

Diversity of rhizobacteria associated with *Vigna subterranea* communally produced in Mpumalanga, KwaZulu-Natal, and Limpopo provinces, South Africa

Rivoningo Ubisi

201806150



<https://orcid.org/0000-0003-1391-1092>

A research dissertation submitted in fulfilment of the requirements for a Master of Science in Agriculture Degree

Supervisor: Dr. Z. P Dube (University of Mpumalanga)

**Co-Supervisor: Dr. T. Suinyuy (University of Mpumalanga)
: Ms. E. Kola (University of Mpumalanga)**

School of Agricultural Sciences

Faculty of Agriculture and Natural Sciences

May 2025



**UNIVERSITY OF
MPUMALANGA**

Table of Contents

DECLARATION	vii
LIST OF FIGURES	xi
LIST OF TABLES	xiv
LIST OF ABBREVIATIONS.....	xvii
GENERATED PUBLICATIONS FROM THIS DISSERTATION.....	xix
ABSTRACT.....	xx

CHAPTER ONE

INTRODUCTION

1.1 Background	1
1.2. Problem statement	2
1.3. Justification	3
1.4. Purpose of the study	4
1.4.1 Aim and Objective.....	4
1.4.2. Hypotheses.....	5
1.5. Reliability, validity, and objectivity	5
1.6. Bias.....	6
1.7. Significance of the study	6
1.8. General overview of chapters in the dissertation	7
1.9. References	8

CHAPTER TWO

LITERATURE REVIEW

2.1. Overview	12
2.2. Significance of Bambara groundnut.....	13

2.2.1. Nutritional advantages	13
2.2.2. Agronomical advantage	14
2.2.3. Medical Significance	14
2.3. Bambara groundnut production in South Africa	15
2.3.1. Production of Bambara groundnut in Mpumalanga province	16
2.3.2. Production of Bambara groundnut in Kwazulu-Natal province	17
2.3.3. Production of Bambara groundnut in Limpopo province.....	17
2.4. Production constraints of BG in South Africa	17
2.4.1. Biotic stresses	17
2.4.2. Abiotic stress	19
2.4.3. Socio-economic challenges	20
2.5. Biological nitrogen fixation and its significance.....	21
2.6. Factors that affect metabolism, survival, and functioning of BNF in the soil	22
2.6.1. Soil pH.....	22
2.6.2. Temperature.....	22
2.6.3. Nitrate	23
2.6.4. Moisture stress.....	23
2.7. Symbiotic interaction in Bambara groundnut rhizosphere.....	24
2.8. Importance of identifying Indigenous rhizobia strains	25
2.8.1. Biodegradation.....	26
2.8.2. Bio-fertilizers and protectors	26
2.8.3. Bio-composition	26
2.8.4. Bioleaching of heavy metals.....	27
2.8.5. Bio-mediation	27

2.9. Plant growth-promoting rhizobacteria (PGPR).....	27
2.10. Mechanism of plant growth promoting rhizobacteria (PGPR)	29
2.10.1. Direct mechanism	32
2.10.2. Indirect mechanism.....	38
2.11. Factors limiting commercial inoculum application and performance in agriculture ..	41
2.12. References	42

CHAPTER THREE

INCIDENCE AND DIVERSITY OF RHIZOBACTERIA ASSOCIATED WITH COMMUNALLY PRODUCED BAMBARA GROUNDNUT ROOT NODULES IN LIMPOPO, KWAZULU-NATAL, AND MPUMALANGA PROVINCE AND THEIR N- CYCLING AND P-CYCLING EFFICIENCY

3.1. Introduction	63
3.2. Material and methods	65
3.2.1. Sample collection	65
3.2.2. Planting procedure	66
3.2.3. Data collection	67
3.2.4. Sterilization and cleaning of root nodules	67
3.2.5. Preparation of NA media	68
3.2.6. Root nodule bacteria extraction and identification	68
3.2.7. Morphological characterization	70
3.2.8. Molecular identification of isolates	70
3.2.9. Sequence alignment and phylogenetic analyses	71
3.2.10. Data analysis	72
3.3. Results	73

3.3.1. Morphological characterization of isolates.....	73
3.3.2. Molecular identification of selected isolates	74
3.3.3. Phylogenetic tree	89
3.4. Discussion	108
3.5. Conclusion	114
3.6. Recommendations	114
3.7. References.....	115

CHAPTER FOUR

ASSESSMENT OF SOIL NUTRIENT STATUS AND ENZYME ACTIVITIES OF BAMBARA GROUNDNUT RHIZOSPHERE SOIL IN LIMPOPO, KWAZULU-NATAL, AND MPUMALANGA PROVINCE

4.1. Introduction	125
4.2 Material and Methods.....	126
4.2.1. Sampling site	126
4.2.2. Soil physico-chemical properties analysis.....	127
4.2.3. Soil enzyme activity	130
4.2.4. Nitrate reductase activities.....	131
4.2.5. Data analysis.....	132
4.3. Results	132
4.3.1. Soil physico-chemical properties.....	132
4.3.2. Soil Chemical Properties	133
4.3.3. Correlation between sample sites and soil nutrients.....	140
4.4. Discussion	141
4.5. Conclusion	144

4.6. Recommendations	145
4.7. References	146

CHAPTER FIVE

STUDY SUMMARY, SIGNIFICANCE OF FINDINGS, FUTURE RESEARCH AND CONCLUSION

5.1. Summary	152
5.2. Significance of findings	153
5.3. Future research	154
5.4. Conclusions	154
5.5. Recommendation.....	154
APPENDICES	156

DECLARATION

I, Rivoningo Ubisi, student number 201806150, hereby declare that the dissertation titled ‘Diversity of rhizobacteria associated with *Vigna subterranea* communally produced in Mpumalanga, KwaZulu-Natal, and Limpopo provinces, South Africa submitted for Master of Science in Agriculture is my own work and that it has not previously been submitted for assessment or completion of any postgraduate qualification to another university or for another qualification.



Ubisi, R. (Ms)

15 April 2025

Date

Supervisor(s):



Dube, ZP. (Dr)

15 April 2025

Date

Suinyuiy, T. (Dr)

Date

DEDICATION

To my loving mother, Elsie Dikeledi Ubisi, my late father, Eckson Thuthani Ubisi, siblings Lucia Ubisi, Adolf Ubisi, Lodrick Ubisi, Melvin Ubisi, Follow Ubisi, and my sons Trinity and Clinton.

ACKNOWLEDGEMENTS

First, I would like to thank the Almighty God for his guidance and presence throughout this academic journey of my life.

My sincere heartfelt gratefulness to my supervisor, Dr Zakheleni Dube from the Faculty of Agriculture and Natural Sciences for his generous tireless encouragement, guidance, valuable suggestions, and constructive criticisms throughout my study period. He has contributed considerably to my achievement. He has provided me with a tremendous amount of support, without him this project would not have been initiated. A special thanks to my Co-Supervisor, Dr Terence Suinyuy from the Faculty of Agriculture and Natural Sciences for the support, eagerness, expertise, guidance, and kindness.

I am very much thankful to the Vice-Chancellor Scholarship and Academic Talented Stewardship Program (ATSP) for funding my studies. A crucial element of this research project was the interaction with the Bambara groundnut farmers across the different provinces, which was facilitated by the agricultural extension officers from Department of Agriculture, Maruleng Municipality, Mr Malumane in Limpopo province and Mr Thulane in Boschfontein. Special thanks are sincerely directed to all the communal farmers in the three provinces for giving me the opportunity to undertake this study in their farms.

I further extend my gratitude to the Head of School of Agricultural Sciences, Prof F Kutu for admitting me to the master's degree at the University of Mpumalanga, your support is highly appreciated.

A special thanks to Tshwane Mongwadi, for your assistance with soil nutrient and enzyme activity analysis, you are highly appreciated.

I also wish to thank my fellow Master's and PhD colleagues in Plant Protection, Mr. Moses Timana and most specially Ms. Gugu Maseko. Their daily encouragement and moral support had made my work much easier.

Last but not least, I would like to express my deepest gratitude with special thanks to Thobile Mkhwanazi for being a wonderful friend, your encouragement, criticism, and support during good and hard times throughout this research project was incomparable.

Special appreciation to my cousin Donald Khumalo for the support, guidance, and motivation.

Thank you for always assisting me when I needed help throughout this research project.

LIST OF FIGURES

Figure 2.1: The role of plant growth promoting rhizobia (PGPR) on the growth and development of plant (PlantWorks, 2013).	13
Figure 2.2: Reddish spots on the leaves of Bambara groundnut caused by <i>Cercospora</i> sp. (Ouoba <i>et al.</i> , 2019).	18
Figure 2.3: Effect of prolonged drought period on Bambara groundnut production (Fleibrer, 2006).	19
Figure 2.4: Interaction between a legume and beneficial microbes (Jaiswal <i>et al.</i> , 2021).	22
Figure 2.5: Model of symbiotic fixation in plant by <i>Rhizobium</i> sp. (Lindström & Mousavi, 2019).	25
Figure 2.6: Interaction between symbiotic bacteria and plant in the root nodules (Nefronus, 2019).	33
Figure 2.7: PGPR direct mechanism on plant growth, nutrient solubilization, biological fertilizers, and phyto-stimulation (Dos Santos, Dias, Lobo & Rigobelo, 2020).	36
Figure 2.8: Indirect mechanism beneficial for plant growth and development from PGPR interaction, siderophores, antibiosis, induced systemic resistance (ISR), and interaction with the quorum sensing (Dos Santos <i>et al.</i> , 2020).	41
Figure 3.1: Farmers at Gabaza village, Limpopo province (A); Bambara groundnut pods (B); Roots nodules (C).	66
Figure 3.2: Bambara groundnut farm at Boschfontein, Mpumalanga province (A); Growth of BG plants on soil collected from KZN (B); Abundance of root nodules (C).	67
Figure 3.3: Prepared NA in an autoclave (A); NA poured into 90 mm Petri dish in the Laminar flow (B).	68
Figure 3.4: Root nodules (A); Nodules removed from Bambara groundnut roots (B); Isolate incubation for bacteria growth (C).	69

Figure 3.5: Bambara groundnut selected rhizobacterial isolates on Nutrient Agar.....	74
Figure 3.6: Agarose gel electrophoresis of the DNA pattern of rhizobia isolates; M (DNA marker); 1-15 (gene fragments of isolated bacteria from Bambara groundnut root nodules...)	77
Figure 3.7: Phylogenetic tree indicating the evolutionary history between identified rhizobia isolates from Bambara groundnut root nodule isolated in Mpumalanga, KwaZulu-Natal, and Limpopo province and the NCBI GenBank <i>Enterobacter asburiae</i> (A) and <i>Leucobacter chromiiresistens</i> (B) species isolates.	89
Figure 3.8: Phylogenetic tree indicating the evolutionary history between identified rhizobia isolates from Bambara groundnut root nodule isolated in Mpumalanga, KwaZulu-Natal, and Limpopo province and the NCBI GenBank <i>Bacillus pumilus</i> (C) and <i>Sphingobacterium faecium</i> (D) species isolates.....	90
Figure 3.9: Phylogenetic tree indicating the evolutionary history between identified rhizobia isolates from Bambara groundnut root nodule isolated in Mpumalanga, KwaZulu-Natal, and Limpopo province and the NCBI GenBank <i>Stenotrophomonas lactitubi</i> (E) and <i>Stenotrophomonas pavanii</i> (F) species isolates.	91
Figure 3.10: Phylogenetic tree indicating the evolutionary history between identified rhizobia isolates from Bambara groundnut root nodule isolated in Mpumalanga, KwaZulu-Natal, and Limpopo province and the NCBI GenBank <i>Stenotrophomonas maltophilia</i> (G) and <i>Lysinibacillus sphaericus</i> (H) species isolates.....	92
Figure 3.11: Phylogenetic tree indicating the evolutionary history between identified rhizobia isolates from Bambara groundnut root nodule isolated in Mpumalanga, KwaZulu-Natal, and Limpopo province and the NCBI GenBank <i>Bacillus licheniformis</i> (I) and <i>Cellulosimicrobium cellulans</i> (J) species isolates.	93
Figure 3.12: Phylogenetic tree indicating the evolutionary history between identified rhizobia isolates from Bambara groundnut root nodule isolated in Mpumalanga and KwaZulu-Natal	

province and the NCBI GenBank <i>Kaistella daneshvariae</i> (K) and <i>Stenotrophomonas geniculate</i> (L) species isolates.	94
Figure 3.13: Phylogenetic tree indicating the evolutionary history between identified rhizobia isolates from Bambara groundnut root nodule isolated in Mpumalanga and KwaZulu-Natal province and the NCBI GenBank <i>Neorhizobium petrolearium</i> (M) and <i>Proteus columbae</i> (N) species isolates.	95
Figure 3.14: Phylogenetic tree indicating the evolutionary history between identified rhizobia isolates from Bambara groundnut root nodule isolated in Mpumalanga and Limpopo province and the NCBI GenBank <i>Micrococcus yunnanensis</i> (O) and <i>Lysinibacillus pakistanensis</i> (P) species isolates.	96
Figure 3.15: Phylogenetic tree indicating the evolutionary history between identified rhizobia isolates from Bambara groundnut root nodule isolated in KwaZulu-Natal province and the NCBI GenBank <i>Mammaliicoccus scuri</i> (Q) and <i>Sphingobacterium multivorum</i> (R) species isolates.....	97
Figure 3.16: Nitrogen cycling bacteria isolates indicated by colour change; negative (A), change of isolates from green to blue by slow grower (B), and positive fast grower (C).	98
Figure 3.17: Isolates tested for phosphate solubilization indicated no zone formed around the colony on Pikovskaya agar plates.	98
Figure 4.1: Soil sample from Hazyview (A), Casteel (B), and Gabaza village (C).....	127
Figure 4.2: Correlation between soil nutrient and sample locations in Mpumalanga, KwaZulu-Natal, and Limpopo province.	140
Figure 4.3: Soil nitrate reductase enzyme activities in $\text{nmol h}^{-1} \text{ g}^{-1}$	141

LIST OF TABLES

Table 2.1: Effect of plant growth promoting microbes (PGPM) on plant well-being (Naik <i>et al.</i> , 2019).	30
Table 2.2: Soil enzymes involved in nutrient mobilization of N, P, and S.....	39
Table 3.1: Bambara groundnut root nodule and soil sample sites in Mpumalanga, KwaZulu Natal, and Limpopo province.	65
Table 3.2: Rhizobia isolates from Bambara groundnut root nodules showing sequence similarity (%) with the NCBI database bacterial strains after 16S rRNA gene sequencing....	78
Table 3.3: Nitrogen cycling and phosphate cycling plant growth promoting activities of rhizobia isolates from Bambara groundnut root nodules.	99
Table 3.4: Functional bacterial diversity index of Bambara groundnut root nodule samples in Mpumalanga, KwaZulu-Natal, and Limpopo province.	107
Table 4.1: Soil physico-chemical properties of studied soil in Mpumalanga, Kwa-Zulu Natal Limpopo province.	138

LIST OF APPENDINCES

Appendix 3.1: Morphological features of 209 root nodule bacteria colonies isolated from Bambara groundnut.....	156
Appendix 4.1: Summary of soil analysis result on Bambara groundnut rhizosphere soil.....	179
Appendix 4.2: Shapiro-Wilk normal distribution test for soil properties and enzyme activities on Bambara groundnut rhizosphere soil.	181
Appendix 4.3: Analysis of variance (ANOVA) for phosphorus on Bambara groundnut rhizosphere soil.	182
Appendix 4.4: Analysis of variance (ANOVA) for exchangeable acidity ($\text{cmol}_c \text{ kg}^{-1}$) on Bambara groundnut rhizosphere soil.	182
Appendix 4.5: Analysis of variance (ANOVA) for acid saturation (%) on Bambara groundnut rhizosphere soil.	182
Appendix 4.6: Analysis of variance (ANOVA) for manganese (mg kg^{-1}) on Bambara groundnut rhizosphere soil.	183
Appendix 4.7: Analysis of variance (ANOVA) for pH on Bambara groundnut rhizosphere soil.	183
Appendix 4.8: Analysis of variance (ANOVA) for Zinc (mg kg^{-1}) on Bambara groundnut rhizosphere soil.	183
Appendix 4.9: Analysis of variance (ANOVA) for copper (mg kg^{-1}) on Bambara groundnut rhizosphere soil.	184
Appendix 4.10: Analysis of variance (ANOVA) for organic carbon (%) on Bambara groundnut rhizosphere soil.	184
Appendix 4.11: Analysis of variance for organic matter (%) on Bambara groundnut rhizosphere soil.....	184

Appendix 4.12: Analysis of variance (ANOVA) for nitrogen (mg kg^{-1}) on Bambara groundnut rhizosphere soil.	185
Appendix 4.13: Analysis of variance (ANOVA) for clay (%) on Bambara groundnut rhizosphere soil.	185
Appendix 4.14: Analysis of variance (ANOVA) for soil density (g L^{-1}) on Bambara groundnut rhizosphere soil.	185
Appendix 4.15: Analysis of variance (ANOVA) for potassium ($\text{cmol}_c \text{ kg}^{-1}$) on Bambara groundnut rhizosphere soil.	186
Appendix 4.16: Analysis of variance (ANOVA) for calcium ($\text{cmol}_c \text{ kg}^{-1}$) on Bambara groundnut rhizosphere soil.	186
Appendix 4.17: Analysis of variance (ANOVA) for magnesium ($\text{cmol}_c \text{ kg}^{-1}$) on Bambara groundnut rhizosphere soil.	186
Appendix 4.18: Analysis of variance (ANOVA) for soil acid phosphatase enzyme activity $\text{nmol h}^{-1} \text{ g}^{-1}$ of Bambara groundnut rhizosphere soil.	187
Appendix 4.19: Analysis of variance (ANOVA) for soil alkaline phosphatase enzyme activity in $\text{nmol h}^{-1} \text{ g}^{-1}$ of Bambara groundnut rhizosphere soil.	187
Appendix 4.20: Analysis of variance (ANOVA) for soil β -glucosaminidase enzyme activity in $\text{nmol h}^{-1} \text{ g}^{-1}$ of Bambara groundnut rhizosphere soil.	187
Appendix 4.21: Analysis of variance (ANOVA) for β -glucosidase soil enzyme activity in $\text{nmol h}^{-1} \text{ g}^{-1}$ of Bambara groundnut rhizosphere soil.	188

LIST OF ABBREVIATIONS

ABA- Absciscic Acid

ANOVA- Analysis of Variance

ATP- Adenosine Triphosphate

BG- Bambara groundnut

BNF- Biological Nitrogen Fixation

Ca- Calcium

Ca²⁺-Calcium ion

CO₂- Carbon dioxide

DAFF- Department of Agriculture, Forestry and Fisheries

DARDLA- Department of Agriculture, Rural Development and Land Administration

DNA- Deoxyribose Nucleic Acid

ePGPR-Extracellular Plant Growth Promoting Rhizobacteria

GE- Gross Energy

HCN- Hydrocyanic Acid

IAA- Indole Acetic Acid

iPGPR- Intracellular Plant Growth Promoting Rhizobacteria

ISFM- Integrated Soil Fertility Management

ISO- International Standard Organization

K⁺- Potassium ion

K- Potassium

LSD- Least Significant Difference

Mg²⁺-Magnesium ion

N- Nitrogen

NA- Nutrient Agar

Na⁺ - Sodium ion

NH₃- Ammonia

OC- Organic Carbon

OM- Organic Matter

P- Phosphorus

PGPR- Plant Growth Promoting Rhizobacteria

RCBD- Randomised Complete Block Design

SSA- Sub-Saharan Africa

Zn- Zinc

GENERATED PUBLICATIONS FROM THIS DISSERTATION

1. Oral Conference presentations

- i. **Ubisi, R.**, Maseko, G., Mkhwanazi T P., Timana, M., Mnyambo, N., Kgotse, L., Sebati, M., Kola, E. and Dube, ZP. 2022. Symbiosis and ecological adaptation of the commercial nitrogen fixing bacteria on underutilised Bambara groundnut crop. One Health Student International Conference 2022.

2. Published journal articles.

- i. **Ubisi, R.**, Maseko, G., Mkhwanazi T. P., Timana, M., Mnyambo, N., Kgotse, L., Sebati, M., Kola, E. and Dube, Z. P. 2023. Symbiosis and ecological adaptation of the commercial nitrogen fixing bacteria on underutilized Bambara groundnut crop. *Horticulture*, LXVII(2): 2285-5653.

ABSTRACT

Bambara groundnut (*Vigna subterranea* L. Verdc) is a pulse crop cultivated mostly by smallholder farmers in Africa and ranked the third most important legume crop after groundnut (*Arachis hypogaea* L.) and cowpea (*Vigna unguiculata* (L.) Walp.). Grain legumes such as Bambara groundnut form nitrogen-fixing association with bacteria in the roots collectively called rhizobia in a process that supplies sufficient nitrogen (N) for legumes and other crops under crop rotation and intercropping systems. Sustainable agriculture depends greatly on these mutualistic relations, especially a balanced interaction between a diversity of rhizosphere microorganisms, plants, and soil physical and chemical properties. Rhizosphere microorganisms perform a variety of functional processes that enhance the soil and promote plant growth, while the plant reciprocates this by providing the microbial communities with conducive endo and exo-microenvironment. The diversity and functional roles of rhizobia associated with Bambara groundnut rhizosphere have not been extensively studied. Hence, the aim of the current study was to (i) determine the incidence and diversity of the rhizobacterial associated with communally produced Bambara groundnut in Mpumalanga, KwaZulu Natal, and Limpopo provinces, South Africa, (ii) identify nutrient-cycling activities of rhizobacteria, and (iii) assess nutrient status, and enzyme activities of Bambara groundnut rhizosphere soil. Roots and soil samples from Bambara groundnut were collected from communal fields in Mpumalanga and Limpopo provinces. In KwaZulu-Natal province, soil samples were gathered from Bambara groundnut farmers' fields before planting the crop. Bambara groundnut root nodules were sterilized, crushed, and streaked on Nutrient Agar (NA) media to isolate bacteria, which were identified using morphological traits and 16S rRNA gene sequencing. The gene sequences were confirmed via BLASTn at NCBI. Nitrogen and phosphorus cycling activities of the bacterial isolates were tested, and the diversity of rhizobia in the samples was assessed using Shannon-Wiener, Simpson, and Pielou's indices. Morphological characterization of the

isolates resulted in the identification of 209 isolates, with 43 different isolates identified in all three provinces. About 89 % (186) of isolates from the root nodules tested positive for nitrogen cycling and 11 % (23) tested negative, while all isolates obtained from nodules in all three provinces tested negative for phosphate solubilization. The 16S rRNA gene sequences of the selected 153 isolates revealed a distinct evolutionary lineage mostly related to the genera *Enterobacter*, *Leucobacter*, *Bacillus*, *Sphingobacterium*, *Lysinibacillus*, *Stenotrophomonas*, *Cellulosimicrobium*, *Kaistella*, *Neorhizobium*, *Proteus*, *Micrococcus*, and *Mammalicoccus*. with a significant *E*-value (≤ 0.000). In this study, 22 bacterial species (14.38%) were closely associated with *Enterobacter absuriae*, 19 (12.42%) with *Leucobacter chromiirens*, 18 (11.76%) with *Bacillus pumilus*, 14 (9.15%) with *Sphingobacterium faecium*, 13 (8.50%) each with *Lysinibacillus sphaericus* and *Stenotrophomonas lactitubi*, and 11 (7.19%) with *Stenotrophomonas pavanii*. Other species were associated with varying percentages, with the least common being *Lysinibacillus pakistanensis*, *Mammalicoccus sciuri*, and *Sphingobacterium multivorum* at 0.65% each. The N-cycling tests indicated that 186 (89 %) of isolates from the root nodules could fix nitrogen and 23 (11 %) could not, whereas all isolates from root nodules could not solubilize phosphate. Mpumalanga province had the highest number of bacterial isolates (107) from root nodules, followed by KwaZulu-Natal province (87) and last was Limpopo province (15). In Mpumalanga province, the University of Mpumalanga had the highest bacterial isolates at 40 and the highest species richness of 21, while Casteel and Mkhuhlu had 5 isolates each and lowest in species abundance. Hazyview had the least species richness (3). In KwaZulu-Natal province, the University of Zululand had higher number of isolates, 64, and 23 species richness when compared to Nhlanguyuke that had 23 isolates and 14 richness score. In Limpopo province, Gabaza village had 15 isolates and 10 richness score. In Mpumalanga province, the University of Mpumalanga had the highest genetic diversity index (H') score of 2.81, followed by Hlamalani at 1.79, Nkomazi at 1.77,

Boschfontein and Bushbuckridge at 1.63, and Hazyview had the lowest score of 1.01. The trend was different for Simpson index (D) scores, with Hazyview having the highest score index of 0.27, while the lowest diversity score of 0.05 was recorded at the University of Mpumalanga. When rhizobia evenness (J) was studied, Casteel had a score closest to even level at 0.83, while Hazyview and Boschfontein both had scores of 0.56. In KwaZulu-Natal province genetic diversity (H') of the microbial population was higher at the University of Zululand at 2.85 and Nhlanguyuke had lower H' index of 0.5. Simpson index also indicated higher diversity (D) score of 0.94 at the University of Zululand and lower at Nhlanguyuke with a diversity (D) score of 0.04. The physicochemical properties of soil samples collected from Bambara groundnut fields in three provinces were assessed at the KwaZulu-Natal Department of Agriculture and Rural Development's Analytical Services Unit, Cedara, South Africa. Phosphorus (P), total nitrogen (N), potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), zinc (Zn), manganese (Mn), copper (Cu), pH, organic carbon (OC), organic matter (OM), clay, exchangeable acidity, and acid saturation in the soils were determined using previously described methods. Phosphorus and nitrogen enzyme cycling activities (acid phosphatase, β -glucosidase, β -glycosaminidase, and alkaline phosphatase) were determined according to the method adapted from Jackson, Tyler, and Millar (2013) and conveyed in $nmolh^{-1} g^{-1}$ using 5 g of each soil sample, while nitrate reductase activities method was adapted from Bruckner, Wright, Kampichler, Bauer and Kandeler (1995). Collected data were subjected to analysis of variance (ANOVA) using Statistix 10 software. Mean separation was achieved using Fisher's Least Significant Differences (LSD) at 5 % probability. All physico-chemical properties such as pH, total nitrogen, organic clay content, magnesium, manganese, soil density, exchangeable bases, effective cation exchange capacity (ECEC), zinc, and copper were statistically ($P \leq 0.05$) different among localities, except for phosphorus, exchangeable acidity, acid saturation and the soil enzyme activities (acid phosphatase, β -glucosaminidase, and alkaline phosphatase). The

pH of the soil in all sample sites was acidic, ranging from 4.5 to 5.8 with Hazyview, Bushbuckridge, and Nkomazi being slightly acidic, and University of Zululand and Mpumalanga pH were rated as strongly acidic. Nhlangenyuke had the highest percentage of soil organic carbon and organic matter of 1.86 % and 3.20 %, respectively. Nhlangenyuke soil had a high K^+ value of $0.45 \text{ cmol}_c \text{ kg}^{-1}$ when compared to the other sample sites. Hazyview had the highest clay content, Ca^{2+} , and effective cation exchangeable capacity (ECEC) of 38.00 %, $4.98 \text{ cmol}_c \text{ kg}^{-1}$, and $6901.9 \text{ cmol}_c \text{ kg}^{-1}$, respectively. Copper (Cu), magnesium (Mg^{2+}), and manganese (Mn) of 9.39 mg kg^{-1} , 2.11 mg kg^{-1} , and 49.55 mg kg^{-1} , respectively, were high in soils collected from Gabaza village whereas Boschfontein had the lowest Cu, Mg^{2+} , Ca^{2+} , K^+ , ECEC, and clay content of 0.30 mg kg^{-1} , 0.23 mg kg^{-1} , $0.99 \text{ cmol}_c \text{ kg}^{-1}$, $0.10 \text{ cmol}_c \text{ kg}^{-1}$, $1344 \text{ cmol}_c \text{ kg}^{-1}$, and 7.50 %, respectively. The University of Zululand had the lowest Mn at 6.01 mg kg^{-1} . Soil collected from Casteel had a high Zn level of 28.47 mg kg^{-1} and the lowest Zn of 0.22 mg kg^{-1} was found in Hlamalani. Nhlangenyuke soil had the highest N of 1.10 mg kg^{-1} whereas the lowest N of 0.43 mg kg^{-1} was in Hlamalani. Soil enzyme activities; N-cycling and P-cycling, which involves acid phosphatase, β -glucosidase, β -glycosaminidase, and alkaline phosphatase were not different among localities. Hlamalani soil had the highest nitrate reductase of $19710 \text{ nmol h}^{-1} \text{ g}^{-1}$ and Bushbuckridge was the lowest nitrate reductase of $6243.33 \text{ nmol h}^{-1} \text{ g}^{-1}$. In conclusion, rhizobia isolates identified in root nodules varied amongst the locations with Mpumalanga province having the highest number of isolates followed by KwaZulu-Natal province and the least was Limpopo Province. In all three provinces nodules had both nitrogen cycling bacteria and non-cycling bacteria with no isolates able to solubilize phosphate. The physico-chemical soil properties varied with the location. Hlamalani had the highest nitrate reductase and Bushbuckridge the lowest. The current study indicated that there is a huge diversity of rhizobacterial organisms associated with Bambara groundnuts with potential for commercialisation after testing their efficiency in nutrient cycling.

Keywords: Bambara groundnut, biological nitrogen fixation, Enzyme activities, Plant growth promoting rhizobacteria (PGPR), Rhizosphere, Soil properties, Symbiosis

CHAPTER ONE

INTRODUCTION

1.1 Background

Globally, food and nutritional security are among the greatest challenges that need to be overcome, especially in the 21st century (Temegne *et al.*, 2020; Cook, 2017). In South Africa, 20 % of households have inadequate access to food and nutrition daily (Cook, 2017). According to Smyth (2020), ‘food security happens when all individuals at all times have access to safe, sufficient, and nutritious food to meet their dietary needs for a healthy and active lifestyle’. Food insecurity may be caused by low yield due to soil nutrient deficiency in agricultural farming systems, especially in communal areas (Unigwe, Gerrano, Adebola & Pillay, 2016). The rapid reduction of soil nutrients most importantly nitrogen (N) and phosphorus (P) in agricultural land is caused by human activities that include overcultivation, uncontrolled burning, and overgrazing (Blair, Nippert & Briggs, 2014). To increase yield, most farmers rely heavily on N fertilizers, however, most resource-poor smallholder farmers in Sub-Saharan Africa cannot afford these chemical fertilizers (Oruru, Njeru, Pasquet & Runo, 2018). In addition to being expensive, fertilizers also contribute extensively to environmental pollution (Adesemoye & Kloepper, 2009).

Legumes can be included in agricultural farming systems as they promote the conversion of atmospheric N to the reduced form of ammonia, which is easily accessible to plants, through biological nitrogen fixation (BFN) (Jaiswal & Dakora 2019; Oruru *et al.*, 2018). *Vigna subterranea* (L.) Verdc, commonly known as Bambara groundnut, is an indigenous African legume crop that can be incorporated into cropping systems due to its ability to fix atmospheric N. It is mainly grown by subsistence farmers in sub-Saharan Africa (Cook, 2017; Unigwe *et al.*, 2016). Moreover, it is highly rich in carbohydrates (55.6 %), protein (21.2 %), fats (7 %),

and fiber (6.3 %) making it a comprehensive meal (Cook, 2017). Bambara groundnut grows well in acidic soil, and it is highly tolerant to severe drought conditions (Cook, 2017). Moreover, can form a symbiotic and non-symbiotic relationship with phosphate solubilizing, nitrogen-fixing, and Nitrogen cycling bacteria (Oruru & Njeru 2016). The symbiotic association involves the interaction between soil microbes and plants such as Rhizobia bacteria and leguminous plants and Frankia with non-leguminous plants (Zoundji, Houngnandan, Boko & Toukourou, 2020). In a symbiotic association, these bacteria infect the roots and stems of leguminous plants, causing lumps and nodules (Mir, Nagabhushanam, Quadriya, Kumar & Hameeda, 2020). In addition, microorganisms found in the soil also produce extracellular enzymes that transform and hydrolyze polymeric compounds into readily available nutrient assimilation of microbes and plants (Lucas *et al.*, 2008). The extracellular enzyme plays a role in mineral regulation and the cycling of nutrients such as carbon, phosphate, and nitrogen. Enzymes such as asparaginase and β -glycosaminidase hydrolyze chitooligosaccharides and convert asparagine into ammonia and aspartic acid (Nanda, Andrio, Marino, Pauly & Dunand, 2010). This influences nitrogen bioavailability, increasing assimilation of nitrogen by plants (Henriksson, Sild, Szabó, Pettersson & Johansson, 1988). For instance, *Bacillus* has been isolated from root nodules and shown to solubilize phosphate and synthesize hydrolytic enzymes, polyamines, and lipopeptides (Maymon *et al.*, 2015). For example, *Bacillus* isolated from root nodules shown to synthesize hydrolytic enzymes, lipopeptides, polyamines, and solubilize phosphate (Maymon *et al.*, 2015).

1.2. Problem statement

Bambara groundnut, like other legume crops, develops some root nodules, which host phosphate solubilization, nitrogen-fixing, and nitrogen-cycling bacteria that enhance the growth of plants and improve soil fertility (Sharma, Kaur & Sharma, 2020). In South Africa,

Bambara groundnut is mainly cultivated in KwaZulu-Natal, Mpumalanga, and Limpopo provinces (DAFF, 2016). Moreover, BG thrives in nutrient deficient soils because of its symbiosis with nitrogen fixation, phosphate solubilization, and nutrient-cycling bacteria (Sharma *et al.*, 2020). However, less is known about the rhizosphere microbes that form a symbiont with nutrient cycling functions in legumes such as Bambara groundnut in the natural soil of Mpumalanga, KwaZulu-Natal, and Limpopo province, and their contribution to soil fertility is not well understood. Hence, Therefore, there is a need to characterize and identify soil microbes that are mainly associated with Bambara groundnut and the role they significantly play in the enhancement of nutrient acquisition. There is a clear enticement to exploit the diversity of microbes and to isolate, at the same time developing functional microbes that can be used, in effect, as targeted fertilizers as an alternative to traditional fertilizer applications (Zilli *et al.*, 2019). Furthermore, no studies have assayed enzyme activities in Bambara groundnut rhizosphere soil in the three different provinces.

1.3. Justification

It has been found that production of high yield tends to decline with poor soil fertility, especially N resulting in poor crop production (Jaiswal *et al.*, 2019; Valentine, Kleinert & Benedito, 2017). Studies have shown that the productivity of soils in South Africa is restricted by insufficiency of nutrients such as phosphorous (P), nitrogen (N), and potassium (K), the problem compounded by low organic matter (Mulinganya, 2016). Small-scale farmers have increased land use while using minimal nutrients, resulting in a decrease in crop yields and an increase in nutrient removal (Nyaauthii, 2017; Mulinganya, 2016). Biological nitrogen fixation by legume plants might enhance fertility of soil sustainably and more cheaply. The use of synthetic fertilizer to increase soil fertility is very costly for small-scale farmers and harms the environment (Ali, Rahman, Khatun, Yasmin & Rashid, 2019; Li *et al.*, 2017). The efficient use

of biological nitrogen fixation is hindered by a shortage of P in the soil which characterizes most subtropical and tropical ecosystems (Valentine, Benedito & Kang, 2018). Since there are high deficiencies of N, P, and K in soil due to nutrient mining in agricultural production land, there is a need to supplement these nutrient elements by using biological fertilizers to optimize crop production because they are less expensive and environmentally friendly (Ramakrishnan, 2015). Moreover, the use of biofertilizers is a practice that supports the three pillars of sustainable agriculture: social, environmental, and economic. Characterizing naturally occurring rhizobia associated with Bambara groundnuts in the root and rhizosphere soil of Bambara groundnut, analyzing the fertility status of different soil, and assessing enzymatic activities will therefore aid in guiding the greatest strategy to boost agricultural production.

1.4. Purpose of the study

1.4.1 Aim and Objective

This study seeks to characterize naturally occurring rhizobia associated with Bambara groundnut root and rhizosphere soil, analyze physicochemical properties of the rhizosphere as a potential influence of rhizobia diversity, and quantify enzymatic activity in N-cycling and P-cycling to improve Bambara groundnut yields in the communal farming communities of Mpumalanga, Kwazulu-Natal, and Limpopo province. With high deficiencies of N, P, and K in soil due to nutrient mining in agricultural production land, it is necessary to supplement these nutrient elements by using biological fertilizers to optimize crop production. The study will provide information on the most efficient nitrogen fixing bacteria associated with Bambara groundnut (Ramakrishnan, 2015).

For this purpose, the following objectives are devised:

- i. To determine the incidence and diversity of rhizobacteria associated with communally produced Bambara groundnut roots in Mpumalanga, KwaZulu-Natal, and Limpopo Province and their N-cycling and P-cycling ability.
- ii. To assay soil nutrient status and enzyme activities of Bambara groundnut rhizosphere soil in Mpumalanga, KwaZulu-Natal, and Limpopo Province.

1.4.2. Hypotheses

- i. The incidence, diversity, N-cycling, and P-cycling ability of root nodule inhibiting bacteria associated with communally produced Bambara groundnut will differ in Mpumalanga, KwaZulu-Natal, and Limpopo province.
- ii. Soil physico-chemical properties and enzyme activities on Bambara groundnut rhizosphere will differ in Mpumalanga, KwaZulu-Natal, and Limpopo province.

1.5. Reliability, validity, and objectivity

Reliability refers to a variable measured repeatedly without any changes and the instrument used to measure produces consistent results (Leedy & Ormrod, 2005). Statistical analysis provides different reliability levels. Various reliability checks on the data are provided by statistical analysis (Berenson & Levine, 1996). Reliability was guaranteed in this study's numerous experiments by assessing the variance explained by models as measured by coefficients of determination and through the application of proper statistical significance levels for mean separation. According to Leedy and Ormrod (2005), the degree to which a measuring device measures what it was intended to measure is known as validity. The validity of a research investigation pertains to the accuracy with which the outcomes among the study subjects reflect genuine findings among comparable individuals beyond the study's scope. To guarantee credibility, this study adopted methodologies established by other researchers, samples were correctly randomized and replicated accordingly. Little and Hills (1981), further

added that validity could be ensured by experimenting with the same location over time. Objectivity is described as striving, as far as possible or practicable, to reduce or eliminate biases, prejudices, or subjective evaluations by relying on verifiable data (Leedy & Ormrod, 2005). The objectivity of the study was achieved by discussion of the results based on empirical evidence displayed by statistical analysis, with the comparison and contrasting of results with other results obtained from other studies (Little & Hills, 1981).

