and methanol extract	109
6.3.5 Analysis of GC-MS chromatograms for <i>B. thuringiensis</i>	114
6.3.5.1 Benzene and butanol extract	114
6.3.5.2 Chloroform and ethyl acetate extract	114
6.3.5.3 Hexane and methanol extract	114
6.3.6 Analysis of GC-MS chromatograms for <i>Bacillus sp.</i>	115
6.3.6.1 Butanol and ethyl acetate extract.....	115
6.3.6.2 Hexane extract.....	116
6.3.6.3 Methanol and petroleum ether extract	116
6.3.7Antibacterial potential of bioactive compounds	116
6.4 Discussion	118
6.5 Conclusion.....	120
CHAPTER 7	121
16S NGS ANALYSIS OF BAMBARA GROUNDNUT RHIZOSPHERIC SOIL AT DIFFERENT GROWTH STAGES	121
Abstract.....	121

7.1 Introduction.....	122
7.2 Methods and Materials.....	123
7.2.1 Collection of soil samples from Bambara groundnut root rhizosphere	123
7.2.2 DNA extraction from soil samples	123
7.2.3 PCR amplification of Bambara groundnut soil 16S rRNA gene.....	125
7.2.4 Data processing Sequences	125
7.2.5 Sequence Analytical Pipeline	125
7.2.6 Statistical and Diversity Analysis.....	126
7.3 Results	126
7.3.1 Overview of Bambara groundnut bacterial community	126
7.3.2 Relationship of rhizosphere bacterial community	127
7.3.3 Effect of the growth stages of Bambara groundnut on the alpha diversity	130
7.3.4 Bacterial responses to plant growth.....	132
7.3.5 Effects of plant growth on the bacterial community beta diversity.....	133
7.4 Discussion	137
7.5 Conclusion	139
CHAPTER EIGHT	140
FIELD EXPERIMENT OF RHIZOBACTERIA WITH BIOFERTILIZER POTENTIAL ON THE GROWTH OF BAMBARA GROUNDNUT	140
Abstract	140
8.1 Introduction	141
8.2 Materials and Methods	142
8.2.1 Description of experimental site	142
8.2.2 Collection of soil for analysis.....	142
8.2.3 Experimental Design	143
8.2.4 Preparation of rhizobacteria from Bambara groundnut rhizosphere for seed inoculation	143
8.2.4.1 Preparation of inoculum	143
8.2.4.2 Bambara groundnut seed propagation.....	143
8.2.4.3 Determination of growth parameters	144
8.2.5 Isolation of genomic DNA.....	144
8.2.6 PCR Amplification targeting the 16S rRNA	145

8.2.7 Nucleotide sequence determination	145
8.2.8 Molecular taxonomy determined by sequences and Phylogenetic analysis.....	146
8.2.9 Nucleotide sequence accession numbers	146
8.2.10 Data analysis	146
8.3 Results	147
8.3.1 Effect of bacterial isolates on the number of stems	147
8.3.2 Effect of bacterial isolates on number of leaves	151
8.3.3 Effect of bacterial isolates on the length of stem	152
8.3.4 Effect of bacterial isolates on the length of leaves	155
8.3.5 Effect of bacterial isolates on the breadth of leaves	157
8.3.6 Effect of bacterial isolates on number of seeds	159
8.3.7 Effect of bacterial isolates on length of shoot	161
8.3.8 Effect of bacterial isolates on length of roots	163
8.3.9 Molecular identification of selected isolates	165
8.4 Discussion	166
8.5 Conclusion.....	168
CHAPTER NINE.....	169
GENERAL CONCLUSION.....	169
REFERENCES	172
APPENDIX.....	207
IAA produced by each bacteria isolate calculated from the standard curve ($y=0.3395x-0.5203$)	207
Carbon source utilization profile of Bambara soil samples at different growth stages corresponding to WAP	208
GC-MS chromatograms of <i>Bacillus</i> spp using different extracts.....	215

LIST OF ABBREVIATIONS

ACC	1-Aminocyclopropane-1-Carboxylate
AgNO₃	Silver nitrate
ANOVA	Analysis of Variance
CEC	Cation Exchangeable Capacity
CLPP	Community-Level Physiologic Properties
CSUP	Carbon Source Utilization Profile
DAP	Days After Planting
DAPG	2,4-diacetylphloroglucinol
Eh	Redox Potential
ESI-MS	Electrospray Ionization Mass Spectrometry
ISO	International Standard Organization
GC	Gas Chromatography
HCN	Hydrogen Cyanide Production
HPLC	High Performance Liquid Chromatography
IAA	Indole Acetic Acid
MALDI	Matrix-Assisted Laser Desorption Ionization
MS	Mass Spectrometry
NGS	Next Generation Sequencing
NUS	Neglected and underutilized Species
OTU	Operational Taxonomic Units
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PGPR	Plant Growth Promoting Rhizobacteria

PGP	Plant Growth Properties
PS	Phosphate Solubilization
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SIFT-MS	Selected Ion Flow Tube Mass Spectrometry
VOC	Volatile Organic Compounds
WAP	Weeks After Planting

LIST OF FIGURES

Figure 2.1: (a) Bambara groundnut seeds in different colours and sizes; (b) Uprooted bambara groundnut plant showing lateral roots and nodules attached to them.	9
Figure 2.2: Associations involved in bambara groundnut-bacterial interaction for food security	13
Figure 2.3: Bambara groundnut root showing nodules and network of lateral roots	17
Figure 2.4: Bambara groundnut and maize in mixed cropping	23
Figure 4.1: Plot showing Bambara groundnut 2 weeks after planting (a and b)	39
Figure 4.2: Comparison of different physical and chemical properties of soil samples between 4 and 20 weeks after planting (WAP).	50
Figure 4.3: Physical and chemical analysis of soil samples at 4 WAP.	51
Figure 4.4: Physical and chemical analysis of soil samples	52
Figure 4.5: (a) Average pH values of soil samples from original soil to the time harvest.	53
Figure 4.6: Carbon and organic matter content of soil samples from 0 WAP to harvest (20 WAP)	54
Figure 4.7a: Standard curve of ACC concentrations	57
Figure 4.7b: Absorbance of bacterial isolates at different concentration of ACC	57
Figure 4.8a: Spectrophotometric measurement of absorbance of IAA in Isolates in the presence or absence of tryptophan at optical density of 530nm	59
Figure 4.8b: Standard graph of IAA at optical density of 530nm	59
Figure 4.9: Antifungal activities of BAMji, BAMr, BAMli and BAMhi against <i>F. graminearum</i>	61
Figure 4.10 Antibacterial activities of isolates BAMui, BAMli, BAMoi, BAMyi, BAMhi, and BAMpii against <i>B. cereus</i> and <i>E. faecalis</i>.	62
Figure 4.11: Phylogenetic tree based on 16S rRNA sequences using neighbour-joining method for bacterial isolates and their closely related type strains.	65
Figure 5.1: The BIOLOG system uses a 96-well microliter plate with 95 different carbon sources.	79
Figure 5.2: Dendrogram illustrating the differences in microbial richness between landraces soil samples over time.	86
Figure 5.3: Dendrogram illustrating the differences in microbial evenness between landraces soil samples over time.	88
Figure 5.4: Dendrogram illustrating the differences in microbial functional diversity between different soil samples of landraces over time	89
Figure 5.5: (a) Box and whisker plot of carbon source utilisation profiles for microbial communities present in different landraces soil samples over time	90
Figure 5.5: (b) Box and whiskers plot constructed to determine the significant difference in the functional diversity of the bulk soil (control) and the various landraces at the different growth stages	92

Figure 6.1: Total number of metabolites detected by GC-MS in <i>B. thuringiensis</i> , BAMhi, BAMr using 7 different extraction solvents	106
Figure 6.3: Structures of compounds from hexane fraction for all isolates	111
Figure 6.4: (a) Structures of compounds from butanol fraction for all isolates.....	112
Figure 6.4: (b): Structures of compounds from chloroform fraction for all isolates	112
Figure 6.5: Structures of compounds from ethyl acetate fraction for all isolates	113
Figure 6.6: Structures of compounds from methanol fraction and petroleum ether for all isolates	113
Figure 7.1: Relative abundance of major bacterial phyla present in the bulk soil and rhizospheric soil of growth stages of Bambara groundnut as detected using the next generation sequencing (NGS).....	128
Figure 7.2: PCoA analysis of bulk soil (O1 and O4) and rhizosphere microbial community associated with Bambara groundnut growth stages from 4 WAP to 16 WAP, based on the Bray distance metric.	129
Figure 7.3: The diversity of rhizosphere microbial community of Bambara groundnut (F1, F4, J1, J4, D1, D4, N1, N4) and bulk soil (O1 and O4) indicated by OTU richness, sequencing depth, Shannon index, and Simpson index in rhizosphere across plant growth stages and bulk soil....	131
Figure 7.4: (a) Average number of OTU-species in Bambara groundnut rhizobiome at growth stages. Abundance of bacterial 16S rRNA genes in bulk and rhizosphere soils associated with bambara groundnut at different growth stages data are presented as mean ($P=0.05$).....	132
Figure 7.5: (a) Heatmap and hierarchical cluster analysis of bacteria based on the relative abundances of dominant genera from the different growth stages of bambara groundnut rhizospheric soil.....	134
Figure 7.5: (b) Heatmap and hierarchical cluster analysis of bacteria based on the relative abundances of dominant genera from the different growth stages of bambara groundnut rhizospheric soil.....	135
Figure 7.5: (c) Heatmap and hierarchical cluster analysis of bacteria based on the unweighted data beta diversity absent or presence of dominant genera from the different growth stages of bambara groundnut rhizospheric soil.....	136

**NWU
LIBRARY**

LIST OF TABLES

Table 2.1: Table showing variation in nitrogen fixed by Bambara groundnut due to cropping system, soil type and location	22
Table 4.1: Total number of different isolates by morphology at different growth stages	55
Table 4.2: Plant growth-promoting activities of rhizobacteria isolates from the above-mentioned soil samples	58
Table 4.3: Biochemical activities of bacterial isolates from Bambara groundnut rhizosphere	60
Table 4.4: Results of 16S rDNA gene sequence similarities of rhizobacteria isolates and GenBank accession numbers using BLASTn algorithm isolate code.....	63
Table 5.1: Main groups of carbon sources utilized by soil microbial communities in the various soil samples of landraces over time.	82
Table 5.2: The Shannon-Weaver diversity index and the Evenness Index between the bambara groundnut landraces and specific sampling times.....	84
Table 6.1: Results of 16S rDNA gene sequence similarities of bacterial isolates and GenBank accession numbers using BLASTn algorithm.....	103
Table 6.3: VOCs profile of <i>B. amyloliquefaciens</i> , <i>B. thuringiensis</i> , <i>Bacillus sp.</i> detected by GC-MS.....	108
Table 8.2: ANOVA for comparison of treatments and their effect on the number of stems	150
Table 8.3: ANOVA for comparison of treatments and their effect on the number of leaves	152
Table 8.4: ANOVA for comparison of treatments and their effect on the length of stem	154
Table 8.5: ANOVA for comparison of treatments and their effect on the length of leaves	156
Table 8.6: ANOVA for comparison of treatments and their effect on the breadth of leaves.....	158
Table 8.7: ANOVA for comparison of treatments and their effect on the number of seeds	160
Table 8.8: ANOVA for comparison of treatments and their effect on the length of shoots	162
Table 8.9: ANOVA for comparison of treatments and their effect on the length of roots	164
Table 8.10: Results of 16S rDNA gene sequence similarities of bacterial isolates and GenBank accession numbers using BLASTn algorithm.....	165

LIST OF PUBLICATIONS

Bambara groundnut-bacteria interaction; source of food security (Submitted to Canadian Journal of Soil Science-under review)

Metabolic diversities of rhizospheric bacteria from legumes for biotechnological processes (Formatted for Frontiers of Environmental Science and engineering)

Evaluation of PGPR and biocontrol activities of bacteria isolated from Bambara groundnut rhizosphere (Abstract already published in New Biotechnology, July 2016)

Effect of growth stages on community dynamics of Bambara groundnut (*Vigna Subterranea*) rhizospheric bacteria (Formatted for European Journal of Soil Biology)

GC-MS analysis of volatile organic compounds from Bambara groundnut rhizobacteria and their antibacterial properties (Formatted for Current Research in Bacteriology)

16S NGS analysis of Bambara groundnut rhizospheric soil at different growth stages (Formatted for Soil Biology and Biochemistry)

Molecular characterization of rhizobacteria with biofertilizer potential on the growth of Bambara groundnut (Formatted for European Journal of Soil Biology)

CHAPTER ONE

1.1 General Introduction

In Africa, bambara groundnut (*Vigna subterranean* (L.) Verdc) is the third most commonly eaten legume after groundnut (*Arachis hypogaea*) and cowpea (*Vigna unguiculata*) (Omoikhoje, 2008). Bambara groundnut is a legume and an important food crop with the ability of forming symbionts and fixing nitrogen in the soil (Nyemba and Dakora, 2010). It can be eaten boiled or milled into flour before cooking. Its leaves are fed as fodder to animals (Brink et al., 2006). It is resistant to drought and produces a relatively high yield in sandy soils especially soils where groundnut cannot thrive and soil with low fertility receiving little rainfall. Its tap root goes deeply into the soil bearing many nodules associated with nitrogen fixing bacteria. It is nutritionally comparable to other legumes, such as soybean, having the essential amino acids of lysine, methionine and cysteine (Bamishaiye et al., 2011). Most of the literature published on bambara groundnut is country specific on its nutritional value or studies based on its germplasm (Azam-Ali and Squire, 2002). Not much research has been carried out on the bacterial community from the rhizosphere of bambara groundnut.

Rhizospheric bacteria represent an important group of soil organisms interacting with plants; some influence plant nutrition through a symbiotic relationship, where the bacteria fix atmospheric nitrogen into a suitable form used by the plant as nutrients (Appuhn and Joergensen, 2006). The bacteria mediated activities in the rhizosphere include properties perceived by humans as beneficial, such as the key role they play in the biogeochemical cycles of the main elements, carbon, nitrogen and sulphur; and of the trace elements iron, nickel and mercury and are therefore, heavily implicated in energy and exchanges within the soil. Bacteria also synthesise vitamins, auxins and growth factors. Bacterial communities in the rhizosphere are influenced by the soil

characteristics, type of plant species they associate with and plant growth development stages (Babalola, 2007).

The rhizosphere of bambara groundnut harbour many genetically diverse bacteria. Some of these bacteria are known to be symbionts and nitrogen fixers. These nitrogen-fixers which are members of the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* and or members of other non-symbiotic bacteria. They form nodules on either the roots or stems of the plants. Therefore, understanding the ecology of rhizobacteria is both of scientific and economic importance.

Despite the importance of bambara groundnut as a source of food security, research attention towards this crop has been quite limited. Most of the information and knowledge about the crop is held by the producers themselves, who in most cases are subsistence farmers or captured in unpublished materials and a few published materials.

1.2 Problem Identification

The interaction between bacteria, plant and rhizosphere is complex and a detailed understanding of their interaction is limited. This is due to limited studies on methods that facilitate rhizosphere bacterial community density and composition. Since bambara groundnut is one of the neglected and underutilized species, not much work has been done on the different and diverse bacterial communities in the rhizosphere. This research builds a body of knowledge on these bacterial communities; reports on possible novel microorganisms present and their metabolites that may be harnessed for biotechnological processes.

1.3 Research Questions

- Is there any similarity or difference between rhizospheric soil from bambara groundnut and those on which bambara groundnut was not planted (bulk soil)?
- What is the bacterial and metabolite structure of bambara groundnuts' rhizosphere?

- Are the bacterial communities in the rhizosphere at different growth stages of bambara groundnut similar?
- Are bacteria isolated from bambara groundnut rhizosphere capable of producing novel metabolites?
- How effective are the metabolites in antagonizing pathogens?

1.4 Research Aims and Objectives

The aim of this research was to carry out molecular analysis of the diverse bacterial community in the rhizosphere of bambara groundnut; and to screen these bacteria for novel metabolites that could be useful for biocontrol, plant growth promotion and other antimicrobial interventions (agriculture, pharmaceutical, environmental and climate related interventions)

The objectives were to

1. Assay for PGPR activities of bacteria isolated from the bambara groundnut rhizosphere;
2. Carry out diversity study of bambara rhizosphere community structure using 16S rRNA next generation sequencing (NGS);
3. Carry out metabolic study of the relationship of bacterial communities using BIOLOG for carbon source utilization profile (CSUP) analysis;
4. Screen and identify novel secondary metabolites from bambara groundnut rhizobacteria and assay for their antagonistic properties; and
5. Field-test identified bacterial isolates for plant-growth promoting potentials.

CHAPTER TWO

BAMBARA GROUNDNUT-BACTERIA INTERACTION; SOURCE OF FOOD

SECURITY

Abstract



With the rise in world population and decrease in food supply due to global climate change, food security becomes very pertinent. Malnutrition, food scarcity and poverty have consistently affected population growth. This issue has driven scientists to seek out other plants that have been under-studied but have potential for food security. African soils now contain essential nutrients in very low quantities leading to low fertility. This makes it unable to support plant growth as efficiently as it used to due to continuous land use without a proper soil management programme. The use of underutilized and neglected food crops has been observed to be the way out of over-use and dependence on staple foods. This review aims to determine the effect of using soil environment of underutilized leguminous crops to be able to accomplish maximum yields to improve crop yield and invariably food security. The problem of low yield from continuous farming has led to more cultivation of land and less use of mineral fertilizers due to the inability to afford such fertilizers cum its hazardous effect on soil and crop. Planting of legumes that are able to increase the nitrogen content of soils by nodulation with rhizobacteria is a non-chemical solution. The use of rhizobacteria is very important to improving crop yield and most especially rhizobacteria from legumes like bambara groundnut. Bambara groundnut and its interaction with various rhizobacteria in the soil could play a vital role in biocontrol and biofertilization. This, in turn, will help to increase crop yield by resisting pests and disease and improving plant growth and productivity.

Keywords: Bambara, biofertilizer, food security, rhizobacteria, rhizobium

2.1 Introduction

Diversification in sourcing for food is very important to the increasing world population (Massawe et al., 2016). Such diversification should increase accessibility to and availability of food to this growing population. Plant-bacterial interaction is a source of food security that has not been fully explored. Rhizospheric bacteria represent an important group of soil organisms interacting with plants; some influence the plant nutrition through a symbiotic relationship, where the bacteria fix atmospheric nitrogen into a form used by the plant as nutrients (Appuhn and Joergensen, 2006). The bacterial mediated activities in the rhizosphere include those beneficial to humans, such as the key role they play in the biogeochemical cycles of the main elements (carbon, nitrogen and sulphur) and of trace elements (iron, nickel and mercury). These activities increases the involvement of bacteria in energy exchanges within the soil (Haichar et al., 2014). Bacteria also synthesise vitamins, auxins and other growth factors (Babalola and Akindolire, 2011; Leeuwenhoek et al., 2012). Bacterial communities in the rhizosphere are influenced by the soil characteristics, type of plant species they associate with and plant growth developmental stages (Babalola, 2007). Some of these bacteria are known to be symbionts and nitrogen fixers (Andrews and Andrews, 2017). These nitrogen-fixing bacteria belong to different bacterial lineages (*Rhizobium*, *Bradyrhizobium* and *Azorhizobium*) that are related to other non-symbiotic bacteria. They form nodules in the roots or stems of leguminous plants such that they form a relationship with cowpea, pigeon pea and Bambara groundnut.

Bambara groundnut (*Vigna subterranean* L. Verdc) is a food security crop (Massawe et al., 2016). It is available, accessible and affordable and it is a source of security for farmers in Africa due to its ability to tolerate drought and fix atmospheric nitrogen (Mkandawire, 2007). It has also been

found to cross-nodulate with isolates from other leguminous plants like cowpea and can also resist pests and diseases caused by some pathogens (Laurette et al., 2015).

In Africa, it is the third most commonly eaten legume after groundnut (*Arachis hypogaea*) and cowpea (*Vigna unguiculata*) (Omoikhoje, 2008). In South Africa, it is known as *ditloo* in Sepedi and *phonda voandzou* or *nzama* by Tshivenda. It is a complete food having different composition of carbohydrate, protein and fat enough to serve as a balanced diet. It is nutritionally comparable to other legumes, such as soybean, in the essential amino acids of lysine, methionine and cysteine (Bamishaiye et al., 2011). Compared to pigeon pea, lentils and cowpea, Bambara groundnut seed has a higher gross energy value (Hillocks et al., 2012). The protein of Bambara groundnut seed is about 16-25%; and competes favourably with other legumes such as groundnut, cowpea and pigeon pea and it is found to be superior to the protein of other legumes (Masindeni, 2006). It can be eaten boiled or milled into flour before cooking. In Senegal, different concoctions made from the leaf, roots and leaf sap have been used to treat infected wounds and abscesses, as an aphrodisiac and to treat epilepsy respectively (Brink et al., 2006). The plant has been used by the Ibo tribe in Nigeria to treat venereal disease while the seeds that were pounded and mixed with water were used to treat cataracts. Its leaves were used as fodder to feed animals (Brink et al., 2006).

Not much work has been done on the bacterial community from the rhizosphere of Bambara groundnut and their interactions with the plant itself. This review examines the symbiotic and non-symbiotic relationship of Bambara groundnut with bacteria, especially in relation to food security in Africa.

2.2 History of Bambara groundnut

The origin of Bambara groundnut can be traced to Africa (Opoku, 2010). Its history dates back to ancient Mali near Timbuctoo from where the English name Bambara was derived from the

Bambara tribe even though they do not lay claim to the plant (Masindeni, 2006). Its centre of origin can be traced to North Central and North Eastern Nigeria all the way to Northern Cameroon and the Central African Republic (Olukolu et al., 2012). Beyond Africa, it is seen to grow in other tropical nations like Greece, Middle East, Malaysia, Indonesia and, most especially, Brazil and Tropical America, where it is supposed that slaves must have helped to transport it to these nations (Brink et al., 2006).

It is an indigenous African crop that is common to many African countries from Sudan in the North to South Africa in the South; from Kenya in the East to Nigeria in the West and even to Madagascar (Bamishaiye et al., 2011). It is one of the many underutilized and under-researched indigenous grain legumes. The fact that it is being underutilized can be seen in the group of people involved in its planting. Female subsistence farmers in Sub-Saharan Africa are the major growers of Bambara groundnut (Mkandawire, 2007).

2.3 World Bambara production

The annual production of Bambara groundnut was estimated at 330, 000t in 1982 with about 50% coming from West Africa (William et al., 2016). Currently, worldwide production is quite low compared to its high demand (Bamishaiye et al., 2011). In most of the semi-arid lands, yields from the farm pods vary between 650 and 850 kg ha⁻¹ (Sangare, 2012). Since the crop is being produced at the subsistence level and not so much on a large scale, worldwide production figures have been difficult to collate but Zambia is still the most extensive producer (Opoku, 2010) while Nigeria, Burkina Faso, Niger, Mali, Ghana, Cote D'Ivoire and Chad are the major producers with only Burkina Faso, Chad, Mali, Niger and Senegal as the main exporting countries (Brink *et al.*, 2006).

2.4 Agronomy and morphology

According to a report by Bamishaiye et al. (2011), Bambara groundnut (*Vigna subterranea*) is a member of the family Fabaceae. It is a small plant like groundnut which grows to a height of between 0.30-0.35 m. It is an intermediate plant with branched stems forming a bunch just above the ground. The plant grows as a small herb with compound leaves of three leaflets which are trifoliate and alternate from erect petioles. The peduncle of the leaves bear the one or two flowers and are auxiliary branching from the stems. The stem begins to branch out very early after planting (Sangare, 2012). After fertilization, the flowers of Bambara groundnut are pale yellow in colour and they hang on the branching stems; these stems then grow downwards into the soil, taking the developing seed with it. The seeds form pods encasing seeds just below the ground in a similar fashion to peanut. Bambara groundnut pods are of different shapes, sizes and colours such as round, wrinkled, smooth; over 1.27 cm long; and white, cream, dark-brown, red or black and may be speckled or patterned with combination of these colours respectively (Fig. 2.1). The roots with numerous nitrogen-fixing nodules grow from the short internodes of the stem to form a thick taproot with lateral roots developing as outgrowths towards the tip (Tweneboah, 2000). It is referred to as an autogamous plant (Baudoin and Mergeai, 2001). The structure of this plant shows that it helps to conserve space and more seeds can be planted on a small expanse of land without fear of low harvest. Its autogamy helps it to be available all through the year since it can be cultivated without the need for external pollination before flowering and seeding. Its availability throughout the year represents one of the core values of food security (Onwubiko et al., 2011b).



Figure 2.1: (a) Bambara groundnut seeds in different colours and sizes; (b) Uprooted bambara groundnut plant showing lateral roots and nodules attached to them.

2.5 Importance of bambara groundnut cultivation worldwide

Bambara groundnut is cultivated for various reasons. It is known to have both agronomic and nutritional advantages

2.5.1 Agronomic advantages

Bambara groundnut is quite important in Agriculture and an extremely drought-tolerant crop (Masindeni, 2006). It can perform well and have good crop yield on marginal soils and soils that have undergone water stress compared to other legumes (Brink et al., 2006). It grows well even in poor and infertile soils (Opoku, 2010). Also, it can grow on soils of both high and low nitrogen content with high and low temperature conditions (Baudoin and Mergeai, 2001). Because it tolerates poor soil, farmers with poor resource especially with respect to purchasing fertilizer to increase yield, are encouraged to farm more with Bambara groundnut. Report shows that the ability of bambara groundnut to tolerate poor soil is advantageous against being planted in nitrogen-rich

soil. This is because nitrogen-rich soils increase vegetative growth of bambara groundnut as against crop pod and seed productivity (Baudoin and Mergeai, 2001). As a legume, root nodules bacteria form symbiotic association with bambara groundnut roots. This helps to increase the nitrogen content of the soil in the sense that the bacteria assimilate atmospheric nitrogen, trap it and make it available to the plant in the soil. This process in turn helps to increase soil fertility that leads to increased crop yield (Masindeni, 2006). It aids crop protection against diseases and pests (Ajayi and Lale, 2001).

2.5.2 Nutritional advantages

Bambara groundnut is a complete food having different composition of carbohydrate, protein and fat enough to serve as a balanced diet (Mahala and Mohammed, 2010; Ijarotimi and Esho, 2009). Fermentation was also found to improve its nutritive and mineral components (Murevanhema and Jideani, 2013). Compared to pigeon pea, lentils and cowpea, bambara groundnut's seed has a higher gross energy value (Bamishaiye et al., 2011). The protein content is found to be of a higher quality (16-25%) compared to other legumes (Brough and Azam-Ali, 1992). Its carbohydrate and fat composition are 65% and 6.5% respectively (Mazahib et al., 2013). Its fat content is higher than that of cowpea ($1.0 \pm 1.6\%$) and pigeon pea ($1.2 \pm 1.5\%$) but lower than that of groundnut (peanut) ($45.3 \pm 47.7\%$) with an estimate of between 5% (Sangare, 2012) and 6.3% (Omoikhoje, 2008). The composition of its protein is superior in essential amino acids and includes phenylalanine, lysine, valine, methionine, leucine, threonine and isoleucine. Its fatty acid composition is also high in palmitic, linolenic and linoleic acids (Minka and Bruneteau, 2000). The protein in bambara groundnut is rich in lysine and methionine comprising 6.6% and 1.3% of the total protein respectively. It is also a rich source of iron, potassium, calcium and fibre (Omoikhoje, 2008; Hillocks et al., 2012).

2.5.2.1 Consumption

Bambara groundnut is eaten when not matured by boiling it with salt and pepper. In many West African countries, it is consumed as a snack. It can also be made into flour when it is dry and matured because the seeds are hard (Tweneboah, 2000). The flour is used to prepare soup in East Africa with or without condiments while the flour has also been used to make bread in Zambia (Opoku, 2010). The seeds can also be roasted after which they are boiled, crushed and eaten as snack. Furthermore, the ground seeds can be used to make “akara” and “moinmoin” or the popular “okpa” in Nigeria (Sangare, 2012).

GIHOC cannery in Nsawam, Ghana, was involved in canning bambara groundnut seeds in gravy. Over 40,000 cans of various sizes were prepared and made available throughout the year (Baudoin and Mergeai, 2001) and was comparable to Heinz baked beans even though its production declined due to competition with high yielding groundnut varieties and pest resistant cowpea (Doku, 1996; Opoku, 2010).

Vegetable milk extracted from bambara groundnut has been found to compete favourably with vegetable milk from soyabean, cowpea and pigeon pea (Brough et al., 1993; Murevanhema and Jideani, 2013). The milk when properly processed has also been used as a weaning complementary food for children (Bamishaiye et al., 2011). The seeds were used as feed for poultry and piggery while its haulm and leaves which are rich in phosphorus and protein are used as fodder for cattle (Brink et al., 2006). Bambara groundnut mixed with other leaf proteins has been used as an aquaculture feed with distinct growth in the fish (Adeparusi and Agbede, 2005).

2.5.2.2 Medicinal

Different preparations from bambara groundnut have been shown to have medicinal properties (Murevanhema and Jideani, 2013). Preparations from the leaves have been applied to infected

wounds and abscess; extracts from the leaves have been applied to the eyes to cure epilepsy (Mkandawire, 2007) while leaf extracts pounded with that of *Lanfana trifolia* L. have been used as an insecticide to wash livestock (Mkandawire, 2007). Venereal disease is treated by the Igbo tribe in Nigeria using the plant (Brink et al., 2006). Grounded bambara seeds when mixed with water have been used to treat cataracts in Senegal and the root has been used as an aphrodisiac (Brink et al., 2006). Water from boiled maize and bambara groundnut when drunk is used by the Luo tribe of Kenya to treat diarrhoea while in Botswana, the black seeded landraces have been used to treat impotency (Mkandawire, 2007). In South Africa, nausea was controlled in pregnant women who chewed and swallowed the seed (Bamishaiye et al., 2011). In Ghana, pounded bambara seeds have been used to treat skin rashes and the powder mixed with the meat of guinea fowl have been used as a treatment against diarrhoea in children (Akpalu et al., 2013).

2.6 Bambara groundnut-bacterial interactions

Plants and microorganisms interact in diverse ways such that nutrients from plants are used by microorganisms who in turn make available the bioactive substances for the growth and development of plants (Dakora, 2003) (Fig. 2.2).

The rhizosphere is one of the most complex environments with thousands of interactions that play crucial roles in plant's health. Plants secrete up to 40% of photosynthates that have access to roots in the rhizosphere (Berendsen et al., 2012). Because most of the soils are carbon deficient, these hot spots of carbon increase the microbial densities from 10 to 1000 times, compared to bulk soil (Smalla et al., 2006). The elevated concentration of microorganisms in this particular region is due to an exchange of nutrients between the plant and the different communities surrounding the root, which allows different types of associations. A number of factors have been shown to influence the quantity and quality of root exudates including; plant species (Dennis et al., 2010), soil type

(Bulgarelli et al., 2012; Berg and Smalla, 2009), developmental stage (Houlden et al., 2008), and nutritional status (Carvalhais et al., 2011). If specific elements associated with the release of such exudates are better understood, novel approaches to enhance beneficial microbial communities could be proposed.

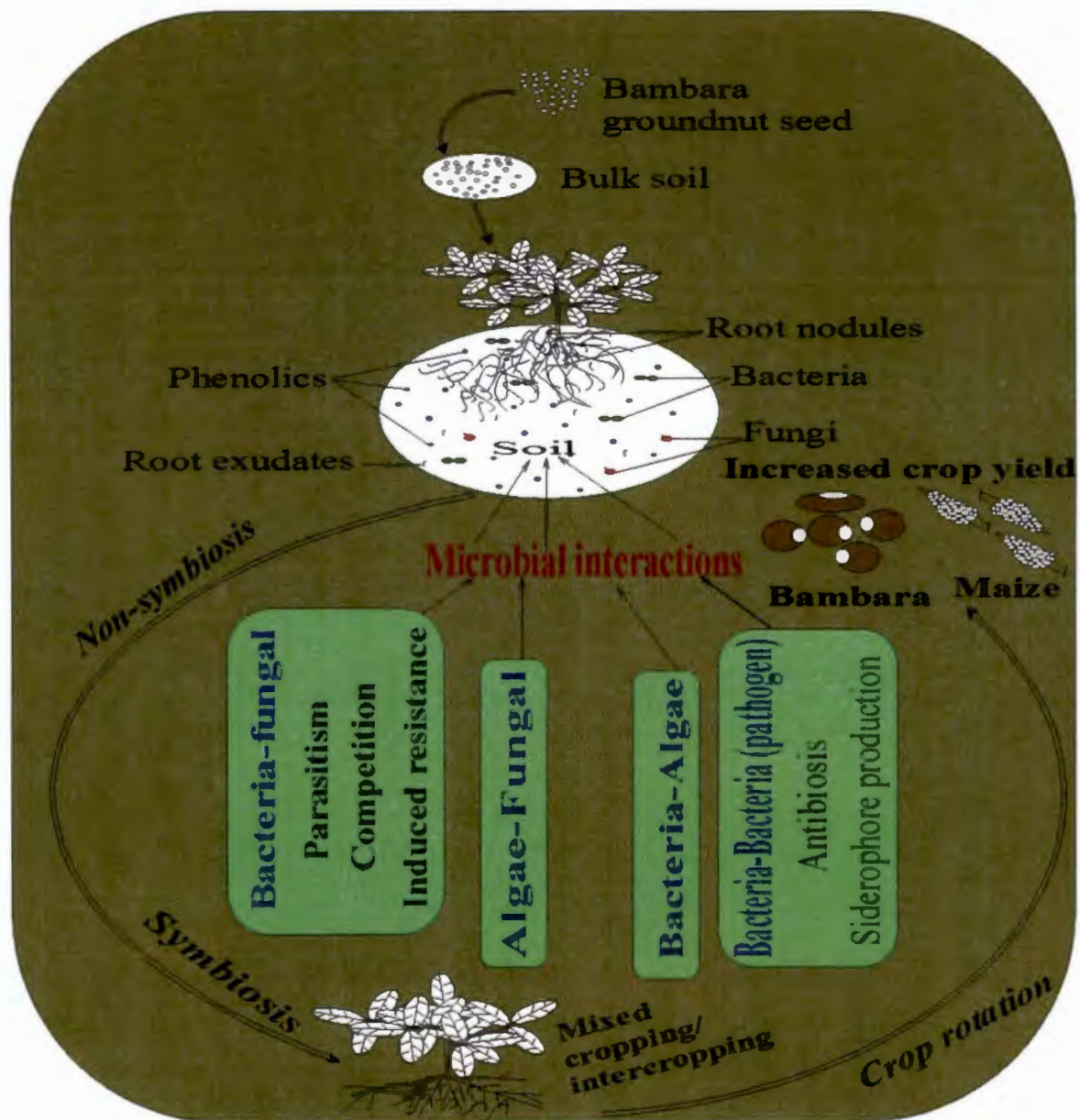


Figure 2.2: Associations involved in bambara groundnut-bacterial interaction for food security

Plant roots release exudates containing phenolics, sugars, organic acids, and amino acids that may attract microbes. In exchange, they protect the plant against pathogens releasing antimicrobial compounds; or increase nutrient uptake (Baetz and Martinoia, 2014). On the other hand, these carbon-containing compounds can also attract pathogens. They can compete for nutrients, infect the plant, and affect the rhizosphere microbial community.

Recent studies have revealed that plants are able to shape their rhizosphere microbiome (Badri and Vivanco, 2009; Lundberg et al., 2012; Berendsen et al., 2012). Some plant species have been demonstrated to host specific communities and attract protective microorganisms to suppress pathogens in the rhizosphere (Mendes et al., 2011). Soil physical, chemical, and biological properties will also play an important role in the establishment of such plant-microbe interactions (Berendsen et al., 2012). Although pathogens can severely affect plant health, certain beneficial bacteria and fungi that also thrive in the rhizosphere, or inside plant tissues, also known as endophytes, can compete with these pathogens for space and nutrients; therefore exerting an antagonistic effect on them (Nihorimbere et al., 2011; Raaijmakers et al., 2009). Root-associated beneficial soil bacteria are generally known as plant growth-promoting rhizobacteria (PGPR).

They grow in, on, or around root plant tissue and enhance plant growth, increase yield, protect plant against pathogens, and/or reduce abiotic or biotic stress (Vessey, 2003). Growth promotion can be achieved directly by the interaction between the microbe and the host, as well as indirectly, due to antagonistic activities against plant pathogens. Various interacting microbes produce phytohormones, which have been shown to inhibit or promote root growth, protect plants against biotic or abiotic stress, and improve nutrient acquisition by roots (Berg, 2009). PGPR represent an environmentally sustainable alternative to increase crop production and plant health as they have the potential to at least partially replace chemical fertilizers and pesticides.

An interesting example of the role of microbial communities in plant nutrition and health is the interaction between rhizospheric fluorescent *Pseudomonas* and plants. Plants reduce soil iron (Fe) availability by acquiring iron and releasing exudates which attract the rhizospheric microbes that also utilize Fe. In Fe-stressed environments, siderophore-producing bacterial populations are enriched, which then suppress pathogens such as fungi e.g.oomycetes through competition for Fe. The plants, however, are able to utilize siderophores-bound iron, which enhances their growth (Lemanceau et al., 2013).

Another instance applied to plant disease suppression is the ability of resident microbiota in suppressive soils or compost to prevent pathogen infection (Hadar and Papadopoulou, 2012). In a soil suppressive to the fungal pathogen *Rhizoctonia solani*, Proteobacteria, Firmicutes and Actinobacteria were prominent taxa found to be involved in disease suppression (Yin et al., 2013). There is also evidence to suggest that plants may use microbial communities to their own benefit to avoid infections (Mendes et al., 2011). The presence of potentially toxic compounds, low availability of essential minerals and pathogens in the soil often restrict crop production (Rincon-Florez et al., 2013).

2.6.1 Symbiotic interaction

Rhizobia species (*Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium*, *Sinorhizobium* and *Mesorhizobium*) have been known to suppress growth of plant pathogens and also form nodules in symbiotic relationship with legumes (Dakora, 2003). The symbiotic relationship also results in the production of nitrogen rich soil. bambara groundnut was found to form nodules (Fig. 2.3.) and fix nitrogen in partnership with *Bradyrhizobium* strain (Laurette et al., 2015). Nodule formation is important in bambara-microbe interaction; this process starts with production of compounds such as betaines, flavonoids and aldonic acid in the root exudates of the plants. These compounds then

have to signal to the rhizobia in a compatible relationship with the compounds. This in turn enhances the production of the nod gene that induces nodulation by interacting with the nodD protein of the cell wall of the rhizobia (Phillips, 2000). The rhizobia react to this inducement by producing and releasing the lipo-chito-oligosaccharide Nod factors, which bring about morphological changes in the root hair of the legume. This leads to the formation of an infection thread and development of nodules that finally enhances fixation of nitrogen (Dakora, 2003). Some of the molecules found in root exudates/flavonoids of bambara groundnut include genistein, coumestrol and daidzein (Dakora, 2000). Nod factors produced by rhizobia is important in plant growth as it promotes germination of seeds and development of seedlings (Kidaj et al., 2012). Zhang and Smith (2001) and Smith et al. (2002) reported that in culture media most of the rhizobial strains produced indole acetic acid (IAA), organic acid and siderophore which help them to obtain nutrients from the environment.



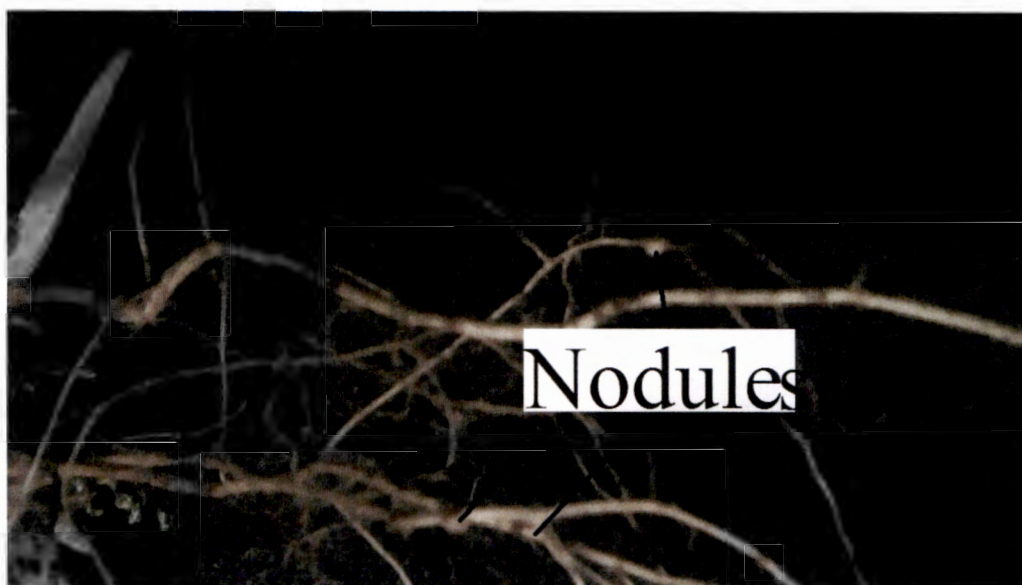


Figure 2.3: Bambara groundnut root showing nodules and network of lateral roots

2.6.2 Non symbiotic interaction

Apart from the symbiotic relationship between plants and rhizobia, the production of phytohormones by these rhizobia such as nod factors, riboflavin, and lipo-chito-oligosaccharide can also stimulate plant growth and increase grain yield (Dakora, 2003). Also legumes generally produce phenolics that help to suppress activities of pathogens, make nutrients available to plants and promote growth of microorganisms with beneficial properties (Dakora, 2003).

Production of phenolic compounds is stimulated by exudation from plant roots. This helps the production of nod-gene inducers whose excess concentration leads to excess production of nod factors around the root (Smith et al., 2002). When this is accumulated in the rhizosphere, it leads to biosynthesis of flavonoids which also lead to increased level of phytoalexin that is important for plant protection against pathogens (Dakora et al., 2015; Jeandet et al., 2013)

Rhizobia are also known to produce riboflavin (De Bruijn, 2015). It is a vitamin that is converted photochemically or by actions of enzymes to lumichrome. This was evidenced in culture preparation from rhizobial cells. In its purified state, it was able to stimulate growth in maize, soybean and sorghum (Dakora et al., 2002). Rhizobia are important in suppressing growth of pathogens of bacterial and fungal origins that infested sunflower, soybean, mungbean and okra (Gopalakrishnan et al., 2015). Legumes constitute very important crop in agricultural and ecological practices. They are known to produce phenolics that are also important in suppressing soil pathogens, promoting growth of plants and other mutualistic organisms (Dakora, 2003). Root exudates such as phytosiderophores and organic acid anions are important in making sure that minerals are available and circulate within the soil and agricultural systems (Dakora, 2003) that is the reason why they are important in mixed cropping. Studies have shown that there was continuous increase in yields and quality of seeds when bambara groundnut was inoculated with local strains of Bradyrhizobia, this is as a result of the increase in the symbiotic nitrogen fixation activity (Laurette et al., 2015).

2.6.3 Nitrogen fixation and food security

Nitrogen is one of the most important minerals found in the atmosphere and it occurs naturally. It is also found in the cells of plants in form of amino acid which is the building block of proteins (Egbe et al., 2013). Most of the African soils are deficient in nitrogen; and availability of nitrogen in the soil is a determining factor for increased production; availability of crops and invariably food security (Dakora and Keya, 1997). Increased use of nitrogen fertilizer has been correlated to an increase in crop yield, but in Africa compared to the other world regions, the use of nitrogen fertilizer is the lowest because of its cost among other reasons (FAO, 1990). Alternative to the purchase of chemical nitrogen fertilizer, is the use of atmospheric nitrogen fixed by some bacteria

in the nodules of leguminous plants (Asei, 2015). This nitrogen fixation helps to reduce the competition for nitrogen in the soil and contributes about half the global amount of chemical nitrogen needed for agriculture (Smil, 2005). This form of nitrogen is the cheapest, most readily available and an effective form of nitrogen in sustainable agriculture.

Nitrogen fixation by bambara groundnut has both direct and indirect beneficial effects in agriculture. Indirectly as a legume, it has a symbiotic relationship with bacteria that form root nodules (Gueye, 1992). These bacteria make use of the free nitrogen from the air and store it in the plant root tissue. This in turn helps to increase the level of the nitrogen in the soil directly and as well increase the yields of the cereal or any other crop that may be planted (Masindeni, 2006). A report by Gueye (1992) revealed that, provided that all plants' nutrient requirements other than nitrogen are met, bambara groundnut fixes nitrogen at a very high level when inoculated with *Rhizobium* strains.

According to FAO (2008), between 1990 and 2007, the number of people who experienced chronic hunger as a result of high cost of food due to low food production increased exponentially. In part, this hunger could also be caused by political instability in countries which have led to wars and invariably no man power to cultivate the land for food production (FAO, 2008).

The World Food Summit of 1996 defined food security as existing "when all people at all times have access to sufficient, safe, nutritious food to maintain a healthy and active life" (FAO 1996). In lay-man's language, it has been defined as the ability of individual people to be able to access food that is sufficient and useful for daily activities on a consistent basis. Food security at household level is food availability in one's home in such a situation where there is no form of hunger or fear of starvation.

Food security is based on the three pillars of, availability of food consistently, easy access to food and being able to utilise the food in such a way that nutritional needs of people are met. Food security is linked to every sector of human life such as health through malnutrition; economic development and trade through agriculture. It is also linked to the environment through the effect of global climate change on food produced and its effect on diet-related diseases and environmental pollution (Mentan, 2014). Food security is a complex, multifaceted world issue faced with multilinked challenges (Kusch et al., 2016). Many neglected and underutilized crops have been researched and shown to have potential to solve the challenge of food security (Mayes et al., 2012). This is because many of them are able to resist adverse environmental and climatic changes that the normal staple foods cannot withstand and they have been found to be able to compete favourably with such foods. One of such underutilised and neglected crop is bambara groundnut.

Bambara groundnut as a food security crop is available, accessible and affordable and it is a source of security for farmers in Africa due to its ability to tolerate drought and fix atmospheric nitrogen (Opoku, 2010; Egbe et al., 2013). It can grow and nodulate in poor soils and resist pests and diseases. Because of its nitrogen fixation properties, it can be used to enrich the soil where other cereals like maize, sorghum, millet and wheat can be grown as an intercrop, mixed crop or in a crop rotation (Opoku, 2010).

2.7 Cultural cultivation of Bambara groundnut and food security

Bambara groundnut fits into different cultural cropping systems of rural farmers such that the farming systems practised among subsistence farmers help the cultivation of Bambara groundnut to thrive with little or no yield loss (Mshelia et al., 2004). The space needed by Bambara groundnut in order to grow is small and so enables it to accommodate the planting of other crops. It grows in

poor soils and known as the legume that thrives where other legumes fail (Tweneboah, 2000) (Table 2.1).

2.7.1 Mixed cropping

Bambara groundnut has been mixed with peanuts before broadcasting for planting in Southern Ghana while in Northern Ghana, it is grown as a mixed crop with maize (Fig. 2.4), millet and sorghum (Doku, 1996; Opoku, 2010). Also, it has been mixed with yam in this case, after yam has been planted in yam mound, the bambara seeds were planted on the mound instead of using straw or grass to cover the surface of the mound. This helped to conserve moisture in the mound, prevent erosion and also maintain temperature. Crop yield was not assessed and so production yield on yam was not determined (Bamishaiye et al., 2011).

Table 2.1: Table showing variation in nitrogen fixed by Bambara groundnut due to cropping system, soil type and location

S/n	Soil type	Cropping system	Location	Nitrogen fixed-final kg/ha	References
1	Sandy loam	Sole cropping	Makurdi Nigeria	12.10	(Egbe et al., 2013)
2	Sandy loam	Intercropping with cowpea	Makurdi Nigeria	10.20	(Egbe et al., 2013)
3	Sandy loam	Intercropping with maize	Makurdi Nigeria	10.16	(Egbe et al., 2013)
4	Sandy loam	Sole cropping with biofertilization	Maiduguri Nigeria	23.59, 58.39	(Yakubu et al., 2010)
5	NK†	Sole cropping	Ghana	40-56	(Dakora, 1985)
6	Sandy clay loam	Crop rotation	Malaysia	83.2, 31.9, 49.5	(Musa et al., 2016)
7	Sandy arenosol soil	Intercropped with peanut	Israel	1170mg/plant	(El-Mohamedy, 2009)

† NK is not known from the article

2.7.2 Intercropping

Performance of bambara groundnut when intercropped with other crops depends on its planting density and also the density of the other crop. If the planting density of maize is low when intercropped with bambara groundnut, then bambara groundnut performs better (Ngugi, 1997). Bambara groundnut has been intercropped in many traditional cropping systems with other tuber and root crops (Brink et al., 2006). Traditionally, it has also been intercropped with sorghum, millet, maize and even vegetables such as pumpkin and okra (De Kock, 2013; Alhassan and Egbe, 2014). It has been shown to be ideal as an intercrop because it does not occupy a vast space for planting and it also gives room and space to the other crops that are thought to be more lucrative (De Kock, 2013).



Figure 2.4: Bambara groundnut and maize in mixed cropping

2.7.3 Crop rotation

Due to its ability to fix nitrogen from the air and improve the nitrogen content of the soil, it is important in crop rotation. Thus, it increases the fertility of the soil which in turn leads to higher crop yields (De Kock, 2013). When legume green manure was included in sole cropping and intercropping with maize during crop rotation, large amounts of nutrients were recycled and this helped to fix biological nitrogen. Also, higher yields of maize were harvested when soya bean was in rotation with maize. This did not only help to recycle nutrients but also controlled nematodes (Baldwin, 2006). Crop rotation has also been shown to disrupt the life cycle of pests and disease. This indirectly helps the healthy growth of the plant and also increase crop yield (Spehar and Souza, 1995; Wright et al., 2015; Hopkins et al., 2004).

2.8 Conclusion

Interactions between soil rhizobacteria and bambara groundnut are diverse and varied. It has led to increase in soil organic matter involved in nutrient and mineral cycling and thus healthy plant life. Cultivation of bambara groundnut alongside other staple crops will also help to increase their crop yields making nutritious food available. Bambara groundnut is a legume with a promising future if it is given the right publicity and the support it deserves, both as a crop and food. Creation of awareness on its importance and its need in agricultural practices is to be encouraged especially through policy. The health of the soil or quality of soil is both directly and indirectly proportional to crop yield and invariably food security.

CHAPTER THREE

METABOLIC DIVERSITIES OF RHIZOSPHERIC BACTERIA FOR BIOTECHNOLOGICAL PROCESSES

Abstract

Secondary metabolites including volatile organic compounds (VOCs) are by products of bacterial metabolism. Volatile organic compounds are very small molecular weight compounds making them easily diffusible in the atmosphere and the rhizosphere. Rhizospheric bacterial VOCs are essential for plant growth promotion and are good antagonists of fungal and bacterial pathogens of crops. This property makes them suitable as ideal candidates to combat crop diseases caused by pathogens. Most VOCs have a characteristic smell which makes them easily noticeable as was observed in the first instance of this being discovered in fungal organisms. These VOCs were discovered as researchers worked on metabolites and became interested in the emissions from the metabolites. Volatile organic compounds are of different functional groups ranging from amines, alcohols, ketones, aldehydes to sulfur-containing compounds. They are mostly lipophilic which is noticed in their extraction as they tend not to mix with water and other solvents. Antifungal, antibacterial and plant growth promoting properties exhibited by these compounds have put more interest in them being the target of major biocontrol research with the help of new biotechnology techniques such as gas chromatography-mass spectrometry, nuclear magnetic resonance, and high pressure liquid chromatography making it easier and realistic to extract and identify these compounds. Volatile organic compounds are released by various microbes from the rhizosphere of cereals in particular. This review provides an overview of metabolites released from the rhizospheric bacteria of legumes.

Key words: Bambara groundnut, metabolites, volatile organic compound, GC-MS,

3.1 Introduction

Rhizobacteria are microbes found in the rhizosphere or soil part closest to plant roots. They are known to be diverse and varied, this span from fungi to bacteria (Antoun, 2013). They are important to plant growth and development directly by enhancing the availability of nutrients to the plants (biofertilization) and indirectly helping to suppress plant pathogens and diseases (biocontrol). They are able to carry out these functions by producing compounds (metabolites and VOCs) that are secreted into the soil environment. Those compounds secreted that are not involved in the normal functioning of the organisms but useful in the environment as means of survival for the bacteria that produced them are secondary metabolites with VOCs inclusive (Demain and Fang, 2000; Bitas et al., 2013). Metabolites are produced as a result of metabolic reactions in the microbial system and are intermediate products catalyzed by various enzymes. Metabolites and VOCs are compounds secreted as intermediate products of metabolic reactions which have been catalyzed by activities of enzymes.

They exhibit different functions in crops ranging from growth promotion to biocontrol which can be in the form of aiding plant disease resistance, inhibiting fungal and bacterial pathogens (Raaijmakers and Mazzola, 2012). They have also been found to be actively involved in cell-to-cell signaling in the rhizosphere as they play a key role in attraction of microorganisms to each other as well as microbe-plant interactions in the rhizosphere (Dorokhov et al., 2014; Fischbach and Segre, 2016; Ping and Boland, 2004; Van Loon and Bakker, 2003; Ryu et al., 2003; Chung et al., 2015; Schmidt et al., 2015). They are produced from microbial primary and secondary metabolism during the catabolism of food for nutrient uptake. They are released as by-products in the process of competition for nutrients in secondary metabolism which lead to the production of VOCs (Schöller et al., 2002; Insam and Seewald, 2010). Rhizobacterial VOCs are composed of

low molecular weight, high vapor pressure and low water solubility amines, ketones, alcohols, aldehydes, aromatic and non-aromatic hydrocarbons, and sulfur –containing compounds (Kanchiswamy et al., 2015; Lemfack et al., 2014). Their major trait is their smell which is very easily detected due to their low threshold (Schmidt et al., 2015; Schulz and Dickschat, 2007). These properties and low odor threshold helps to facilitate evaporation and diffusion of VOCs through both water- and gas-filled pores in soil and rhizosphere environments (Schmidt et al., 2015).

Legumes are from the family Fabaceae and are known to produce a wide variety of secondary metabolites as plants (Wink, 2013). Leguminous plants have also been known to exude secondary metabolites into the rhizosphere that are used by rhizobacteria. For example, flavonoids in the soil can be modified or metabolized by rhizobacteria. Rhizobia were observed to modify flavonoids that induce nod gene to produce more or less active flavonoid inducers (Hassan and Mathesius, 2012). Metabolites from legumes can be grouped into nitrogen and non-nitrogen containing metabolites. Nitrogen-containing metabolites include alkaloids and amines (Wink, 2013). The nitrogen containing amines and alkaloids among others are tyramine, and tryptamine derivatives, pyrrolidine, phenylethylamine, simple indole, pyridine, isoquinoline, imidazole alkaloids and pyrrolizidine. The non-nitrogen containing metabolites among others include polyketides, such as anthraquinone, phenolics such as flavonoids, tannins and terpenoids such as tetraterpenes (Wink, 2008). In this review, our focus is mainly on the functions of some secondary metabolites and VOCs together with their effect in sustainable agriculture.

3.2 Metabolism and volatile organic compounds (VOCs)

The metabolome is the final stage of downstream production from the genome and it is referred to as the sum total of all the small molecular weight compounds in a cell, tissue or organism. Its

atomic arrangement varies leading to the diversity of its chemical and physical properties (Dunn et al., 2005; Hall, 2006). Metabolism is the total sum of all the biochemical activities going on in an organism. All living organisms go through a form of metabolism or the other either through the primary metabolic pathways or the secondary metabolic pathways (Khanam, 2007). The primary metabolic pathways produce primary metabolites that are used for the primary growth and development as well as cellular functioning of the organisms (Khanam, 2007). In rhizobacteria, primary metabolites include ethylene which is produced endogenously and it is important for growth and development of plants (Ahemad and Kibret, 2014). Rapid analyses of complex data and identification of novel compounds is made possible through the metabolomics approach as it helps to classify metabolites based on the different metabolite-profiling studies (Rochfort, 2005; Want et al., 2005).

3.2.1 Microbial volatile organic compounds

Microbial volatile organic compounds (mVOCs) are small chemical molecules released by both bacteria and fungi. They evaporate fast due to their low boiling range. The boiling range of volatile organic compounds lies between 60°C and 250°C. Furthermore, mVOCs are characterized by a molecular weight of between 100 and 300 Dalton, a high vapour pressure and a lipophilic character (Lemfack et al., 2014).

3.2.1.1 Volatile organic compounds (VOCs)

Volatile organic compounds are parts of secondary metabolites and they are a mix of low-molecular weight lipophilic compounds that are secreted by all living organisms especially microorganisms (Schenkel et al., 2015). The use of volatiles is being encouraged in agriculture as a potent biological alternative to pesticide (Bitas et al., 2013; Morath et al., 2012). They have been seen as info-chemicals and are important in long and short distance intercellular interactions and

they can travel through different media ranging from air, water to soil (Kanchiswamy et al., 2015). Their functions have been seen as biomarkers in spoilage of food and even in disease incidence in human beings (Lemfack et al., 2014; Abdullah et al., 2015). Also, their importance has been emphasized in plant growth promotion. Two *Bacillus* spp, were observed to release VOC which were novel compounds and involved with conferring induced systemic resistance (ISR) on *Arabidopsis* that also aided plant growth promotion (Lucy et al., 2004). *Bacillus subtilis* strain GB03 and *B. amyloliquefaciens* strain IN937a were able to significantly promote the growth of *Arabidopsis thaliana*. It also elicited ISR against *Erwinia carotovora* subsp. *carotovora* as a result of VOC secreted especially 2, 3-butanediol as the major component (Ryu et al., 2003). The importance of volatile metabolites in biological control of pathogens is seen in their inhibitory and bactericidal effect on the vital functioning of the pathogenic bacteria (Kanchiswamy et al., 2015). The most frequently emitted volatile organic compounds include acetaldehyde, butanoic acid, camphene, camphor, β -caryophyllene, furfural, geosmin, 5-hydroxy-methyl-furfural, methanol, 2-methylisobornol, 1-octen-3-ol, α -pinene and propanoic acid (Kanchiswamy et al., 2015).

3.2.1.2 Rhizobacteria as sources of volatile organic compounds

Secondary metabolites are not required for any particular function by the organism but are produced in abundance (Bitas et al., 2013). Secondary metabolites from biocontrol agents have been found to help improve the fitness of the bacteria by targeting other rhizobacteria thereby reducing predator pressure (Jousset et al., 2008). They include those with unique structures such as xanthenes, antibiotics, alkaloids, terpenoids, gibberellins, flavonoids, phenolics, toxins, tetralones, benzopyranones, quinones and steroids. (Tan and Zou, 2001). Production of these secondary metabolites lead to the formation of VOCs as by-products although some secondary metabolites are classified as VOCs themselves such as terpenes, hydrogen cyanide (HCN), 2,4-

diacetylphloroglucinol (DAPG) and pyrrolnitrin (Insam and Seewald, 2010; Kanchiswamy et al., 2015).

They are important in agriculture, medicine, industry and pharmaceuticals as immunosuppressants, agrochemicals, antiparasitic, anticancer, antibiotic and antioxidants (Strobel, 2003). Isolates BNM340 (*B. amyloliquefaciens*), BNM296 (*Pseudomonas fluorescens*), and BNM297 (*Pseudomonas* spp) were able to produce the VOC antifungal compounds surfactin and iturin A, pyrrolnitrin (PRN), pyoluteorin (PLT) and HCN respectively as biocontrol agents against damping-off caused by *Pythium ultimum* and increased growth of soy bean (León et al., 2009). *Pseudomonas fluorescens* strain SS101 was also reported to produce VOCs which showed relative plant growth ability (Park et al., 2015). Other antifungal metabolites discovered to be produced by rhizobacteria include tensin, phenazines, 1-butan-3-methyl acetate, isobutyric acid, 1,8-cineole, 2,4-diacetylphloroglucinol and viscosinamide (Bhattacharyya and Jha, 2012; Morath et al., 2012; Ali et al., 2015). Hydrogen cyanide as a secondary metabolite has been implicated in different rhizobacteria such as *Streptomyces* spp (Inbar et al., 2005), *Pseudomonas* spp (Heydari et al., 2008), *Bacillus* spp (Ahmad et al., 2008) and *P. entomophila* (Ryall et al., 2009) in biocontrol activities against weeds, other bacteria and soil borne plant pathogens. Rhizobacteria from the rhizosphere of various crops have been proven to be involved in biosynthesis of the phytohormone auxin (indole-3-acetic acid/indole acetic acid/IAA) and able to release it as secondary metabolites (Patten and Glick, 1996).

3.2.1.3 Ability and adaptation of rhizobacteria to produce volatile organic compounds

The ability of *P. fluorescens* to suppress plant pathogens and diseases and also to enable signaling that can bring about gene expression as a result of its interaction with other bacteria is a product of production of secondary metabolites (Paulsen et al., 2005). *Pseudomonas fluorescens* Pf-5

produced four metabolites that are important in biocontrol of fungi and oomycetes. They are pyoluteorin, HCN, DAPG and pyrrolnitrin (Paulsen et al., 2005; Ali et al., 2015).

3.2.1.4 Contribution of rhizobacteria from legumes to production of volatile organic compounds

Rhizobacteria from leguminous crops are important producers of VOCs. As natural nitrogen-fixers, they help trap atmospheric nitrogen and make it available to plants for plant growth and development (Vessey, 2003).

3.3 Functions of metabolites in plant-bacteria interactions

3.3.1 Phenazines

Phenazines are nitrogen based compounds with a heterocyclic structure, over six thousand compounds containing phenazine have been identified with over 100 structural derivative (Pierson III and Pierson, 2010). They are found prominent in bacteria especially the pseudomonads family, also they are obvious among Eubacteria among which are *Nocardia*, *Sorangium*, *Brevibacterium*, *Burkholderia*, *Erwinia*, *Pantoea agglomerans*, *Vibrio*, and *Pelagibacter* (Mavrodi et al., 2006).

Phenazines have bright pigmentation which enables their activity in redox reaction and pH to bring about a change in colour (Pierson III and Pierson, 2010). A very common example is pyocyanin (5-N-methyl-1-hydroxyphenazine), which becomes blue when oxidized (Pierson III and Pierson, 2010). They have been implicated in the management of plant diseases. They are also broad spectrum antibiotics. They are important as the signals from cells used in regulating the way genes are expressed in the cells. They are involved in biofilm production that helps bacteria to persist and survive in their locations (McBain, 2009). *Pseudomonas aeruginosa* was observed to antagonize and suppress the activities of *Aspergillus niger*, *Fusarium oxysporum* and *Helminthosporium* spp. Minimum inhibitory concentration (MIC) against *A. niger* was detected

by its production of bioactive compound Phenazine-1-carboxamide PCN (zag 1) (Saraf et al., 2014). Apart from the functions of phenazine in plant-microbe interactions, they have been implicated as antitumor compounds and their interference with topoisomerase I and II activities in cancer cells can help to stop active division of human cells (Hari et al., 2009)

3.3.2 Toxin production

The ability of some rhizobacteria to produce metabolites extraneously into their environment makes them capable of being biocontrol agents against pathogenic soil-borne bacteria and provides a better replacement for agrochemicals (Weller, 2007). Toxin production in the rhizosphere helps to decrease competition for resources with other soil bacteria and aids rhizosphere colonization of plants (Jousset et al., 2008). Phytotoxin, haterumalide A, produced by *Serratia plymuthica* (strain A153), was able to control weeds such as *Stellaria media*, *Thlaspi arvense* and *Chenopodium album* (Gerhardson et al., 2001).

3.3.3 Hydrogen cyanide

Hydrogen cyanide is a volatile compound and an antibiotic that is effective against other pathogenic organisms and roots of weeds (Ali et al., 2015). It is a common metabolite found in 50% of *Bacillus* spp and over 88% of *Pseudomonas* spp in the rhizospheric soil and plant root nodules (Ahmad et al., 2008).

Hydrogen cyanide produced by rhizospheric bacteria of chickpea was found to improve plant growth *in vitro* either directly, indirectly or synergistically although other PGPR metabolites were also produced (Wani et al., 2007). The growth of Indian mustard (*Brassica campestris*) was enhanced by the production of HCN from *Mesorhizobium loti* MP6 (Chandra et al., 2007). Also, the growth of wheat seedlings which was reflected in its rate of germination, the ability to take up nutrient and increase in plant biomass was improved when seeds were bacterized with

psychrotolerant *P. fragi* CS11RH1 (MTCC 8984), which produced HCN (Selvakumar et al., 2009). Hydrogen cyanide is able to exhibit this property because at pico-molar concentrations, it is highly toxic to aerobic microbes and has the ability to block the pathway of the cytochrome oxidase (Kamei and Apou Kamei, 2014).

3.4 Mechanism of detection of metabolites from rhizobacteria

The production of secondary metabolites such as auxins, IAA, cytokinins, riboflavin and vitamins in rhizobacteria can be used directly by plants (Dakora, 2003) for division of cells, growth and development (Campanoni et al., 2003). They also make nutrients available to plants in forms that can be used (Biswas et al., 2000). Indirectly, secondary metabolites such as siderophores, HCN and antibiotics (Nagarajkumar et al., 2004) are produced in the rhizosphere by bacteria which are able to inhibit or suppress the growth of pathogenic or deleterious microbes.

Mass spectrometry is very important in the detection of metabolites. Since metabolites are small molecules and enzymatic activities within a cell or organisms is detected in its metabolic profile (Want et al., 2005), equipment to be used to carry out profiling becomes important. Detection of metabolites production can be carried out in several ways. Volatile compounds produced by bacterial growth were detected by selected ion flow tube mass spectrometry (SIFT-MS) (Allardyce et al., 2006). The biological effects of secondary metabolites from bacteria can be observed using MALDI (Matrix-Assisted Laser Desorption Ionization) imaging. This helps to observe the interspecies interaction and communication between pathogen and host or symbiotic relationship (Yang et al., 2009).

Fungal strains that were responsible for spoilage in stored cereals were analyzed for their secondary metabolite production using high-performance liquid chromatography (HPLC) diode array detection and flow injection analysis as well as electrospray ionization mass spectrometry

(ES-MS). The metabolic profile of the strain of the genus *Erwinia* was detected using HPLC–ES-MS (Smedsgaard and Frisvad, 1996).

The use of LC-MS to carry out more comprehensive analysis of metabolic profiling has also increased in recent times because it has been observed to produce better metabolic profiles. This is as a result of its ability to detect known and unknown compounds with high sensitivity and throughput.

3.5 Conclusion

Volatile organic compounds are very important secondary metabolites that are being detected from rhizobacteria of legumes. They have been found to be important in biocontrol activities, as PGPR and also in pharmaceutical industries for new drug formulation that are needed in the face of non-reliance on chemicals



CHAPTER FOUR

EVALUATION OF PGPR AND BIOCONTROL ACTIVITIES OF BACTERIA ISOLATED FROM BAMBARA GROUNDNUT RHIZOSPHERE

Abstract

Bambara groundnut, an underutilized crop has been proved to be an indigenous crop in Africa with the potential for food security. The rhizosphere of bambara groundnut like other legumes contains several important bacteria that have not been explored for their plant growth-promoting (PGP) properties. This research study evaluated the rhizobacteria of bambara groundnut for their biofertilization and biocontrol potentials as tools for sustainable agricultural practices. Analyses of bambara groundnut rhizospheric soil samples included physical and chemical analysis, nitrogen content using extractable inorganic nitrogen method as well as cation exchangeable capacity (CEC) using ammonium acetate method. Plant growth-promoting properties of isolated rhizobacteria evaluated include indole acetic acid (IAA), hydrogen cyanide (HCN), phosphate solubilization (PS) and ammonia production (NH_3P) activities using standard methods. In addition, antifungal assay dual culture method was used to analyze the biocontrol properties of the isolates. Phylogenetic analysis using 16S rRNA was also carried out on the isolates. Isolated rhizobacteria from bambara groundnut rhizosphere were cultured. After subculturing, 62.32% of rhizobacteria were viable. From the viable isolates, 41.87% showed positive actions in one or some of the PGP tests, 27.91% were all able to produce the enzymes, catalase, oxidase and protease. All the isolates were able to produce ammonia while 4.65%, 12.28% and 27.91% produced HCN, IAA and solubilized phosphate respectively, making them important targets as biocontrol and biofertilizer agents. Among the isolates, the genera identified include *Bacillus* spp, *Kocuria* spp, *Arthrobacter* spp and *Enterobacter* spp. The growth of *Fusarium graminearum* was suppressed *in vitro* by

6.98% of the isolates. The production of catalase and oxidase reveals the ability of the isolates to be highly resistant against chemical, environmental and mechanical stress while protease which is both a mucolytic and mycolytic enzyme is important in biocontrol technology. Plant growth promoting activities of rhizobacteria from bambara groundnut rhizosphere were comparable to those of other legumes and show that it has great potentials in food security as biofertilizer and biocontrol agent against fungal and bacterial pathogens.

Keywords: Bambara groundnut, biocontrol, biofertilizer, plant growth-promoting rhizobacteria, rhizosphere

4.1 Introduction

The use of chemicals to inhibit the growth of pathogenic microorganisms in plant disease control has been a global issue. Research for more healthy environmental control methods have led to biocontrol and biofertilization. Rhizospheric soils of legume crops have been considered a reservoir for plant growth promoting rhizobacteria (PGPR). Bambara groundnut (*Vigna subterranean* L. Verdc), a legume crop, is one of the neglected and underutilized (NUS) species. The term 'NUS' is used to mean wild species of plant which are non-commodity cultivated. They form part of a large agro biodiversity portfolio that are not used as a result of an array of factors such as agronomic, genetic, economic, social and cultural factors (Giuliani et al., 2012). They are traditionally grown by subsistence farmers in their various localities where they are useful in supporting and securing nutrition in local communities in order to meet their socio-cultural needs and traditional uses. They have been largely ignored by research and development and so there is no competition for them compared to other well-established major crops. This results in the loss of both their diversity and traditional knowledge. It is a food known as a balanced diet as it contains the right proportion of protein (16.25%), carbohydrate (63%) and fats (6.3%) (Celine, 2010). The

protein is high in both lysine (6.6%) and methionine (1.3%) (Brough and Azam-Ali, 1992). Because of its richness in protein and the fact that it is nutritious, it is a source of food security especially for small scale farmers and small households (Hillocks et al., 2012). Bambara groundnut is also very rich in micronutrients such as potassium, calcium and iron with a high proportion of fiber (Mubaiwa et al., 2017). There are different varieties with varying mineral composition for example the red varieties contains iron twice as much as the cream variety making it quite suitable for mineral deficient in iron (Hillocks et al., 2012). It was observed that fermentation of bambara groundnut helped to improve its mineral composition which invariably reduced the different factors that inhibited nutrient utilization such as trypsin, oxalate, phytic and tannic acid (Ijarotimi, 2008).

Bambara groundnut has the ability to grow under different climatic and soil conditions that are harsh and extreme thus making it suitable to be grown in semi-arid lands. The soil rhizosphere of legumes which include bambara groundnut has been indicated to enhance plant growth and also for controlling plant pests and diseases. Such beneficial attributes are associated with a host of rhizobacteria that inhabit this rhizosphere and are also sometimes referred to as PGPR. Examples of such bacteria include *Bacillus* spp, *Actinomycetes* , spp *Pseudomonas* spp, *Burkholderia* spp and *Rhizobium* spp (Pal and Gardener, 2006). The diversity of these microbial communities is driven by plant-microbe activities such as organic compound secreted by plants as well as availability and quantity of nutrients released by microbes. These interactions also play a crucial influence on the health, growth, pest and disease susceptibility of the plant as well as the health of the soil (Pal and Gardener, 2006).

Plant growth-promoting rhizobacteria (PGPR) also promote plant growth directly by producing phytohormones such as IAA and HCN. Sometimes they enhance iron chelation (siderophore

production) and supply of nutrients such as phosphorus (phosphate solubilization) and nitrogen (nitrogen fixation) to also promote plant growth. They are well known to participate in biofertilization, which involves enriching rhizospheric soil, making nutrients available to the plants as well as aiding the plants in nutrient uptake and the subsequent use of the nutrients for metabolic processes by the plants (Maheshwari, 2013). They have also been found to help in biocontrol of plant pests and diseases by suppressing and/or inhibiting the growth of pathogens in/on plants (Compant et al., 2005). As new pathogens causing plant diseases are being identified, there is the need to find better bio-alternatives that have not been harnessed but have prospects. The rhizosphere of bambara groundnut has not been explored like other legumes for rhizobacteria that are important in biofertilization and biocontrol. This study aimed at evaluating the rhizobacteria found in the rhizosphere of bambara groundnut for their biofertilization and biocontrol potentials as a tool for food security.

4.2 Materials and methods

4.2.1 Planting of Bambara groundnut

The propagation of bambara groundnut was through seeds on level seedbed or ridges where the soil is wet. Seeds from two different landraces VL and VBR were planted on seedbeds in plots that are 50 cm apart and spacing between seed holes on each plot was 50 cm apart (Fig. 4.1a and Fig 4.1b). Seeds were planted 3-4 cm deep in the soil. Twenty-five (25) plots were cultivated for each replication and the experiment was repeated thrice.



Figure 4.1: Plot showing Bambara groundnut 2 weeks after planting (a and b)

4.2.2 Soil sampling and collection

Soil samples were collected from field trials during the planting period between October 2014 and March 2016 from the North-West University Agricultural Farm, Mafikeng Campus (Lat. 25°78'91" Long. 25°61'84") Mafikeng, South Africa. Four soil samples were collected randomly from the uprooted Bambara groundnut root rhizosphere at four weeks interval from bulk soil before planting for 16 weeks corresponding to different growth stages of the plant. Twenty four (24) soil samples were collected in all to a depth of 15 cm per sample. Samples were collected in triplicates from the rhizosphere of two different landraces and were stored at 4°C until ready for use.

4.2.3 Soil Analysis

According to the International Standard Organization (ISO) standard 11464, the samples were prepared for analysis by drying at room temperature, pulverized, and sieved through a 2 mm sieve. All glassware used for soil analyzes were washed thoroughly, soaked in 20% nitric acid and rinsed with deionized water to prevent the presence of impurities. The selected physical and chemical parameters of the samples were analyzed using standard laboratory procedures (ISO-11464, 2006). All soil analysis were repeated twice.

4.2.3.1 Physical and chemical analysis of samples



4.2.3.1.1 Determination of CEC and extractable cations

Exchangeable cation was determined using the ammonium acetate method (EPA, 1986). Sample of the soil was aliquot and sieved using a 2-mm screen and allowed to air dry (at a temperature of $\leq 60^{\circ}\text{C}$). Ten (10) g of the air-dried soil was placed in a 500-mL Erlenmeyer flask and 250 mL of neutral, 1 N NH_4OAc was added to it. The flask was thoroughly shaken and allowed to stand overnight. The soil was filtered with light suction using a 55-mm Buchner funnel. The soil was leached with the neutral NH_4OAc reagent until no test for calcium can be obtained in the effluent solution. The soil was then leached four times with neutral 1 N NH_4Cl and once with 0.25 N NH_4Cl .

The electrolytes were washed with 99% isopropyl alcohol having a volume of between 150 to 200 mL. When the test for chloride in the leachate (use 0.10 AgNO_3) became negligible, the soil was allowed to drain thoroughly. The adsorbed NH_4 was determined by the aeration method (Chapman, 1965).

4.2.3.1.2 Nitrogen analysis of soil samples

Soil samples were dried at 80°C, ground to a powder and 1 g analysed for nitrogen (N) by Kjeldahl digestion (Page et al., 1982).

4.2.3.1.3 Determination of nitrate composition

Nitrate contents of the samples were determined using the equilibrium extraction method. Ten (10) g ≤ 2.0 mm of air-dry soil was placed into a 250 mL wide mouth extraction bottle and 100 mL of 0.1 mol dm⁻³ of KCl was added, stoppered and shaken for 30 min on a shaker. The solution obtained was filtered to get a clear extract and the nitrate contents were determined in the clear extract.

4.2.3.1.4 Determination of pH and redox potential

Ten (10) g of soil was weighed and mixed with 25mL of distilled water to obtain ratio 1:2.5 (m:v) soil-water suspension and left to shake for 1 h and left standing overnight for pH measurement with a pH meter Jenway 3520TM (Lasec, South Africa). The pH meter was standardized using calibration buffer 4, 7 and 9. The combined electrode was inserted into supernatant and pH values and redox potential of the samples was recorded, electrode was washed with distilled water after each reading.

4.2.3.1.5 Determination of organic matter

Organic carbon was determined by Walkley-Black method (Abollino et al., 2002). One (1) g of air-dried sample was weighed and 10 mL of 1 N potassium dichromate solution was added followed by addition of 20 mL concentrated sulphuric acid and the beaker was swirled to mix the suspension. The solution was left undisturbed and allowed to stand for 30 min and 200 mL deionized water and 10 mL concentrated orthophosphoric acid was added. Twelve (12) drops of

diphenylamine indicator (1 g diphenylamine in 100 mL concentrated sulfuric acid) was added with continuous stirring on a magnetic stirrer and finally the mixture was titrated with 0.5 M ferrous ammonium sulphate until a colour change from violet-blue to green was observed. A blank solution was also prepared in which no sample was added. The percentage of organic matter was used to calculate the organic carbon content by using the conversion factor 1.724 and the fact that 58% of the soil organic matter is the average content of carbon (Abollino et al., 2002). This calculation is given below;

Percentage organic matter in soil is calculated as written below:

$$\text{Molarity (M)} = \frac{10}{v_{\text{blank}}} = 10/9.6$$

$$=1.041$$

$$\% \text{ oxidizable organic carbon} = \frac{[v_{\text{blank}} - v_{\text{sample}}] \times 0.3 \times M}{wt}$$

(wt=weight of air-dried soil =1g)

$$\% \text{ total organic Carbon (w/w)} = 1.334 \times \% \text{ oxidizable organic carbon}$$

$$\% \text{ organic matter (w/w)} = 1.724 \times \% \text{ total organic Carbon}$$

4.2.4 Preparation of soil samples for bacterial isolation

Soil samples from the rhizosphere of Bambara groundnut were collected. Samples were prepared according to Abdulkadir and Waliyu (2012) and placed into sterile 50-mL Falcon tubes (Becton Dickson Paramus, N.J) and kept on ice or at 4°C until it was needed (within 3 days).

4.2.5 Culturing and isolation of bacteria from soil samples

Isolation and enumeration of bacteria present in the soil sample were performed by serial dilution plate technique using tryptone soy agar (TSA) and the procedure of Cavaglieri et al. (2005). A 10-fold dilution series was prepared from the rhizospheric soil of bambara groundnut in sterile distilled water and 0.5 mL from the selected dilution was spread plated on the already set tryptic

soy agar (TSA). Two (2) loopfuls of each of the bacteria from 3-day old cultures on TSA were each transferred separately to 50 mL tryptic soy broth (TSB) medium and incubated overnight at $28\pm 2^{\circ}\text{C}$. A loopful from each TSB bacterial inoculum was streaked on prepared TSA to have pure isolates from each broth culture. Viability was confirmed by standard plate count method using tryptone soy broth plus 2% agar (TSBA). These inocula were prepared in order to use them *in vitro* for testing the antifungal and biocontrol activities of the isolates.

4.2.6 PGPR and biochemical analysis of bacteria isolates

4.2.6.1 Detection of hydrogen cyanide (HCN) production

Bacterial isolates were screened for the production of hydrogen cyanide (HCN) production according to the methodology previously described by Castric (1975). Bacterial cultures were streaked on nutrient agar medium containing 4.4 g per liter of glycine. Picric acid solution (0.5% in 2% sodium carbonate) was prepared and Whatman filter paper No 1 was soaked in it and was placed inside the lid of a plate which was sealed with parafilm. After plates were incubated at 30°C for 4 days, production of HCN was observed by the light brown to dark brown colour that developed and no colouration development indicated negative activity.

4.2.6.2 Determination of indole acetic acid (IAA) production

Fifty (50) mL of nutrient broth (Merck) containing 0.1% (D) L-tryptophan were inoculated with 500 μL of 24 h old bacterial cultures and incubated in refrigerated incubator Shaker at 30°C and 180 rpm for 48 h in the dark. The bacterial cultures were then centrifuged at 10,000 rpm for 10 min at 4°C (Loper and Schroth, 1986).

An aliquot of 1 mL of supernatant was transferred into a fresh tube to which 50 μL of 10 mM orthophosphoric acid and a 2 mL of Salkowski reagent comprising (1 mL of 0.5 M FeCl_3 in 50 mL of 35% HClO_4) were added. The mixture was incubated at room temperature for 25 min. The

development of a pink colour indicated the presence of indole acetic acid (Bric et al., 1991). The absorbance of the pink solution from each isolate was measured and recorded at 530 nm using spectrophotometer (Thermo Spectronic, Merck, SA).

4.2.6.3 Determination of Phosphate solubilisation (PS)

Bacterial isolates were spot inoculated on Pikovskaya agar medium plates. The plates were incubated at 28°C for 7 days. Phosphate solubilization activity was observed as clear zone around the colonies while no zone was considered negative activity (Katznelson and Bose, 1959).

4.2.6.4 Detection of ammonia (NH₃) production

Peptone water was used to determine ammonia production of bacterial cultures. Freshly grown cultures were inoculated into 10 mL peptone water and incubated for 48–72 h at 30°C. Nessler's reagent (0.5 mL) was added in each tube after incubation and positive test was observed as brown to yellow colour development while negative activity was observed with no colour development (Laslo et al., 2012).

4.2.6.5 Determination of 1-aminocyclopropane-1-carboxylate (ACC)

This procedure was carried out according to the protocol of Li et al., 2011. Ninhydrin reagents were prepared and five working concentrations of ACC were used which are 0.05, 0.15, 0.2, 0.3 and 0.5 mmol⁻¹ for colorimetric assay using the 96-wells PCR plates. Absorbance was read at 570 nm.

4.2.6.6 Catalase activity

A sterile toothpick was used to mix 48 h old bacterial colonies placed on a clean glass slide to which a drop of 3% hydrogen peroxide was added. The effervescence that follows indicated catalase positive activity while no effervescence indicated negative activity.

4.2.6.7 Assay for protease production

Extracellular protease production was assayed according to Maurhofer et al. (1995). Spot inoculation of each bacterial isolate on skim milk agar plate was carried out and incubated at 37°C for 24 h. Development of halo zone around the bacterial colony was considered as a positive test for protease production while absence of halo zone was considered negative test.

4.2.6.8 Oxidase activity

Oxidase activity was determined by using the filter paper spot method (Murray et al., 1981). Kovács oxidase reagent (1-2 drops) was added to 24 h old culture on a small piece of filter paper. Change in colour to dark purple within 60 to 90 s was considered as oxidase positive test while absence of colour change indicated negative activity.

All analysis were repeated twice

4.2.7 Antifungal effect assay

Potato dextrose agar (PDA) medium was used as the medium to assay for the antifungal activities of 8 isolates against *F. graminearum* which is a toxin producing fungi and pathogenic to man, animals and plants. This was carried out by inoculating the pathogenic fungi at the centre of the medium and then streaking the isolates on the medium 3 cm away from the fungi. The clear zones between isolates and fungi after incubation for 4 to 7 days at room temperature indicated antagonist interaction between them.

4.2.8 Antibacterial effect assay

Antagonistic activity of isolates against *B. cereus* and *E. feacalis* was carried out. *B. cereus* is a food poisoning pathogen worldwide with serious health implication, so it is very important in the food industry and agriculture (Tewari and Abdullah, 2015). *E. feacalis* is among the diverse type of bacteria capable of causing infection in man, animals and plants causing death of plant within

7 days (Jha et al., 2005). They were screened by using a perpendicular streak method (Parthasarathi et al., 2010). In perpendicular streak method, Luria Bertani agar (Merck) was used and each plate was streaked with test bacterial isolates at the centre of the plate and incubated at 30°C for 48 h to allow optimum growth. Later, 24 h fresh sub-cultured isolated bacteria were prepared and streaked perpendicular to the test isolates and incubated at 37°C for 24 h. The experiment was carried out in triplicate.

4.2.9 Isolation of genomic DNA



Genomic DNA of all isolates was extracted using ZR soil Microbe DNA MiniPrep™ (Zymo Research, USA) extraction kit. Bacterial cultures were grown in 10 mL of Luria Bertani broth (Merck) at 37°C for 24 h and then centrifuged at 10,000 rpm (Universal Z300K model centrifuge; HERMLE Labortechnik, Germany) for 5 min. The bacterial pellets were resuspended in 200 µL of distilled water and transferred to ZR Bashing Bead™ lysis tube and 750 µL lysis solutions were added to the tube. The bashing bead was secured in a bead beater fitted with a 2-mL tube holder FastPrep® 24 and processed at a maximum speed for 5 min. The ZR BashingBead™ lysis tube was centrifuged in a microcentrifuge at 10,000×g for 1 min, 400 µL of supernatant was transferred to a Zymo-Spin™ IV Spin Filter in a collection tube and was centrifuged at 7,000×g for 1 min and 1,200 µL of Soil DNA Binding Buffer was added to the filtrate in the collection tube. Eight hundred (800) micro litres of the mixture of the binding buffer and filtrate was transferred to a Zymo-Spin™ IIC Column in a collection tube and centrifuged at 10,000×g for 1 min. Two hundred (200) µL of DNA Pre-Wash Buffer was added to the Zymo-Spin™ IIC column in a new collection tube and centrifuged at 10,000×g for 1 min. Five hundred (500) µL soil DNA Wash Buffer was added to the Zymo-Spin™ IIC Column in a new collection tube and centrifuged at 10,000×g for 1 min. The Zymo-Spin™ IIC Column was transferred to a clean 1.5 mL micro-centrifuge tube and

100 μ L DNA Elution Buffer was added directly to the column matrix. The tube was centrifuged at 10,000 $\times$ g for 30 s to elute the DNA.

4.2.10 PCR Amplification targeting the 16S rRNA

PCR was carried out in 25 μ L reaction volumes. Each reaction contained 12.5 μ L of PCR master mix, 0.5 μ L of primer, 11 μ L of nuclease free water and 1.0 μ L of DNA template. The 16S rDNA gene was amplified using the universal bacterial primers F1 (5'-GAGTTTGATCCTGGCTCAG-3') and R2 (5'-GWATTACCGCGGCKGCTG-3') (Maynard et al 2005). PCR was performed using a DNA Engine DYADTM Peltier thermal cycler (Bio-Rad). The PCR program used was an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min.

4.2.11 Agarose gel electrophoresis procedure

One times Tris-Acetate EDTA (1X TAE) buffer was prepared by adding 4900 mL distilled water to 100 mL of 50X TAE (375 mL of Tris-Cl, 28.55 mL of acetic acid, 50 mL of EDTA and 46.45 mL distilled water) and filled in the electrophoresis tank. 1.5% agarose gel (4.5 g in 300 mL of 1X TAE buffer) was prepared by melting in microwave until boiling. Once the agarose was dissolved, it was allowed to cool to ~45°C before pouring into the casting mold. Before pouring the agarose gel, combs of desired sizes were inserted into the tray in such a way that no bubbles were caught under the teeth. After the gel had cooled, the combs were gently removed. The gel was placed in the electrophoresis tank to which 150 μ L ethidium bromide has been added to 1.5 L buffer to cover it to a depth of about 1 cm. DNA samples were prepared by mixing 10 μ L of the PCR reaction mixture with 10 μ L of 1X loading buffer in 1:1 ratio on sterile parafilm. Twenty (20) μ L samples were loaded into each well in the gel with a sterile micropipette and taking care not to cross-contaminate the wells. Six(6) μ L of molecular marker (1 kb DNA ladder (Fermentas)) was loaded

in the first and the last wells of each comb. The voltage was set to 80 V and top cover was attached, making sure that the polarity of the preparation was placed correctly. After about 1 h when the loading dye had migrated to the mid-point of the gel, the power was turned off and the gel removed. DNA fragments were visualized by removing the gel slab from the tray and placing it on a UV trans-illuminator. The outcome of running the gel was recorded/captured using Chemidoc™ MP imaging system (Bio-Rad USA).

4.2.12 Sequencing and phylogenetic analysis

The Sequencing of the purified PCR products was conducted at facilities of Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa using ABI PRISM® 3500XL DNA Sequencer (Applied Biosystems). Only isolates with best activities in terms of biochemical, biofertilization and biocontrol were sequenced.

The chromatograms were edited using Chromas Lite version 2.4 software (Technelysium Pty Ltd 2012) Nucleotide sequences were analyzed and edited by using BioEdit software (Hall, 1999). The obtained 16S rDNA sequences were compared to sequences in the NCBI GenBank database with the Basic Alignment Search Tool (BLAST) (Altschul et al., 1990). Multiple alignments of the sequences were carried out by Mafft programme 7.050 (Katoh, 2013) against corresponding nucleotide sequences of the genus selected isolate retrieved from GenBank. Phylogenetic analyses were conducted using software's in MEGA version 5.2.2 (Tamura et al., 2011). Evolutionary distance matrices were generated as described by (Jukes and Cantor, 1969) and a phylogenetic tree was inferred by the neighbor-joining method (Saitou and Nei, 1987). Tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings of the neighbor-joining data set. Manipulation and tree editing were carried out using TreeView (Page, 1996).

4.2.13 Supporting data

The 16S rDNA gene sequences determined for the bacterial isolates in this study were deposited into the GenBank database and assigned accession numbers (Table 4.4).

4.3 Results

4.3.1 Physical and chemical characterization of samples

4.3.1.1 Cation Exchange Capacity (CEC), Nitrate and Nitrogen analysis of soil samples

In Figure 4.2, it was observed that the exchangeable cations and the nitrate value followed the same trend in the graph. They decreased from the 4 WAP to the 8 WAP and increased again at the 12 WAP which was the peak and decreased again at the 16 WAP only to start increasing gradually to the time of harvest. The nitrate content ranged between 6.28 mg/kg at the 8 WAP and 26.16 mg/kg at the 4 WAP. From the 12 WAP, it decreased again from 19.06 mg/kg to 6.40 mg/kg at the 16 WAP and gradually increased again to the time of harvest to 7.39 mg/kg.

All chemical parameters, N, K, Ca, Na and Mg increased from zero the original bulk soil to 4 WAP. Cation Exchangeable capacity (CEC) and Nitrate followed the same pattern of decreasing from the time of fertilization/flowering (4 WAP) till the 8 WAP and increasing at 12 WAP. They gradually decreased from 12 WAP to 16 WAP and gradually increased again to the time of harvest. Samples at 12 WAP had the highest CEC of 21.64 meq/100g. The lowest was 15.19 meq/100g at 8 WAP. Nitrate (NO₃) was highest at 4 WAP and lowest at 8 WAP. After 4 WAP, all the minerals kept decreasing gradually until the 16 WAP (which is the time of maturity of seeds) and then started increasing gradually again. The only exceptions are the individual cations Mg, Na, K and Ca which all followed the same pattern of gradually decreasing up until the 16th week and then gradually increasing afterwards to the time of harvest (Fig. 4.3 and Fig. 4.4).

Furthermore, Figure 4.4 (a) revealed that CEC and Ca were the highest mineral found in the soil samples and both were found in DVL2 and DVBR2 while NO₃ had decreased and total N is seen to increase from the previous weeks where it was absent in all samples. (b) showed that CEC and NO₃ were the highest mineral found in the soil samples and both were found in JVL1 and JVL2 (c) showed that CEC is highest and found in FVBR1 while NO₃ has decreased again but available in all samples. (d) showed that CEC is highest and found in MVBR1 while NO₃ has gradually increased again and available in all samples. Also total N increased and consistent in b, c, d samples.

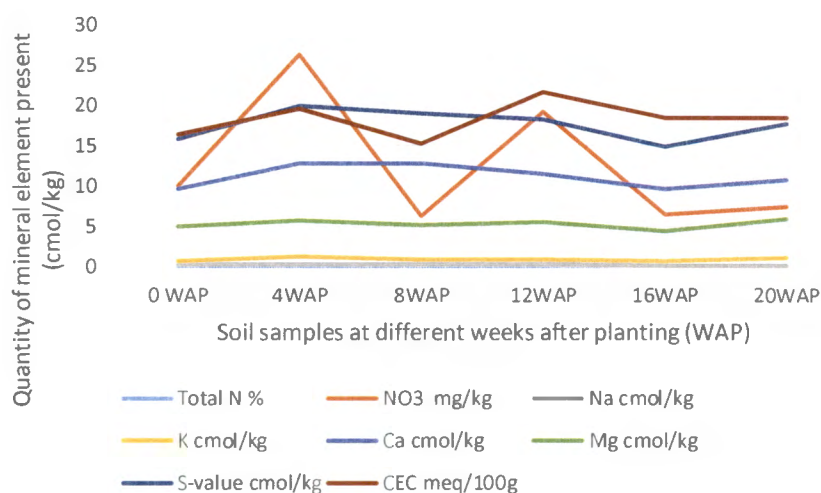


Figure 4.2: Comparison of different physical and chemical properties of soil samples between 4 and 20 weeks after planting (WAP).

N=nitrogen, K=potassium, Ca=calcium, Na=sodium, Mg=magnesium, S-value shows the mean of the whole graph

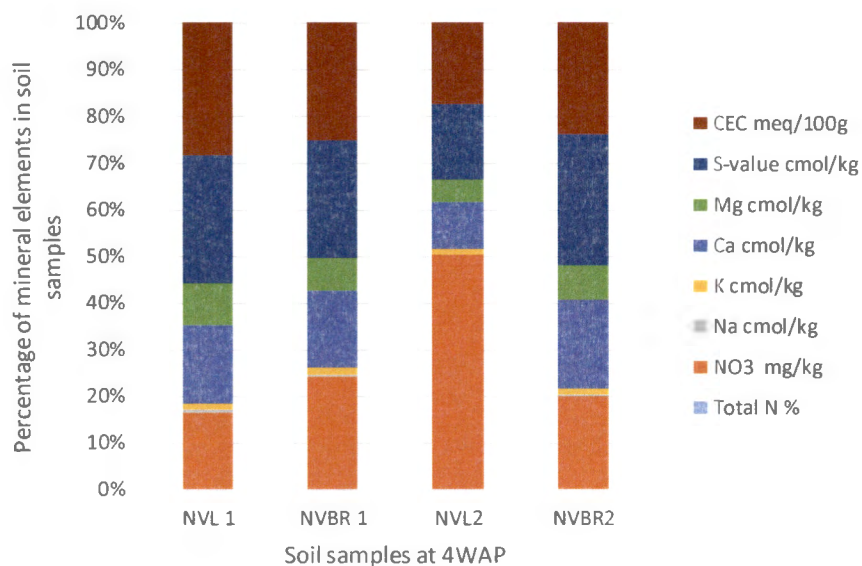


Figure 4 3: Physical and chemical analysis of soil samples at 4 WAP.

Nitrate (NO₃) and CEC were the highest quantity of mineral found in the soil samples and both were found in NVL2 and NVL1 respectively. N=November; VL and VBR=Variety of Bambara from whose root, soil samples (NVL1, NVL2, NVBR1, NVBR2) were taken

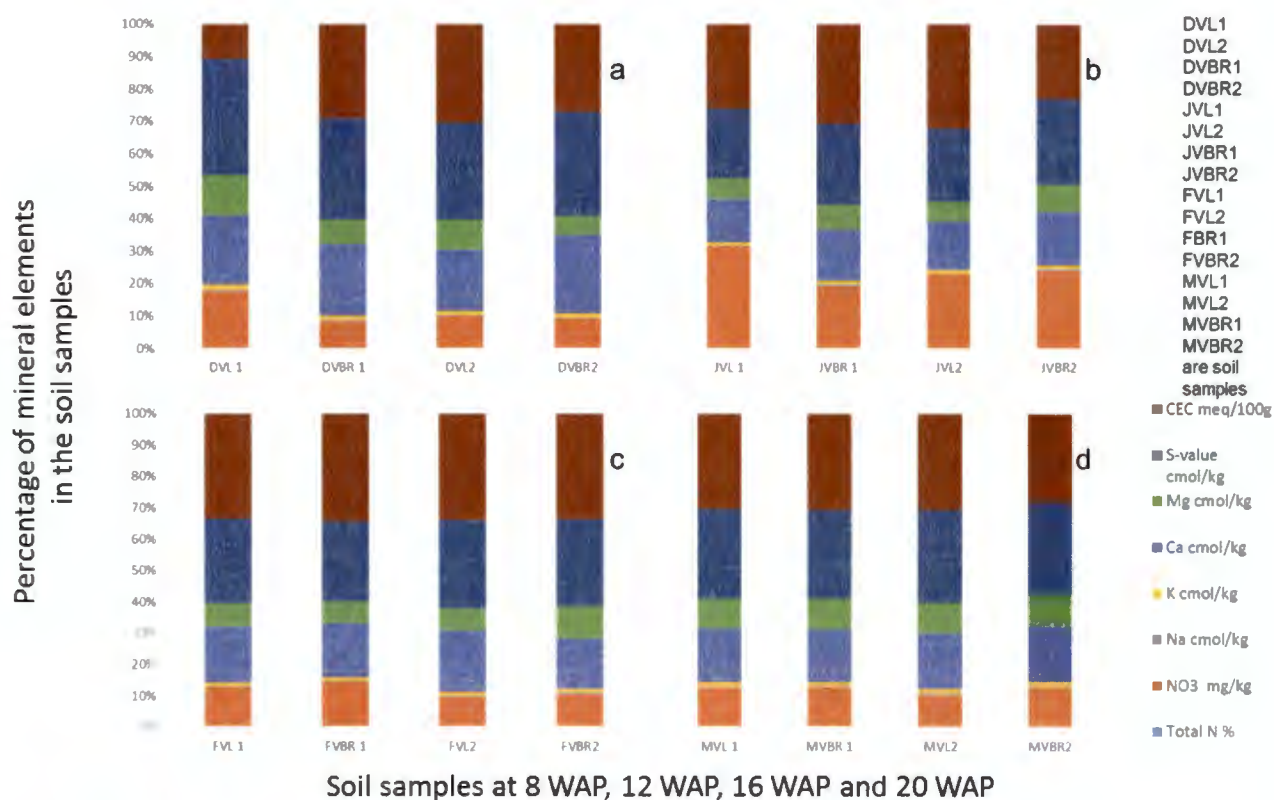


Figure 4 4: Physical and chemical analysis of soil samples at 8, 12, 16 and 20 WAP

DVL1, DVL2, DVBR1, DVBR2 are soil samples from 8 WAP (a), JVL1, JVL2, JVBR1, JVBR2 are soil samples from 12 WAP (b), FVL1, FVL2, FVBR1, FVBR2 are soil samples from 16 WAP (c), MVL1, MVL2, MVBR1, MVBR2 are soil samples from 20 WAP (d), D=December; J=January; F=February; M=March; VL and VBR=Variety of Bambara from whose root soil samples were taken

4.3.1.2 pH and Redox tolerance of soil samples

The different soils at the different growth stages differed in their physical and chemical properties. The pH reduced gradually from the original bulk soil to the soil at the time of harvest. The pH ranged from 2.3 at 16 WAP which was the lowest, to 3.4 at 12 WAP which was the highest. The reduction-oxidation relationship of both living and non-living things is measured by the redox potential (Eh) measured in volts. The Eh in this study ranged from 170.33 mV at 12 WAP to 221.67 mV at 16 WAP. This pattern of Eh and pH shows that they are both negatively correlated. This result reveals that nitrogen fixation by root nodule bacteria and activities of rhizospheric bacteria can affect the condition of the soil from one season to the other (Fig. 4.5a and Fig. 4.5b). Also though redox value, <300 mV can be limiting for plant growth but bambara groundnut has grown and also increased in yield.

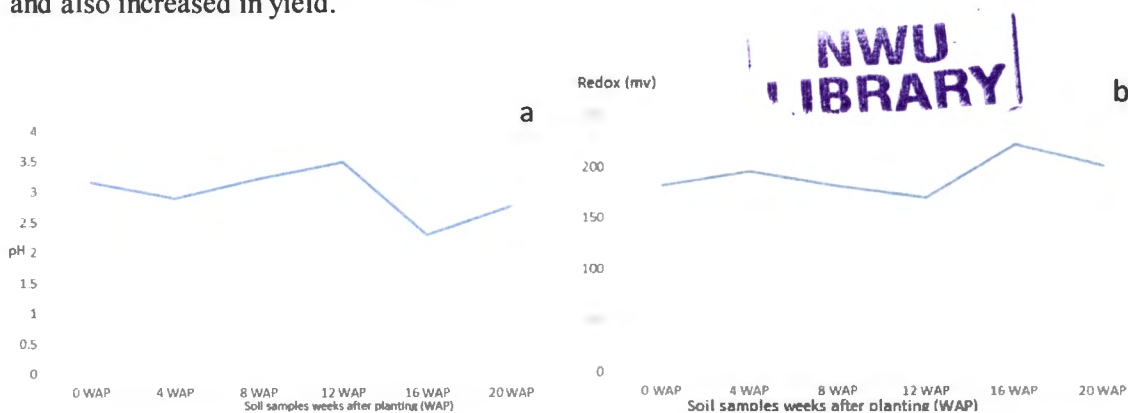


Figure 4.5: (a) Average pH values of soil samples from original soil to the time harvest. The highest pH was at 12 WAP while the least was at 16 WAP. (b) Average Redox value for soil samples at the different growth stages from original soil to the time harvest. The highest value was at 16 WAP while the lowest was at 12 WAP. The line graph for redox and pH are inversely related.

4.3.2 Organic matter content of soil samples

The organic matter of soils varies based on the type of soil. The organic matter in this study ranged from 2.02% at 0 WAP (bulk soil) which is the lowest to 3.46% which is the highest at 16 WAP. The organic matter kept increasing from 4WAP to 16 WAP and reduced at the time of harvest. Total organic carbon also followed the same pattern as the total organic matter. It ranged from 1.18% at 0 WAP which was the lowest to 20.01% at 16 WAP which was the highest.

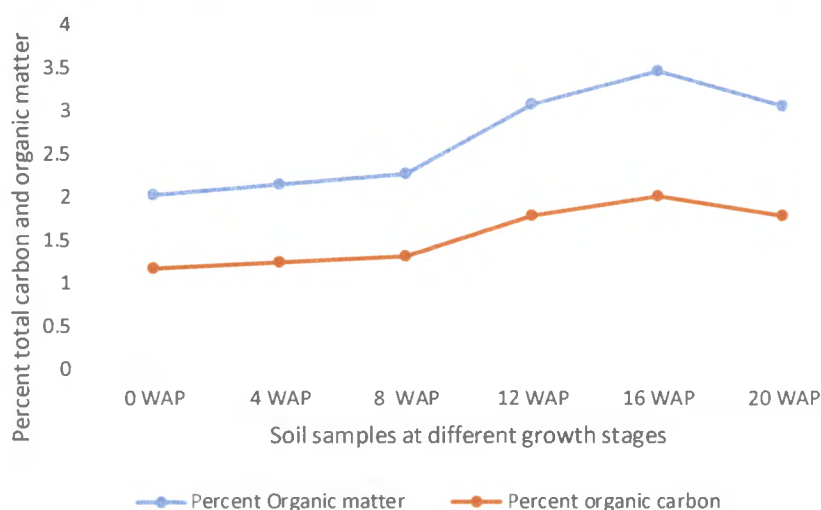


Figure 4.6: Carbon and organic matter content of soil samples from 0 WAP to harvest (20 WAP)

4.3.3 Culturing and isolation of bacteria from soil samples

The 43 isolates subcultured spanned through the different growing seasons. Some isolates that grew at the beginning of the growth period were also isolated at harvest while most of them were not. Most of the organisms isolated at the time of harvest were not the same as those that were there from beginning. The number of isolates increased from 4 WAP to 8 WAP only to decrease at 12 WAP and then continued increasing up till the time of harvest. The period of the 12 WAP corresponded to the pod and seed formation period (Table 4.1).

Table 4.1: Total number of different isolates by morphology at different growth stages

Soil Samples (4 WAP)		Soil Samples (8 WAP)		Soil Samples (12 WAP)		Soil Samples (16 WAP)		Soil Samples (20 WAP/harvest)	
	*NOI		*NOI		*NOI		*NOI		*NOI
NVL 1	3	DVL 1	5	JVL 1	3	FVL 1	3	MVL 1	22
NVBR 1	2	DVBR 1	9	JVBR 1	2	FVBR 1	4	MVBR 1	17
NVL2	8	DVL2	8	JVL2	5	FVL2	11	MVL2	8
NVBR2	-	DVBR2	2	JVBR2	3	FVBR2	4	MVBR2	-
Total	13		24		13		22		47

*NOI is number of isolates

4.3.4 PGPR activities

From the PGPR tests, 41.87% (18) showed positive actions in two or more of the PGP tests. Out of which 4.65% of the isolates were positive for HCN production, all were positive for NH_3 production and ACC with absorbance at 570 nm and standard curve was drawn (Fig 4.7a and Fig 4.7b). Isolates that were positive for IAA production were 16.28%. Absorbance value was recorded for all organisms (Fig 4.8a) and 27.91% solubilized phosphate (Table 4.2). Standard curve for IAA was also plotted (Fig 4.8b). This was used to calculate the quantity of IAA produced by each isolate (Appendix). Of all the isolates, 27.91% showed positive activity for catalase, oxidase and protease production but the isolates that were positive in at least two of the PGP tests were all positive in at least one of the biochemical test while 13.95% of the isolates that were not positive to at least 2 of the PGP tests were positive to all the 3 biochemical tests (Table 4.3). While out of the 18 isolates used in this study, 27.77% were positive to catalase, oxidase and protease production.

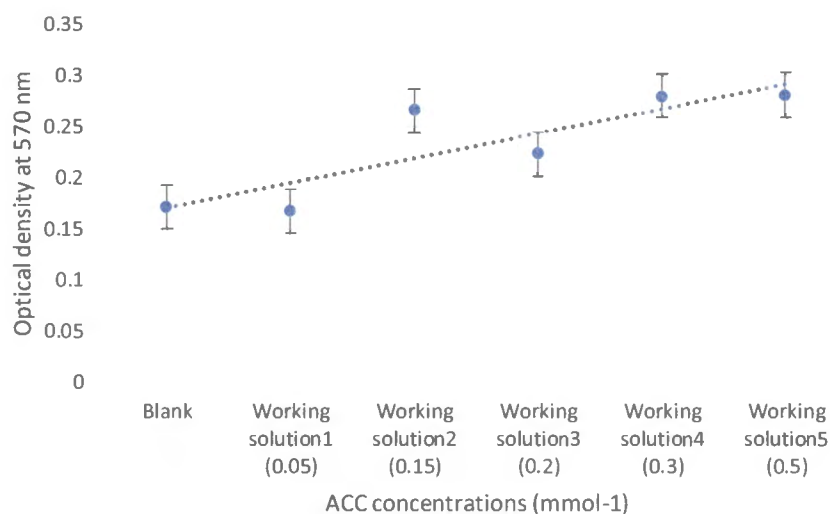


Figure 4.7a: Standard curve of ACC concentrations ranging from 0.05 to 0.5 mmol⁻¹ determined by the 96-well PCR-plate ninhydrin assay ($y=0.0242x+0.1467$, $R^2 = 0.7364$). Each data point represents the mean from triplicate determinations, and the error bar represents the standard error ACC, 1-aminocyclopropane-1-carboxylate.