1.6. Bias

Bias is described as any influence, conditions or set of conditions that singly or altogether distort the data (Leedy & Ormrod, 2005). In this study, bias was minimised by ensuring that the experimental error in each experiment was reduced through increased replications and randomization (Little & Hills, 1981).

1.7. Significance of the study

The screening of rhizobia strains that are adapted to local conditions and searching for greatly effective strains to be used as inoculants, represents an encouraging strategy in overcoming inoculation failure (Chibeba, Kyei-Boahen, de Fátima Guimarães, Nogueira & Hungria, 2017). The efficient rhizobia strains can be used as bio-fertilizers which bear higher efficiency over the chemical nitrogenous fertilizers and contribute to sustainable agriculture, at the same time decreasing the use of synthetic fertilizers which are harmful to the environment (Chibeba *et al.*, 2017). This research will contribute towards the development of cost-effective, easy to use method of improving soil fertility for communal farmers (Temegne *et al.*, 2020). Furthermore, the study contributes to the scientific knowledge of Integrated Soil Fertility Management (ISFM) technology with high potential to improve nitrogen fixing abilities of Bambara groundnuts, increasing soil fertility and enhancing crop yields (Cook, 2017; Mulinganya,

2016). This will result in improved nutrition, food security, and income for the farmers (Mulinganya, 2016). Overall, the findings of the study will be very useful in the development of sustainable practices to increase food security and the management of N flow in farming systems in Mpumalanga, Limpopo, and KwaZulu-Natal provinces and South Africa as a whole.

1.8. General overview of chapters in the dissertation

This dissertation contains six chapters. The summaries in each chapter are mainly offered to allow the readers to understand and follow discussions on issues and research findings were applicable. Chapter one outlines the background of the study, narrating on the challenges of soil nutrient in agriculture and the importance of pulse crop in improving soil nutrient through biological nitrogen fixation. The chapter goes on to describe the adaptation that is exhibited by legume crop to ensure efficient acquisition and utilization of soil nutrients. The research problem, rationale of the study, hypothesis, aim and objective of the study are also highlighted in this chapter. Chapter two, discusses literature review relative to food security, the growth requirement, production, nutritional content, and uses of Bambara groundnut. Furthermore, covering literature relating to biological nitrogen fixation, their significance and restriction factors. This chapter also offered a transitory overview on the importance of rhizobia in cropping systems and plant growth promoting rhizobia (PGPR) in agriculture. A detailed description given on the effect of P stress on biological nitrogen fixation and an overview of the different mechanisms used by pulse crop to improve P acquisition are also discussed. Chapter three, presents and discusses findings on the incidence and diversity of rhizobacteria that are associated with communal produced Bambara groundnut in Mpumalanga, Limpopo, and Kwa-Zulu Natal provinces. Chapter four, reports on soil nutrient status in the different studied provinces and the most restricting macro-nutrients, N, P and K of Bambara groundnut producing areas is discussed. Also, the chapter describes and discusses findings on the

extracellular enzyme activities on carbon cycling, phosphorus solubilisation and nitrogen fixation of the rhizosphere symbiotic PGPR in the three different provinces. Chapter five, gives the summary of the findings of the whole study, significance of the findings, future potential of Bambara groundnut production, general conclusions, and recommendation.

1.9. References

- Adesemoye, A. O., and Kloepper, J. W. 2009. Plant–microbes interactions in enhanced fertilizer-use efficiency. *Applied Microbiology and Biotechnology*, 85: 1-12.
- Ali, S., Rahman, M., Khatun, S., Yasmin, S. and Rashid, H. 2019. Isolation, characterization, and symbiotic performance evaluation of soybean (*Glycine max*) nodulating rhizobia from different districts of Bangladesh. *Journal of Bioscience and Biotechnology Discovery*, 4(1): 10-20.
- Berenson, M. L. and Levine, D. M. 1996. *Basic business statistics: Concepts and applications*. 12th ed. New Jersey: Prentice-Hall.
- Blair, J., Nippert, J., Briggs, J. 2014. Grassland ecology. In: Monson, R. ed. *Ecology and the Environment*. New York: Springer.
- Chibeba, A. M., Kyei-Boahen, S., de Fátima Guimarães, M., Nogueira, M. A. and Hungria, M. 2017. Isolation, characterization, and selection of indigenous *Bradyrhizobium* strain with outstanding symbiotic performance to increase soybean yields in Mozambique. *Journal of Agriculture, Ecosystems, and Environment*, 246: 291-305.
- Cook, D. 2017. Small scale farmer's utilization and perceptions of Bambara groundnut production in South Africa: A case study in a semi-arid region of Limpopo. Master of Philosophy Dissertation, University of Cape Town.
- Department of Agriculture, Forestry, and Fisheries (DAFF), 2016. Production Guideline for Bambara groundnuts (*Vigna subterranea*): National Department of Agriculture and

- Agricultural Research Council Grain Crops Institute. Pretoria. [Online]. Available at: [https://www.google.com/search?q=DAFF%2C+2016,+Production+Guideline+for+Bambara+groundnuts+\(Vigna+subterranea\)%3A+National+Department+of+Agriculture+and+Agricultural+Research+Council+Grain+Crops+Institute.+Pretoria%2C+South+Africa](https://www.google.com/search?q=DAFF%2C+2016,+Production+Guideline+for+Bambara+groundnuts+(Vigna+subterranea)%3A+National+Department+of+Agriculture+and+Agricultural+Research+Council+Grain+Crops+Institute.+Pretoria%2C+South+Africa). [Accessed 24 March 2022].
- Henriksson, G., Sild, V., Szabó, I. G., Pettersson, G. and Johansson, G. 1998. Substrate specificity of cellobiose dehydrogenase from *Phanerochaete chrysosporium*. *Biochim. Biophys. Acta Protein Structure and Molecular Enzymology*, 1383: 48-54.
- Jaiswal, S. K. and Dakora, F. D. 2019. Widespread distribution of highly adapted *Bradyrhizobium* species nodulating diverse legumes in Africa. *Frontiers in Microbiology*, 10: 310.
- Leedy, P. D. and Ormrod, J. E. 2005. *Practical research: Planning and design*. 11th ed. New Jersey: Pearson Education.
- Little, T.M. and Hills, F. J. 1981. *Statistical Methods in Agricultural Research*. University of California, Davis, California.
- Li, H., Singh, R., Singh, P., Song, Q., Xing, Y., Yang, L. and Li, Y. 2017. Genetic diversity of nitrogen-fixing and plant growth promoting *Pseudomonas* species isolated from sugarcane rhizosphere. *Frontiers in Microbiology*, 8: 1-20.
- Lucas, N., Benaïme, C., Belloy, C., Queneudec, M., Silvestre, F. and Nava-Saucedo, J. E. 2008. Polymer biodegradation: Mechanisms and estimation techniques-A review. *Chemosphere*, 73: 429-442.
- Maymon, M., Martínez-Hidalgo, P., Tran, S. S., Ice, T., Craemer, K., Anbarchian, T., Sung, T., Hwang, L. H., Chou, M., Fujishige, N. A. and Villella, W. 2015. Mining the Phyto microbiome to understand how bacterial co-inoculations enhance plant growth. *Frontiers in Plant Science*, 6: 784.

- Mir, M. I., Nagabhushanam, B., Quadriya, H., Kumar B. K. and Hameeda, B. 2020. Morphological, biochemical, and intrinsic antibiotic resistance of rhizobia isolated from root and stem nodules of various leguminous plants. *Cell Biotechnology and Molecular Biology*, 21: 126-138.
- Mulinganya, N. 2016. Rhizobia inoculants combined with organic and inorganic fertilizer amendments effects on nitrogen fixation and yields of beans in South Kivu, Democratic Republic of Congo. Master's degree of science, Kenyatta University.
- Nanda, A. K., Andrio, E., Marino, D., Pauly, N. and Dunand, C. 2010. Reactive oxygen species during plant-microorganism early interactions. *Journal of Integrative Plant Biology*, 52(2): 195-204.
- Nyaaauthii, M. C. 2017. Soybean (*Glycine max*) response to rhizobia inoculation as influenced by soil nitrogen levels. Master of Philosophy, Kenyatta University.
- Oruru, M. B. and Njeru, E. M. 2016. Upscaling arbuscular mycorrhizal symbiosis and related agroecosystems services in Smallholder Farming Systems. *BioMed Research International*, 2016: 4376240.
- Oruru, M. B., Njeru, E. M., Pasquet, R. and Runo, S. 2018. Response of a wild-type and modern cowpea cultivars to arbuscular mycorrhizal inoculation in sterilized and non-sterilized soil. *Journal of Plant Nutrition*, 41: 90-101.
- Puozaa, D., Jaiswal, S. and Dakora, F. 2017. African origin of *Bradyrhizobium* sp population's nodulating Bambara groundnut (*Vigna subterranea* L. Verdc) in Ghanaian and South African soils. *PLOS ONE*, 12(9): 27-59.
- Ramakrishnan, V. V. G. A. 2015. Nitrogen sources and cycling in the ecosystem and its role in air, water, and soil pollution: a critical review. *Journal of Pollution Effects and Control*, 3: 1-28.

- Sharma, V., Kaur, J. and Sharma, S. 2020. Plant growth promoting rhizobacteria: potential for sustainable agriculture. *Vegetal Biotechnology*, 20 (3): 157-166.
- Smyth, S. J. 2020. Regulatory barriers to improving global food security. *Global Food Security*, 26: 100440.
- Temegne, N., Dooh, J., Nbandah, P., Ntsomboh-Ntsefong, G., Taffouo, V. and Youmbi, E. 2020. Cultivation and utilization of Bambara groundnut (*Vigna subterranea* (L.) Verdc.), a neglected plant in Cameroon. *Asian Plant Research Journal*, 4(2): 9-21.
- Unigwe, A., Gerrano, A., Adebola, P. and Pillay, M. 2016. Morphological variation in selected accessions of Bambara groundnut (*Vigna subterranea* L. Verdc) in South Africa. *Journal of Agricultural Science*, 8: 69-80.
- Valentine, A. J., Benedito, V. A. and Kang, Y. 2018. Legume nitrogen fixation and soil abiotic stress: from physiology to genomics and beyond. *Annual Plant Reviews*, 42: 207-248.
- Valentine, A. J., Kleinert, A. and Benedito, V. A. 2017. Adaptive strategies for nitrogen metabolism in phosphate deficient legume nodules. *Plant Science*, 256: 46-52.
- Zilli, J., Alves, B., Rouws, L., Simões-Araujo, J., de Barros Soares, L., Cassán, F., Castellanos, M. and O'Hara, G. 2019. The importance of denitrification performed by nitrogen-fixing bacteria used as inoculants in South America. *Plant and Soil Science Journal*, 451(1-2): 5-24.
- Zoundji, M., Houngnandan, P., Boko, F. and Toukourou, F. 2020. Characterization of indigenous rhizobia strains associated to Soybean [*Glycine max* (L.) Merrill] in Benin. *International Journal of Plant and Soil Science*, 32(2): 35-46.

CHAPTER TWO

LITERATURE REVIEW

2.1. Overview

In 2030, it is projected that there will be greater demand for agricultural products by over 60% (Majola, Gerrano & Shimelis, 2021; Laplaze, Sparvoli, Masmoudi & Hash, 2018; Ojiewo *et al.*, 2015). Soil under crop production, particularly in Sub-Saharan Africa, is nutrient lacking and mostly acidic, leading to low crop yields (Cook, 2017; Abd-Alla, El-Enany, Nafady, Khalaf & Morsy, 2014). Many tropical nations use inorganic manure to increase crop production, even though they have been found to pose potential health risks to both humans and the environment (Babalola *et al.*, 2017). Nevertheless, the high cost of synthetic fertilizers remains a serious challenge to resource-poor smallholder farmers (Oruru, Njeru, Pasquet & Runoet, 2018). Furthermore, continued use of this chemical fertilizer remains a threat to the environment and human health (Ajilogba, Olanrewaju & Babalola, 2021; Valentine, Benedito & Kang, 2018; Abd-Alla *et al.*, 2014). A sustainable approach to achieving food security includes the use of beneficial microbes and improving the breeding of underutilized legumes (Laplaze *et al.*, 2018). Using microbiome-assisted breeding in underutilized legumes offers great potential for improving food security (Hassen, Van Vuuren, Bopape & Gerrano, 2022). Legumes can form a symbiotic association with rhizobia bacterial found in the soil and as a result, atmospheric nitrogen will be fixed into forms that are assimilable by the legumes thereby promoting crop productivity and improving soil mineral nutrition (Hassen *et al.*, 2022; Laurette *et al.*, 2015). Plant growth-promoting rhizobacteria use several mechanisms for the enhancement of plant growth and development (Lugtenberg & Kamilove, 2009). Plant growth-promoting rhizobia are involved in various biological processes within the rhizosphere soil both direct and indirect mechanisms which include nutrient cycling, nitrogen fixation, and mineral transportation (Omara, Hauka, Afify; El-Din & Kassem, 2017) (Figure 2.1). Hence

this review will summarize the significance of Bambara groundnut known as a legume crop, plant growth promoting rhizobia association with legumes and non-legume crops, and the use of rhizobia as biofertilizers in agriculture production (Figure 2.1).

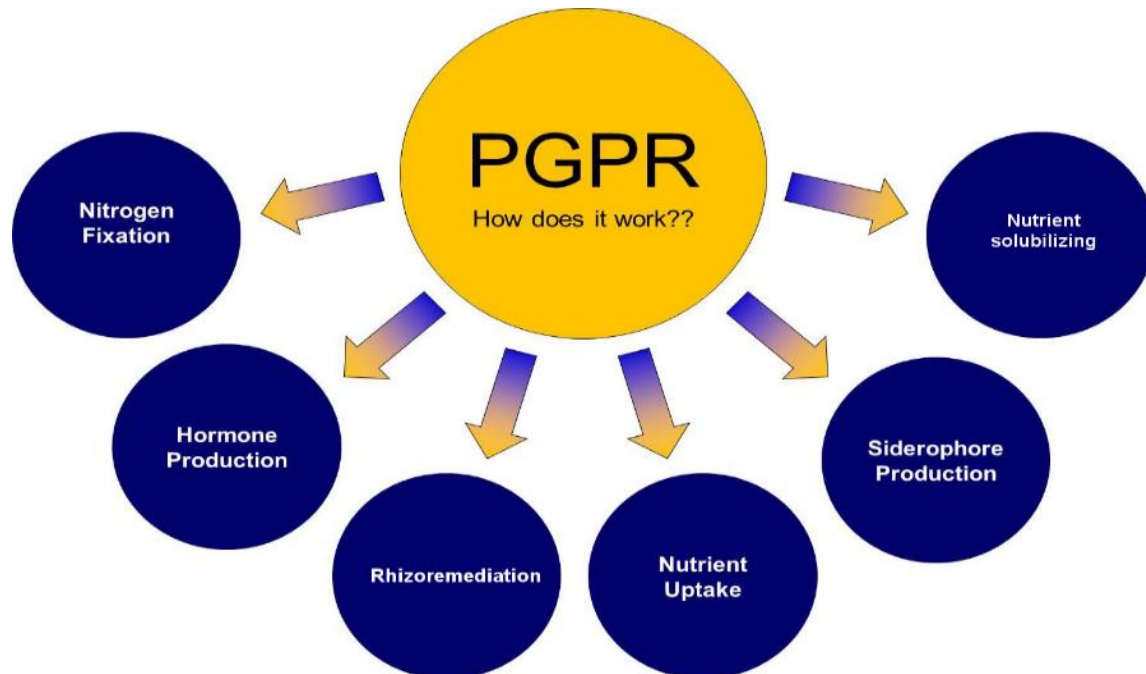


Figure 2.1: Role of plant growth promoting rhizobia (PGPR) on the growth and development of plants (PlantWorks, 2013).

2.2. Significance of Bambara groundnut

2.2.1. Nutritional advantages

The Bambara groundnut is a complete food source that contains proportions of protein, fats, fiber, and carbohydrates that are sufficient to qualify as a balanced diet (Tan *et al.*, 2020; Halimi, Barkla, Mayes & Kinget, 2019; Ijarotimi & Esho, 2009). Its leaves are mainly rich in phosphorus (P) and can be used as feed for livestock. Bambara groundnuts are a low-cost, protein-rich food source that enhances food and nutritional security in rural communities (Azman Halimi, Raymond, Barkla, Mayes & King, 2021). The protein composition is higher in essential amino acids, such as lysine, phenylalanine, valine, isoleucine, methionine, and threonine, with 80 % protein with superior quality compared to soybean (74 %), cowpea (64

%), and groundnut (65 %). (Adegboyega, Shoge & Tamasi, 2021; Mubaiwa, Fogliano, Chidewe, Bakker & Linnemann, 2018). Bambara groundnut seeds have a high Gross Energy (GE) (Ajilogba *et al.*, 2021). Its fat composition is estimated at 6.5 % while carbohydrates are at 65 %, respectively (Ibny, Jaiswal, Mohammed & Dakora, 2019; Mazahib, Nuha, Salawa & Babiker, 2013). Furthermore, it contains micronutrients such as calcium (Ca), zinc (Zn), potassium (K), and iron (Fe) (Cook, 2017). Mubaiwa *et al.* (2018) revealed that the red-seeded cultivars have practically twice as much iron just like the cream white-eye seeds.

2.2.2. Agronomical advantage

Bambara groundnut (BG) is a drought-tolerant legume crop that has significant importance in agricultural systems (Tan *et al.*, 2020; Babalola *et al.*, 2017). Chibarabada (2014) reported that BG is suitable for intercropping with other crops such as maize and cereal. This legume crop is mostly grown by farmers because of its ability to produce in poor soil conditions when compared to other crops such as peanuts, beans, and groundnuts (Olanrewaju, Oyatomi, Babalola & Abberton, 2022; Babalola *et al.*, 2017). Bambara groundnut roots form a symbiotic relationship with nodules of bacteria found on the roots, this symbiotic relationship assists in the fixation of atmospheric nitrogen making it accessible to plants (Babalola *et al.*, 2017).

2.2.3. Medical Significance

Bambara groundnut seeds and leaves have been shown to have some medicinal properties (Murevanhema & Jideani, 2013). The leaves of this legume crop can be applied in infected wounds and abscesses, the juice found from the leaves is mainly applied to the eyes to treat epilepsy (Khan, Bano, Ali & Babar, 2020). Whereas the pounded extracts from the leaves mixed with *Lantana trifolia* are mainly used as insecticides in livestock production (Mkandawire, 2007). Temerge (2018) also stated that grounded seeds of BG can be mixed with

water and administered in treating cataracts mostly in Senegal. In Ghana, crushed seeds of BG have been used to treat a rash on the skin, and a mixture of the powder with some meat of guinea fowl is used as the treatment of diarrhoea in children (Akpalu *et al.*, 2013). In Botswana, the black-seeded cultivar has been used to treat impotence, while the BG with boiled water from maize when drunk is mainly used to treat diarrhoea in Kenya by the Luo tribe (Udeh, Nyila & Kanu, 2020). In South Africa chewing and swallowing of the seeds have been used to control nausea (Khan *et al.*, 2020). Moreover, the seeds of Bambara groundnut contain kaempferol, which is an antioxidant polyphenol that is used to reduce the risk of various chronic diseases including cancer (Temerge *et al.*, 2020). Bambara groundnut in Cameroon has been used in the fight against stomach pains, amoebic dysentery, joint pains, bone decalcification, headaches, and sore throat (Udeh *et al.*, 2020). In Nigeria, the Igbo tribe uses the plant to treat venereal disease (Oluwole *et al.*, 2021). It also helps to stimulate the production of milk in a woman who is breastfeeding and is also given to women who have just given birth to help in the proper healing of the wounds (Temegne, 2018).

2.3. Bambara groundnut production in South Africa

In South Africa, Bambara groundnut is mostly cultivated in KwaZulu-Natal, Mpumalanga, and Limpopo provinces (Minnaar-Ontong, Gerrano & Labuschagne, 2021; DAFF, 2016). It was migrated by the indigenous people from West Africa to South Africa (Nkambule, 2020). However, it is cultivated by smallholder farmers in areas that range between 302- 2500 m² per farmer, with a production yield of approximately 300 kg ha⁻¹ (Nkambule, 2020). Bambara groundnut production figures in South Africa are not reliable and the legume crop is not grown commercially (DAFF, 2016). Common names of Bambara groundnut in South Africa include Ditloo- marapo (Sepedi), Tindluwa (Xitsonga), Jugoboos (Afrikaans), and Phonda (Venda) (DAFF, 2016; Mohammed, 2014). Bambara groundnut is considered the third most significant

legume crop after cowpea and groundnut (Minnaar-Ontong *et al.*, 2021; Cook, 2017). Over the years BG was mostly produced for subsistence use in South Africa, and only later that South Africans started selling the crop (Cook, 2017; DAFF 2016). Cook (2017) argued that the primary reason BG has been less prioritized by farmers compared to other legumes such as cowpea and groundnut is that there is limited research done on the crop, hence there are no enhanced varieties resulting in little attention on the crop. The estimated market size ranges between 1,500 to 4000 tons, with a substantial proportion of the supply met by imports from neighbouring countries such as Zimbabwe (Mubaiwa *et al.*, 2018). Bambara groundnut accessions in South Africa are low yielding when compared to other Bambara groundnut accessions in other countries (Unigwe, Gerrano, Adebola & Pillay, 2016; Karunaratne, Walker & Ruane, 2015).

2.3.1. Production of Bambara groundnut in Mpumalanga province

In Mpumalanga province, Bambara groundnut is the third most significantly cultivated crop in the Lowveld region after groundnut and maize (Cook, 2017), mainly grown by smallholder African farmers in the middle and low veld areas of the province, both as cash and food crops (DARDLA, 2012). Matthews (2013) reported that South Africa's Department of Agriculture and Land Administration identified local accessions in Mpumalanga for great yielding BG with yields of up to 2350- 2355 kg ha⁻¹ (local accessions MPB51 and MPB71). Also, carried out a baseline survey on Bambara groundnut production in the Mpumalanga province revealed that Bambara groundnut is mostly planted on land that is newly cleared (DARDLA, 2012). There is a tradition in the Mpumalanga region that prohibits planting Bambara groundnuts early before January, which reduces its growing season as well as yields (Matthews, 2013).

2.3.2. Production of Bambara groundnut in Kwazulu-Natal province

Bambara groundnut in KwaZulu-Natal is grown in Msinga, Greytown, Nkandla, Nguthuthu, Kosibaai, and Makhati areas. Also grown on a minor scale in Illembe and Umzinyathi districts. Controlled trials of Bambara groundnut have been conducted in Ukulinga Research Farm (KwaZulu-Natal) (DAFF, 2016). Most of BG production is mainly for food and income generation by smallholder farmers (Majola *et al.*, 2021).

2.3.3. Production of Bambara groundnut in Limpopo province

Bambara groundnut production in Limpopo province is relatively low and the major production regions are in the districts of Mopani, Capricorn, Vhembe, and Waterburg (DAFF, 2016). Just like in other provinces, the crop is produced by small-scale farmers, at a small scale. In Venda, the BG crop is planted after the maize crop, with the belief that Bambara groundnut fields probably function as protection of the small, intensively cultivated plots of maize (Cook, 2017; Matthew, 2013).

2.4. Production constraints of BG in South Africa

Bambara groundnut production is affected by numerous production factors. These factors include abiotic stresses (poor soil fertility, extreme temperature, and drought) and biotic stresses such as diseases which are caused by bacteria, viruses, nematodes, fungi, insect pests (Majola *et al.*, 2021).

2.4.1. Biotic stresses

The production of Bambara groundnut is affected by disease and field insect pests (Majola *et al.*, 2021).

Pathogenic organisms

Bambara groundnut crop is attacked by several fungal pathogens under both humid and dry conditions (Majola *et al.*, 2021). These fungal pathogens of more significance are *Cercospora* spp., *Colletotrichum capsici capsica*, and *Fusarium* spp. *Cercospora* leaf spot on Bambara groundnut is characterized by reddish to almost brown circular spots, as well as some lesions on the stem, pods, and petioles (Figure 2.2) (DAFF, 2016). *Colletotrichum capsici capsica* and *Fusarium* the cause of wilt, are the most common fungal pathogens on Bambara groundnuts. Other diseases with minor importance include the leaf-blotch caused by *Phomopsis* sp. and powdery mildew, these two diseases have been reported in Zimbabwe attacking mostly immature leaves of Bambara groundnut (Olanrewaju *et al.*, 2022; Majola *et al.*, 2021).

Viruses that have been reported on the crop include a necrotic-mosaic virus, cowpea mottle virus, two potyviruses, and white-clover mosaic virus, with recent reports indicating significant crop losses (Olanrewaju *et al.*, 2022).



Figure 2.2: Reddish spots on the leaves of Bambara groundnut caused by *Cercospora* sp. (Ouoba *et al.*, 2019).

Insect pests and nematodes

Insect pests generally are less damaging to the Bambara groundnut crop than other legume crops such as cowpea (DAFF, 2016). Storage pests such as the cowpea weevil (*Callosobruchus maculatus*) have been reported to cause severe damage to the grain stored poorly (Olanrewaju

et al., 2022; Kosini & Nukenine, 2017). Seeds stored with high moisture content seem to be the ones most affected by weevils, as the molds growing on them attract attack by weevils (Nyamador *et al.*, 2017). The root-knot nematodes (*Meloidogyne* spp.) documentation indicates that the pest is a major threat to BG in lighter soils, causing direct yield reduction and enabling *Fusarium* infection (DAFF, 2016).

2.4.2. Abiotic stress

Several environmental factors pose some challenges to crop yield, such include drought and temperature (Suzuki, Rivero, Shulaev, Blumwald & Mittler, 2014).

Drought stress

Research indicates that Bambara groundnut legumes are more drought tolerant than most other legume crops, but it has been reported to be sensitive to prolonged drought conditions, mostly during pod setting and grain filling phase (DAFF, 2016). Under moderate drought stress conditions, the crop can thrive and produce a yield (Olanrewaju *et al.*, 2022; DAFF, 2016). The inherent drought tolerance response of Bambara groundnut has been linked to its ability to decelerate the rate of water loss by reducing its leaf area and decreasing canopy size (Figure 3) (Cook, 2017). The crop can withstand heavy rain but excessive rainfall during harvest results in yield losses (Olanrewaju *et al.*, 2022).



Figure 2.3: Effect of prolonged drought on Bambara groundnut production (Fleibrer, 2006).

Temperature

Production of Bambara groundnut is mainly suitable in regions with temperatures ranging between 19 °C and 30 °C and is regarded as the fast-growing crop that requires warm temperature (DARDLA, 2012). The crop cannot tolerate cold temperatures during the growing season (DARDLA, 2012). DAFF (2016) suggested that extreme temperatures may cause leaves to wilt which will result in the decline of biomass yield.

2.4.3. Socio-economic challenges

Bambara groundnut in Sub-Saharan Africa (SSA) is mostly cultivated by female smallholder farmers and is regarded as a minor crop (Unigwe *et al.*, 2016). This legume crop has received little support in research on seed systems, agronomic management, and breeding of new varieties (Majola *et al.*, 2021). Meena *et al.* (2018) stated that the breeding project of BG is mandatory in developing farmers and market preferences and also the development of some superior BG cultivars for food security, excellent returns on investment, and enhanced livelihood feeds. Additionally, smallholder farmers have restricted access to finances for production expanding through the usage of seed varieties that are newly developed, inputs of the crop which includes fertilizers, irrigation systems, resources for crop protection, and post-harvest storing facilities (Majola *et al.*, 2021). Also, smallholder farmers must have the entry to regional markets for economic gains from BG production. Cook (2017) outlined that several people in the Limpopo province mainly are dependent on small-scale farming as a source of living, economic, and food security and are struggling to uphold food security and are resource-poor. Hence, they must be educated and receive support from researchers and the government to improve their farming systems (Cook, 2017).

2.5. Biological nitrogen fixation and its significance

Biological nitrogen fixation (BNF) results from the interaction between a plant and diazotrophic bacteria, these bacteria are either free-living in the soil or live in symbiosis with the plants (Walker *et al.*, 2020). This process occurs in the root nodules of leguminous plants and within the rhizosphere of the non-leguminous plant (Concha & Doerner, 2020). Biological nitrogen fixation is the reduction of nitrogen in the atmosphere into ammonia (NH_4), whilst the rhizobia receive simple sugar from the plant (Babalola *et al.*, 2017). The overall amount of adenosine triphosphate (ATP) required by nitrogen-fixing bacteria is 16 mol either found from the photosynthetic product or other organisms (Jones *et al.*, 2016). Furthermore, simple sugar obtained from photosynthesis is then transferred to the root nodules used by the bacteria for nitrogen fixation (Jones *et al.*, 2016; Courty, Smith, Koegel, Redecker & Wipt, 2015). The production of NH_3 leads to the development of necessary biomolecules that are needed by plants through the production of an amino acid (Concha & Doerner, 2020). Another problem faced by farmers is the cost of synthetic fertilizers as most of them cannot afford them (Courty *et al.*, 2015). Sharma, Kaur and Sharma (2020) stated that rhizobia strains also act as biocontrol agents against fungi through antibiotics, mycolytic enzymes, and hydrocyanic acid (HCN).

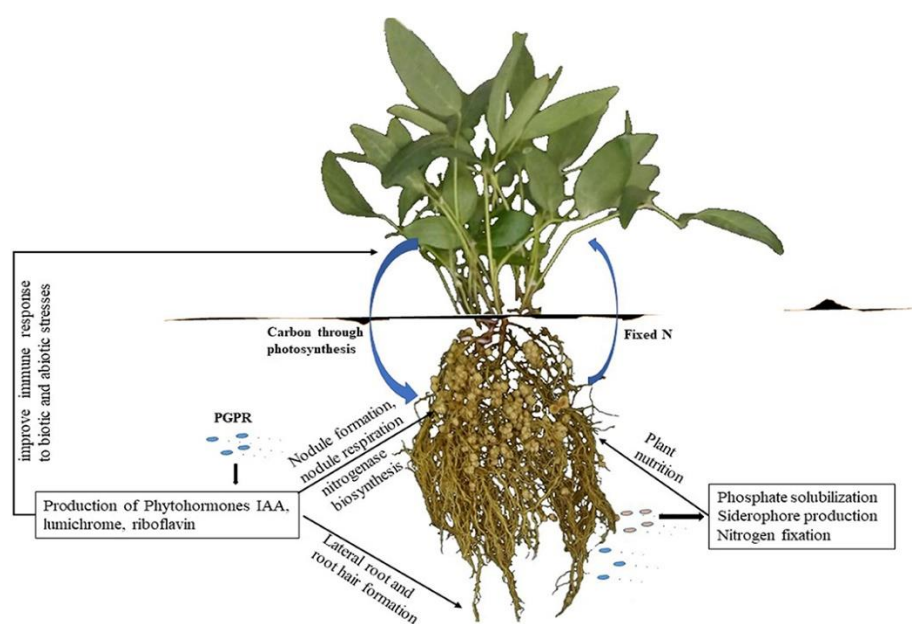


Figure 2.4: Interaction between legume and beneficial microbes (Jaiswal *et al.*, 2021).

2.6. Factors that affect metabolism, survival, and functioning of BNF in the soil

2.6.1. Soil pH

Active survival, growth, and nodulation of rhizobium strains are deleteriously influenced at low pH (Hashmat *et al.*, 2021). A study by Ferreira *et al.* (2016) discovered that the lack of nodulation in *Medicago truncatula* plants in acidic soil was primarily due to the inability of *Rhizobium meliloti* to grow or survive. Ferreira *et al.* (2016) also added that the failure of nodulation is caused by Rhizobia's poor survival in acidic soil, which is particularly likely when inoculation is not practiced every year. The nodulation and nitrogen fixation abilities of alfalfa (*Medicago sativa*. L.) and the Lotus (*Glaber mill*) varied according to pH 4.0 or acid soil conditions, which is due to the host's tolerance to low pH, some strains of *Rhizobium phaseoli* can be multiple at a pH of 3.8 (Ameyu, Tesfaye & Regassa, 2020). Nitrogen fixation improved at a slightly acidic pH of (6-7) and in turn increased the nitrogen content and growth of the plant (Hashmat *et al.*, 2021).

2.6.2. Temperature

Rhizobia strains in soil are highly dependent on temperature for survival and persistence. Nodulation competition is also significantly influenced by the temperature of the soil (Ayalew & Yoseph, 2022). Some of this effect may be attributed to a temperature-induced delay in nodulation or the restriction of nodules to the subsurface (Sita *et al.*, 2017; Kumar Meena, Kumar Singh, Singh, Kumari Meena & Singh Meena, 2015). Nodulated alfalfa plants were studied for their CO₂ exchange and nitrogen fixation under elevated temperature and water availability. It was found that temperature affects plant and nodule dry weight (Ayalew & Yoseph, 2022). Plant growth is reduced by high temperatures due to their negative effects not

only on the photosynthetic performance of plants but also on nodule growth and development, which results in diminished nodule biomass and a depletion of total nodule soluble protein content (Sita *et al.*, 2017; Kumar Meena *et al.*, 2015). Interestingly, N fixation is more sensitive to high temperatures than photosynthesis (Bhandari *et al.*, 2017). Nodulation interruption is a legume-specific phenomenon; for instance, common beans and soybean have a similar threshold, whereas lentils are comparatively cold tolerant such that nodulation is substantially diminished only at temperatures below 10 °C (Bhandari *et al.*, 2017; Siyanga, 2016). Microbes are highly likely to die at freezing temperatures (below zero) due to cellular collapse, which occurs when the DNA and membrane of the cell are damaged (Siyanga, 2016).

2.6.3. Nitrate

Nitrogenase enzyme reduces nitrogen to ammonia, is Oxygen-labile, and hence denatures when the oxygen concentration is great (Shandilya, Kumar, Shrivastava, Varma & Vishwakarma, 2021). Nitrate and ammonium are effective inhibitors of nodule dry-matter supply, nodule formation, and nitrogen fixation (Mbah & Dakora, 2017). Nitrate can combine with leghaemoglobin to form nitrosylleghaemoglobin, which decreases the oxygen supply to Nitrogen-fixing bacteroids (Mbah & Dakora, 2017).

2.6.4. Moisture stress

Soil moisture influences the development and growth of soil microorganisms through the process of mass flow, diffusion, and nutrient concentration (Barbosa, Brito, Fernandes, Fernandes-Júnior, & Lima, 2018). Poor nodulation of legume crops in arid soils is due to reductions in the population levels of rhizobia throughout the dry season (Mweetwa, Chilombo & Gondwe, 2016). Barbosa *et al.* (2018) argue that moisture level was the dominant factor influencing short and long-term survival of *Bradyrhizobium japonicum* strains inoculated into

loamy sand. However, in low water potential rhizobia survival is much better in silt loams, sandy loam, and sandy clay loam than in clay or sandy loam soil (Rodiño, Riveiro & De Ron, 2020). Root nodules are particularly sensitive to changes in soil moisture potential (Ndimbo, Nchimbi-Msolla & Semu, 2015). A drop from about -0.55 to -0.7×10^5 Pa, has resulted in a reduction of nitrogen fixation in soybeans (Rodiño *et al.*, 2020). Moreover, legume crops are mainly intolerant to excess and shortage of moisture and this is mainly due to the ultra-sensitivity of the symbiosis to moisture stress (Mweetwa *et al.*, 2016; Ndimbo *et al.*, 2015). Extreme moisture stress can inhibit nodule initiation or cause nodule shedding in some legume species (Barbosa *et al.*, 2018). Excess moisture can also reduce N_2 fixation potential if insufficient oxygen for rhizobia respiration is available (Kasper, Christoffersen, Soti & Racelis, 2019).

2.7. Symbiotic interaction in Bambara groundnut rhizosphere

Symbiotic nitrogen fixation is an alternative farming system that is resilient to climate change, eco-friendly, and improves soil biodiversity, and soil structure management (Kebede, Amsalu, Argaw & Tamiru, 2020). Rhizobia species such *Bradyrhizobium*, *Sinorhizobium*, and *Rhizobium* can form symbiotic interactions with legumes such as the Bambara groundnut and they can suppress the growth of plant pathogens (Kebede *et al.*, 2020; Babalola *et al.*, 2017). Interactions between legumes and rhizobia are mainly due to the result of a very complex series of several signals that are exchanged amongst the potential rhizobia and plant symbiont in the soil (Concha & Doerner, 2020). Temegne (2018); Concha and Doerner (2020) stated that Bambara groundnut microbe interaction results in nodule formation; this process initiates with compound production which includes flavonoids, betaines, and aldonic acid as the root exudates from the plant (Figure 2.5). These compounds signal to the rhizobia in a compatible relationship with the compounds. Also, the lipochito-oligosaccharide Nod-factors that are

produced cause root hair curling which will provide an entry route into the plant through an infection thread (Figure 5) (Temegne, 2018). Moreover, enhancing the production of the Nod gene that induces nodulation by interacting with the NodD-protein of the cell wall of the rhizobia (Figure 2.5) (Chen, Wang, Ji & Zhang, 2020).

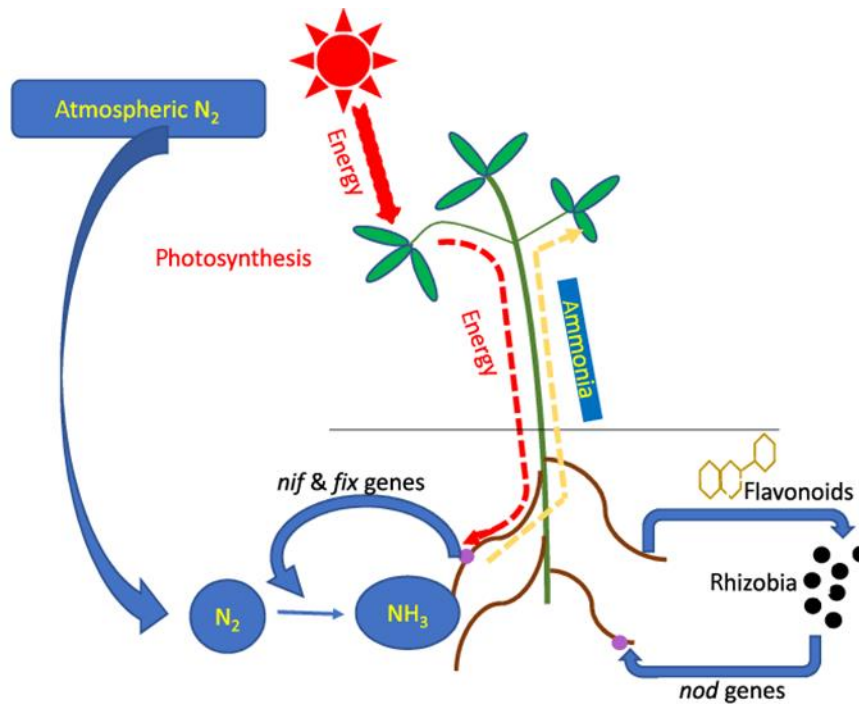


Figure 2.5: Model of symbiotic fixation in plants by *Rhizobium* sp. (Lindström & Mousavi, 2019).