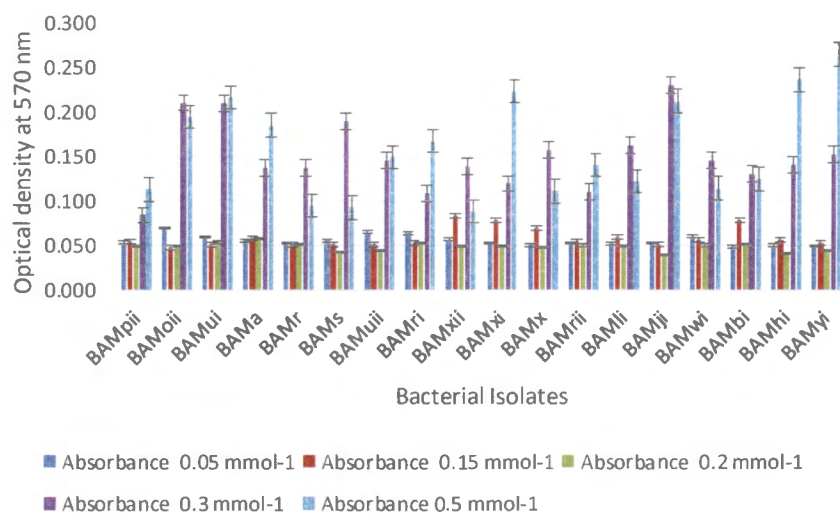


Figure 4.7b: Absorbance of bacterial isolates at different concentration of ACC

Table 4.2: Plant growth-promoting activities of rhizobacteria isolates from the above-mentioned soil samples

Isolates	IAA	NH ₃ production	HCN	P-solubilization
BAMxi	+	++	-	++
BAMyi	-	+	-	++
BAMpii	+	+	-	-
BAMwi	+	+	-	-
BAMri	+	+	-	-
BAMoii	+	+	-	-
BAMxii	+	++	-	++
BAMuii	+	+	-	-
BAMr	-	+	-	+
BAMs	-	+	-	+
BAMhi	-	+	+	+
BAMli	-	++	-	+
BAMx	-	+	-	+
BAMa	-	+	-	++
BAMbi	-	+	-	++
BAMji	-	++	-	+
BAMui	-	+	+	-
BAMrii	-	+	-	+

BAM means isolates are from bambara groundnut rhizosphere while the letters represents the different isolates

+ means positive; - means negative, ++ means very positive

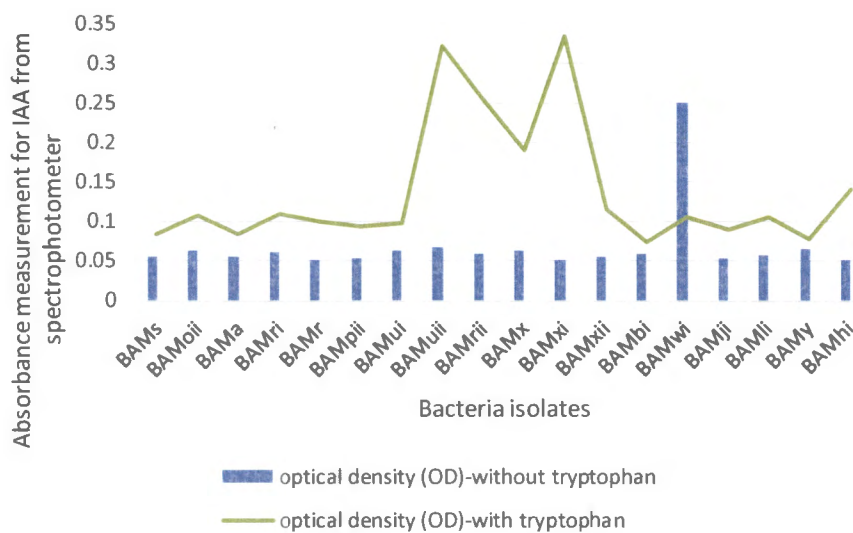


Figure 4.8a: Spectrophotometric measurement of absorbance of IAA in Isolates in the presence or absence of tryptophan at optical density of 530nm

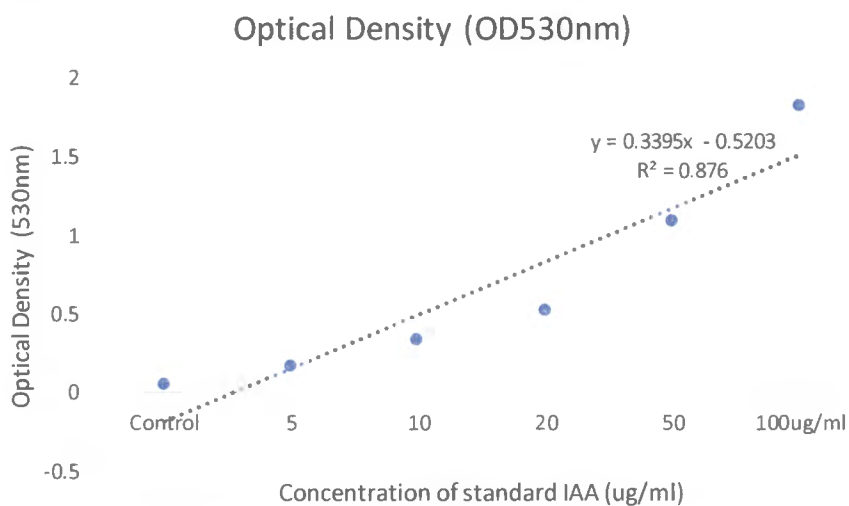


Figure 4.8b: Standard graph of IAA at optical density of 530nm

Table 4.3: Biochemical activities of bacterial isolates from Bambara groundnut rhizosphere

Isolates	Catalase	Oxidase	Protease
BAMxi	+	+	-
BAMyi	++	-	-
BAMpii	+	+	-
BAMwi	+	+	-
BAMri	+	-	-
BAMoii	+	+	-
BAMxii	+	+	-
BAMuii	++	-	-
BAMr	++	+	+
BAMs	++	+	+
BAMhi	+	-	-
BAMli	++	+	+
BAMx	-	+	+
BAMa	+	-	-
BAMbi	+	+	+
BAMji	+	+	+
BAMui	+	-	-
BAMrii	+	-	-

BAM means isolates are from bambara groundnut rhizosphere while the letters represents the different isolates

+ means positive; - means negative, ++ means very positive

4.3.5 Biocontrol activities

Biocontrol activities were carried out in this study to test for the antifungal and antibacterial potentials of the bacterial isolates.

4.3.5.1 Antifungal activities

Isolated bacteria were tested against *Fusarium graminearum* (written as f.g on the plate). BAMji, BAMr, BAMli and BAMhi (9.3%) (*B. cereus*, *B. amyloliquefaciens*, *B. thuringiensis*, *Bacillus* sp.) showed antifungal potential against *F. graminearum* (Fig. 4.9).

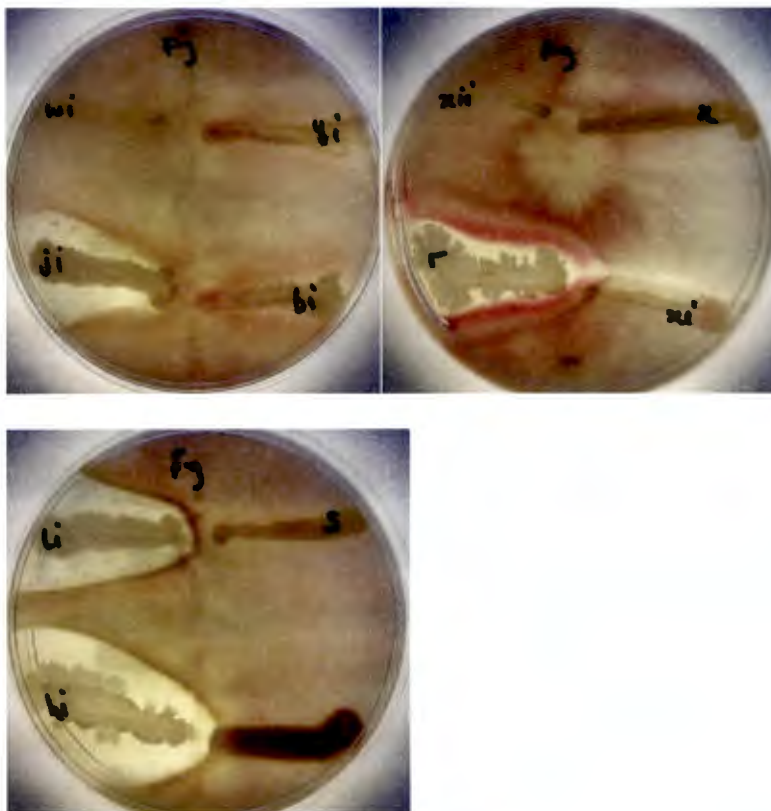


Figure 4.9: Antifungal activities of BAMji, BAMr, BAMli and BAMhi against *F. graminearum*

4.3.5.2 Antibacterial activities

Rhizobacteria from this study were tested against *B. cereus* (written as B.C on the plates) and *E. faecalis* (written as E.F on the plates). BAMui, BAMli, BAMoi, BAMyi,

BAMhi, and BAMpii (16.2%) had antagonistic effects against *B. cereus* and *E. faecalis* as seen in the pattern formed on the streaked pathogen (Fig. 4.10).

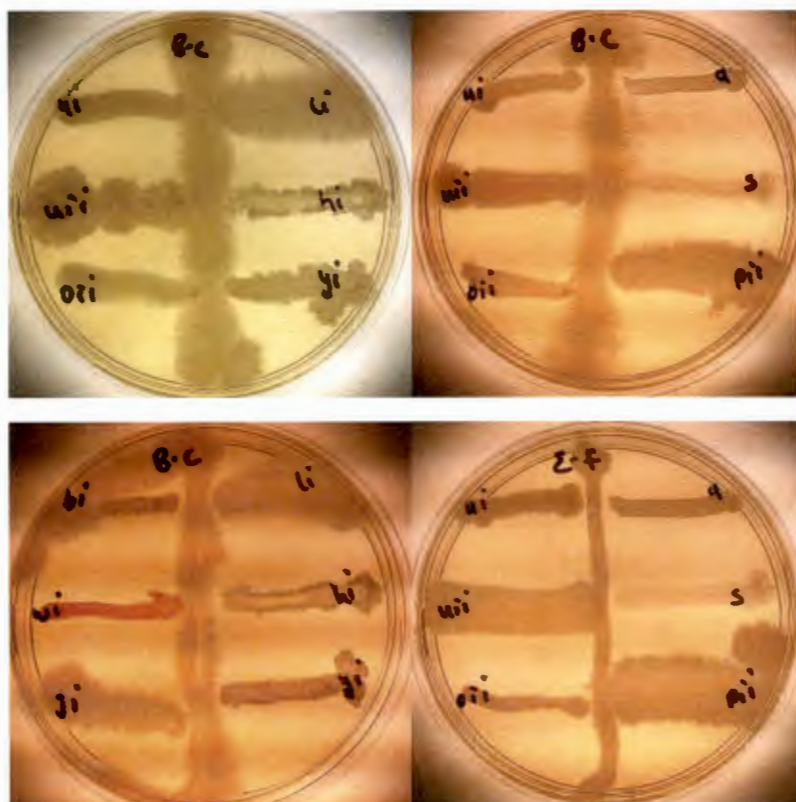


Figure 4.10 Antibacterial activities of isolates BAMui, BAMli, BAMoii, BAMyi, BAMhi, and BAMpii against *B. cereus* and *E. faecalis*

4.3.6 Molecular identification of selected isolates

Bacterial universal primers (F1R2) amplified 1.5 kb fragment from the genomic DNA of the isolates. Computational analysis was used as a means of identifying the isolates. Analysis of the partial sequences of the 16S rDNA gene of the selected isolates was used as a means of identifying them at the genus level. The BLAST tool was used to compare the partial nucleotide sequences of the 16S rDNA gene of the isolates with the nucleotide database of NCBI web server. From the BLAST search, it was inferred that the isolates were members of the GC-rich firmicutes, actinobacteria and proteobacteria. The 16S rDNA gene sequence of the selected isolates was obtained by BLASTn search; however, 27 strains of combination of the phylum firmicutes, actinobacteria and

proteobacteria were selected based on high identity (%) with good E value. Table 4.4 results show that query sequences were best pairwise aligned with 16S rDNA gene sequence of other firmicutes, proteobacteria and actinobacteria with sequence similarity and identity ranged between 96-99%, with E-value of 0.

Table 4.4: Results of 16S rDNA gene sequence similarities of rhizobacteria isolates and GenBank accession numbers using BLASTn algorithm isolate code

Isolate code	Sequence length (bp)	Closest related strain in database	Accession in number	Similarity (%)	E-value
BAMji	1424	<i>B. cereus</i>	KX588094	99	0
BAMr	1058	<i>Bacillus sp.</i>	KX588095	98	0
BAMui	1405	<i>Bacillus sp.</i>	KX588096	97	0
BAMx	1417	<i>B. megaterium</i>	KX588099	99	0
BAMuii	1353	<i>Glutamicibacter</i> (<i>Arthrobacter</i>) <i>bergerei</i>	KX588097	98	0
BAMwi	1335	<i>Kocuria rosea</i>	KX588098	99	0
BAMxi	1377	<i>Enterobacter</i> <i>hormaechei</i>	KX588100	99	0
BAMa	1387	<i>Staphylococcus</i> <i>sp.</i>	KX588093	96	0

4.3.7 Phylogenetic analysis and diversity

The isolates were subjected to phylogenetic analysis. The 16S rDNA sequences of the bacterial isolates were aligned with reference nucleotide sequences obtained from the

GenBank. The phylogenetic position of the bacterial isolates was evaluated by constructing a phylogenetic tree using neighbour joining method (Fig. 4.11). This method placed the bacterial isolates in different clades encompassing members of their genera; this was supported with bootstrap values. Bootstrap values based on 1000 replications were listed as percentages at the branching points.

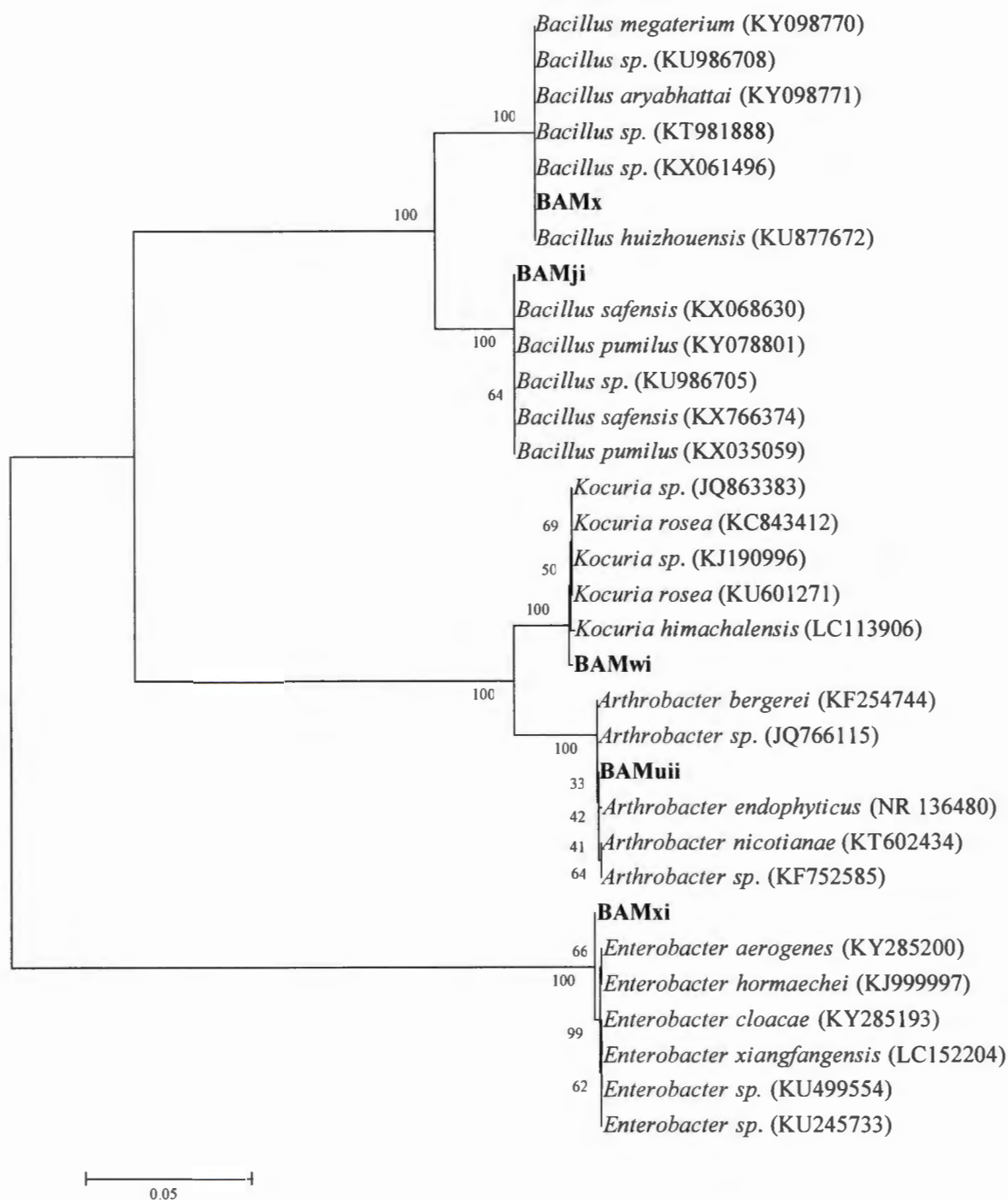


Figure 4.11: Phylogenetic tree based on 16S rRNA sequences using neighbour-joining method for bacterial isolates and their closely related type strains.

Bootstrap values based on 1000 resampled datasets are shown at branch nodes. Bar 1 substitution per 100 nucleotide position

4.4 Discussion

The plants' rhizosphere, especially those of legumes, have been observed to be a microbiome for diverse PGPR. Bambara groundnut as a leguminous plant is not only good for food but its rhizosphere is also very important in promoting plant growth and increasing food production. The rhizosphere is rich in total nitrogen, nitrate and CEC. In this study, the highest nitrate and CEC values were 26.16 cmol/kg at 4 WAP and 21.64 meq/100g at 12 WAP respectively while the least values were 6.28 cmol/kg and 15.19 meq/100g both at 8 WAP respectively (Fig. 4.3). The nitrate available in the soil falls in the same range with the soil nitrate analysed at different growing season from Dobra Voda and Chvalina farms during Spring and Autumn ranging between 4 and 14 ppm N, though some of the values from this study are higher (Vaněk et al., 2003). These higher values of nitrate can be as a result of bambara groundnut's ability to fix nitrogen through symbiosis with the bacteria in the root nodules. According to Vaněk et al. (2003), it can also be as result of the activities of leguminous crops in which case bambara groundnut is one. The CEC of soil samples in this study (between 15.19 and 21.64 meq/100g) fall within the range of CEC of organically and conventionally managed apple orchard ranging from 19.23 to 20.28 cmol/kg (Gasparatos et al., 2011), even though no fertilizer or organic manure was applied to bambara groundnut in this study.



The variation in the physical and chemical composition of the soil at different growth stages of bambara groundnut shows the involvement of these rhizobacteria in nitrification (Fig. 4.3, Fig. 4.4 and Fig. 4.5). This is because as the number of rhizobacteria increases (Table 4.1), the cmol/kg of nitrate decreases, this could be as a result of uptake of nitrate by plants through the rhizobacteria (Mantelin and Touraine, 2004). As the number of rhizobacteria increases, the usage of NO_3 increases by the plant and so its availability in the soil decreases. This is in agreement with the

observation made that with an increase of PGPR in the soil, plant uptake of nitrogen from fertilizer applied to the soil also increases (Adesemoye et al., 2010). This is obvious in 8 WAP and 16 WAP where the number of isolates were high but the volume of NO_3 and other CEC were low. It could also mean that though the rhizobacteria were available in the soil they might not be nitrogen fixers. Nitrogen available in the soil can also be low if plant density is low since both are positively correlated and might not be as a result of poor symbiotic relationship (Mohale et al., 2014). This was contrary to other findings which observed that nitrogen increased with increase in microbial population and activities (Salama 2011). In the 12 WAP, when NO_3 was very high but number of isolates was low, it corresponded to the period of pod and seed formation. This helps to shed more light on the reason for the increase in NO_3 and also if the type of rhizobacterial available were nitrogen fixers or the number of nitrogen fixers increased in the soil at this growth stage. Apart from nitrogen-fixers being in the nodules there are other free living rhizobacteria that are non-symbionts, free living in the rhizosphere and can also fix nitrogen (Ahemad and Kibret, 2014) even though in small amounts (Glick, 2012).

Normally, leguminous plants are sensitive to pH and this is important to their nitrogen-fixing ability because at low pH the soil is acidic and inhibits the activities of nitrogen-fixing bacteria and nitrogen is not released for plant growth (Beegle, n.d). Bambara groundnut grows well on acidic soils (Mkandawire, 2007) which agrees with the result from this study. It was observed that there was an increase in alfalfa yield when there was no additional nitrogen and the pH was high which is in contrast to the observation in this study. Although the pH of the soils throughout the season were acidic as they ranged from 2.3 to 3.49, nitrogen was available in the form of nitrate as mentioned earlier and bambara groundnut was enhanced in growth. Out of 12 isolates from the root nodule of bambara groundnut isolated from Cameroonian soil, 8 of them were able

to grow at pH 3.5 in the soil which was acidic. This also reveals the potential of bambara groundnut to grow under very harsh condition (Laurette et al., 2015). Soil pH and redox potential (Eh) are negatively correlated, as one increases the other decreases. The Eh in this study ranged from 170.33 to 221.66 mV which falls into the category of moderately reduced soils having Eh between +100 and +400 mV and close to cultivated soils with Eh range of between +300 and +500 mV (Husson, 2013). The Eh and pH of the soil help to determine the type of metabolism evident in the bacterial community of the soil and invariably the biological activities of the soil. Growth and development of soil bacteria and their metabolic, enzymatic and microbial activities are directly or indirectly affected by the Eh and/or the pH (Kimbrough et al., 2006; Marino et al., 2009).

Soil organic matter in the soil is very vital and important as it can produce as much carbon found in the combination of that of the atmosphere as carbon dioxide and the biomass of plants (Schimel, 1995). In this study, the soil organic matter increased till 16 WAP and decreased at harvest, this could be as a result of heavy metabolic processes involving pod and seed formation at the 12 and 16 WAP. Assertion from this study points to the fact that between 4 WAP and 16 WAP, the soil of bambara groundnut kept increasing and at 16 WAP experienced the highest level of fertility. At this point, other cereals can be cropped with bambara groundnut to improve their growth.

Bacteria from the rhizosphere are important in auxin production and vitamin synthesis that encourage biofertilization (Babalola and Akindolire, 2011). Bacterial isolates from this study were able to produce IAA and solubilise phosphate which are important in biofertilization to increase crop growth. Ammonia, HCN and siderophore production and phosphate solubilization are also able to contribute to biocontrol potentials of rhizobacteria (Laslo et al., 2012). Seventy eight percent (78%) of the isolates from different monocotyledonic plants' rhizosphere and soil (Laslo et al., 2012), 77.1 % of isolates from chickpea rhizosphere (Joseph et al., 2012), 85.57% of isolates from maize

rhizosphere (Agbodjato et al., 2015) produced ammonia as against 100% ammonia production from this study. This could be as a result of the high nitrogen-fixing capacity of both the nitrogen fixers and free living non-symbionts in the rhizosphere of bambara groundnut.

Soil phosphorous is an important macronutrient needed for plants to grow, hence deficiency of Phosphorus in soil is a major challenge in agricultural food/crop production. Total soil P occurs in either organic or inorganic form. The major form of organic phosphorus in the soil is phytate (salts of phytic acid). As a source of phosphorus, it constitutes about 60% of soil organicphosphorus and this organic form is poorly utilized by plants (Mudge et al., 2003). It either forms a complex with cations or adsorbs to various soil components, so it is not readily available to plants. For phytate-P to be immobilized so that it is easily utilized by plants, phytate has to be solubilized. Phytate can then be dephosphorylated by phosphatases (phytases and phosphatases) before it is assimilated by plants (Richardson et al., 2009). Some soil microorganisms secrete phytases into the soil to be able to make use of organic substances from plants roots and also when they compete with plant roots for elements such as phosphorus (Wasaki et al., 2009). The phytases secreted into the rhizosphere invariably help to immobilize phytate and make it soluble so that it is utilised by the plants (Singh and Satyanarayana, 2011).

Phosphate solubilization is a complex phenomenon and it helps to discriminatively screen the bacteria which are able to break down tricalcium phosphate (TCP) and thereby release inorganic phosphate. According to Laslo et al. (2012), 63.8% of bacteria isolates from different rhizosphere of monocotyledonous plants solubilized phosphate as against 27.91% from this study. It was observed that none of the isolates from fields growing chickpea from West of Allahabad Agricultural Institute, India produced HCN (Joseph et al., 2012) while in this study 4.65% produced HCN. This result is not

comparable to a report by Agbodjato et al. (2015) which revealed that 100% of isolates from maize rhizosphere produced HCN.

Isolates *B. cereus*, *B. amyloliquefaciens*, *B. thuringiensis*, *Bacillus* sp. from this study were able to antagonise the growth of *F. graminearum*, which is an agricultural challenge to barley, wheat and maize in South Africa (Boutigny et al., 2014; van der Lee et al., 2015). It was observed that *B. amyloliquefaciens* produced HCN in addition to ammonia and solubilizing phosphate while *B. cereus*, *B. thuringiensis* and *Bacillus* sp. produced ammonia and solubilised phosphate. Isolates BAMui, BAMoi, BAMyi, BAMpii, *B. amyloliquefaciens* and *B. thuringiensis* were able to antagonise the growth of *B. cereus* and *E. faecalis*. Three of the antifungal isolates also displayed antibacterial activities which show that some rhizobacteria are both antifungal and antibacterial agents while the rest are just purely antibacterial. *Lysobacter* spp strains have been found to carry out both antimicrobial and antifungal activities against *Pythium ultimum*, *Colletotrichum gloeosporioides*, *F. oxysporum*, *Botrytis cinerea*, *Rhizoctonia solani*, *Botryosphaeria dothidea* and *B. subtilis* (Mohale et al., 2014). The ability of these isolates to be able to inhibit and/or suppress the growth of both fungi and bacteria implies the richness of the bambara groundnut rhizosphere and its ability to resist diseases and pests.

It is important to identify bacterial isolates at the species level as vital information concerning the organisms such as its novelty and its ability to produce bioactive compound is provided (Adegboye and Babalola, 2012). The 16S rDNA gene sequence analysis was used to identify selected bacterial isolates in this study. The comparison of the bacterial isolates sequences revealed 96-99% identification similarities with 16S rDNA gene sequence of the genus *Bacillus*, *Enterobacter*, *Arthrobacter* and *Kocuria*. The 16S rDNA gene sequences analysis has been shown to be a very effective tool in phylogenetic characterization of microorganisms (Thenmozhi and Kannabiran, 2010).

This analysis is important as it helps to explain the evolutionary relationship that exist among microorganisms. The phylogenetic relationship of the potent bacterial isolates to known *Bacillus*, *Arthrobacter*, *Enterobacter*, and *Kocuria* spp was first estimated through a BLAST search of the GenBank database. In order to have analysis that is robust and reliable, the strains that are closest to the selected isolates were chosen and used for comparison of pairwise sequence and also for the phylogenetic tree construction. The selected bacterial isolates were grouped distinctly in different branches. Sometimes strains of bacteria that are grouped distinctly produce distinct microbial agents (Intra et al., 2011).

The analysis of 16S rRNA nucleotide sequences is used to determine higher taxonomic relationships of microorganisms (Yarza et al., 2014). The nucleotide sequences comparison of the bacterial isolates showed 99-100% identification similarities with those of reference nucleotide sequences from the GenBank. The phylogenetic tool is a powerful method that helps to elucidate the evolutionary relationship among organisms (Lau et al., 2014). The tree revealed that the bacterial isolates have a phylogeny that is well supported and completely resolved. It also shows high resolution of all inner branches. Overall, this phylogenetic tree with its high-level branching is in consonance with traditional systematic divisions. This is because organisms that belong to the same family or genus taxonomically are grouped into different species using the traditional systematic divisions.

Relationship between Bambara groundnut physical and chemical analyses, pH, Eh, PGPR and biocontrol

Bambara groundnut physical and chemical analyses in this study reveal that the soil is an acidic soil and the growth of bambara groundnut in the soil makes it more acidic and that it can thrive in acidic soil. It has been observed that redox reaction is very important to the biocontrol activity of plant pathogen by rhizobacteria (Husson, 2013). This, they

do by generating reactive oxygen species (ROS) in the plant and this serves as an antagonistic response of the plant to the pathogen and indirectly stresses the pathogen (Hartmann et al., 2009). Hydrogen peroxide and other signals like salicylic and glutathione have been observed to increase the resistance of plants to pathogens (Van Camp et al., 1998). The isolates in this study were also able to release hydrogen peroxide in the catalase reaction, which might have also enhanced their ability to resist pathogen growth.

4.5 Conclusion

The rhizosphere of bambara groundnut is very rich in terms of biotic and abiotic components. It is quite interesting that most studies on bambara groundnut have been on its food production but not much in-depth study has gone into its rhizosphere which is able to enhance its food production potential and then take it to the next level. This study revealed that the physical and chemical properties of soil at different growth stages are different and they affected the number, types and diversities of bacteria of bambara groundnut rhizosphere. The Eh and pH of the soil were very important in the diversity of the bacterial isolates from the rhizosphere. They affected the type and abundance of the bacterial isolates at each growth stage. They were also important in the biocontrol potential of bacterial isolates. It is also observed from this study that PGP activities of rhizobacteria from bambara groundnut's rhizosphere is comparable to those of other legumes and crops. Also, that bambara groundnut has great potentials in food security as biofertilizer and biocontrol agent against fungal and bacterial pathogens. These bacteria would be explored for their VOCs and how they can be used in other biotechnological processes.

CHAPTER FIVE

THE INFLUENCE OF BAMBARA GROUNDNUT GROWTH STAGES AND LANDRACES ON SOIL BACTERIAL COMMUNITIES

Abstract

The diversity of microbes in the soil of plants is important for sustainable agriculture as these microbes are important in carrying out different functional processes to improve the soil and invariably plant growth. Inversely the presence of the crop also affects the types of microbial communities in the soil. In this study, bambara groundnut was grown during the planting season in South Africa, from November to March and soil samples were taken after every four weeks. Soil samples were taken 15 cm deep from 2 different landraces VBR and VL. Microbial diversity was determined by soil microbial Carbon Source Utilization Profiles (CSUP) using BIOLOG[™] GN2 plates. The soil microbial richness and abundance were measured using the Shannon-Weaver and Evenness diversity indices.

A cluster analysis revealed a shift in soil microbial community diversity over the plant growth stages. Bacterial abundance and diversity were higher at 4 and 8 WAP. The microbial abundance (richness index) in this study ranged from 0.64 to 0.94 with cultivar VL2 at 8 WAP being the highest while bulk soil (control), R2 was the lowest. The Shannon-Weaver index varied between 2.19 and 4.00 with the lowest corresponding to control while the highest was VL2 at 8 WAP. Carbon sources utilized by bacterial communities spread across the 96 carbon sources. The highest utilization of alcohols, amides, amines, aromatic chemicals, brominated chemicals and phosphorylated chemicals was found in the control landraces. The highest utilization of carboxylic acids, ester, amino acids and polymers and carbohydrates was found in the bacterial communities of the different landraces across growth stages. This implies that

the soil samples between 4 WAP and 12 WAP were richer in diversity of microbial species and their abundance. This soil diversity and richness enhance the fertility of the soil and also make the soil to be more important in crop production.

Keywords: Bambara groundnut, BIOLOG, growth stages, carbon source

5.1 Introduction

Bambara groundnut, is an annual crop with a root system comprising a well-developed tap root. Numerous stems about 20 cm long having very short internodes from where the roots grow out with the stems bearing trifoliolate compound leaves emanating from the tap root (Hillocks et al., 2012). These stems can have branches as many as twenty after the first few weeks of germination (Opoku, 2010). Flowers are borne on auxiliary peduncle on branching stems from where they grow downwards taking with them the developing seeds. The pods develop first about 1 cm beneath the surface of the soil and then the seeds develop inside the pods (Hillocks et al., 2012). Pollination in flowers can either be self or by ants depending on if they are the spreading or branching types respectively (Bamishaiye et al., 2011).

Growth stages in bambara groundnut can be categorized into 5 stages. After 7-15 days of planting it germinates. This is followed by flowering which is between four and eight weeks (30-55 days) (Brink et al., 2006) and may continue throughout the plant's life. For nodule formation in bambara groundnut, the flowering stage is critical. At twelve weeks, there is seed development and by sixteenth week, the nuts are matured for harvest (Trivedi, 2006). Growth stages in the plant have different effect on soil environment and also on the microbial community of the soil. In the rhizosphere, bacterial communities are dynamic and liable to changes in plant conditions (Inceoglu et al., 2010).

This dynamism of bacteria in the rhizosphere can be analyzed as a measure of the level of richness and abundance of the different species present in the soil. A range of microbiological and molecular techniques are available to study soil microbial populations. However, the methods selected to study these changes, should be both cost-effective and useful (valuable) in order to contribute to a better understanding of the effect of management practices such as cropping systems, for example crop rotation and tillage on the biological component of soils (Das and Chakrabarti, 2013). This is because cropping systems involving legume species have been observed to stimulate the microbial community due to the association between the soil microbes and the rhizosphere. This leads to the release of exudates that improve cycling of plant nutrient, its availability to the plants, plant growth promotion and also diseases and pest resistance (Kong et al., 2011). This process leads to improvement of soil health and quality and thus improved crop health, crop yield and food security. Functional diversity in the soil can be measured to determine the biological status of soil microbial populations, since it relates to the actual or potential activities of organisms that contribute to ecosystem dynamics (Pignataro et al., 2012). BIOLOGTM has been used in comparing the different metabolic diversity of microbial communities including water, soil and wheat rhizosphere (Stefanowicz, 2006). The catabolic diversity of bacterial populations can be determined based on sole-carbon substrate utilisation, using the BIOLOG[®] system (Wang et al., 2007). Its principle rests on the utilization of carbon sources by environmental samples in which case the carbon sources have been dried in 95 wells to which colourless tetrazolium dye had been added. Upon utilization/oxidation of the substrate by the bacterial communities in the samples, the dyes in the wells are reduced to violet formazan (Insam et al., 2004; Zhang et al., 2016; Stefanowicz, 2006; Campbell et al., 1997; Garland, 1999).

In this context, soil microbial carbon source utilisation profiles (CSUP), i.e. profiling the different food sources utilised by the microbial species within a population, are frequently used as indicators of soil quality. This involves the ability of the microbial population to utilise a specific substrate (“food source”). Populations of organisms will give a characteristic reaction pattern, called a metabolic fingerprint. This method has been frequently used as an indicator of soil quality under different land-use and management practices (Habig and Swanepoel, 2015).

So far this is the first study where the metabolic diversity of bambara groundnut is analyzed using BIOLOG™. Therefore, this study was carried out to observe the impact of the different growth stages of bambara groundnut and the crop itself on soil bacterial communities of bambara groundnut rhizosphere.

5.2 Materials and Methods



5.2.1 Preparation of Bambara groundnut landraces for sample collection

Two bambara groundnut landraces VBR and VL were collected from the International Institute of Agriculture (IITA) and planted in the agricultural farm of the North-West University Mafikeng. Soil samples were collected before planting and referred to as the control (bulk soil) and after planting referred to as VBR1, VBR2, VL1 and VL2.

5.2.2 Sample collection

Soil samples were collected from field trials during the planting period between October 2014 and March 2016 from the North-West University agricultural farm, Mafikeng campus (Lat. 25°78'91" Long. 25°61'84") Mafikeng, South Africa. Apart from the bulk soil that was collected before planting, four soil samples each were collected randomly from the uprooted bambara groundnut root rhizosphere at four weeks interval for 16 weeks corresponding to different growth stages of the plant. Twenty four (24) soil

samples were collected in all to a depth of 15 cm per sample and were stored at 4°C until ready for use.

5.2.3 Analysis of bacterial community in the soil samples /microbial activities

5.2.3.1 Soil Chemical Analysis

Chemical analysis were conducted on soil samples at each growth stage (4 weeks interval). Using a soil auger, composite soil samples were collected from four sampling points. The sampled soils were air dried and sieved through a 2 mm sieve. Standard laboratory procedures were followed to analyze for P (Bray 1), total N (Dumas method), and organic C (Walkley–Black) (Ajilogba et al., 2016).

5.2.4 Soil Microbial Functional Diversity

5.2.4.1 Sample preparation

Three replicates were made per soil making a total of 72 soil samples to be analyzed. Ten (10) g of each soil sample were measured into 90 ml of sterile distilled water (SDW). The resultant solutions were shaken on the rotary shaker for 45- 60 min at 160 rpm and were allowed to settle for another 45 min so that solid particles are not transferred during dilution. Thereafter, serial dilution was carried out on all samples by taking 1 ml from the resultant supernatant and adding to 2 ml of SDW. To 9.9 ml of SDW, 0.1 ml of the previous supernatant was added and finally, 2 ml of this supernatant was added to 18 ml of SDW.

5.2.4.2 Inoculation of soil samples in BIOLOG GN2 Plates

Each supernatant from the 72 samples was used to inoculate the 96 wells of BIOLOG GN2 Plates (Biolog Inc., Hayward, CA, USA) each plate containing 95 sources of C and a control well by aliquoting 150 µl of supernatant into each well. The samples were incubated at 28°C. Respiration of C sources by microbial populations reduced the tetrazolium dye within each BIOLOG GN2 well, causing a colour change. This colour

change was spectrophotometrically determined by taking readings every 8 h for 7 days using ThermoFisher MultiSkan EFX at absorbance of 590 nm. This was in order to determine the average well colour development (AWCD) within each plate (Habig and Swanepoel, 2015).

5.2.4.3 Determination of functional diversity

The optical density (OD) values obtained from each plate were analysed using the average well colour development (AWCD) technique (Gryta et al., 2014; Wang et al., 2007). Standardized patterns were obtained by blanking the absorbance values for the wells with C sources against the absorbance value of the control well without a C source. Any negative values were converted to zero, and any variance in the inoculum density was accounted for by dividing the absorbance of each well by the average absorbance for the whole plate, giving the standardized OD.

5.2.4.4 Analysis of Carbon source utilization profiles (CSUP)

Carbon source utilization profiles (CSUP), *viz.* functional diversity, of soil microbial populations were determined by using the amount and equitability of carbon substrates metabolized as indicators of richness and evenness, respectively (Habig and Swanepoel, 2015; Wang et al., 2007).

Biodiversity was determined by using the Shannon-Weaver substrate diversity index. The functional diversity of soil microbial communities was thus, quantified with Shannon-Weaver's substrate diversity index (H') by using the number of different substrates utilized by the microbial communities (Habig and Swanepoel, 2015). Bacterial functional diversity of substrate utilization was also determined by using substrate richness and substrate evenness (E) (Lupwayi et al., 2001).

5.2.5 Statistical Analyses

Raw data on carbon source utilisation from the reading of the spectrophotometer were transposed on Excel. Data was subjected to non-parametric statistical analyses using STATISTICA 12 (StatSoft, Inc. ©). This program has four blocks. The raw data were fed into the first block. The second block subtracted the control from each of the well. The third block got rid of all the negative values. AWCD was calculated from the fourth block at OD 0.25nm. Soil microbial diversity was statistically analysed using Principal Component Analysis (PCA) and cluster analyses (vertical hierarchical tree plots). Homogenous grouping with Fisher Least Significant Difference (LSD) was also determined, and calculated at $p < 0.05$. The Fisher LSD test is considered to be a less conservative post hoc test than the Tukey Honest Significant Difference (HSD) (Habig and Swanepoel, 2015).

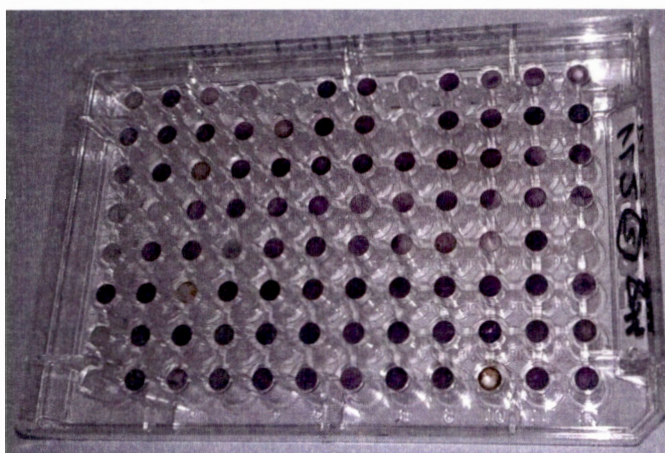


Figure 5.1: The BIOLOG system uses a 96-well microliter plate with 95 different carbon sources.

5.3 Results

5.3.1 Soil chemical analysis

The soil used in this study was the same soil whose chemical analysis had been carried out by Ajilogba et al 2016 (Fig. 4.2-Fig 4.4 and discussed in section 4.3.1).

5.3.2 AWCD analysis

The mechanism of colour development in GN2 MicroPlates™ is related to differences in carbon source utilization (CSU), i.e. food source consumption, which, in turn, appears to relate to the number of viable microorganisms able to utilize the substrates (“food sources”) within the wells of the MicroPlate™ as a sole carbon source (Fig. 5.1).

Main groups of carbon sources utilized by soil microbial communities in the different landraces over time are summarized in Table 5.1. Significant differences in carbon source utilization by soil microbial communities in the different landraces are evident. The highest utilization of alcohols, amides, amines, aromatic chemicals, brominated chemicals and phosphorylated chemicals was found in the control (bulk soil). The highest utilization of carboxylic acids was found in the VBR1 cultivar, while the highest utilization of esters was found in the VBR2 cultivar. Soil microbial communities in the VL1 landraces demonstrated the highest utilization of amino acids and polymers, while the microbial communities in the VL2 landraces were responsible for the highest utilization of carbohydrates. On the other hand, the lowest utilization of carbohydrates and carboxylic acids was exhibited in the control (bulk soil). The VBR1 landraces exhibited the lowest utilization of amino acids and polymers, while the VBR2 cultivar exhibited the lowest utilization of amines and aromatic chemicals. The lowest utilization of amides and brominated chemicals was found in the VL1 cultivar, whereas, the lowest utilization of alcohols and phosphorylated chemicals was found in the VL2 cultivar.

On average, carbohydrates, amino acids and carboxylic acids were the most utilized carbon sources, whereas, amides, brominated chemicals and phosphorylated chemicals were the least utilized. Considering the different landraces, soil microbial communities in the VBR1 and the control exhibited (on average) the lowest overall carbon source

utilization, whereas, microbial communities in the VL1 and VL2 landraces exhibited the highest overall carbon source utilization (Appendix). The number of bacterial species in the soil sample able to utilize specific carbon sources is indicated in the intensity of the color change. This implies that several bacterial species in each soil samples utilize more than one carbon source. This is because each soil sample is inoculated in a carbon source plate having 95 carbon sources. The bacterial species utilize the carbon sources resulting into different intensity of color change as observed in the plate in Fig. 5.1.

Table 5.1: Main groups of carbon sources utilized by soil microbial communities in the various soil samples of landraces over time.

Soil samples	Alcohols	Amides	Amines	Amino Acids	Aromatic chemicals	Brominated chemicals	Carbohydrates	Carboxylic acids	Esters	Phosphorylated chemicals	Polymer
R2	3,091 ab	6,596 b	9,340 b	5,146 a	15,468 e	1,742 ab	35,073 abcde	14,739 abc	0,394 a	0,000 a	3,066 ab
R3	1,526 ab	0,309 a	2,612 a	21,654 abc	4,643 abcd	1,529 ab	29,304 abcd	13,189 abc	11,481 c	5,250 cd	3,153 abc
R4	3,949 ab	1,118 a	0,775 a	24,775 bc	3,668 abcd	0,917 a	32,080 abcde	15,538 abc	1,904 ab	1,605 abcd	8,541 abcde
R5	4,164 ab	0,000 a	5,662 ab	23,399 abc	3,627 abcd	3,732 ab	21,359 ab	16,370 abcd	0,920 ab	5,408 d	10,359 bcde
VBR1_4w	0,772 a	1,420 a	0,573 a	19,366 abc	2,419 abc	1,626 ab	38,779 bcde	17,726 abcd	3,218 ab	3,630 abcd	3,771 abcd
VBR1_8w	1,151 a	0,856 a	0,384 a	21,228 abc	3,683 abcd	1,489 ab	31,583 abcde	23,388 abcde	4,695 abc	2,158 abcd	4,384 abcd
VBR1_12w	4,655 ab	2,564 a	0,703 a	14,861 ab	1,470 ab	1,877 ab	41,141 cde	24,397 bcde	1,104 ab	0,211 ab	2,017 a
VBR1_16w	2,573 ab	0,423 a	3,750 ab	23,962 bc	4,883 abcd	0,023 a	18,934 a	35,199 e	1,538 ab	2,045 abcd	1,670 a
VBR1_20w	1,979 ab	0,676 a	5,409 ab	11,489 ab	2,270 abc	2,624 ab	35,953 abcde	21,341 abcde	4,038 abc	4,208 abcd	5,013 abcd
VBR2_4w	1,580 ab	3,736 ab	0,733 a	18,541 abc	3,606 abcd	0,816 a	39,107 bcde	18,900 abcde	3,356 abc	0,300 ab	3,678 abcd
VBR2_8w	0,533 a	1,243 a	0,288 a	18,432 ab	1,830 abc	0,499 a	33,452 abcde	20,545 abcde	6,894 abc	0,505 ab	10,777 cde
VBR2_12w	6,660 b	2,150 a	1,621 a	20,212 abc	1,037 a	3,630 ab	21,818 ab	32,218 de	1,175 ab	2,523 abcd	1,958 a
VBR2_16w	1,736 ab	0,195 a	2,454 a	20,131 abc	7,002 abcd	0,981 a	28,875 abcd	15,489 abc	8,669 bc	4,857 bcd	4,610 abcd
VBR2_20w	1,535 ab	0,027 a	0,834 a	28,066 bc	0,973 a	1,114 a	36,130 abcde	15,107 abc	0,111 a	0,882 abcd	10,221 bcde
VL1_4w	2,867 ab	1,118 a	1,139 a	36,814 c	2,929 abcd	1,596 ab	29,909 abcd	7,995 a	3,274 ab	3,365 abcd	4,794 abcd
VL1_8w	1,806 ab	0,383 a	0,799 a	17,459 ab	8,192 cd	2,318 ab	34,931 abcde	15,761 abc	2,577 ab	0,712 abcd	10,063 bcde
VL1_12w	3,246 ab	0,077 a	0,798 a	11,527 ab	9,052 de	0,766 a	41,570 de	11,165 ab	0,112 a	2,338 abcd	14,352 e
VL1_16w	4,523 ab	0,488 a	5,230 ab	21,184 abc	2,869 abcd	0,230 a	25,798 abcd	29,104 cde	1,809 ab	1,846 abcd	1,919 a
VL1_20w	0,619 a	1,756 a	0,159 a	20,282 abc	7,826 bcd	1,796 ab	27,890 abcd	21,328 abcde	1,477 ab	0,635 abc	11,232 de
VL2_4w	2,932 ab	0,849 a	1,468 a	15,524 ab	4,867 abcd	0,472 a	39,388 bcde	19,360 abcde	0,700 ab	1,803 abcd	7,637 abcde
VL2_8w	0,559 a	0,570 a	3,127 ab	24,068 bc	2,723 abcd	0,595 a	34,597 abcde	19,881 abcde	2,755 ab	2,751 abcd	3,375 abc
VL2_12w	1,618 ab	0,367 a	1,137 a	11,685 ab	3,110 abcd	0,027 a	49,999 e	18,629 abcd	1,994 ab	1,252 abcd	5,181 abcd
VL2_16w	2,229 ab	2,365 a	1,129 a	22,772 abc	2,006 abc	5,401 b	22,216 abc	26,385 bcde	3,085 ab	1,043 abcd	6,371 abcd
VL2_20w	2,415 ab	0,943 a	1,168 a	26,991 bc	2,121 abc	1,434 ab	23,197 abcd	27,289 bcde	2,650 ab	1,686 abcd	5,106 abcd

* a,b,c,d,e are the mean values of the absorbance from the colour change (average well colour development) after subjecting the values to analysis using STATISTICA

* Means values of AWCD within columns which have a common letter suffix do not differ at a probability of 5%

R-bulk soil, VBR and VL-Landraces

5.3.3 Diversity Indices

The Shannon-Weaver diversity index was used to quantify the functional diversity of soil microbial populations. This was based on the amount of different carbon sources utilised by soil microbial populations in Biolog EcoPlates™, i.e., comparable to species richness in the soil. Depending on the soil samples from the two landraces of bambara groundnut sampled, varying percentages of carbon sources were utilized, with values ranging from 2.19 to 4.00 as seen in Table 5.2. These values show a higher diversity. Substrate evenness assumes a value between 0 and 1, with 1 representing a situation in which all species were equally abundant within a microbial population present in the samples. This means less variation in microbial populations between species, thus, less dominance, and higher diversity. Substrate evenness indices obtained in this analysis were moderately high and ranged between 0.64 and 0.94 as seen in Table 5.2.

Taking a closer look at the microbial richness (Shannon-Weaver Index) and microbial abundance (Evenness index) in Table 5.2, it is clear that microbial diversity was significantly changed by crops present, as well as the difference in crop growth stages. No significant changes in microbial richness were observed between the VBR1 and VBR2 landraces, irrespective of sampling time. Microbial richness in the VBR1 cultivar changed significantly over time from the fourth and eighth week to the twelfth and sixteenth week; whereas no significant change could be observed between the fourth and twentieth week. Microbial richness in the VBR2 landraces did not change significantly between sampling times.

Table 5.2: The Shannon-Weaver diversity index and the Evenness Index between the bambara groundnut landraces and specific sampling times

Treatment	Shannon-Weaver Index	Evenness Index
R2	2,187 ^a	0,643 ^a
R3	2,980 ^{ab}	0,769 ^a
R4	3,323 ^b	0,793 ^a
R5	2,562 ^{ab}	0,746 ^a
VBR1_4w	3,861 ^b	0,879 ^b
VBR2_4w	3,582 ^{ab}	0,819 ^{ab}
VL1_4w	3,162 ^a	0,773 ^a
VL2_4w	3,233 ^a	0,818 ^{ab}
VBR1_8w	3,863 ^b	0,879 ^{ab}
VBR2_8w	3,295 ^a	0,817 ^a
VL1_8w	3,283 ^a	0,828 ^a
VL2_8w	3,997 ^b	0,941 ^b
VBR1_12w	2,634 ^a	0,816 ^a
VBR2_12w	3,197 ^a	0,765 ^a
VL1_12w	3,063 ^a	0,843 ^a
VL2_12w	3,025 ^a	0,741 ^a
VBR1_16w	2,836 ^a	0,741 ^a
VBR2_16w	2,991 ^a	0,745 ^a
VL1_16w	3,188 ^a	0,794 ^a
VL2_16w	3,371 ^a	0,772 ^a
VBR1_20w	3,542 ^a	0,798 ^a
VBR2_20w	2,800 ^a	0,801 ^a
VL1_20w	3,444 ^a	0,859 ^a
VL2_20w	3,370 ^a	0,803 ^a

*Means followed by similar letters do not differ significantly ($p > 0.05$).

R-bulk soil, VBR and VL-landraces

Microbial richness and evenness in the VL1 cultivar did not change significantly over time, but microbial richness in the VL2 landraces differed significantly between the eighth and twelfth week. Microbial evenness in the VBR1 cultivar, on the other hand, changed significantly over time from the fourth and eighth week to the sixteenth week,

but no significant change could be observed between the fourth and twentieth week. Microbial evenness in the VBR2 landraces did not change significantly between sampling times.

Considering the various landraces, the highest average microbial diversity was observed in the VBR1 and VL2 landraces, while the lowest average microbial diversity was observed in the control (bulk soil). Between the VBR and VL landraces, the highest overall microbial diversity was observed in the VL landraces, while the overall lowest microbial diversity was observed in the VBR landraces.

According to the Shannon-Weaver and Evenness Indices, the highest microbial species richness and evenness was associated with the VBR1 landraces during the fourth and eighth week, and the VL 2 cultivar during the eighth week. Microbial species richness and evenness were the lowest in the VBR1 and VBR2 landraces during the twelfth and twentieth week, respectively, as well as in the twelfth week of both the VL1 and VL2 landraces.