2.8. Importance of identifying Indigenous rhizobia strains

Indigenous microorganisms are a group of innate microbial that inhabit the soil and the surfaces of all living things inside, and outside they are important in biodegradation, bioleaching, bio composting, nitrogen fixation, improving soil fertility, and as well in the production of plant growth hormones (Kumar & Gopal, 2015; Zahid, Abbasi, Hameed & Rahim, 2015).

2.8.1. Biodegradation

An organic compound can be degraded by indigenous bacteria without any artificial enhancement (Kumar & Gopal, 2015). This process is called intrinsic bioremediation and is one of the best remedial actions for soil contamination. Biodegradation involves a variety of microbial enzymes transforming both natural and artificial hydrocarbons into intermediate compounds that may be less or equally hazardous than their parent compounds (Joshi, duttand, Choudhary & Mundra, 2019; Kumar & Gopal, 2015).

2.8.2. Bio-fertilizers and protectors

Indigenous microorganisms contain a variety of beneficial microorganisms and not just one culture (Joshi *et al.*, 2019). By utilizing these microorganisms, host plants can obtain more nutrients and hold more water, so they are always hydrated (Kumar & Gopal, 2015). As a result, the plant roots are effectively aerated, and the exchange of gases is effective in preventing soil erosion (Zahid *et al.*, 2015). Native microorganisms protect the normal host from the invasion of microorganisms that are more likely to cause disease (Joshi *et al.*, 2019). By producing bacteriocins and other inhibitory substances, they compete with pathogens for essential nutrients and receptors on host cells, making the environment hostile to pathogen colonization (Kumar & Gopal, 2015).

2.8.3. Bio-composition

Wastes generated by the agro-industrial sector have become a major environmental and health concern because most farmers burn them, endangering human and environmental health, and the danger to soil erosion (Joshi *et al.*, 2019). Composting is one of the most attractive disposal alternatives because of its low environmental impact, low cost, and capacity for generating a

valuable product that can be used both in agriculture and horticulture (Kumar & Gopal, 2015; Zahid *et al.*, 2015).

2.8.4. Bioleaching of heavy metals

Bioleaching is the process of removing metals using microorganisms, which can be done by exogenous, indigenous, and genetically manipulated microorganisms (Zahid *et al.*, 2015). In general, the efficiency of metal removal depends greatly on the affinity between the metal and the microbial cell wall; this can be achieved using indigenous microorganisms isolated from mine sites (Kumar & Gopal, 2015).

2.8.5. Bio-mediation

Wastewater treatment is economically and environmentally friendly using indigenous microorganisms to reduce pollution from brewery effluents by evaluating their bio-mediation potential (Kumar & Gopal, 2015). Oljira, Muleta and Jida (2018) also suggested that brewery wastewater may pose environmental concerns such as biochemical oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS), pH, nutrients (nitrogen and phosphorus) concentration, and temperature. Wastewater from industry contains organic waste whose pollution level varies with the product production process and the quantity of water consumed (Pardamean, Islamy, Hasan, Herawati & Mutmainnah, 2021; Oljira *et al.*, 2018).

2.9. Plant growth-promoting rhizobacteria (PGPR)

The rhizosphere is a narrow soil that is influenced by the root system of plants and is mostly rich in sugar, amino acids, nutrients for the growth of bacteria, and provides a source of energy (Sharma *et al.*, 2020). A diverse group of microorganisms populate it and colonize the habitat. Microorganisms can adapt to the environment and are mutual partners associated with plant

growth. Additionally, it can promote and support the overall growth of plants leading to high crop yield (Lugtenberg & Kamilove, 2009). They are now integrated into biofertilizers and biopesticides to increase soil fertility and control plant pathogens (Souza, Ambrosini & Passaglia, 2015). PGPRs are used as biocontrol agents and biofertilizers to improve crop productivity as well as soil fertility (Sharma *et al.*, 2020). Diverse bacterial genera exhibiting plant growth-stimulating activities improve nutrients and sustainable crop production by fixing nitrogen gas, providing inorganic forms of zinc, potassium, silicon, phosphate, and synthesizing hormones such as gibberellins, cytokinin, and IAA that affect root elongation (Lindström & Mousavi, 2019). Examples include *Azotobacter*, *Bacillus*, *Microbacterium*, *Flavobacterium*, *Pseudomonas*, *Mesorhizobium*, *Achromobacter*, etc (Narozna *et al.*, 2014). *Azospirillum* are free-living nitrogen fixers found to play a significant role in enhancing the growth of non-leguminous crops (Lin *et al.*, 2015). According to Lin *et al.* (2015) *Pseudomonas fluorescens* and *Pseudomonas putida* groups stimulate the growth of plants as biocontrol agents aiding in phosphorus solubilization and nitrogen fixation. *Azotobacter* and *Azospirillum* are effective in enhancing production (Rueda *et al.*, 2016). Phosphorus, a crucial soil nutrient that is present in a complex inaccessible form, is made available by phosphate-solubilizing microbes that make them available for plant uptake. A study in similar lines on an eggplant was reported (Souza *et al.*, 2015) with *Bacillus mucilaginosus* and *Bacillus megaterium*. Co-inoculation of two or more organisms may result in improved yield and growth when compared to monoseptic inoculation as they provide diversified benefits to the plants (Souza *et al.*, 2015). Furthermore, PGPR are further classified into two groups: intracellular plant growth-promoting rhizobacteria (iPGPR) and extracellular plant growth-promoting rhizobacteria (ePGPR). The ePGPR are mainly found in the rhizosphere, on the rhizoplane, or in the area between the cells' root cortexes. Bacterial examples include *Agrobacterium*, *Bacillus*, *Azospirillum*, and *Azotobacter* (Rathore, 2015; Ahmed & Kilbert,

2014). intracellular plant growth-promoting rhizobacteria (iPGPR) intracellular plant growth-promoting rhizobacteria (iPGPR). The iPGPRs belong to the family of rhizobiaceae, *Bradyrhizobium*, *Mesorhizobium*, , and *Allorhizobium*.

2.10. Mechanism of plant growth promoting rhizobacteria (PGPR)

Plant growth-promoting rhizobacteria use several mechanisms for the enhancement of plant growth and development (Lugtenberg & Kamilove, 2009). These can be categorized as direct and indirect mechanisms (Koskey, Mburu, Awino, Njeru & Maingi, 2021). PGPR directly promotes the growth of plants by their capability for nutrient supply P, N, essential minerals, and K and the production of hormones or indirectly by decreasing inhibitory effects of pathogens on plant growth and development in the form of biocontrol agents, root colonizer, and environmental protectors (Lugtenberg & Kamilove, 2009; Bhardwaj, Ansari, Sahoo & Tuteja, 2014). This is achieved through nitrogen fixation, phytohormone activities, siderophores production, mineral and phosphorus solubilization, enhancement of soil characterization, etc (Naik, Mishra, Srichandan, Singh, & Sarangi, 2019). Enzymatic activity is another plant growth rhizobacteria mechanism to enhance plant growth that certain enzymes such as proteases, kitinase, phosphatase, dehydrogenase, beta-glucanase attack pathogens by excreting cell wall hydrolysis (Sharma *et al.*, 2020). The hydrolytic enzyme degrades the cell wall of pathogens that act indirectly for the growth mechanism of plants. Plant growth-promoting rhizobacteria through the activity of these enzymes play a significant role in protecting plants by suppressing pathogens fungi including *Phytophthora*. Table 2.2 represents ways through which microbes indirectly and directly associate themselves with the crops and enhance plant growth.

Table 2.1: Effect of plant growth promoting microbes (PGPM) on plant well-being (Naik *et al.*, 2019).

Microbial group		Mechanism of action	Representative species	Mode of Inhabitation	Beneficial (+) or detrimental (-)	Type of association	Reference(s)
Bacteria	P-solubilising microbe	P-solubilisation	<i>Bacillus</i> and <i>Pseudomonas</i> (bacteria)	Soil/rhizosphere	+	Symbiotic association	Sharma <i>et al.</i> (2013)
Bacteria and Fungi	PGPR and AMF	N-fixation	<i>Pseudomonas reactans</i> , <i>Chryseobacterium humi</i> , <i>Rhizophagus irregularis</i>	Rhizosphere/soil	+	Symbiotic association	Moreira <i>et al.</i> (2016)
Bacteria	PGPR	N-fixation	<i>Rhizobium</i> , <i>Sinorhizobium</i> , <i>Bradyrhizobium</i> , <i>Azorhizobium</i> , <i>Mesorhizobium</i> , <i>Allorhizobium</i>	Rhizosphere soil	+	Symbiotic N ₂ - fixers	Hayat <i>et al.</i> (2010)
Bacteria	PGPR Isolates	Biocontrol agent, plant growth substances, Antagonistic activity against phytopathogen	<i>Azospirillum</i> , <i>Enterobacter</i> , <i>Klebsiella</i> , <i>Pseudomonas</i> , <i>Pseudomonas fluorescens</i> , <i>Bacillus subtilis</i>	Rhizosphere soil	–	Free-living N ₂ -fixers Symbiotic association	Sivasakthi <i>et al.</i> (2014)
Bacteria	PGPR	–	<i>P. fluorescens</i> , <i>B. subtilis</i> , <i>P. putida</i>	soil	+	Symbiotic association	Del <i>et al.</i> (2017)
Bacteria	PGPR	Antagonistic activity, IAA, GA3	<i>Bacillus amyloliquefaciens</i>		–	–	Yuan <i>et al.</i> (2013)
Bacteria	Microbial pest control agent, plant growth promotion	Root growth development, biocontrol agent	<i>Bacillus subtilis</i> , <i>Azospirillum brasilense</i> Sp245, <i>Rhizobacterium</i> (PGPR)	Rhizosphere	+/-	Symbiotic association	Felici <i>et al.</i> (2008)
Fungi and bacteria	AMF and PGPB	Sugar and vitamin production, sweetness to tomato	<i>Pseudomonas</i> , AM fungi	soil	+	Symbiotic association	Bona <i>et al.</i> (2017)
Fungi	AM fungi	Phosphatase activity	<i>Glomus fasciculatum</i> , <i>Glomus fasciculatum</i> , <i>Glomus mossae</i> ,	Soil	+	Symbiotic association	Bona <i>et al.</i> (2017)

Actinomycetes	Endophytic actinomycetes	Antimicrobial activity against phytopathogen	<i>Gigaspora margarita</i> , <i>Acaulospora laevis</i> <i>Streptomyces</i> , <i>Streptosporangium</i> , <i>Microbispora</i> , <i>Streptoverticillium</i> , <i>sacchromonospora</i> , <i>Nocardia</i>	<i>Azadirachta indica</i> A. juss (stem, root, leaf)	–	Symbiotic association	Verma <i>et al.</i> (2009)
Bacteria and actinomycetes	PGP agents	ACC deaminase (stress-buster) and IAA, N ₂ -fixing, PO ₄ solubilising, siderophore producing	<i>Microbispora</i> sp., <i>Streptomyces</i> sp.	Soil	+/-	Symbiotic association	Souza <i>et al.</i> (2015)
Bacteria	Halotolerant endophyte	Salt tolerance	<i>Bacillus flexus</i> , <i>Enterobacter</i> sp. UPMR18	Halophyte <i>Limonium sinense</i> (PGPR)	–	Symbiotic association	Wang <i>et al.</i> (2017)
Bacteria, fungi and actinomycetes	PGPR (PGPB), fungi, actinomycetes	Soil conditioner, plant pathogen suppressor, biofertiliser, plant straightener, phytostimulator, biopesticide	<i>Azospirillum</i> , <i>Rhizobium</i> , <i>Bacillus</i> , <i>Pseudomonas</i> , <i>Serratia</i> , <i>Stenotrophomonas</i> , <i>Streptomyces</i> , <i>Coniothyrium</i> , <i>Ampelomyces</i> , <i>Trichoderma</i>	soil	+/-	Symbiotic association	Berg (2009)
Bacteria and fungi	PGPB and Endophytic fungi	Biocontrol agent against pathogen, pest	<i>P. aeruginosa</i> , <i>Trichoderma viride</i>	Soil and rhizosphere	–	Symbiotic association	Afzal <i>et al.</i> (2013)
Fungi	–	suppress fungal infections	<i>Trichoderma harzianum</i>	<i>Trichoderma</i> -enriched compost extracts	–	Symbiotic association	Siddiqui <i>et al.</i> (2008)
Bacteria and Fungi	AM fungi and PGPB	Stimulate plant growth, drought tolerance, IAA production	<i>Pseudomonas putida</i> , <i>Bacillus megaterium</i> , AM fungi (<i>Glomus coronatum</i> , <i>Glomus constrictum</i> or <i>Glomus claroideum</i>)	Abiotic (water)-stress condition	+/-	Symbiotic association	Marulanda <i>et al.</i> (2009)

2.10.1. Direct mechanism

Direct plant growth mechanisms differ upon the use of particular plant species and microbial strains. It mainly involves soil improvement and the production of substances that are needed for the growth of plants, which increases fertility by soil mineral mobilization (Naik *et al.*, 2019). The inhabiting root surface intensifications individual ion fluxes and improves direct mechanisms (Lugtenberg & Kamilove, 2009). Moreover, these improvements may include growth regulators supply and essential minerals such as phosphorus and potassium (Tabassum *et al.*, 2017).

Nitrogen fixation

Nitrogen is a common nutrient that is required for plant development, growth, and productivity. Moreover, it forms part of essential biomolecules (Figure 2.6). More than 80 percent of nitrogen in the atmosphere is present as inert gas unavailable to plants. Nitrogen fixation is a process by which nitrogen that is present in the atmosphere is converted into related nitrogenous compounds or ammonia (Satyanarayana, Krishna & Kumar, 2018; Darnajoux *et al.*, 2016). Furthermore, essential to live because inorganic that is fixed are required for the biosynthesis of all N-containing compounds essential for the manufacturing of fertilizer and agriculture (Puozaa *et al.*, 2017). N-fixation is environmentally friendly and economically alternative to chemical or synthetic fertilizer, referred to as Biological N-fixation (Tamagno & Ciampitti, 2017). It mainly occurs, at warm temperature, by N-fixing microorganisms that are naturally widely distributed, changing N to amino by using a complex enzyme known as nitrogenase (Puozaa *et al.*, 2017). Plant growth-promoting rhizobacteria (PGPR) fix atmospheric N and make it available to the plant through two mechanisms - Symbiotic and non-symbiotic interaction (Figure 2.6). Symbiotic N-fixation involves the interaction that occurs between plants and microbes such as *Rhizobium* and *Bradyrhizobium* that forms symbiosis association with leguminous plants such as Bambara groundnut and *Frankia* with

non-leguminous plants (Zahran, 2001) (Figure 2.6). Non-symbiotic N-fixation includes bacteria such as cyanobacteria like *Nostoc*, *Anabaena*, *Azotobacter*, *Enterobacter*, *Azospirillum*, and *Pseudomonas* (Zahran, 2001).

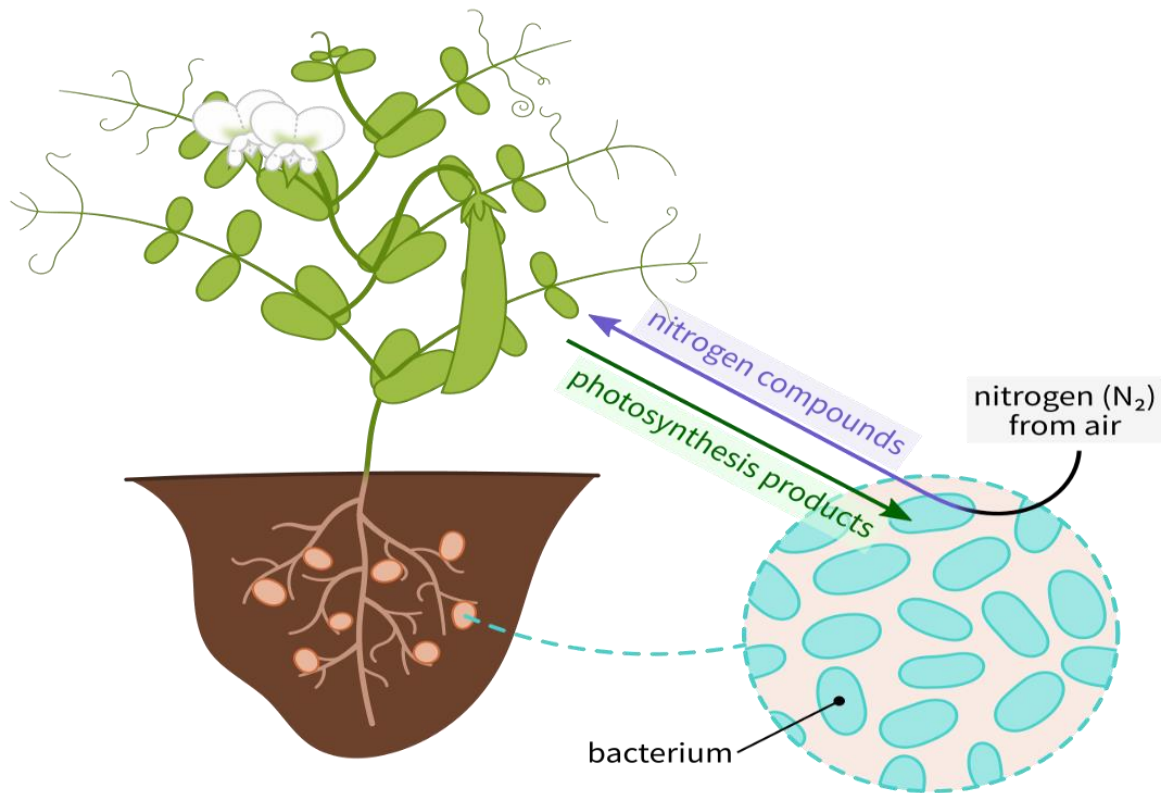


Figure 2.6: Interaction between symbiotic bacteria and plants in the root nodules (Nefronus, 2019).

Production of phytohormone

Phytohormones play a significant role in the growth and development of plants which allows the plant to tolerate diverse stress conditions (Shrestha, Kim & Park, 2014). Most rhizobacteria can produce phytohormones; auxins, ethylene, gibberellins, abscisic acid (ABA), and cytokinin (Figure 2.7), which play diverse roles in plant growth processes including cell multiplication (Glick, 2014).

Phosphate solubilization

Phosphorus (P) is an essential nutrient needed by plants and plays a significant role in all major metabolic processes which include the transfer of energy, respiration, macromolecular

biosynthesis, signal transduction, and photosynthesis (Figure 2.7) (Kalayu, 2019; Anand, Kumari & Mallick, 2016). Microorganisms found in the soil play a significant role in the transformation of P, including the solubilization of P that is required for the growth of plants. The capacity to mineralize and solubilize P by phosphate-solubilizing bacteria is of significant characterization (Oteino *et al.*, 2015) furthermore members with the ability to solubilize phosphate are of the genera *Erwinia*, *Pseudomonas*, *Bacillus*, *Rhizobium*, *Microbacterium*, *Burkholderia*, *Beijerinckia*, *Flavobacterium*, *Serratia*, *Mesorhizobium*, and *Rhodococcus* (Oteino *et al.*, 2015). Moreover, phosphate solubilization is mainly based on the secretion of organic acids by microorganisms in the soil due to the metabolism of sugar and the organisms within the rhizosphere use sugar from the exudates (Goswami, Dhandhukia, Patel & Thakker, 2014). Organic P solubilization plays a significant role in phosphorus cycling in an agricultural system, it is released from organic compounds in the soil by some enzymes such as phytases, C-P lyases, phosphatases, and phosphonates (Sharma, Sayyed, Trivedi & Gobi, 2013). It mainly accounts for about 0.2 % to 0.8 % of the total dry weight of the plants (Kumar, Kumar & Patel, 2018) and is contained within the enzymes, nucleic acids, coenzymes, phospholipids, nucleotides, and phospholipids. P is an essential aspect of plant growth and development, from the molecular level to various biochemical and physiological plant activities (Sharma *et al.*, 2013). This includes root development and growth, crop maturity, cell division and enlargement, storage and transfer reactions, energy production, formation of flowers and seeds, resistance to plant diseases, N fixation in legumes, crop maturity and quality of the crop, strengthening the stalks and stems, and photosynthesis (Satyaprakash, Nikitha, Reddi, Sadhana & Vani, 2017), sugar to starch transformation, and genetic traits transportation (Satyaprakash *et al.*, 2017; Santana, Marques & Dias, 2016). Moreover, it is the second most significant macronutrient that is required by plants after N. Yet, the availability of P-soluble forms for plants in the soil is restricted because of its fixation as an insoluble phosphate ion, calcium, and

aluminum in the soil (Walpola & Yoon, 2012). In the soil P does not exist as an element, P in the soil solution exists as insoluble inorganic P and insoluble P (Figure 6) (Walpola & Yoon, 2012). There is no interchange in the atmosphere hence its cycle in the biosphere can be referred to as “sedimentary” (Santana *et al.*, 2016). Consequently, P deficiency mainly restricts the growth and yield of the crops (Kalayu, 2019). The cell of the plant might take up several P forms, but the greatest part is absorbed in the form of dibasic ions (HPO_4^{2-}) and monobasic (H_2PO_4^-) depending upon the pH of the soil (Perez-Montano *et al.*, 2014). The released acid acts as a good chelator of divalent Ca^{2+} ions that will follow phosphate release from the insoluble compounds.

Potassium solubilization

Potassium (K) is one of the essential macronutrients and plays a significant role in the development, growth, and metabolism of plants (Chen *et al.*, 2020) (Figure 2.7). An insufficient amount of K results in poor development of roots, plant growing slowly, lower yield, and production of small seeds (Parmar & Sindhu, 2018). It constitutes about 2.5 % of the lithosphere but the actual concentration of soil of this nutrient differs ranging from 0.04 % to 3.0 %. Moreover, in plants, it improves cold, stress, and drought resistance and promotes photosynthesis in plants (Zhang & Kong, 2014). Potassium solubilizing bacteria (KSB) can solubilize rock K, synthetic K mineral powder through the excretion and production of some enzymes and organic acids (Jiang *et al.*, 2017; Ahmad, Nadeem, Naveed & Zahir, 2016; Zhang & Kong, 2014). Furthermore, it produces an array of bio-active compounds and is used as biological control of Phytopathogens. A large number of bacterial strains such as *Burkholderia* spp., *Pseudomonas* spp., *Bacillus edaphicus*, *Bacillus mucilaginosus*, *Acidithiobacillus* spp., and *Paenibacillus* spp. have been recently studied in releasing K in available form from K bearing minerals in the soils. About 90 % of K is present in the form of insoluble minerals of silicate and rock, the concentration of soluble is very low in soils (Bahadur, Maurya, Roy &

Kumar, 2019; Ahmad *et al.*, 2016; Parmar & Sindhu, 2013) and have been reported for their action of solubilizing K into assimilable forms from K minerals in the soil (Liu, Xing, Ma, Du & Ma, 2013). The primary mechanism of K solubilization is through the production of protons (acidolysis mechanism), organic acids, and inorganic acids and protons (Maurya, Meena & Meena, 2014; Parmar & Sindhu, 2013), which can convert insoluble K into soluble forms of K that are be easily absorbed by the plants (Figure 6). Several organic acids involved in the solubilization of insoluble K including citric acid, tartaric acid, oxalic acid, succinic acid, and α -ketogluconic acid are the most essential ones that are released by K-solubilizing bacteria (Meena *et al.*, 2014).

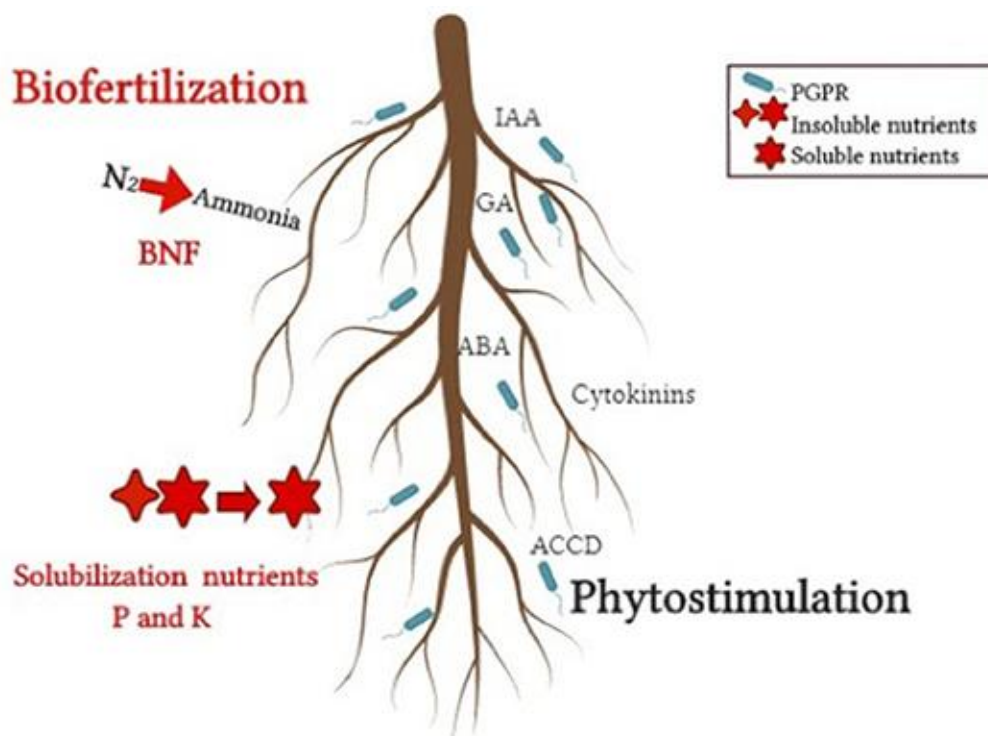


Figure 2.7: PGPR direct mechanism of plant growth, nutrient solubilization, biological fertilizers, and phyto-stimulation (Dos Santos, Dias, Lobo & Rigobelo, 2020).

Carbon cycling

The soil carbon cycle is a dynamic balance between the respiration of decomposing organisms, photosynthesis, and the stabilization of carbon (Falkowski, Fenchel & Delong, 2008). During the process of carbon cycling the plant takes in carbon dioxide through the process of

photosynthesis and uses it to build its roots, stems and leaves, the energy of sunlight is then trapped in the carbon-to-carbon bonds of organic molecules (Lu & Conrad, 2005). Microbes are critical in the process of breaking down and transforming dead organic material into forms that can be reused by other organisms (Falkowski *et al.*, 2008). This is why the microbial enzyme systems involved are viewed as key ‘engines’ that drive the Earth’s biogeochemical cycles. The terrestrial carbon cycle is mostly dominated by the balance that occurs between respiration and photosynthesis (Trumbore, 2006). Moreover, carbon is transferred from the atmosphere to the soil through carbon-fixation autotrophic organisms, mostly by photosynthesizing plants, photoautotrophic, and chemoautotrophic microbes that mainly synthesize atmospheric CO₂ into organic material (Trumbore, 2006; Lu & Conrad, 2005). Fixed carbon is returned to the atmosphere by a diversity of different paths that account for the respiration of autotrophic and heterotrophic organisms (Trumbore, 2006). The reverse route includes the decomposition of organic material by organic carbon-consuming heterotrophic microorganisms that utilize the carbon of either animal, plant, or microbial origin as a substrate for metabolism, retaining some carbon in their biomass and releasing the rest as metabolites or as CO₂ back to the atmosphere (Liang & Balser, 2011). Soil microbes essentially transfer carbon between environmental compartments to fulfill their fundamental goal: survival through reproduction. Thus, microbes utilize different organic and inorganic forms of carbon as carbon and energy sources. However, the carbon cycle does not operate independently; it is closely joined with essential elements for microbial metabolism. Hence the availability of other key elements essential for life, mainly P and N, and some environmental factors which include pH, temperature, soil texture, mineralogy, temperature, and soil water content control the rate at which microbes consume and respire carbon (Davidson & Janssens, 2006).

2.10.2. Indirect mechanism

The indirect mechanism includes the production of biological control agents that kill or inactivate pathogens, providing an environment that is healthier for the plant (Naik, Mishra, Srichandan, Singh & Sarang, 2019) such as exopolysaccharides, antibiotics, and enzyme activity. Antibiosis, production, and competition of lytic enzymes (glucanases and chitinases with the ability to hydrolyze the cell wall of fungi are regarded as indirect mechanisms of plant growth promotion (Bhattacharyya & Jha, 2012). Also, bacteria improve the growth of plants by suppressing pathogens and increasing plant innate immunity against the pathogens (Tabassum *et al.*, 2017).

Microbial processes in the soil catalyzed by enzymes

Soil enzymes are the most important components of biological soil processes which include organic compounds degradation, their mineralization, and nutrient cycling including phosphorus, sulfur, nitrogen, and other essential metals (Baldrian & Valášková, 2009). Enzymatic activity is a mechanism for growth enhancement by PGPR whereby enzymes such as proteases, dehydrogenase, lipase, phosphatases, kitinase, and betaglucanase attack pathogen through the excretion of cell wall hydrolysis (Hayat, Ali & Ahmed, 2010). The activities of enzyme hydrolytic and ligninolytic oxidases and peroxidases directly affect the transformation rate of soil biopolymers into compounds that are accessible to microorganisms and plants (Ramadan, Abdelhafez, Hassan & Saber, 2016). Enzyme activities in environmental samples such as litter, soil, lignocellulose, and other matrices are a useful tool for assessing the functional diversity of soil microbial communities' turnover. Hydrolytic enzymes degrade pathogens or virulence factors, cell wall components acting indirectly for the mechanism of plant growth (Giacometti *et al.*, 2014). For example, PGPR through the activities of these enzymes plays a significant role in protecting plants by suppressing pathogenic fungi such as *Fusarium oxysporum*, *Botrytis cinerea*, and *Phytophthora* sp (Baldrian & Valášková, 2009).

Chitin and β -1,4-N-acetylglucoseamine are fungal cell wall components, therefore the PGPRs producing chitinase and β -1,3-glucanase can control their growth (Islam, Akanda, Prova, Islam, & Hossain, 2016). Hydrolytic enzymes play a significant role in decomposing organic matter and the rate of decomposition is influenced by agricultural management (Giacometti *et al.*, 2014). Primary enzymes involved in carbon cycling include β -glucosidase or β -xylosidase, they exhibit higher activity with the application of sewage and manure due to their microbial growth-promoting cellulose, lignin, and hemicellulose (Kracmarova *et al.*, 2020). Other enzymes have also been reported to be influenced by fertilization and are monitored in soils to determine biological quality β -N-acetylglucosaminidase (NAG), involved in the nitrogen cycle, catalyses chitin breakdown to amino sugars and is a major source of mineralizable nitrogen in the soil (Kracmarova *et al.*, 2020). Moreover, soil enzyme activities have a long tradition of evaluating soil fertility and quantifying processes in seminatural and natural ecosystems with a great turnover of organic compounds (Naik *et al.*, 2019). Table 2 is a summary of enzymes targeted in soils which includes enzymes that are involved in nutrient mobilization of N, S, or P from complex organic substrates and an intricate array of enzymes that mainly participate in the transformation of biopolymers, which includes plant cell wall polymers such as hemicellulose and cellulose and some other polysaccharides that are available in litter.

Table 2.2: Soil enzymes activities involved in nutrient mobilization of N, P, and S.

Process	Enzymes	References
Cellulose degradation	endoglucanase	Baldrian and valášková (2009)
	cellobiohydrolase	
	β -glucosidase	
Degradation hemicellulose	Endoxylanase	Biely and Puchart (2006)

	Endomannanase	
	β -glycosidases	
	esterases	Biely and Puchart (2006)
Polysaccharide degradation	endochitinase	Seidl (2008)
	n-acetylglucosaminidase	Seidl (2008)
	α -glucosidase	Seidl (2008)
Lignin transformation	Mn-peroxidase	Hofrichter (2002)
	lignin	Martinez <i>et al.</i> (2005)
	peroxidase	Baldrian (2006)
	laccase (phenoloxidase)	Martinez <i>et al.</i> (2005)
	H ₂ O ₂ -producing enzymes	
n acquisition	Proteases	Rao <i>et al.</i> (1998)
	Aminopeptidases	Kilcawley <i>et al.</i> (2002)
	Urease	Martinez <i>et al.</i> (2005)
p acquisition	Hosphomonoesterase	Hayes <i>et al.</i> (2000)
	Phosphodiesterase	

Exopolysaccharides production

Exopolysaccharides are polymer carbohydrate polymers of great molecular weight that are mainly secreted by a wide variety of PGPRs (Sharma *et al.*, 2020) (Figure 2.8). Moreover, essential in root colonization, biofilm formation, bioremediation, cellular function maintenance, and gelling availability (Etesami, Emami & Alikhani, 2017) (Figure 2.8). Biofilm is referred to as a complex of bacterial cells that are attached to different abiotic and biotic surfaces that can retain moisture and also protect the roots of plants from several pathogens (Quarashi & Sabn, 2012). Exo-polysaccharides producing PGPR include *Azotobacter* sp.,

Rhizobium sp., *Bacillus*, *Pseudomonas*, *Xanthomonas* sp., and *Agrobacterium* sp., assist in enhancing soil fertility and also contributing to sustainable agriculture (Quarashi & Sabn, 2012). Additionally, exopolysaccharides are involved in cell aggregation and their synthesis may result in increased chances of bacterial survival under desiccation and mainly helps in nitrogen fixation by preventing great oxygen tension (Sharma *et al.*, 2020).

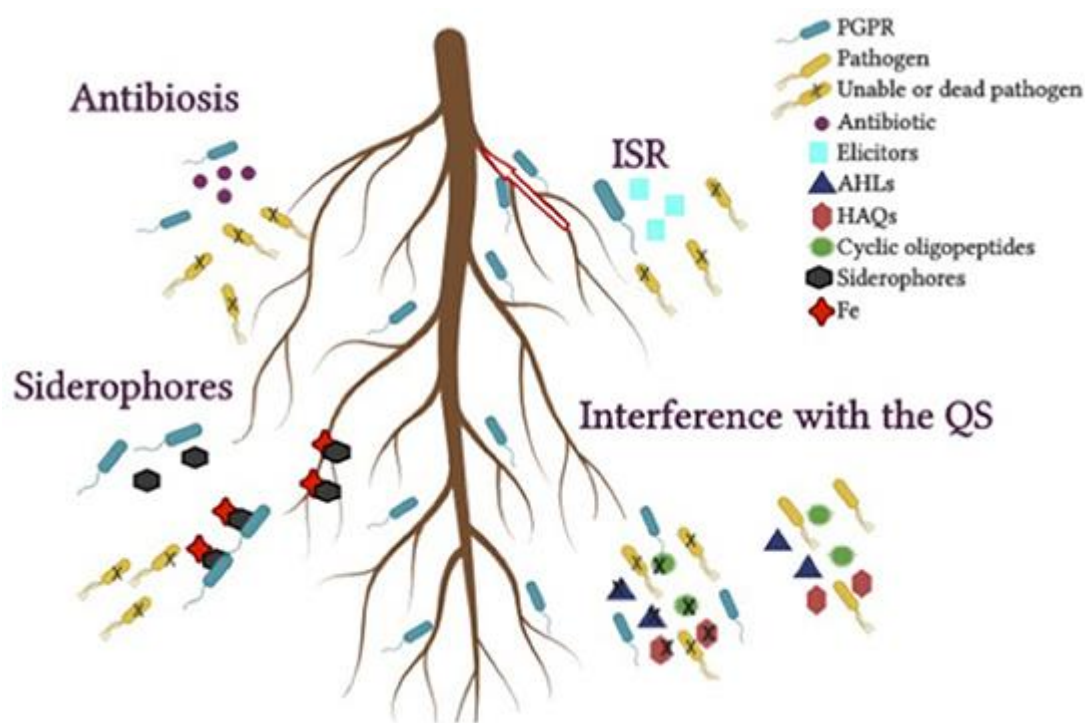


Figure 2.8: Indirect mechanism that is beneficial for plant growth and development from PGPR interaction, siderophores, antibiosis, induced systemic resistance (ISR), and interaction with the quorum sensing (Dos Santos *et al.*, 2020).

2.11. Factors limiting commercial inoculum application and performance in agriculture

The weak point of commercialization is often the performance of the microbial inoculum. The most shared barriers of inoculants are formulation inadequacies (Soumare *et al.*, 2020a). Commercial inoculum can be effective in the greenhouse and/or laboratory conditions but formulating that organism into an adequate inoculant is challenging (Soumare *et al.*, 2020a).

Gabasawa (2020), also added that inoculants are prone to some contamination after formulations that can decrease the shelf-life of the inoculant after autoclaving. Inoculants are the individual distribution of live bacteria from the unit of production to individual plants in the field (Gabasawa, 2020). Hence, an inoculant should be: (i) able to carry a greater number of microbes, (ii) simply sterilized and pulverized, (iii) harmless to other organisms, (iv) cost-effective and easily accessible, and (v) have excellent absorption capacity (Soumare *et al.*, 2020b). Inoculants should keep viability throughout storage time in the farmer's warehouse to avoid drying and should have an elongated shelf-life and stability (Soumare *et al.*, 2020b). Additional factors limiting inoculant effectiveness include poor quality of inoculants accompanied by low viability, its inability to compete with indigenous rhizobia, and its inability to tolerate the inherent physical and chemical conditions of the soil to which it is introduced (Gavit Pavankumar, Chaudhari Ambalal, Shelar Rajendra & Dandi Navin, 2019). The success of commercial inoculants is dependent on the number of viable bacteria available to participate in the infection process at the point of use (Sissay, Adesola, Massia & Taddesse, 2015).

2.12. References

- Abd-Alla, M. H., El-Enany, A. W. E., Nafady, N. A., Khalaf, D. E. and Morsy. F. M. 2014. Synergistic interaction of *Rhizobium leguminosarum* bv. *viciae* and arbuscular mycorrhizal fungi as a plant growth promoting biofertilizers for faba bean (*Vicia faba* L.) in alkaline soil. *Microbiological Research*, 169: 49-58.
- Adegboyega, T. T., Shoge, M. O. and Tamasi, A. A. 2021. Proximate and antinutrient composition of selected West African Bambara groundnut (*Vigna subterranea* (L.) Verdc.) Accessions. *Journal of Underutilized Legume*, 3: 13-25.