5.3.4 Dendrogram constructed to compare Microbial richness and evenness over time

Differences in active bacterial functional diversity were determined by means of Principal Component Analysis (PCA), but since clear distinction between groups were challenging, cluster analysis was performed as an alternative measure to enable a 2-D visualisation of possible differentiation between landraces. Dendrograms were constructed to assign microbial functions within different landraces into groups, so that similar microbial function will appear in the same cluster, compared to landraces in other clusters as illustrated by the dendrogram in Fig. 5.2.

Nutrients released by decomposing plant material, as well as the composition of plant root exudates (that serve as food sources to soil microbial populations in the immediate vicinity of the plant roots), were greatly determined by the plants/crops present in the

sampled landraces. The composition of the nutrients released through decomposition or the plant root exudates attract microbial populations that were especially well-adapted to utilise the specific compounds rapidly, thus, contributing to the difference in CSUP, i.e. microbial functions, of soil microbial populations. From the dendrogram (Fig. 5.2), two main clusters can be distinguished: groups containing mainly the control samples (green block) and group containing the different bambara groundnut soils (red block). Furthermore, the red block contains two sub-clusters: one representing mainly the VL landraces (blue block), and the other sub-cluster representing mainly the VBR landraces (purple block). Based on the results obtained, the dendrogram thus, indicates that microbial richness present in the VL and VBR landraces are more similar to each other, compared to the microbial richness present mainly in the bulk soil samples.

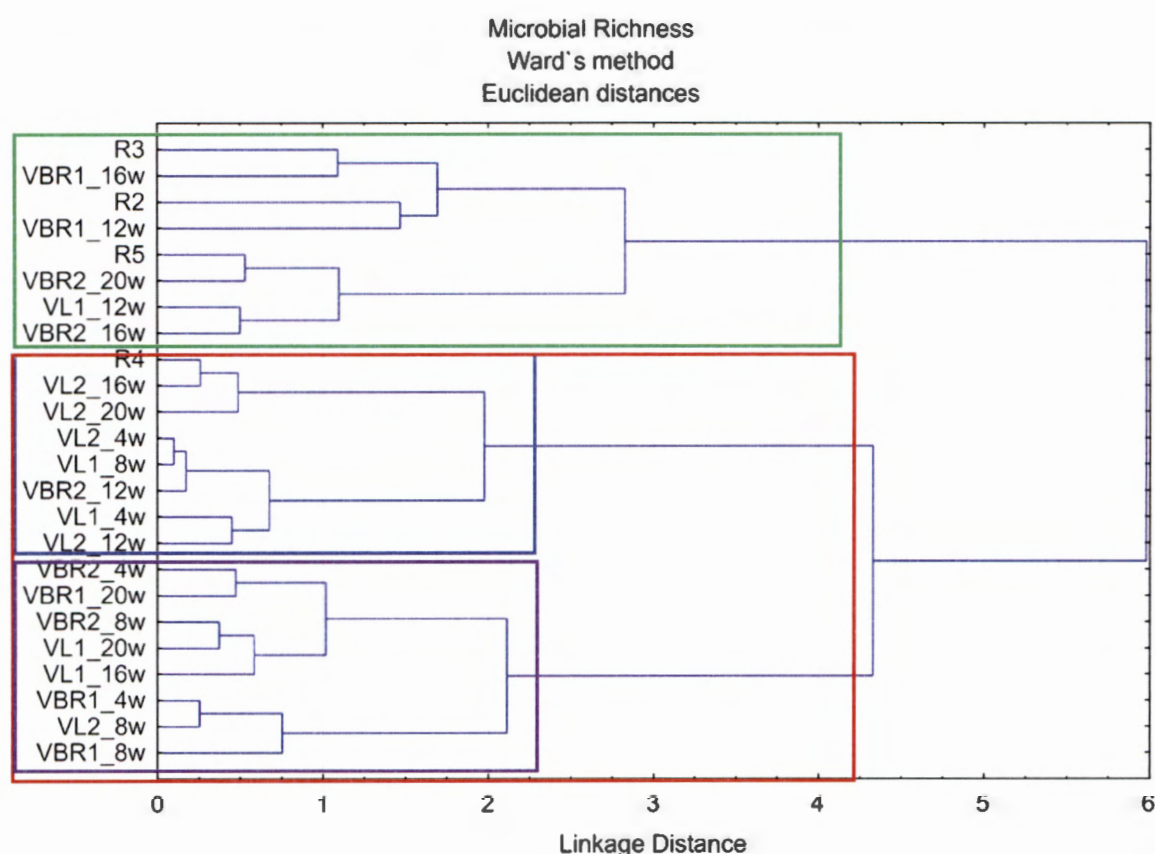


Figure 5.2: Dendrogram illustrating the differences in microbial richness between landraces soil samples over time.

When considering the microbial abundance found in the soil samples of the different landraces, two main clusters can be distinguished in the dendrogram (Fig. 5.3).

The top main cluster (green block) consists mainly of the VL1 samples, while most of the control (bulk soil) are present in the bottom main cluster (red block) with most of the VBR2 and VL2 landraces. Furthermore, the green block contained mostly the samples taken during the fourth, eighth and twentieth week, whereas, the red block contained mostly samples taken during the twelfth and sixteenth week. Considering the results obtained, the dendrogram thus, indicates that microbial evenness in landraces such as VL1, stabilised, i.e. occurred in a single main cluster, after the eighth week, whereas VL2 stabilised after the twelfth week. It is interesting to note that both the VBR1 and VBR2 cultivars fourth and twentieth weeks occurred in the same main cluster. The microbial evenness in the VBR1 cultivar's initial and final evenness differed slightly from each other since they were present in the same main cluster, but different sub-clusters. The VBR2 cultivar's initial and final evenness indices were present in the same sub-cluster in the same main cluster. This implies a change in microbial evenness during the different plant growth stages, but might be that microbial evenness returned to its original state.

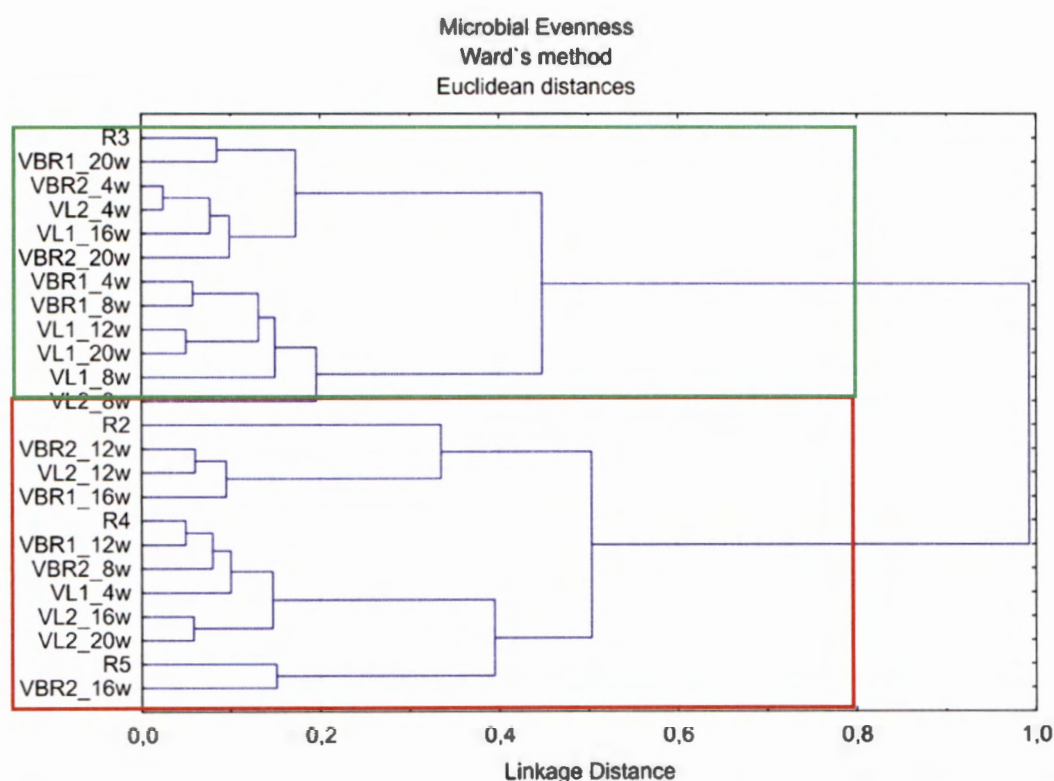


Figure 5.3: Dendrogram illustrating the differences in microbial evenness between landraces soil samples over time.

5.3.5 Cluster analysis to compare Microbial functional diversity

From the dendrogram (Fig. 5.4), no clearly distinguishable clusters, according to landraces or sampling times, could be observed. Most of the VBR landraces' fourth and eighth week sampling cluster were found mainly in the red block while the remaining sampling times were found in the green block. Furthermore, the red block also contains most of the VL landraces initial and final sampling times while the intermediate sampling times clustered in the green block. Based on the results obtained, the dendrogram thus indicates that the functions performed by the soil microbial communities in the VBR landraces changed over time as the crop matured. Also, functions performed by the microbial communities in the VL landraces reverted back to their original functions after changing according to the needs of the maturing crops.

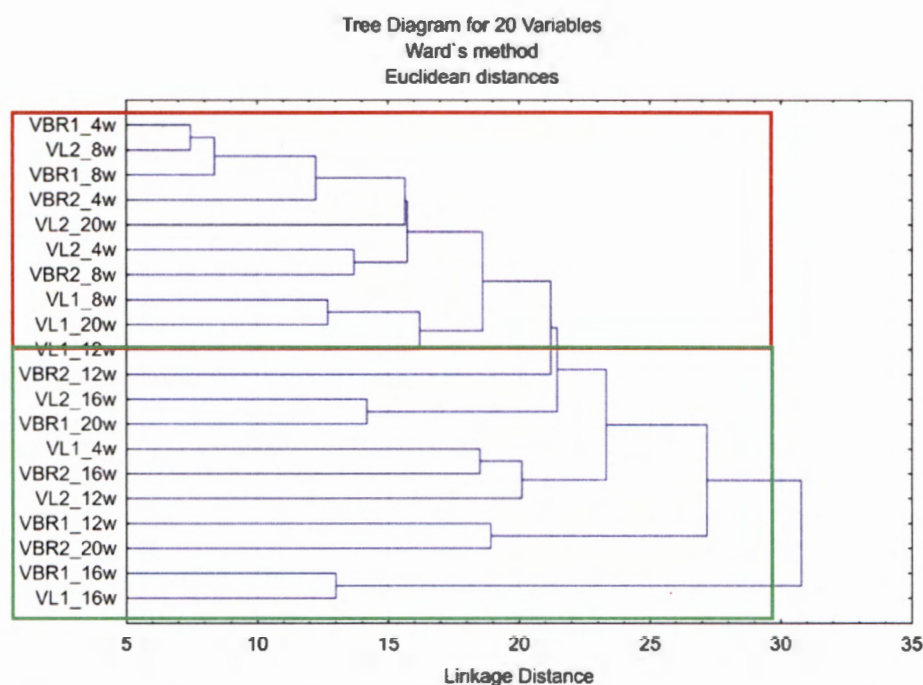


Figure 5.4: Dendrogram illustrating the differences in microbial functional diversity between different soil samples of landraces over time

To determine significant differences in functional diversity of microbial communities present in the different landraces at various sampling times, a box and whisker plot was constructed (Fig. 5.5a). From the diagram, significant differences in microbial functions were observed between VBR1 and VBR2 during the fourth week, while the microbial function changed significantly from the eighth and twelfth, as well as from the fourth to the twentieth week in the VBR1 cultivar. No significant differences in soil microbial functions were observed in either the VBR2 or VL1 landraces from the fourth to the twentieth week. The VL2 cultivar, on the other hand, differed significantly from the VL1 cultivar during the eighth week. Within the VL2 cultivar, significant differences in microbial functions were observed between the fourth and the eighth week, and again between the eighth and the sixteenth week. It should be noted that, at the twentieth week, no significant difference could be observed in the microbial functions between the VBR1, VBR2, VL1, or VL2 landraces.

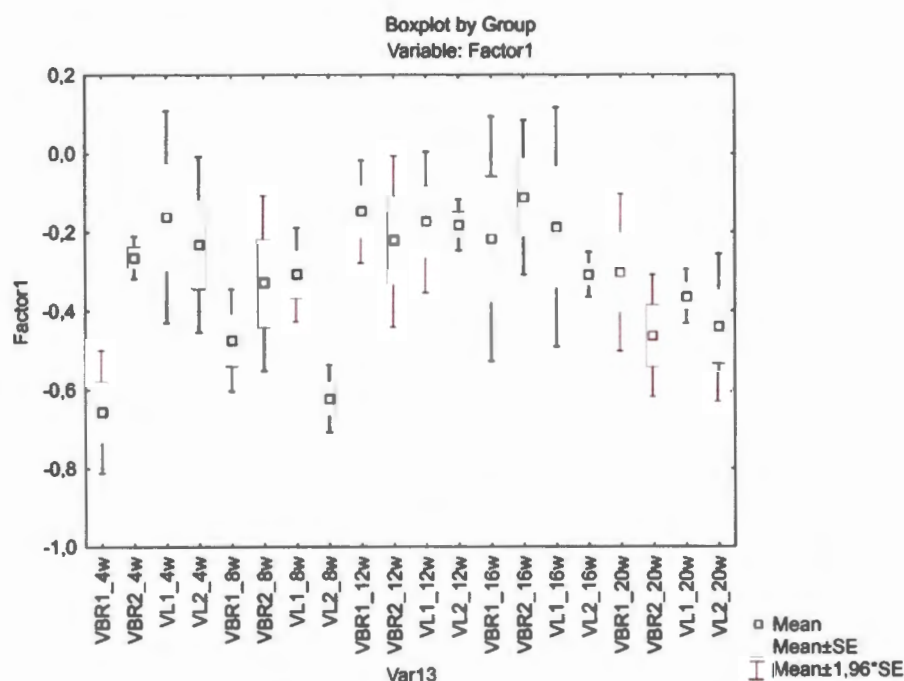


Figure 5.5: (a) Box and whisker plot of carbon source utilisation profiles for microbial communities present in different landraces soil samples over time

Figure 5.5 b, represents the box and whiskers plot constructed to determine the significant difference in the functional diversity of the various landraces at the different growth stages corresponding to the monthly time interval. It was observed that, there was a significant difference between the landrace VBR1 and the others while at 12 weeks there was no significant difference observed in the soil microbial functional diversity. In week 8, there was significant difference between the VL2 landrace and others while in week 16, the significant difference between landraces was not appreciable. In week 20, there was significant difference in the soil microbial function of VBR1 compared with the others while among the control samples or bulk soil, R4 was significantly different from the others. Among the VBR landrace across the sampling time or growth stages, VBR1 at week 4 was significantly different from the rest while VL2 cultivar at week 8 was also different from the others. This box and whiskers plot showed that the microbial activities at weeks 4 and 8 across the growth

stages were quite significant compared to others. Microbial activities in bambara groundnut rhizosphere was found to be diverse at both 4 WAP and 8 WAP which relates to the vegetative and reproductive phase of the plant. The box and whiskers plot also revealed groups or clusters found among cultivars across the growth stages. The bulk soil clustered together except for R4, the landraces (VBR and VL) weeks 4 and 8 clustered together, the landraces in weeks 12 and 16 also clustered together while week 20 cultivar clustered on its own. This helps to show the dynamics of diversity across landraces and across growth stages.

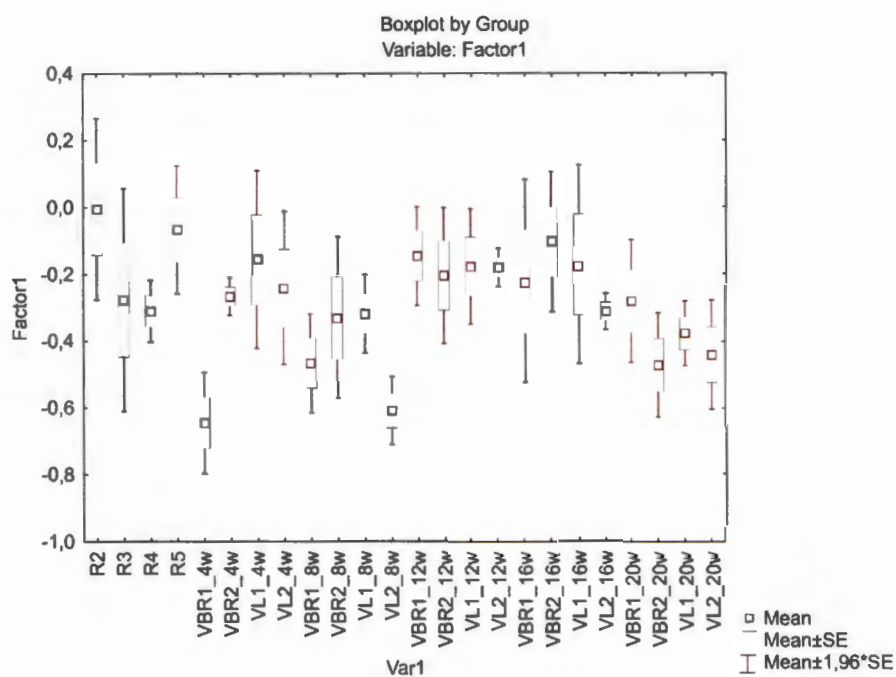


Figure 5.5: (b) Box and whiskers plot constructed to determine the significant difference in the functional diversity of the bulk soil (control) and the various landraces at the different growth stages

5.4 Discussion

This study is the first of its kind in South Africa where the influence of bambara groundnut plant growth stages on metabolic activities of microbial community structure is investigated. Other studies have been carried out on maize plants (Baudoin et al., 2002), potato (Van Overbeek and Van Elsas, 2008), sugar beet, wheat and pea (Houlden et al., 2008). Functional diversity of microbes in soil is directly related to the types of agricultural practices carried out. These agricultural practices include, the cropping system practiced, application of fertilizer, the types of crops grown and the growth stages of the crop. This functional diversity is evidenced in the type and abundance of carbon utilized in the soil (Habig and Swanepoel, 2015).

Carbon source utilization at different growth stages of Bambara groundnut

Average well colour development observed in carbon sources inoculated with soil samples reflects the ability of the microbes to utilize them and it is directly related to the number of species present in the soil as observed in this study and previous studies (Yang et al., 2013; Chen et al., 2008).

The highest carbon sources utilised in the bulk soil of this study were 6 out of the 11 classes while landraces VBR1, VBR2, VL1 and VL2 utilised the highest carboxylic acid, esters, amino acids/polymers and carbohydrates respectively. This study shows a decrease in the quantity of carbon utilized from the rhizosphere soil of growth stages to the bulk soil. This was in agreement with Sugiyama et al. (2014) whose metabolic and microbiological activities were higher in the rhizosphere soil compared to the bulk soil. This is because it has been reported that in the rhizosphere higher number of bacteria flourish as a result of availability of nutrients (Bakker et al., 2012).

The bulk soil in this study had the lowest average microbial diversity while the highest average microbial diversity was observed in the VBR1 and VL2. The Shannon-Weaver index in this study varied between 2.19 and 4.00 and agrees with Magurran (1988) whose index fluctuated between 1.5 and 3.5 but seldomly goes beyond 4.5. The index was 4.00 for soil samples from growth stages VL2 at 8 WAP which corresponds to seeding and podding time and was the highest while that of bulk soil was the least 2.19. These results suggest that the bacterial community present in the rhizosphere at the different growth stages increased in complexity from the bulk soil. This result is in contrast with the study from García-Salamanca et al. (2013) where the bulk soil was more complex than the rhizosphere.

The Evenness index, on the other hand, is used as an indicator of how abundant species are within a soil microbial community, i.e., how close in “numbers” each of the microbial species is in a soil microbial community. If abundances/quantities of different species in a community are measured, it will invariably be found that some species are rare, whereas, others are more abundant/dominant (Habig and Swanepoel, 2015).

The microbial abundance (richness index) in this study ranged from 0.64 and 0.94 with cultivar VL2 at 8 week being the highest while bulk soil (control), R2 was the lowest. This value varied from 0.64-0.79 in the bulk soil (control) to 0.77-0.87, 0.81-0.94, 0.74-0.84, 0.74-0.79 and 0.79-0.85 across landraces at 4, 8, 12, 16 and 20 WAP respectively in agreement with Magurran (1988). Between the 4 WAP and 12 WAP, the abundance of microbial species was high which shows that the different microbial species were reaching a point where they were all present in almost equal amounts – there is a very low level of dominance (Habig and Swanepoel, 2015).

Singh et al. (2007), observed that there was no significant impact of the species of crop planted and the bacterial community, this was only observed in this study with microbial evenness using

cultivar VBR2 at the 4 WAP and harvest. There was significant difference in cultivar VL1 and VL2, showing its impact on the bacterial community (Lamb et al., 2011). Ideally, a healthy soil is characterised by high microbial species richness, as well as an equal distribution of species throughout a soil microbial community, i.e. a combination of high Shannon-Weaver and Evenness indices (Thompson and Kao-Kniffin, 2016). Plant species or cultivars have been observed to influence the composition of the microbes in soil the community (Eisenhauer et al., 2010; Berg and Smalla, 2009). Also, the richness of a plant species is seen in the different influences exerted on soil nutrients and mechanisms of root exudation (Berg and Smalla, 2009). This ability of plants to change the diversity, distribution, quantity and functions of microbes in the rhizosphere could be attributed to the ability of microbes to create a microenvironment within the rhizosphere. This microenvironment is rich in carboxylic acid, carbohydrates and amino acids as a result of the diverse exudates from plants (Uroz et al., 2010).



Plant developmental growth stages were observed not to have any detectable influence on the bacterial community of maize at the first sample (Cavaglieri et al., 2009). But in this study, there was significant change overtime which corresponds to growth stages in microbial evenness from the 4 WAP to the 16 WAP in cultivar VBR1.

Generally, in this present study, the metabolic functions of the soil significantly changed through the plant growth stages with the lowest in the bulk soil and the highest found between 4 WAP and 8 WAP of landraces VBR1 and VL2, this also corresponds to the podding and seeding time. This is in agreement with the study on maize developmental growth stages by Cavaglieri et al. (2009). Microbial richness and evenness varied by growth stages and by landraces in this study, this was also observed in the study by Inceoglu et al. (2011), as the relative abundance of microbes in the soil increased from the bulk soil to all the growth stages of potato.

In general, it would seem that the presence of bambara groundnut played a bigger role than the changes in microbial richness over time. The fact that the microbial richness in the near-harvest samples of the VBR landraces grouped with the microbial richness of the control (bulk soil), might indicate that microbial richness in the VBR landraces returned to its original state.

5.5 Conclusion

It is evident that carbohydrates, carboxylic acids and amino acids were the most utilised carbon sources and the soil microbial functions was greatly affected by the presence of the bambara groundnut and their development stages. Some of the landraces differed significantly from their duplicate parts (i.e. VL1 and VL2) during the initial weeks and changed during the course of the study. Finally, the physiological activities of the rhizosphere bacterial communities of bambara groundnut measured by BIOLOGTM assay suggested that the metabolic capabilities of the rhizosphere were higher in the soil during the growth stages compared with the control. This capability was also more obvious during the flowering stage (4 to 8 WAP). Although statistically insignificant, the VL treatments exhibited the highest carbon source utilisation, whereas, the VBR treatments exhibiting the lowest carbon source utilisation – even lower than the control treatments. This observation is important in agriculture in the sense that planting of landrace VL if encouraged, could increase microbial diversity of the soil which invariably increases soil fertility and crop production.

Although carbon source utilization profiles can be used as indicators of soil quality, these indicators are sensitive to changes in various environmental factors, crops planted, and soil and agricultural management practices. It is inadvisable to analyze composite soil samples, since it could give rise to statistical constraints, which, in turn, could result in limited analyses and incorrect conclusions. It is therefore, recommended that trends in carbon source utilization profiles

be monitored over time in order to attain a more complete reflection that different agricultural practices might have on microbial diversity as an indicator of soil fertility and health.

CHAPTER SIX

GC-MS ANALYSIS OF VOLATILE ORGANIC COMPOUNDS FROM BAMBARA GROUNDNUT RHIZOBACTERIA AND THEIR ANTIBACTERIAL PROPERTIES

Abstract

Bacterial metabolites have been observed to be important in new drug formulation for both plant, animals and human beings. The aim of this study was to identify the different bioactive compounds found in three rhizobacterial isolates (*B. amyloliquefaciens*, *B. thuringiensis* and *Bacillus* sp) from the rhizosphere of bambara groundnut and to assay for their antibacterial properties. Gas chromatography mass spectrometry (GC-MS) was used to carry out the analysis using seven extraction solvents. Solvents used were benzene, hexane, petroleum ether, chloroform ethyl acetate, butanol and ethanol. In the GC-MS analysis, 68 compounds were identified based on peak area percentage, retention time, molecular weight and formula. Among the bioactive compounds produced by the three selected isolates were carbaxazide, tropone, phthalan and p-xylene. Antibacterial analysis revealed that compounds of butanol extracts are more potent and inhibited the growth of the 4 test organisms, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Microbacterium cryophilus* and *Enterococcus faecalis*. These results suggest that the 3 isolates are quite rich in the production of bioactive compounds that are also very effective antibacterial agents. These volatile organic compounds are promising compounds for more antibacterial bioactivity development.

Keywords: Antibacterial, Bambara groundnut, GC-MS, secondary metabolites,

6.1 Introduction

Many rhizobacterial species are involved in plant growth promotion and biocontrol activities. Diverse mechanisms are applied directly and or indirectly such as indole acetic acid production

and phosphate solubilisation which have been used to achieve this PGP potential (Ajilogba and Babalola, 2013). Others are able to suppress or antagonise the growth of competing or pathogenic microbes that can cause disease in or/and around the plant by the production of hydrogen cyanide (HCN), siderophores, antibiotics and other antibacterial compounds such as 2,4-diacetylphloroglucinol and phenazine derivatives (Szentés et al., 2013).

Rhizobacteria are recognized as potential sources of bioactive substances and they are therefore involved in the production of secondary metabolites in the rhizosphere (Kanchiswamy et al., 2015). The genera involved in secondary metabolite production in the rhizosphere include *Pseudomonas*, *Bacillus*, *Klebsiella*, *Azotobacter*, *Azospirillum*, *Azomonas*, *Rhizobium*, *Bradyrhizobium* and *Mesorhizobium* (Ahemad and Kibret, 2014). Secondary metabolites secreted by rhizobacteria are involved in different biological activities such as antimicrobial, antiprotozoal, antihelminthic, antitumor, anticancer, antifungal and immunosuppressant (Graça et al., 2013; Sadrati et al., 2013).

Bacillus spp have been known to secrete secondary metabolites such as antifungal, antibacterial and siderophores. Antibiotics produced by *Bacillus* spp include a wide variety of bacteriocin and have been found to be important as antibiotic precursors, biocontrol agents of plant pathogens and as biopreservatives of beverages and food systems (Sansinenea and Ortiz, 2011). An important example of bacteriocin includes megacin secreted by *B. megaterium*. Khalil et al. (2009) observed that bacteriocin was produced and secreted by *B. megaterium* in both solid and liquid medium and it was able to inhibit the growth of pathogenic bacteria thereby serving as a biopreservative.

Hydrogen cyanide and 2,4-DAPG were observed to function as nematicides and contributed to the toxicity of the bacteria *P. fluorescens* CHA0 against nematode *Caenorhabditis elegans* by repelling the nematode (Neidig et al., 2011). Bacilysin, a simple peptide antibiotic was observed

to possess both antifungal and antibacterial properties. It is a biocontrol agent against *Erwinia amylovora*. It was isolated from several strains of *B. pumilus*, *B. subtilis* and *B. amyloliquefaciens* (Steinborn et al., 2005; Arguelles-Arias et al., 2009). Other secondary metabolites have been documented to be secreted by rhizobacteria from different crops for biocontrol and plant growth activities.

However, bioactive substance and secondary metabolites emitted from the rhizobacteria of bambara groundnut have not been studied. The main aim of the study was to identify and extract bioactive compounds with the use of diverse solvent and then assay for their antibacterial activities.

6.2 Materials and Methods

6.2.1 Cultivation of the isolates

Each of the selected *Bacillus* spp (*B. amyloliquefaciens*, *B. thuringiensis* and *Bacillus* sp) was isolated from bambara groundnut rhizospheric soil samples collected from North-West University agriculture farm, Mmabatho, Mafikeng, South Africa. The isolates were maintained on Luria Bertani agar medium (Merck). Each actively growing pure culture of the isolates was used to inoculate 100 ml of Luria Bertani broth (Merck) in a 250-ml Erlenmeyer flask. The 3 *Bacillus* spp from where the VOCs were extracted were not grown in medium based on root exudates in the laboratory. The seeds grown on the field were soaked in *Bacillus* inoculum, which means the *Bacillus* spp grew in the rhizosphere having root exudates on the field which is as good as and/or more effective than using root exudates medium in the laboratory.

6.2.2 Extraction and partial purification of the crude extracts from the rhizobacteria isolates

The fermentation medium was centrifuged for 20 min at 8000× g to remove the bacterial cells. The supernatant was shared in 4 equal volumes of 60 ml, and then each was extracted with 60 ml of organic solvent. A range of extraction solvents was screened for effectiveness, including

petroleum ether, n-hexane, chloroform, ethyl acetate, benzene, ethanol and n-butanol. The organic extracts were evaporated to dryness using a rotary evaporator (Stuart RE300) and lyophilized using a BenchTop Pro 8L ZL-105 lyophilizer (Sp Scientific, USA). The resulting dry extracts were recuperated in 1 ml of methanol and subjected to biological assay against test organisms.

6.2.3 Biological assay

Sensitivity test of the partially purified fractions obtained from the crude extracts of the isolates were tested against the test organisms by agar-well diffusion method (Cappuccino and Sherman, 2011) using Mueller Hinton agar (Sigma Aldrich). Different concentrations of compounds (0 to 1000 µg/ml) were prepared in dimethyl sulphoxide (DMSO) and assayed against the test organisms. The organisms used in this assay are *B. cereus*, *P. aeruginosa*, *E. feacalis* and *M. cryophilus* which are locally isolated organisms (LIO). *B. cereus* is a food poisoning pathogen worldwide with serious health implication, so it is very important in the food industry and agriculture (Tewari and Abdullah, 2015). *E. feacalis* and *P. aeruginosa* are among the diverse type of bacteria capable of causing infection in man, animals and plants causing plants death 7 days post inoculation (Jha et al., 2005; Walker et al., 2004). The bacteria used for the tests were resistant to at least one known antimicrobial agent (Adegboye and Babalola, 2013). Inhibition zones were measured after 24 h incubation at 37°C.

6.2.4 Antagonism assay against phytopathogenic fungi and bacteria

Potato dextrose agar (PDA) medium was used as the medium to assay for the antifungal activities of 8 isolates against *Fusarium graminearum*. This was carried out by inoculating the pathogenic fungi at the centre of the medium and then streaking the isolates on the medium 3 cm away from the fungi. The clear zones between isolates and fungi after incubation for 4 to 7 days at room temperature indicated antagonist interaction between them.

Antagonistic activity of isolates against Gram-negative and Gram-positive bacteria was screened by using perpendicular streak method (Parthasarathi et al., 2010). In perpendicular streak method, Luria Bertani agar (Merck) was used and each plate was streaked with test bacteria isolates at the centre/diameter of the plate and incubated at 30°C for 48 h to allow optimum growth. Later, 24 h fresh sub-cultured isolated bacteria were prepared and streaked perpendicular to the test isolates and incubated at 37°C for 24 h. The experiment was carried out in triplicate.

6.2.5 Gas chromatography-mass spectrometry (GC-MS) analysis

The partially purified active fractions were analyzed by GC-MS. The analyses of the compounds in the active fractions were run on a GC-MS system (Agilent GC: 6890, with a 7683B Autosampler). The fused-silica Rxi-5Sil MS capillary column (30 m 0.25 mm ID, film thickness of 0.25 mm) was directly coupled to an Agilent variant. Oven temperature was programmed (35°C for 5 min, then 35-300°C at 10 °C/min) and subsequently, held isothermal for 20 min. The injector port; was 250°C, the transfer line: 290°C, splitless. Volume injected: 0.2 ml and the column flow rate was 1 ml/min of 1 mg/ml solution (diluted in chloroform). The peaks of components in gas chromatography were subjected to mass-spectral analysis.

The MS was a LECO Pegasus 4D recording with a EI-source at -70 eV; the solvent delay was 9 min. scan time 1.5 s; acquisition rate 10 spectra/second; mass range 50-1000 amu; detector voltage 1800 V, and Ion source temperature: 250°C. Data were recorded in TIC mode. The software adopted to handle the mass spectra and chromatograms was an Agilent chemstation software. The constituents were identified after comparing with available data in the GC-MS library in the literatures.

6.2.6 Mass spectrometer

The GC-MS mass spectrum data were analyzed using Mnova 11.0.1 and the database of National Institute Standard and Technology (NIST) was used to interpret analyzed data. Comparison of the mass spectrum of the unidentified components released by the bacterial isolates was carried out against the mass spectrum of already known components available in the NIST library. The name and structure of the components of the test materials were confirmed.

6.3 Results

6.3.1 Cultivation of Rhizobacteria isolates

Three rhizobacterial isolates used in the field were BAMhi, BAMli and BAMr. These have been identified as *Bacillus* strains and they have been deposited in the GenBank (Table 6.1).

Table 6.1: Results of 16S rDNA gene sequence similarities of bacterial isolates and GenBank accession numbers using BLASTn algorithm

Isolate code	Sequence length (bp)	Closest related strain in database	Accession number	Similarity (%)	E-value
BAMhi	1443	<i>B. amyloliquefaciens</i>	KX809652	99	0
BAMli	1388	<i>B. thuringiensis</i>	KX809653	99	0
BAMr	1058	<i>Bacillus sp.</i>	KX588095	98	0

6.3.2 Antibacterial assay of Volatile Organic Compounds (VOCs)

Antagonistic assay against selected bacterial pathogens was carried out on the crude extracts of VOCs reveals that butanol extracts showed more antagonism against the selected pathogens (Table 6.2).

Table 6.2: Antibacterial activities of the active fractions against test organisms

Test organisms	Zone of inhibition (mm)													
	Butanol		Hexane				Petroleum ether				Ethyl acetate			
	<i>Bacillus sp</i>	<i>B. amyloliquefaciens</i>	<i>B. thuringiensis</i>	<i>B. thuringiensis</i>	<i>B. thuringiensis</i>	<i>B. thuringiensis</i>	<i>B. thuringiensis</i>	<i>B. thuringiensis</i>	<i>B. thuringiensis</i>	<i>B. thuringiensis</i>	<i>B. thuringiensis</i>	<i>B. thuringiensis</i>	<i>B. thuringiensis</i>	<i>B. thuringiensis</i>
<i>E. faecalis</i>	18	21	23	6	8	4	4	0	0	0	0	12	7	
<i>B. cereus</i>	18	28	16	6	12	8	0	0	0	6	12	8	4	
<i>M. cryophilus</i>	12	24	19	8	8	12	7	0	0	0	10	18	8	
<i>P. aeruginosa</i>	15	15	26	12	0	0	0	0	0	4	17	2	6	

6.3.3 GC-MS analysis carried out on the rhizobacteria isolates

The GC-MS results revealed that from the three *Bacillus* strains used in the field, 31 VOCs were identified from isolate *B. thuringiensis* which was highest number of metabolites from the 7 different extraction solvents used. *B. amyloliquefaciens* had the least with 15 VOCs identified while isolate *Bacillus sp.* had 22 VOCs identified (Table 6.3). With *B. amyloliquefaciens*, the solvent with the highest metabolites is hexane with 5 VOCs while benzene and chloroform were the least with 1 VOC each. With *B. thuringiensis*, the solvent with the highest VOC was methanol having 15 while chloroform was the least with only 1. In *Bacillus sp.*, the solvent with the highest VOCs was also hexane with 13 while benzene and chloroform had no hits. This shows that hexane detected the highest number of VOCs (22) from the 3 isolates while chloroform detected the least (2) while a total of 68 VOCs were detected from the isolates (Fig 6.1 and Table 6.3) in which case none of them has been identified before from the bacterial isolates of bambara groundnut rhizosphere but some have been identified from rhizobacteria of other plants (All spectra drawn showing peaks and compound structure and relative content (%) are in the Appendix).

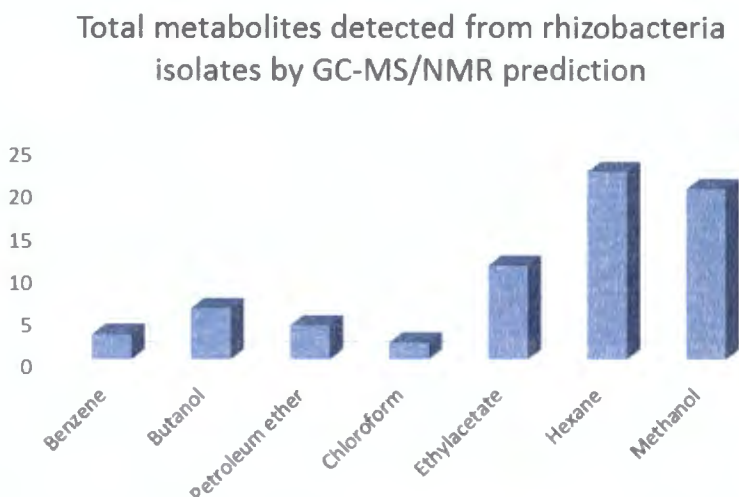


Figure 6.1: Total number of metabolites detected by GC-MS in *B. thuringiensis*, BAMhi, BAMr using 7 different extraction solvents

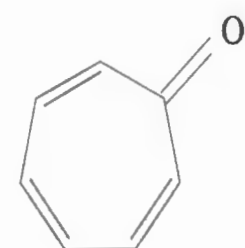
Some VOCs were commonly produced by the three isolates and they include tropone, p-xylene, ethylbenzene, carbazic acid and, isocarbaxazide (5). Others common to *B. thuringiensis* and *B. amyloliquefaciens* are formic acid butyl ester, paraldehyde and dimethylfulvene (3). Those common to *B. amyloliquefaciens* and BAMr are 2,2-dimethylhexane-3-one and formic acid 2-methylpropyl ester (2). While those common to *B. thuringiensis* and BAMr are fumaronitrile, phenylethylalcohol, benzyl 2-chloroethyl sulfone, N-hydroxymethylmethyl-2-phenylacetamide, tropeolin, benzene butyl, n-propylbenzene, phthalan, carbobenzoxyhydrazide and 1,2-dimethylbenzene (10). Only 15 were not common across the 3 isolates and from *B. amyloliquefaciens*, they are, s-(+)-1,2 propanediol and 2(3H)-furanone dihydro-5-methyl (2). From *B. thuringiensis*, they are, 2, 2-dimethylhexanone, 2-oxopropanoic acid, 2,4-dimethylhexane, dihexylether, tridecylamine, 2,4-dimethylhexane, cetane and hexane,1,1'-oxybis

(8). Also from *Bacillus sp.*, they are Acetic butyl ester, isooctanol, benzene, tridecane and 1,3-dimethylbenzene (5). (Table 6.3).

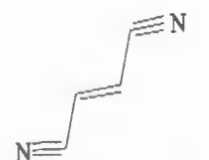
6.3.4 Analysis of GC-MS chromatograms for *B. amyloliquefaciens*

6.3.4.1 Benzene and Butanol extract

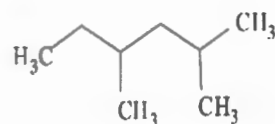
The GC-MS chromatograms of *B. amyloliquefaciens* benzene extract reveals a prominent peak which correspond to tropone on comparison with the mass spectra of the NIST library. Its intensity or peak area was 24.06% and 5.45 retention time (RT). The characterized peak and RT of the VOCs from butanol crude extract that is formic acid butyl ester and 2,2-dimethylhexane-3-one were 19.98%/5.64 and 41.08%/7.72 respectively (Table 6.3 and Fig. 6.2).



Tropone



Fumaronitrile



2,4-Dimethylhexane

Figure 6.2: Structures of compounds from benzene fraction for all isolates

Table 6.3: VOCs profile of *B. amyloliquefaciens*, *B. thuringiensis*, *Bacillus* sp. detected by GC-MS

Extraction solvents	VOCs detected by GCMS from <i>B. amyloliquefaciens</i>	VOCs detected by GCMS from <i>B. thuringiensis</i>	VOCs detected by GCMS from <i>Bacillus</i> sp.
Benzene	Tropone	Fumaronitrile Tropone	
Butanol	Formic acid butyl ester 2, 2-dimethylhexane-3-one	Formic acid butyl ester 2, 2-dimethylhexanone	Acetic acid, butyl ester 2, 2-dimethyl-3-hexanone
Chloroform	Trichloromethane	Bromodichloromethane	
Ethylacetate	Paraldehyde Ethylbenzene Dimethylfulvene p-Xylene	Paraldehyde 2-oxopropanoic acid Ethylbenzene Dimethylfulvene p-Xylene	P-xylene 1,2-Dimethylbenzene
Hexane	2(3H)-furanone dihydro-5-methyl Formic acid 2-methylpropyl ester Ethylbenzene Carbazic acid Isocarboxazid	2,4-Dimethylhexane Dihexylether Ethylbenzene Tridecylamine	Phenylethylalcohol Benzyl 2-chloroethyl sulfone N-hydroxymethylmethyl-2-phenylacetamide Tropolin Benzene, butyl n-propylbenzene Phthalan Carbobenzoxyhydrazide Isooctanol Formic acid, 2-methylpropylester Ethylbenzene Isocarboxazid Carbazic acid Tropone
Methanol	Paraldehyde s-(+)-1,2 propanediol	Phenylethylalcohol Benzyl 2-chloroethyl sulfone N-hydroxymethylmethyl-2-phenylacetamide Tropolin Benzene, butyl-n-propylbenzene	Benzene Fumaronitrile

Extraction solvents	VOCs detected by GCMS from <i>B. amyloliquefaciens</i>	VOCs detected by GCMS from <i>B. thuringiensis</i>	VOCs detected by GCMS from <i>Bacillus sp.</i>
Petroleum ether		Phthalan	
		Carbobenzoxyhydrazide	
		Cetane	
		Hexane, 1,1'-oxybis	
		Ethylbenzene	
		Isocarboxazid	
		Carbazic acid	
		p-Xylene	
		1,2-Dimethylbenzene	
		2,4-Dimethylhexane	1,3-Dimethylhexane
		Ethylbenzene	Tridecane

6.3.4.2 Chloroform and ethyl acetate extract

The characterized and identified VOCs from chloroform extract that is trichloromethane and that of ethyl acetate extract that is paraldehyde, ethylbenzene, dimethylfuvene and p-xylene had RT corresponding to 5.4, 3.46, 5.05, 5.35 and 6.14 respectively. Dimethylfulvene exhibited the highest intensity or peak area with 89.11% while trichloromethane exhibited the lowest with 27.37% and paraldehyde, ethylbenzene and p-xylene were 83.79%, 52.37% and 47.92% respectively (Table 6.3, Fig. 6.4 and Fig. 6.5).

6.3.4.3 Hexane and methanol extract

The GC-MS chromatograms of hexane extract revealed that even though there were 6 peaks, only 4 peaks indicated the presence of secondary VOCs though the 2 peaks without VOCs presence were higher than some of the peaks with VOCs presence (100% and 39.94%). On comparison of the 4 peaks with the mass spectra from NIST library, the highest peak area of 86.72% and RT 3.06 picked 8 VOCs that were characterized and identified as phenylethylalcohol, benzyl 2-chloroethyl sulfone, N-hydroxymethylmethyl-2-phenylacetamide, tropeolin, benzene, butyl-, n-

propylbenzene, phthalan and carbobenzoxyhydrazide. The combination of VOCs at this specific RT and peak area was also noticed with the isolate *Bacillus sp.* using hexane extract and BAMli using methanol extract. The other 2 peaks were compared with the mass spectra from NIST library and were characterized and identified as 2(3H)-furanone dihydro-5-methyl and Formic acid 2-methylpropyl ester with the RT of 3.47 and 3.76 respectively and peak area of 9.65% and 6.25% respectively. The 4th peak also had a combination of VOCs ethylbenzene, carbazic acid, isocarboxazid with the RT of 5.05 and peak area of 32.72%. The GC-MS chromatogram of methanol extract showed 3 peaks out of which one of them though with peak area of 100% had no hit. The other 2 peaks indicated presence of VOCs that were compared with the NIST library and where characterized and identified as paraldehyde and s-(+)-1,2 propanediol. Retention time and peak area were highest in s-(+)-1,2 propanediol (3.47 and 71.53) while paraldehyde was 3.28 and 56.22% respectively (Table 6.3, Fig. 6.3 and Fig. 6.6).

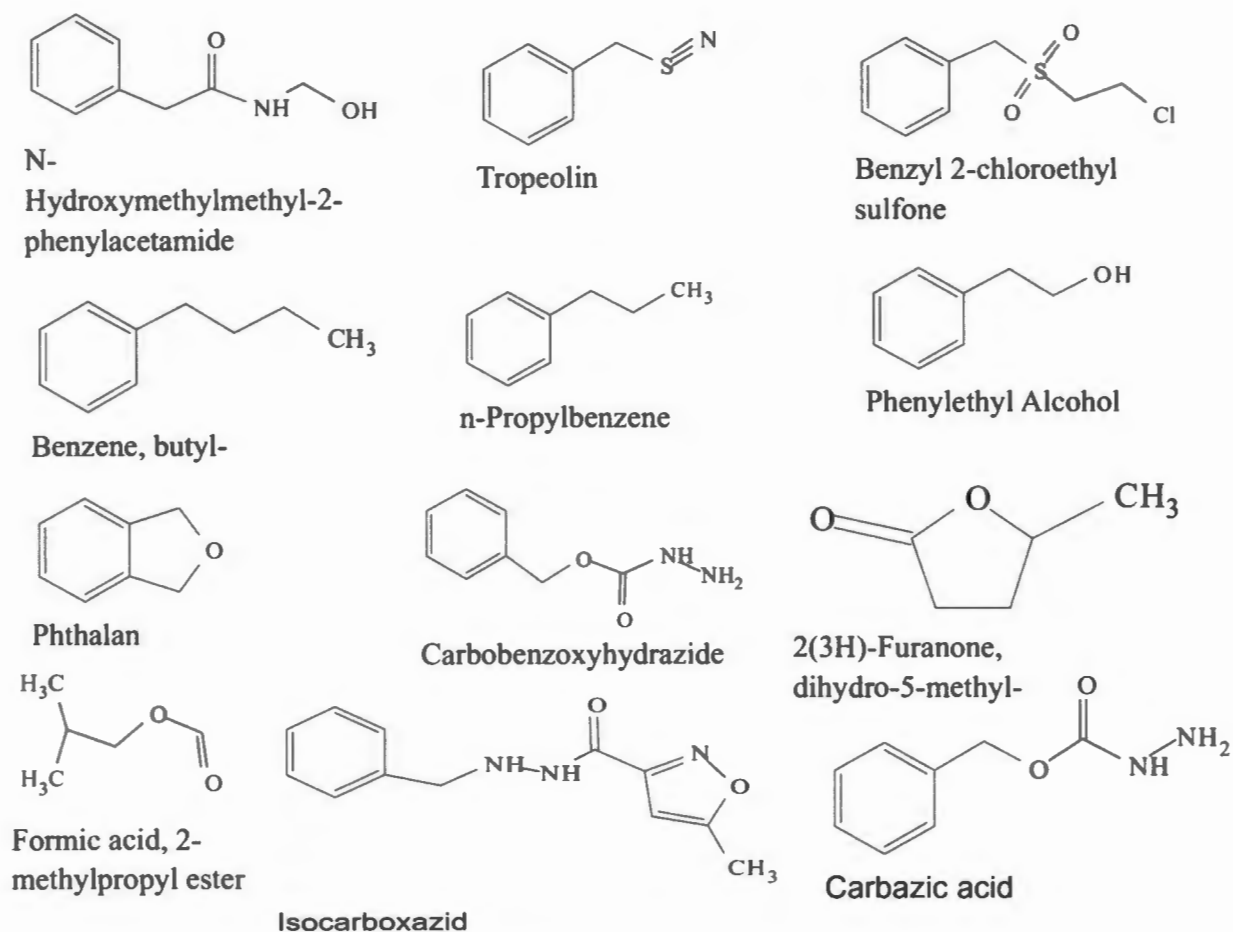
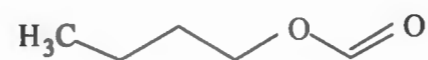
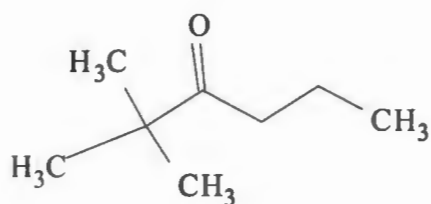


Figure 6.3: Structures of compounds from hexane fraction for all isolates

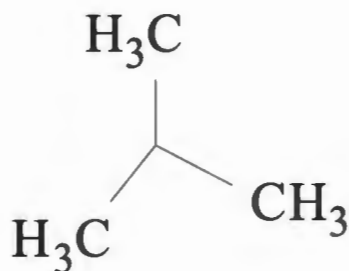


Formic acid, butyl
ester

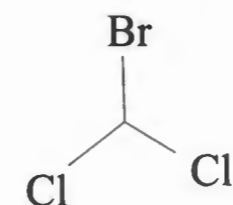


2,2-
Dimethylhexan-3-one

Figure 6.4: (a) Structures of compounds from butanol fraction for all isolates

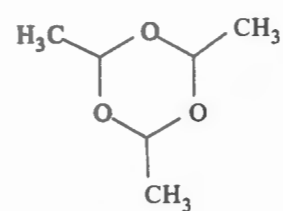


Trichloromethane

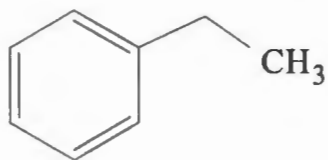


Bromodichloromethane

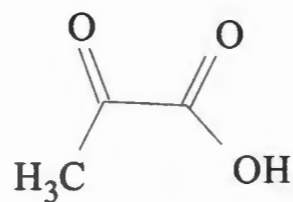
Figure 6.4: (b): Structures of compounds from chloroform fraction for all isolates



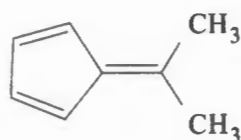
Paraldehyde



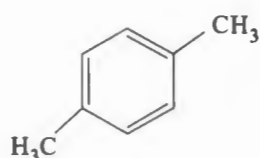
Ethylbenzene



2-oxopropanoic acid

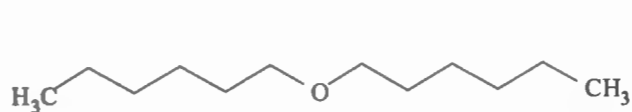


Dimethylfulvene

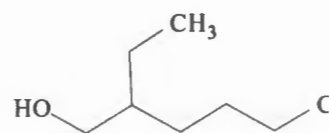


p-Xylene

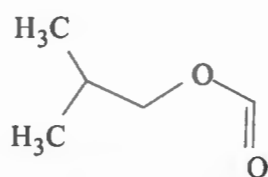
Figure 6.5: Structures of compounds from ethyl acetate fraction for all isolates



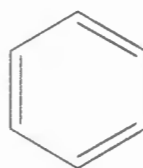
Hexane, 1,1'-oxybis-



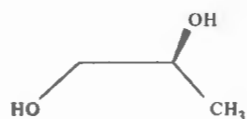
Isooctanol



Formic acid, 2-methylpropyl ester



Benzene



(s)-(+)-1,2
Propanediol



Tridecane

Figure 6.6: Structures of compounds from methanol fraction and petroleum ether for all isolates

6.3.5 Analysis of GC-MS chromatograms for *B. thuringiensis*

6.3.5.1 Benzene and butanol extract

The GC-MS chromatogram of benzene extract from *B. thuringiensis* did not show very sharp peaks but two of the peaks indicated the presence of VOCs. On comparison to the mass spectra from the NIST library, the compounds were characterized and identified as fumaronitrile and tropone (Fig. 6.2) with RT of 4.37 and 5.34 respectively. While fumaronitrile had the highest peak area of 35.44% compared to tropone with 25.23%. Two peaks of GC-MS chromatograms of butanol extract from isolate *B. thuringiensis* were characterized and identified by comparing with the NIST library as formic acid butyl ester and 2, 2-dimethylhexanone (Fig. 6.4a) in the RT of 5.36 and 7.70 respectively. The highest peak area of 43.70% was exhibited by 2, 2-dimethylhexanone while formic acid butyl ester showed the lowest peak area of 15.88% (Table 6.3 and Fig. 6.2).

6.3.5.2 Chloroform and ethyl acetate extract

The GC-MS chromatogram of chloroform extract revealed only 1 peak which was characterized and identified as bromodichloromethane (Fig. 6.4b) from the NIST library. The RT and peak area were observed as 4.25, 43.38% respectively. The characterized and identified VOCs from ethyl acetate extract of *B. thuringiensis* which were observed as 5 peaks when compared with NIST library. The mass spectra corresponded with paraldehyde, 2-oxopropanoic acid, ethylbenzene, dimethylfulvene, p-xylene (Fig. 6.5) at RT 3.46, 4.45, 5.05, 5.35 and 6.14 respectively. The compound dimethylfulvene had the highest peak area of 89.11% while 2-oxopropanoic acid had the lowest at 41.55% (Table 6.3, Fig. 6.4b and Fig. 6.5).