- Afzal, S. A. I. M. A., Tariq, S., Sultana, V., Ara, J. and Ehteshamul-Haque, S. 2013. Managing the root diseases of okra with endo-root plant growth promoting *Pseudomonas* and *Trichoderma viride* associated with healthy okra roots. *Pakistan Journal of Botany*, 45: 1455-1460.
- Ahmad, M., Nadeem, S. M., Naveed, M. and Zahir, Z. A. 2016. Potassium-Solubilizing Bacteria and Their Application in Agriculture. In: Meena, V., Maurya, B., Verma, J. and Meena, R. eds. *Potassium Solubilizing Microorganisms for Sustainable Agriculture*. New Delhi: Springer.
- Ahmed, M. and Kilbert, M. 2014. Mechanisms and applications of plant growth promoting rhizobacteria: current perspective. *Journal of King Saud University Science*, 26: 1-20.
- Ajilogba, C., Olanrewaju, O. and Babalola, O. 2022. Improving Bambara groundnut production: insight into the role of omics and beneficial bacteria. *Frontiers in Plant Science*, 13: 1-17.
- Akpalu, M., Atubilla, I. and 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.
- Ameyu, T., Tesfaye, A. and Regassa, A. 2020. Response of Soybean genotypes for root, nodulation, growth, yield, and yield components to lime and phosphorus application at Mettu, Southwestern Ethiopia. *Academic Research Journal of Agricultural Science and Research*, 8(2): 82-97.
- Anand, K., Kumari, B. and Mallick, M. A. 2016. Phosphate solubilizing microbes: an effective and alternative approach as biofertilizers. *Journal of Pharmacy and Pharmaceutical Sciences*, 8: 37-40.

- Ayalew, T. and Yoseph, T. 2022. Cowpea (*Vigna unguiculata* L. Walp.): A choice crop for sustainability during the climate change periods. *Cogent Food and Agriculture*, 10(3): 154-162.
- Azman Halimi, R., Raymond, C., Barkla, B., Mayes, S. and King, G. 2021. Development of selection indices for improvement of seed yield and lipid composition in Bambara groundnut (*Vigna subterranea* (L.) Verdc.). *Foods*, 11(1): 86.
- Babalola, O. O., Olanrewaju, O. S., Dias, T., Ajilogba, C. F., Kutu, F. R. and Cruz, C. 2017. Biological nitrogen fixation: The role of underutilized leguminous plants. In: Panpatte, D., Jhala, Y., Vyas, R. and Shelat, H. eds. *Microorganisms for Green Revolution*. Singapore: Springer.
- Bahadur, I., Maurya, R., Roy, P. and Kumar, A. 2019. Potassium-Solubilizing Bacteria (KSB): A Microbial Tool for K-Solubility, Cycling, and Availability to Plants. In: Kumar, A. and Meena, V. eds. *Plant Growth Promoting Rhizobacteria for Agricultural Sustainability*. Singapore: Springer.
- Baldrian, P. 2006. Fungal laccases – occurrence and properties. *FEMS Microbiology Reviews*, 30: 215-242.
- Baldrian, P. and Valášková, V. 2009. Degradation of cellulose by basidiomycetous fungi. *FEMS Microbiology Reviews*, 32: 501-521.
- Barbosa, D., Brito, S., Fernandes, P., Fernandes-Júnior, P. and Lima, L. 2018. Can *Bradyrhizobium* strains inoculation reduce water deficit effects on peanuts. *World Journal of Microbiology and Biotechnology*, 34(7): 1-8.
- Biely, P. and Puchart, V. 2006. Recent progress in the assays of xylanolytic enzymes. *Journal of Science in Food and Agriculture*, 86: 1636-1647.

- 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.
- Bhandari, K., Sharma, K., Hanumantha Rao, B., Siddique, K., Gaur, P., Agrawal, S., Nair, R. and Nayyar, H. 2017. Temperature sensitivity of food legumes: a physiological insight. *Acta Physiologiae Plantarum*, 39(3): 2-22.
- Bhardwaj, D., Ansari, M. W., Sahoo, R. K. and Tuteja, N. 2014. Biofertilizers function as key player in sustainable agriculture by improving soil fertility, plant tolerance and crop productivity. *Microbial Cell Factories*, 13(1): 1.
- Bhattacharyya, P. N. and Jha, D. K. 2012. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World Journal of Microbiology and Biotechnology*, 28: 1327-1350.
- Bona, E., Cantamessa, S., Massa, N., Manassero, P., Marsano, F., Copetta, A., Lingua, G., Agostino, G. D., Gamalero, E. and Berta, G. 2017. Arbuscular mycorrhizal fungi and plant growth-promoting *Pseudomonads* improve yield, quality, and nutritional value of tomato: a field study. *Mycorrhiza*, 27: 1-11.
- Chen, W., Wang, E., Ji, Z. and Zhang, J. 2020. Recent development and new insight of diversification and symbiosis specificity of legume rhizobia: mechanism and application. *Journal of Applied Microbiology*, 131(2): 553-563.
- Chibarabada, T. P. 2014. Seed quality and water use characteristics of a Bambara groundnut (*Vigna subterranea* L.) landrace differing in seed coat colour. Master of Philosophy Dissertation, University of Kwazulu-Natal.
- Concha, C. and Doerner, P. 2020. The impact of the rhizobia-legume symbiosis on host root system architecture. *Journal of Experimental Botany*, 71(13): 3902-3921.

- Cook, D. 2017. Small scale farmer's utilization and perceptions of Bambara groundnut production in South Africa: a case study in a semi-arid region of Limpopo. Master of Philosophy Dissertation, University of Cape Town.
- Courty, P. E., Smith, P., Koegel, S., Redecker, D. and Wipf, D. 2015. Inorganic nitrogen uptake and transport in beneficial plant root-microbe interactions. *Critical Reviews in Plant Sciences*, 34(1-3): 4-16.
- Darnajoux, R., Zhang, X., McRose, D., Miadlikowska, J., Lutzoni, F., Kraepiel, A. and Bellenger, J. 2016. Biological nitrogen fixation by alternative nitrogenases in boreal cyanolichens: the importance of molybdenum availability and implications for current biological nitrogen fixation estimates. *New Phytologist*, 213(2): 680-689.
- Davidson, E. A. and Janssens, I. A. 2006. Temperature sensitivity of soil carbon decomposition and feedback to climate change. *Nature*, 440: 165-173.
- Del, R., Cappellari, L., Chiappero, J., Santoro, M. V., Giordano, W. and Banchio, E. 2017. Inducing phenolic production and volatile organic compounds emission by inoculating *Mentha piperita* with plant growth-promoting rhizobacteria. *Scientia Horticulturae*, 220: 193-198.
- Department of Agriculture, Forestry, and Fisheries (DAFF), 2016. Production Guideline for Bambara groundnuts (*Vigna subterranea*): National Department of Agriculture and Agricultural Research Council Grain Crops Institute. Pretoria. [Online]. Available at: [https://www.google.com/search?q=DAFF%2C+2016.+Production+Guideline+for+Bambara+groundnuts+\(Vigna+subterranea\)%3A+National+Department+of+Agriculture+and+Agricultural+Research+Council+Grain+Crops+Institute.+Pretoria%2C+South+Africa](https://www.google.com/search?q=DAFF%2C+2016.+Production+Guideline+for+Bambara+groundnuts+(Vigna+subterranea)%3A+National+Department+of+Agriculture+and+Agricultural+Research+Council+Grain+Crops+Institute.+Pretoria%2C+South+Africa). [Accessed 24 March 2022].
- Department of Agriculture, Rural Development and Land Administration. 2012. Bambara groundnut *Vigna subterranea*. [Online]. Available at:

https://dardlea.mpg.gov.za/publications/tips/ Bambara_groundnuts. pdf [Accessed 24 March 2022].

- Dos Santos, R. M., Diaz, P. A., Lobo, L. L. and Rigobelo, E. C. 2020. Use of plant growth-promoting rhizobacteria in maize and sugarcane: Characteristics and applications. *Frontiers in Sustainable Food Systems*, 4: 1-15.
- Etesami, H., Emami. S. and Alikhani, H. A. 2017. Potassium solubilizing bacteria (KSB): mechanisms, promotion of plant growth, and future prospects- A review. *Journal of Soil Science and Plant Nutrition*, 17: 897-911.
- Falkowski, P. G., Fenchel, T. and Delong, E. F. 2008. The microbial engines that drive Earth's biogeochemical cycles. *Science*, 320: 1034-1039.
- Felici, C., Vettori, L., Giraldi, E., Forino, L. M. C., Toffanin, A., Tagliasacchi, A. M. and Nuti, M. 2008. Single and co-inoculation of *Bacillus subtilis* and *Azospirillum brasilense* on *Lycopersicon esculentum*: effects on plant growth and rhizosphere microbial community. *Applied Soil Ecology*, 40: 260-270.
- Ferreira, T., Aguilar, J., Souza, L., Justino, G., Aguiar, L. and Camargos, L. 2016. pH effects on nodulation and biological nitrogen fixation in *Calopogonium mucunoides*. *Brazilian Journal of Botany*, 39(4): 1015-1020.
- Fleibrer, K. W. E. 2006. The improvement of Bambara groundnut production in Northern Namibia is achieved through breeding strategies and agronomic investigations. Doctor of Agricultural Science, Technical University of Munich.
- Gabasawa, A. I. 2020. Prospects for Developing Effective and Competitive Native Strains of Rhizobium Inoculants in Nigeria. In: Abia, A. and Lanza, G. eds. *Current Microbiological Research in Africa*. Zaria: Springer.
- Gavit Pavankumar, M., Chaudhari Ambalal, B., Shelar Rajendra, D. and Dandi Navin, D. 2019. Microbial augmentation of salt-affected soils: emphasis on haloalkalitolerant PGPR.

- In: Singh, D., Gupta, V. and Prabha, R. eds. *Microbial Interventions in Agriculture and Environment*. Singapore: Springer.
- Giacometti, C., Cavani, L., Baldoni, G., Ciavatta, C., Marzadori, C. and Kandeler, E. 2014. Microplate-scale fluorometric soil enzyme assays as tools to assess soil quality in a long-term agricultural field experiment. *Applied Soil Ecology*, 75: 80-85.
- Glick, B. R. 2014. 'Bacteria with ACC deaminase can promote plant growth and help to feed the world'. *Microbiological Research*, 169(1): 30-39.
- Goswami, D., Dhandhukia, P., Patel, P. and Thakker, J. N. 2014. Screening of PGPR from saline desert of kutch: Growth promotion in *Arachis hypogea* by *bacillus licheniformis* A2. *Microbiological Research*, 169(1): 66-75.
- Halimi, R. A., Barkla, B. J., Mayes, S. and King, G. J. 2019. The potential of the underutilized pulse Bambara groundnut (*Vigna subterranea* (L.) Verdc.) for nutritional food security. *Journal of Food Composition and Analysis*, 77: 47-59.
- Hashmat, S., Shahid, M., Tanwir, K., Abbas, S., Ali, Q., Niazi, N., Akram, M., Saleem, M. and Javed, M. 2021. Elucidating distinct oxidative stress management, nutrient acquisition, and yield responses of *Pisum sativum* L. fertigated with diluted and treated wastewater. *Agricultural Water Management*, 247: 2-19.
- Hassen, A. I., Vuuren, A. van, Bopape, F. L. and Gerrano, A. S. 2022. Nodulation compatibility and symbiotic performance of rhizobia spp. with different landraces of Bambara groundnut (*Vigna subterranea* (L.) Verdc.) Collections. [Online]. Available at: <https://assets.researchsquare.com/files/rs-1233082/v1/44eda8a6-8479-44d8-a1a6-3007f2aa0ce0.pdf?c=1654071340> [Accessed 12 March 2022].
- Hayat, R., Ali, S. and Ahmed, I. 2010. Soil beneficial bacteria and their role in PGPR. *Annals of Microbiology*, 60: 579-598.

- Hayes, J. E., Richardson, A. E. and Simpson, R. J. 2000. Components of organic phosphorus in soil extracts that are hydrolysed by phytase and acid phosphatase. *Biology and Fertility of Soils*, 32: 279-286.
- Hofrichter, M. 2002. Review: lignin conversion by manganese peroxidase (MnP). *Enzyme and Microbial Technology*, 30: 454-466.
- Ibny, F., Jaiswal, S., Mohammed, M. and Dakora, F. 2019. Symbiotic effectiveness and ecologically adaptive traits of native rhizobial symbionts of Bambara groundnut (*Vigna subterranea* L. Verdc.) in Africa and their relationship with phylogeny. *Scientific Reports*, 9(1): 1-2.
- Ijarotimi, S. O. and 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.
- Islam, S., Akanda, A. M., Prova, A., Islam, Md. T. and Hossain, M. M. 2016. Isolation and identification of plant growth promoting rhizobacteria from cucumber rhizosphere and their effect on plant growth promotion and disease suppression. *Frontiers in Microbiology*, 6: 2-12.
- Jaiswal, S., Mohammed, M., Ibny, F. and Dakora, F. 2021. Rhizobia as a source of plant growth-promoting molecules: potential applications and possible operational mechanisms. *Frontiers in Sustainable Food Systems*, 4: 2-7.
- Jiang, J. H., Peng, X. W., Yan, Z. X., He, B. W., Zhu, C. X., Guo, H. and Genhg, B. 2017. Isolation and identification of potassium-solubilizing bacteria from rhizosphere soil of apple tree. *Chinese Journal of Agrometeorology*, 38: 738-748.
- Jones, F., Clark, I., King, R., Shaw, L., Woodward, M. and Hirsch, P. 2016. Novel European free-living, non-diazotrophic *Bradyrhizobium* isolates from contrasting soils that lack

- nodulation and nitrogen fixation genes – a genome comparison. *Scientific Reports*, 6(1): 1-11.
- Joshi, H., Duttand, S., Choudhary, P. and Mundra, S. 2019. Role of effective microorganisms (EM) in sustainable agriculture. *International Journal of Current Microbiology and Applied Sciences*, 8(03): 172-181.
- Kalayu, G. 2019. Phosphate solubilizing microorganisms: Promising approach as biofertilizers. *International Journal of Agronomy*, 2019: 1-7.
- Karunaratne, A. S., Walker, S. and Ruane, A. C. 2015. Modeling Bambara groundnut yield in Southern Africa: towards a climate-resilient future. *Climate Research*, 65: 193-203.
- Kasper, S., Christoffersen, B., Soti, P. and Racelis, A. 2019. Abiotic and biotic limitations to nodulation by leguminous cover crops in South Texas. *Agriculture*, 9(10): 209.
- Kebede, E., Amsalu, B., Argaw, A. and Tamiru, S. 2020. Symbiotic effectiveness of cowpea (*Vigna unguiculata* (L.) Walp.) nodulating rhizobia isolated from soils of major cowpea producing areas in Ethiopia. *Cogent Food and Agriculture*, 6(1): 1-16.
- Kilcawley, K. N., Wilkinson, M. G. and Fox, P. F. 2002. Determination of key enzyme activities in commercial peptidase and lipase preparations from microbial or animal sources. *Enzyme and Microbial technology*, 31: 310-320.
- Khan, M. M., Rafii, M. Y., Ramlee, S. I., Jusoh, M and Mamun, A. 2020. Genetic variability, heritability, and clustering pattern exploration of Bambara groundnut (*Vigna subterranea* L. Verdc) accessions for the perfection of yield and yield-related traits. *BioMed Research International*, 2020: 1-31.
- Kosini, D. and Nukenine, E. N. 2017. Bioactivity of novel botanical insecticide from *Gnidia kaussiana* (Thymeleaceae) against *Callosobruchus maculatus* (Coleoptera: Chrysomelidae) in stored *Vigna subterranea* (Fabaceae) grains. *Journal of Insect Science*, 17: 31-37.

- Koskey, G., Mburu, S. W., Awino, R., Njeru, E. M. and Maingi, J. M. 2021. Potential use of beneficial microorganisms for soil amelioration, phytopathogen biocontrol, and sustainable crop production in smallholder, agroecosystems. *Frontiers in Sustainable Food System*, 5: 1-20.
- Kracmarova, M., Kratochvilova, H., Uhlik, O., Strejcek, M., Szakova, J., Cerny, J., Tlustos, P., Balik, J., Demnerova, K. and Stiborova, H. 2020. Response of soil microbes and soil enzymatic activity to 20 years of fertilization. *Agronomy*, 10(10): 1542.
- Kumar, A., Kumar, B. and Patel, H. 2018. Role of microbes in phosphorus availability and acquisition by plants. *International Journal of Current Microbiology and Applied Sciences*, 7(5): 1344-1347.
- Kumar, B. and Gopal, D. 2015. Effective role of indigenous microorganisms for sustainable environment. *Three Biotech*, 5(6): 867-876.
- Kumar Meena, R., Kumar Singh, R., Pal Singh, N., Kumari Meena, S. and Singh Meena, V. 2015. Isolation of low temperature surviving plant growth – promoting rhizobacteria (PGPR) from pea (*Pisum sativum* L.) and documentation of their plant growth promoting traits. *Biocatalysis and Agricultural Biotechnology*, 4(4): 806-811.
- Laplaze, L., Sparvoli, F., Masmoudi, K. and Hash, C. 2018. Editorial: harvesting plant and microbial biodiversity for sustainably enhanced food security. *Frontiers in Plant Science*, 9: 1-2.
- Laurette, N. N., Maxémilienne, N. B., Henri, F., Souleymanou, A., Kamdem, K., Albert, N., Dieudonné, N. and 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 Microbiology Research*, 3(2): 65-75.

- Liang, C. and Balser, T. C. 2011. Microbial production of recalcitrant organic matter in global soils: implications for productivity and climate policy. *Nature Reviews Microbiology*, 9: 75.
- Lin, S. Y., Hameed, A., Liu, Y. C., Hsu, Y. H., Lai, W. A., Shen, F. T. and Young, C. C. 2015. *Azospirillum soli* sp. nov, A nitrogen-fixing species isolated from agricultural soil. *International Journal of Systematic and Evolutinary Microbiology*, 65: 4601-4607.
- Lindström, K. and Mousavi, S. A. 2019. Effectiveness of nitrogen fixation in rhizobia. *Microbial Biotechnology*, 13(5): 1314-1335.
- Liu, F., Xing, S., Ma, H., Du, Z. and Ma, B. 2013. Cytokinin-producing, plant growth-promoting rhizobacteria that confer resistance to drought stress in *Platycladus orientalis* container seedlings. *Applied Microbiology and Biotechnology*, 97(20): 9155-9164.
- Lugtenberg, B. and Kamilova, F. 2009. Plant-growth-promoting rhizobacteria. *Annual Review of Microbiology*, 63(1): 541-556.
- Lu, Y. and Conrad, R. 2005. In situ stable isotope probing of methanogenic archaea in the rice rhizosphere. *Science*, 309: 1088-1090.
- Majola, N., Gerrano, A. and Shimelis, H. 2021. Bambara Groundnut (*Vigna subterranea* (L.) Verdc.) production, utilisation, and genetic improvement in Sub-Saharan Africa. *Agronomy*, 11(7): 1345.
- Martínez, A. T., Speranza, M., Ruiz-Duenas, F. J., Ferreira, P., Guillén, F., Martínez, M. J., Gutiérrez, A. and Rio, C. J. D. 2005. Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. *International Microbiology*, 8: 195-204.
- Marulanda, A., Barea, J. M. and Azcon, R. 2009. Stimulation of plant growth and drought tolerance by native microorganisms (AM fungi and bacteria) from dry environments:

- mechanisms related to bacterial effectiveness. *Journal of Plant Growth Regulation*, 28: 115-124.
- Matthews. C. 2013. An overview of indigenous crop development by the Mpumalanga Department of Agriculture and Land Administration (DALA). *South African Journal of Plant and Soil*, 27: 337-340.
- Maurya, B. R., Meena, V. S. and Meena, O. P. 2014. Influence of inceptisol and Alfisol's potassium solubilizing bacteria (KSB) isolates on release of K from waste mica. *Vegetos- An International Journal of Plant Research*, 27(1): 181.
- Mazahib, A., Nuha, M., Salawa, I. and Babiker, E. 2013. Some nutritional attributes of Bambara groundnut as influenced by domestic processing. *International Food Research Journal*, 20 (3): 1165-1171.
- Mbah, G. and Dakora, F. 2017. Nitrate inhibition of N₂ fixation and its effect on micronutrient accumulation in shoots of soybean (*Glycine max* L. Merr.), Bambara groundnut (*Vigna subterranea* L. Verc) and Kersting's groundnut (*Macrotyloma geocarpum* Harms.). *Symbiosis*, 75(3): 205-216.
- Meena, H., Meena, R., Lal, R., Yadav, G., Mitran, T., Layek, J., Patil, S., Kumar, S. and Verma, T. 2018. Response of sowing dates and bio regulators on yield of cluster bean under current climate in alley cropping system in Eastern U.P., India. *Legume Research*, 41(00): 1-10.
- Minnaar-Ontong, A., Gerrano, A. and Labuschagne, M. 2021. Assessment of genetic diversity and structure of Bambara groundnut [*Vigna subterranea* (L.) verdc.] landraces in South Africa. *Scientific Reports Journal*, 11(1): 7408.
- Mkandawire, C. H. 2007. Review of Bambara groundnut (*Vigna subterranea* (L.) Verdc.) production in sub-Saharan Africa. *The Journal of Agricultural Science*, 2: 464-470.

- Mohammed, M. S. 2014. Pre-breeding of Bambara groundnut (*Vigna subterranea* (L.) Verdc). Master of Philosophy Dissertation. Abubakar Tafawa Balewa University.
- Moreira, H., Pereira, S. I., Marques, A. P., Rangel, A. O. and Castro, P. M. 2016. Mine land valorisation through energy maize production enhanced by the application of plant growth-promoting rhizobacteria and arbuscular mycorrhizal fungi. *Environmental Science and Pollution Research*, 23: 6940-6950.
- Mubaiwa, J., Fogliano, V., Chidewe, C., Bakker, E. and Linnemann, A. 2018. Utilization of Bambara groundnut (*Vigna subterranea* (L.) Verdc.) for sustainable food and nutrition security in semi-arid regions of Zimbabwe. *PLOS ONE*, 13(10): 1-19.
- Murevanhema, Y. Y. and 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.
- Mweetwa, A., Chilombo, G. and Gondwe, B. 2016. Nodulation, nutrient uptake, and yield of common bean inoculated with rhizobia and Trichoderma in an acid soil. *Journal of Agricultural Science*, 8(12): 61.
- Naik, K., Mishra, S., Srichandan, H., Singh, P. K. and Sarangi, P. K. 2019. Plant growth promoting microbes: Potential link to sustainable agriculture and environment. *Biocatalysis and Agricultural Biotechnology*, 21: 1-12.
- Narozna, D., Pudelko, K., Kroliczak, J., Golinska, B., Sugawara, M., Madrazak, C. J. and Sadowsky, M. J. 2014. Survival and competitiveness of *Bradyrhizobium japonicum* strains 20 yrs after introduction into field location in Poland. *Applied Environmental Microbiology*, 81: 1-10.
- Ndimbo, M. A., Nchimbi-Msolla, S. and Semu, E. 2015. Effects of moisture stress levels at different growth stages on nodulation and nitrogen fixation in common bean (*Phaseolus*

vulgaris L.) Genotype. *Asian Journal of Agriculture and Rural Development*, 5(8): 187-201.

Nefronus. 2019. Root nodules. Available at: https://www.google.com/search?sca_esv=099d353e2e922eb4&rlz=1C1CHBF_enZA1019ZA1019&q=Interaction+between+symbiotic+bacteria+and+plant+in+the+root+nodules&uds=AMwkrPtNxDP2fwrHG71Vw9iACnxG02CWhMXUZfQvNWMv73Zf-LjlxWMw9TLTYWsAsS2Cs6iFtJZgvSVdlUFJCRxJ7jZIGdkZtnldUGaRsjcE8PB1sURrBa6oeE2lkm0scKKrxhncw7D-bQnXRdgSSXAvR1q7Cw8ja6ne9xcJk6BuNpH8exMOImZK-NF3k8xkTu4Tnq7FGwM_zNP8J8egMAE90mQY1mWgyVjVRFCOzyto87cXsWde29JCHgD0j7n4yvu3DH5oN6Kq7VO9DNkyRDL_R274ZyA33KfCFBKafSbp2rucFEZf0GcvxPka-Ho3fPezG_ewYeVVQwR_hwyI3NvF88SgmKE91hfFdMlN-TyqxPgp7bMJu34&udm=2&prmd=ivs nbmtz&sa=X&ved=2ahUKEwjU3cD-8ZeFAxUAhv0HHZ3FC58QtKgLegQIChA B&biw=1536&bih=730&dpr=1.25#vhid=TcQcRhn2hjDPRM&vssid=mosaic
[Accessed 31 October 2022].

Nkambule, S. N. 2020. The development of a sheller for Bambara groundnuts. Master of Philosophy Dissertation, University of KwaZulu-Natal.

Nyamador, S. W., Mondedji, A. D., Kasseney, B. D., Ketoh, G. K., Koumaglo, H. and Glitho, I. 2017. Insecticidal activity of four essential oils on the survival and oviposition of two sympatric bruchid species: *Callosobruchus maculatus* F. and *Callosobruchus subinnotatus* PIC. (Coleoptera: Chrysomelidea: Bruchinae). *Journal of Stored Production*, 8: 103-112.

Ojiewo, C., Keatinge, D., Hughes, J., Tenkouano, A., Nair, R., Varshney, R., Siambi, M., Monyo, E., Ganga-Rao, N. and Silim, S. 2015. The role of vegetables and legumes in assuring food, nutrition, and income security for vulnerable groups in Sub-Saharan Africa. *World Medical and Health Policy*, 7(3): 187-210.

- Olanrewaju, O., Oyatomi, O., Babalola, O. and Abberton, M. 2022. Breeding potentials of Bambara groundnut for food and nutrition security in the face of climate change. *Frontiers in Plant Science*, 12: 1-14.
- Oljira, T., Muleta, D. and Jida, M. 2018. Potential applications of some indigenous bacteria isolated from polluted areas in the treatment of brewery effluents. *Biotechnology Research International*, 2018: 1-13.
- Oluwole, O. B., Nicholas-Okpara, V. A. N., Elemo, G., Adeyoju, O., Ibekwe, D. and Adegboyega, M. O. 2021. Medicinal uses, nutraceutical potentials and traditional farm production of Bambara beans and Pigeon pea. *Global Journal of Epidemiology Public Health*, 6: 41-50.
- Omara, A., Hauka, F., Aida, A., Nour El-Din. and Kassem, M., 2017. The role of some PGPR strains to biocontrol *Rhizoctonia solani* in soybean and enhancement the growth dynamics and seed yield. *Environment, Biodiversity and Soil Security*, 1: 47-59.
- Oruru, M. B., Njeru, E. M., Pasquet, R. and Runo. S. 2018. Response of a wild-type and modern cowpea cultivars to arbuscular mycorrhizal inoculation in sterilized and non-sterilized soil. *Journal of Plant Nutrition*, 41: 90-101.
- Oteino, N., Lally, R. D., Kiwanuka, S., Lloyd, A., Ryan, D., Germaine, K. J. and Dowling, D. N. 2015. Plant growth promotion induced by phosphate solubilizing endophytic pseudomonas isolates. *Frontiers in Microbiology*, 6: 2-10.
- Ouoba, A., Zida, E., Soalla, R., Bangratz, M., Essowè, P., Konaté, M., Konaté, M., Nandkangré, H., Ouédraogo, M. and Sawadogo, M. 2019. Molecular characterization of the main fungi associated to Bambara groundnut foliar diseases in Burkina Faso. *Journal of Applied Biosciences*, 133(1): 13574.
- Parmar, P. and Sindhu, S. S. 2018. The novel and efficient method for isolating potassium solubilizing bacteria from rhizosphere soil. *Geomicrobiology Journal*, 36(2): 130-136.

- Parmar, P. and Sindhu, S. S. 2013. Potassium solubilization by rhizosphere bacteria: influence of nutritional and environmental conditions. *Journal of Microbiology Research*, 3: 25-31.
- Pardamean, M., Islamy, R., Hasan, V., Herawati, E. and Mutmainnah, N. 2021. Identification and physiological characteristics of potential indigenous bacteria as bio-remediation agent in the wastewater of sugar factory. *Sains Malaysiana*, 50(2): 279-286.
- Pérez-Montañó, F., Alías-Villegas, C., Bellogín, R. A., del Cerro, P., Espuny, M. R., Jiménez-Guerrero, I., López-Baena, F. J., Ollero, F. J. and Cubo, T. 2014. Plant growth promotion in cereal and leguminous agricultural important plants: From microorganism capacities to crop production. *Microbiological Research*, 169 (5-6): 325-336.
- PlantWork. 2013. Plant growth promoting rhizobia (PGPR). [Online]. Available at: <https://smart.plantworksuk.co.uk/about-us/> [Accessed 18 May 2021].
- Quarashi, A. W. and Sabn, A. N. 2012. Bacterial exopolysaccharide and biofilm formation in chickpea. *Brazilian Journal of Microbiology*, 43: 1183-1191.
- Ramadan, E. M., Abdelhafez, A. A., Hassan, E. A. and Saber, F. M. 2016. Plant growth promoting rhizobacteria and their potential for biocontrol of phytopathogens. *African Journal of Microbial Research*, 10: 486-504.
- Rao, M. B., Tanksale, A. M., Ghatge, M. S. and Deshpande, V. V. 1998. Molecular and biotechnological aspects of microbial proteases. *Microbiology and Molecular Biology Reviews*, 62: 597-635
- Rathore, P. 2015. A review on approaches to develop plant growth promoting rhizobacteria. *Journal of Plant Physiology*, 176: 47-54.
- Rodiño, A., Riveiro, M. and De Ron, A. 2020. Implications of the symbiotic nitrogen fixation in common bean under seasonal water stress. *Agronomy*, 11(1): 70.

- Rueda, D., Valencia, G., Soria, N., Rueda, B. B., Manjunatha, B., Kundapur, R. R. and Selvanayagam, M. 2016. Effect of *Azospirillum* spp. and *Azotobacter* spp. on the growth and yield of strawberry (*Fragaria vesca*) in hydroponic system under different nitrogen levels. *Journal of Applied Pharmaceutical Science*, 6: 48-54.
- Santana, E. B., Marques, E. L. S. and Dias, J. C. T. 2016. Effects of phosphate-solubilizing bacteria, native microorganisms, and rock dust on *Jatropha curcas* L. growth. *Genetics and Molecular Research*, 15 (4): 1-18.
- Satyanarayana, S., Krishna, M. and Kumar, P. 2018. Identification of upregulated nitrogen fixing bacteria for *Arachis hypogaea* by exploring natural combination: A physical, biochemical, and in silico approach. *Journal of Pure and Applied Microbiology*, 12(1): 73-83.
- Satyaprakash, M., Nikitha, T., Reddi, T. E. U., Sadhana, B. and Vani, S. S. 2017. “A review on phosphorous and phosphate solubilising bacteria and their role in plant nutrition. *International Journal of Current Microbiology and Applied Sciences*, 6: 2133-2144.
- Seidl, V. 2008. Chitinases of filamentous fungi: a large group of diverse proteins with multiple physiological functions. *Fungal Biology Reviews*, 22: 36-42.
- Shandilya, C., Kumar, N., Shrivastava, N., Varma, A. and Vishwakarma, K. 2021. Tools for characterization of nitrogen fixing microbes. In: Cruz, C., Vishwakarma, K., Choudhary, D. K. and Varma, A. eds. *Soil nitrogen ecology*. Cham: Springer.
- Sharma, S. B., Sayyed, R. Z., Trivedi, M. H. and Gobi, T. A. 2013. Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. *Springerplus*, 2: 587.
- Sharma, V., Kaur, J. and Sharma, S. 2020. Plant growth promoting rhizobacteria: potential for sustainable agriculture. *Biotechnología Vegetal*, 20 (3): 157-166.

- Shrestha, A., Kim, B. S. and Park, D. H. 2014. Biological control of bacterial spot disease and plant growth-promoting effects of lactic acid bacteria on pepper. *Biocontrol Science and Technology*, 24(7): 763-779.
- Siddiqui, Y., Meon, S., Ismail, R., Rahmani, M. and Ali, A. 2008. Bio-efficiency of compost extracts on the wet rot incidence, morphological and physiological growth of okra (*Abelmoschus esculentus*) [(L.) Moench]. *Scientia Horticulturae*, 117: 9-14.
- Sinyanga, K. Y. 2016. Phenotypic characterization of rhizobia isolates and distribution of Burkholderia rhizobia in the Core Cape Subregion. Master of Science. University of Cape Town.
- Sissay, B., Adesola, O., Massia, J. and Taddesse, W. 2015. Microbial and physico-chemical evaluation of soils from different farming systems practicing fields in Lesotho and the adaptive capacity of Machobane Farming System to climate change. *African Journal of Agricultural Research*, 10(40): 3851-3859.
- Sivasakthi, S., Usharani, G. and Saranraj, P. 2014. Biocontrol potentiality of plant growth promoting bacteria (PGPR) – *Pseudomonas fluorescens* and *Bacillus subtilis*: a review. *African Journal of Agricultural Research*, 9: 1265-1277
- Sita, K., Sehgal, A., HanumanthaRao, B., Nair, R., Vara Prasad, P., Kumar, S., Gaur, P., Farooq, M., Siddique, K., Varshney, R. and Nayyar, H. 2017. Food legumes and rising temperatures: effects, adaptive functional mechanisms specific to reproductive growth stage and strategies to improve heat tolerance. *Frontiers in Plant Science*, 8: 1-31.
- Soumare, A., Boubekri, K., Lyamlouli, K., Hafidi, M., Ouhdouch, Y. and Kouisni, L. 2020a. From isolation of phosphate solubilizing microbes to their formulation and use as biofertilizers: Status and Needs. *Frontiers in Bioengineering and Biotechnology*, 7: 4-18.

- Soumare, A., Diedhiou, A., Thuita, M., Hafidi, M., Ouhdouch, Y., Gopalakrishnan, S. and Kouisni, L. 2020b. Exploiting biological nitrogen fixation: a route towards a sustainable agriculture. *Plants*, 9(8): 1011.
- Souza, R. D., Ambrosini, A. and Passaglia, L. M. 2015. Plant growth-promoting bacteria as inoculants in agricultural soils. *Genetic Molecule Biology*, 38: 401-419.
- Suzuki, N., Rivero, R. M., Shulaev, V., Blumwald, E. and Mittler, R. 2014. Abiotic and biotic stress combinations. *New Phytologist*, 203: 32-43.
- Tabassum, B., Khan, A., Tariq, M., Ramzan, M., Khan, M. S. I., Shahid, N., Aaliya, K. and Khan, I. 2017. Bottlenecks in commercialisation and future prospects of PGPR. *Applied Soil Ecology*, 121: 102-117.
- Tamagno, S. and Ciampitti, I. 2017. Seed yield and biological nitrogen fixation for historical soybean genotypes. *Kansas Agricultural Experiment Station Research Reports*, 3(6): 2-8.
- Tan, X., Azam-Ali, S., Goh, E., Mustafa, M., Chai, H., Ho, W., Mayes, S., Mabhaudhi, T., Azam-Ali, S. and Massawe, F. 2020. Bambara Groundnut: an underutilized leguminous crop for global food security and nutrition. *Frontiers in Nutrition*, 7: 3-5.
- Temegne, N. C. 2018. Improvement in the performances of Voandzou (*Vigna subterranea* (L.) Verdc.) In response to phosphate deficiency through chemical and biological fertilization. Doctor of Philosophy Dissertation, University of Yaounde.
- Trumbore, S. 2006. Carbon respired by terrestrial ecosystems – recent progress and challenges. *Global Change Biology*, 12: 141-153.
- Unigwe, A., Gerrano, A., Adebola, P. and Pillay, M. 2016. Morphological variation in selected accessions of Bambara groundnut (*Vigna subterranea* L. Verdc) in South Africa. *Journal of Agricultural Science*, 8: 69-80.

- Udeh, E. L., Nyila, M. A. and Kanu, S. A. 2020. Nutraceutical and antimicrobial potentials of Bambara Groundnut (*Vigna Subterranean*): A Review. *Heliyon*, 6(10): 1-5.
- Valentine, A. J., Benedito, V. A. and Kang, Y. 2018. Legume nitrogen fixation and soil abiotic stress: from physiology to genomics and beyond. In Foyer, C. H. and Zhang, H. eds. *Annual Plant Reviews online*. Hoboken: Wiley-Blackwell.
- Verma, M., Brar, S. K., Tyagi, R. D., Surampalli, R. Y. and Valero, J. R. 2007. Antagonistic fungi, *Trichoderma* sp. panoply of biological control. *Biochemical Engineering Journal*, 37: 1-20.
- Walker, L., Lagunas, B. and Gifford, M. 2020. Determinants of host range specificity in legume-rhizobia symbiosis. *Frontiers in Microbiology*, 11: 1-10.
- Walpola, B. C. and Yoon, M. 2012. "Prospectus of phosphate solubilizing microorganisms and phosphorus availability in agricultural soils: a review. *African Journal of Microbiology Research*, 6: 6600-6605.
- Wang, T. T., Ding, P., Chen, P., Xing, K., Bai, J. L., Wan, W., Jiang, J. H. and Qin, S. 2017. Complete genome sequence of endophyte *Bacillus flexus* KLBMP 4941 reveals its plant growth promotion mechanism and genetic basis for salt tolerance. *Journal of Biotechnology*, 260: 38-41.
- Yuan, J., Ruan, Y., Wang, B., Zhang, J., Waseem, R., Huang, Q. and Shen, Q. 2013. Plant growth-promoting rhizobacteria strain *Bacillus amyloliquefaciens* NJN-6-enriched bio-organic fertilizer suppressed Fusarium wilt and promoted the growth of banana plants. *Journal of Agriculture and Food Chemistry*, 61: 3774-3780.
- Zahid, M., Abbasi, M., Hameed, S. and Rahim, N. 2015. Isolation and identification of indigenous plant growth promoting rhizobacteria from Himalayan region of Kashmir and their effect on improving growth and nutrient contents of maize (*Zea mays* L.). *Frontiers in Microbiology*, 6: 1-10.

- Zahran, H. H. 2001. Rhizobia from wild legumes: diversity, taxonomy, ecology, nitrogen fixation and biotechnology. *Journal of Biotechnology*, 91: 143-153.
- Zhang, C. and Kong, F. 2014. Isolation and identification of potassium-solubilizing bacteria from tobacco rhizospheric soil and their effect on tobacco plants. *Applied Soil Ecology*, 8: 18-25.