6.3.5.3 Hexane and methanol extract

The characterized and identified VOCs from hexane extract of *B. thuringiensis* were observed as 3 major peaks (Fig. 6.3). When compared with NIST library, the mass spectra of the first peak

corresponded to ethyl benzene (Fig. 6.3) in RT of 5.03 and peak area of 26.98%. The second peak corresponded to tridecylamine (Fig. 6.3) with 3.57 RT and peak area of 12.76%. The third peak was identified as combination of 2,4-dimethylhexane and dihexylether (Fig. 6.3) in RT of 3.47 and peak area of 55.54%. GC-MS chromatogram of methanol extract showed 5 peaks with the first peak having a combination of VOCs as was mentioned with *B. amyloliquefaciens*, hexane extract (Fig. 6.3 and Fig. 6.6). The second peak also had a combination of 2 peaks identified as cetane and hexane, 1,1'-oxybis and 3.85 in RT and peak area of 7.36%. The third peak was identified as a combination of ethylbenzene, isocarboxazid and carbazic acid with RT in 5.04 and peak area of 32.23%. The fourth peak corresponded to p-xylene in 5.34 RT and had the highest peak area of 100%. The fifth metabolite identified was 1,2-dimethylbenzene in 6.14 RT and 40.58% peak area (Table 6.3, Fig. 6.3 and Fig. 6.6).

6.3.6 Analysis of GC-MS chromatograms for *Bacillus* sp.

6.3.6.1 Butanol and ethyl acetate extract

The GC-MS chromatogram of butanol extract of isolate *Bacillus* sp. shows 2 sharp peaks, indicating the presence of VOCs characterized and identified as acetic acid, butyl ester and 2,2-dimethyl-3-hexanone (Fig. 6.4) in RT 3.84 and 7.69 respectively. Acetic acid, butyl ester has the highest peak area of 60.41% while 2,2-dimethyl-3-hexanone has a peak area of 47.28%. The GC-MS chromatogram of ethyl acetate shows 2 peaks of characterized and identified VOCs p-xylene and 1,2-dimethylbenzene (Fig. 6.4) in RT of 5.35 and 6.15 respectively. The highest peak area of 64.33% was observed in p-xylene while 1,2-dimethylbenzene had a peak area of 36.89% (Table 6.3 and Fig. 6.5).

6.3.6.2 Hexane extract

Hexane extract produced GC-MS chromatograms of 4 peaks indicating the presence of microbial VOCs. The first peak had combination of VOCs as was mentioned in *B. amyloliquefaciens*, hexane extract and *B. thuringiensis* methanol extract in RT of 3.06 and the highest peak area of 86.72%. The third peak was identified as a combination of ethylbenzene, isocarboxazid and carbazic acid which was the same with the third peak of methanol extract of *B. thuringiensis* (Fig. 6.3) with RT in 5.05 and peak area of 32.23%. The remaining 2 peaks were characterized and identified as isooctanol and formic acid, 2-methylpropyl ester in RT 3.47 and 3.76 respectively. Isooctanol showed the lowest peak area of 7.46%, while formic acid, 2-methylpropyl ester also showed a low peak area of 8.78% (Table 6.3, Fig. 6.3 and Fig. 6.6).

6.3.6.3 Methanol and petroleum ether extract

The GC-MS chromatogram of petroleum ether extract showed 2 peaks indicating the presence of microbial VOCs. In comparison with the mass spectra of VOCs in the NIST library, the compounds were characterized and identified as tridecane and 1,3-dimethylbenzene (Figure 6.7) in RT of 3.48 and 5.34 respectively. Tridecane showed the highest peak area of 61.72% while 1,3-dimethylbenzene showed peak area value of 52.57%. Chromatogram from methanol extract only picked one peak indicating the presence of microbial VOCs which were characterized and identified as fumaronitrile, tropone and benzene (Fig. 6.7) in RT 3.81 and peak area of 34.22% (Table 6.3, Fig. 6.3 and Fig. 6.6).

6.3.7 Antibacterial potential of bioactive compounds

The antibacterial activities of the different extract tested against *B. cereus*, *P. aeruginosa*, *M. cryophilus* and *E. faecalis* was so obvious with butanol extract from bioactive compounds of *Bacillus sp.*, *B. amyloliquefaciens* and *B. thuringiensis* inhibiting the growth of all test organisms.

Petroleum ether extract from bioactive compounds of isolate *B. amyloliquefaciens* had the least antagonism activity against test organisms. While the butanol extract of bioactive compounds from *B. amyloliquefaciens* had the highest antagonism activity against test organisms (Table 6.2). It may show highest antagonism against some selected pathogens because butanol has four carbon atom in its structure and this prevent it from penetrating into the cell walls compare with ethanol that has two carbon atom. One solvent performs better than the other because of the difference in polarity. Butanol extract may show antagonism against some selected pathogens because it has four carbon atom in its structure and allows to penetrate into the cell walls compare with ethanol that has two carbon atom. Antimicrobial activities increase as the polarity of the solvents increase (Pradesh and Chanda 2007). According to Bakht et al., (2011), butanol extract also had the highest activity against bacterial pathogens *E. carotovora*.



6.4 Discussion

Rhizobacteria have been observed to produce secondary metabolites especially antibiotics (Raaijmakers and Mazzola, 2012). Gas Chromatography-Mass Spectrometry (GC-MS) analysis of crude extract from the 3 isolates in this study detected 68 compounds using 7 extraction solvents while *Streptomyces* spp isolated from the rhizosphere of chili pepper was observed to detect 77 compounds using ethyl acetate and based on RT and peak area percentage (Jalaluldeen et al., 2015). A total of 22 volatile compounds were secreted by 6 bacterial strains from the rhizosphere of lemon plant (Gutiérrez-Luna et al., 2010) while a total of 38 volatiles were released by 4 PGPR strains and were used to trigger growth in *Arabidopsis thaliana* (Farag et al., 2006). This difference in bioactive compounds detected could be as a result of the volatility of the compounds or extraction solvent (Groenhagen et al., 2013). Rhizobacterial strains very close to *B. thuringiensis*, *B. amyloliquefaciens* and *Bacillus* sp. have been involved in the production of one or more bioactive compounds (Arguelles-Arias et al., 2009; Bacon et al., 2015; Baysal et al., 2008). *Bacillus* spp are able to withstand harsh environmental conditions in the soil because of the production of spores that are able to withstand extreme weather conditions. They are also able to compete favourable with other microbes as a result of VOCs production (Amin et al., 2015). *Bacillus* spp have been observed to be able to produce diverse VOCs which are effective against human beings, plant and animal pathogens (Kai et al., 2007).

Antagonistic activities have been reported from many origins including bacteriocins, enzymes and volatiles while the effect of volatiles have been understudied but not given the relevant attention it deserves. In this study, it was shown that the rhizosphere of bambara groundnut was able to attract organisms which could be important pesticide-producing agents as well as useful in drug production and other industries. The GC-MS result from the three isolates showed that mostly the

same compound was present and this could be attributed to the 3 organisms being members of the same genera. A number of pesticides and antidepressants are notable compounds produced such as Phthalan. The derivative of phthalan can be found in the drug Citalopram, which is an antidepressant drug in the category selective serotonin reuptake inhibitor (SSRI) and is used to treat major depressions (Ramesh Rao, 2013).

It was observed that the bioactive compounds extracted using butanol were the potent antibacterial compounds from the isolates. They showed considerably larger zone of inhibition compared to other selected solvent extracts while the least zones were recorded in the petroleum extract solvents (Table 6.3). One solvent performs better than the other because of the difference in polarity. Butanol extract may show antagonism against some selected pathogens because it has four carbon atom in its structure and allows to penetrate into the cell walls compared with ethanol that has two carbon atom and the other solvents. Antimicrobial activities increase as the polarity of the solvents increase (Pradesh and Chanda, 2007). Also, according Bakht et al., (2011), butanol extract also had the highest activity against bacterial pathogens *E. carotovora*.

The GC-MS result is in the order hexane>methanol>ethyl acetate> benzene, chloroform> petroleum ether (Fig. 6.2-Fig 6.6). Benzene and chloroform have the same number of notable compounds while petroleum ether had the least.

Some of the bioactive compounds like tropolone have been shown to have a strong inhibitory activity on plant growth and are known to have antimicrobial and insecticidal activity. Strong antifungal activity and broad antimicrobial spectrum of tropolone have led to their wide utilization in agriculture, clinical products, cosmetics and other areas (Saniewski et al., 2007). They are able to chelate iron and make it available for plant growth promotion (Saniewski et al., 2007).

Another compound, p-xylene which is a chemical feedstock in the chemical industry was secreted by the three selected isolates, these were all *Bacillus* spp. Other bioactive compounds were tridecane, fumaronitrile, isocarboxazid and tridecylamine.

6.5 Conclusion

Apart from antibiotics, VOCs are another class of secondary metabolites that have showed great promise in combating pathogenic organisms. The VOCs crude extracts from this study were antagonistic against test organisms, thus, making them good antibacterial compounds for the agricultural, medical and pharmaceutical industries. These three isolates (*Bacillus* sp., *B. thuringiensis* and *B. amyloliquefaciens*) are good sources of bioactive compounds and the compounds can be modelled into antibacterial agents. Since not much work has been done to exploit these compounds, it means basic research should be conducted more to exploit the hidden potentials of these compounds and the organisms producing them as this might lead to the discovery of new world of metabolism in bacteria.

CHAPTER 7

16S NGS ANALYSIS OF BAMBARA GROUNDNUT RHIZOSPHERIC SOIL AT DIFFERENT GROWTH STAGES

Abstract

Bambara groundnut is one of the neglected and underutilized crops (NUS) commonly found in Sub-Saharan Africa and commonly known as a complete food. Several studies have been carried out on the nutritional properties of bambara groundnut but very little is known about the changes that occur between the microbial communities of the rhizosphere of the root at different growth stages of the plant and the bulk soil. This study reports on the bacterial communities at the different growth stages of bambara groundnut and the bulk soil. Paired end illumina-Miseq sequencing of 16S rRNA was carried on the soil samples of the bacterial community with the phyla dominated by Actinobacter (30.1%), Proteobacter (22%), Acidobacter (20.9%), Bacteroides (8.4%), Chloroflex (4.5%) and Firmicutes (4.4%) in all the soil samples. Samples from the bulk soil had the least average percent phyla (O1 and O4) while samples at 16 WAP (F1 and F4) had the highest average percent phyla. The alpha diversity at $p=0.05$ was highest at F1 and F4 (which corresponds to the seed maturity stage) compared to the others and the control. *Rubrobacter* was the most predominant genera, after which is *Acidobacterium* and *Skermanella*. It was observed from the analysis of operational taxonomic units that there was significant change in the bacterial structure of the rhizosphere with a higher abundance of potential plant growth promoting rhizobacteria, at the different growth stages which includes the genera *Bacillus* and *Acidobacterium*. These results demonstrate that the bacterial communities of bambara groundnut rhizosphere in the field are dynamic and changes with abundance of the bacterial communities at growth stages of the plant.

Keywords: Bacterial community, Bambara groundnut, diversity, growth stages, NGS, OTU

7.1 Introduction

Plants are very important to the survival of animals and human beings and so their health and growth become very relevant in sustainable agricultural practices in cropping and farming systems (Johns, 1996). This includes their ability to withstand and suppress pests and diseases, biotic and abiotic stress and utilization of available nutrients for their growth and development. Plant's rhizosphere has been observed to be able to accommodate microorganisms that are able to perform these activities without the influence of chemicals and achieve results. Plant-microbe interaction at the rhizosphere level is important to the growth and development of plants (Sugiyama et al., 2014; Berendsen et al., 2012).

The rhizosphere is a beehive of activities and interactions between plants, soil and microorganisms in the soil (Nihorimbere et al., 2011). The plants via their roots release a wide range of chemical compounds and roots exudates into the environment which are used to attract beneficial microbes. These root exudates help to mediate these interactions between plants, microorganisms and the environment (Chaparro et al., 2013; Badri et al., 2013). As much as root exudates affect the types of microbes in the rhizosphere as a result of the category of compounds secreted, microbes also have a way of determining the compounds in the rhizosphere by the type of volatiles secreted into the plant-root environment (Huang et al., 2014). This means that the activities of plant roots alter the biochemical environment of the soil which in turn affect the categories of microbes in the soil at different seasons in the growth stages of plant (Shi et al., 2015). High-throughput sequencing (e.g., Illumina and 454 pyrosequencing) of 16S rRNA gene amplicons have been observed to enhance exploratory analysis of structure of microbial communities, their taxa compositions and

phylogeny. The description of diversity of the microbial communities in the root associated soil is also better enhanced using high-throughput sequencing (Fierer et al., 2012; Caporaso et al., 2011; Berendsen et al., 2012; Philippot et al., 2013; Peiffer et al., 2013). This study profiled the rhizosphere taxa of the soil from the different growth stages of bambara groundnut in order to determine if the rhizosphere is richer at some point compared to others and whether rhizobacteria can be targeted for biotechnological processes. In order to facilitate the management of sustainable agricultural practices and to encourage formulation of agricultural products such as biofertilizers, biopesticides, bioherbicides and biocontrol agents, the interaction between plants and the microbes inhabiting the rhizosphere of plants especially legumes during different growth stages should be given more attention.

7.2 Methods and Materials

7.2.1 Collection of soil samples from Bambara groundnut root rhizosphere

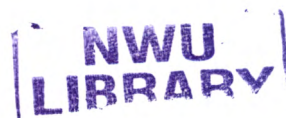
Soil samples were collected from the rhizosphere of bambara groundnut at the different growth stages and were kept on ice or at 4°C until they were needed (within 3 days). This has been discussed in section 5.2.2.

7.2.2 DNA extraction from soil samples

DNA extraction from soil samples was carried out using MOBIO PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) and following the manufacturer's instructions.

Soil samples (0.25) g from bambara groundnut rhizosphere were added to the powerbead tubes and gently vortexed to mix the components of the powerbead which should have helped to lyse and disperse the soil particles. Sixty (60) µl of solution C1 was added to the powerbead tube, inverted and vortexed at maximum speed for 10 min. The powerbead tube was then centrifuged at

10,000 x g for 30 s at room temperature. The supernatant is transferred into a 2 ml collection tube provided. Two hundred and fifty (250) µl of solution C2 was added to the solution in the powerbead and vortexed for 5 s after which they were incubated at 4°C for 5 min. The tubes were centrifuged at room temperature for 1 min at 10,000 x g. Six hundred (600) µl of supernatant was transferred to a clean 2 ml collection tube and the pellets were avoided while transferring. Two hundred (200) µl of solution C3 was added, vortexed briefly and incubated at 4°C for 5 min. The tubes were then centrifuged at room temperature for 1 min at 10,000 x g. Up to 750 µl of supernatant were transferred into each clean 2 ml collection Tube. Solution C4 was shaken to mix it before adding 1.2 ml to the supernatant and vortexed for 5 s. Approximately 675 µl of supernatant were loaded onto a spin filter and centrifuged at 10,000 x g for 1 min at room temperature. The flow through was discarded and another additional 675 µl were added and the process was repeated thrice for each sample. Five hundred (500) µl of solution C5 were added to the spin filter and centrifuged at room temperature for 30 s at 10,000 x g. This was to help clean the DNA that was bound to the silica filter membrane and the flow through was discarded from the 2 ml collection tube. The spin filter was then centrifuged at room temperature for 1 min at 10,000 x g and placed in a clean 2 ml collection tube. One hundred (100) µl of solution C6 were added to the centre of the white filter membrane to elute the DNA. This was also centrifuged at room temperature for 30 s at 10,000 x g. Finally, the spin filters were discarded and the DNA collected in the collection tube. DNAs in collection tube from all samples were kept frozen until further analysis to be performed at Molecular Research DNA laboratory (MR DNA, Shallowater, Texas, USA).



7.2.3 PCR amplification of Bambara groundnut soil 16S rRNA gene

PCR primers 515/806 with barcoding on the forward primer were used in a 28 cycle PCR (5 cycle used on PCR products) using the HotStar Taq Plus Master Mix Kit (Qiagen, USA) to target the 16S rRNA gene for region V3 and V4. The following conditions; 94°C for 3 min, followed by 28 cycles of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min, after which a final elongation step at 72°C for 5 min was used to perform the PCR amplification.

7.2.4 Data processing Sequences

In order to determine the success of amplification, PCR products were checked in 2% agarose gel in order to determine the relative intensity of bands. Illumina DNA library was prepared by using the pooled and purified PCR products. This was done by pooling multiple samples in equal proportions on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq following the manufacturer's guidelines. Sequence data were processed using MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA).

7.2.5 Sequence Analytical Pipeline

Sequenced data were derived by sequencing the V3–V4 region of the 16S rRNA gene as described at MR DNA Laboratory (www.mrdnalab.com). Sequences were joined together and cleaned by removing barcodes and primers, sequences less than 150bp and sequences with ambiguous base calls. Homopolymer runs exceeding 6bp were also removed from the data set. Sequences were then denoised, operational taxonomic units (OTU's) generated after which chimeric sequences and all abnormal sequences removed. Filtered species-level OTUs were defined by clustering at 3% divergence (97% similarity). Finally, these OTUs were taxonomically classified using BLASTn against a curated database derived from RDP11 and NCBI

(www.ncbi.nlm.nih.gov, <http://rdp.cme.msu.edu>) and compiled by taxonomic level into both “counts” and “percentage” files. Sequences were considered to be at the species level if they have more than 97% identity to annotated rRNA gene sequenced. They were considered to be at the genus level, family level, order level, class level and phylum level if the sequences have identities between 95 and 97%; between 90 and 95%; between 85 and 90%; between 80 and 85%; and those between 77 and 80% respectively (Mills et al., 2012) .

7.2.6 Statistical and Diversity Analysis

All statistical analyses were run in the statistical environment R (version 2.15.0; R Development Core Team, 2011), and significant differences were defined at P values of <0.05. Alpha diversity (measures of microbiota diversity within each soil sample) was calculated using the Shannon index of diversity (H) and Simpson index. Evenness was calculated on the basis of the Shannon index calculations, and richness was based on taxonomic identities in each sample. These were performed using the “vegan” package in R. Beta diversity (measures of microbiota differences and similarities among soil samples from growth stages) was also carried out to measure association matrix using Bray-Curtis. The overall structure of microbial communities was ordinated by principal coordinate analysis (PCoA) based on the Bray distance metric.

7.3 Results

7.3.1 Overview of Bambara groundnut bacterial community

The rhizospheric bacterial community of bambara groundnut was observed to have 5 kingdoms, namely bacteria, fungi, eukaryote, metazoa and viridiplantae. There were 30 phyla, 604 genera, 1154 species and 759902 sequences in all 10 samples. Figure 7.1 shows the different phyla captured in the growth season N1, D1, J1, F1 and the bulk soil O1. The most abundant phyla which

were obvious in all samples were Actinobacteria, Proteobacteria and Acidobacteria. Others that followed include Bacteroides, Chloroflex, Firmicutes, Planctomycetes and Verrucomicrobia.

7.3.2 Relationship of rhizosphere bacterial community

From the 10 samples across the growth stages of bambara groundnut, 1173 species operational taxonomic units (OTU) were obtained from the illumina 16S rRNA sequencing. The dynamics of the bacterial community structure over the growth stages was assessed by carrying out a principal coordinate analysis (PCoA) based on the Bray distance matrix (Fig. 7.2). The bacterial community from the bulk soil (O) clustered partially with samples from 4 WAP to 12 WAP (N, D, J). Samples from 16 WAP clustered differently apart from the bulk soil and the other samples.

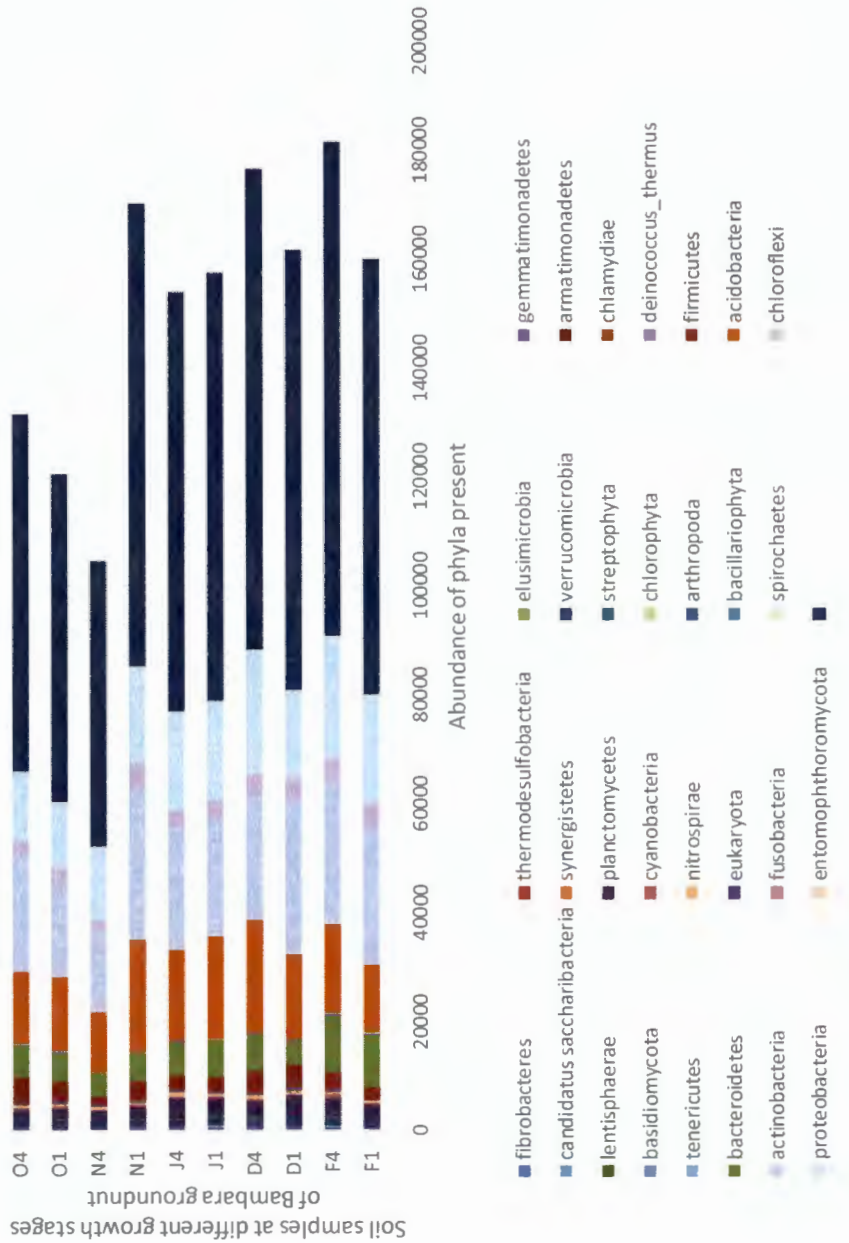


Figure 7.1 : Relative abundance of major bacterial phyla present in the bulk soil and rhizospheric soil of growth stages of Bambara groundnut as detected using the next generation sequencing (NGS). F1 and F4 represent the rhizosphere at 16 WAP which corresponds to the stage of seed maturation. J1 and J4 represent the rhizosphere at 12 WAP which correspond to the stage of seed formation. D1 and D4 is the rhizosphere at 8 WAP which correspond to the flowering stage, N1 and N4 represent the rhizosphere at 4 WAP which corresponds to vegetative stage while O1 and O4 represent the rhizosphere of the bulk soil.

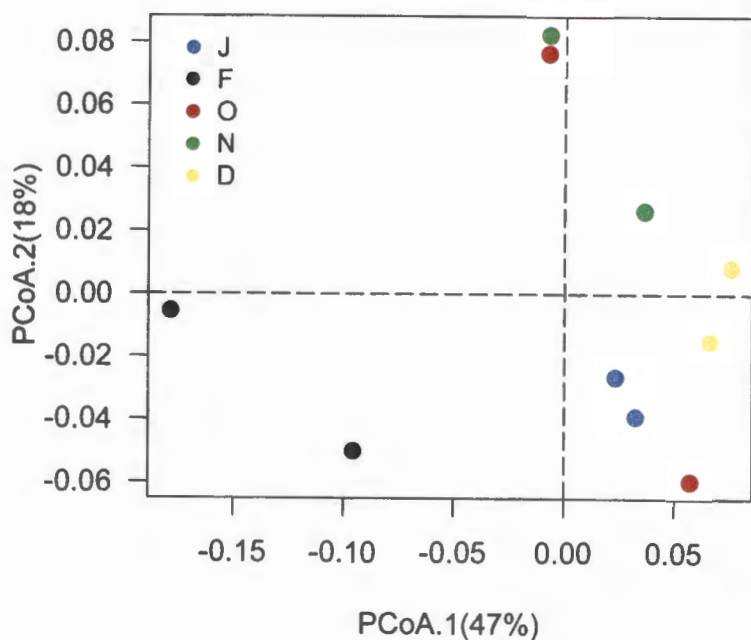


Figure 7.2: PCoA analysis of bulk soil (O1 and O4) and rhizosphere microbial community associated with Bambara groundnut growth stages from 4 WAP to 16 WAP, based on the Bray distance metric. The percent value for each axis represents the proportion of total variation explained. Circles represent soil samples from all growth stages. Samples collected at weeks 0, 4, 8, 12, and 16 are shown in red, green, yellow, blue and black respectively.

7.3.3 Effect of the growth stages of Bambara groundnut on the alpha diversity

Alpha diversity was expressed both as richness (N.obs) and diversity using both Shannon-Weaver index and Simpsons index. The depth of the sequencing (number of reads obtained/samples) was also calculated (N.obs.rar). These yielded similar result with the PCoA. Indices of bacterial alpha-diversity in bambara groundnut gradually increased from the bulk soil through the growth stages except for the 4 WAP (N) where it was lower than the bulk soil (Fig. 7.3). The Shannon-Weaver index was between 3.4 at N1 (4 WAP) and 4.04 (16 WAP) with the bulk sample at 3.82 and 3.68. This shows that the samples were quite diverse and the numbers of species present were evenly distributed. The measure of richness and evenness also followed the same trend with samples from F1 and F4 being the most even and richest while samples N1, N4, O1 and O4 were the least.

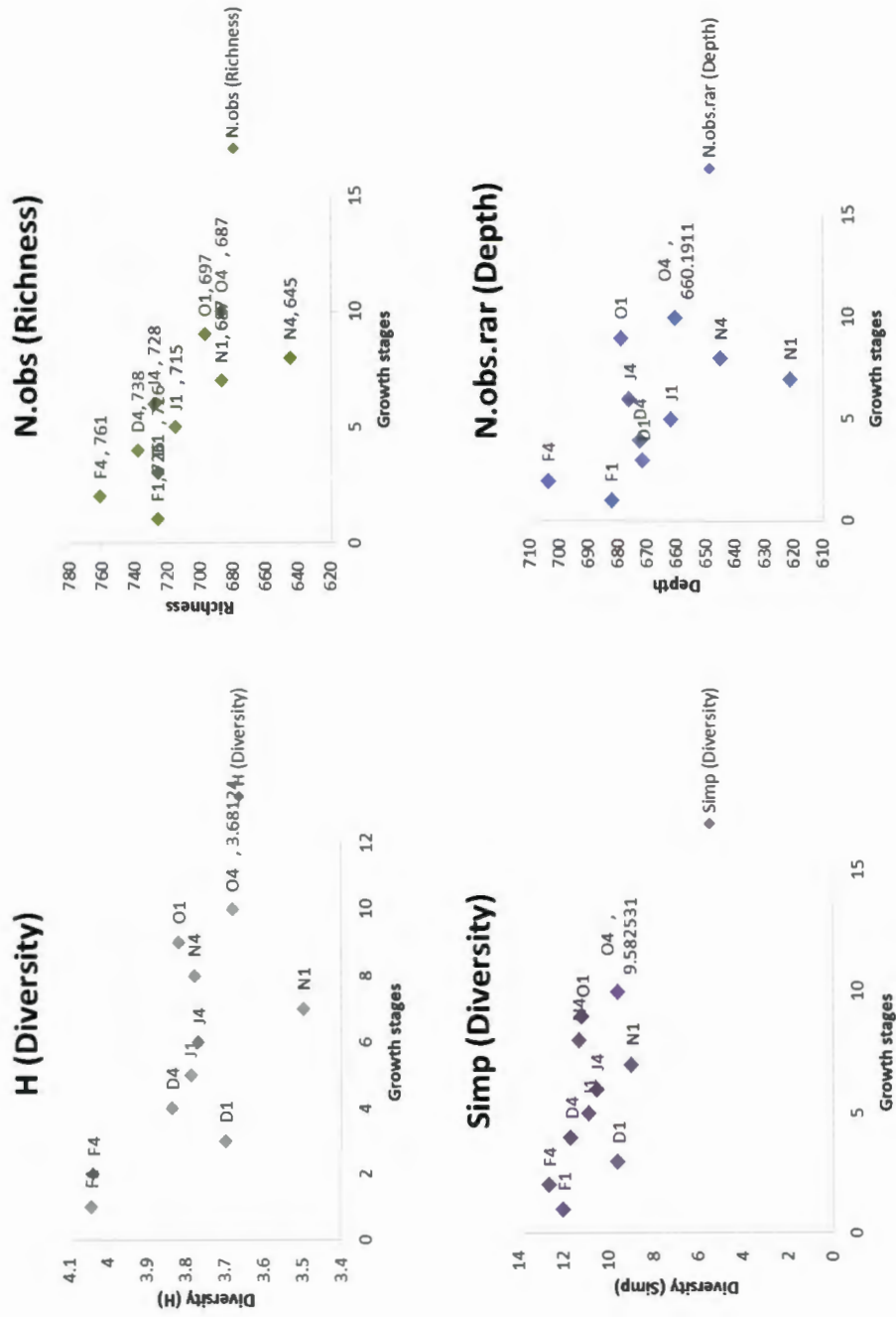


Figure 7.3: The diversity of rhizosphere microbial community of Bambara groundnut (F1, F4, J1, J4, D1, D4, N1, N4) and bulk soil (O1 and O4) indicated by OTU richness, sequencing depth, Shannon index, and Simpson index in rhizosphere across plant growth stages and bulk soil

7.3.4 Bacterial responses to plant growth.

The abundance of bacterial 16S rRNA gene in Bambara groundnut rhizosphere soil gradually increased over time beginning at week 4 ($P=0.05$ [Fig. 7.4]). The abundance of 16S rRNA gene was significantly higher in rhizosphere soils than in bulk. The highest average abundant phyla represented in soil samples across growth stages are Proteobacteria, Actinobacteria and Acidobacteria.

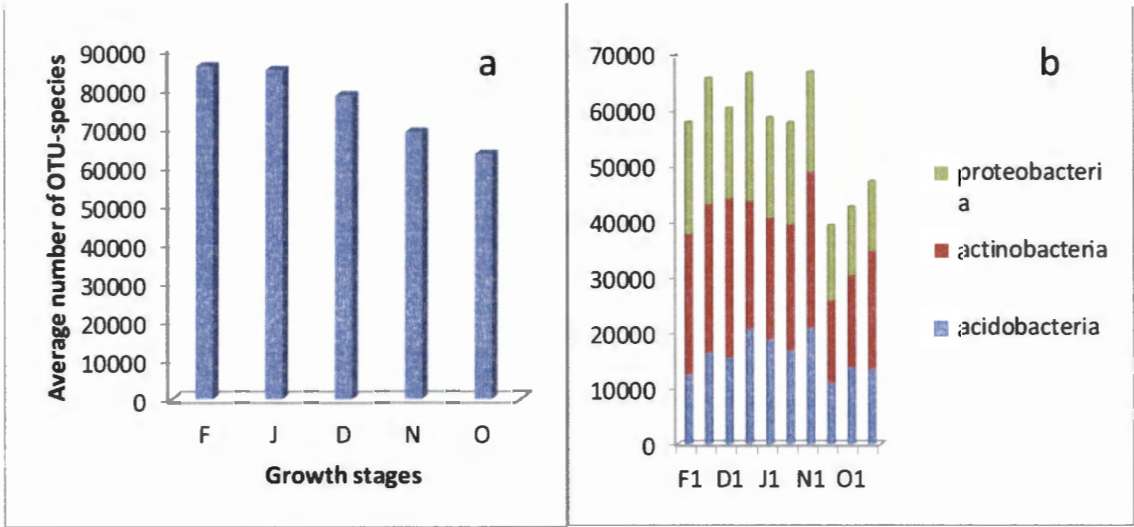


Figure 7.4: (a) Average number of OTU-species in Bambara groundnut rhizobiome at growth stages. Abundance of bacterial 16S rRNA genes in bulk and rhizosphere soils associated with bambara groundnut at different growth stages data are presented as mean ($P=0.05$). (b) The highest abundant phyla present at all the growth stages and bulk soil where F1, J1, D1, N1 and O1 are the mean values.

7.3.5 Effects of plant growth on the bacterial community beta diversity

In order to measure the differential diversity between the ecosystem of the rhizosphere at different growth stages, beta diversity was determined using Bray-Curtis matrix. This matrix is developed based on relative abundance of species (Fig. 7.5a) while the difference between unweighted data and untransformed data were also seen in the heatmap (Fig. 7.5b and Fig. 7.5c). From the heat map (Fig. 7.5a), it was observed that F1 and F4 which are the samples from seed maturation (16 WAP) clustered together, though O1 and O4 were the samples from the bulk soil, but they are seen to cluster together and with samples from 8 WAP during flowering stage. Samples N4 (4 WAP) from vegetative stage clustered with J4 (12 WAP) at seed and pod formation while sample D4 (8 WAP) during flowering and J1 (12 WAP) during seed and pod formation also clustered together. This clustering between samples could be as a result of the nature of bambara groundnut in which case when it starts flowering and producing seeds, it does so throughout its life span until harvest.

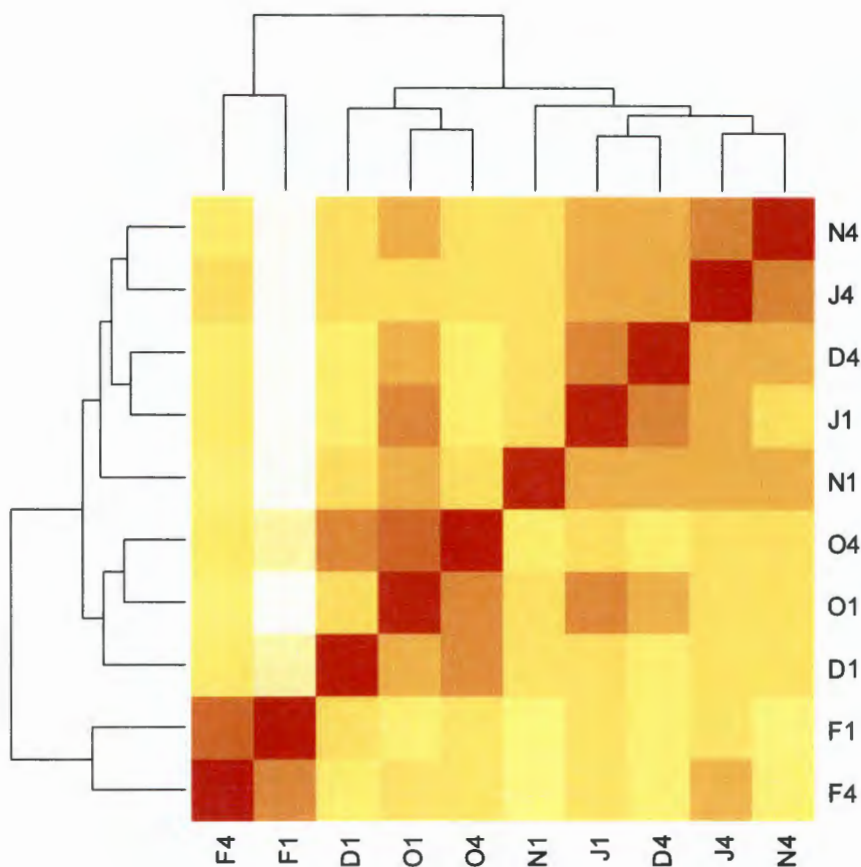


Figure 7.5: (a) Heatmap and hierarchical cluster analysis of bacteria based on the relative abundances of dominant genera from the different growth stages of bambara groundnut rhizospheric soil. O1 and O4 are samples from bulk soil. N1, N4, D1, D4, J1, J4, F1 and F4 indicate soil sample from growth stages. Transformed data-relative abundance (species table was transformed to relative abundance using decostand function in vegan). This is for weighted beta diversity measures i.e., Bray-Curtis dissimilarity matrix seen as presence /absence and relative abundance.

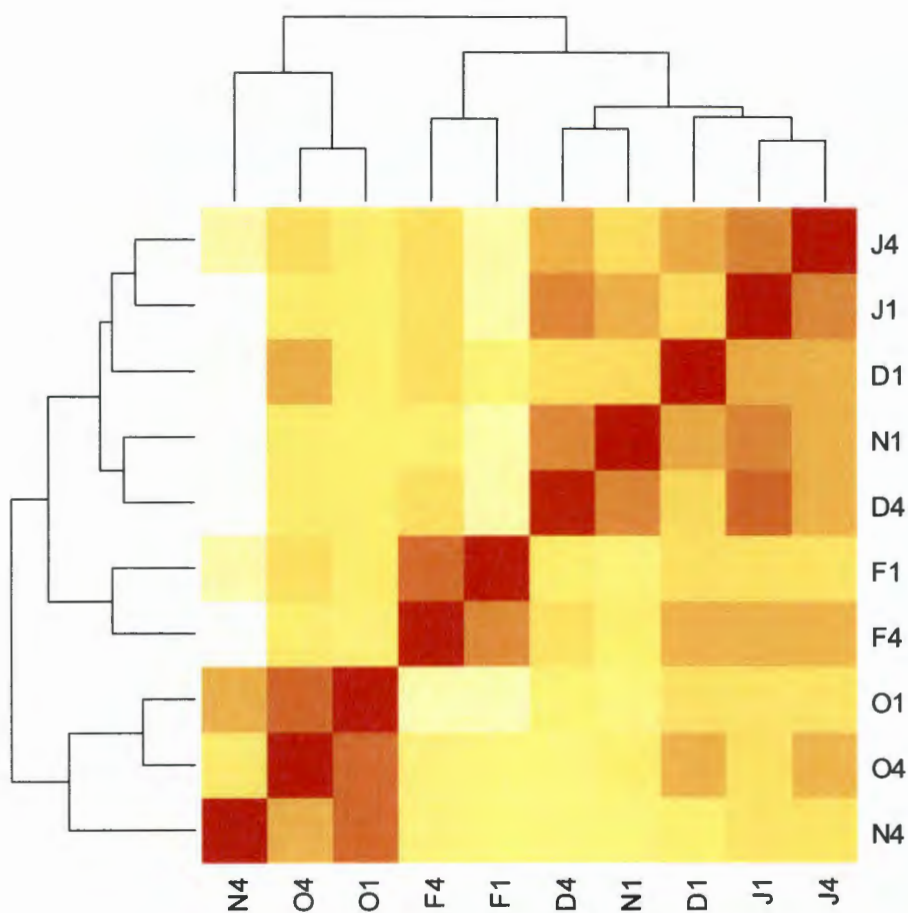


Figure 7.5: (b) Heatmap and hierarchical cluster analysis of bacteria based on the relative abundances of dominant genera from the different growth stages of bambara groundnut rhizospheric soil. O1 and O4 are samples from bulk soil. N1, N4, D1, D4, J1, J4, F1 and F4 indicate soil sample from growth stages. Bray-Curtis distance matrix for untransformed data of the soil samples (the more intense the colour, the more similar a pair of samples are).

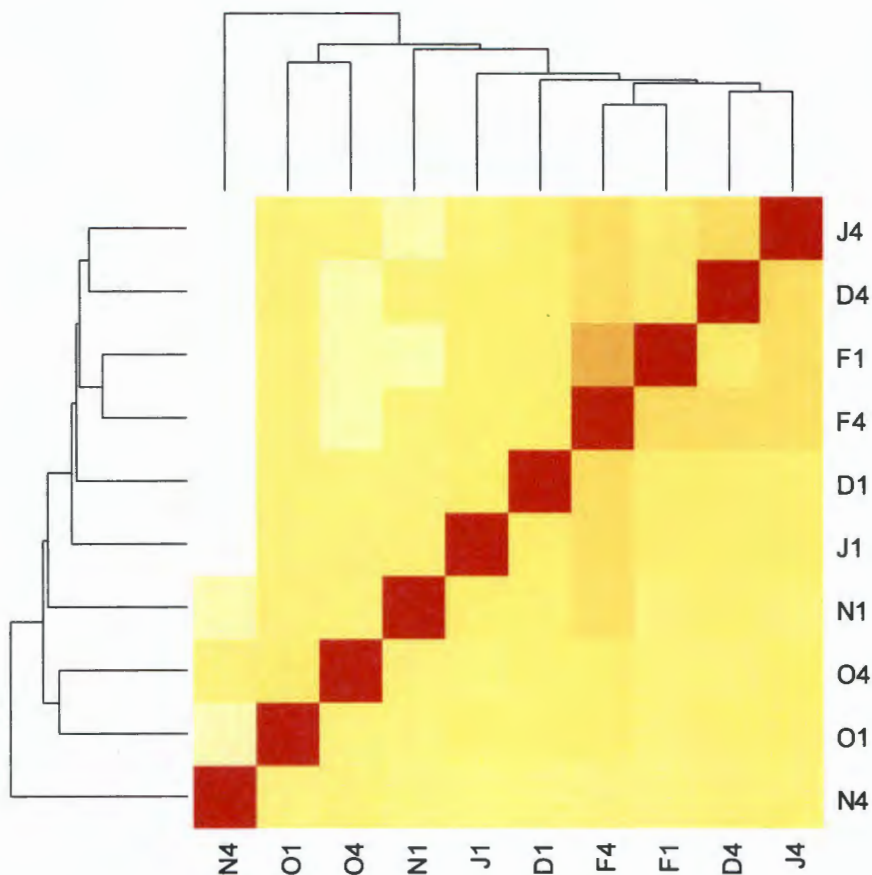


Figure 7.5: (c) Heatmap and hierarchical cluster analysis of bacteria based on the unweighted data beta diversity absent or presence of dominant genera from the different growth stages of bambara groundnut rhizospheric soil. O1 and O4 are samples from bulk soil. N1, N4, D1, D4, J1, J4, F1 and F4 indicate soil sample from growth stages.

The bacterial community of the bulk soil differed from those of bambara groundnut during its growth (Fig. 7.5b). It clustered differently from them except with one of the samples during 4 WAP and 12 WAP corresponding to its vegetative and flowering stage. The soil samples at F corresponding to the 16 WAP also clustered differently from the other treatments and from the bulk soil. This shows that the bacterial communities were modified and changed during the growth of bambara groundnut. The five most abundant rhizospheric bacterial community OTU (species) of bambara groundnut are *Rubrobacter*, *Acidobacterium*, *Skermanella*, *Conexibacter* and *Bacillus*.

7.4 Discussion

It has been observed that plants do have effects on soil bacterial community which further influence plant growth and invariably crop yield (Berendsen et al., 2012; Schreiter et al., 2015). In this present study, 16S rRNA metagenomics was used to analyze the rhizospheric bacterial communities of bambara groundnut. It has been reported that a greater number of microbes reside and flourish in the rhizosphere of plants probably due to the release of root exudates by plants providing a nutritious environment to rhizobacteria (Gopal et al., 2015; Mendes et al., 2013; Sugiyama et al., 2014). Our study demonstrated that at 4 WAP, the average abundance of rhizobacteria started increasing, showing the effect of growth of bambara groundnut on the bulk soil. This was similar to the report by Shi et al. (2015) who observed that the growth of plants drove the succession of rhizobacteria in the rhizosphere of *Avena fatua*. Maize rhizosphere was observed to change between early and late growth stages which was due in part to either the growth stages or seasonal effects (Li et al., 2014). Rhizospheric bacterial communities of *Arabidopsis thaliana* at the seedling growth stage was distinct and different compared to the other growth stages (Chaparro et al., 2014). There was difference between the bacterial community of the rhizosphere of soybean during growth, evident after 6 weeks corresponding to the vegetative stage compared to the bulk soil using pyrosequencing analysis at the phylum level (Sugiyama et al., 2014). It is commonly assumed that the changes in the rhizosphere bacterial community is plant-driven as a result of the production of plant root exudates, whose quality and or quantity can alter the structure of the rhizosphere bacterial community at different growth stages (Chaparro et al., 2013; Singh and Mukerji, 2006).

We found that there was change in the community bacterial structure of bambara groundnut rhizosphere after 4 WAP (N1 and N4) corresponding to the vegetative growth stage all the way to the flowering, podding and seeding stages. The structure at 16 WAP (F1 and F4) corresponding to the seeding was quite different compared to all the other stages and also the

bulk soil. This is due likely to the biochemical process that takes place during seeding in legumes. Plants release flavonoids into the soil which form part of root exudates in the rhizosphere (Cesco et al., 2012) that is used as carbon source by soil microbes which enhance their ability to solubilize phosphate and make it available to the plant for growth promotion. This also helps to chelate iron and enhance its availability for plant growth promotion including seeding (Cesco et al., 2010). At this point of seeding, the activities of the rhizospheric bacterial community are increased resulting in the different cluster formation at these growth stages (F1 and F4).



The most abundant phyla throughout the growth stages and bulk soil were Actinobacteria (30.1%), Proteobacteria (22%), Acidobacteria (20.9%), Bacteroides (8.4%), Chloroflex (4.5%) and Firmicutes (4.4%) (Fig. 7.1). The phylum Actinobacteria is one of the largest phylum within bacteria and comprised mainly of Gram-positive organisms that have a high G+C content (>55 mol% in genomic DNA) (Gao and Gupta, 2012). It is known to be one of the richest sources of nitrogen-fixing bacteria, antibiotics, natural products and other compounds that are of biotechnological importance (Gao and Gupta, 2012). It is also known to be a group of bacteria with potentials as biological control agents, plant growth promotion and plant-bacterial interaction agents (Palaniyandi et al., 2013). *Rubrobacter* spp which is the largest species in this study is a subclass in the phylum Actinobacteria (Takarada et al., 2008; Garrity and Holt, 2001). Some of the organisms under this phylum thrive under harsh conditions like *Kocuria rhizophila*. (Takarada et al., 2008). This is quite obvious because Bambara groundnut is a legume that can thrive under very harsh condition like drought (Bamishaiye et al., 2011). Actinobacteria are also known to produce plant growing hormones and exudates that can enhance the growth of plants (Sousa and Olivares, 2016). They are also important as biocontrol agents wading off pathogenic microbes to indirectly increase plant growth by suppressing disease. The seeming decrease in the relative abundance at some point 4 WAP could be as a

result of acclimatization by the new rhizobacteria just trying to colonize the rhizosphere and competing with other resident microbes (Halder and Sengupta, 2015; Hibbing et al., 2010).

In this study, *Rubrobacter* spp was the most abundant species in the rhizosphere of bambara groundnut. This could be due to the fact that they are mostly prevalent in desert soil and are not common in soil culture collections (Aislabie et al., 2013; Holmes et al., 2000). It was found that out of the OTU's from actinobacteria phyla isolated from soils of Atacama desert in Chile, the second most abundant subclass was Rubrobacteridae (Neilson et al., 2012). The ability of bambara groundnut to grow in harsh weather could be attributed to the presence of the abundance of drought-tolerant bacteria (Mkandawire, 2007). Further studies on being able to isolate drought-tolerant bacteria and their genes should be harnessed for further biotechnological processes that can enhance sustainable agriculture.

7.5 Conclusion

This study revealed the changes that occur in the bacterial community of bambara groundnut rhizosphere grown in the field. The changes were distinct as it was observed that the bacterial communities from the growth stages contained potential PGPR genera that are specific to each growth stage and are highly abundant. The changes observed in the bacterial communities are in part due to the growth stage because of the different metabolic and physiological activities going on at those growth stages in the rhizosphere and also the influence of bambara groundnut is also perceived.

Further studies on both metabolic activities of bambara groundnut such as root exudation and the physiological functions of these rhizobacteria on plant growth are warranted to elucidate the reciprocal interactions between plants and rhizosphere microbes on the fields for better utilization of rhizospheric bacteria in sustainable agriculture.

CHAPTER EIGHT
FIELD EXPERIMENT OF RHIZOBACTERIA WITH BIOFERTILIZER
POTENTIAL ON THE GROWTH OF BAMBARA GROUNDNUT

Abstract

Development of biofertilizers that can withstand and thrive well in harsh conditions is important to withstand the challenges of climate change. Rhizobacteria from the rhizosphere of legumes such as bambara groundnut with the ability to grow, adapt and colonize their surroundings even in unfavourable conditions was adopted. A 2-year field study planting was carried out to determine the effect of plant growth promoting bacteria BAMhi, BAMli and BAMr, as biofertilizers for bambara groundnut (*Vigna subterranea*). The study was carried out in complete randomized block experimental design with three replications. The Null hypothesis stated that there is no significant difference between the treatments BAMli, BAMhi, BAMr and control while the alternative hypothesis emphasized that there exist a significant difference between the treatments. Bambara groundnut seeds were bacterized with inoculum prepared from *Bacillus* isolates and left for 48 hours before they were planted in the field. Growth parameters such as length of plant, number of leaves, number of branches and number of seeds were measured. The results were significant for both the treatment/bacterial effect and the block/week effect at a 5% level of significance (treatment: $F = 12.028$, $p = 0.001$; block: $F = 105.350$, $p = 0.000$). Since the p-value is less than 0.05, the null hypothesis is rejected in favour of the alternative hypothesis. However, the overall model used for this data was significant ($F = 196.068$, $p = 0.000$) signifying that the model fits the data. Characterization of BAMhi, BAMli and BAMr using the 16S rRNA gene reveals their identity as *B. amyloliquefaciens*, *B. thuringiensis* and *Bacillus* sp respectively. These *Bacillus* isolates have proved to be plant growth-promoting agents that can be used as biofertilizers to enhance the growth of crops and consequent improved yield. This is the first time the rhizobacteria from the bambara groundnut rhizosphere is applied as biofertilizer.

Keywords: *Bacillus* sp, Bambara groundnut, biofertilizer, plant growth-promoting rhizobacteria

8.1 Introduction

Increasing crop yield in the face of climate change and an ever-growing global population is important to agricultural productivity and food security. Crop yields have decreased as a result of infestation of plants with pests and diseases together with over-use of land leading to decreased fertility (Esitken et al., 2006). The use of chemicals to control pests and diseases and also to enhance plant growth through the use of chemical fertilizer has had a diminishing-return, effect on environmental pollution and also hazardous effects (Ajilogba and Babalola, 2013). Biofertilizers have been defined as the application of microbial inoculants or beneficial microorganisms especially plant growth-promoting rhizobacteria (PGPR) to soil, plants and/or seeds to increase plant growth and development and invariably crop yield (Condolji and Yarnia). This is achieved directly by aiding the supply of nutrients to plants for growth and indirectly by suppressing plant diseases which invariably create a friendly environment to enhance plant growth.

Several studies have been conducted to show the effect of PGPR on plant growth and crop yield. Different bacterial species have been termed PGPR and they include strains in the genera *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Rhizobium* and *Bacillus* (Arıkan et al., 2013; Glick, 1995). *Bacillus* spp have been observed to be effective in promoting growth of various crops including legumes, tomato, pepper (Ajilogba et al., 2013; Dastager et al., 2011; López-Bucio et al., 2007; Agrawal and Agrawal, 2013). They are known to be endospore-producing microorganisms which make them stable and very important in microbial product formulation (Schisler et al., 2004; Kanjanamaneesathian et al., 2013). They are able to secrete antimicrobial components that indirectly enhance plant growth and able to solubilize components like

phosphate which makes nutrients available to the plant and invariably increase plant growth (Calvo et al., 2010).

Bambara groundnut, an underutilized leguminous crop is very important in helping with soil conservation by its ability to make use of the microbes in its nodules to fix nitrogen that enrich the soil fertile and then increase crop yield (Bamishaiye et al., 2011). Its drought-tolerant ability also makes it an all season crop with or without water and in harsh weather (Mabhaudhi and Modi, 2013; Vurayai et al., 2011). It is an excellent crop that has been proved to have agricultural, environmental and medicinal relevance. It is a nutritionally-rich crop and considered as a balanced diet being a readily-available source of protein. (Bamishaiye et al., 2011). The objective of this study was to determine the effect of *Bacillus* spp isolated from the rhizosphere of bambara groundnut on its plant growth and also to characterise the isolates. This is the first field study on biofertilizer effect of PGPR on the growth of bambara groundnut.

8.2 Materials and Methods

8.2.1 Description of experimental site

The experiment was conducted at the Agricultural farm of the North-West University, Mafikeng campus under field condition. The experiment area covered Lat. 25°78'91" Long. 25°61'84" with average amount of rain as 360 mm.

8.2.2 Collection of soil for analysis

Soil samples were collected from the rhizospheric soil around the root of the plant (0–20 cm) and the soils were sieved with a 2 mm mesh to remove gravel and plant roots. Sterile polythene bags were used to collect soil samples and kept in ice cooler boxes to transfer from farm to the laboratory for further analysis.

8.2.3 Experimental Design

This experimental design was a completely randomized design (CRD) with four treatments replicated three times. The treatments were with *B. amyloliquefaciens*, *B. thuringiensis*, *Bacillus sp.* and the control.