CHAPTER THREE

INCIDENCE AND DIVERSITY OF RHIZOBACTERIA ASSOCIATED WITH COMMUNALLY PRODUCED BAMBARA GROUNDNUT ROOT NODULES IN LIMPOPO, KWAZULU-NATAL, AND MPUMALANGA PROVINCE AND THEIR N- CYCLING AND P-CYCLING EFFICIENCY

3.1. Introduction

Rhizobacteria are bacteria that are found in the rhizosphere and have a positive effect on the plant's growth and development (Bontemps *et al.*, 2015). These bacteria occur either in the root nodules or free-living, both groups are reported to stimulate the health and growth of plants, hence referred to as plant growth promoting rhizobacteria (PGPR) (Khan, Sayyed, & Seifi, 2019). The rhizobacteria usually supply plants with nutrients, suppress activities of plant pathogens, and nutrient cycling (Jeyanthi & Kanimozhi, 2018), improving soil structure, bioaccumulation, and microbial leaching of inorganic compounds. Rhizobia symbionts involved in nitrogen fixation are usually capable of inducing the formation of stem or root nodules on leguminous plants in which atmospheric N is primarily reduced to ammonia for plant benefit (Jeyanthi & Kanimozhi, 2018). The distribution and diversity of rhizobia are affected greatly by the geographical locality and determining their phylogeny could highlight their evolutionary origin (Liu, Xiong, Wu, Ling & Kong, 2023). Taxonomically, the diverse heterogenous groups of rhizobia comprise the Alpha-proteobacteria and Beta-proteobacteria group. The alpha group forms the majority of the rhizobia species which includes *Allorhizobium*, *Azorhizobium*, *Blastobacter*, *Bradyrhizobium*, *Mesorhizobium*, *Methylobacterium*, *Rhizobium*, and *Sinorhizobium* (Koskey *et al.*, 2018; Lemaire *et al.*, 2014). Some legumes such as those in the large genus *Mimosa*, are nodulated predominately by members of the class Betaproteobacteria in the genera *Burkholderia* and *Cupriavidus* (Lemaire

et al., 2014). However, there are other nitrogen-fixing bacteria, which have been recently identified from beta and gamma Proteobacteria, that form symbiotic relationships with legumes (Jeyanthi & Kanimozhi, 2018).

Bambara groundnut is the third most important grain legume indigenous to Africa after cowpea (*Vigna unguiculata*) and groundnut (*Arachis hypogaea*) and belongs to the family Leguminosae (DAFF, 2016). In Limpopo, Mpumalanga, and KwaZulu-Natal province, South Africa, Bambara groundnut has gained prominence as an alternate dietary protein, carbohydrate, fat, and fiber source with several agronomic advantages to communal farmers (Onyango & Ogolla, 2019; Sprent, Odee & Dakora, 2010). Moreover, it is drought tolerant and grown in intercropping systems with zero fertilizer added because of its ability to form symbiotic associations with root nodule bacteria (Onyango & Ogolla, 2019). Information on rhizobia symbiotic to Bambara groundnut in the soil of this region is largely unexplored yet they have a great potential in soil fertility management and in improving crop yield (Puozaa, Jaiswal & Dakora, 2017). In the last few years, few studies investigating rhizobia isolated from legume crops in South Africa have revealed considerable phenotypic and genotypic diversity among strains, and several distinct groups have been identified and novel species described. Studies on Bambara groundnut symbioses in other parts of the world have indicated that it is non-selective in its rhizobia nodulating bacteria (Onyango *et al.*, 2015). Sprent *et al.* (2010), listed five α -proteobacteria members, including *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Ensifer*, and *Mesorhizobium*, as possible nodulators of Bambara groundnuts (Lemaire *et al.*, 2015). Mohale, Belane and Dakora (2013) reported the highly ‘promiscuous’ nature of Bambara groundnuts forming a nitrogen-fixing symbiosis with a wide range of bacteria, including some members of the β -proteobacteria such as the N-fixing *Burkholderia*. The present study aimed to determine the incidence and diversity of rhizobacteria associated with

communally produced Bambara groundnut roots in Mpumalanga, KwaZulu-Natal, and Limpopo province and their N-cycling and P-cycling ability.

3.2. Material and methods

3.2.1. Sample collection

Bambara groundnut root nodules were collected from farming fields in Mpumalanga and Limpopo Province (Table 3.1). Due to financial constraints only, soil samples were collected in KwaZulu-Natal province at the University of Zululand and Nhlangenyuke, in fields with long history of growing Bambara groundnuts, and taken to the University of Mpumalanga farm, Mbombela campus, South Africa for planting, under greenhouse conditions (Table 3.1). The temperature and humidity in the greenhouse were set at 25 ± 2 °C and 70 ± 10 %, respectively.

Table 3.1: Bambara groundnut root nodule and soil sample site in Mpumalanga, KwaZulu-Natal, and Limpopo province.

Province	Sample site	GPS coordinate
Mpumalanga	Boschfontein	-25° 73' 17" S 31° 60 '41 "E
	Hlamalani	-24° 77' 05.37" S 31° 05' 93.17" E
	Casteel	-24° 73' 87.13" S 31° 02' 27.23" E
	Nkomazi	-25° 73' 26" S 31° 64 '91" E
	University of Mpumalanga	25° 27' 06.18" S 30 °58 '5.21" E
	Bushbuckridge	-24° 46' 31.7" S 31° 08' 13.0" E
	Hazyview	-25° 14' 20.5" S 31° 01' 49.5" E
	Mkhuhlu	24° 57' 50" S 31° 18' 43" E
KwaZulu-Natal	University of Zululand	27° 88' 72" S 31° 44' 56" E
	Nhlangenyuke	-28° 52' 33.2" S 31° 45' 46.8" E

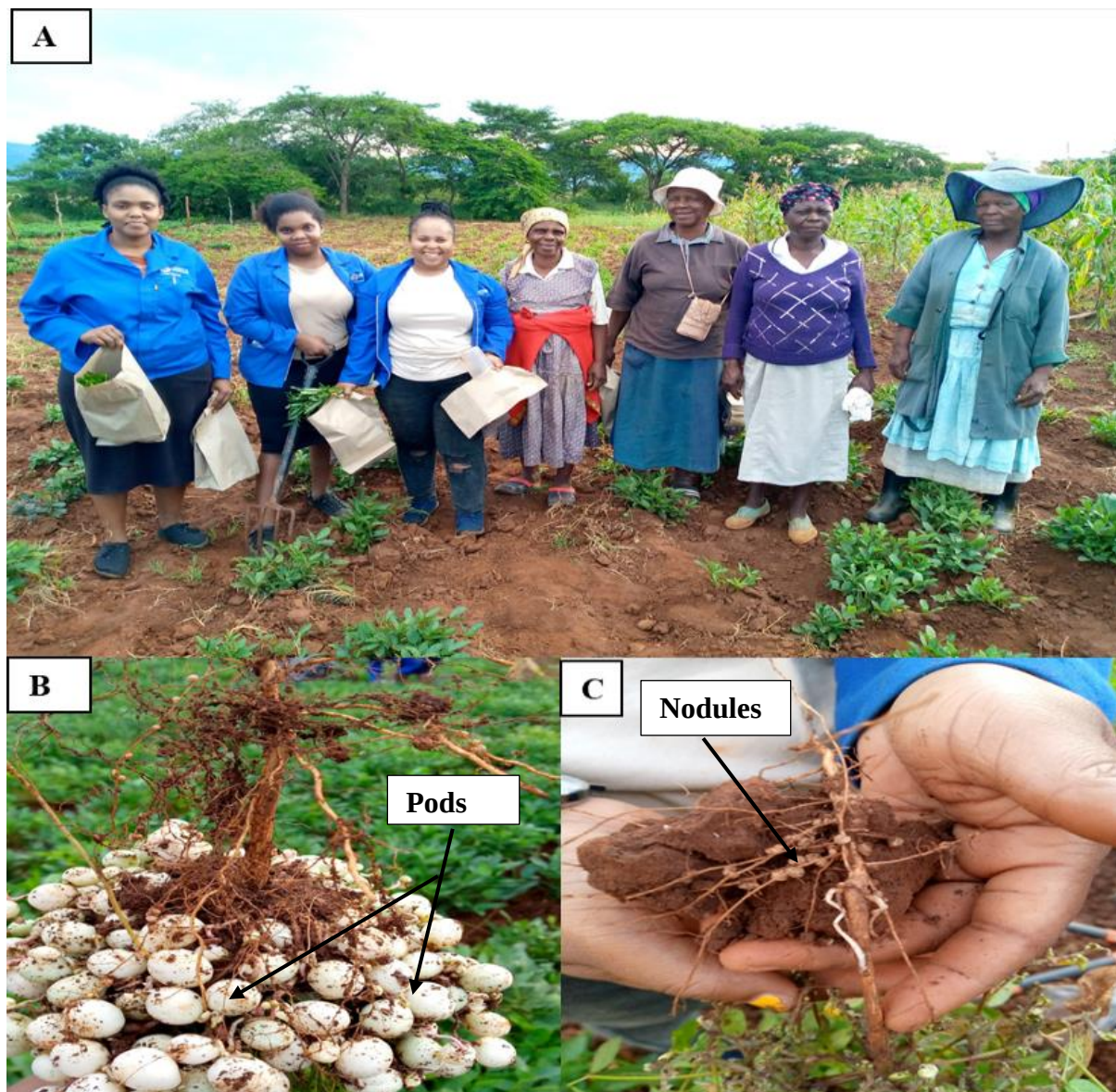


Figure 3.1: Farmers at Gabaza village, Limpopo province (A); Bambara groundnut pods (B); roots nodules (C).

3.2.2. Planting procedure

Due to management challenges, KwaZulu Natal province soils where Bambara groundnuts are grown were collected and brought to the University of Mpumalanga farm. Twenty-five-centimetre diameter pots were filled with the collected soil and four seeds of farmer-retained

Bambara groundnut seeds were sown in each pot and then irrigated with 300 ml of tap water every other day. Thinning was done at two true-leaf stages to leave one healthy and vigorous plant per pot (Figure 3.2B).



Figure 3.2: Bambara groundnut farm at Boschfontein, Mpumalanga province (A); Growth of BG plants on soil collected from KZN (B); Abundance of root nodules (C).

3.2.3. Data collection

At 56 days after sowing during flowering stage, the roots of Bambara groundnut plants were removed from the pots, immersed in sterile distilled water to wash off soil particles, and then blotted dry using a paper towel (Figure 3.2C). The root nodules that were intact, mostly pinkish, and fresh from greenhouse (Figure 3.1B) and those collected from the fields (Figure 3.1A) were taken to the University of Mpumalanga laboratory for further tests.

3.2.4. Sterilization and cleaning of root nodules

The research study was conducted under aseptic environmental conditions in a laminar-flow cabinet. Lamina flow surface disinfecting was done using 70 % ethanol for 15 minutes before work commencement. All equipment to be used, such as inoculation loops, blades, and forceps were surface sterilized employing flaming over the Bunsen burner.

3.2.5. Preparation of NA media

A 28 g of nutrient Agar was weighed and then dissolved in 1000 mL of distilled water and autoclaved at 121°C at the University of Mpumalanga Biology laboratory, Block D (Figure 3.3) (Lawless *et al.*, 2018).

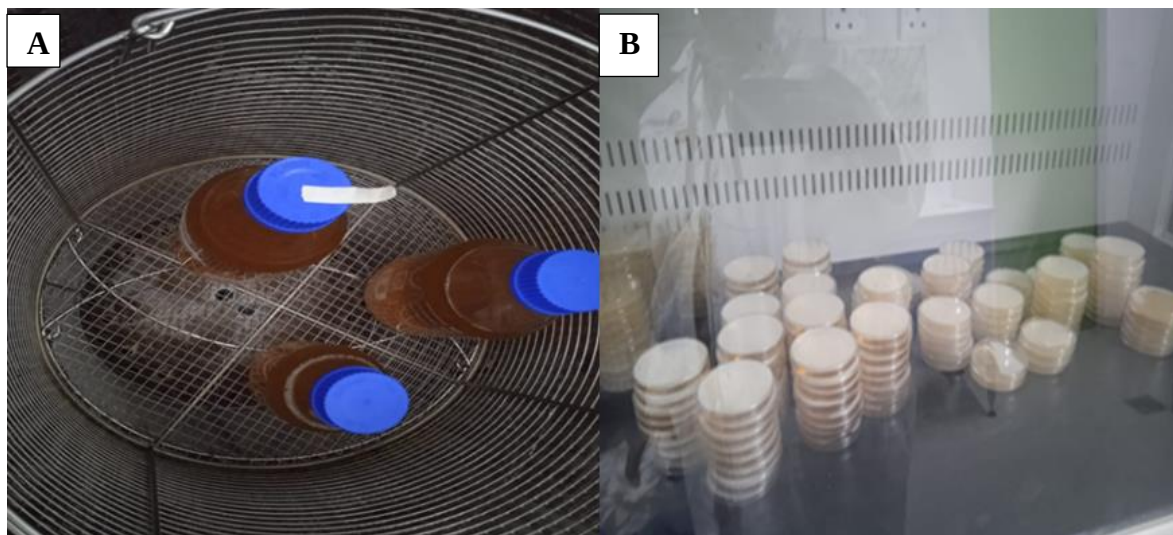


Figure 3.3: Prepared NA in an autoclave (A); NA poured into 900 mm Petri dish in the Laminar flow cabin (B).

3.2.6. Root nodule bacteria extraction and identification

All root nodules were collected at the plant flowering stage, with intact and juvenile nodules carefully removed from plant roots (Figure 3.4B) before surface sterilizing them with 70 % (v/v) alcohol for 45 seconds, followed by 3.5 % (v/v) sodium hypochlorite (NaOCI) for three minutes (Figure 3.4B). Surface sterilised root nodules were then rinsed 6 times in sterilized and double distilled water, to remove excess alcohol (Hassen, Vuuren, Bopape & Gerrano, 2022). Surface sterilized nodules were squashed in distilled water (50 μ L/nodule) using a sterile homogenizer. A loopful of suspension from each crushed nodule was streaked onto NA media. The plates were incubated at 30 °C for 3 to 7 days (Vincent, 1970) with the growth of the colony

monitored daily. After growth, single colonies were picked and then purified by repeat streaking.

Bacterial isolates were named using the collection site as prefix (GAB = Gabaza, ZULU = University of Zululand, HLAM = Hlamalani, CAST = Casteel, BUSH = Bushbuckridge, BF = Boschfontein, NHLANG = Nhlangenyuke, UMP = University of Mpumalanga, NK = Nkomazi, HAZYW = Hazyview, and MKHLU = Mkhuhlu) (Figure 3.4C)

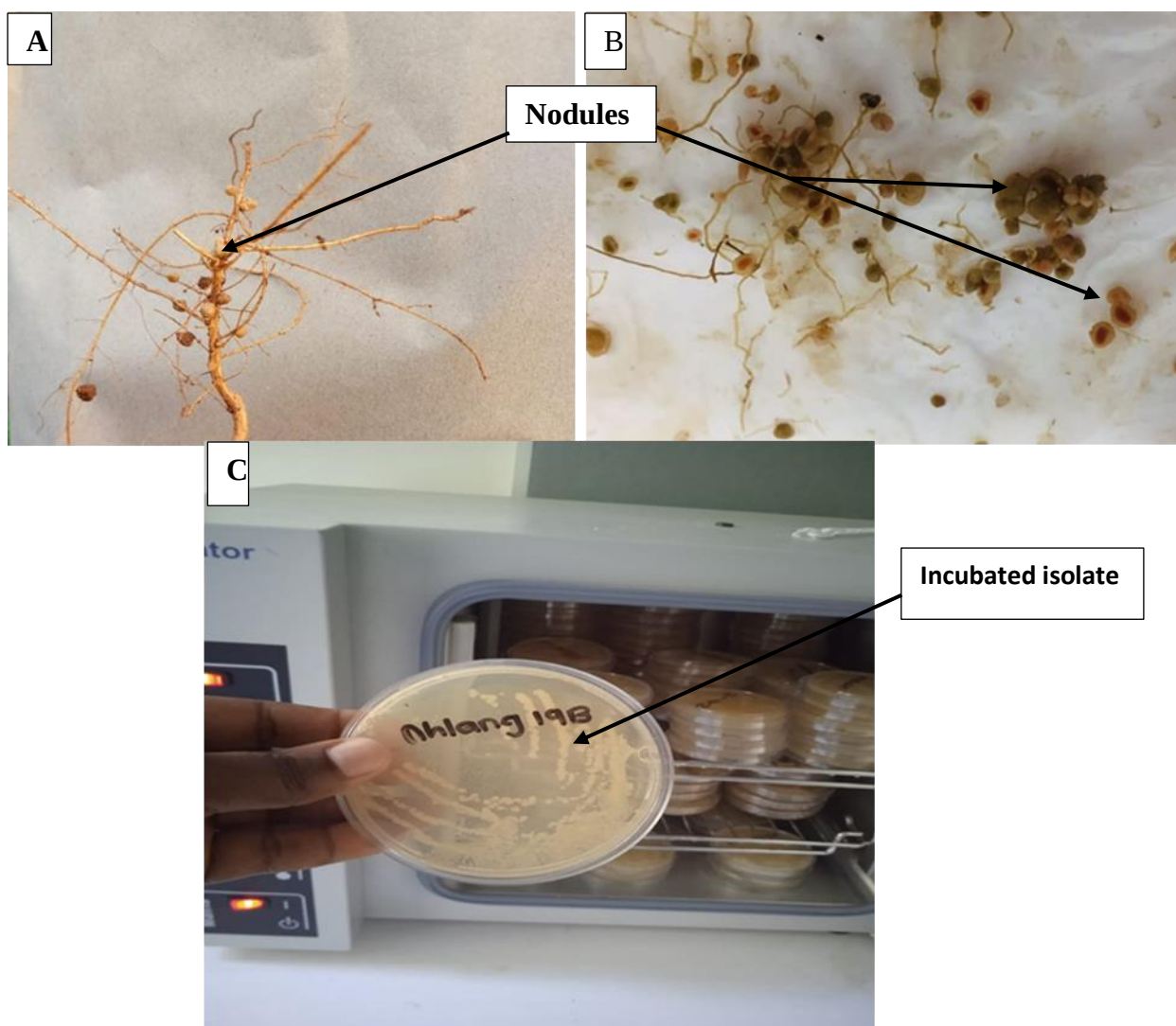


Figure 3.4: Root nodules (A); Nodules removed from Bambara groundnut roots (B); Isolate incubation for bacteria growth (C).

3.2.7. Morphological characterization

The morphological characterization of the isolates was performed after having grown pure bacterial isolate for 2-3 days on NA medium as described by Shirling and Gottlieb (1966) and Somasegaran and Hoben (1994). A single colony of each isolate was characterized based on colony appearance; texture, shape of colony surface, margin, elevation, and colour of the isolate's colonies under a light microscope (Shirling & Gottlieb, 1966).

3.2.8. Molecular identification of isolates

Total DNA was obtained by extraction with RNase treatment and Phenol-chloroform from the pure culture in the phase of exponential growth in NA medium. The isolation of DNA pure culture was carried out according to a method developed by Chen & Kuo (1993) in a volume of 300µL of bacterial lysis buffer: (40mM Tris-acetate (pH of 7.8) and RNase at 20mg/ml) and 100 µL of 5M NaCl. After purification with Phenol-Chloroform mixture (v/v), the pellet resultant from the centrifugation was surfaced and sterilized with 70% and 100% ethanol, then suspended in 55µL of TE (pH 7.8, 10mM Tris, 1mM EDTA) and stored at -20°C. The quality and quantity of the DNA extract were evaluated using the Nanodrop™ Spectrophotometer at an absorbance of 260nm (DNA) and 280 nm (Protein).

The genomic DNA was extracted from the culture received using the Quick-DNA™ bacterial Miniprep kit (NEB, Catalogue No. M0486) performed using the following primers: 16S-27F Forward primer (5' AGAGTTTGATCMTGGCTCAG 3') and 16S-1492R Reverse primer (5' CGGTTACCTTGTTACGACTT 3'). The PCR amplification runs on a gel, the gel is extracted with the Zymoclean™ Gel DNA recovery kit (Zymo Research, Catalogue No. D4001). The size of the amplicons is 1550 bp long. The PCR reaction was prepared as a standard 25µL reaction containing 12.5µL of 2X DreamTaq Green Master Mix; 0.25µL of each oligonucleotide primer (27F and 1492R); 11µL RNase-DNase free water and 1µL template

DNA. All PCR reagents used were Fermentas, USA products supplied by Inqaba Biotechnical Industry Ltd, Sunnyside, South Africa. PCR amplification was performed using the C1000 Touch™ Thermal Cycler (Bio-Rad, Hercules, California, USA) using initial denaturing (95 °C for 5 minutes), denaturing (95 °C for 1 minute), annealing (55 °C for minute), extension (72 °C for 1 minute) and final extension (72 °C for 10 minutes). All PCR products were resolved by 2 % agarose gel electrophoresis and then stored at 4 °C for future use. The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, and BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D40500). The purified fragments were then analyzed on the ABI 3500xl Genetic Analyser (Applied Biosystems, ThermoFisher Scientific) for each reaction for every sample. CLC bio-Main workbench v7.6 was used to analyze files that are generated by the ABI 3500XL Genetic Analyser and then subjected to BLAST search (NCBI; <http://www.ncbi.nlm.nih.gov/BLAST/>).

3.2.9. Sequence alignment and phylogenetic analyses

The 16S gene sequencing quality of each bacterial isolate was then verified using Chromas LITE version 2.1, Nucleotide sequences were then analyzed and edited using BioEdit software to obtain the consensus sequence (Normand, Ponsonnet, Nesme, Neyra & Simmonet, 1996). Similar sequence for each different haplotype was searched in a GenBank database using the BLAST program for sequence similarity (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). A maximum likelihood (ML) approach was achieved using software MEGA version 6 (Tamura, Stecher, Peterson, Filipski & Kumar, 2013), which includes the choice of the best model of molecular evolution implemented in MEGA, was applied. Evolutionary histories were inferred using the Maximum Composite Likelihood (Tamura & Kumar, 2004). Bootstrap test (1000 replicates) was used to cluster associated taxa and replicate trees with above 50 % likelihoods indicated

on the branches (Altschul, Gish, Miller, Myers & Lipma, 1990). All the trees were drawn to scale, with some branch lengths in the same units as those of the evolutionary distance used to infer the phylogenetic tree.

3.2.11. Screening of isolates for plant growth-promoting (PGPR) traits

Two growth-promoting traits were investigated because of their importance in South African soils, nitrogen, and phosphorus solubilization. To determine the isolated bacteria's nitrogen cycling efficiency, they were grown on Simmons citrate agar (SCA) containing citrate as a carbon (C) source and inorganic ammonium salts as the only N source (Hill *et al.*, 1967; cited in Ndlovu, Suinyuy, Pérez-Fernández & Magadlela, 2023). A single colony was picked in each isolate and slightly streaked on the slant surface. Three replications were made for each isolate. The nitrogen cycling ability (Citrate positive) of the isolate was indicated by a visible intense Prussian blue colour change on bacteria growing on the surface of the slanted media (Figure 3.16). Citrate negative or no nitrogen cycling ability was indicated by no growth or a media retaining its deep green colour (Figure 3.16).

The phosphate solubilization ability of bacteria was tested by spotting 10 µL of each bacterial isolate on Pikovskaya's agar plates and then incubating at 28 °C for seven days as described by Nautiyal (1999). Phosphate solubilization (PS) activity was observed as a clear zone around the bacterial colonies, while no zone was considered negative activity (Suleman *et al.*, 2018).

3.2.10. Data analysis

Diversity, evenness, and richness of species were measured using the Simpson index (D) (Simpson, 1949) and Shannon-Weiner (H') index (Shannon & Weaver, 1949) to determine variation in the diversity of bacterial species among the different Bambara groundnut localities and the plant roots. Therefore, the diversity of species in a community was computed using the formula below:

$$H' = -\sum (p_i) [\ln(p_i)]$$

H' = Shannon-Wiener index of species diversity

$\ln$ = natural logarithm

p_i = proportion of total abundance represented by i^{th} species

The abundance of species was estimated using Pielou's evenness index (J) (Pielou, 1966).

Pielou's evenness index (J) was calculated using the below formula:

$$J = \frac{H'}{\ln(s)}$$

Where H' = Shannon-Wiener Index

s = number of species in a given area.

The Simpson Index (D) was used to measure the diversity which studies a number of species present and the relative abundance of each species. It was measured using the formula below:

$$D = 1 - \left(\frac{\sum n(n-1)}{N(N-1)} \right)$$

Whereby: n = total number of Bambara groundnuts of a particular species

N = the total number of Bambara groundnuts of all species.

3.3. Results

3.3.1. Morphological characterization of isolates

The colony colour of the isolates varied, it included yellow, bright yellow, golden yellow, cream, cream white, white, opaque, red, orange, bright orange, and brown observed which had either rough or smooth surfaces (Appendix 3.1; Figure 3.5). Colony margins varied from entire, irregular, lobate, undulate, serrated, curled, or filamentous (Appendix 3.1). The colony shapes were round, irregular, filamentous, punctiform, rhizoid, and curled (Appendix 3.1 and Figure 3.5). A total of 209 rhizobacterial strains were obtained from the interior of Bambara groundnut root nodules. Among the 209 isolates, 43 unique isolates were found in all three provinces (Appendix 3.1).

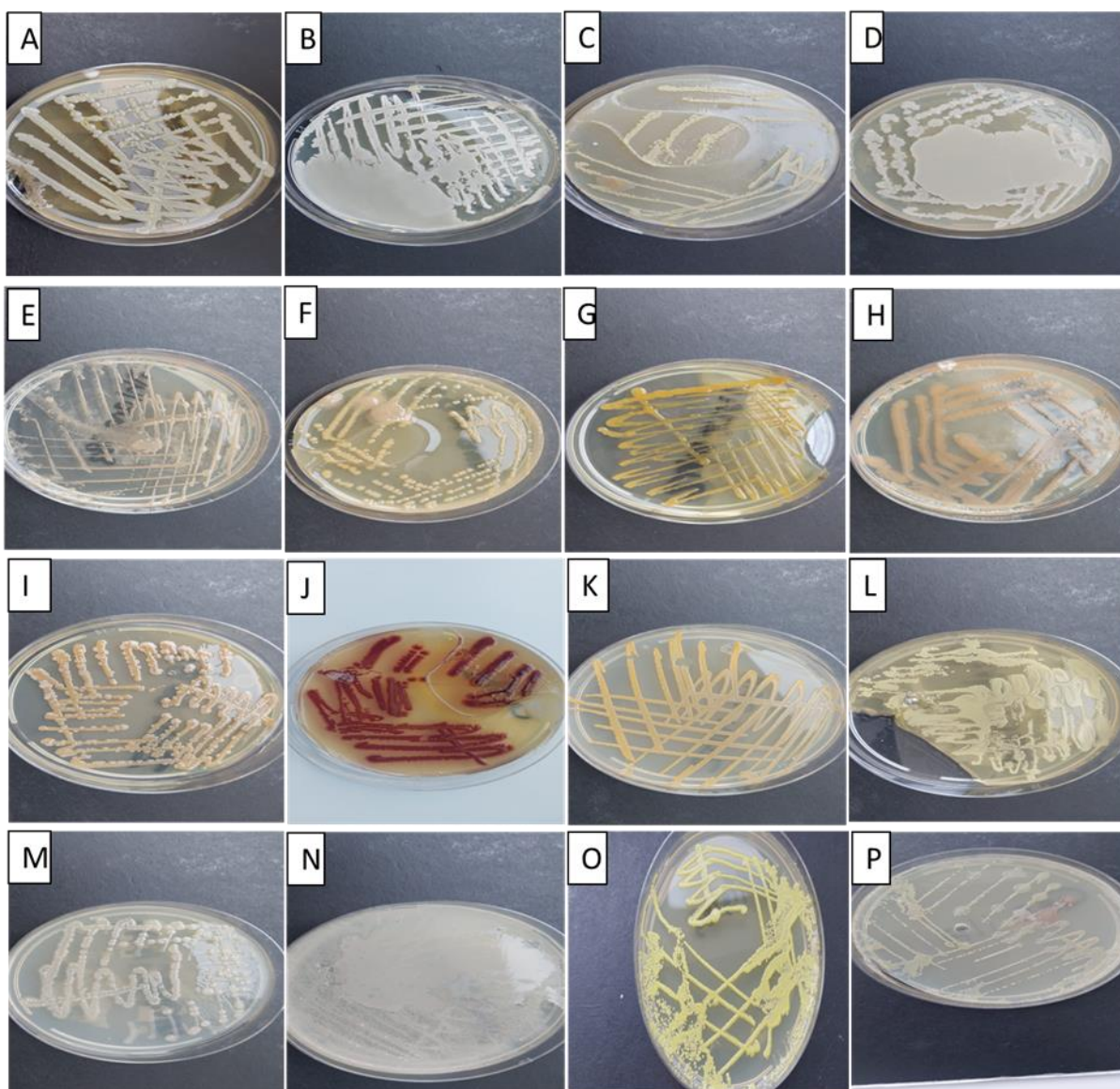


Figure 3.5: Bambara groundnut selected rhizobacterial isolates on Nutrient Agar.

3.3.2. Molecular identification of selected isolates

BLASTn search of the 16S rRNA sequence on the NCBI GenBank database resulted in 12 different rhizobia belonging to the genera *Enterobacter*, *Leucobacter*, *Bacillus*, *Spingobacterium*, *Lysinibacillus*, *Stenotrophomonas*, *Neorhizobium*, *Cellulosimicrobium*, *Kaistella*, *Proteus*, *Micrococcus*, and *Mammalicoccus* (Table 3.2). From each genus bacterial species that were obtained in this study were *Enterobacter absuriae* 22 (14.38 %), *Leucobacter chromiiresistens* 19 (12.42 %), *Bacillus pumilus* 18 (11.76 %), *Spingobacterium faecium* 14

(9.15 %), *Stenotrophomonas lactitubi* 13 (8.50 %), *Stenotrophomonas pavanii* 11 (7.19 %), *Stenotrophomonas maltophilia* 10 (6.54 %), *Lysinibacillus sphaericus* 10 (6.54 %), *Bacillus licheniformis* 9 (5.88 %), *Cellulosimicrobium cellulans* 7 (4.58 %), *Kaistella daneshvariae* 5 (3.27 %), *Stenotrophomonas geniculate* 4 (2.61 %), *Neorhizobium petrolierum* 4 (2.61 %), *Proteus columbae* 2 (1.31 %), *Micrococcus yunnanensis* 2 (1.31 %), *Lysinibacillus pakistanensis* 1 (0.65 %), *Mammalicoccus sciuri* 1 (0.65 %), and *Sphingobacterium multivorum* 1 (0.65 %) (Table 3.2). *Enterobacter absuriae* and *Leucobacter chromiiresistens* species were found in all sample sites, except for Hlamalani, Bushbuckridge, Hayview, and Mkhuhlu, while *Leucobacter chromiiresistens* species was not observed in Boschfontein and least species that were only found in one sample site were *Lysinibacillus pakistanensis*, *Mammalicoccus sciuri*, and *Sphingobacterium* (Table 3.2). *Lysinibacillus pakistanensis* rhizobia species was only found at Bushbuckridge (Table 3.2). *Mammalicoccus sciuri* and *Sphingobacterium multivorum* species were found only at the University of Zululand (Table 3.2). Twenty-two (22) isolates submitted to the GenBank showed 99.93 % homology with *Enterobacter absuriae* with accession number NZCP083403.1 (Table 3.2). Nineteen (19) isolates submitted to the GenBank had 86.83 % homology with *Leucobacter chromiiresistens*, accession number NZFNK301000001.1 (Table 3.2). Eighteen (18) isolates submitted to the GenBank had 90.08 % homology with *Bacillus pumilus*, accession number NZPTXV01000013.1 (Table 3.2). Fourteen (14) isolates submitted to the GenBank database showed 90.34 % homology with *Spingobacterium faecium* with accession number NZQBKH010000017.1 (Table 3.2). Thirteen (13) isolates submitted to the GenBank database indicated 78.33 % homology with *Lysinibacillus sphaericus* with accession number FJ528593.1 (Table 3.2). Thirteen (13) isolates submitted to GenBank showed 85.89 % homology with *Stenotrophomonas lactitubi* with accession number NZFZPB01000013.1 (Table 3.2). Eleven (11) isolates submitted to the GenBank database indicated 91.12 %

homology with *Stenotrophomonas pavanii* with accession number NZAP024684.1 (Table 3.2). Ten (10) isolates also submitted to the GenBank database showed 93.55 % homology with *Stenotrophomonas maltophilia* with accession number NZLS483377.1 (Table 3.2). Nine (9) isolates submitted to GenBank showed 81.22 % homology with *Bacillus licheniformis* with accession number NZCP014842.1 (Table 3.2). Seven (7) isolates submitted to GenBank showed 80.21 % homology with *Cellulosimicrobium cellulans* with accession number NZCP072387.1 (Table 3.2). Five (5) isolates submitted to the GenBank database showed 81.62 % homology with *Kaistella daneshvariae* species with accession number NZRJUG01000006.1 (Table 3.2). Four (4) isolates submitted to GenBank showed 93.97 % homology with *Stenotrophomonas geniculate* with accession number NZCP140571.1 (Table 3.2). Four (4) isolates submitted to the Genebank Database had 83.67 % homology with *Neorhizobium petrolierum* with accession number NZCP123000.1 (Table 3.2). Two (2) isolates each submitted to GenBank had 91.47 % and 87.60 % homology with *Proteus columbae* and *Micrococcus yunnanensis* with accession number NZNGVR010000010.1 and KT44390.1 (Table 3.2). Every single isolate submitted to the GenBank database had 78.93 %, 88.16 %, and 82.65 % homology with *Lysinibacillus pakistanensis*, *Mammalicoccus sciuri*, and *Sphingobacterium multivorum* with accession number NZCP126101.1, NZCP022046.1, and CP068088.1 (Table 3.2)

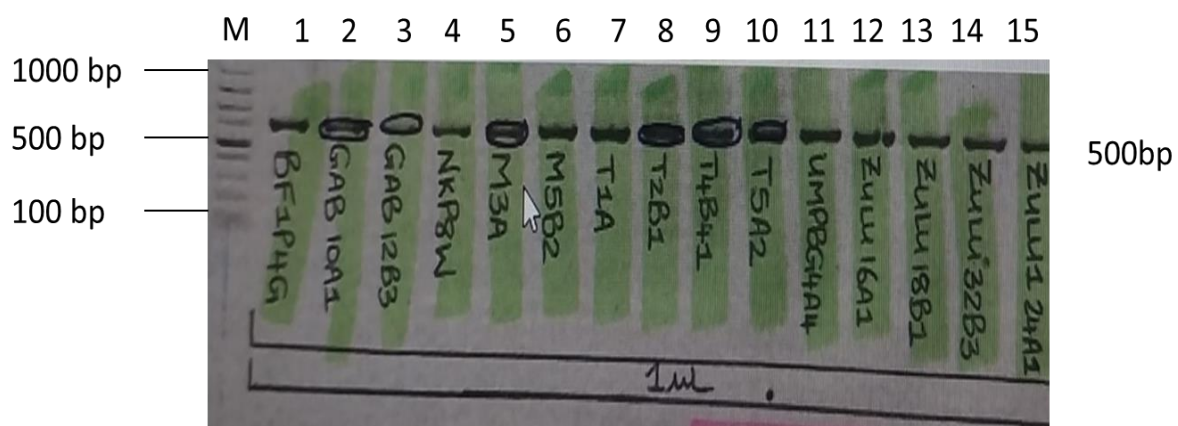


Figure 3.6: Agarose gel electrophoresis of the DNA pattern of rhizobia isolates; M (DNA marker); 1-15 (gene fragments of isolated bacteria from Bambara groundnut root nodules).

Table 3.2: Rhizobia isolates from Bambara groundnut root nodules showing sequence similarity (%) with the NCBI database bacterial strains after 16S rRNA gene sequencing

Isolates name	Accession no.	Probable organisms	%	E-value	Isolates name	GenBank Accession no.	Probable organisms	%	E-value
BF2P6G1	NZCP 014842.1	<i>Bacillus licheniformis</i>	81.22	0.0	BUSHP1P4	FJ528593.1	<i>Lysinibacillus sphaericus</i>	78.33	2e-151
BF2P3P	NZCP 014842.1	<i>Bacillus licheniformis</i>	81.22	0.0	BUSHP1P5	FJ528593.1	<i>Lysinibacillus sphaericus</i>	78.33	2e-151
BF1P11P	NZPTXV 01000013.1	<i>Bacillus pumilus</i>	90.08	0.0	HAZYW4B	NZLS 483377.1	<i>Stenotrophomonas maltophilia</i>	93.55	0.0
BF1P3P	NZPTXV 01000013.1	<i>Bacillus pumilus</i>	90.08	0.0	HAZYW4A	NZLS 483377.1	<i>Stenotrophomonas maltophilia</i>	93.55	0.0
BF1P3G	NZPTXV 01000013.1	<i>Bacillus pumilus</i>	90.08	0.0	MKHLUP1A1	NZCP 014842.1	<i>Bacillus licheniformis</i>	81.22	0.0

BF2P11G	NZPTXV 01000013.1	<i>Bacillus pumilus</i>	90.08	0.0
BF2P3G	NZPTXV 01000013.1	<i>Bacillus pumilus</i>	90.08	0.0
BF1P4G	NZPTXV 01000013.1	<i>Bacillus pumilus</i>	90.08	0.0
BF1P12P	NZPTXV 01000013.1	<i>Bacillus pumilus</i>	90.08	0.0
BF1P8G	NZAP 019630.1	<i>Enterobacter absuriae</i>	83.12	0.0
BF1P9G	NZAP 024684.1	<i>Stenotrophomonas pavanii</i>	91.12	0.0
BF1P12PA	NZAP 024684.1	<i>Stenotrophomonas pavanii</i>	91.12	0.0
BF1P13G	NZCP 140571.1	<i>Stenotrophomonas geniculate</i>	93.97	0.0

MKHLUP2A2	NZCP 014842.1	<i>Bacillus licheniformis</i>	81.22	0.0
MKHLUP2A1	NZFZPB 01000005.1	<i>Stenotrophomonas lactitubi</i>	85.89	0.0
MKHLUP2A3	NZFZPB 01000005.1	<i>Stenotrophomonas lactitubi</i>	85.89	0.0
ZULU30A4	NZPTXV 01000013.1	<i>Bacillus pumilus</i>	90.08	0.0
ZULU9A3	NZPTXV 01000013.1	<i>Bacillus pumilus</i>	90.08	0.0
ZULU12A3	NZPTXV 01000013.1	<i>Bacillus pumilus</i>	90.08	0.0
ZULU27B5	NZPTXV 01000013.1	<i>Bacillus pumilus</i>	90.08	0.0
ZULU9A1	NZCP 083403.1	<i>Enterobacter asburiae</i>	99.93	0.0

BF2P9G	NZFZPB 01000005.1	<i>Stenotrophomonas lactitubi</i>	85.89	0.0	ZULU9A8	NZCP 083403.1	<i>Enterobacter asburiae</i>	99.93	0.0
HLAM6B1	NZCP 014842.1	<i>Bacillus licheniformis</i>	81.22	0.0	ZULU18B4	NZCP 083403.1	<i>Enterobacter asburiae</i>	99.93	0.0
HLAM1B2	NZPTXV 01000013.1	<i>Bacillus pumilus</i>	90.08	0.0	ZULU30A3	NZCP 083403.1	<i>Enterobacter asburiae</i>	99.93	0.0
HLAM1A4	NZPTXV 01000013.1	<i>Bacillus pumilus</i>	90.08	0.0	ZULU4B4	NZCP 083403.1	<i>Enterobacter asburiae</i>	99.93	0.0
HLAM3B4	NZFNK 301000001.1	<i>Leucobacter chromiiresistens</i>	86.83	4e-177	ZULU11A2	NZCP 083403.1	<i>Enterobacter asburiae</i>	99.93	0.0
HLAM3B3	NZFNK 301000001.1	<i>Leucobacter chromiiresistens</i>	86.83	4e-177	ZULU27A5	NZCP 083403.1	<i>Enterobacter asburiae</i>	99.93	0.0
HLAM3B1	NZFNK	<i>Leucobacter chromiiresistens</i>	86.83	4e-177	ZULU27A1	NZFNK 301000001.1	<i>Leucobacter chromiiresistens</i>	86.83	4e-177

	301000001.1			
HLAM3B5	NZAP 024684.1	<i>Stenotrophomonas pavanii</i>	91.12	0.0
HLAM3B2	NZQBKH 01000017.1	<i>Sphingobacterium faecium</i>	90.34	0.0
CAST4B2	NZCP 014842.1	<i>Bacillus licheniformis</i>	81.22	0.0
CAST2B1	NZCP 083403.1	<i>Enterobacter asburiae</i>	99.93	0.0
CAST4B1	NZCP 083403.1	<i>Enterobacter asburiae</i>	99.93	0.0
CAST3A1	MT533900.1	<i>Leucobacter chromiiresistens</i>	86.83	4e-177
CAST1B2	NZAP 024684.1	<i>Stenotrophomonas pavanii</i>	91.12	0.0

ZULU18B2	NZFNK 301000001.1	<i>Leucobacter chromiiresistens</i>	86.83	4e-177
ZULU2A5	NZFNK 301000001.1	<i>Leucobacter chromiiresistens</i>	86.83	4e-177
ZULU9B3	NZFNK 301000001.1	<i>Leucobacter chromiiresistens</i>	86.83	4e-177
ZULU11A1	NZFNK 301000001.1	<i>Leucobacter chromiiresistens</i>	86.83	4e-177
ZULU20A4	NZFNK 301000001.1	<i>Leucobacter chromiiresistens</i>	86.83	4e-177
ZULU30A2	NZFNK 301000001.1	<i>Leucobacter chromiiresistens</i>	86.83	4e-177
ZULU20A3	NZFNK 301000001.1	<i>Leucobacter chromiiresistens</i>	86.83	4e-177

NKP8W	NZPTXV 01000013.1	<i>Bacillus pumilus</i>	90.08	0.0	ZULU12B4	NZAP 024684.1	<i>Stenotrophomonas pavanii</i>	91.12	0.0
NKP1W1	NZPTXV 01000013.1	<i>Bacillus pumilus</i>	90.08	0.0	ZULU32B1	NZAP 024684.1	<i>Stenotrophomonas pavanii</i>	91.12	0.0
NKP6G	NZPTXV 01000013.1	<i>Bacillus pumilus</i>	90.08	0.0	ZULU9B5	NZFZPB 01000005.1	<i>Stenotrophomonas lactitubi</i>	85.89	0.0
NKP4G	NZCP 083403.1	<i>Enterobacter asburiae</i>	99.93	0.0	ZULU2A1	NZLS 483377.1	<i>Stenotrophomonas maltophilia</i>	93.55	0.0
NKP10G	NZCP 083403.1	<i>Enterobacter asburiae</i>	99.93	0.0	ZULU9A6	NZFZPB 01000005.1	<i>Stenotrophomonas lactitubi</i>	85.89	0.0
NKP5G	NZCP 083403.1	<i>Enterobacter asburiae</i>	99.93	0.0	ZULU9B8	NZLS 483377.1	<i>Stenotrophomonas maltophilia</i>	93.55	0.0
NKP3G	KT44390.1	<i>Micrococcus yunnanensis</i>	87.60	0.0	ZULU27A7	NZQBKH 01000017.1	<i>Sphingobacterium faecium</i>	90.34	0.0
NKP5P	NZCP 083403.1	<i>Enterobacter asburiae</i>	99.93	0.0	ZULU30A5	NZQBKH 01000017.1	<i>Sphingobacterium faecium</i>	90.34	0.0

NKP65G	NZLS 483377.1	<i>Stenotrophomonas maltophilia</i>	93.55	0.0	ZULU4B3	NZQBKH 01000017.1	<i>Sphingobacterium faecium</i>	90.34	0.0
NKP4P	NZFZPB 01000005.1	<i>Stenotrophomonas lactitubi</i>	85.89	0.0	ZULU32B2	NZQBKH 01000017.1	<i>Sphingobacterium faecium</i>	90.34	0.0
NKF10WB	NZFZPB 01000005.1	<i>Stenotrophomonas lactitubi</i>	85.89	0.0	ZULU27A3	NZQBKH 01000017.1	<i>Sphingobacterium faecium</i>	90.34	0.0
NKP12F	NZLS 483377.1	<i>Stenotrophomonas maltophilia</i>	93.55	0.0	ZULU9B4	NZRJUG 01000006.1	<i>Kaistella daneshvariae</i>	81.62	0.0
UMPP2PB6	NZPTXV 01000013.1	<i>Bacillus pumilus</i>	90.08	0.0	ZULU24A1	NZRJUG 01000006.1	<i>Kaistella daneshvariae</i>	81.62	0.0
UMPP9G5	NZPTXV 01000013.1	<i>Bacillus pumilus</i>	90.08	0.0	ZULU9B2	NZCP 123000.1	<i>Neorhizobium petrolearium</i>	83.67	0.0
UMPBG4B 4	NZCP 083403.1	<i>Enterobacter asburiae</i>	99.93	0.0	ZULU9B1	NZCP 123000.1	<i>Neorhizobium petrolearium</i>	83.67	0.0
UMP1P3PB 5	NZCP 083403.1	<i>Enterobacter asburiae</i>	99.93	0.0	ZULU27B2	NZRJUG 01000006.1	<i>Kaistella daneshvariae</i>	81.62	0.0

UMPBG9A 2	NZCP 083403.1	<i>Enterobacter asburiae</i>	99.93	0.0	ZULU9A5	NZCP 072387.1	<i>Cellulosimicrobium cellulans</i>	80.21	2e-105
UMPP2PB7	NZCP 083403.1	<i>Enterobacter asburiae</i>	99.93	0.0	ZULU24A5	NZCP 072387.1	<i>Cellulosimicrobium cellulans</i>	80.21	2e-105
UMPP6PB1	NZFNK 301000001. 1	<i>Leucobacter chromiiresistens</i>	86.83	4e- 177	ZULU18B5	NZCP 072387.1	<i>Cellulosimicrobium cellulans</i>	80.21	2e-105
UMPP7GA 2	NZFNK 301000001. 1	<i>Leucobacter chromiiresistens</i>	86.83	4e- 177	ZULU27B4	NZCP 072387.1	<i>Cellulosimicrobium cellulans</i>	80.21	2e-105
UMPBGPA 3	NZFNK 301000001. 1	<i>Leucobacter chromiiresistens</i>	86.83	4e- 177	ZULU18B1	NZNGVR 01000010.1	<i>Proteus columbae</i>	91.47	0.0
UMPP9GA 2	NZAP 024684.1	<i>Stenotrophomonas pavanii</i>	91.12	0.0	ZULU16A1	NZCP 022046.1	<i>Mammaliicoccus sciuri</i>	88.16	0.0

UMPBG5A 2	NZAP 024684.1	<i>Stenotrophomonas pavanii</i>	91.12	0.0	ZULU27B1	CP068088.1	<i>Sphingobacterium multivorum</i>	82.65	2e-153
UMPP9G4	NZLS 483377.1	<i>Stenotrophomonas maltophilia</i>	93.55	0.0	NHLANG7A2	NZCP 014842.1	<i>Bacillus licheniformis</i>	81.22	0.0
UMP1P3PB 3	KT44390.1	<i>Micrococcus yunnanensis</i>	87.60	0.0	NHLANGE2 B2	NZCP 083403.1	<i>Enterobacter asburiae</i>	99.93	0.0
UMPP2PB2	NZFZPB 01000005.1	<i>Stenotrophomonas lactitubi</i>	85.89	0.0	NHLANGE17 B1	NZCP 083403.1	<i>Enterobacter asburiae</i>	99.93	0.0
UMPP2PB5	NZFZPB 01000005.1	<i>Stenotrophomonas lactitubi</i>	85.89	0.0	NHLANGE15 B1	NZCP 083403.1	<i>Enterobacter asburiae</i>	99.93	0.0
UMPP9PA	NZFZPB 01000005.1	<i>Stenotrophomonas lactitubi</i>	85.89	0.0	NHLANGE1 B5	NZFNK 301000001.1	<i>Leucobacter chromiiresistens</i>	86.83	4e-177
UMPP7GA 3	NZCP 140571.1	<i>Stenotrophomonas geniculate</i>	93.97	0.0	NHLANGE5 A1	NZFNK 301000001.1	<i>Leucobacter chromiiresistens</i>	86.83	4e-177
UMPP9G3	NZQBKH 01000017.1	<i>Sphingobacterium faecium</i>	90.34	0.0	NHLANGE5 A5	NZFNK 301000001.1	<i>Leucobacter chromiiresistens</i>	86.83	4e-177

UMPBG1B 1	NZQBKH 01000017.1	<i>Sphingobacterium faecium</i>	90.34	0.0
UMPBG4A 5	NZQBKH 01000017.1	<i>Sphingobacterium faecium</i>	90.34	0.0
UMPP1P3P B2	NZQBKH 01000017.1	<i>Sphingobacterium faecium</i>	90.34	0.0
UMPP7GA 1	NZCP 123000.1	<i>Neorhizobium petrolearium</i>	83.67	0.0
UMP1P3PB 2	NZRJUG 01000006.1	<i>Kaistella daneshvariae</i>	81.62	0.0
UMPBG4A 1	NZCP 072387.1	<i>Cellulosimicrobium cellulans</i>	80.21	2e- 105
UMPP9GA 1	NZCP 072387.1	<i>Cellulosimicrobium cellulans</i>	80.21	2e- 105
UMPP2PA2	FJ528593.1	<i>Lysinibacillus sphaericus</i>	78.33	2e- 151

NHLANG1B1	NZAP 024684.1	<i>Stenotrophomonas pavanii</i>	91.12	0.0
NHLANGE17 B2	NZCP 140571.1	<i>Stenotrophomonas geniculata</i>	93.97	0.0
NHLANGE7 B4	NZLS483377 .1	<i>Stenotrophomonas maltophilia</i>	93.55	0.0
NHLANGE17 A1	NZQBKH 01000017.1	<i>Sphingobacterium faecium</i>	90.34	0.0
NHLANGE6 B	NZRJUG 01000006.1	<i>Kaistella daneshvariae</i>	81.62	0.0
NHLANGE2 B1	NZCP 123000.1	<i>Neorhizobium petrolearium</i>	83.67	0.0
NHLANGE8 B	FJ528593.1	<i>Lysinibacillus sphaericus</i>	78.33	2e-151
GAB1B1	NZCP 083403.1	<i>Enterobacter asburiae</i>	99.93	0.0

UMPBG8B	FJ528593.1	<i>Lysinibacillus sphaericus</i>	78.33	2e- 151	GAB2B1	NZFNK 301000001.1	<i>Leucobacter chromiirestiens</i>	86.83	4e-177
UMPP4GB	FJ528593.1	<i>Lysinibacillus sphaericus</i>	78.33	2e- 151	GAB6B2	NZAP 024684.1	<i>Stenotrophomonas pavanii</i>	91.12	0.0
UMPP3PB3	NZNGVR 01000010.1	<i>Proteus columbae</i>	91.47	0.0	GAB7A1	NZAP 024684.1	<i>Stenotrophomonas pavanii</i>	91.12	0.0
BUSHP1A	NZCP 140571.1	<i>Stenotrophomonas geniculate</i>	93.97	0.0	GAB10A1	FJ528593.1	<i>Lysinibacillus sphaericus</i>	78.33	2e-151
BUSHP2B1	NZFZPB 01000005.1	<i>Stenotrophomonas lactitubi</i>	85.89	0.0	GABA6B1	NZFZPB 01000005.1	<i>Stenotrophomonas lactitubi</i>	85.89	0.0
BUSHP2B3	NZFZPB 01000005.1	<i>Stenotrophomonas lactitubi</i>	85.89	0.0	GAB5A1	NZLS 483377.1	<i>Stenotrophomonas maltophilia</i>	93.55	0.0
BUSHPA2	NZLS 483377.1	<i>Stenotrophomonas maltophilia</i>	93.55	0.0	GAB12B2	NZQBKH 01000017.1	<i>Sphingobacterium faecium</i>	90.34	0.0
BUSHPA7	NZCP 014842.1	<i>Bacillus licheniformis</i>	81.22	0.0	GAB13A1	NZQBKH 01000017.1	<i>Sphingobacterium faecium</i>	90.34	0.0

BUSHPA9	NZCP 014842.1	<i>Bacillus</i> <i>licheniformis</i>	81.22	0.0	GAB4B3	NZCP 072387.1	<i>Cellulosimicrobium</i> <i>cellulans</i>	80.21	2e-105
BUSHPA1	NZQBKH 01000017.1	<i>Sphingobacterium</i> <i>faecium</i>	90.34	0.0	GAB12A	FJ528593.1	<i>Lysinibacillus</i> <i>sphaericus</i>	78.33	2e-151
BUSHPA1	FJ528593.1	<i>Lysinibacillus</i> <i>sphaericus</i>	78.33	2e- 151	GAB11A	NZCP126101 .1	<i>Lysinibacillus</i> <i>pakistanensis</i>	78.93	1e-92
BUSHPA3	FJ528593.1	<i>Lysinibacillus</i> <i>sphaericus</i>	78.33	2e- 151*					

^x E-value (≤ 0.0) = highly significant; E-value (0.0) = significant; E-value (≥ 0.0) = random alignment event.

3.3.3. Phylogenetic tree

The phylogenetic trees were used to confirm the morphological identity of the isolates. Figure 3.7 to 3.15 indicates the different phylogenetic trees of the species: *Enterobacter asburiae*, *Leucobacter chromiirensistens*, *Bacillus pumilus*, *Spingobacterium faecium*, *Stenotrophomonas lactitubi*, *Lysinibacillus sphaericus*, *Stenotrophomonas pavanii*, *Stenotrophomonas maltophilia*, *Bacillus licheniformis*, *Cellulosimicrobium cellulans*, *Kaistella daneshvariae*, *Stenotrophomonas geniculate*, *Neorhizobium petrolierum*, *Proteus columbae*, *Micrococcus yunnanensis*, *Lysinibacillus pakistanensis*, *Mammalicoccus sciuri*, and *Sphingobacterium multivorum*.

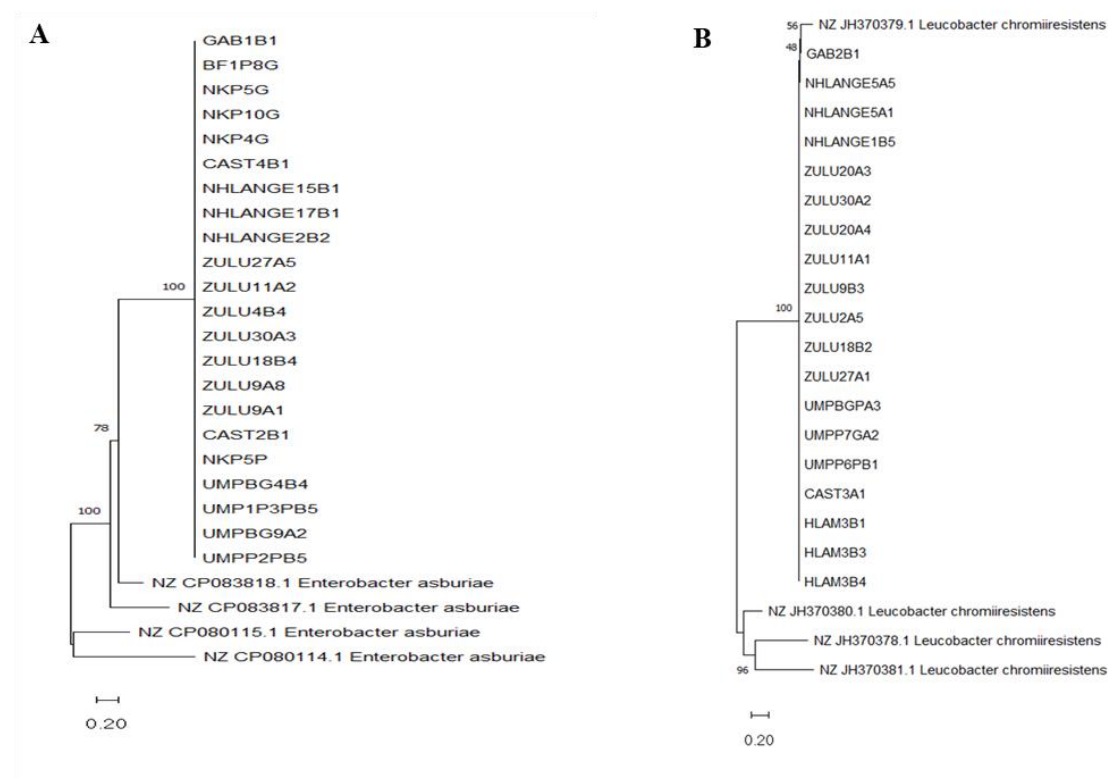


Figure 3.7: Phylogenetic tree indicating the evolutionary history between identified rhizobia isolates from Bambara groundnut root nodule isolated in Mpumalanga, KwaZulu-Natal, and Limpopo province and the NCBI GenBank *Enterobacter asburiae* (A) and *Leucobacter chromiirensistens* (B) species isolates.

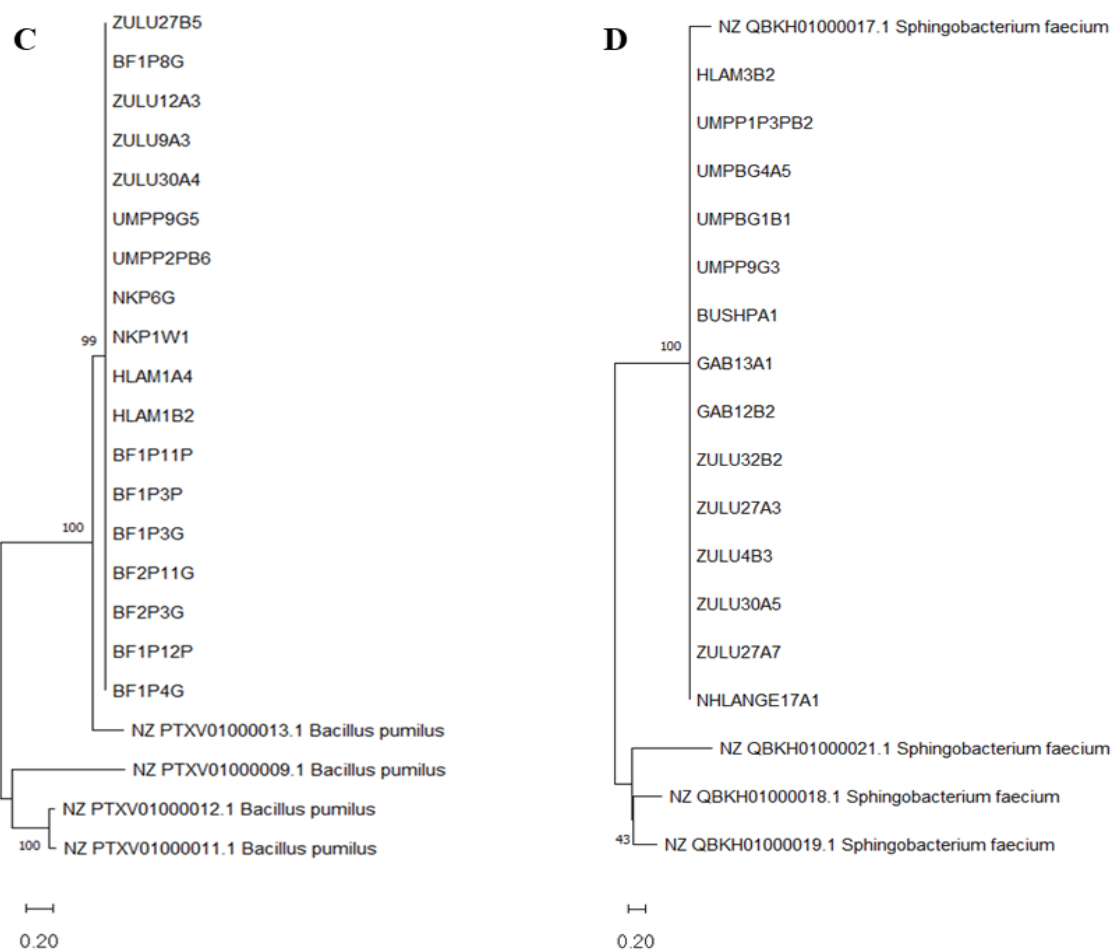


Figure 3.8: Phylogenetic tree indicating the evolutionary history between identified rhizobia isolates from Bambara groundnut root nodule isolated in Mpumalanga, KwaZulu-Natal, and Limpopo province and Limpopo province and the NCBI GenBank *Bacillus pumilus* (C) and *Sphingobacterium faecium* (D) species isolates.

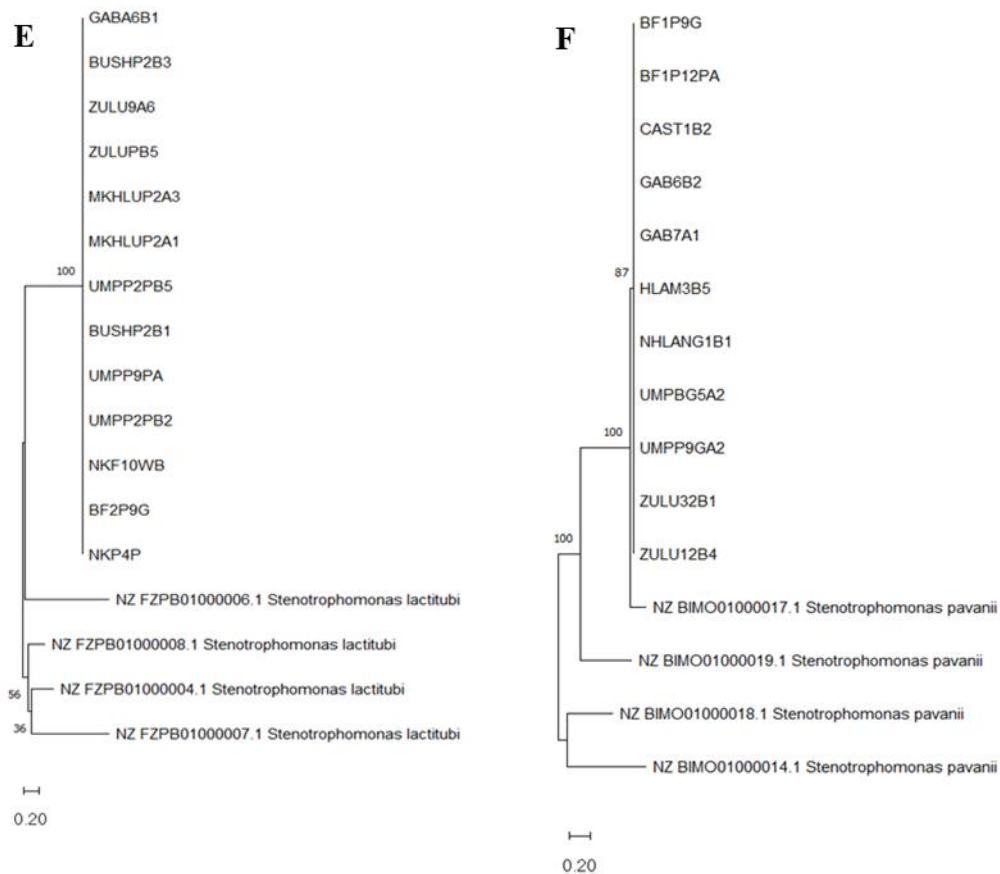


Figure 3.9: Phylogenetic tree indicating the evolutionary history between identified rhizobia isolates from Bambara groundnut root nodule isolated in Mpumalanga, KwaZulu-Natal, and Limpopo province and the NCBI GenBank *Stenotrophomonas lactitubi* (E) and *Stenotrophomonas pavanii* (F) species isolates.

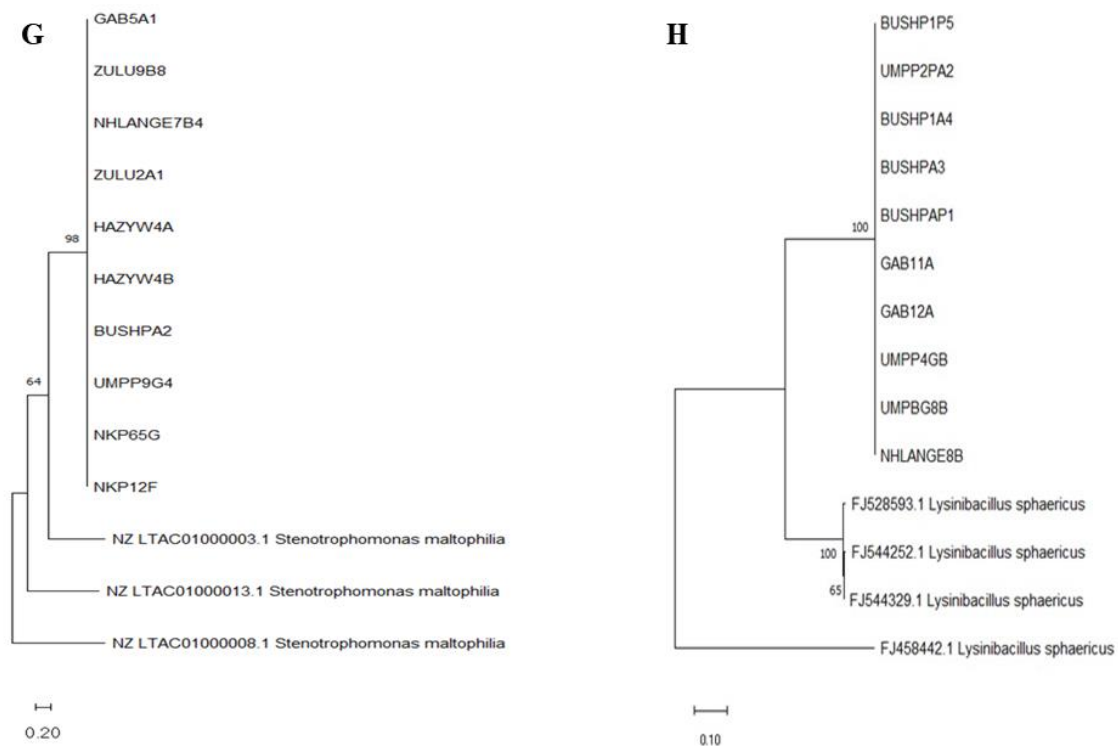


Figure 3.10: Phylogenetic tree indicating the evolutionary history between identified rhizobia isolates from Bambara groundnut root nodule isolated in Mpumalanga, KwaZulu-Natal, and Limpopo province and the NCBI GenBank *Stenotrophomonas maltophilia* (G) and *Lysinibacillus sphaericus* (H) species isolates.

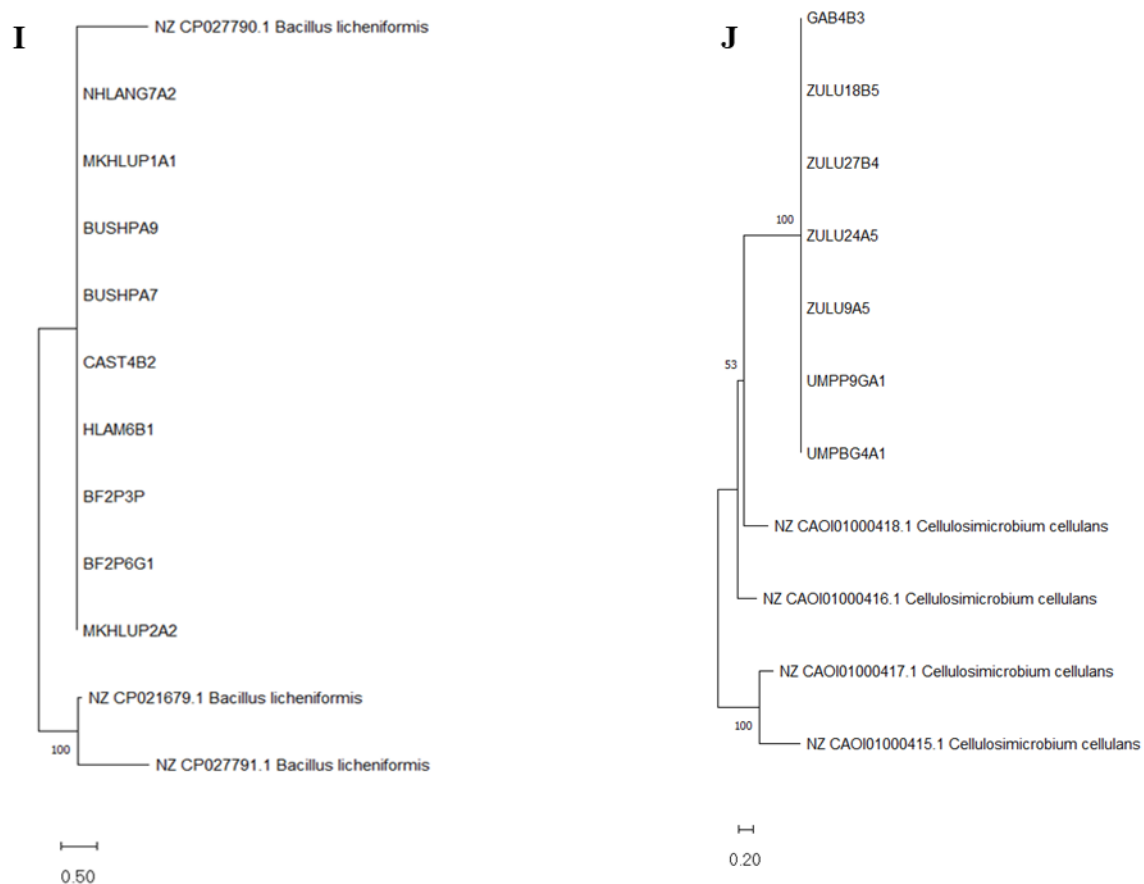


Figure 3.11: Phylogenetic tree indicating the evolutionary history between identified rhizobia isolates from Bambara groundnut root nodule isolated in Mpumalanga, KwaZulu-Natal, and Limpopo province and the NCBI GenBank *Bacillus licheniformis* (I) and *Cellulosimicrobium cellulans* (J) species isolates.

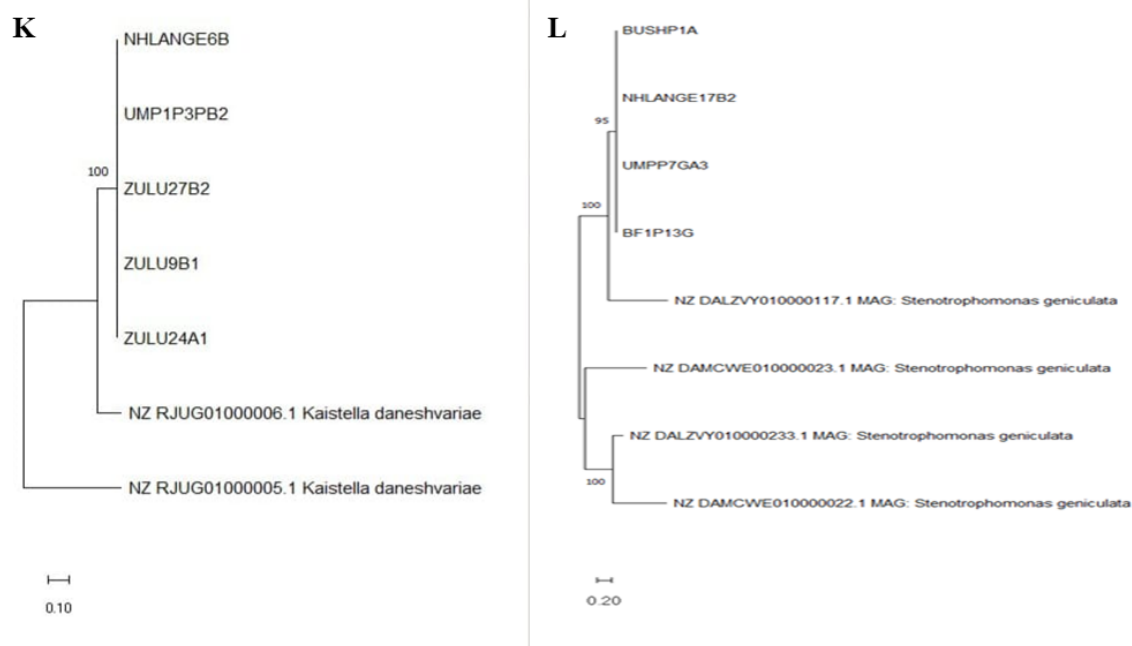


Figure 3.12: Phylogenetic tree indicating the evolutionary history between identified rhizobia isolates from Bambara groundnut root nodule isolated in Mpumalanga and KwaZulu-Natal province and the NCBI GenBank *Kaistella daneshvariae* (K) and *Stenotrophomonas geniculata* (L) species isolates.

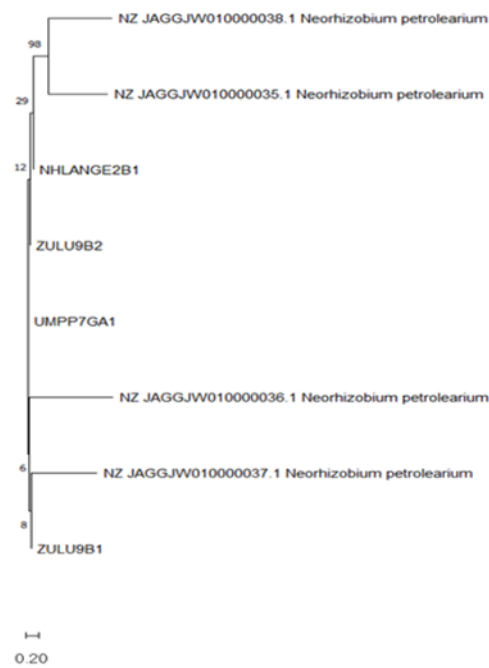
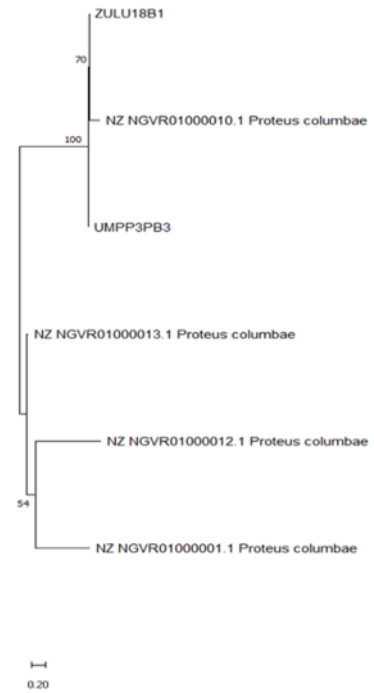
M**N**

Figure 3.13: Phylogenetic tree indicating the evolutionary history between identified rhizobia isolates from Bambara groundnut root nodule isolated in Mpumalanga and KwaZulu-Natal province and the NCBI GenBank *Neorhizobium petrolearium* (M) and *Proteus columbae* (N) species isolates.

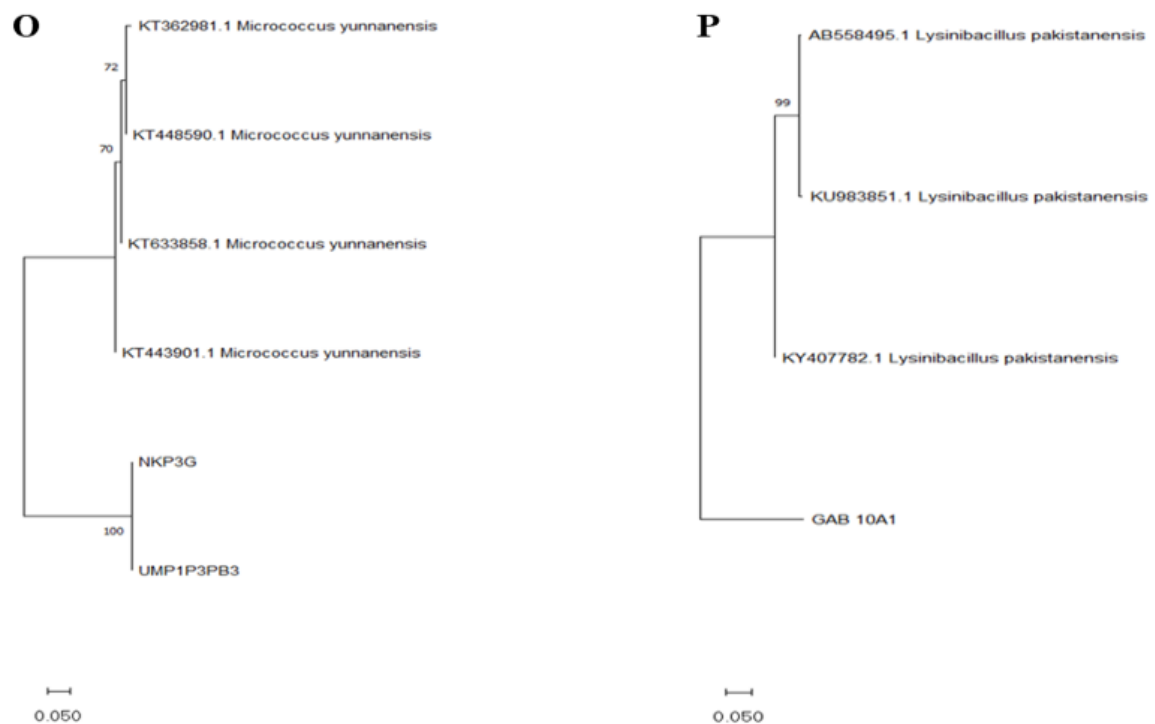


Figure 3.14: Phylogenetic tree indicating the evolutionary history between identified rhizobia isolates from Bambara groundnut root nodule isolated in Mpumalanga and Limpopo province and the NCBI GenBank *Micrococcus yunnanensis* (O) and *Lysinibacillus pakistanensis* (P) species isolates.