8.2.4 Preparation of rhizobacteria from Bambara groundnut rhizosphere for seed inoculation

8.2.4.1 Preparation of inoculum

Isolation of rhizobacteria from soil samples have been previously described (Ajilogba et al., 2016). Preparation of rhizobacteria inoculum was carried out according to Cavaglieri et al. (2005). Two loopfuls of each of the bacteria from 3-day old cultures on tryptic soy agar (TSA) were transferred separately to 50 mL tryptic soy broth (TSB) medium and incubated overnight at $28\pm 2^{\circ}\text{C}$. Viability was confirmed by standard plate count method using tryptone soy broth plus 2% agar (TSBA) (Ajilogba et al., 2013)

The inocula for use in the field were prepared from a 24 h culture of each of the *Bacillus* isolate incubated at $28\pm 2^{\circ}\text{C}$. Ten-fold serial dilution was carried out and each serial dilution was plated using Luria Bertani agar and incubating at room temperature for two days until colonies were formed and a concentration of 5×10^7 was achieved. Five (5) ml of *Bacillus* suspension containing 5×10^7 cfu/ml was used to inoculate bambara groundnut seeds according to Wang (2012).

8.2.4.2 Bambara groundnut seed propagation

The propagation of bambara groundnut was through seeds on level seedbed or ridges where the soil is wet. Bambara groundnut seeds were planted on seedbeds in plots that are 50 cm apart and spacing between seed holes on each plot was 50 cm apart. Bambara groundnut seeds were planted 3-4cm deep in the soil (2-3 seeds per hole). Twenty-five (25) plots were cultivated for

each replication and the experiment was repeated thrice for the first planting season (2014/2015). By the second planting season the second year, 2015/2016, treatments with *B. amyloliquefaciens*, *B. thuringiensis* and *Bacillus* sp were applied to bambara groundnut seeds before planting on a freshly cultivated soil.

Samples were taken from different ridges or seed beds each having 3 rows per ridge, each row having 5 seed holes and having 3 seeds per seed hole making a total of 15 seeds by row and 45 seeds by ridge or 45 plants per ridge.

8.2.4.3 Determination of growth parameters

The following growth parameters were analysed in the field; they are leaf number, length of leaf, breadth of leaf, number of stems, length of stem, root and shoot length and number of seeds. At the end of 4, 8, 12, 16 and 20 WAP, one plant from each planting hole was carefully scooped with all its roots intact using a trowel and hand washed over a fine sieve with tap water collecting all roots one at a time. A meter rule was used to measure plant growth parameters while the seeds were counted.

8.2.5 Isolation of genomic DNA

Genomic DNA of all isolates was extracted using ZR soil Microbe DNA MiniPrep™ (Zymo Research, USA) extraction kit. It was used to obtain genomic DNA from all *Bacillus* strains. Bacterial cultures were grown in 10 ml of Luria Bertani broth (Merck) at 37°C for 24 h and then centrifuged at 10,000 rpm (Universal Z300K model centrifuge; HERMLE Labortechnik, Germany) for 5 min. The bacterial pellets were resuspended in 200 µl of distilled water and transferred to ZR Bashing Bead™ lysis tube and 750 µl lysis solutions were added to the tube. The bashing bead was secured in a bead beater fitted with a 2-ml tube holder FastPrep® 24 and processed at a maximum speed for 5 min. The ZR BashingBead™ lysis tube was centrifuged in a microcentrifuge at 10,000×g for 1 min, 400 µl of supernatant was transferred to a Zymo-Spin™ IV Spin Filter in a collection tube and was centrifuged at 7,000×g for 1 min and 1,200

μL of Soil DNA Binding Buffer was added to the filtrate in the collection tube. Eight hundred (800) μl of the mixture of the binding buffer and filtrate was transferred to a Zymo-Spin™ IIC Column in a collection tube and centrifuged at $10,000\times g$ for 1 min. Two hundred (200) μL of DNA Pre-Wash Buffer was added to the Zymo-Spin™ IIC column in a new collection tube and centrifuged at $10,000\times g$ for 1 min. Five hundred microliters Soil DNA Wash Buffer was added to the Zymo-Spin™ IIC Column in a new collection tube and centrifuged at $10,000\times g$ for 1 min. The Zymo-Spin™ IIC Column was transferred to a clean 1.5 ml micro-centrifuge tube and 100 μl DNA Elution Buffer was added directly to the column matrix. The tube was centrifuged at $10,000\times g$ for 30 s to elute the DNA.

8.2.6 PCR Amplification targeting the 16S rRNA

The 16S rRNA gene was amplified from genomic DNA obtained from bacterial cultures by PCR with previously described primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') (Piterina et al., 2010). PCR was performed in a total volume of 50 μl containing 30-50 ng DNA, 100 mM of each primer, 0.05 U/ μl *Taq* DNA polymerase, 4 mM MgCl_2 , and 0.4 mM of each dNTP. The amplification reaction was performed with a DNA Engine DYAD Peltier thermal cycler (Bio-Rad, USA). The thermal cycling condition used was an initial denaturation at 96°C for 5 min, followed by 30 cycles of denaturation at 96°C for 45 s, annealing at 56°C for 30 s and extension at 72°C for 2 min, followed by a final extension at 72°C for 5 min. The PCR amplicons were analyzed by electrophoresis in 1% (w/v) agarose gel. The gel containing ethidium bromide (10 $\mu\text{g}/\text{ml}$) was viewed under Syngene Ingenious Bioimager (UK) to confirm the expected size of the product. The remaining mixture was purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany).

8.2.7 Nucleotide sequence determination

PCR purified products of the 16S rDNA of the strains were analyzed for nucleotide sequence determination by using ABI PRISM® 3500XL DNA Sequencer (Applied Biosystems) at Inqaba

Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa. Nucleotide sequence of the 16S rDNA of the strains were determined and compared for similarity level with the reference species of bacteria contained in genomic database banks, using the NCBI Blast[®] available at the ncbi.nlm.nih.gov website (Altschul et al., 1990).

8.2.8 Molecular taxonomy determined by sequences and Phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using software. Nucleotide sequences were analyzed and edited by using BioEdit software (Hall, 1999). The partial 16S rRNA gene sequences were used to search the GenBank database with the Blastn algorithm to determine relative phylogenetic positions (Altschul et al., 1990). Multiple alignments of the sequences were carried out by Mafft program 6.864 (Kato and Toh, 2010) against corresponding nucleotide sequences retrieved from GenBank. Evolutionary distance matrices were generated as described by Jukes and Cantor (1969). Phylogenetic analyses were conducted using MEGA version 5.10 (Tamura et al., 2011) and neighbor-joining (Saitou and Nei, 1987); minimum evolution (Rzhetsky and Nei, 1992); maximum likelihood; UPGMA and maximum parsimony (Fitch, 1986) trees was constructed. The methods were used in order to expatiate on the phylogeny and for better comparison. The robustness of the tree topology was evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings. Putative chimeric sequences were identified using the ChimeraBuster 1.0 software. Manipulation and tree editing were carried out using TreeView (Page, 1996).

8.2.9 Nucleotide sequence accession numbers

The 16S rRNA gene sequences obtained in this study have been submitted to the GenBank database and assigned accession numbers (Table 8.9).

8.2.10 Data analysis

On growth parameters, analysis of variance (ANOVA) was performed for comparison of means. Significant means was separated using the Tukey-Kramer honest significant difference (HSD) test at 5% level, and correlation analysis was performed to determine the relationship

between variables at 5% level. On the bacterial isolates, phylogenetic tree construction was carried out from the sequences of DNA and the generational distances between isolates were calculated.

8.3 Results

Mean of the growth parameter measured per plant is given on Table 8.1. This is a summary of the measurement taken per treatment on the field. The yield per plant of bambara groundnut on field varied per treatment and compared with control as follows; *Bacillus sp*, *B. amyloliquefaciens*, *B. thuringiensis* and control produced average of 41, 36, 25 and 20 seeds respectively. Treatment with *Bacillus sp* yielded more than double seeds compared with the control.

8.3.1 Effect of bacterial isolates on the number of stems

A Randomized Block Design was employed to investigate the effect of the different treatments of the bacteria on the number of stems (Table 8.2). The results were significant for both the treatment/bacterial effect and the block/week effect at a 5% level of significance (treatment: $F = 12.028$, $p = 0.001$; block: $F = 105.350$, $p = 0.000$). Since the p-value is less than 0.05, the null hypothesis was rejected in favour of the alternative hypothesis. However, the overall model used for this data was significant ($F = 196.068$, $p = 0.000$) signifying that the chosen model fits the data.

Furthermore, A Post-Hoc Analysis was conducted using the Tukey-Kramer technique to determine which treatment means are similar and which are different. Comparing treatments *B. amyloliquefaciens*, *B. thuringiensis* and *Bacillus sp* with the control, it is observed that only *B. amyloliquefaciens* and *Bacillus sp* treatments were significantly different from the control (diff. = 16.40, $p = 0.023$ and diff. = 27.40, $p = 0.001$ respectively).

Table 8.1: Mean measurement characteristics of the treatment per plant

TREATMENT		MEASUREMENT CHARACTERISTICS							
		number of stems	number of leaves	length of stem	length of leaves	breadth of leaves	number of seeds	length of shoots	length of roots
WEEK 4	<i>B. thuringiensis</i>	12	54	8.1	1.5	0.9	1	1.5	9.5
	<i>B. amyloliquefaciens</i>	18	60	8	1.8	0.8	1	2	10
	<i>Bacillus sp</i>	18	78	8.4	1.9	1	2	2.2	10.1
	control	10	50	6	1	0.5	0.8	1	7
WEEK 8	<i>B. thuringiensis</i>	19	68	8.5	3.3	0.9	5	2	10.3
	<i>B. amyloliquefaciens</i>	20	75	8.9	3.2	1	4	2.9	10.5
	<i>Bacillus sp</i>	39	119	9.2	4.7	1.2	6	3	13.8
	control	15	60	7	3	0.6	3	1.5	8
WEEK 12	<i>B. thuringiensis</i>	51	117	10.5	4.8	1	11	2.9	13.1
	<i>B. amyloliquefaciens</i>	55	135	10.1	4.3	1.2	10	3.6	15.2
	<i>Bacillus sp</i>	69	177	12.9	5.5	1.4	14	4.1	16.4
	control	45	100	8	3.5	0.8	8	2	10
WEEK 16	<i>B. thuringiensis</i>	82	228	11.6	5.3	1.4	16	3.5	21.5
	<i>B. amyloliquefaciens</i>	91	246	12.7	6.6	1.8	19	4.4	20.8
	<i>Bacillus sp</i>	101	294	14.3	6.8	1.7	25	5	22.4
	control	70	200	10	4	1	12	3	15
HARVEST	<i>B. thuringiensis</i>	93	276	12.8	6.8	2.1	25	5.3	25.8
	<i>B. amyloliquefaciens</i>	118	353	13.9	7.5	2.5	36	5.7	27.2
	<i>Bacillus sp</i>	130	421	16	8	3	41	6.1	30.5
	control	80	260	10	5	2	20	5	20

Table 8.2: ANOVA for comparison of treatments and their effect on the number of stems

Dependent Variable: Number of Stem

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	91093.100 ^a	8	11386.638	196.068	.000
WEEK	24472.700	4	6118.175	105.350	.000
TREATMENT	2095.600	3	698.533	12.028	.001
Error	696.900	12	58.075		
Total	91790.000	20			

Multiple Comparisons

Dependent Variable: Number of Stems

Tukey HSD

(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
<i>B. thuringiensis</i>	<i>B. amyloliquefaciens</i>	-9.00	4.820	.292	-23.31	5.31
	<i>Bacillus</i> sp	-20.00*	4.820	.006	-34.31	-5.69
	Control	7.40	4.820	.448	-6.91	21.71
<i>B. amyloliquefaciens</i>	<i>B. thuringiensis</i>	9.00	4.820	.292	-5.31	23.31
	<i>Bacillus</i> sp	-11.00	4.820	.157	-25.31	3.31
	Control	16.40*	4.820	.023	2.09	30.71
<i>Bacillus</i> sp	<i>B. thuringiensis</i>	20.00*	4.820	.006	5.69	34.31
	<i>B. amyloliquefaciens</i>	11.00	4.820	.157	-3.31	25.31
	Control	27.40*	4.820	.001	13.09	41.71
Control	<i>B. thuringiensis</i>	-7.40	4.820	.448	-21.71	6.91
	<i>B. amyloliquefaciens</i>	-16.40*	4.820	.023	-30.71	-2.09
	<i>Bacillus</i> sp	-27.40*	4.820	.001	-41.71	-13.09

Based on observed means.

The error term is Mean Square (Error) = 58.075.

**The mean difference is significant at the .05 level.*

8.3.2 Effect of bacterial isolates on number of leaves

From Table 8.3, the results were significant for both the treatment/bacterial effect and the block/week effect at a 5% level of significance (block: $F = 89.561$, $p = 0.000$; treatment: $F = 11.745$, $p = 0.001$). Since the p-value is less than 0.05, the null hypothesis was rejected in favour of the alternative hypothesis. The overall model used for this data was significant ($F = 172.925$, $p = 0.000$) signifying that the model fits the data.

The Post-Hoc Analysis revealed that only treatment *Bacillus sp.* was significantly different from the control and the other treatments (*B. amyloliquefaciens*, *B. thuringiensis*) (diff. = 83.80, $p = 0.001$). Treatments *B. amyloliquefaciens*, *B. thuringiensis* were statistically the same when compared with each other and the control. Therefore, if treatment *B. amyloliquefaciens*, *B. thuringiensis*, are applied to the plant, there will not be any significant change but on the other hand, if treatment *Bacillus sp.* is applied to the plant an increase in the number of leaves would be expected.

Table 8.3: ANOVA for comparison of treatments and their effect on the number of leaves*Dependent Variable: Number of Leaves*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	794027.400 ^a	8	99253.425	172.925	.000
WEEK	205621.200	4	51405.300	89.561	.000
TREATMENT	20224.150	3	6741.383	11.745	.001
Error	6887.600	12	573.967		
Total	800915.000	20			

*Multiple Comparisons**Dependent Variable: Number of Leaves**Tukey HSD*

(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
<i>B. thuringiensis</i>	<i>B. amyloliquefaciens</i>	-25.20	15.152	.383	-70.19	19.79
	<i>Bacillus</i> sp	-69.20*	15.152	.003	-114.19	-24.21
	Control	14.60	15.152	.772	-30.39	59.59
<i>B. amyloliquefaciens</i>	<i>B. thuringiensis</i>	25.20	15.152	.383	-19.79	70.19
	<i>Bacillus</i> sp	-44.00	15.152	.056	-88.99	.99
	Control	39.80	15.152	.090	-5.19	84.79
<i>Bacillus</i> sp	<i>B. thuringiensis</i>	69.20*	15.152	.003	24.21	114.19
	<i>B. amyloliquefaciens</i>	44.00	15.152	.056	-.99	88.99
	Control	83.80*	15.152	.001	38.81	128.79
Control	<i>B. thuringiensis</i>	-14.60	15.152	.772	-59.59	30.39
	<i>B. amyloliquefaciens</i>	-39.80	15.152	.090	-84.79	5.19
	<i>Bacillus</i> sp	-83.80*	15.152	.001	-128.79	-38.81

*Based on observed means.**The error term is Mean Square (Error) = 573.967.***The mean difference is significant at the .05 level.***8.3.3 Effect of bacterial isolates on the length of stem**

From Table 8.4, the results were significant for both the treatment/bacterial effect and the block/week effect at a 5% level of significance (block: $F = 11.775$, $p = 0.000$; treatment: $F = 4.244$, $p = 0.029$). Since p -value is less than 0.05, the null hypothesis was rejected in favour of

the alternative hypothesis. The overall model used for this data was significant ($F = 147.807$, $p = 0.000$) signifying that the chosen model fits the data.

From the Post-Hoc analysis conducted, all the treatments *B. amyloliquefaciens*, *B. thuringiensis*, *Bacillus sp.* including the control produced the same results in term of the length of stem. Although, treatment has a significant effect when applied to the plants, there was however, no statistical difference between each of the individual treatments.

Table 8.4: ANOVA for comparison of treatments and their effect on the length of stem*Dependent Variable: Length of Stem*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	2254.451 ^a	8	281.806	147.807	.000
WEEK	89.797	4	22.449	11.775	.000
TREATMENT	24.274	3	8.091	4.244	.029
Error	22.879	12	1.907		
Total	2277.330	20			

Multiple Comparisons

Dependent Variable: Length of Stem

Tukey HSD

(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
<i>B. thuringiensis</i>	<i>B. amyloliquefaciens</i>	-2.040	.8733	.144	-4.633	.553
	<i>Bacillus</i> sp	-2.000	.8733	.155	-4.593	.593
	Control	.340	.8733	.979	-2.253	2.933
<i>B. amyloliquefaciens</i>	<i>B. thuringiensis</i>	2.040	.8733	.144	-.553	4.633
	<i>Bacillus</i> sp	.040	.8733	1.000	-2.553	2.633
	Control	2.380	.8733	.076	-.213	4.973
<i>Bacillus</i> sp	<i>B. thuringiensis</i>	2.000	.8733	.155	-.593	4.593
	<i>B. amyloliquefaciens</i>	-.040	.8733	1.000	-2.633	2.553
	Control	2.340	.8733	.082	-.253	4.933
Control	<i>B. thuringiensis</i>	-.340	.8733	.979	-2.933	2.253
	<i>B. amyloliquefaciens</i>	-2.380	.8733	.076	-4.973	.213
	<i>Bacillus</i> sp	-2.340	.8733	.082	-4.933	.253

Based on observed means.

The error term is Mean Square (Error) = 1.907.

8.3.4 Effect of bacterial isolates on the length of leaves

From Table 8.5, the results were significant for both the treatment/bacterial effect and the block/week effect at a 5% level of significance (block: $F = 30.002$, $p = 0.000$; treatment: $F = 4.961$, $p = 0.018$). Since p -value is less than 0.05, the null hypothesis was rejected in favour of the alternative hypothesis. The overall model used for this data was significant ($F = 106.612$, $p = 0.000$) signifying that the chosen model fits the data.

From the Post-Hoc analysis conducted, only treatment *Bacillus sp.* was significantly different from the control (diff. = 1.580, $p = 0.000$). The other treatments *B. amyloliquefaciens*, *B. thuringiensis* had no statistical difference when compared with the control and when compared with each other.

Table 8.5: ANOVA for comparison of treatments and their effect on the length of leaves

Dependent Variable: Length of Leaves

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	465.185 ^a	8	58.148	106.612	.000
WEEK	65.455	4	16.364	30.002	.000
TREATMENT	8.117	3	2.706	4.961	.018
Error	6.545	12	.545		
Total	471.730	20			

Multiple Comparisons

Dependent Variable: Length of Leaves

Tukey HSD

		Mean Difference	Std. Error	Sig.	95% Confidence Interval	
(I) TREATMENT	(J) TREATMENT	(I-J)			Lower Bound	Upper Bound
<i>B. thuringiensis</i>	<i>B. amyloliquefaciens</i>	-.840	.4671	.321	-2.227	.547
	<i>Bacillus</i> sp	-1.060	.4671	.160	-2.447	.327
	Control	.520	.4671	.689	-.867	1.907
<i>B. amyloliquefaciens</i>	<i>B. thuringiensis</i>	.840	.4671	.321	-.547	2.227
	<i>Bacillus</i> sp	-.220	.4671	.964	-1.607	1.167
	Control	1.360	.4671	.055	-.027	2.747
<i>Bacillus</i> sp	<i>B. thuringiensis</i>	1.060	.4671	.160	-.327	2.447
	<i>B. amyloliquefaciens</i>	.220	.4671	.964	-1.167	1.607
	Control	1.580*	.4671	.024	.193	2.967
Control	<i>B. thuringiensis</i>	-.520	.4671	.689	-1.907	.867
	<i>B. amyloliquefaciens</i>	-1.360	.4671	.055	-2.747	.027
	<i>Bacillus</i> sp	-1.580*	.4671	.024	-2.967	-.193

Based on observed means.

The error term is Mean Square (Error) = .545.

*The mean difference is significant at the .05 level.

8.3.5 Effect of bacterial isolates on the breadth of leaves

From Table 8.6, the results were significant for only the block/week effect ($F = 19.789, p = 0.000$) while the treatment/bacterial effect remained insignificant (block: $F = 19.789, p = 0.075$) at a 5% level of significance. This means that the treatment has no effect on the breadth of leaves. This is to say that no matter the treatment being applied, the results in term of the breadth of the leaves, remained the same. Since p-value is less than 0.05, the null hypothesis is rejected in favour of the alternative hypothesis. The overall model used for this data was significant ($F = 54.747, p = 0.000$) signifying that the chosen model fits the data.

Table 8.6: ANOVA for comparison of treatments and their effect on the breadth of leaves

Dependent Variable: Breadth of Leaves

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	42.301 ^a	8	5.288	54.747	.000
WEEK	7.645	4	1.911	19.789	.000
TREATMENT	.856	3	.285	2.954	.075
Error	1.159	12	.097		
Total	43.460	20			

Multiple Comparisons

Dependent Variable: Breadth of Leaves

Tukey HSD

		Mean Difference			95% Confidence Interval	
(I) TREATMENT	(J) TREATMENT	(I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
<i>B. thuringiensis</i>	<i>B. amyloliquefaciens</i>	-.160	.1966	.847	-.744	.424
	<i>Bacillus</i> sp	-.320	.1966	.400	-.904	.264
	Control	.240	.1966	.626	-.344	.824
<i>B. amyloliquefaciens</i>	<i>B. thuringiensis</i>	.160	.1966	.847	-.424	.744
	<i>Bacillus</i> sp	-.160	.1966	.847	-.744	.424
	Control	.400	.1966	.229	-.184	.984
<i>Bacillus</i> sp	<i>B. thuringiensis</i>	.320	.1966	.400	-.264	.904
	<i>B. amyloliquefaciens</i>	.160	.1966	.847	-.424	.744
	Control	.560	.1966	.061	-.024	1.144
Control	<i>B. thuringiensis</i>	-.240	.1966	.626	-.824	.344
	<i>B. amyloliquefaciens</i>	-.400	.1966	.229	-.984	.184
	<i>Bacillus</i> sp	-.560	.1966	.061	-1.144	.024

Based on observed means.

The error term is Mean Square (Error) = .097.

In the Table 8.6 above, it was discovered that treatment was insignificant ($p = 0.075$) i.e. The application of the bacteria to the plants has no effect or does not influence the growth of the breadth of the leaves in any way. Therefore, the Post-Hoc analysis revealed no significant difference between each of the individual treatments. When treatments *B. amyloliquefaciens*, *B. thuringiensis*, *Bacillus sp.* were compared with the control or were compared against one another, there was no significant differences between them.

8.3.6 Effect of bacterial isolates on number of seeds

From Table 8.7 below, the results were significant for both the treatment/bacterial effect and the block/week effect at a 5% level of significance (block: $F = 35.505$, $p = 0.000$; treatment: $F = 4.548$, $p = 0.024$). Since p-value is less than 0.05, the null hypothesis was rejected in favour of the alternative hypothesis. The overall model used for this data was significant ($F = 46.800$, $p = 0.000$) signifying that the chosen model fits the data.

The Post-Hoc analysis revealed that treatment was significantly different from the control (diff. = 8.84, $p = 0.018$) but when compared with other treatments the output remained the same. Other treatments *B. amyloliquefaciens* and *B. thuringiensis* had no significant difference when compared with the control and when compared with one another.

Table 8.7: ANOVA for comparison of treatments and their effect on the number of seeds

Dependent Variable: Number of Seeds

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	5776.496 ^a	8	722.062	46.800	.000
WEEK	2191.208	4	547.802	35.505	.000
TREATMENT	210.486	3	70.162	4.548	.024
Error	185.144	12	15.429		
Total	5961.640	20			

Multiple Comparisons

Dependent Variable: Number of Seeds

Tukey HSD

		Mean Difference			95% Confidence Interval	
(I) TREATMENT	(J) TREATMENT	(I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
<i>B. thuringiensis</i>	<i>B. amyloliquefaciens</i>	-2.40	2.484	.771	-9.78	4.98
	<i>Bacillus</i> sp	-6.00	2.484	.127	-13.38	1.38
	Control	2.84	2.484	.671	-4.54	10.22
<i>B. amyloliquefaciens</i>	<i>B. thuringiensis</i>	2.40	2.484	.771	-4.98	9.78
	<i>Bacillus</i> sp	-3.60	2.484	.495	-10.98	3.78
	Control	5.24	2.484	.205	-2.14	12.62
<i>Bacillus</i> sp	<i>B. thuringiensis</i>	6.00	2.484	.127	-1.38	13.38
	<i>B. amyloliquefaciens</i>	3.60	2.484	.495	-3.78	10.98
	Control	8.84*	2.484	.018	1.46	16.22
Control	<i>B. thuringiensis</i>	-2.84	2.484	.671	-10.22	4.54
	<i>B. amyloliquefaciens</i>	-5.24	2.484	.205	-12.62	2.14
	<i>Bacillus</i> sp	-8.84*	2.484	.018	-16.22	-1.46

Based on observed means.

The error term is Mean Square (Error) = 15.429.

**The mean difference is significant at the .05 level.*

8.3.7 Effect of bacterial isolates on length of shoot

From Table 8.8 below, the results were significant for both the treatment/bacterial effect and the block/week effect at a 5% level of significance (block: $F = 98.076$, $p = 0.000$; treatment: $F = 25.253$, $p = 0.000$). Since p -value was less than 0.05, the null hypothesis was rejected in favour of the alternative hypothesis. The overall model used for this data was significant ($F = 362.670$, $p = 0.000$) signifying that the chosen model fits the data.

The Post-Hoc analysis revealed that both treatment *B. amyloliquefacien* and *Bacillus sp.* were significantly different from the control (diff. = 1.240, $p = 0.000$ and diff. = 1.440, $p = 0.00$ respectively) and there was a significant difference between treatment *B. amyloliquefaciens*, *B. thuringiensis* (diff. = 0.820, $p = 0.005$) as well as a significant difference between treatments *B. thuringiensis* and *Bacillus sp.* (diff. = 1.020, $p = 0.001$).

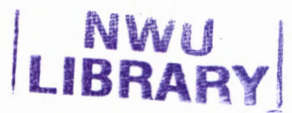


Table 8.8: ANOVA for comparison of treatments and their effect on the length of shoots

Dependent Variable: Length of Stems

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	265.233 ^a	8	33.154	362.670	.000
WEEK	35.863	4	8.966	98.076	.000
TREATMENT	6.925	3	2.308	25.253	.000
Error	1.097	12	.091		
Total	266.330	20			

Multiple Comparisons

Dependent Variable: Length of Shoots

Tukey HSD

(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
<i>B. thuringiensis</i>	<i>B. amyloliquefaciens</i>	-.820*	.1912	.005	-1.388	-.252
	<i>Bacillus</i> sp	-1.020*	.1912	.001	-1.588	-.452
	Control	.420	.1912	.179	-.148	.988
<i>B. amyloliquefaciens</i>	<i>B. thuringiensis</i>	.820*	.1912	.005	.252	1.388
	<i>Bacillus</i> sp	-.200	.1912	.727	-.768	.368
	Control	1.240*	.1912	.000	.672	1.808
<i>Bacillus</i> sp	<i>B. thuringiensis</i>	1.020*	.1912	.001	.452	1.588
	<i>B. amyloliquefaciens</i>	.200	.1912	.727	-.368	.768
	Control	1.440*	.1912	.000	.872	2.008
Control	<i>B. thuringiensis</i>	-.420	.1912	.179	-.988	.148
	<i>B. amyloliquefaciens</i>	-1.240*	.1912	.000	-1.808	-.672
	<i>Bacillus</i> sp	-1.440*	.1912	.000	-2.008	-.872

Based on observed means.

The error term is Mean Square (Error) = .091.

*The mean difference is significant at the .05 level.

8.3.8 Effect of bacterial isolates on length of roots

From Table 8.9 below, the results were significant for both the treatment/bacterial effect and the block/week effect at a 5% level of significance (treatment: $F = 10.975$, $p = 0.001$; block: $F = 59.539$, $p = 0.00$). Since p -value was less than 0.05, the null hypothesis was rejected in favour of the alternative hypothesis. The overall model used for this data was significant ($F = 219537$, $p = 0.000$) signifying that the chosen model fits the data.

The Post-Hoc analysis revealed that treatment *B. amyloliquefaciens* and *Bacillus sp* were significantly different from the control (diff. = 5.400, $p = 0.003$ and diff. = 5.980, $p = 0.001$ respectively). When the treatments were compared with each other, there was no significant difference existed between them.

Table 8.9: ANOVA for comparison of treatments and their effect on the length of roots

Dependent Variable: Length of Roots

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	5829.589 ^a	8	728.699	219.537	.000
WEEK	790.505	4	197.626	59.539	.000
TREATMENT	109.284	3	36.428	10.975	.001
Error	39.831	12	3.319		
Total	5869.420	20			

Multiple Comparisons

Dependent Variable: Length of Roots

Tukey HSD

(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
<i>B. thuringiensis</i>	<i>B. amyloliquefaciens</i>	-1.980	1.1523	.357	-5.401	1.441
	<i>Bacillus</i> sp	-2.560	1.1523	.172	-5.981	.861
	Control	3.420	1.1523	.050	-.001	6.841
<i>B. amyloliquefaciens</i>	<i>B. thuringiensis</i>	1.980	1.1523	.357	-1.441	5.401
	<i>Bacillus</i> sp	-.580	1.1523	.957	-4.001	2.841
	Control	5.400*	1.1523	.003	1.979	8.821
<i>Bacillus</i> sp	<i>B. thuringiensis</i>	2.560	1.1523	.172	-.861	5.981
	<i>B. amyloliquefaciens</i>	.580	1.1523	.957	-2.841	4.001
	Control	5.980*	1.1523	.001	2.559	9.401
Control	<i>B. thuringiensis</i>	-3.420	1.1523	.050	-6.841	.001
	<i>B. amyloliquefaciens</i>	-5.400*	1.1523	.003	-8.821	-1.979
	<i>Bacillus</i> sp	-5.980*	1.1523	.001	-9.401	-2.559

Based on observed means.

The error term is Mean Square (Error) = 3.319.

*The mean difference is significant at the .05 level.

8.3.9 Molecular identification of selected isolates

A 1.5 kb fragment was amplified from the genomic DNA with the bacterial universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'CGGTTACCTTGTTACGACTT-3'). Identification of the isolates was confirmed by computational analysis. The BLAST tool was used to compare the partial nucleotide sequences of the 16S rDNA gene of the isolates with the nucleotide database of NCBI web server. From the BLAST search, it was inferred that the isolates were members of the GC-rich firmicutes. Four of the isolates in this study were from the genus *Bacillus* which are known to be spore-forming *Bacillus* spp and have been involved in plant growth promotion and also biocontrol of plants from pests and diseases. Three (3) of them were selected for plant growth promotion activities.

The genus *Microbacterium* are mostly endophytic bacteria but in this study, they were found in the soil rhizosphere. They are part of the phylum Actinobacteria and are gram positive closer to *Bacillus* spp while *Acinetobacter parvus* is from the phylum proteobacteria and gram negative bacteria, which are not too closely related to the phylum proteobacteria and firmicutes.

Table 8.10: Results of 16S rDNA gene sequence similarities of bacterial isolates and GenBank accession numbers using BLASTn algorithm

Isolate code	Sequence length (bp)	Closest related strain in database	Accession number	Similarity (%)	E-value
BAMbi	1403	<i>B. safensis</i>	KX809651	99	0
BAMhi	1443	<i>B. amyloliquefaciens</i>	KX809652	99	0
BAMli	1388	<i>B. thuringiensis</i>	KX809653	99	0
BAMr	1058	<i>Bacillus</i> sp.	KX588095	98	0
BAMpii	1414	<i>M. hydrocarbonoxydans</i>	KX809654	99	0
BAMrii	1416	<i>M. testaceum</i>	KX809655	99	0
BAMxii	1389	<i>Acinetobacter parvus</i>	KX809656	98	0

8.4 Discussion

The impact of plant growth promoting rhizobacteria on plants in the same environment from where they were isolated is important in biofertilization. This is because the natural environment of the rhizobacteria helps them to establish in such an environment and enable their quick PGPR activities. When transferred to a different environment, the rhizobacteria requires time to establish after competition with other resident bacteria. Rhizobacteria *B. amyloliquefaciens*, *B. thuringiensis*, *Bacillus sp.* were able to stabilize in the soil and affect plant growth within the expected growth stage of bambara groundnut. The number of stems throughout all the treatments and in comparison with the control increased significantly with the highest number of stems from the treatment with *Bacillus sp.* and the lowest as the control. The number of stems of bambara groundnut recorded in this study ranged from 10 in the control at 4 WAP to 130 in harvest with treatment with *Bacillus sp.* This is very high and comparable with those reported by other authors which range from average of 23.97 to 28.87 recorded by Onwubiko et al. (2011a) and number of branches/nodes/stem ranging from 2 to 25 in the study by Goli et al. (1997).

The length of shoot in this study ranged between 1 cm in control and 6.1 cm at harvest with the *Bacillus sp.* treatment. The length of shoots in this study falls within the range of 1cm to 20 cm long, as recommended by Murevanhema and Jideani (2013). Also, studies by Chibarabada et al. (2015) was observed with range between 11.68 cm and 17.8 cm, the difference might be as a result of different genotypes, varieties of landraces or cultivars planted and/or the condition under which they were grown. The root length ranged from 7 cm at 4 WAP in control to 30.5 cm at harvest in the treatment BAMr compared to the range between 4.36 cm and 6.31 cm also observed by Chibarabada et al. (2015).

In this study, the mean number of leaves per plant ranged from 50 leaves in the control at 4 WAP to 421 leaves in treatment with *Bacillus sp.* at harvest, while Onuh and Christo (2011)

recorded that the mean number of leaves ranged from 9 in the control at 28 days after planting (DAP) to 14.1 at 14 DAP in the treatment with 500 ml of water. The number of leaves in this study far exceeds what has been recorded as number of leaves in bambara groundnut.

The number of seeds in this study ranged from 0.8 in the control at 0 WAP to 41 seeds in treatment with *Bacillus sp.* at harvest. It was also observed that at 4 and 8 WAP, some of the plants had started producing pods and seeds which were also reported by Swanevelde (1998) that flowering and seeding in bambara groundnut can start as early as 30 DAP. There was an increase in seed yield in contrast with the report by Onuh and Christo (2011) whose mean seed yield ranged between 2.3 and 3.3 seeds. This increase can be linked to the *Bacillus* isolates treatment of the seeds as was recorded by Iqbal Hussain et al. (2013) who observed that treatment of maize with *Bacillus* spp yielded increase from 36.7 g/plant to 53.87 g/plant after 16 weeks compared with control which increased from 34.4 g/plant to 40.6 g/plant at 16 weeks. Increase in yield of runner beans was recorded as ranging from 641.25 kg/ha in the control to 906.7 kg/ha in treatment with S7 (Stefan et al., 2013).

The results obtained from application of the biofertilized seeds before planting is significantly different from the control. This shows that biofertilization improved the soil composition so that minerals were easily made available to plants and then plant growth and crop yield were enhanced. When the composition of microbial communities in the soil is altered, it is as a result of the alteration of the chemical composition of the soils (Rengel and Marschner, 2005) and this can be due to the secretion of exudates by the plant and or bacterial communities (Dakora and Phillips, 2002). The ability of the *Bacillus* isolates to increase bambara groundnut growth could be as a result of the PGP potentials of the isolates (Ajillogba et al 2016). They were able to solubilise phosphate, produce indole acetic acid, ammonia and hydrogen cyanide. These potentials have been harnessed by different bacterial isolates to improve growth.

Rhizobacterial isolates were characterized using 16Sr RNA. The molecular relationship of the isolates shows that 57.14% are from the phylum Firmicutes, 28.57% are from phylum Actinobacteria while 14.28% are from the phylum Proteobacteria. The Actinobacteria and Firmicutes are closer together as they are both gram-positive and contain high guanine and cytosine content in their DNA while Proteobacteria are gram negative bacteria. In this study, there were more Firmicutes compared to Actinobacteria and Proteobacteria. This is in agreement with the community structure of the rhizosphere of vascular plant from the antarctics having Firmicutes as the most abundant phyla while Firmicutes, Actinobacteria and Proteobacteria as the 3 most abundant phyla (Teixeira et al., 2010). These 3 groups of bacteria, Firmicutes, Actinobacteria and Proteobacteria have been described as important plant growth promoting groups of bacteria (Lagos et al., 2015). This ability to promote growth has also been proved in this study as the 3 *Bacillus* strains increased plant growth.

8.5 Conclusion

The 3 *Bacillus* isolates used in field planting of Bambara groundnut in this study showed that they improved growth compared to the control. *Bacillus sp.* improved growth more than the other two *B. amyloliquefaciens* and *B. thuringiensis*. These isolates have been proved to have potential as biofertilizers under field conditions being adapted to all the environmental challenges. Hence, they can be used as a substitute for chemical fertilizers.

CHAPTER NINE

GENERAL CONCLUSION

The health of the soil or quality of soil is both directly and indirectly related to crop yield and invariably food security. Interactions between soil rhizobacteria and bambara groundnut are diverse and varied. They have led to an increase in soil organic matter and are involved in nutrient and mineral cycling and thus promote a healthy plant life. Cultivation of bambara groundnut alongside other staple crops will also help to increase their crop yields making nutritious food available. Bambara groundnut is a legume with a promising future if it is given the right publicity and the support it deserves, both as a crop and food

This study revealed that the physical and chemical properties of soil at different growth stages vary and affect the number, types and diversities of bacteria around/in bambara groundnut rhizosphere. The Eh and pH of the soil were important in the diversity of the bacterial isolates from the rhizosphere. The Eh and pH affected the type and abundance of the bacterial isolate at each growth stage. The Eh and pH also contributed to the biocontrol potential of bacterial isolates. Out of 43 isolates cultured in this study, 18 showed either plant growth-promoting or biocontrol activities against at least 1 out of 4 test organisms.

In this study, soil microbial carbon source utilization and diversity were greatly affected by the presence of bambara groundnut and their development stages. Carbohydrates, carboxylic acids and amino acids were the most utilized carbon sources. It was evident that physiologic activities of the rhizosphere bacterial communities of bambara groundnut measured by BIOLOG assay suggested that the metabolic capabilities of the bacterial/microorganism communities in the rhizosphere were higher in the soil during the growth stages when compared with the bulk soil control. This capability was particularly evident during the flowering stage (4 to 8 WAP). This may be important to farmers and crop breeders involved in mixed or intercropping as planting

of other crops during the flowering stage of bambara groundnut can support better plant growth due to the diverse bacterial activities.

Some of the bioactive compounds identified in this study have been identified already but from different sources. This was the first time secondary metabolites were screened from the rhizobacteria of bambara groundnut and it is obvious that the rhizobacteria are hosts to many metabolites. Tropone and tropoline have been found as lipid solubilizing agents and are important as anti-cancer, antibacterial and antifungal compounds. For example, thiotropocin and tropodithietic acid (TDA) are both tropone antibiotics isolated from marine *Roseobacter* spp and soil *Pseudomonas* spp with thiotropocin having a broad spectrum antimicrobial activity against Gram positive, Gram negative bacteria and mycoplasma. Another compound, p-xylene which is a chemical feedstock in the chemical industry was secreted by the three selected isolates which were all *Bacillus* spp. Other bioactive compounds were tridecane, fumaronitrile, isocarboxazid and tridecylamine.

This study revealed that changes occurred in the rhizobacterial community of bambara groundnut grown in the field. The changes were distinct as it was observed that the bacterial communities from the growth stages contained potential PGPR genera that are specific to each growth stage and are highly abundant. The changes observed in the bacterial communities are in part due to the growth stage because of the different metabolic and physiological activities taking place during those growth stages in the rhizosphere and also the influence of Bambara groundnut as a legume. This knowledge is important to agriculturist in particular, so that particular stages of plant growth are targeted for interactions between plants and rhizosphere microbes in the field for better utilization of rhizobacteria in sustainable agriculture.

The 3 *Bacillus* strains used to inoculate the seeds of Bambara groundnut before planting in this study shows that they improved its growth compared to the control. *Bacillus* sp improved

growth more than the other two *Bacillus* strains (*B. amyloliquefaciens* and *B. thuringiensis*). Their growth parameters especially crop yield were found to exceed those that have been reported earlier on without bio-treatment. As part of the study carried out in this research earlier, it was observed that these *Bacillus* isolates were able to suppress the growth the *F. graminearum* and bacteria *B. cereus* and *E. faecalis* that are pathogenic to man, animals and plants. This suppression was made possible by their ability to produce HCN which is important in antagonising pathogens and indirectly support plant growths. They were able to produce indole acetic acid, ACC deaminase and solubilise phosphate which are very important in improving plant growths. These isolates have been established in this study to have great potentials as biofertilizers on the field in spite of all the environmental challenges. Hence, they can be used as biocontrol to substitute for chemical fertilizers and also to act as biofertilizers.

Relationship between the microbial carbon source utilization and metagenomics diversity

Carbon utilization by the microbial communities in bambara groundnut rhizosphere depicted by the box and whiskers plot showed that the microbial activities at the 4WAP and 8WAP across the growth stages were significantly different compared to the other stages. This is in agreement with the result from the NGS which showed the bacterial phyla of soil samples from 4WAP, 8WAP and 12 WAP were significantly diverse compared to that of the bulk soil and 16WAP. The significant difference did not affect the species in each sample but only the abundance. This is also true with the carbon source utilization observed throughout the growth stages but only different in the depth of utilization of the carbon sources as observed in the Shannon-Weaver diversity index.

REFERENCES

- Abdulkadir, M. & Waliyu, S. 2012. Screening and isolation of the soil bacteria for ability to produce antibiotics. *European Journal of Applied Sciences*, 4, 211-215.
- Abdullah, A. A., Altaf-Ul-Amin, M., Ono, N., Sato, T., Sugiura, T., Morita, A. H., Katsuragi, T., Muto, A., Nishioka, T. & Kanaya, S. 2015. Development and mining of a volatile organic compound database. *BioMed Research International*, 2015, 13.
- Abollino, O., Aceto, M., Malandrino, M., Mentasi, E., Sarzanini, C. & Petrella, F. 2002. Heavy metals in agricultural soils from Piedmont, Italy. Distribution, speciation and chemometric data treatment. *Chemosphere*, 49, 545-557.
- Adeparusi, E. & Agbede, J. 2005. Evaluation of *Leucaena* and *Gliricidia* leaf protein concentrate as supplements to Bambara groundnut (*Vigna subterranea* (L. Verdc) in the diet of *Oreochromis niloticus*. *Aquaculture Nutrition*, 12, 335-342.
- Adesemoye, A., Torbert, H. & Kloepper, J. 2010. Increased plant uptake of nitrogen from ¹⁵N-depleted fertilizer using plant growth-promoting rhizobacteria. *Applied Soil Ecology*, 46, 54-58.
- Agbodjato, N. A., Noumavo, P. A., Baba-Moussa, F., Salami, H. A., Sina, H., Sèzan, A., Bankolé, H., Adjanooun, A. & Baba-Moussa, L. 2015. Characterization of potential plant growth promoting rhizobacteria Isolated from maize (*Zea mays* L.) in Central and Northern Benin (West Africa). *Applied and Environmental Soil Science*, 2015.
- Agrawal, D. P. K. & Agrawal, S. 2013. Characterization of *Bacillus* sp. strains isolated from rhizosphere of tomato plants (*Lycopersicon esculentum*) for their use as potential plant growth promoting rhizobacteria. *International Journal of Current Microbiology and Applied Science*, 2, 406-417.
- Ahemad, M. & Kibret, M. 2014. Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. *Journal of King Saud University-Science*, 26, 1-20.

- Ahmad, F., Ahmad, I. & Khan, M. 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiological Research*, 163, 173-181.
- Aislabie, J., Deslippe, J. R. & Dymond, J. 2013. Soil microbes and their contribution to soil services. In: DYMOND, J. (ed.)(eds.) *Ecosystem Services in New Zealand: Conditions and Trends*. Lincoln, New Zealand: Manaaki Whenua Press.
- Ajayi, F. A. & Lale, N. E. S. 2001. Susceptibility of unprotected seeds of local Bambara groundnut cultivars protected with insecticidal essential oils to infestation by *Callosbrucuss maculates*. *Journal of Stores Production*, 37, 47-62.
- Ajilogba, C., Babalola, O., Adebola, P. & Adeleke, R. 2016. Evaluation of PGPR and biocontrol activities of bacteria isolated from Bambara groundnut rhizosphere. *New Biotechnology*, S128-S129.
- Ajilogba, C. F. & Babalola, O. O. 2013. Integrated management strategies for tomato *Fusarium* wilt. *Biocontrol Science* 18, 117-127.
- Ajilogba, C. F., Babalola, O. O. & Ahmad, F. 2013. Antagonistic effects of *Bacillus* species in biocontrol of tomato *Fusarium* wilt. *Studies on Ethno-Medicine*, 7, 205-216.
- Akpalu, M., Atubilla, I. & Oppong-Sekyere, D. 2013. Assessing the level of cultivation and utilization of Bambara groundnut (*Vigna subterranea* (L.) Verdc.) In the Sumbrungu community of Bolgatanga, Upper East Region, Ghana. *International Journal of Plant, Animal and Environmental Sciences*, 3, 68-75.
- Alhassan, G. A. & Egbe, M. O. 2014. Bambara groundnut/maize intercropping: Effects of planting densities in Southern guinea savanna of Nigeria. *African Journal of Agricultural Research*, 9, 479-486.
- Ali, G. S., Norman, D. & El-Sayed, A. S. 2015. Chapter Ten-soluble and volatile metabolites of plant growth-promoting rhizobacteria (PGPRs): Role and practical applications in

- inhibiting pathogens and activating induced systemic resistance (ISR). *Advances in Botanical Research*, 75, 241-284.
- Allardyce, R. A., Langford, V. S., Hill, A. L. & Murdoch, D. R. 2006. Detection of volatile metabolites produced by bacterial growth in blood culture media by selected ion flow tube mass spectrometry (SIFT-MS). *Journal of Microbiological Methods*, 65, 361-365.
- Amin, M., Rakhisi, Z. & Zarei Ahmady, A. 2015. Isolation and identification of *Bacillus* species from soil and evaluation of their antibacterial properties. *Avicenna Journal of Clinical Microbiology and Infection*, 2, e23233.
- Andrews, M. & Andrews, M. E. 2017. Specificity in legume-rhizobia symbioses. *International Journal of Molecular Sciences*, 18, 705.
- Antoun, H. 2013. Plant growth-promoting rhizobacteria. In: HUGHES, S. M. (ed.)(eds.) *Brenner's Encyclopedia of Genetics (Second Edition)*. San Diego: Academic Press.
- Appuhn, A. & Joergensen, R. G. 2006. Microbial colonisation of roots as a function of plant species. *Soil Biology and Biochemistry*, 38, 1040-1051.
- Arguelles-Arias, A., Ongena, M., Halimi, B., Lara, Y., Brans, A., Joris, B. & Fickers, P. 2009. *Bacillus amyloliquefaciens* GA1 as a source of potent antibiotics and other secondary metabolites for biocontrol of plant pathogens. *Microbial Cell Factories*, 8, 1.
- Arıkan, Ş., İpek, M. & Pırlak, L. 2013. Effects of plant growth promoting rhizobacteria (PGPR) on yield and fruit quality of quince. *International Proceedings of Chemical, Biological & Environmental Engineering*, 60, 97.
- Asei, R. 2015. *Evaluation of some microbial inoculants and micronutrient fertilizers on the performance of soybean and maize in the northern savannah zones of Ghana*. Master of Science in Soil Science (Microbiology Option), Kwame Nkrumah University of Science and Technology, Kumasi.

- Azam-Ali, S. N. & Squire, G. R. 2002. *Principles of tropical agronomy*, Wallingford, United Kingdom, CABI.
- Babalola, O. O. 2007. Pectinase and cellulase enhance the control of *Abutilon theophrasti* by *Colletotrichum coccodes*. *Biocontrol Science and Technology*, 17, 53-61.
- Babalola, O. O. & Akindolire, A. M. 2011. Identification of native rhizobacteria peculiar to selected food crops in Mmabatho municipality of South Africa. *Biological Agriculture and Horticulture* 27, 294-309.
- Bacon, C. W., Palencia, E. R. & Hinton, D. M. 2015. Abiotic and biotic plant stress-tolerant and beneficial secondary metabolites produced by endophytic *Bacillus* species. *Plant Microbes Symbiosis: Applied Facets*. Springer.
- Badri, D. V., Chaparro, J. M., Zhang, R., Shen, Q. & Vivanco, J. M. 2013. Application of natural blends of phytochemicals derived from the root exudates of *Arabidopsis* to the soil reveal that phenolic-related compounds predominantly modulate the soil microbiome. *Journal of Biological Chemistry*, 288, 4502-4512.
- Badri, D. V. & Vivanco, J. M. 2009. Regulation and function of root exudates. *Plant Cell Environment*, 32, 666–681.
- Baetz, U. & Martinoia, E. 2014. Root exudates: the hidden part of plant defense. *Trends in Plant Science*, 19, 90-98.
- Bakker, M. G., Manter, D. K., Sheflin, A. M., Weir, T. L. & Vivanco, J. M. 2012. Harnessing the rhizosphere microbiome through plant breeding and agricultural management. *Plant and Soil*, 360, 1-13.
- Baldwin, K. R. 2006. Crop rotations on organic farms. *Center for Environmental Farming Systems*. North Carolina North Carolina A&T State University.

- Bamishaiye, O., Adegbola, J. & Bamishaiye, E. 2011. Bambara groundnut: An under-utilized nut in Africa. *Advances in Agricultural Biotechnology*, 1, 60-72.
- Baudoin, E., Benizri, E. & Guckert, A. 2002. Impact of growth stage on the bacterial community structure along maize roots, as determined by metabolic and genetic fingerprinting. *Applied Soil Ecology*, 19, 135-145.
- Baudoin, J. P. & Mergeai, G. (eds.) 2001. *Bambara groundnut*, Brussels, Belgium: DGIC, Ministry of foreign affairs, External Trade and International development.
- Baysal, Ö., Çalışkan, M. & Yeşilova, Ö. 2008. An inhibitory effect of a new *Bacillus subtilis* strain (EU07) against *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Physiological and Molecular Plant Pathology*, 73, 25-32.
- Beegle, D. n.d. Soil fertility management for forage crops -Pre-establishment. In: UNIVERSITY, P. S. (ed.) *Agronomy Facts 31-A*. Pennsylvania, USA: Pennsylvania State University College of Agricultural Sciences
- Berendsen, R. L., Pieterse, C. M. & Bakker, P. A. 2012. The rhizosphere microbiome and plant health. *Trends in Plant Science*, 17, 478-486.
- Berg, G. 2009. Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Applied Microbiology and Biotechnology*, 84, 11-18.
- Berg, G. & Smalla, K. 2009. Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiology Ecology*, 68, 1-13.
- Bhattacharyya, P. & Jha, D. 2012. Plant growth-promoting rhizobacteria (PGPR): Emergence in agriculture. *World Journal of Microbiology and Biotechnology*, 28, 1327-1350.
- Biswas, J., Ladha, J. & Dazzo, F. 2000. *Rhizobia* inoculation improves nutrient uptake and growth of lowland rice. *Soil Science Society of America Journal*, 64, 1644-1650.

- Bitas, V., Kim, H.-S., Bennett, J. W. & Kang, S. 2013. Sniffing on Microbes: Diverse roles of microbial volatile organic compounds in plant health. *Molecular Plant-Microbe Interactions*, 26, 835-843.
- Boutigny, A.-L., Ward, T. J., Ballois, N., Iancu, G. & Ioos, R. 2014. Diversity of the *Fusarium graminearum* species complex on French cereals. *European journal of plant pathology*, 138, 133-148.
- Bric, J. M., Bostock, R. M. & Silverstone, S. E. 1991. Rapid in situ assay for indole acetic acid production by bacteria immobilized on a nitrocellulose membrane. *Applied and Environmental Microbiology*, 57, 535-538.
- Brink, M., Ramolemana, G. M. & Sibuga, K. P. 2006. *Vigna subterranea* (L.) Verdc. Record from Protabase. In: BRINK, M. & BELAY, G. (eds.) *PROTA (Plant Resources of Tropical Africa / Ressources végétales de l'Afrique tropicale)*. Wageningen, Netherlands: PROTA
- Brough, S. H. & Azam-Ali, S. N. 1992. The effect of soil moisture on the proximate composition of Bambara groundnut (*Vigna subterranea* (L) Verdc). *Journal of the Science of Food and Agriculture*, 60, 197-203.
- Brough, S. H., Taylor, A. J. & Azam-Ali, S. N. 1993. The potential of Bambara groundnut (*Vigna subterranea*) in vegetable milk production and basic protein functionality systems. *Food Chemistry*, 47, 277-283.
- Bulgarelli, D., Rott, M., Schlaeppi, K., Van Themaat, E. V. L., Ahmadinejad, N., Assenza, F., Rauf, P., Huettel, B., Reinhardt, R. & Schmelzer, E. 2012. Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. *Nature*, 488, 91–95.