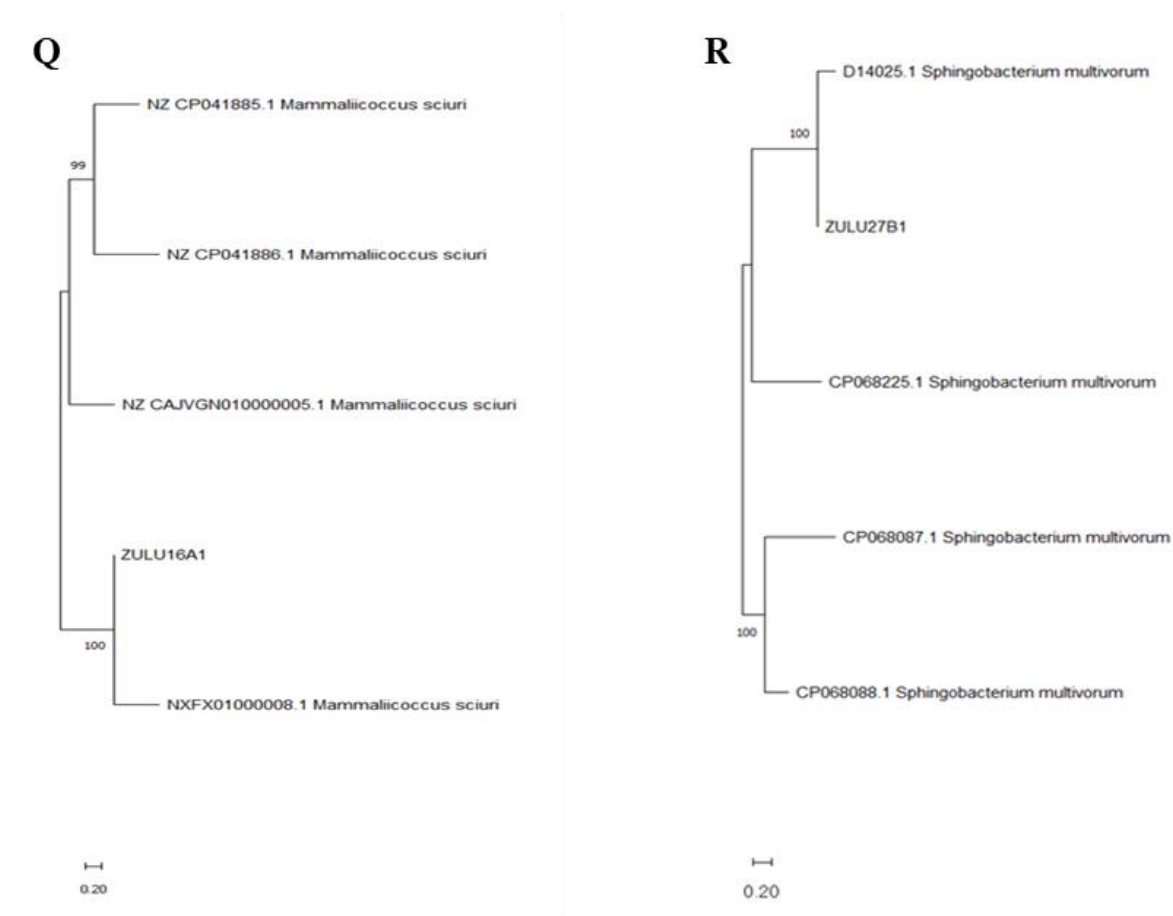


Figure 3.15: Phylogenetic tree indicating the evolutionary history between identified rhizobia isolates from Bambara groundnut root nodule isolated in KwaZulu-Natal province and the NCBI GenBank *Mammaliicoccus sciuri* (Q) and *Sphingobacterium multivorum* (R) species isolates.

3.3.4. Screening of isolates for plant growth-promoting (PGPR) traits

The N-cycling tests indicated that 89 % (186) of isolates from the root nodules had ability to fix nitrogen and 11.00 % (23) tested negative for N-cycling in all three provinces (Table 3.3). All studied location isolates in Mpumalanga province tested positive for N-cycling except for Hlamalani (1), Nkomazi (1), and Bushbuckridge (1) (Table 3.3). In KwaZulu-Natal province, 17 isolates from the University of Zululand tested negative, whereas 3 tested negative in

Nhlangenyuke (Table 3.3). All isolates obtained from roots in Gabaza village, Limpopo province tested positive for N-cycling (Table 3.3). All isolates tested negative (100 %) for phosphate solubilization which means no zone was formed around the colony (Figure 3.17).

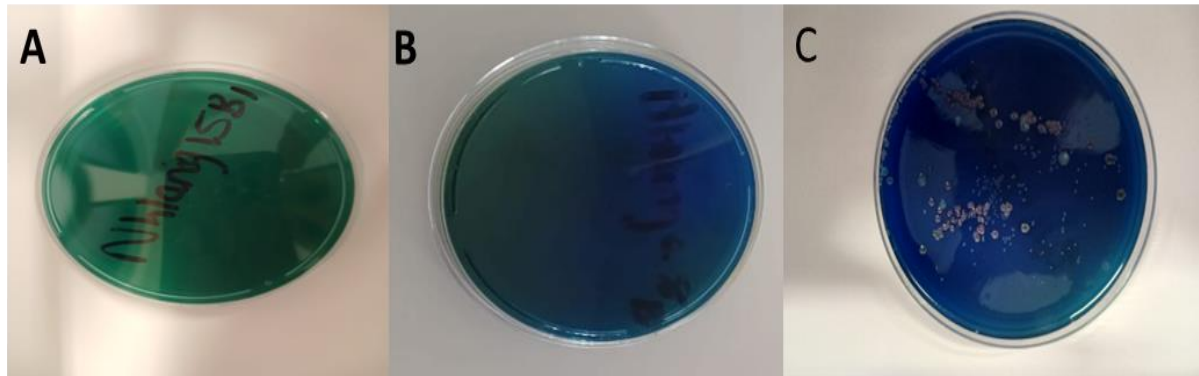


Figure 3.16: Nitrogen cycling bacteria isolates indicated by colour change; negative (A), change of isolates from green to blue by slow grower (B), and positive fast grower (C).



Figure 3.17: Isolates tested for phosphate solubilization indicated no zone formed around the colony on Pikovskaya agar plates.

Table 3.3: Nitrogen cycling and phosphate cycling plant growth promoting activities of rhizobia isolates from Bambara groundnut root nodules.

Province	Location	Isolate name/code	N-cycling	P-cycling	Province	Location	Isolate name/code	N-cycling	P-cycling
Mpumalanga	Boschfontein	BF2P6G1	+	-	KwaZulu-Natal	University of Zululand	ZULU30A4	+	-
		BF2P3P	+	-			ZULU9A3	+	-
		BF1P11P	+	-			ZULU12A3	+	-
		BF1P3P	+	-			ZULU27B5	+	-
		BF1P3G	+	-			ZULU8A2	+	-
		BF2P11G	+	-			ZULU18A2	+	-
		BF2P3G	+	-			ZULU2B1	+	-
		BF1P4G	+	-			ZULU276	+	-
		BF1P12P	+	-			ZULU9A9	+	-
		BF1P8G	+	-			ZULU10B1	+	-
		BF1P4G2	+	-			ZULU27A3	+	-
		BF1P9G	+	-			ZULU9A1	+	-
		BF1P12PA	+	-			ZULU9A8	+	-

		BF1P13G	+	-			ZULU18B4	+	-
		BF2P9G	+	-			ZULU30A3	+	-
	Hlamalani	HLAM6B1	+	-			ZULU4B4	+	-
		HLAM1B2	+	-			ZULU11A2	+	-
		HLAM1A4	+	-			ZULU27A5	+	-
		HLAM2B2	+	-			ZULU11A3	+	-
		HLAM3B4	+	-			ZULU9B8	+	-
		HLAM3B3	+	-			ZULU27A1	+	-
		HLAM3B1	+	-			ZULU18B2	+	-
		HLAM3B5	+	-			ZULU2A5	+	-
		HLAM3B2	-	-			ZULU9B3	+	-
		HLAM2A1	+	-			ZULU11A1	+	-
	Casteel	CAST4B2	+	-			ZULU20A4	+	-
		CAST2B1	+	-			ZULU30A2	+	-
		CAST4B1	+	-			ZULU20A3	+	-
		CAST3A1	+	-			ZULU12B4	+	-

		CAST1B2	+	-
	Nkomazi	NKP8W	+	-
		NKP1W1	+	-
		NKP6G	+	-
		NK10WA	+	-
		NKP4G	+	-
		NKP10G	+	-
		NKP5G	+	-
		NKP11G	+	-
		NKP3G	-	-
		NKP5P	+	-
		NKP65G	+	-
		NKP4P	+	-
		NKF10WB	+	-
		NKP12F	+	-
		UMPP2PB6	+	-

		ZULU32B1	+	-
		ZULU9B5	+	-
		ZULU2A1	+	-
		ZULU9A6	+	-
		ZULU9B8	+	-
		ZULU27A7	-	-
		ZULU30A5	-	-
		ZULU4B3	-	-
		ZULU32B2	-	-
		ZULU28A3	-	-
		ZULU9B4	-	-
		ZULU24A1	-	-
		ZULU9B2	-	-
		ZULU9B1	-	-
		ZULU27B2	-	-
		ZULU24A3	-	-

University of Mpumalanga	UMPP9G5	+	-
	UMPP2PB3	+	-
	UMPBG1B2	+	-
	UMPBG1A	+	-
	UMPBG9A3	+	-
	UMPBG6A2	+	-
	UMPBG4B4	+	-
	UMP1P3PB5	+	-
	UMPBG9A2	+	-
	UMPP2PB7	+	-
	UMP1P3PB4	+	-
	UMPP2PA3	+	-
	UMPP6PB1	+	-
	UMPP7GA2	+	-
	UMPBGPA3	+	-
	UMPP9GA2	+	-
	ZULU27A4	-	-
	ZULU32B4	-	-
	ZULU9A1A	-	-
	ZULU9A2	-	-
	ZULU8A3	-	-
	ZULU8A1	+	-
	ZULU9A5	+	-
	ZULU24A5	+	-
	ZULU18B5	+	-
	ZULU27B4	+	-
	ZULU32B3	+	-
	ZULU18B1	+	-
	ZULU27A2	+	-
	ZULU16A1	+	-
	ZULU12B2	+	-
	ZULU7B1	+	-

		UMPBG5A2	+	-
		UMPP9G4	+	-
		UMP1P3PB3	+	-
		UMPP2PB2	+	-
		UMPP2PB5	+	-
		UMPP9PA	+	-
		UMPP7GA3	+	-
		UMPP9G3	+	-
		UMPBG1B1	+	-
		UMPBG4A5	+	-
		UMPP1P3PB2	+	-
		UMPP7GA1	+	-
		UMP1P3PB2	+	-
		UMPBG4A1	+	-
		UMPP9GA1	+	-
		UMPP2PA2	+	-
		ZULU27B1	+	-
		ZULU27B9	+	-
		ZULU2A4	+	-
	Nhlangenyuke	NHLANGE2A2	+	-
		NHLANGE22A	+	-
		NHLANG7A1	+	-
		NHLANG7A2	+	-
		NHLANGE2B2	+	-
		NHLANGE17B1	+	-
		NHLANGE15B1	+	-
		NHLANGE7B2	+	-
		NHLANGE1B5	-	-
		NHLANGE5A1	+	-
		NHLANGE5A5	+	-
		NHLANG1B1	+	-
		NHLANGE17B2	+	-

		UMPBG8B	+	-			NHLANGE7B4	+	-
		UMPP4GB	+	-			NHLANGE17A1	+	-
		UMPP3PB3	+	-			NHLANGE6B	+	-
		UMPBG4B	+	-			NHLANGE2B1	+	-
		UMPP2PA1	+	-			NHLANGE2A1	-	-
		UMPP9PB	+	-			NHLANGE7B3	+	-
		UMPPBG4A4	+	-			NHLANGE14A	-	-
	Bushbuckridge	BUSHP1A	+	-			NHLANGE8B	+	-
		BUSHP2B1	+	-			NHLANGE7B1	+	-
		BUSHP2B3	+	-			NHLANG7A2A	+	-
		BUSHPA2	+	-		Limpopo	GAB12B4	+	-
		BUSHPA7	+	-			GAB1B1	+	-
		BUSHPA9	+	-			GAB13B1	+	-
		BUSHPA1	+	-			GAB2B1	+	-
		BUSHPAP1	-	-			GAB6B2	+	-
		BUSHPA3	+	-			GAB7A1	+	-
						Gabaza			

		BUSHP1P4	+	-
		BUSHP1P5	+	-
		BUSHP2P1	+	-
	Hazyview	HAZYW1B1	+	-
		HAZYW4B	+	-
		HAZYW4A	+	-
		HAZYW4B1	+	-
		HAZYW2B	+	-
		HAZYW2B1	+	-
	Mkhuhlu	MKHLUP1A1	+	-
		MKHLUP2A2	+	-
		MKHLUP2A1	+	-
		MKHLUP2A3	+	-
		MKHLUP2B1	+	-
		MKHLUP2B1	+	-

		GAB10A1	+	-
		GABA6B1	+	-
		GAB5A1	+	-
		GAB12B2	+	-
		GAB13A1	+	-
		GAB4B3	+	-
		GAB1	+	-
		GAB12A	+	-
		GAB11A	+	-

3.4. Diversity, evenness, and abundance of bacterial isolates

There was high diversity of bacterial isolates found in the Bambara groundnut root nodules from the three different provinces and it varied with location. Mpumalanga province had the highest number of bacterial isolates (107), followed by KwaZulu-Natal province (87) and last was Limpopo province (15). From the sampled locations in the three different provinces, the University of Zululand in KwaZulu-Natal province had the highest number of isolates (64) and 23 richness score index followed by the University of Mpumalanga province with 40 isolates and 21 richness index whereas Casteel and Mkhuhlu had the least isolate (5) while Hazyview and Mkhuhlu had the least number of richness index (3) (Table 3.4). Species diversity (H') and (D) were high at the University of Zululand, with scores of 2.85 and 0.94 followed by the University of Mpumalanga (H') and Hazyview (D) scores of 2.81 and 0.27 and the lowest was Nhlanguyuke (H') and (D) score of 0.56 and 0.04. Casteel had the highest evenness (J) score of 0.83 followed by Hlamalani with a score of 0.78, while Hazyview and Boschfontein had the lowest (J) score of 0.56 each (Table 3.4).

In Mpumalanga province, the University of Mpumalanga had the highest isolates number of 40, followed by Boschfontein with 15, and the least was Casteel and Mkhuhlu with 6 species each (Table 3.4). The highest genetic diversity was recorded in the University of Mpumalanga with an H' score of 2.81 and Hazyview had the lowest genetic diversity (H') score of 1.01. However, Hazyview had the highest diversity (D) score of 0.27, with the University of Mpumalanga having the least diversity (D) score of 0.05. Casteel had the highest evenness (J) score of 0.83 followed by Hlamalani with an evenness (J) score of 0.78, while Hazyview and Boschfontein had the lowest evenness (J) score of 0.56 each (Table 3.4).

In KwaZulu-Natal province, the University of Zululand had a higher number of isolates (64) and species richness index (23) compared to Nhlanguyuke with 23 isolates and 14 species richness index (Table 3.4). The University of Zululand had a higher genetic diversity (H') score

of 2.85 and D score of 0.94 compared to Nhlangenyuke (H') with a score of 0.56 and D score of 0.04 (Table 3.4). Nhlangenyuke had a higher species evenness (J) score of 0.81 compared to the University of Zululand evenness (J) score of 0.68 (Table 3.4).

In Limpopo province, Gabaza village had 15 isolates and 10 species richness index. Gabaza also had a genetic diversity (H') score was 2.21, a D score of 0.06, and a species evenness (J) score of 0.81 (Table 3.4).

Table 3.4: Functional bacterial diversity index of Bambara groundnut root nodule samples in Mpumalanga, KwaZulu-Natal, and Limpopo province.

Province	Sample sites	Number of isolates	Species richness	Shannon diversity index (H')	Simpson Index (D)	Pielou index (J)
MP	Hlamalani	10	6	1.79	0.16	0.78
	Boschfontein	15	6	1.63	0.23	0.56
	Nkomazi	14	7	1.77	0.13	0.67
	Bushbuckridge	12	6	1.63	0.15	0.66
	Mkhuhlu	5	3	1.05	0.20	0.66
	Casteel	5	4	1.33	0.10	0.83
	Hazyview	6	3	1.01	0.27	0.56
	UMP	40	21	2.81	0.05	0.76
KZN	UniZululand	64	23	2.85	0.94	0.68
	Nhlangenyuke	23	14	0.5	0.04	0.81
LP	Gabaza	15	10	2.21	0.06	0.82
Total		209	43			

3.4. Discussion

Plant growth-promoting rhizobia colonizes the root nodule and plays a beneficial significant role that directly and indirectly influences the development and growth of plants (Gerhardt, Huang, Glick & Greenberg, 2009). Two hundred and nine bacterial isolates were identified from Bambara groundnut root nodules in this study with 43 unique rhizobia isolates. From the 209 identified isolates only 153 were molecular identified using 16S rRNA gene sequence. Findings in this study corroborated various studies, which discovered a high level of heterogeneity in the populations of legume nodulating rhizobia (McInroy *et al.*, 1999; Ndiaye, 1996). Isolate pigmentation varied from cream, cream white, white, yellow, golden yellow, bright yellow, orange, bright orange, red, and brown with smooth or rough surfaces. The shape also varied from irregular, round, curled, filamentous, rhizoid, and punctiform with either flat, raised, convex, or crateriform elevation and the margin also varied from lobate, entire, undulate, filamentous, and irregular. Singh, Jaiswal and Akhouri Vaishampayan (2013) reported the differences in colony morphology of nodule bacteria in soybeans. The variation of the 43 rhizobia isolates showed the diverse nature of the isolates colonizing nodules of Bambara groundnut in the different provinces and sample locations. The findings of this study on morphological characteristics of native rhizobia isolates on Bambara groundnut root nodules agree with a similar study done on another legume, common bean, in Ecuador (Torres-Gutiérrez *et al.*, 2017). Although native Bambara groundnut isolates occur in the soils, the nodulation potential of different sites may differ greatly. This could be due to factors such as soil mineral composition and pH (Berrada, 2012), this could have contributed to the observed low rhizobia isolates in the soil from Nkomazi, Mkhuhlu, Bushbuckridge, Hlamalani, Casteel, Gabaza, Boschfontein, Nhlangenyuke, and Hazyview.

The NCBI GenBank database indicated that Bambara groundnut forms a symbiotic relationship with diverse bacteria, 12 genera which are *Enterobacter*, *Leucobacter*, *Bacillus*, *Spingobacterium*, *Lysinibacillus*, *Stenotrophomonas*, *Cellulosimicrobium*, *Kaistella*, *Neorhizobium*, *Proteus*, *Micrococcus*, and *Mammalicoccus*. The diversity of micro-symbionts of Bambara groundnut observed in this study concurs with a report by (Pohajda, Huic Babi, Rajnovi, Kaji & Sikora, 2016; Sprent *et al.*, 2010). Sprent *et al.* (2010) indicating the capacity of Bambara groundnut to freely nodulate with various rhizobia groups making it a promiscuous host. These findings of the study are supported by earlier reports by Ngeno, Chemining'wa and Hutchinson (2018); Santos, Kandasamy and Rigobelo (2018) who demonstrated the ability of promiscuous legumes such as cowpeas to trap various rhizobia from the soils under different agricultural systems. The findings also support the “promiscuous” nature of Bambara groundnut to nodulate with diverse rhizobia which aids the plant in thriving in different environmental conditions (Santos *et al.*, 2018). High genetic diversity in cultivated soils can be a result of high demand for N by the plants, which in turn stimulates nodulation, leading to rhizobia proliferation (Lima *et al.*, 2009). Wasike *et al.* (2009) reported a higher diversity of indigenous *Bradyrhizobia* in Western Kenya compared to Eastern Kenya because of agroecological differences between the two locations. Other factors such as host genotype, cropping history, and land usage might have contributed to the difference in the diversity of rhizobia in different parts of KwaZulu-Natal, Mpumalanga, and Limpopo provinces, supported by a report on Central highlands of Kenya (Mwenda, O'Hara, De Meyer, Howieson & Terpolilli, 2018). The University of Zululand had the highest species richness compared to the other sample locations, this may be due to the light sandy loam soils in the area. The report on this study contradicted the findings by Ajilogba, Olanrewaju and Babalola (2022), who observed Bambara groundnut root nodules being colonised mostly by *Bacillus* (57.14 %). In the current study, the dominant species on Bambara groundnut root nodules was *Enterobacter*

asburiae (14.4 %). A study by Wang *et al.* (2016), on biogeography and biodiversity of rhizobia that are associated with the common bean in Shaanxi province, China, linked gene transfer of symbiotic genes among diverse strains of nitrogen-fixing bacteria (*Ensifer*, *Agrobacterium*, *Rhizobium*, and *Bradyrhizobium*). Similar findings were reported by Ayangbenro, Adem and Babalola (2023) that root nodule bacteria of several genera, such as *Chryseobacterium*, *Stenotrophomonas*, and *Pseudomonas* symbiotically co-exist with Bambara groundnuts. The finding of this study agrees with the study on soybeans done by Xu *et al.* (1995) that the diversity of nodulating rhizobia is affected by host specificity and the narrow range of rhizobia species, which forms an effective symbiosis with soybean. Soil properties and climatic conditions also play a role in the diversity of bacteria found in the soil (Adhikari *et al.*, 2012; Han, Wang, Han & Liu, 2009; Suzuki *et al.*, 2008). Different land use and management of planted crops (Yan *et al.*, 2014), geographical location (Shiro *et al.*, 2013) all play a role on diversity. The 16S rRNA gene sequence also indicated the presence of non-rhizobia bacteria in the different provinces associated with root nodules such as genera *Bacillus* and *Sphingomonas* in the ecological niche (Martínez-Hidalgo & Hirsch, 2017; Deng *et al.*, 2011). It has been suggested that root nodules of legume plants may develop a niche that will allow non-rhizobia bacteria to thrive and survive. When rhizobia bacteria are present and infected, these non-rhizobia strains can infiltrate the root nodules of legumes (Etesami, 2022). However, phylogenetically not all the isolates identified in this study were closely related. This suggests that rhizobia bacteria with the potential to nodulate Bambara groundnuts are mainly not restricted to a phylogenetic group (Arora, Khare, Singh & Tewari, 2018). Consequently, these rhizobia isolates might have divergently evolved to colonize the root nodules of diverse Bambara groundnut genotypes, while at the same time retaining their critical genes that code for the nodulation of Bambara groundnut. Furthermore, genetic changes also result in pleiotropic effects on various traits at the same time affecting the resulting phenotypes (Gratten,

& Visscher, 2016). The occurrence of diverse isolates from different genera on the Bambara groundnut root nodules in Mpumalanga, KwaZulu-Natal province soils could be attributed to the horizontal gene transfer between the α and β proteobacteria (Ramírez *et al.*, 2020). The high diversity of the genus *Enterobacter* and *Leucobacter* observed in this study could be due to a high level of tolerance and can dominate in environments with low soil fertility and low pH (Dall'Agnol *et al.*, 2016). Isolates GAB10A1, ZULU16A1, and ZULU27B1 identified as *Lysinibacillus pakistanensis*, *Mammalicoccus sciuri*, and *Sphigobacterium multivorum* were unique in the sample site Gabaza and University of Zululand. To our knowledge, this is the first report of genus *Lysinibacillus* and *Mammalicoccus* that nodulates Bambara groundnut in South Africa. The non-rhizobia isolates observed in the Bambara groundnut nodules in this study belonged to the genera *Strenotrophomonas*, *Bacillus*, *Micrococcus*, and *Enterobacter*. These findings of this study support earlier reports by Chidebe, Jaiswal and Dakora (2018) and Leite *et al.* (2017) who documented the diversity of non-rhizobia endophytes associated with root nodules of cowpea. Leite *et al.* (2017) added that these non-rhizobia bacteria strains have previously been isolated from *Vigna unguiculata* root nodules in Brazil and *Phaesolus vulgaris* (Kawaka *et al.*, 2018) in Western Kenya. Moreover, the occurrence and diversity of the non-rhizobia bacterial in this study might be attributed to the compatibility of the microbes with either the host plant or that each microbe occupies a diverse ecological niche in the root nodules of the host plant (De Meyer, De Beuf, Vekeman & Willems, 2015). This means that the presence of the non-rhizobacteria on Bambara groundnut rhizosphere soil was not accidental and similar results were documented by Castro *et al.* (2017). Moreover, the rhizobia *nod* and *nif* genes are symbiotic and adaptive genes. Earlier studies suggested that they very repeatedly have an evolutionary history independent of the house-keeping genes explained by a lateral transfer of *nod* loci across divergent-chromosomal lineages, like in the case of rhizobia representing the genus *Rhizobium* and *Ensifer* (Wang *et al.*, 2007). The horizontal transfer of the nodulating

genes adapts rhizobia to a new host plant and then enables the bacteria with a different genomic background but similar *nod* genes to enter symbiotic association with the same legume plants. Furthermore, studies of genus *Mesorhizobium* strains suggested that the broad host range of these bacteria may be attributed to the convergence of a distinct *nod* genotype into the same nodulation phenotype (Wernegreen & Riley 1999; Haukka, Lindstrom & Young, 1998).

Rhizobacteria play a significant role in plant growth promotion, nutrient recycling, and soil structure maintenance (Anderson, Hamonts, Clough & Condron, 2014). Studies have demonstrated that indigenous plant growth-promoting rhizobia contributes to P-solubilization and N-cycling, thus making N and P bioavailable for plant uptake (Marler & Krishnapillai, 2018). The root nodules of Bambara groundnut predominated N-cycling bacteria, which indicated poor N nutrients in the soil which triggers biological N-cycling to ensure plant survival and nutrient uptake. Rhizosphere N-cycling bacterial diversity and structure influence the nitrogen fixation of legumes. Limpopo province isolates all tested positive for N-cycling and Mpumalanga province had few isolates that tested negative. This might be attributed to the different planting cultivations that affect soil microbial quantity, microbial community structure, and microbial activity. All N-cycling rhizobia isolates observed at Gabaza village, in Limpopo province is as a result of intercropping system. Previous studies done earlier indicated that the abortion of the intercropping system can have varying effects on the N-cycling bacteria communities' diversity dependent on the specific soil condition and crop combinations. For instance, peanut and cassava intercropping increased microbial diversity when compared to peanut monoculture (Tang *et al.*, 2020), while legume and oat intercropping improved the diversity of oat N-fixing bacteria communities (Yang, Feng, Hu, Ren & Zeng, 2007).

However, the present study indicated that isolates observed from the three provinces could not solubilize phosphate which includes bacteria that belong to the genera *Leucobacter*, *Bacillus*, *Lysinibacillus*, *Micrococcus*, and *Mammalicoccus* known to solubilize phosphate. The finding

might be attributed to certain factors which include ecological condition, climatic zone, land use system, agronomic practices, soil pH, soil organic matter, microbial interaction with other soil microbes, the extent of vegetation, soil type, type of plant, and soil physico-chemical properties (Seshachala & Tallapragada, 2012).

Mpumalanga province had the highest abundance of isolate followed by KwaZulu-Natal and the least was Limpopo province. University of Zululand in KwaZulu-Natal province had the highest species richness compared to all sample sites whereas a few species richness was estimated in Casteel and Hazyview. The highest rhizobia population that was observed at the University of Zululand might be because these soils had earlier been used to cultivate legumes belonging to the same cross-inoculation groups as the tree legumes (Koskey *et al.*, 2018). In KwaZulu-Natal, University of Zululand Bambara groundnut roots nodules had the highest genetic diversity (H') and (D) compared to Nhlangenyuke whereas Nhlangenyuke species were evenly distributed compared to the University of Zululand isolates. In Mpumalanga, the University of Mpumalanga had the highest (H') and the lowest (D) compared to the other sample site, Hazyview had the lowest genetic (H') and the highest (D), and Casteel had high evenness (J) and Hazyview and Boschfontein with the lowest. This could be due to the difference in soil and agroclimatic conditions of the three different provinces and the studied sites in each province. The finding might be attributed to certain factors which include ecological condition, climatic zone, land use system, agronomic practices, soil pH, soil organic matter, microbial interaction with other soil microbes, the extent of vegetation, soil type, type of plant, and soil physico-chemical properties (Seshachala & Tallapragada, 2012). The abundance of this species in acidic soil has been attributed to its adaptation to acidic conditions (Howieson *et al.*, 2013; Sprent Ardley & James, 2013).

3.5. Conclusion

This study successfully isolated and identified a total of 209 rhizobacterial strains from the root nodules of Bambara groundnut collected from three different provinces. Through molecular analysis, the strains were classified into 18 different bacterial genera, with *Enterobacter absuriae*, *Leucobacter chromiirensiensis*, and *Bacillus pumilus* being the most predominant. Most of the isolates showed a high percentage of homology with their respective species in the GenBank database, confirming their identities. Phylogenetic analysis also validated these results. The ability of these rhizobacterial isolates to participate in nitrogen cycling was evident, as 89% of them tested positive, suggesting their potential role in enhancing soil fertility and plant growth. However, none of the isolates exhibited phosphate solubilization capabilities, indicating a limitation in their multifunctional potential.

3.6. Recommendations

Future research should focus on investigating methods to enhance the phosphate-solubilizing abilities of these rhizobacterial isolates, possibly through co-inoculation with other beneficial microbes. Additionally, since certain species like *Lysinibacillus pakistanensis*, *Mammalicoccus sciuri*, and *Sphingobacterium multivorum* were found in only one location, further studies should explore their distribution and potential functions in different soil environments. Understanding the full range of plant growth-promoting traits in these isolates can contribute to developing efficient biofertilizers to improve crop productivity and sustainable agriculture practices.

3.7. References

- Adhikari, D., Kaneto, M., Itoh, K., Suyama, K., Pokharel, B. B. and Gaihre, Y. K. 2012. Genetic diversity of soybean-nodulating rhizobia in Nepal concerning climate and soil properties. *Plant Soil*, 357: 131-145.
- Ajilogba, C., Olanrewaju, O. and Babalola, O. 2022. Improving Bambara groundnut production: insight into the role of omics and beneficial bacteria. *Frontiers in Plant Science*, 13: 1-17.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipma, D. J. 1990. Basic local alignment search tool. *Journal of Molecular Biology*, 215: 403-410.
- Anderson, C. R., Hamonts, K., Clough, T. J. and Condron, L. M. 2014. Biochar does not affect soil N-transformations or microbial community structure under ruminant urine patches but does alter relative proportions of nitrogen cycling bacteria. *Agriculture, Ecosystem and Environment*, 191: 63-72.
- Arora, N. K., Khare, E., Singh, S. and Tewari, S. 2018. Phenetic, genetic diversity and symbiotic compatibility of rhizobial strains nodulating pigeon pea in northern India. *Three Biotech*, 8(1): 52.
- Ayangbenro, A. S., Adem, M. R. and Babalola, O. O. 2023. Bambara nut root-nodules bacteria from a semi-arid region of South Africa and their plant growth-promoting traits. *International Journal of Microbiology*, 2023: 1-8.
- Berrada, H. 2012. Phenotypic and genotypic characterizations of rhizobia isolated from root nodules of multiple legume species native of Fez, Morocco. *African Journal of Microbiology Research*, 6(25): 1505.
- Bontemps, C., Rogel, M. A., Wiechmann, A., Mussabekova, A., Moody, S., Simon, M. F., Moulin, L., Elliott, G. N., Lacercat-Didier, L., Dasilva, C., Grether, R., Camargo-Ricalde, S. L., Chen, W., Sprent, J. I., Martínez-Romero, E., Young, J. P. W. and James,

- E. K. 2015. Endemic *mimosa* species from Mexico prefer alphaproteobacterial rhizobial symbionts. *New Phytologist*, 209(1): 319-333.
- Castro, J. L., Souza, M. G., Rufini, M., Guimarães, A. A., Rodrigues, T. L. and Moreira, F. M. S. 2017. Diversity and efficiency of rhizobia communities from iron mining areas using cowpea as a trap plant. *Revista Brasileira de Ciência do Solo*, 41: 1-20.
- Chidebe, I. N., Jaiswal, S. K. and Dakora, F. D. 2018. Distribution and phylogeny of microsymbionts associated with cowpea (*Vigna unguiculata*) nodulation in three agroecological regions of Mozambique. *Applied and Environmental Microbiology*, 84(2): 1-21.
- Dall'Agnol, R. F., Plotegher, F., Souza, R. C., Mendes, I. C., dos Reis Junior, F. B., Béna, G., Moulin, L. and Hungria, M. 2016. *Paraburkholderia nodosa* is the main N₂-fixing species trapped by promiscuous common bean (*Phaseolus vulgaris* L.) in the Brazilian 'cerradão.' *FEMS Microbiology Ecology*, 92(8): 1-18.
- De Meyer, S. E., De Beuf, K., Vekeman, B. and Willems, A. 2015. A large diversity of non-rhizobial endophytes found in legume root nodules in Flanders (Belgium). *Soil Biology and Biochemistry*, 83: 1-11.
- Deng, Z. S., Zhao, L. F., Kong, Z. Y., Yang, W. Q., Lindström, K., Wang, E. T. and Wei, G. H. 2011. Diversity of endophytic bacteria within nodules of the *Sphaerophysa salsula* in different regions of Loess Plateau in China. *FEMS Microbiology Ecology*, 76(3): 463-475.
- Etesami, H. 2022. Root nodules of legumes: a suitable ecological niche for isolating non-rhizobia bacteria with biotechnological potential in agriculture. *Current Research in Biotechnology*, 4: 78-86.

- Gerhardt, K. E., Huang, X. D., Glick, B. R. and Greenberg, B. M. 2009. Phytoremediation and rhizoremediation of organic soil contaminants: Potential and challenges. *Plant Science*, 176(1): 20-30.
- Gratten, P. and Visscher, M. 2016. Genetic pleiotropy in complex traits and diseases: implications for genomic medicine. *Genome Medicine*, 8 (1): 78.
- Han, L. L., Wang, E. T., Han, T. X. and Liu, J. 2009. Unique community structure and biogeography of soybean rhizobia in the saline-alkaline soils of Xinjiang, China. *Plant Soil*, 324: 291-305.
- Hassen, A. I., Vuuren, A. van, Bopape, F. L. and Gerrano, A. S. 2022. Nodulation compatibility and symbiotic performance of rhizobia spp. with different landraces of Bambara groundnut (*Vigna subterranea* (L.) Verdc.) Collections. [Online]. Available at: <https://assets.researchsquare.com/files/rs-1233082/v1/44eda8a6-8479-44d8-a1a6-3007f2aa0ce0.pdf?c=1654071340> [Accessed 12 March 2022].
- Haukka, K., Lindstrom, K. M. and Young, P. J. W. 1998. Three phylogenetic groups of *nodA* and *nifH* genes in *Sinorhizobium* and *Mesorhizobium* isolates from leguminous trees growing in Africa and Latin America. *Applied and Environmental Microbiology*, 64: 419-426.
- Hill, J. M., Roberts, J., Loeb, E., Khan, A., MacLellan, A. and Hill, R. W. 1967. L-asparaginase therapy for leukemia and other malignant neoplasms: remission in human leukemia. *JAMA*, 202: 882-888.
- Howieson, J. G., De Meyer, S. E., Vivas-Marfisi, A., Ratnayake, S., Ardley, J. K. and Yates, R. J. 2013. Novel Burkholderia bacteria isolated from *Lebeckia ambigua* – a perennial suffrutescent legume of the Fynbos. *Soil Biology and Biochemistry*, 60: 55-64.

- Jeyanthi, V. and Kanimozhi, S. 2018. Plant growth promoting rhizobacteria (PGPR) - perspective and mechanisms: A review. *Journal of Pure and Applied Microbiology*, 12(2): 733-749.
- Kawaka, F., Makonde, H., Dida, M., Opala, P., Ombori, O., Maingi, J. and Muoma, J. 2018. Genetic diversity of symbiotic bacteria nodulating common bean (*Phaseolus vulgaris*) in western Kenya. *PLOS ONE*, 13(11): 1-13.
- Khan, A., Sayyed, R. and Seifi, S. 2019. Rhizobacteria: Legendary soil guards in abiotic stress management. In: Reddy, M. S., Sayyed, R. Z. and Arora, N. K. eds. *Plant growth promoting rhizobacteria for sustainable stress management*. Singapore: Springer.
- Koskey, G., Mburu, S. W., Kimiti, J. M., Ombori, O., Maingi, J. M. and Njeru, E. M. 2018. Genetic characterization and diversity of *Rhizobium* isolated from root nodules of mid-altitude climbing bean (*Phaseolus vulgaris* L.) varieties. *Frontiers in Microbiology*, 9: 1-12.
- Kumar, S., Stecher, G. and Tamura, K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 33(7): 1870-1874.
- Lawless, C., Zedonek, M., Curtis, D., Scharringhausen, J., Bryant, J., Dustman, W. A., Kurtz, A. M. and Ward, B. 2018 "Identification and Characterization of Nitrogen Fixing Bacteria Associated with Kudzu Root Nodules. *Georgia Journal of Science*, 76(2): 1-12.
- Leite, J., Fischer, D., Rouws, L. F., Fernandes-Júnior, P. I., Hofmann, A., Kublik, S., Schlöter, M., Xavier, G. R. and Radl, V. 2017. Cowpea nodules harbor non-rhizobial bacterial communities that are shaped by soil type rather than plant genotype. *Frontiers in Plant Science*, 7: 2064.