- Calvo, P., Ormeño-Orrillo, E., Martínez-Romero, E. & Zúñiga, D. 2010. Characterization of *Bacillus* isolates of potato rhizosphere from andean soils of Peru and their potential PGPR characteristics. *Brazilian Journal of Microbiology*, 41, 899-906.
- Campanoni, P., Blasius, B. & Nick, P. 2003. Auxin transport synchronizes the pattern of cell division in a tobacco cell line. *Plant Physiology*, 133, 1251-1260.
- Campbell, C., Grayston, S. & Hirst, D. 1997. Use of rhizosphere carbon sources in sole carbon source tests to discriminate soil microbial communities. *Journal of Microbiological Methods*, 30, 33-41.
- Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., Fierer, N. & Knight, R. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences*, 108, 4516-4522.
- Carvalhais, L. C., Dennis, P. G., Fedoseyenko, D., Hajirezaei, M. R., Borriss, R. & Von Wirén, N. 2011. Root exudation of sugars, amino acids, and organic acids by maize as affected by nitrogen, phosphorus, potassium, and iron deficiency. *Journal of Plant Nutrition and Soil Science*, 174, 3–11.
- Castric, P. A. 1975. Hydrogen cyanide, a secondary metabolite of *Pseudomonas aeruginosa*. *Canadian Journal of Microbiology*, 21, 613-618.
- Cavaglieri, L., Orlando, J. & Etcheverry, M. 2009. Rhizosphere microbial community structure at different maize plant growth stages and root locations. *Microbiological Research*, 164, 391-399.
- Cavaglieri, L., Orlando, J., Rodriquez, M. I., Chulze, S. & Etcheverry, M. 2005. Biocontrol of *Bacillus subtilis* against *Fusarium verticillioides* *in vitro* and at maize root level. *Research Microbiology*, 156, 748-754.

- Celine, M. 2010. *Molecular, environmental and nutritional evaluation of bambara groundnut (Vigna subterranea (L) Verdc.) for food production in Namibia.*
- Cesco, S., Mimmo, T., Tonon, G., Tomasi, N., Pinton, R., Terzano, R., Neumann, G., Weisskopf, L., Renella, G., Landi, L. & Nannipieri, P. 2012. Plant-borne flavonoids released into the rhizosphere: Impact on soil bio-activities related to plant nutrition. A review. *Biology and Fertility of Soils*, 48, 123-149.
- Cesco, S., Neumann, G., Tomasi, N., Pinton, R. & Weisskopf, L. 2010. Release of plant-borne flavonoids into the rhizosphere and their role in plant nutrition. *Plant and Soil*, 329, 1-25.
- Chandra, S., Choure, K., Dubey, R. C. & Maheshwari, D. K. 2007. Rhizosphere competent *Mesorhizobium loti* MP6 induces root hair curling, inhibits *Sclerotinia sclerotiorum* and enhances growth of Indian mustard (*Brassica campestris*). *Brazilian Journal of Microbiology*, 38, 124-130.
- Chaparro, J. M., Badri, D. V., Bakker, M. G., Sugiyama, A., Manter, D. K. & Vivanco, J. M. 2013. Root exudation of phytochemicals in *Arabidopsis* follows specific patterns that are developmentally programmed and correlate with soil microbial functions. *PloS one*, 8, e55731.
- Chaparro, J. M., Badri, D. V. & Vivanco, J. M. 2014. Rhizosphere microbiome assemblage is affected by plant development. *The ISME Journal*, 8, 790-803.
- Chapman, H. D. 1965. Cation-exchange Capacity. *American Society of Agronomy, Method of soil analysis, Part 2: Chemical and microbiological properties*, 891-900.
- Chen, X. P., Kong, W. D., He, J. Z., Liu, W. J., Smith, S. E., Smith, F. A. & Zhu, Y. G. 2008. Do water regimes affect iron-plaque formation and microbial communities in the rhizosphere of paddy rice? *Journal of Plant Nutrition and Soil Science*, 171, 193-199.

- Chibarabada, T. P., Modi, A. T. & Mabhaudhi, T. 2015. Water use characteristics of a Bambara groundnut (*Vigna subterranea* L. Verde) landrace during seedling establishment. *Water South Africa*, 41, 472-482.
- Chung, J.-H., Song, G. C. & Ryu, C.-M. 2015. Sweet scents from good bacteria: Case studies on bacterial volatile compounds for plant growth and immunity. *Plant Molecular Biology*, 1-11.
- Compant, S., Duffy, B., Nowak, J., Clément, C. & Barka, E. A. 2005. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied and environmental microbiology*, 71, 4951-4959.
- Condolji, R. J. & Yarnia, M. The response of yield and related traits of pinto-bean cultivars towards the use of nitrogen chemical and biological fertilizers. *Journal of Biodiversity and Environmental Sciences* 5, 469-478.
- Dakora, F. & Keya, S. 1997. Contribution of legume nitrogen fixation to sustainable agriculture in Sub-Saharan Africa. *Soil Biology and Biochemistry*, 29, 809-817.
- Dakora, F. D. 2000. Commonality of root nodulation signals and nitrogen assimilation in tropical grain legumes belonging to the tribe Phaseoleae. *Functional Plant Biology*, 27, 885-892.
- Dakora, F. D. 2003. Defining new roles for plant and rhizobial molecules in sole and mixed plant cultures involving symbiotic legumes. *New Phytologist*, 158, 39-49.
- Dakora, F. D., Matiru, V., King, M. & Phillips, D. A. 2002. Plant growth promotion in legumes and cereals by lumichrome, a rhizobial signal metabolite. In: FINAN, T. M., O'BRIAN, M. R., LAYZELL, D. B., VESSEY, K. & NEWTON, W. E. (eds.) *Nitrogen fixation: global perspectives*. Wallingford, UK: CABI Publishing.



- Dakora, F. D., Matiru, V. N. & Kanu, A. S. 2015. Rhizosphere ecology of lumichrome and riboflavin, two bacterial signal molecules eliciting developmental changes in plants. *Frontiers in Plant Science*, 6, 700.
- Dakora, F. D. & Phillips, D. A. 2002. Root exudates as mediators of mineral acquisition in low-nutrient environments. *Plant and Soil*, 245, 35-47.
- Das, B. & Chakrabarti, K. 2013. Assessment of community level physiological profiles and molecular diversity of soil bacteria under different cropping systems. *Turkish Journal of Agriculture and Forestry*, 37, 468-474.
- Dastager, S. G., Deepa, C. & Pandey, A. 2011. Growth enhancement of black pepper (*Piper nigrum*) by a newly isolated *Bacillus tequilensis* NII-0943. *Biologia*, 66, 801-806.
- De Bruijn, F. J. 2015. Biological nitrogen fixation. *Principles of Plant-Microbe Interactions*, 1 215-224.
- De Kock, C. 2013. Bambara groundnut. Harare, Zimbabwe: Specialty Foods of Africa Pvt Ltd.
- Demain, A. L. & Fang, A. 2000. The natural functions of secondary metabolites. *Advances in Biochemical Engineering*, 69, 1-39.
- Dennis, P. G., Miller, A. J. & Hirsch, P. R. 2010. Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities? *FEMS Microbiology Ecology*, 72, 313–327.
- Doku, E. V. 1996. Problems and prospects for the improvement of Bambara groundnut. *Proceedings of the International Bambara Groundnut Symposium*. University of Nottingham UK.
- Dorokhov, Y. L., Komarova, T. V. & Sheshukova, E. V. 2014. Chapter 13 - Volatile organic compounds and plant virus–host interaction. In: SHARMA, R. K., GAUR, T. & HOHN, P. (ed.)(eds.) *Plant Virus–Host Interaction*. Boston: Academic Press.

- Dunn, W. B., Bailey, N. J. & Johnson, H. E. 2005. Measuring the metabolome: Current analytical technologies. *Analyst*, 130, 606-625.
- Egbe, M. O., Alhassan, G. A. & Ijoyah, M. 2013. Nodulation, nitrogen yield and fixation by Bambara groundnut (*Vigna Subterranea* (L.) Verdc.) Landraces intercropped with cowpea and maize in Southern Guinea Savanna of Nigeria. *Agricultural Sciences*, 1, 15-28.
- Eisenhauer, N., Beßler, H., Engels, C., Gleixner, G., Habekost, M., Milcu, A., Partsch, S., Sabais, A., Scherber, C. & Steinbeiss, S. 2010. Plant diversity effects on soil microorganisms support the singular hypothesis. *Ecology*, 91, 485-496.
- Epa. 1986. *Environmental Protection Agency, 1986. Method 9080 — Cation exchange capacity of soils (ammonium acetate)* [Online]. Available: www.epa.gov/epawaste/hazard/testmethods/sw846/.../methindx.pdf. [Accessed].
- Esitken, A., Pirlak, L., Turan, M. & Sahin, F. 2006. Effects of floral and foliar application of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrition of sweet cherry. *Scientia Horticulturae*, 110, 324-327.
- Fao 1990. *Production Year Book*, Rome, Italy, FAO.
- Fao 2008. *State of food security in the world*, Rome, Italy.
- Farag, M. A., Ryu, C.-M., Sumner, L. W. & Paré, P. W. 2006. GC–MS SPME profiling of rhizobacterial volatiles reveals prospective inducers of growth promotion and induced systemic resistance in plants. *Phytochemistry*, 67, 2262-2268.
- Fierer, N., Leff, J. W., Adams, B. J., Nielsen, U. N., Bates, S. T., Lauber, C. L., Owens, S., Gilbert, J. A., Wall, D. H. & Caporaso, J. G. 2012. Cross-biome metagenomic analyses of soil microbial communities and their functional attributes. *Proceedings of the National Academy of Sciences*, 109, 21390-21395.

- Fischbach, M. A. & Segre, J. A. 2016. Signaling in host-associated microbial communities. *Cell*, 164, 1288-1300.
- Gao, B. & Gupta, R. S. 2012. Phylogenetic framework and molecular signatures for the main clades of the phylum Actinobacteria. *Microbiology and Molecular Biology Reviews*, 76, 66-112.
- García-Salamanca, A., Molina-Henares, M. A., Dillewijn, P., Solano, J., Pizarro-Tobías, P., Roca, A., Duque, E. & Ramos, J. L. 2013. Bacterial diversity in the rhizosphere of maize and the surrounding carbonate-rich bulk soil. *Microbial Biotechnology*, 6, 36-44.
- Garland, J. Potential and limitations of BIOLOG for microbial community analysis. Proceedings of the 8th International Symposium on Microbial Ecology Atlantic Canada Society for Microbial Ecology, 1999. Society for Microbial Ecology, 1-7.
- Garrity, G. M. & Holt, J. G. 2001. The road map to the manual. *Bergey's Manual® of Systematic Bacteriology*. Springer.
- Gasparatos, D., Roussos, P., Christofilopoulou, E. & Haidouti, C. 2011. Comparative effects of organic and conventional apple orchard management on soil chemical properties and plant mineral content under Mediterranean climate conditions. *Journal of Soil Science and Plant Nutrition*, 11, 105-117.
- Gerhardson, B., Thaning, C., Weissmann, R., Borowicz, J., Welch, C. & Hedman, R. 2001. New bacterial isolate and its active metabolites, including new compound Haterumalide X, useful for controlling weeds and treating fungal diseases in plants, human and animals. *SE Pat. 9904334-A*.
- Giuliani, A., Hintermann, F., Rojas, W. & Padulosi, S. 2012. Biodiversity of Andean grains: balancing market potential and sustainable livelihoods. *Bioversity International, Rome*.
- Glick, B. R. 1995. The enhancement of plant growth by free-living bacteria. *Canadian Journal of Microbiology*, 41, 109-117.

- Glick, B. R. 2012. Plant growth-promoting bacteria: mechanisms and applications. *Scientifica*, 2012, 15 pages.
- Goli, A., Begemann, F. & Ng, N. 1997. Characterization and evaluation of IITA's Bambara groundnut collection. *Proceedings of the workshop on conservation and improvement of bambara groundnut*.
- Gopal, M., Gupta, A. & Thomas, G. V. 2015. Bespoke microbiome therapy to manage plant diseases. *Frontiers in Microbiology* 4, 355.
- Gopalakrishnan, S., Sathya, A., Vijayabharathi, R., Varshney, R. K., Gowda, C. L. & Krishnamurthy, L. 2015. Plant growth promoting rhizobia: challenges and opportunities. *Biotechnology*, 5, 355-377.
- Graça, A. P., Bondoso, J., Gaspar, H., Xavier, J. R., Monteiro, M. C., De La Cruz, M., Oves-Costales, D., Vicente, F. & Lage, O. M. 2013. Antimicrobial activity of heterotrophic bacterial communities from the marine sponge *Erylus discophorus* (Astrophorida, Geodiidae). *PLoS One*, 8, e78992.
- Groenhagen, U., Baumgartner, R., Bailly, A., Gardiner, A., Eberl, L., Schulz, S. & Weisskopf, L. 2013. Production of bioactive volatiles by different *Burkholderia ambifaria* strains. *Journal of Chemical Ecology*, 39, 892-906.
- Gryta, A., Frąc, M. & Oszust, K. 2014. The application of the Biolog EcoPlate approach in ecotoxicological evaluation of dairy sewage sludge. *Applied Biochemistry and Biotechnology*, 174, 1434-1443.
- Gueye, M. 1992. Effect of *Rhizobium* and *Rhizobium-Glomus* inoculation on nitrogen fixation in Bambara groundnut. In: MULONGOY, K., GUEYE, M. & SPENCER, D., S, C (ed.)(eds.) *Biological nitrogen fixation and sustainability of tropical agriculture*. Chichester: Wiley.

- Gutiérrez-Luna, F. M., López-Bucio, J., Altamirano-Hernández, J., Valencia-Cantero, E., De La Cruz, H. R. & Macías-Rodríguez, L. 2010. Plant growth-promoting rhizobacteria modulate root-system architecture in *Arabidopsis thaliana* through volatile organic compound emission. *Symbiosis*, 51, 75-83.
- Habig, J. & Swanepoel, C. 2015. Effects of conservation agriculture and fertilization on soil microbial diversity and activity. *Environments*, 2, 358-384.
- Hadar, Y. & Papadopoulou, K. K. 2012. Suppressive composts: microbial ecology links between abiotic environments and healthy plants. *Annual Review of Phytopathology*, 50, 133-153.
- Haichar, F.-E. Z., Santaella, C., Heulin, T. & Achouak, W. 2014. Root exudates mediated interactions belowground. *Soil Biology and Biochemistry*, 77, 69-80.
- Haldar, S. & Sengupta, S. 2015. Plant-microbe cross-talk in the rhizosphere: Insight and biotechnological potential. *The Open Microbiology Journal*, 9, 1.
- Hall, R. D. 2006. Plant metabolomics: From holistic hope, to hype, to hot topic. *New phytologist*, 169, 453-468.
- Hari, N. M. N., Karthikeyan, C. & Trivedi, P. 2009. Synthesis, cytotoxic evaluation and in silico pharmacokinetic prediction of some benzo [a] phenazine-5-sulfonic acid derivatives. *Medicinal chemistry (Sharjah (United Arab Emirates))*, 5, 549-557.
- Hartmann, A., Schmid, M., Van Tuinen, D. & Berg, G. 2009. Plant-driven selection of microbes. *Plant and Soil*, 321, 235-257.
- Hassan, S. & Mathesius, U. 2012. The role of flavonoids in root-rhizosphere signalling: opportunities and challenges for improving plant-microbe interactions. *Journal of Experimental Botany*, 63, 3429-3444.
- Heydari, S., Rezvani-Moghadam, P. & Arab, M. 2008. Hydrogen Cyanide Production Ability by *Pseudomonas fluorescence* Bacteria and their Inhibition Potential on Weed

Germination. *Competition for Resources in a Changing World: New Drive for Rural Development*. Hohenheim: Tropentag.

- Hibbing, M. E., Fuqua, C., Parsek, M. R. & Peterson, S. B. 2010. Bacterial competition: surviving and thriving in the microbial jungle. *Nature Reviews Microbiology*, 8, 15-25.
- Hillocks, R., Bennett, C. & Mponda, O. 2012. Bambara nut: A Review of utilisation, market potential and crop improvement. *African Crop Science Journal*, 20, 1-16.
- Holmes, A. J., Bowyer, J., Holley, M. P., O'donoghue, M., Montgomery, M. & Gillings, M. R. 2000. Diverse, yet-to-be-cultured members of the Rubrobacter subdivision of the Actinobacteria are widespread in Australian arid soils. *FEMS Microbiology Ecology*, 33, 111-120.
- Hopkins, B., Hutchinson, P., Patterson, P., Miller, J., Thornton, M., Hafez, S. & Alvarez, J. 2004. Cropping Sequence and Rotation: Impact on Potato Production and Soil Condition. In: COPELAND, K. (ed.) *Winter Commodity Schools*. Moscow, ID: UI-Cooperative Extension System.
- Houlden, A., Timms-Wilson, T. M., Day, M. J. & Bailey, M. J. 2008. Influence of plant developmental stage on microbial community structure and activity in the rhizosphere of three field crops. *FEMS Microbiology Ecology* 65, 193–201.
- Huang, X.-F., Chaparro, J. M., Reardon, K. F., Zhang, R., Shen, Q. & Vivanco, J. M. 2014. Rhizosphere interactions: root exudates, microbes, and microbial communities 1. *Botany*, 92, 267-275.
- Husson, O. 2013. Redox potential (Eh) and pH as drivers of soil/plant/microorganism systems: a transdisciplinary overview pointing to integrative opportunities for agronomy. *Plant and Soil*, 362, 389-417.
- Ijarotimi, O. 2008. Nutritional composition, microbial status, functional and sensory properties of infant diets formulated from cooking banana fruits (*Musa* spp, ABB genome) and

- fermented Bambara groundnut (*Vigna subterranean* L. Verdc) seeds. *Nutrition & Food Science*, 38, 325-340.
- Ijarotimi, S. O. & Esho, R. T. 2009. Comparison of nutritional composition and anti-nutrient status of fermented, germinated and roasted Bambara groundnut seeds (*Vigna subterranea*). *British Food Journal*, 111, 376-386.
- Inbar, E., Green, S. J., Hadar, Y. & Minz, D. 2005. Competing factors of compost concentration and proximity to root affect the distribution of streptomycetes. *Microbial Ecology*, 50, 73-81.
- Inceoğlu, Ö., Al-Soud, W. A., Salles, J. F., Semenov, A. V. & Van Elsas, J. D. 2011. Comparative analysis of bacterial communities in a potato field as determined by pyrosequencing. *PLOS ONE*, 6, e23321.
- Inceoğlu, Ö., Salles, J. F., Van Overbeek, L. & Van Elsas, J. D. 2010. Effects of plant genotype and growth stage on the betaproteobacterial communities associated with different potato cultivars in two fields. *Applied and Environmental Microbiology*, 76, 3675-3684.
- Insam, H., Goberna, M., Kowalchuk, G., De Bruijn, F., Head, I., Akkermans, A. & Van Elsas, J. 2004. Use of Biolog® for the community level physiological profiling (CLPP) of environmental samples. *Molecular Microbial Ecology Manual*, 1, 853-860.
- Insam, H. & Seewald, M. S. 2010. Volatile organic compounds (VOCs) in soils. *Biology and Fertility of Soils*, 46, 199-213.
- Iqbal Hussain, M., Naeem Asghar, H., Javed Akhtar, M. & Arshad, M. 2013. Impact of phosphate solubilizing bacteria on growth and yield of maize. *Soil and Environment*, 32, 71-78.
- Iso-11464 2006. *Soil quality — Pretreatment of samples for physico-chemical analysis*, Switerland, ISO.

- Jalaluldeen, A. M., Sijam, K., Othman, R. & Ahmad, Z. a. M. 2015. Growth characteristics and production of secondary metabolites from selected *Streptomyces* species isolated from the Rhizosphere of Chili Plant. *International Journal of Enhanced Research in Science Technology and Engineering*, 4, 1-8.
- Jeanet, P., Clément, C., Courot, E. & Cordelier, S. 2013. Modulation of phytoalexin biosynthesis in engineered plants for disease resistance. *International Journal of Molecular Sciences*, 14, 14136-14170.
- Jha, A. K., Bais, H. P. & Vivanco, J. M. 2005. Enterococcus faecalis Mammalian Virulence - Related Factors Exhibit Potent Pathogenicity in the Arabidopsis thaliana Plant Model. *Infection and Immunity*, 73, 464-75.
- Joseph, B., Ranjan Patra, R. & Lawrence, R. 2012. Characterization of plant growth promoting rhizobacteria associated with chickpea (*Cicer arietinum* L.). *International Journal of Plant Production*, 1, 141-152.
- Jousset, A., Scheu, S. & Bonkowski, M. 2008. Secondary metabolite production facilitates establishment of rhizobacteria by reducing both protozoan predation and the competitive effects of indigenous bacteria. *Functional Ecology*, 22, 714-719.
- Kai, M., Effmert, U., Berg, G. & Piechulla, B. 2007. Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia solani*. *Archives of Microbiology*, 187, 351-360.
- Kamei, A. K. D. & Apou Kamei, A. 2014. Role of hydrogen cyanide secondary metabolite of plant growth promoting rhizobacteria as biopesticides of weeds. *Global Journal of Science Frontier Research*, 14, 108-112.
- Kanchiswamy, C. N., Malnoy, M. & Maffei, M. E. 2015. Chemical diversity of microbial volatiles and their potential for plant growth and productivity. *Frontiers in Plant Science*, 6, 151.

- Kanjanamaneesathian, M., Wiwattanapatapee, R., Rotniam, W., Pengnoo, A., Wongpetkhiew, W. & Tanmala, V. 2013. Application of a suspension concentrate formulation of *Bacillus velezensis* to control root rot of hydroponically-grown vegetables. *New Zealand Plant Protection*, 66, 229-234.
- Katznelson, H. & Bose, B. 1959. Metabolic activity and phosphate-dissolving capability of bacterial isolates from wheat roots, rhizosphere, and non-rhizosphere soil. *Canadian Journal of Microbiology*, 5, 79-85.
- Khalil, R., Djadouni, F., Elbahloul, Y. & Omar, S. 2009. The influence of cultural and physical conditions on the antimicrobial activity of bacteriocin produced by a newly isolated *Bacillus megaterium* 22 strain. *African Journal of Food Science*, 3, 011-022.
- Khanam, S. 2007. Pharmacognosy: General study of formation of secondary metabolites. *Dept. of Pharmacognosy, Al-Ameen college of Pharmacy. Hosur Road, Opp. Lalbagh main Gate, Bangalore-560, 27*, 24-5.
- Kidaj, D., Wielbo, J. & Skorupska, A. 2012. Nod factors stimulate seed germination and promote growth and nodulation of pea and vetch under competitive conditions. *Microbiological Research*, 167, 144-150.
- Kimbrough, D. E., Kouame, Y., Moheban, P. & Springthorpe, S. 2006. The effect of electrolysis and oxidation–reduction potential on microbial survival, growth, and disinfection. *International Journal of Environment and Pollution*, 27, 211-221.
- Kong, A. Y., Scow, K. M., Córdova-Kreylos, A. L., Holmes, W. E. & Six, J. 2011. Microbial community composition and carbon cycling within soil microenvironments of conventional, low-input, and organic cropping systems. *Soil Biology and Biochemistry*, 43, 20-30.
- Kusch, S., Aysha, F., Nicholas, C.-H., Nele, S., Laura, P., Jess, V., Michelle, L., Ali, K., Chijioke, J. E., Perrine, H., Silke, B., James, P., Carolina, A., Wilma, W., Ai, S., Anna,

- M. A., Atul, D., Nibedita, M., Sheila, O., Fawzi, M., Alice, C. H., Kathryn, B., Antonio, T., Kiah, S., Steven, H. & Chibuike, C. U. 2016 Sustainability in a changing world: integrating human health and wellbeing, urbanisation, and ecosystem service. *In: ZODROW, D. & KAREN, V. E. (eds.) Organic Production publication series* Centre for Environmental Farming, North Carolina State University.
- Lagos, L., Maruyama, F., Nannipieri, P., Mora, M. L., Ogram, A. & Jorquera, M. A. 2015. Current overview on the study of bacteria in the rhizosphere by modern molecular techniques: A mini review. *Journal of Soil Science and Plant Nutrition*, 15, 504-523.
- Lamb, E. G., Kennedy, N. & Siciliano, S. D. 2011. Effects of plant species richness and evenness on soil microbial community diversity and function. *Plant and Soil*, 338, 483-495.
- Laslo, É., György, É., Mara, G., Tamás, É., Ábrahám, B. & Lányi, S. 2012. Screening of plant growth promoting rhizobacteria as potential microbial inoculants. *Crop Protection*, 40, 43-48.
- Lau, M. C., Cameron, C., Magnabosco, C., Brown, C. T., Schilkey, F., Grim, S., Hendrickson, S., Pullin, M., Sherwood Lollar, B. & Van Heerden, E. 2014. Phylogeny and phylogeography of functional genes shared among seven terrestrial subsurface metagenomes reveal N-cycling and microbial evolutionary relationships. *Frontiers in Microbiology*, 5, 531.
- Laurette, N. N., Maxémilienne, N. B., Henri, F., Souleymanou, A., Kamdem, K., Albert, N., Dieudonné, N. & François-Xavier, E. 2015. Isolation and screening of indigenous Bambara groundnut (*Vigna Subterranea*) nodulating bacteria for their tolerance to some environmental stresses. *American Journal of Microbiological Research*, 3, 65-75.

- Leeuwenhoek, A. V., Garrido, J. F. & Lugo, D. M. 2012. Bacterial community structure in the rhizosphere of three *Cactus* species from semi-arid highland in central Mexico. *Antonie Van Leeuwenhoek*, 101, 891-904.
- Lemanceau, P., Mazurier, S., Avoscan, L., Robin, A. & Briat, J. F. 2013. Reciprocal interactions between plants and fluorescent *Pseudomonas* in relation to iron in the rhizosphere. In: DE BRUIJN, F. J. (ed.)(eds.) *Reciprocal interactions between plants and fluorescent Pseudomonas in relation to iron in the rhizosphere*. Hoboken, NJ, USA: Wiley-Blackwell.
- Lemfack, M. C., Nickel, J., Dunkel, M., Preissner, R. & Piechulla, B. 2014. mVOC: a database of microbial volatiles. *Nucleic Acid Research*, 42, D744-D748.
- León, M., Yaryura, P., Montecchia, M., Hernández, A., Correa, O., Pucheu, N., Kerber, N. & García, A. 2009. Antifungal activity of selected indigenous *Pseudomonas* and *Bacillus* from the soybean rhizosphere. *International Journal of Microbiology*, 2009, 572049.
- Li, X., Rui, J., Mao, Y., Yannarell, A. & Mackie, R. 2014. Dynamics of the bacterial community structure in the rhizosphere of a maize cultivar. *Soil Biology and Biochemistry*, 68, 392-401.
- Loper, J. & Schroth, M. 1986. Importance of siderophores in microbial interactions in the rhizosphere. *Iron, Siderophores, and Plant Diseases*. Springer.
- López-Bucio, J., Campos-Cuevas, J. C., Hernández-Calderón, E., Velásquez-Becerra, C., Farías-Rodríguez, R., Macías-Rodríguez, L. I. & Valencia-Cantero, E. 2007. *Bacillus megaterium* rhizobacteria promote growth and alter root-system architecture through an auxin-and ethylene-independent signaling mechanism in *Arabidopsis thaliana*. *Molecular Plant-Microbe Interactions*, 20, 207-217.
- Lucy, M., Reed, E. & Glick, B. 2004. Applications of free living plant growth-promoting rhizobacteria. *Antonie van Leeuwenhoek*, 86, 1-25.



- Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., Tremblay, J., Engelbrektson, A., Kunin, V. & Del Rio, T. G. 2012. Defining the core *Arabidopsis thaliana* root microbiome. *Nature*, 488, 86–90.
- Lupwayi, N., Arshad, M., Rice, W. & Clayton, G. 2001. Bacterial diversity in water-stable aggregates of soils under conventional and zero tillage management. *Applied Soil Ecology*, 16, 251-261.
- Mabhaudhi, T. & Modi, A. T. 2013. Growth, phenological and yield responses of a Bambara groundnut (*Vigna subterranea* (L.) Verdc.) landrace to imposed water stress under field conditions. *South African Journal of Plant and Soil*, 30, 69-79.
- Magurran, A. E. 1988. *Why diversity?*, Springer.
- Mahala, A. G. & Mohammed, A. a. A. 2010. Nutritive evaluation of Bambara groundnut (*Vigna subterranea*) pods, seeds and Hulls as Animal Feeds. *Journal of Applied Sciences Research*, 6, 383-386.
- Maheshwari, D. K. 2013. *Bacteria in agrobiolgy: disease management*, Springer Science & Business Media.
- Mantelin, S. & Touraine, B. 2004. Plant growth-promoting bacteria and nitrate availability: Impacts on root development and nitrate uptake. *Journal of Experimental Botany*, 55, 27-34.
- Marino, D., Pucciariello, C., Puppo, A. & Frendo, P. 2009. The redox state, a referee of the legume–rhizobia symbiotic game. *Advances in Botanical Research*, 52, 115-151.
- Masindeni, D. R. 2006. *Evaluation of Bambara groundnut (Vigna subterranea) for yield stability and yield related characteristics*. MSc dissertation M.Sc.Thesis, University of the Free State.
- Massawe, F., Mayes, S. & Cheng, A. 2016. Crop diversity: An unexploited treasure trove for food security. *Trends in plant science*, 21, 365-368.

- Maurhofer, M., Keel, C., Haas, D. & Défago, G. 1995. Influence of plant species on disease suppression by *Pseudomonas fluorescens* strain CHAO with enhanced antibiotic production. *Plant Pathology*, 44, 40-50.
- Mavrodi, D. V., Blankenfeldt, W. & Thomashow, L. S. 2006. Phenazine compounds in fluorescent *Pseudomonas* spp. biosynthesis and regulation. *Annual Review of Phytopathology*, 44, 417-445.
- Mayes, S., Massawe, F., Alderson, P., Roberts, J., Azam-Ali, S. & Hermann, M. 2012. The potential for underutilized crops to improve security of food production. *Journal of Experimental Botany*, 63, 1075-1079.
- Mazahib, A., Nuha, M., Salawa, I. & Babiker, E. 2013. Some nutritional attributes of bambara groundnut as influenced by domestic processing. *International Food Research Journal*, 20, 1165-1171.
- Mcbain, A. J. 2009. In vitro biofilm models: An overview. *Advances in Applied Microbiology*, 69, 99-132.
- Mendes, R., Garbeva, P. & Raaijmakers, J. M. 2013. The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. *FEMS Microbiology Reviews*, 37, 634-663.
- Mendes, R., Kruijt, M., De Bruijn, I., Dekkers, E., Van Der Voort, M., Schneider, J. H., Piceno, Y. M., Desantis, T. Z., Andersen, G. L. & Bakker, P. A. 2011. Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science*, 332, 1097-1100.
- Mentan, T. 2014. *Africa: Facing Human Security Challenges in the 21st Century*, Langaa RPCIG.
- Mills, H. J., Reese, B. K. & Peter, C. S. 2012. Characterization of microbial population shifts during sample storage.

- Minka, S. R. & Bruneteau, M. 2000. Partial chemical composition of Bambara pea [*Vigna subterranea* (L.) Verde]. *Food Chemistry*, 68, 273-276.
- Mkandawire, C. H. 2007. Review of Bambara groundnut (*Vigna subterranea* (L.) Verdc.) production in Sub-Sahara Africa. *Agricultural Journal*, 2, 464-470.
- Mohale, K. C., Belane, A. K. & Dakora, F. D. 2014. Symbiotic N nutrition, C assimilation, and plant water use efficiency in Bambara groundnut (*Vigna subterranea* L. Verdc) grown in farmers' fields in South Africa, measured using ¹⁵N and ¹³C natural abundance. *Biology and Fertility of Soils*, 50, 307-319.
- Morath, S. U., Hung, R. & Bennett, J. W. 2012. Fungal volatile organic compounds: A review with emphasis on their biotechnological potential. *Fungal Biology Reviews*, 26, 73-83.
- Mshelia, J. S., Kuchinda, N. C. & Aliyu, L. 2004. Performance of Maize/Bambara groundnuts crops as affected by sowing date and planting pattern. *Journal of Sustainable Agriculture and Environment*, 6, 120-127.
- Mubaiwa, J., Fogliano, V., Chidewe, C. & Linnemann, A. R. 2017. Hard-to-cook phenomenon in bambara groundnut (*Vigna subterranea* (L.) Verdc.) processing: Options to improve its role in providing food security. *Food Reviews International*, 33, 167-194.
- Mudge, S. R., Smith, F. W. & Richardson, A. E. 2003. Root-specific and phosphate-regulated expression of phytase under the control of a phosphate transporter promoter enables *Arabidopsis* to grow on phytate as a sole P source. *Plant Science*, 165, 871-878.
- Murevanhema, Y. Y. & Jideani, V. A. 2013. Potential of Bambara groundnut (*Vigna subterranea* (L.) Verdc) milk as a probiotic beverage—A review. *Critical Reviews in Food Science and Nutrition*, 53, 954-967.
- Murray, R., Costilow, R. N., Nester, E., Wood, W. A., Krieg, N. R. & Phillips, G. 1981. *Manual of methods for general bacteriology*, Washington, DC, American Society for Microbiology.

- Nagarajkumar, M., Bhaskaran, R. & Velazhahan, R. 2004. Involvement of secondary metabolites and extracellular lytic enzymes produced by *Pseudomonas fluorescens* in inhibition of *Rhizoctonia solani*, the rice sheath blight pathogen. *Microbiological Research*, 159, 73-81.
- Neidig, N., Paul, R. J., Scheu, S. & Jousset, A. 2011. Secondary metabolites of *Pseudomonas fluorescens* CHA0 drive complex non-trophic interactions with bacterivorous nematodes. *Microbial Ecology*, 61, 853-859.
- Neilson, J. W., Quade, J., Ortiz, M., Nelson, W. M., Legatzki, A., Tian, F., Lacombe, M., Betancourt, J. L., Wing, R. A. & Soderlund, C. A. 2012. Life at the hyperarid margin: novel bacterial diversity in arid soils of the Atacama Desert, Chile. *Extremophiles*, 16, 553-566.
- Ngugi, G. W. 1997. Bambara Groundnut production in Kenya. . In: BEGEMANN, F., MUSHONGA, J. N. & HELLER, J. (ed.)(eds.) *Bambara Groundnut Vigna Subterranea (L.) Verdc: Proceedings of the Workshop on Conservation and Improvement of Bambara Groundnut (Vigna Subterranea (L.) Verdc.): 14-16 November 1995*. Harare, Zimbabwe: Bioversity International.
- Nihorimbere, V., Ongena, M., Smargiassi, M. & Thonart, P. 2011. Beneficial effect of the rhizosphere microbial community for plant growth and health. *Biotechnology Agronomy Society Environment* 15, 327-337.
- Nyemba, R. C. & Dakora, F. D. 2010. Evaluating N₂ fixation by food grain legumes in farmers' fields in three agro-ecological zones of Zambia, using ¹⁵N natural abundance. *Biology and Fertility of Soils*, 46, 461-470.
- Olukolu, B. A., Mayes, S., Stadler, F., Quat Ng, N., Fawole, I., Dominique, D., Azam-Ali, S. N., Abbott, A. G. & Kole, C. 2012. Genetic diversity in Bambara groundnut (*Vigna*

- subterranea* (L.) Verdc.) as revealed by phenotypic descriptors and DArT marker analysis. *Genetic Resources and Crop Evolution*, 59, 347-358.
- Omoikhoje, S. O. 2008. Assessment of the nutritive value of Bambara groundnut as influenced by cooking time. *Livestock Research for Rural Development*, 20, 55.
- Onuh, M. D. & Christo, I. 2011. Agronomic and physiological performance of bambara groundnut (*Vigna subterranea* (L.) Verdc) in Southeastern Nigeria. *World Journal of Agricultural Sciences*, 7, 166-171.
- Onwubiko, N., Odum, O., Utazi, C. & Poly-Mbah, P. 2011a. Studies on the Adaptation of Bambara Groundnut [*Vigna subterranea* (L.) Verdc] in Owerri Southeastern Nigeria. *Agricultural Journal*, 6, 60-65.
- Onwubiko, N., Uguru, M., Ngwuta, A., Inyang, E. & Nnajieme, O. 2011b. Floral biology of Bambara groundnut [*Vigna subterranea* (L.) Verdc]. *Journal of Plant Breeding and Crop Science*, 3, 293-295.
- Opoku, M. K. 2010. *Response of Bambara groundnut landraces to heat and drought stress in the Guinea Savanna and transition zones of Ghana*. Master Of Science (Agronomy), Kwame Nkrumah University Of Science And Technology.
- Page, A. L., Miller, R. H. & Keeney, D. R. (eds.) 1982. *Methods of Soil Analysis, Part II*, Madison, WI, USA: American Society of Agronomy – Soil Science Society of America.
- Pal, K. K. & Gardener, B. M. 2006. Biological Control of Plant Pathogens. *The Plant Health Instructor*, 10, 1094.
- Palaniyandi, S. A., Yang, S. H., Zhang, L. & Suh, J.-W. 2013. Effects of actinobacteria on plant disease suppression and growth promotion. *Applied Microbiology and Biotechnology*, 97, 9621-9636.

- Park, Y.-S., Dutta, S., Ann, M., Raaijmakers, J. M. & Park, K. 2015. Promotion of plant growth by *Pseudomonas fluorescens* strain SS101 via novel volatile organic compounds. *Biochemical and Biophysical Research Communications*, 461, 361-365.
- Patten, C. L. & Glick, B. R. 1996. Bacterial biosynthesis of indole-3-acetic acid. *Canadian Journal of Microbiology*, 42, 207-220.
- Paulsen, I. T., Press, C. M., Ravel, J., Kobayashi, D. Y., Myers, G. S., Mavrodi, D. V., Deboy, R. T., Seshadri, R., Ren, Q. & Madupu, R. 2005. Complete genome sequence of the plant commensal *Pseudomonas fluorescens* Pf-5. *Nature Biotechnology*, 23, 873-878.
- Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Tringe, S. G., Dangl, J. L., Buckler, E. S. & Ley, R. E. 2013. Diversity and heritability of the maize rhizosphere microbiome under field conditions. *Proceedings of the National Academy of Sciences*, 110, 6548-6553.
- Philippot, L., Raaijmakers, J. M., Lemanceau, P. & Van Der Putten, W. H. 2013. Going back to the roots: the microbial ecology of the rhizosphere. *Nature Reviews Microbiology*, 11, 789-799.
- Phillips, D. A. 2000. Biosynthesis and release of rhizobial nodulation gene inducers by legumes. In: TRIPLETT, E. P. (ed.)(eds.) *Prokaryotic nitrogen fixation: A model system for the analysis of a biological process*. Wymondham, UK: Horizon Scientific Press.
- Pierson Iii, L. S. & Pierson, E. A. 2010. Metabolism and function of phenazines in bacteria: impacts on the behavior of bacteria in the environment and biotechnological processes. *Applied Microbiology and Biotechnology*, 86, 1659-1670.
- Pignataro, A., Moscatelli, M. C., Mocali, S., Grego, S. & Benedetti, A. 2012. Assessment of soil microbial functional diversity in a coppiced forest system. *Applied Soil Ecology*, 62, 115-123.

- Ping, L. & Boland, W. 2004. Signals from the underground: bacterial volatiles promote growth in *Arabidopsis*. *Trends in Plant Science*, 9, 263-266.
- Piterina, A. V., Bartlett, J. & Pembroke, J. T. 2010. Molecular analysis of bacterial community DNA in sludge undergoing autothermal thermophilic aerobic digestion (ATAD): pitfalls and improved methodology to enhance diversity recovery. *Diversity*, 2, 505-526.
- Raaijmakers, J. M. & Mazzola, M. 2012. Diversity and natural functions of antibiotics produced by beneficial and plant pathogenic bacteria. *Annual Review of Phytopathology*, 50, 403-424.
- Raaijmakers, J. M., Paulitz, T. C., Steinberg, C., Alabouvette, C. & Moëgne-Loccoz, Y. 2009. The rhizosphere: A playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant and Soil*, 321, 341-361.
- Rengel, Z. & Marschner, P. 2005. Nutrient availability and management in the rhizosphere: exploiting genotypic differences. *New Phytologist*, 168, 305-312.
- Richardson, A. E., Barea, J.-M., Mcneill, A. M. & Prigent-Combaret, C. 2009. Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant and Soil*, 321, 305-339.
- Rincon-Florez, V. A., Carvalhais, L. C. & Schenk, P. M. 2013. Culture-independent molecular tools for soil and rhizosphere microbiology. *Diversity*, 5, 581-612.
- Ryall, B., Mitchell, H., Mossialos, D. & Williams, H. D. 2009. Cyanogenesis by the entomopathogenic bacterium *Pseudomonas entomophila*. *Letters in Applied Microbiology*, 49, 131-135.
- Ryu, C.-M., Farag, M. A., Hu, C.-H., Reddy, M. S., Wei, H.-X., Paré, P. W. & Kloepper, J. W. 2003. Bacterial volatiles promote growth in *Arabidopsis*. *Proceedings of the National Academy of Sciences*, 100, 4927-4932.

- Sadrati, N., Daoud, H., Zerroug, A., Dahamna, S. & Bouharati, S. 2013. Screening of antimicrobial and antioxidant secondary metabolites from endophytic fungi isolated from wheat (*Triticum durum*). *Journal of Plant Protection Research*, 53, 128-136.
- Salama, O. a. E. B. a. E. A. 2011. *Utilization of Biofertilizers Utilization of Biofertilizers and Organic Sources in and Organic Sources in Arable Soils under Saline Conditions Using Tracer Technique*. The Degree of Doctor of Philosophy in Microbiology Research, Al-Azhar University.
- Sangare, S. S. 2012. *Effect of different seed primng methods on germination, seedling establishment and vigour in sorghum Sorghum bicolor (L.) Moench.) and Bambara groundnut (Vigna subterrenea (L.) Verdc.)* MSc (Seed Science and Technology), Kwame Nkrumah University of Science and Technology, Kumasi.
- Saniewski, M., Saniewska, A. & Kanlayanarat, S. 2007. Biological activities of tropolone and hinokitiol: The tools in plant physiology and their practical use. 755 ed.: International Society for Horticultural Science (ISHS), Leuven, Belgium.
- Sansinenea, E. & Ortiz, A. 2011. Secondary metabolites of soil *Bacillus* spp. *Biotechnology Letters*, 33, 1523-1538.
- Saraf, M., Pandya, U. & Thakkar, A. 2014. Role of allelochemicals in plant growth promoting rhizobacteria for biocontrol of phytopathogens. *Microbiological Research*, 169, 18-29.
- Schenkel, D., Lemfack, M. C., Piechulla, B. & Splivallo, R. 2015. A meta-analysis approach for assessing the diversity and specificity of below ground root and microbial volatiles. *Frontiers in Plant Science*, 6, 707.
- Schimel, D. S. 1995. Terrestrial ecosystems and the carbon-cycle. *Global Change Biology*, 1, 77-91.

- Schisler, D., Slininger, P., Behle, R. & Jackson, M. 2004. Formulation of *Bacillus* spp. for biological control of plant diseases. *Phytopathology*, 94, 1267-1271.
- Schmidt, R., Cordovez, V., De Boer, W., Raaijmakers, J. & Garbeva, P. 2015. Volatile affairs in microbial interactions. *The ISME Journal*, 9, 2329-2335.
- Schöller, C. E., Gürtler, H., Pedersen, R., Molin, S. & Wilkins, K. 2002. Volatile metabolites from actinomycetes. *Journal of Agricultural and Food Chemistry*, 50, 2615-2621.
- Schreiter, S., Ding, G.-C., Heuer, H., Neumann, G., Sandmann, M., Grosch, R., Kropf, S. & Smalla, K. 2015. Effect of the soil type on the microbiome in the rhizosphere of field-grown lettuce. *The Plant Microbiome and Its Importance for Plant and Human Health*, 5, 144.
- Schulz, S. & Dickschat, J. S. 2007. Bacterial volatiles: the smell of small organisms. *Natural Product Reports*, 24, 814-842.
- Selvakumar, G., Joshi, P., Nazim, S., Mishra, P., Bisht, J. & Gupta, H. 2009. Phosphate solubilization and growth promotion by *Pseudomonas fragi* CS11RH1 (MTCC 8984), a psychrotolerant bacterium isolated from a high altitude Himalayan rhizosphere. *Biologia*, 64, 239-245.
- Shi, S., Nuccio, E., Herman, D. J., Rijkers, R., Estera, K., Li, J., Da Rocha, U. N., He, Z., Pett-Ridge, J. & Brodie, E. L. 2015. Successional trajectories of rhizosphere bacterial communities over consecutive seasons. *MBio*, 6, e00746-15.
- Singh, B. & Satyanarayana, T. 2011. Microbial phytases in phosphorus acquisition and plant growth promotion. *Physiology and molecular biology of plants : an international journal of functional plant biology*, 17, 93-103.

- Singh, B. K., Munro, S., Potts, J. M. & Millard, P. 2007. Influence of grass species and soil type on rhizosphere microbial community structure in grassland soils. *Applied Soil Ecology*, 36, 147-155.
- Singh, G. & Mukerji, K. G. 2006. Root exudates as determinant of rhizospheric microbial biodiversity. *Microbial Activity in the Rhizosphere*. Springer.
- Smalla, K., Sessitsch, A. & Hartmann, A. 2006. The rhizosphere: "Soil compartment influenced by the root". *FEMS Microbiology Ecology* 56, 165.
- Smedsgaard, J. & Frisvad, J. C. 1996. Using direct electrospray mass spectrometry in taxonomy and secondary metabolite profiling of crude fungal extracts. *Journal of Microbiological Methods*, 25, 5-17.
- Smil, V. 2005. The next 50 years: unfolding trends. *Population and Development Review*, 31, 605-643.
- Smith, D. L., Prithiviraj, B. & Zhang, F. 2002. Rhizobial signals and control of plant growth. In: Finan, T. M., O'Brian, M. R., Layzell, D. B., Vessey, K. & Newton, W. E. (eds.) *Nitrogen fixation: global perspectives*. Wallingford, UK: CABI Publishing.
- Sousa, J. a. D. J. & Olivares, F. L. 2016. Plant growth promotion by streptomycetes: ecophysiology, mechanisms and applications. *Chemical and Biological Technologies in Agriculture*, 3, 24.
- Spehar, C. & Souza, P. 1995. Developing sustainable cropping systems for the Brazilian savannas. *Moist Savannas of Africa (KANG, BT, et al., Eds.)*, IITA, Ibadan, 325-356.
- Stefan, M., Munteanu, N., Stoleru, V. & Mihasan, M. 2013. Effects of inoculation with plant growth promoting rhizobacteria on photosynthesis, antioxidant status and yield of runner bean. *Romanian Biotechnological Letters*, 18, 8132-8143.
- Stefanowicz, A. 2006. The Biolog plates technique as a tool in ecological studies of microbial communities. *Polish Journal of Environmental Studies*, 15, 669.

- Steinborn, G., Hajirezaei, M.-R. & Hofemeister, J. 2005. Bac genes for recombinant bacilysin and anticapsin production in *Bacillus* host strains. *Archives of Microbiology*, 183, 71-79.
- Strobel, G. A. 2003. Endophytes as sources of bioactive products. *Microbes and Infection*, 5, 535-544.
- Sugiyama, A., Ueda, Y., Zushi, T., Takase, H. & Yazaki, K. 2014. Changes in the Bacterial Community of Soybean Rhizospheres during Growth in the Field. *PLOS ONE*, 9, e100709.
- Swanevelder, C. 1998. Bambara—food for Africa. *National Department of Agriculture, Government Printer, Republic of South Africa*.
- Szentes, S., Radu, G.-L., Laslo, É., Lányi, S. & Mara, G. 2013. Selection and evaluation of potential biocontrol rhizobacteria from a raised bog environment. *Crop Protection*, 52, 116-124.
- Takarada, H., Sekine, M., Kosugi, H., Matsuo, Y., Fujisawa, T., Omata, S., Kishi, E., Shimizu, A., Tsukatani, N. & Tanikawa, S. 2008. Complete genome sequence of the soil actinomycete *Kocuria rhizophila*. *Journal of Bacteriology*, 190, 4139-4146.
- Tan, R. X. & Zou, W. X. 2001. Endophytes: A rich source of functional metabolites. *Natural Product Reports*, 18, 448-459.
- Teixeira, L. C., Peixoto, R. S., Cury, J. C., Sul, W. J., Pellizari, V. H., Tiedje, J. & Rosado, A. S. 2010. Bacterial diversity in rhizosphere soil from Antarctic vascular plants of Admiralty Bay, maritime Antarctica. *The ISME Journal*, 4, 989-1001.
- Tewari, A. & Abdullah, S. 2015. *Bacillus cereus* food poisoning: international and Indian perspective. *Journal of food science and technology*, 52, 2500-2511.

- Thompson, G. L. & Kao-Kniffin, J. 2016. Diversity Enhances NPP, N Retention, and Soil Microbial Diversity in Experimental Urban Grassland Assemblages. *PLoS ONE*, 11, e0155986.
- Trivedi, P. C. 2006. *Advances in Plant Physiology*, New Delhi, IK International Pvt Ltd.
- Tweneboah, C. 2000. Modern Agriculture in the Tropics. *Food crops. Co-wood Publishers*.
- Uroz, S., Buée, M., Murat, C., Frey-Klett, P. & Martin, F. 2010. Pyrosequencing reveals a contrasted bacterial diversity between oak rhizosphere and surrounding soil. *Environmental Microbiology Reports*, 2, 281-288.
- Van Camp, W., Van Montagu, M. & Inzé, D. 1998. H₂O₂ and NO: Redox signals in disease resistance. *Trends in Plant Science*, 3, 330-334.
- Van Der Lee, T., Zhang, H., Van Diepeningen, A. & Waalwijk, C. 2015. Biogeography of *Fusarium graminearum* species complex and chemotypes: a review. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*, 32, 453-60.
- Van Loon, L. & Bakker, P. 2003. Signalling in rhizobacteria-plant interactions. *Root ecology*. Springer.
- Van Overbeek, L. & Van Elsas, J. D. 2008. Effects of plant genotype and growth stage on the structure of bacterial communities associated with potato (*Solanum tuberosum* L.). *FEMS Microbiology Ecology*, 64, 283-296.
- Vaněk, V., Šilha, J. & Němeček, R. 2003. The level of soil nitrate content at different management of organic fertilizers application. *Plant Soil and Environment*, 49, 197-202.
- Vessey, J. K. 2003. Plant growth promoting rhizobacteria as biofertilizers. *Plant and Soil*, 255, 571-586.



- Vurayai, R., Emongor, V. & Moseki, B. 2011. Effect of water stress imposed at different growth and development stages on morphological traits and yield of Bambara Groundnuts (*Vigna subterranea* L. Verdc). *American Journal of Plant Physiology*, 6, 17-27.
- Walker, T. S., Bais, H. P., Déziel, E., Schweizer, H. P., Rahme, L. G., Fall, R. & Vivanco, J. M. 2004. *Pseudomonas aeruginosa*-Plant Root Interactions. Pathogenicity, Biofilm Formation, and Root Exudation. *Plant Physiol*, 134, 320-31.
- Wang, G., Jin, J., Chen, X., Liu, J., Liu, X. & Herbert, S. 2007. Biomass and catabolic diversity of microbial communities with long-term restoration, bare fallow and cropping history in Chinese Mollisols. *Plant Soil and Environment*, 53, 177.
- Wang, J. 2012. *The effect of combining two biological control microbes on seed and root colonization*. MSc, Swedish University of Agricultural Science.
- Wani, P., Khan, M. & Zaidi, A. 2007. Co-inoculation of nitrogen-fixing and phosphate-solubilizing bacteria to promote growth, yield and nutrient uptake in chickpea. *Acta Agronomica Hungarica*, 55, 315-323.
- Want, E. J., Cravatt, B. F. & Siuzdak, G. 2005. The expanding role of mass spectrometry in metabolite profiling and characterization. *European Journal of Chemical Biology, Synthetic Biology and Bio-Nanotechnology*, 6, 1941-1951.
- Wasaki, J., Maruyama, H., Tanaka, M., Yamamura, T., Dateki, H., Shinano, T., Ito, S. & Osaki, M. 2009. Overexpression of the LASAP2 gene for secretory acid phosphatase in white lupin improves the phosphorus uptake and growth of tobacco plants. *Soil science and plant nutrition*, 55, 107-113.
- Weller, D. M. 2007. *Pseudomonas* biocontrol agents of soilborne pathogens: Looking back over 30 years. *Phytopathology*, 97, 250-256.

- William, A., George, N., O'reilly Patrick, J., Sean, M., Aryo, F. & Halimi, A. 2016. Adoption of Bambara groundnut production and its effects on farmers welfare in Northern Ghana. *African Journal of Agricultural Research*, 11, 583-594.
- Wink, M. 2008. Evolutionary advantage and molecular modes of action of multi-component mixtures used in phytomedicine. *Current Drug Metabolism*, 9, 996-1009.
- Wink, M. 2013. Evolution of secondary metabolites in legumes (Fabaceae). *South African Journal of Botany*, 89, 164-175.
- Wright, P., Falloon, R. & Hedderley, D. 2015. Different vegetable crop rotations affect soil microbial communities and soilborne diseases of potato and onion: Literature review and a long-term field evaluation. *New Zealand Journal of Crop and Horticultural Science*, 43, 85-110.
- Yang, Q., Wang, X. & Shen, Y. 2013. Comparison of soil microbial community catabolic diversity between rhizosphere and bulk soil induced by tillage or residue retention. *Journal of Soil Science and Plant Nutrition*, 13, 187-199.
- Yang, Y.-L., Xu, Y., Straight, P. & Dorrestein, P. C. 2009. Translating metabolic exchange with imaging mass spectrometry. *Nature Chemical Biology*, 5, 885-887.
- Yarza, P., Yilmaz, P., Pruesse, E., Glöckner, F. O., Ludwig, W., Schleifer, K.-H., Whitman, W. B., Euzéby, J., Amann, R. & Rosselló-Móra, R. 2014. Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. *Nature Reviews Microbiology*, 12, 635-645.
- Yin, C., Hulbert, S. H., Schroeder, K. L., Mavrodi, O., Mavrodi, D., Dhingra, A., Schillinger, W. F. & Paulitz, T. C. 2013. Role of bacterial communities in the natural suppression of *Rhizoctonia solani* bare patch disease of wheat (*Triticum aestivum* L.). *Applied and Environmental Microbiology*, 79, 7428-7438.

- Zhang, F. & Smith, D. L. 2001. Interorganismal signalling in suboptimum environments: the legume–rhizobia symbiosis. *Advances in Agronomy*, 76, 125–161.
- Zhang, H., Pang, H., Ao, H., Li, X., Teng, Z., Hu, J., Sun, G. & Zhang, X. 2016. Effects of forest degradation on soil microbial communities in Daxing'an mountains. *Sustainable Development: Proceedings of the 2015 International Conference on Sustainable Development (ICSD2015)*. World Scientific.

APPENDIX

IAA produced by each bacteria isolate calculated from the standard curve ($y=0.3395x-0.5203$)

Bacteria isolates	Absorbance at 530nm		IAA quantified (ug/ml)	
	Without tryptophan	With tryptophan	Without tryptophan	With tryptophan
BAMs	0.055	0.084	1.69	1.78
BAMoii	0.063	0.107	1.72	1.85
BAMa	0.055	0.083	1.69	1.78
BAMri	0.061	0.109	1.71	1.85
BAMr	0.052	0.099	1.69	1.82
BAMpii	0.053	0.093	1.69	1.82
BAMui	0.063	0.097	1.72	1.82
BAMuii	0.068	0.322	1.73	2.43
BAMrii	0.059	0.256	1.71	2.29
BAMx	0.063	0.19	1.72	2.09
BAMxi	0.052	0.333	1.69	2.51
BAMxii	0.055	0.116	1.69	1.87
BAMbi	0.059	0.075	1.71	1.75
BAMwi	0.25	0.105	2.27	1.84
BAMji	0.053	0.089	1.69	1.79
BAMli	0.058	0.105	1.7	1.84
BAMy	0.066	0.078	1.73	1.76
BAMhi	0.052	0.142	1.69	1.95

Carbon source utilization profile of Bambara soil samples at different growth stages corresponding to WAP

20th week	VBR2#1	VBR2#2	VBR2#3	VBR1#1	VBR1#2	VBR1#3	VL2#1	VL2#2	VL2#3	VL1#1	VL1#2	VL1#3	Total
Alcohols	0.574	0.000	4.032	1.159	2.330	2.448	1.688	5.063	0.4935	0.8619	0.754	0.2419	19.645
Amides	0.000	0.000	0.080	0.257	0.024	1.746	0.646	0.586	1.5966	0.278	3.0093	1.9801	10.204
Amines	0.737	0.000	1.764	5.063	0.241	10.924	1.030	0.703	1.7708	0.4379	0	0.0382	22.710
Amino Acids	41.682	18.197	24.320	3.905	16.824	13.739	17.023	28.549	35.402	17.086	27.665	16.095	260.488
Aromatic chemicals	1.270	0.000	1.650	4.463	0.345	2.001	3.188	3.000	0.174	9.460	12.249	1.770	39.570
Brominated chemicals	0.055	0.000	3.288	4.763	1.888	1.220	0.633	0.070	3.5997	0.2224	2.9167	2.2475	20.903
Carbohydrates	33.488	42.683	32.218	26.046	47.813	34.000	42.824	10.946	15.821	29.167	26.197	28.307	369.510
Carboxylic acids	9.738	16.056	19.526	41.364	12.357	10.302	19.281	38.956	23.63	20.464	18.452	25.066	255.193
Esters	0.1093	0	0.2234	1.0727	1.8238	9.2177	2.024	2.1799	3.7448	0.0556	0	4.374	24.825
Phosphorylated chemicals	0.997	0.000	1.650	1.631	10.043	0.949	3.674	1.383	0	1.849	0	0.0573	22.232
Polymer	6.351	18.063	6.249	5.278	1.310	8.452	2.990	3.563	8.767	15.119	3.7566	14.822	94.719

16th week	VBR2#1	VBR2#2	VBR2#3	VBR1#1	VBR1#2	VBR1#3	VL2#1	VL2#2	VL2#3	VL1#1	VL1#2	VL1#3	Total
Alcohols	1.493	0.672	3.042	4.618	0.933	2.169	2.520	3.538	0.6291	9.8239	0.9367	2.8098	33.184
Amides	0.480	0.105	0.000	0.000	0.944	0.326	6.391	0.454	0.2493	0	0.8249	0.6378	10.412
Amines	0.675	0.798	5.889	4.508	6.136	0.606	1.643	1.078	0.6648	3.4545	11.115	1.1205	37.689
Amino Acids	17.322	26.570	16.502	4.288	35.487	32.110	22.696	20.680	24.94	4.1023	39.901	19.548	264.147
Aromatic chemicals	12.544	3.739	4.724	9.786	1.225	3.638	2.246	2.157	1.614	7.017	1.314	0.276	50.279
Brominated chemicals	2.363	0.063	0.518	0.000	0.000	0.070	1.552	0.132	14.518	0	0	0.6895	19.906
Carbohydrates	38.008	25.709	22.909	9.346	27.239	20.217	20.578	31.957	14.114	15.114	21.908	40.372	287.470
Carboxylic acids	5.843	30.918	9.707	53.218	19.058	33.322	27.918	30.500	20.738	45.557	15.589	26.168	318.535
Esters	4.3371	0.3151	21.356	1.9792	2.5172	0.1166	1.1138	0.8514	7.2885	2.0511	1.7196	1.6549	45.300
Phosphorylated chemicals	10.830	0.441	3.300	3.958	1.056	1.119	1.662	0.908	0.5579	3.0227	1.2583	1.2584	29.372
Polymer	1.104	5.671	7.054	3.299	0.405	1.306	6.683	2.743	9.6864	4.858	0.4334	0.4654	43.707

12th week	VBR2#1	VBR2#2	VBR2#3	VBR1#1	VBR1#2	VBR1#3	VL2#1	VL2#2	VL2#3	VL1#1	VL1#2	VL1#3	Total
Alcohols	3.183	3.399	13.398	0.000	0.000	13.964	3.711	0.653	0.4912	0.0984	8.4753	1.1636	48.537
Amides	2.122	0.025	4.301	0.000	0.000	7.692	0.907	0.193	0	0.0861	0	0.1435	15.470
Amines	3.537	0.534	0.791	2.110	0.000	0.000	2.804	0.608	0	0.2889	0	2.104	12.777
Amino Acids	38.905	17.132	4.598	4.996	18.252	21.335	2.886	24.499	7.671	20.661	9.631	4.2878	174.854
Aromatic chemicals	0.566	1.407	1.137	4.044	0.000	0.366	5.855	2.076	1.398	8.723	16.219	2.216	44.007
Brominated chemicals	0.000	5.451	5.438	0.000	0.000	5.631	0.082	0.000	0	0.6393	0	1.6577	18.900
Carbohydrates	20.514	31.517	13.423	48.958	50.886	23.578	58.468	43.407	48.124	28.868	29.278	66.564	463.584
Carboxylic acids	20.655	27.126	48.872	27.537	24.502	21.152	9.896	10.470	35.521	22.001	2.3114	9.1812	259.225
Esters	0.5659	2.0684	0.8899	3.3112	0	0	1.237	4.7456	0	0	0	0.3347	13.153
Phosphorylated chemicals	1.698	5.154	0.717	0.000	0.314	0.320	2.639	1.097	0.0189	5.7539	0	1.2592	18.971
Polymer	3.254	1.187	1.434	4.044	1.046	0.961	6.515	7.252	1.7761	7.8808	29.086	6.0889	70.524

8th week	VBR2#1	VBR2#2	VBR2#3	VBR1#1	VBR1#2	VBR1#3	VL2#1	VL2#2	VL2#3	VL1#1	VL1#2	VL1#3	total
Alcohols	0.062	1.225	0.312	1.336	1.382	0.737	0.277	0.174	1.22829	0.5213	0.42366	4.47212	12.149
Amides	3.426	0.000	0.304	0.179	2.170	0.220	0.176	0.160	1.37291	0	1.14773	0	9.155
Amines	0.000	0.466	0.397	0.945	0.000	0.207	6.116	3.031	0.23377	0.82795	1.17855	0.39077	13.793
Amino Acids	11.369	20.246	23.681	26.196	19.824	17.665	30.877	17.929	23.3969	27.2509	19.758	5.36944	243.562
Aromatic chemicals	0.000	0.479	5.010	6.099	0.911	4.038	5.186	1.875	1.107	0.746	8.735	15.095	49.282
Brominated chemicals	0.000	0.399	1.099	2.550	1.328	0.590	0.801	0.336	0.64782	3.28115	3.54334	0.13026	14.706
Carbohydrates	37.954	28.925	33.479	28.308	31.248	35.192	21.614	42.110	40.0679	37.7179	39.0692	28.005	403.689
Carboxylic acids	20.816	18.489	22.331	16.923	27.398	25.844	22.831	20.271	16.5403	11.438	3.1659	32.6798	238.728
Esters	6.64348	8.61216	5.42776	2.50852	3.9266	7.64947	3.72084	2.78654	1.75724	1.02216	6.67842	0.02895	50.762
Phosphorylated chemicals	0.000	1.304	0.211	3.490	2.713	0.272	1.760	3.218	3.27477	0.5213	0.12325	1.49071	18.378
Polymer	14.729	14.855	2.748	6.467	4.099	2.586	1.641	3.109	5.37276	11.6731	11.1769	7.33775	85.795

4th week carbon source utilization	VBR2#1	VBR2#2	VBR2#3	VBR1#1	VBR1#2	VBR1#3	VL2#1	VL2#2	VL2#3	VL1#1	VL1#2	VL1#3	Total
Alcohols	2.197	0.356	2.185	0.167	0.323	1.825	5.17536	1.1741	2.4471	0.445	6.275	1.88021	24.450
Amides	0.393	10.617	0.198	3.721	0.119	0.419	0.04779	2.49901	0	0.269	2.987	0.09896	21.369
Amines	0.302	0.454	1.444	0.032	0.030	1.658	0.15704	0.2262	4.02024	0.750	0.937	1.73177	11.741
Amino Acids	18.219	20.242	17.162	23.106	16.494	18.499	23.4701	18.7318	4.36983	24.370	37.632	48.4401	270.737
Aromatic chemicals	1.251	6.388	3.179	1.080	3.720	2.458	1.140	1.400	12.061	4.162	1.856	2.771	41.466
Brominated chemicals	0.517	0.185	1.747	1.805	0.340	2.732	1.30067	0.02693	0.0874	2.439	2.003	0.34635	13.530
Carbohydrates	47.008	35.903	34.409	30.917	47.551	37.870	42.13	35.3954	40.6394	32.766	29.104	27.8568	441.550
Carboxylic acids	14.121	17.909	24.670	20.004	13.649	19.524	13.1262	28.0869	16.8675	7.170	13.301	3.51302	191.942
Esters	6.735	0.802	2.531	4.670	1.921	3.064	0.82273	0.84018	0.43698	9.337	0.088	0.39583	31.644
Phosphorylated chemicals	0.113	0.072	0.716	2.252	5.291	3.348	1.48502	0.25313	3.67065	6.354	1.317	2.42448	27.296
Polymer	4.268	0.700	6.068	3.546	3.433	4.332	6.14489	6.36601	10.4002	7.522	1.317	5.54167	59.639

Control	R3#1	R3#2	R3#3	R2#1	R2#2	R2#3	R4#1	R4#2	R4#3	R5#1	R5#2	R5#3	Total
Alcohols	0.000	4.223	0.355	0.171	2.056	7.047	0.507	11.118	0.22172	0.13775	2.16612	10.187	38.190
Amides	0.000	0.000	0.926	0.000	8.883	10.904	1.563	1.770	0.02195	0	0	0	24.069
Amines	4.272	3.208	0.355	0.342	27.678	0.000	0.309	0.769	1.24469	2.42948	2.47557	12.0823	55.166
Amino Acids	11.748	28.976	24.238	6.507	5.506	3.425	24.858	33.507	15.9592	14.9525	54.772	0.47382	224.922
Aromatic chemicals	2.067	4.651	7.210	6.027	17.987	22.390	0.309	3.462	7.232	0.050	3.249	7.581	82.216
Brominated chemicals	0.517	0.160	3.911	1.747	3.083	0.396	0.710	1.039	1.00248	0.01252	1.46987	9.71322	23.760
Carbohydrates	29.043	29.297	29.573	70.959	5.726	28.535	27.964	25.601	42.6749	28.3898	19.3404	16.3466	353.449
Carboxylic acids	11.662	8.180	19.725	6.301	16.959	20.956	22.036	9.906	14.6713	29.2664	6.57573	13.2668	179.504
Esters	33.1079	0.53461	0.80013	0.23973	0.88099	0.06182	1.79809	0.15388	3.76113	0	0.15472	2.60599	44.099
Phosphorylated chemicals	1.723	13.365	0.661	0.000	0.000	0.000	0.096	2.712	2.00569	0.25046	2.70765	13.2668	36.787
Polymer	0.861	1.818	6.779	2.705	5.873	0.618	14.822	4.597	6.20513	19.5109	2.08876	9.47631	75.355

GC-MS chromatograms of *Bacillus* spp using different extracts

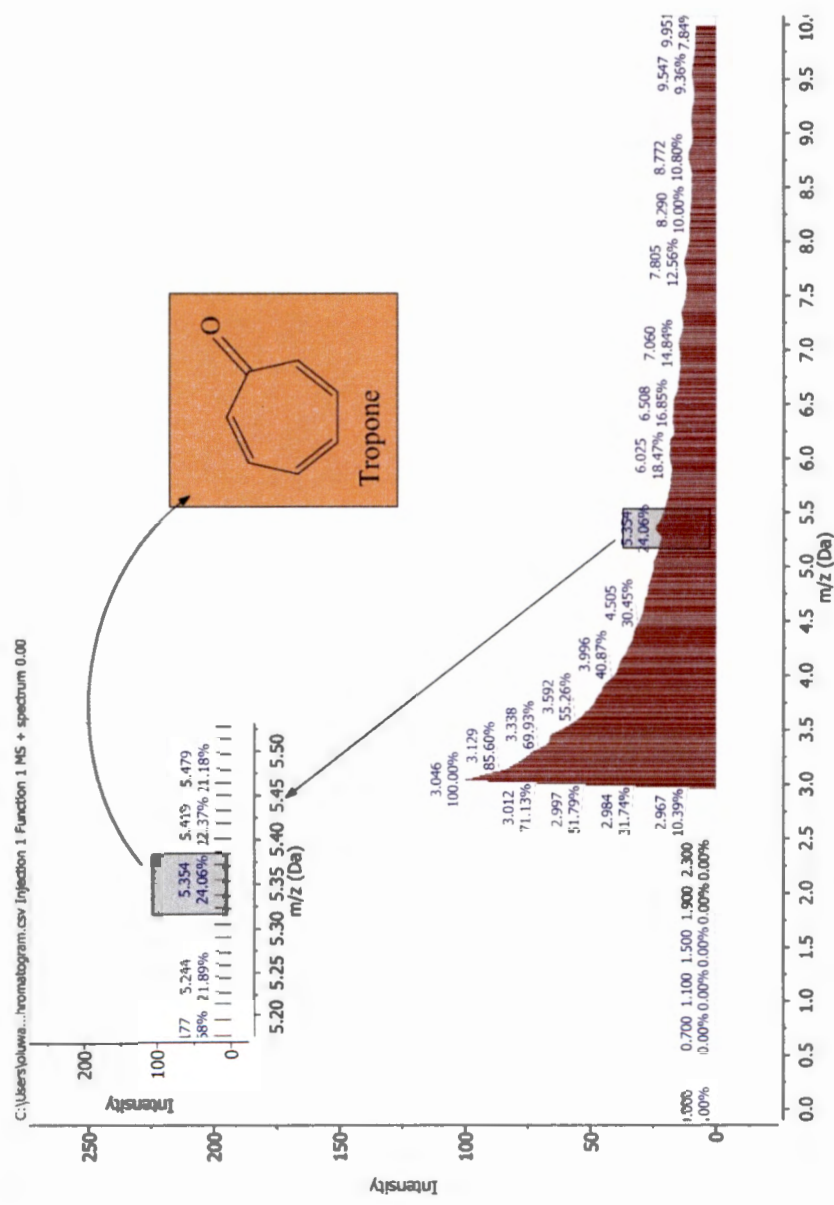
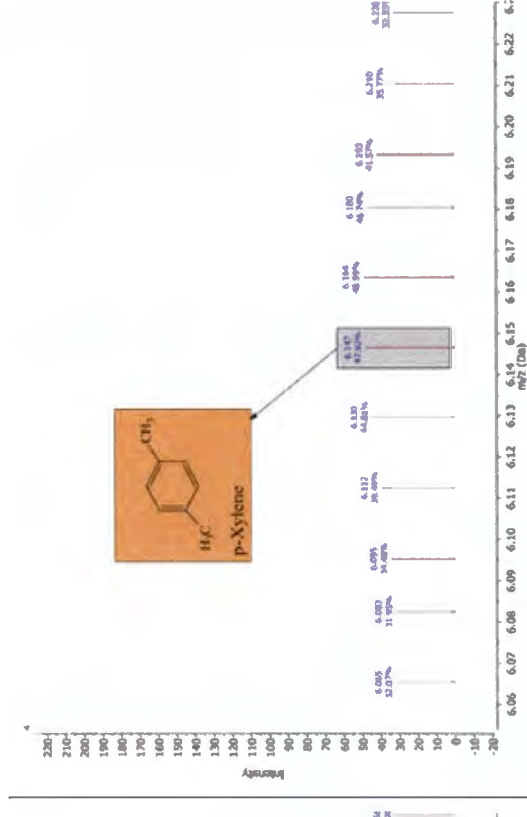
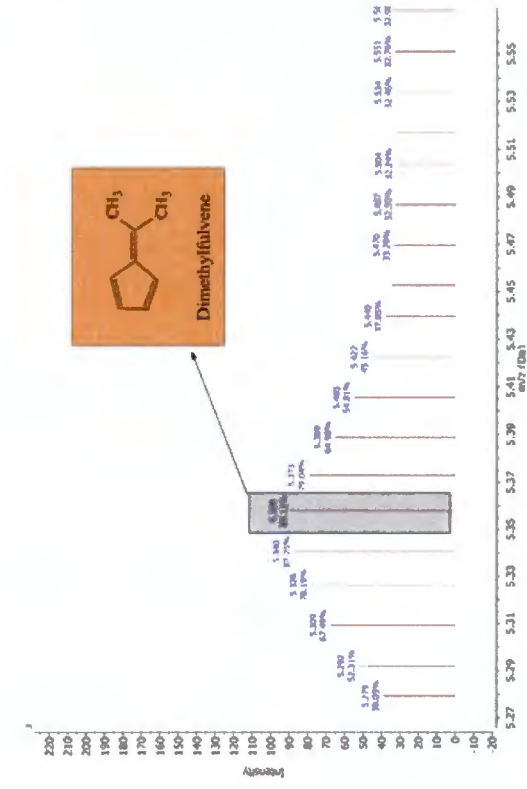
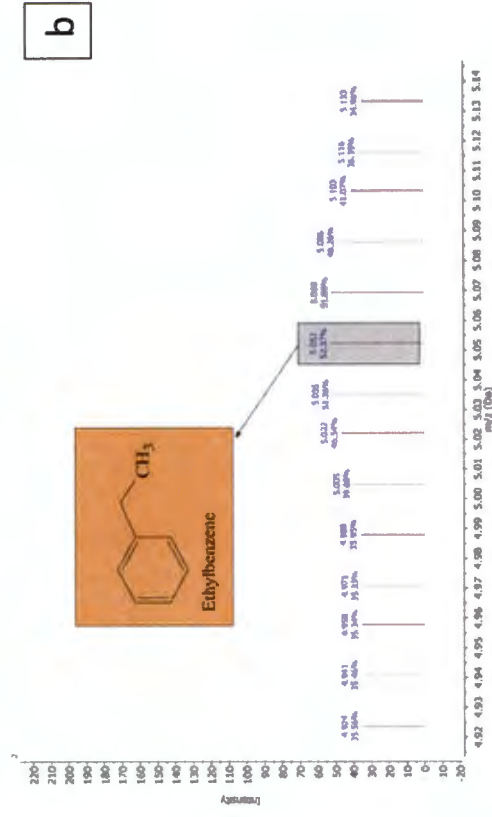
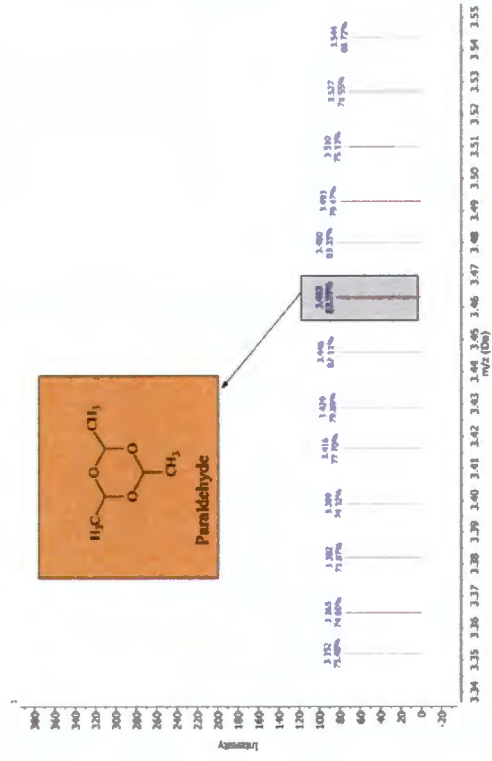


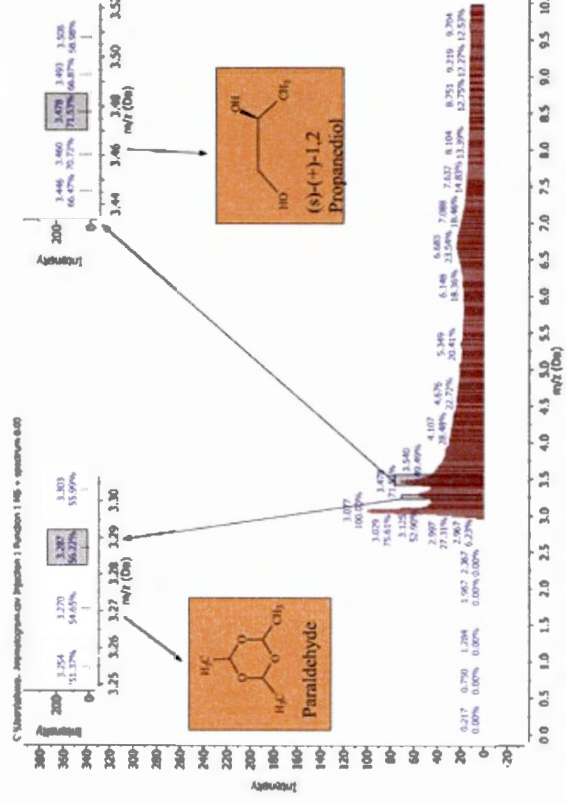
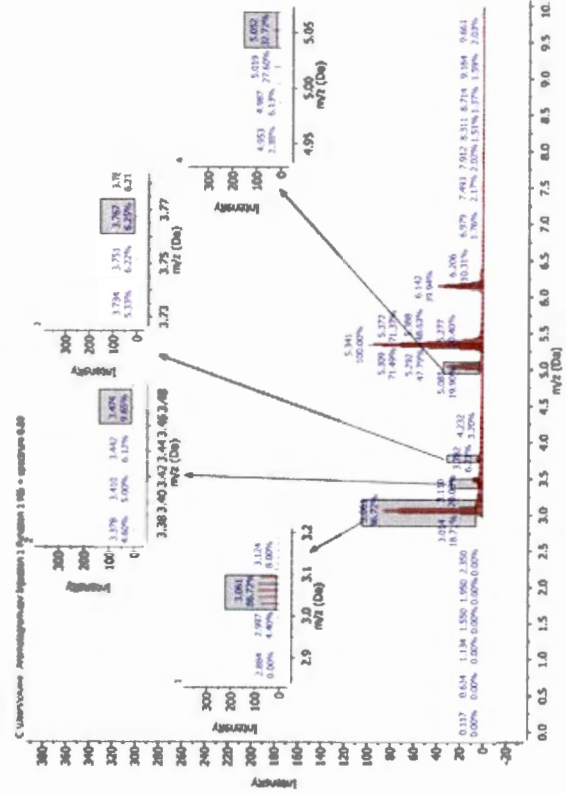
Figure 1: GC-MS chromatogram of *Bacillus* spp (Benzene extract)



BAMhi-hexane

BAMhi-methanol

a



BAMhi-hexane

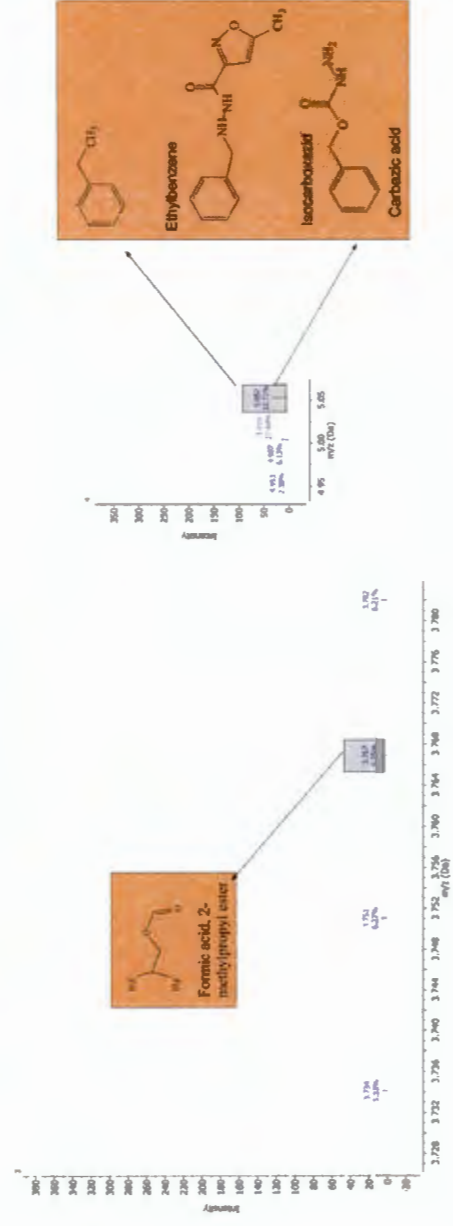
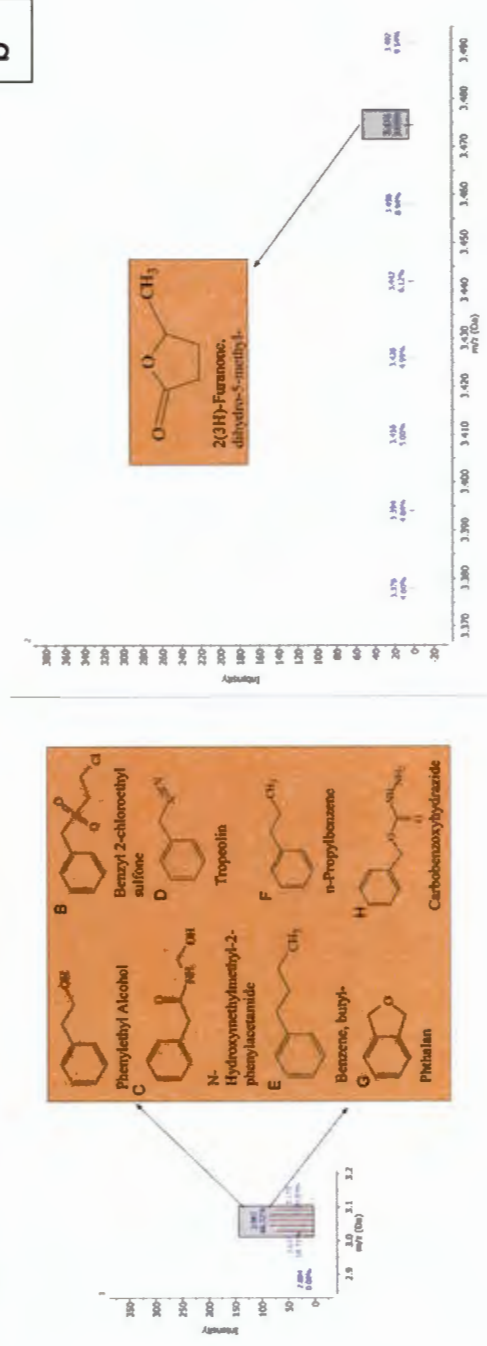
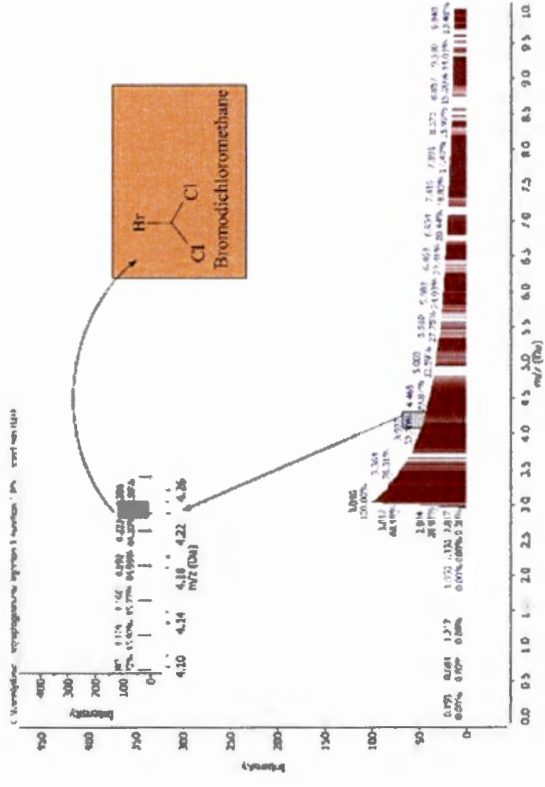


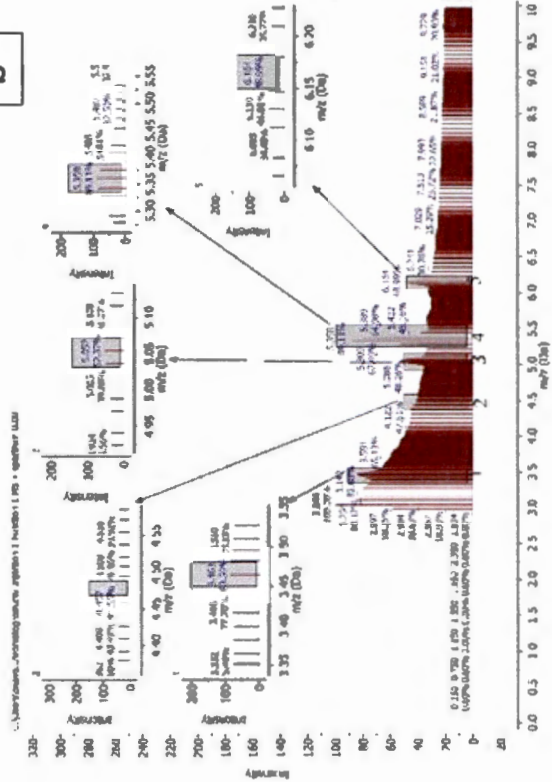
Figure 3: a and b GC-MS chromatogram of BAMhi comparing extracts using hexane and methanol

BAMli-chloroform



BAMli-ethylacetate

b



BAMli ethylacetate

C

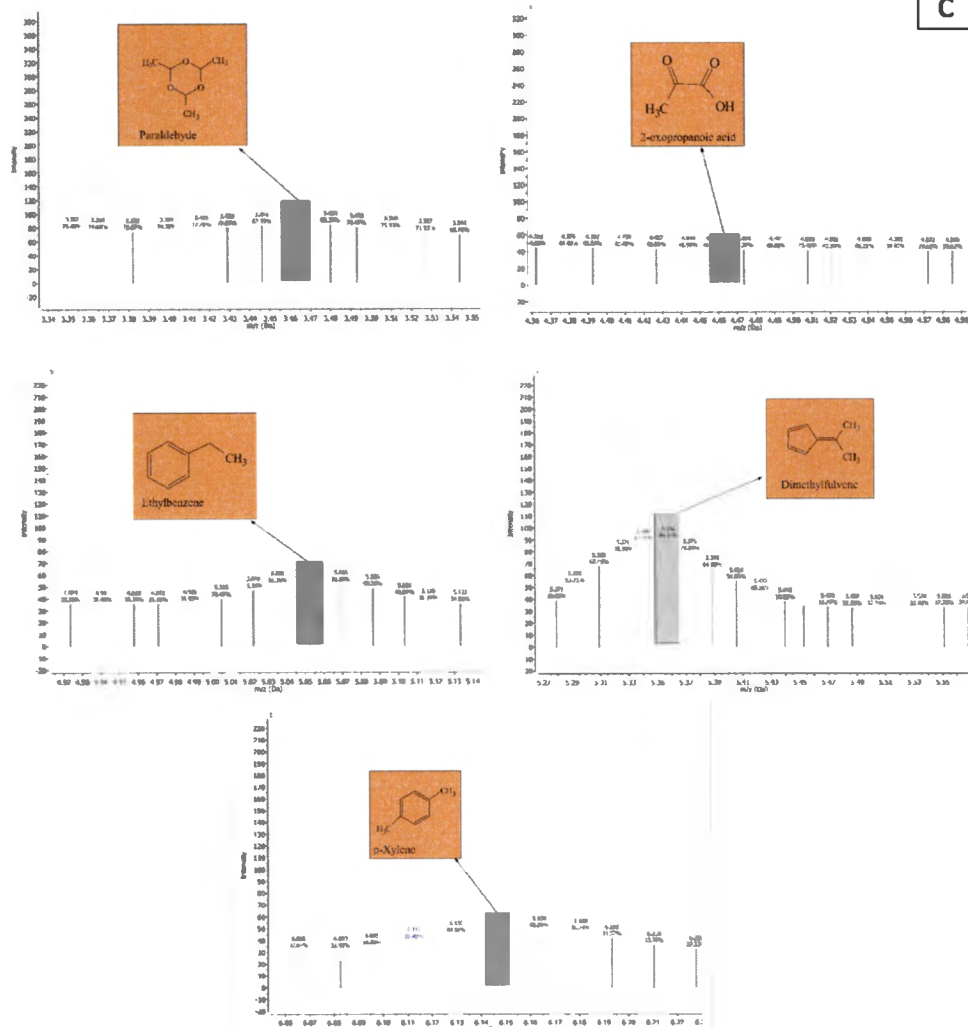
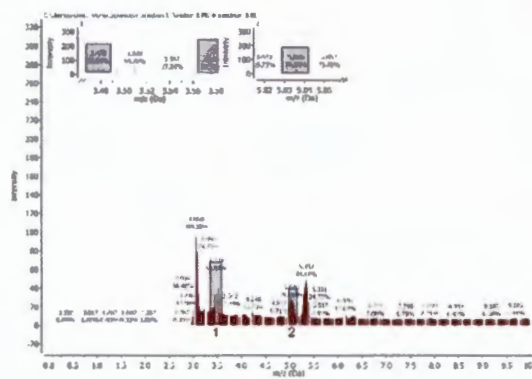
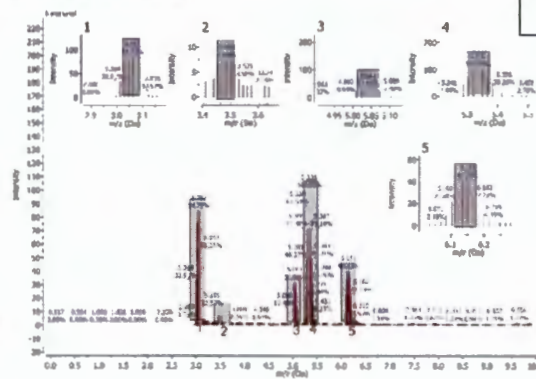


Figure 4: a, b and c GC-MS chromatogram of BAMli comparing extracts of benzene, butanol, chloroform and ethylacetate extract

BAMli-hexane

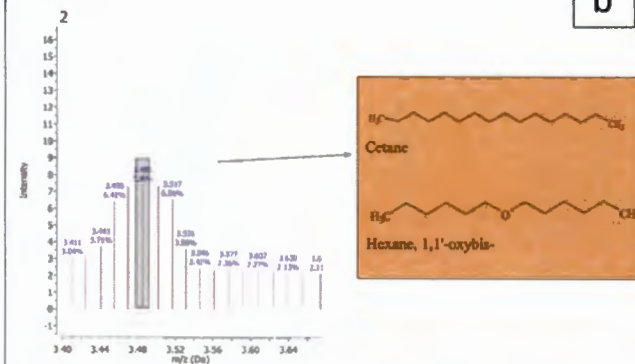
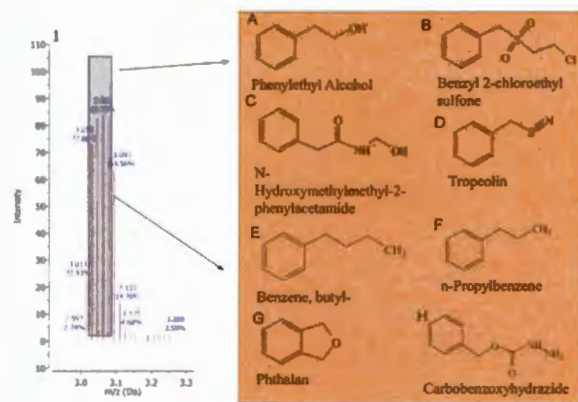


BAMli-methanol



a

BAMli-methanol



b

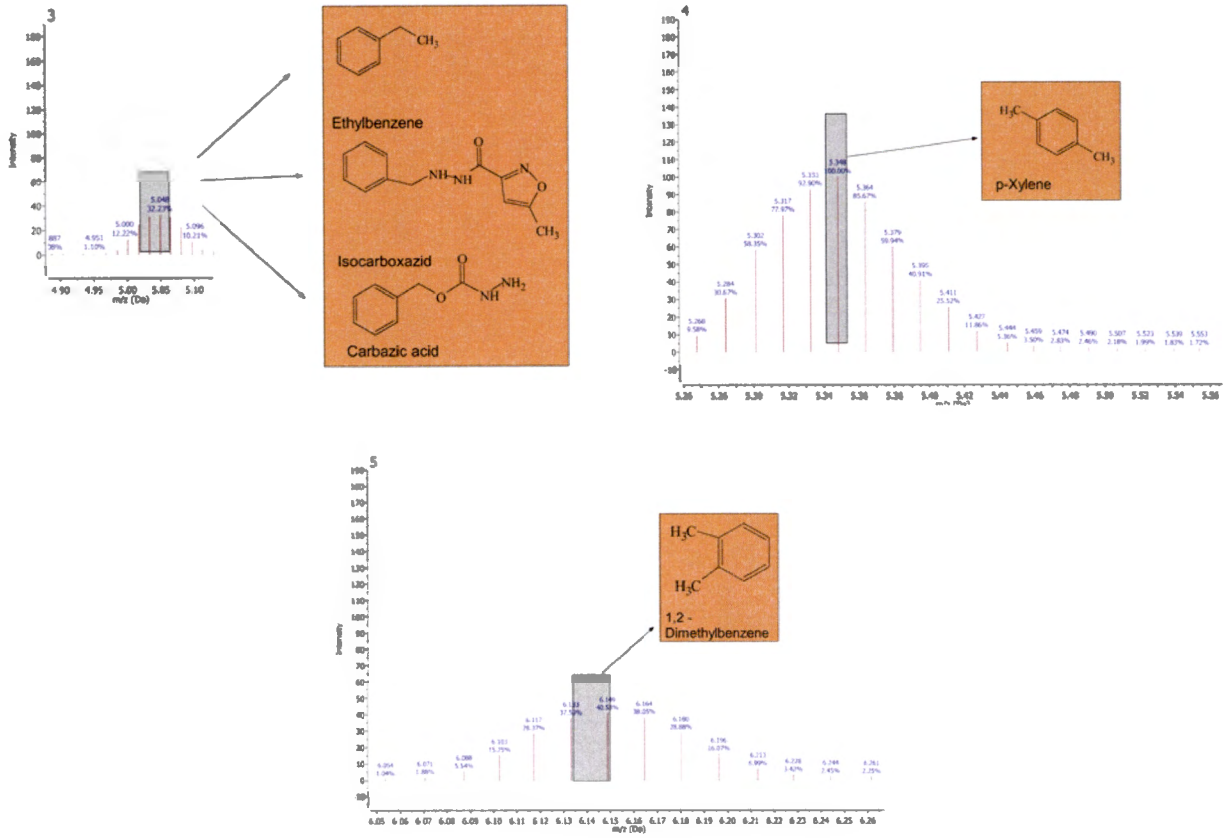


Figure 5: a, b and c GC-MS chromatogram of BAMhi comparing extracts from methanol and hexane

BAMli-petroleum ether

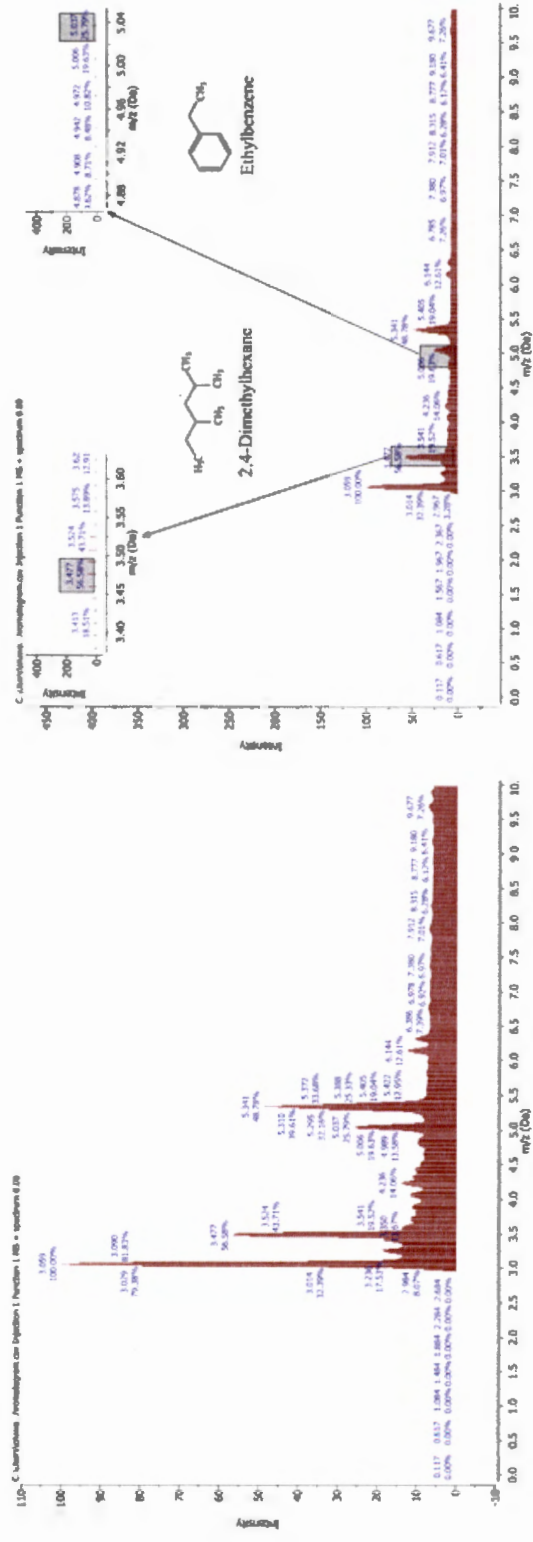
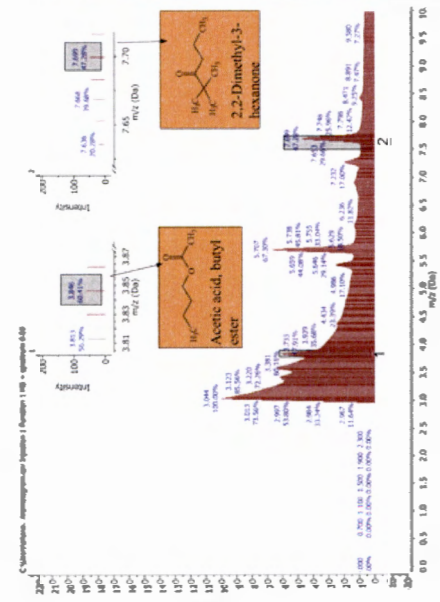


Figure 6: GC-MS chromatogram of BAMli (petroleum ether extract)

BAMr-butanol



BAMr-ethyl acetate

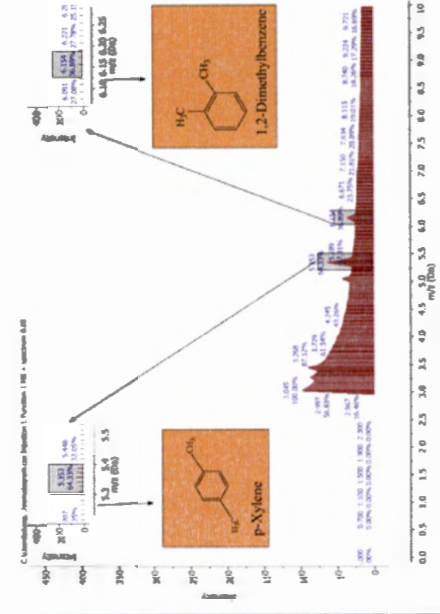
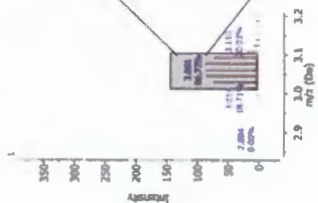
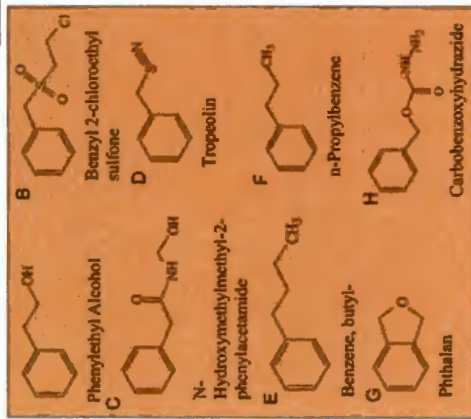
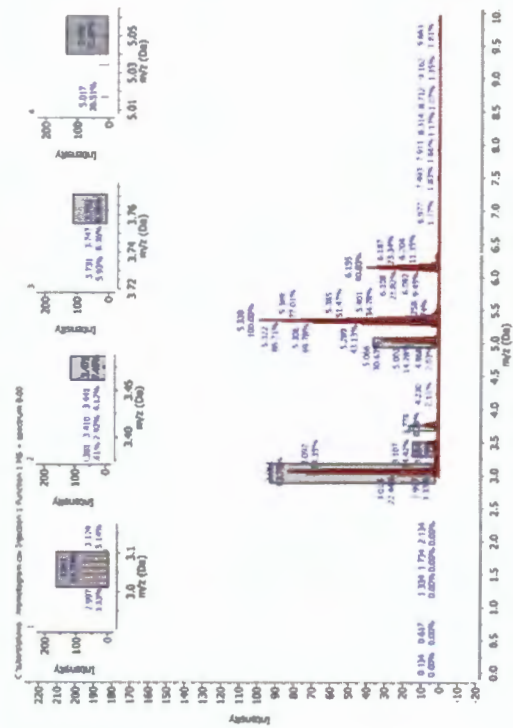


Figure 7: GC-MS chromatogram of BAMr comparing extracts using butanol and ethylacetate

BAMr-hexane

BAMr-hexane

a



BAMr-hexane

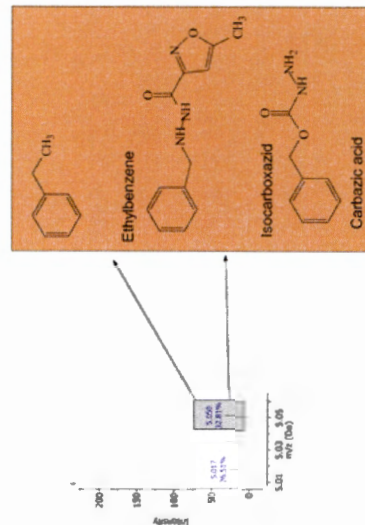
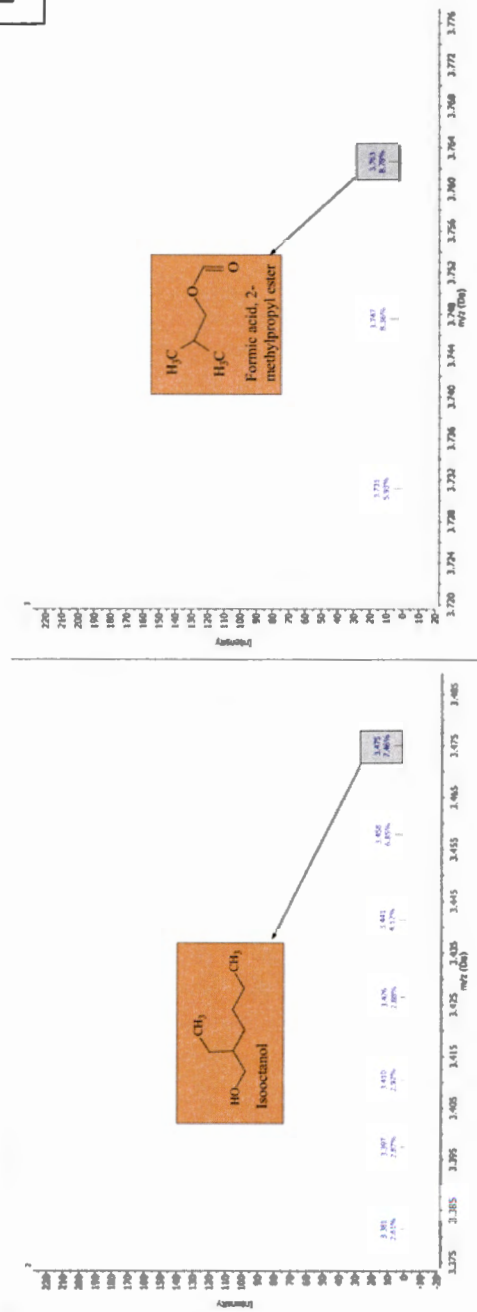
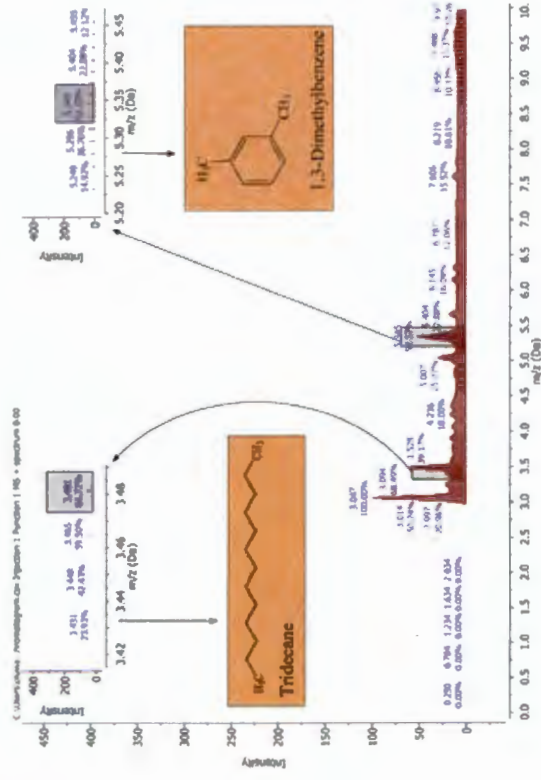


Figure 8: a and b GC-MS chromatogram of BAMr (hexane extract)

BAMr-petroleum ether



BAMr-methanol

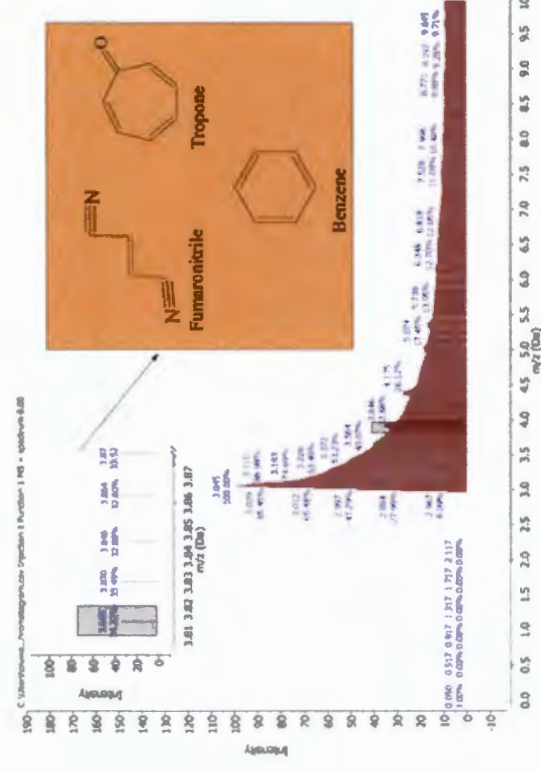


Figure 9: GC-MS chromatogram of BAMr comparing extracts using petroleum ether and methanol