- Lemaire, B., Dlodlo, O., Chimphango, S., Stirton, C., Schrire, B., Boatwright, J. S., Honnay, O., Smets, E., SPRENT, J., James, E. K. and Muasya, A. M. 2014. Symbiotic diversity, specificity and distribution of rhizobia in native legumes of the core Cape Subregion (South Africa). *FEMS Microbiology Ecology*, 91(2): 1-17.
- Lima, A. S., Nóbrega, R. S., Barberi, A., da Silva, K., Ferreira, D. F. and Moreira, F. M. 2009. Nitrogen-fixing bacteria communities occurring in soils under different uses in the Western Amazon region as indicated by nodulation of Siratro (*Macroptilium atropurpureum*). *Plant and Soil*, 319(1-2): 127-145.
- Liu, Y., Xiong, Z., Wu, W., Ling, H.Q. and Kong, D. 2023. Iron in the symbiosis of plants and microorganisms. *Plants*, 12(10): 1958.
- Marler, T. E. and Krishnapillai, M. V. 2018. *Cycas micronesica* trees alter local soil traits. *Forests*, 9: 565.
- Martínez-Hidalgo, P. and Hirsch, A. M. 2017. The nodule microbiome: N₂-fixing rhizobia do not live alone. *Phytobiomes Journal*, 1(2): 70-82.
- McInroy, S. G., Campbell, C. D., Haukka, K. E., Odee, D. W., Sprent, J. I., Wang, W. J. and Sutherland, J. M. 1999. Characterisation of rhizobia from African acacias and other tropical woody legumes using Biology and partial 16S rRNA sequencing. *FEMS Microbiology Letters*, 170(1): 111-117.
- Mohale, K. C., Belane, A. K. and Dakora, F. D. 2013. 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(2): 307-319.
- Mwenda, G. M., O'Hara, G. W., De Meyer, S. E., Howieson, J. G. and Terpolilli, J. J. 2018. Genetic diversity and symbiotic effectiveness of *Phaseolus vulgaris* nodulating rhizobia in Kenya. *Systematic and Applied Microbiology*, 41(4): 291-299.

- Nautiyal, C. S. 1999. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiology Letters*, 170(1): 265-270.
- Ndiaye, A. A. 1996. Diversity and nitrogen fixation of rhizobia from. DEA. Cheikh Anta Diop University, Dakar, Senegal.
- Ndlovu, S., Suinyuy, T. N., Pérez-Fernández, M. A. and Magadlela, A. 2023. *Encephalartos natalensis*, their nutrient-cycling microbes and enzymes: A story of successful trade-offs. *Plants*, 12(5): 1-15.
- Ngeno, J. K., Chemining'wa, G. N. and Hutchinson, M. J. 2018. Symbiotic efficiency of commercial and native rhizobia of cowpea (*Vigna unguiculata* L.) in Southwestern Kenya, *Asian Journal of Advances in Agricultural Research*, 8(1): 1-17.
- Normand, P., Ponsonnet, C., Nesme, X., Neyra, M. and Simmonet, P. 1996. ITS analysis of prokaryotes. In: Akkermans, A. D. L., van Elsas, J. D. and Bruijn, F.J. eds. *Molecular microbiology ecology manual*. Dordrecht. Academic publisher.
- Onyango, B., Anyango, B., Nyunja, R., Koech, P.K., Robert A. Skilton, R.A. and Stomeo, F. 2015. Morphological, genetic, and symbiotic characterization of root nodule bacteria isolated from Bambara groundnuts (*Vigna subterranea* L. Verdc) from soils of Lake Victoria basin, western Kenya. *Journal of Applied Biology and Biotechnology*, 3 (01): 001-010.
- Onyango, O. B. and Ogolla, O. F. 2019. Assessment of rhizobia strains isolates of soils around Lake Victoria Basin for their effectiveness in nodulation and symbiotic efficiency on soybeans and Bambara groundnuts. *International Journal of Environment, Agriculture and Biotechnology*, 4(5): 1469-1481.
- Pielou, E. C. 1966. The measurement of diversity in different types of biological collections. *Journal of Theoretical Biology*, 13: 131-144.

- Pohajda, I., Huic Babi, C. K., Rajnovi, C. I., Kaji, C. S. and Sikora, S. 2016. Genetic ´ diversity and symbiotic efficiency of indigenous common bean rhizobia in Croatia. *Food Technology Biotechnology*, 54: 468-474.
- Puozaa, D., Jaiswal, S. and Dakora, F. 2017. African origin of Bradyrhizobium sp population's nodulating Bambara groundnut (*Vigna subterranea* L. Verdc) in Ghanaian and South African soils. *PLOS ONE*, 12(9): 27-59.
- Ramírez, M. D., España, M., Sekimoto, H., Okazaki, S., Yokoyama, T. and Ohkama-Ohtsu, N. 2021. Genetic diversity and characterization of symbiotic bacteria isolated from endemic phaseolus cultivars located in contrasting agroecosystems in Venezuela. *Microbes and Environments*, 36(2): 1-16.
- Santos, R. M., Kandasamy, S. and Rigobelo, E. C. 2018. Sugarcane growth and nutrition levels are differentially affected by the application of PGPR and cane waste. *Microbiology Open*, 7(6): 1-9.
- Seshachala, U. and Tallapragada, P. 2012. Phosphate solubilizers from the rhizosphere of *Piper nigrum* L. in Karnataka, India. *Chilean Journal of Agricultural Research*, 72(3): 397-403.
- Shannon, C. and Weaver, W. 1949. The mathematical theory of communication. Urbana: University of Illinois Press.
- Shirling, E. and Gottlieb, D. 1966. Method for characterization of *Streptomyces* species. *International Journal of Systematic Bacteriology*, 16(3): 313-340.
- Shiro, S., Matsuura, S., Saiki, R., Sigua, G. C., Yamamoto, A., Umehara, Y. and Saeki, Y. 2013. Genetic diversity and geographical distribution of indigenous soybean-nodulating bradyrhizobia in the United States. *Applied and Environmental Microbiology*, 79(12): 3610-3618.
- Simpson, E. H. 1949. Measurement of diversity. *Nature*, 163(4148): 688-688.

- Singh, S. K., Jaiswal, S. K. and Akhouri Vaishampayan, D. B. 2013. Physiological behavior and antibiotic response of soybean (*Glycine max* L.) nodulating rhizobia isolated from Indian soils. *African Journal of Microbiology Research*, 7(19): 2093-2102.
- Somasegaran, P. and Hoben, H. J. 1994. *Handbook for Rhizobia. Methods in Legume-Rhizobium Technology*. New York, Springer-Verlag: 240-58.
- Sprent, J. I., Ardley, J. K. and James, E. K. 2013. From north to South: A latitudinal look at legume nodulation processes. *South African Journal of Botany*, 89: 31-41.
- Sprent, J. I., Odee, D. W. and Dakora, F. T. 2010. African legumes: A vital but under-utilized resource. *Journal of Experimental Botany*, 61(5): 1257-1265.
- Suleman, M., Yasmin, S., Rasul, M., Yahya, M., Atta, B. M. and Mirza, M. S. 2018. Phosphate solubilizing bacteria with glucose dehydrogenase gene for phosphorus uptake and beneficial effects on wheat. *PLOS ONE*, 13(9): 1-28.
- Suzuki, K., Oguro, H., Yamakawa, T., Yamamoto, A., Akao, S. and Saeki, Y. 2008. Diversity and distribution of indigenous soybean-nodulating rhizobia in the Okinawa islands, Japan. *Soil Science and Plant Nutrition*, 54: 237-246.
- Tamura, K., Nei, M. and Kumar, S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences*, 101(30): 11030-11035.
- Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Journal of Molecular Biology Evolution*, 30: 2725-2729.
- Tang, X., Zhong, R., Jiang, J., He, L., Huang, Z., Shi, G., Wu, H., Liu, J., Xiong, F., Han, Z., Tang, R. and He, L. 2020. Cassava/peanut intercropping improves soil quality via rhizospheric microbes increased available nitrogen contents. *BMC Biotechnology*, 20: 13.

- Torres-Gutiérrez, R., Granda-Mora, K. I., Alvarado-Capó, Y., Rodríguez, A. S., Mogollón, N. G. and Almeida, J. R. 2017. Genetic and phenotypic diversity of *Rhizobium* isolates from southern Ecuador. *Ciências Agrotecnologia*, 41(6): 634-647.
- Vincent, J. M. 1970. A Manual for the Practical Study of root nodule bacteria. *Journal of Basic Microbiology*, 12(5): 440.
- Wang, F. Q., Wang, E. T., Liu, J., Chen, Q., Sui, X. H., Chen, W. F. and Chen, W. X. 2007. *Mesorhizobium albiziae* sp. nov., a novel bacterium that nodulates *Albizia kalkora* in a subtropical region of China. *International Journal of System and Evolutionary Microbiology*, 57: 1192-1199.
- Wang, L., Cao, Y., Wang, E. T., Qiao, Y. J., Jiao, S., Liu, Z. S., Zhao, L. and Wei, G. H. 2016. Biodiversity and biogeography of rhizobia associated with common bean (*Phaseolus vulgaris* L.) in Shaanxi Province. *System Applied Microbiology*, 39(3): 211-219.
- Wasike, V. W., Lesueur, D., Wachira, F. N., Mungai, N. W., Mumera, L. M., Sanginga, N., Mburu, H. N., Mugadi, D., Wango, P. and Vanlauwe, B. 2009. Genetic diversity of indigenous *Bradyrhizobium* nodulating promiscuous soybean [*Glycine max* (L) Merr.] varieties in Kenya: impact of phosphorus and lime fertilization in two contrasting sites. *Plant Soil*, 322: 151-163.
- Wernegreen, J. J. and Riley, M. A. 1999. Comparison of the evolutionary dynamics of symbiotic and housekeeping loci: a case for the genetic coherence of rhizobia lineages. *Molecular Biology and Evolution*, 16: 98-113.
- Xu, L. M., Ge, C., Cui, Z., Li, J. and Fan, H. 1995. *Bradyrhizobium Liaoningense* sp. nov., isolated from the root nodules of soybeans. *International Journal of Systematic Bacteriology*, 45(4): 706-711.

- Yan, J., Han, X. Z., Ji, Z. J., Li, Y., Wang, E. T., Xie, Z. H. and Chen, W. F. 2014. Abundance and diversity of soybean-nodulating rhizobia in black soil are impacted by land use and crop management. *Applied and Environmental Microbiology*, 80(17): 5394-5402.
- Yang, Y. D., Feng, X. M., Hu, Y. G., Ren, C. Z. and Zeng, Z. H. 2007. Effects of legume-oat intercropping on abundance and community structure of soil N₂ -fixing bacteria. *Journal of Applied Ecology*, 28: 957-965.

CHAPTER FOUR

ASSESSMENT OF SOIL NUTRIENT STATUS AND ENZYME ACTIVITIES OF BAMBARA GROUNDNUT RHIZOSPHERE SOIL IN LIMPOPO, KWAZULU-NATAL, AND MPUMALANGA PROVINCE

4.1. Introduction

Soil is the key source of nutrients that are assimilated by plant root systems to promote the growth and development of plants (Zungu, Egbewale, Olaniran, Pérez-Fernández & Magadlela, 2020). Zungu *et al.* (2020) added that the availability of these nutrients in the soil is mainly regulated by factors such as microbial composition, pH, and soil enzyme activities. Poor agricultural practices, increased population, nutrient mining, and uncontrolled burning have resulted in the drastic degradation of most soils (Sanderson *et al.*, 2013). Consequently, there has been a high loss of soil nutrients, with reports pointing to about 90 % of soil nutrients depletion, soil structure, and increased soil acidity in these soils (Parihar *et al.*, 2020). Of all the nutrients, N, and P levels in the soil are the biggest restriction to agricultural production in sub-Saharan Africa (SSA) (Pasley, Cairns, Camberato & Vyn, 2019; Mmbaga, Mtei & Ndakidemi, 2014). The unavailability of N in agricultural soils results in poor growth and development of plants. While insufficient uptake of P by plants is mainly because P has formed insoluble complexes with cations such as aluminum (Al_3) and iron (Fe^{3+}) in mostly acidic soils (Dabessa, Abebe & Bekele, 2018). The decreased pH in these soils and decreased cation-exchange capacity reduce the availability of nutrients such as potassium (K^+), calcium (Ca^{2+}), and ammonium (NH_4^+) (Aprile & Lorandi, 2012). Despite soil acidity and poor nutrition, the rhizospheric soils of legumes have been considered to host bacteria that play an important role in the cycling of nutrients, which includes genera such as *Bacillus*, *Rhizobium*, *Azotobacter*, *Bradyrhizobium*, *Paenibacillus*, and *Pseudomonas* (Jaiswal & Dakora, 2019).

Bambara groundnut (*Vigna subterranea*) is an underutilized and neglected legume crop, rich in nutrients such as calcium, potassium, and iron with a greater proportion of fiber (Mubaiwa, Fogliano, Chidewe & Linnemann, 2017). It is traditionally grown by subsistence farms in various localities where they are useful in securing and supporting nutrition in local communities to meet their socio-cultural traditional uses and their needs (Sprent, Odee & Dakora, 2010). Bambara groundnut rhizosphere bacteria are involved in crucial processes which include the decomposition of organic matter, formation of soil structure, and cycling of significant nutrients such as nitrogen, phosphorus, potassium, carbon, and sulfur through mineralization, mobilization, nitrogen fixation, and the secretion of enzymes in the soil (Billah *et al.*, 2019; Pontigo *et al.*, 2018; Youssef, El-Azab, Mahdy, Essa & Mohammed, 2017). Moreover, soil enzymes help to enhance soil fertility by breaking down organic matter into assimilable forms and they facilitate the processes of cycling and mineralization of essential nutrients including P, C, and N (Martínez-Hidalgo & Hirsch 2017; Veres *et al.*, 2015). The activity of soil enzymes, often influenced by rhizobacteria, contributes to maintaining soil fertility. These enzymes help in the degradation of pollutants and the stabilization of soil organic matter, leading to improved soil structure, moisture retention, and overall soil health. Hence, the objective of this study was to assay soil nutrient status and enzyme activities of Bambara groundnut rhizosphere soil in Limpopo, KwaZulu-Natal, and Mpumalanga Province.

4.2 Material and Methods

4.2.1. Sampling site

Bambara groundnut rhizosphere soil samples were collected from varying altitudes in Mpumalanga, Limpopo, and KwaZulu-Natal provinces (Table 3.1). The selection of farmers was based on Bambara groundnut production interest and fields that had no manure and fertilizer application in the previous years. Soil samples collected in the Bambara groundnut

soil rhizosphere at each point was thoroughly mixed (Figure 4.1). To determine the enzyme activities, a portion of each Bambara groundnut rhizosphere soil sample was stored in sterile plastic bags in a refrigerator at 4 °C until biological and chemical analyses were conducted.

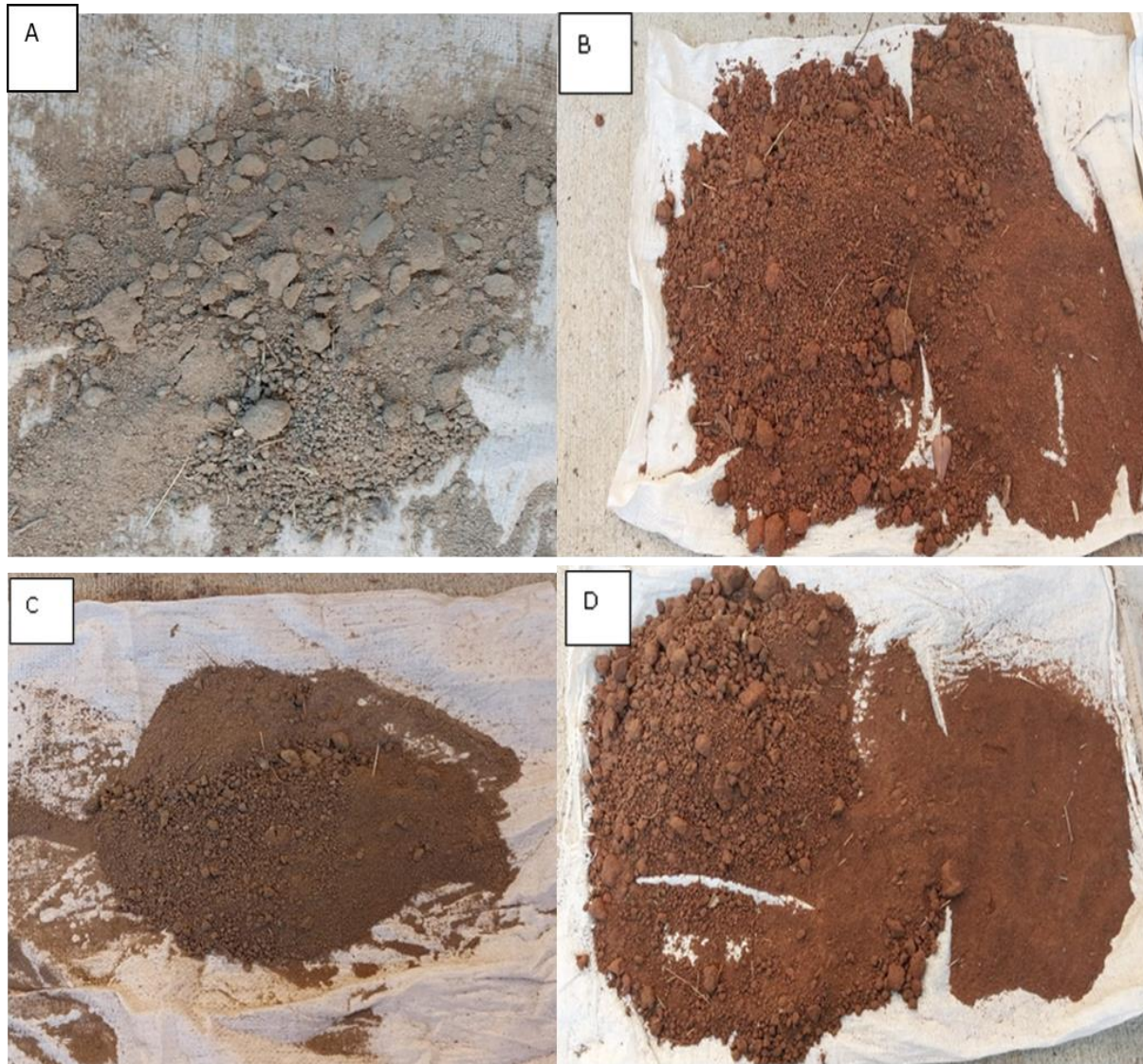


Figure 4.1: Soil sample from collected from Hazyview (A), Casteel (B), Gabaza village (C) and Zululand (D).

4.2.2. Soil physico-chemical properties analysis

Soil samples were prepared for analysis according to the International Standard Organization (ISO) standard 64, which involved drying at room temperature before sieving through a 2 mm sieve and pulverizing. All glassware used for analysis were washed thoroughly, soaked in 20

% nitric acid, and cleansed with deionized water to eliminate all potential contaminants. The selected physical and chemical parameters of the soil samples were analysed using standard laboratory procedures (Gavrić *et al.*, 2019). To determine the total nutrient analysis, soil samples were assayed at KwaZulu-Natal Department of Agriculture and Rural Development's Analytical Services Unit, Cedara, South Africa for total sample density, clay content, pH, N, Ca^{2+} , Mg^{2+} , P, K^{+} , Zn, Cu, Mn, acid saturation, exchangeable acidity, effective cation exchangeable capacity (ECEC), organic carbon, and organic matter following the below stated methods:

Sample density.

Soil samples were put in a flask with distilled water before homogenisation. The homogenised soil samples were then boiled to remove all air from the sample. Then allowed to cool, water was added to the mixture. The mixture's weight was subsequently assessed. Next, the weight of the water was subtracted from the combined mass of the soil and water. Soil sample density was analyzed on a volume bases. To enable the conversion of the results to a mass basis, the mass of a 10 mL scoop of a dried and milled sample was determined.

Clay content

Clay content was measured by first air-drying soil samples and determining their weight. The soil was then mixed with a dispersing agent (sodium hexametaphosphate) and distilled water to form a suspension. After the suspension had settled, the height of clay layer was measured and percentage volume computed relative to the volume of suspension pipetted.

Determination of soil pH

Ten (10) mL of soil sample was homogenized with 25 mL of 1 M potassium chloride (KCl) solution at 400 r.p.m. using multiple stirrers for five minutes and then allowed to stand for 30 minutes. The pH was measured using a gel-filled combination electrode glass while stirring.

Determination of total nitrogen

Total nitrogen was determined by using the micro-Kjedahl digestion-distillation method as explained by Bremner and Mulvaney (1982) in (Ameen, Han & Xie, 2019). A sample of soil dried at 80 °C and then ground to powder; 1 g was used to analyse for N (Page, Miller & Keeney, 1982). This method involves a three-step approach for the quantification of protein: distillation, digestion, and titration (Page *et al.*, 1982).

Determination of calcium (Ca^{2+}) and magnesium (Mg^{2+})

Sample soil cups were filled with 2.5 mL of soil, 25 mL of 1 M KCl solution was then added and stirred at 400 r.p.m for a total of 10 min using a multiple stirrer. Whatman No. 1 paper was then used to filter the extracts. A 5 mL of the filtrate was diluted with 20 mL of 0.0346 M SrCl_2 . Mg^{2+} , and Ca^{2+} were then determined by atomic absorption.

Determination of phosphorus (P), potassium (K^+), Zinc (Zn), Copper (Cu), and manganese (Mn)

The extracting solution of Ambic-2 consisted of 0.25 M NH_4CO_3 + 0.01 M Na_2EDTA + 0.01 M NH_4F + 0.05 g L^{-1} Super-floc (N100) which was adjusted to pH of 8 with ammonia solution concentration. A 25 mL of the solution was then added to 2.5 mL soil and the suspension was stirred using multiple stirrers for 10 min at 400 r.p.m. Whatman No.1 paper was used to filter the extracts. Phosphorus (P) was determined on a 2 mL aliquot of filtrate using a modification of the Murphy and Riley (1962) molybdenum blue procedure (Hunter, 1974), while potassium is determined by atomic absorption on a 5 mL aliquot of the filtrate after dilution with 20 mL deionized H_2O and micronutrients zinc (Zn), copper (Cu), and manganese (Mn) described by Lindsay and Norvell (1978) were determined by atomic absorption on the remaining undiluted filtrate.

Acid saturation and effective cation exchangeable capacity (ECEC)

The ECEC was calculated by adding the sum of KCl- extractable Mg, Ca, acidity, and Ambic-2 extractable K. The Acid saturation percentage of the ECEC is calculated as "extractable acidity" x 100 / (Mg + K + Ca + "extractable acidity").

Determination of organic carbon (OC) and organic matter (OM)

Organic carbon was determined by the Walkley-Black wet oxidation method (Nelson & Sommer, 1996). One kg of air-dried samples were oxidized to carbon dioxide with 10 mL of 1 N with potassium dichromate solution in the presence of 20 mL of sulphuric acid concentration and allowed to stand for 30 minutes, then 10 mL of orthophosphoric acid concentration and 200 mL of deionized H₂O was added. Twelve drops of 1 g of diphenylamine indicator in 100 mL of sulfuric acid concentration was also added with continuous stirring on a magnetic stirrer and later the mixture was titrated with 0.5 M ferrous ammonium sulphate until colour change from violet-blue to green was observed. Considering that the average content of carbon in soil organic matter is equal to 58 %, the conversion factor 1.724 was used to calculate the percentage of organic matter from the content of organic carbon.

4.2.3. Soil enzyme activity

Phosphorus and nitrogen enzyme cycling activities (acid phosphatase, β -glucosidase, β -glycosaminidase, and alkaline phosphatase) were determined according to the method that was adapted from Jackson *et al.* (2013) and conveyed in units of nmol h⁻¹ g⁻¹. Briefly, 5 g of each soil sample collected in various farms were homogenized at low speed in a 50 mL ultrapure at 4 °C for 2 hrs. Resultant supernatants were then transferred into black 96-well microplates before adding the substrates. For P-cycling enzyme activity, 4-MUB-phosphate substrate was added, while for N-cycling enzyme activity 4-MUB-N-acetyl- β -D-glucosaminide was added. The substrates were prepared using a solution of 200 μ M of MUB-linked which was dissolved in a sterilized distilled H₂O as described by Jackson *et al.* (2013). Samples run consisted of 200

μL aliquot plus 50 μL substrate and then incubated alongside reference standards (200 μl buffer plus 50 μl standard), standard quench (200 μL soil aliquot plus 50 μL standard), with sample control (200 μl soil aliquot plus 50 μl buffer), blanks (250 μL buffer), and negative controls (200 μL buffer plus 50 μL substrate). After incubation at 30 °C for 2 hours the reaction was then stopped with 0.5 M NaOH (sodium hydroxide). Later, a Glomax Multi Plus microplate reader (Bio Tek, USA) was used to measure fluorescent absorbance at 450 nm. Both standard and buffer were adjusted to a pH of 5 before determining the phosphate activity.

4.2.4. Nitrate reductase activities

Method adapted from Bruckner *et al.* (1995) was used to measure nitrate reductase activities. A 5 g of soil sample was transferred in a solution that comprised 4 mL of 0.9 mM 2,4-dinitrophenol, 1 ml of 25 Mm KNO_3 , and 5 mL of ultrapure water (H_2O) in a sealed 50 mL centrifuge tube. The mixture was homogenized and then incubated in the dark at 30 °C for 24 hours. After the incubation period, about 10 mL of 4 M KCl of solution was then added to each of the samples and vigorously mixed. Subsequently, they were allowed to pass through Whatman number 1 filter paper. The enzymatic reaction started by adding 2 mL of the filtrate to 1.2 mL of 0.19 M ammonium chloride buffer (pH 8.5) and 800 μL of the colour reagent (1 % sulphanilamide in 1 N HCl and 0.2 % N-(1-naphthyl) ethylenediamine dihydrochloride (NEDD) before incubation for 30 minutes in a dark at a temperature of 30 °C. The absorbance was then measured at 520 nm using an Agilent Cary 60 UV-Vis spectrophotometer (Agilent, Santa Clara, CA, USA). The amount of nitrite (NO_2) released into the medium was expressed as $0.1 \mu\text{molh}^{-1}\text{g}^{-1}$.

4.2.5. Data analysis

Macro and intermediate nutrients as well as pH, total cation, and exchange activity in all study sites soil of Mpumalanga, Limpopo, and KwaZulu-Natal provinces, were analyzed using one-way analysis of variance (ANOVA) through Statistix 10 software. The mean separation was achieved using Fisher's Least Significant Differences (LSD) at a 5 % probability level. The data that was not normally distributed was transformed using $\text{Log}_{10}(x+1)$. The measured soil nutrients of all sample sites were examined by Principal Component Analysis (PCA), statistical procedure version 4.02 was used. Correlations matrix of the variables were then determined by Pearson co-efficient ($p \leq 0.05$ and $p \leq 0.01$).

4.3. Results

All physico-chemical soil analyses were done at the KwaZulu-Natal Department of Agriculture and Rural Development's Analytical Services Unit, Cedara, South Africa (Appendix 4.1). According to Shapiro-Wilk normality tests, all tested enzyme activities and soil physico-chemical properties were not normally distributed ($P \leq 0.05$) except for effective cation exchange capacity (ECEC), hence the data were transformed accordingly (Appendix 4.2).

All physico-chemical soil properties were highly significant ($P \leq 0.01$), except for pH, and nitrogen (N) which were significant ($P \leq 0.05$), while phosphorus (P), exchangeable acidity, and acid saturation were not significant. The soil physico-chemical properties of the studied site varied across all farms (Table 4.1).

4.3.1. Soil physico-chemical properties

Soil density (SD)

Boschfontein had the highest soil density of 1455 g L^{-1} , followed by Hlamalani with 1400 g L^{-1} , and least was Hazyview with 1070 g L^{-1} (Table 4.1). According to Hazelton and Murphy

(2007), soil density is a significant physical property of soils used as a measure of soil compactness, available water capacity, root penetration, soil structure, and soil aeration. The tested soil density of the studied soil was low. The result on SD indicates that Hlamalani and Boschfontein were not different, Casteel, Nkomazi, Bushbuckridge, and Mkhuhlu were also the same and Hazyview, Gavaza, Nhlangenyuke, University of Mpumalanga, and Zululand was not different.

Clay content

The clay content in the study areas ranged from 7.50 % to 38.00 %. Hazyview had the highest clay content of 38.00 %, followed by Gabaza village with 37.00 % and the least was Boschfontein with 7.50 % (Table 4.1). The clay content of Hazyview, and Gabaza was not different from Nhlangenyuke when compared to the other tested soil sites (Table 4.1). Casteel clay content was different from the University of Zululand and Mpumalanga, which was not different from Bushbuckridge. While Bushbuckridge was not different from Nkomazi, Mkhuhlu, and Hlamalani and different from Boschfontein. Moreover, Boschfontein was also not different from Hlamalani and Mkhuhlu (Table 4.1).

4.3.2. Soil Chemical Properties

Soil pH

The studied soil pH ranged from 4.41-5.58. The soil pH value for the University of Zululand and Mpumalanga was 4.41 (< 4.5) and is rated strongly acidic (Table 4.1) (Table 4.1). Soil sample pH of Nhlangenyuke, Gabaza, Boschfontein, Mkhuhlu, Hlamalani, and Casteel were 4.55, 4.76, 8.84, 4.85, 5.12, and 5.20, respectively, are rated as medium to slightly acid (Table 4.1). Bushbuckridge, Hazyview, and Nkomazi are rated as being very slightly acidic with pH of 5.51, 5.55, and 5.58 respectively (Table 4.1). University of Zululand soil pH was not different from Gabaza, Nhlangenyuke, Mkhuhlu, Hlamalani, University of Mpumalanga, and

Boschfontein, while Hazyview, Nkomazi, Casteel, and Bushbuckridge were different from the University of Zululand and Mpumalanga (Table 4.1).

Total nitrogen (N)

In this present study, N value in the studied soil samples ranged from 0.44 mg kg⁻¹ to 1.10 mg kg⁻¹, with Gabaza having the highest and Mkhuhlu the lowest (Table 4.1). These values are classified as very low to low nitrogen levels (Landon, 1991) adapted from Metson (1961). Mkhuhlu, Nhlangenyuke, Boschfontein, and Gabaza had low nitrogen levels that were not significantly different, while Bushbuckridge, University of Zululand, University of Mpumalanga, Hazyview, Casteel, and Nkomazi had very low N level (Table 4.1). On the total N value Nhlangenyuke, Casteel, Bushbuckridge, University of Zululand, and Mpumalanga, Mkhuhlu, and Boschfontein were not different, while the value of N for Casteel, Bushbuckridge, University of Zululand and Mpumalanga, and Boschfontein were not different from Hazyview, Gabaza, Nkomazi, and Hlamalani (Table 4.1).

Exchangeable bases Mg²⁺, Ca²⁺, and K⁺

The amount of exchangeable cations namely: Ca²⁺, K⁺, and Mg²⁺ of the studied soil sample represented in Table 4.1 varied amongst and within the soil sample. The exchangeable Ca²⁺ level was the highest when compared to the other exchangeable cations. Hazyview had the highest level of Ca²⁺ of 4.98 cmol_c kg⁻¹, Nhlangenyuke had the highest k⁺ of 0.45 cmol_c kg⁻¹, and Gabaza village had the highest Mg²⁺ of 2.11 cmol_c kg⁻¹, and Boschfontein lowest on Ca²⁺, K⁺, and Mg²⁺ with 0.98 cmol_c kg⁻¹, 0.097 cmol_c kg⁻¹, and 0.23 cmol_c kg⁻¹ (Table 4.1). Soil Ca²⁺ content values were rated as very low to moderate by Landon (1991). It was also observed that the Hazyview site has moderate Ca²⁺. However, the rhizosphere soil exchangeable Ca²⁺ level indicated that Hazyview was not different from Gabaza soil and different from the other sample sites. Nhlangenyuke Ca²⁺ exchangeable level was not different from Casteel, Nkomazi,

Bushbuckridge, University of Zululand, Mkhuhlu, Hlamalani, and University of Mpumalanga, while Hlamalani was not different from Boschfontein (Table 4.1).

According to Landon's (1991), categorization of K^+ , all studied sites had low K^+ content of <0.15 except for study site Hazyview ($0.25 \text{ cmol}_c \text{ kg}^{-1}$), Gabaza ($0.23 \text{ cmol}_c \text{ kg}^{-1}$), Nhlangenyuke ($0.45 \text{ cmol}_c \text{ kg}^{-1}$), Casteel ($0.18 \text{ cmol}_c \text{ kg}^{-1}$), Nkomazi ($0.22 \text{ cmol}_c \text{ kg}^{-1}$), University of Zululand ($0.21 \text{ cmol}_c \text{ kg}^{-1}$), and Mpumalanga ($0.23 \text{ cmol}_c \text{ kg}^{-1}$) had moderate K^+ (Table 4.1). This result shows that K^+ was problematic in these areas and its application should be done to boost yield. Nhlangenyuke K^+ content was different from the other tested soil sites, while Hazyview was not different from Gavaza, University of Mpumalanga, Casteel, Nkomazi, and University of Zululand and different from Bushbuckridge, Mkhuhlu, Hlamalani, and Boschfontein, which were not different from Casteel (Table 4.1). According to Landon (1991) rates of Mg^{2+} are as follows: 0.3-1.0 as low, 1.0-3.0 as moderate, 3.0-8.0 as high, and >8 as very high. Therefore, Mg^{2+} was low in study sites Casteel ($0.45 \text{ cmol}_c \text{ kg}^{-1}$), Nkomazi ($0.55 \text{ cmol}_c \text{ kg}^{-1}$), Bushbuckridge ($0.52 \text{ cmol}_c \text{ kg}^{-1}$), Mkhuhlu ($0.78 \text{ cmol}_c \text{ kg}^{-1}$), Hlamalani ($0.31 \text{ cmol}_c \text{ kg}^{-1}$), Boschfontein ($0.23 \text{ cmol}_c \text{ kg}^{-1}$), and University of Mpumalanga ($0.23 \text{ cmol}_c \text{ kg}^{-1}$) (Table 4.1). Studied sites with moderate Mg^{2+} content were Hazyview ($1.58 \text{ cmol}_c \text{ kg}^{-1}$), Gabaza ($2.11 \text{ cmol}_c \text{ kg}^{-1}$), Nhlangenyuke ($1.89 \text{ cmol}_c \text{ kg}^{-1}$), and University of Zululand ($1.78 \text{ cmol}_c \text{ kg}^{-1}$) (Table 4.1). The level of Mg^{2+} of Hazyview, Gabaza, Nhlangenyuke, and the University of Zululand were not different from each other but different from Casteel, Nkomazi, Bushbuckridge, Mkhuhlu, Hlamalani, and Boschfontein. While Mkhuhlu was not different from Boschfontein and the University of Mpumalanga (Table 4.1).

Soil micronutrients manganese, copper, and zinc

Manganese (Mn), Zn, and Cu were rated high, all micronutrients became available to plants in acidic soil except for molybdenum. Mn ranged from 6.01 mg kg^{-1} to 49.55 mg kg^{-1} , the highest of 49.55 mg kg^{-1} was found in Gabaza village followed by Boschfontein with 48.12 mg kg^{-1}

and the lowest was 6.01 mg kg⁻¹ (Table 4.1). The Manganese (Mn) level in Gabaza soil was significant to Boschfontein and Hazyview. Hazyview was not different from Nhlangenyuke, Casteel, the University of Mpumalanga, and Bushbuckridge (Table 4.1). Casteel was also not different from Nkomazi, Mkhuhlu, and Hlamalani and different from the University of Zululand which was not different from Hlamalani (Table 4.1). Copper (Cu) ranged from 0.39 mg kg⁻¹ to 9.39 mg kg⁻¹, the highest Cu was in Gabaza village with 9.39 mg kg⁻¹, followed by Nhlangenyuke with 7.51 mg kg⁻¹, and the lowest was Boschfontein with 0.39 mg kg⁻¹ (Table 4.1). Gavaza and Nhlangenyuke Cu were not different and different from the other tested soil sites (Table 4.1). Hazyview was also different from the tested soil sites while Casteel, Bushbuckridge, University of Zululand and Mpumalanga, and Mkhuhlu, were not different (Table 4.1). Mkhuhlu and the University of Mpumalanga were also not different from Nkomazi and Hlamalani, while Hlamalani was not different from Boschfontein (Table 4.1). Zinc (Zn) ranged from 0.31 mg kg⁻¹ to 28.46 mg kg⁻¹. The level of Zn was rated low to high, the highest was found in Casteel and the lowest was in Boschfontein, according to Landon (1991). The zinc (Zn) level of Casteel was different from all tested soil samples, while Bushbuckridge and University of Mpumalanga soil was not different from Nhlangenyuke, Nkomazi, and Hlamalani which were not different from Hazyview, Gabaza, University of Zululand, Mkhuhlu, and Boschfontein (Table 4.1).

Effective cation exchange capacity (ECEC)

The effective Cation exchange capacity (ECEC) of the studied soil ranged from 1344.0 cmol_c kg⁻¹ to 6901.9 cmol_c kg⁻¹ (Table 4.1). According to Metson (1961), rating of CEC, all sample areas have high ECEC. According to Maier and Pepper (2009), the average ECEC of soils ranges from 15 to 20 meq/100 g (cmol_c kg⁻¹) and ECEC values of 15 meq/100 g are regarded as very low. The highest ECEC was in Hazyview of 6901.9 cmol_c kg⁻¹, followed by Gabaza village with 6736.2 cmol_c kg⁻¹ and the lowest was in Boschfontein with 1344.0 cmol_c kg⁻¹

(Table 4.1). The ECEC values of Hazyview, Gabaza, and Nhlangenyuke were different from the other soil sample sites, while Nhlangenyuke was not different from the University of Zululand and Mpumalanga, which were not different from Casteel, Nkomazi, and Bushbuckridge. Nkomazi soil (Table 4.2). While Casteel and Bushbuckridge were not different from Hlamalani and Mkhuhlu whereas Hlamalani was not different from Boschfontein (Table 4.1).

Organic carbon (OC) and organic matter (OM)

According to Landon (1991), the amount of OC and OM in all sites ranged from 0.50 % to 1.86 % for OC and 0.86 % to 3.20 % for OM, which is described as very low (Table 4.1). The organic matter of the soil plays a significant role in nutrient availability such as P, N, and CEC. The organic carbon of the soil varied based on the type of soil. Nhlangenyuke had the highest OC of 1.86 % and OM of 3.20 % while Hlamalani had the lowest OC and OM of 0.50 % and 0.50 % (Table 4.1). However, the OC of Nhlangenyuke was not different from the University of Zululand and Mkhuhlu and different from all other soil samples (Table 4.1). Soil OM of the tested sample area as shown in Table 4.1 that Nhlangenyuke was not different from Mkhuhlu, University of Zululand, and Mpumalanga, which were the same as Gabaza. Mkhuhlu was also not different from Boschfontein and Boschfontein which was significant from Hazyview, Gabaza, Casteel, Nkomazi, and Bushbuckridge. Hlamalani was not different from Bushbuckridge, Nkomazi, Casteel, and Hazyview (Table 4.1).

Table 4.1: Soil physico-chemical properties of studied soil in Mpumalanga, Kwa-Zulu Natal Limpopo province.

Soil Sample site	Density	Ph	Zn	Cu	Mn	N	OC	OM	Clay	Ca ²⁺	K ⁺	Mg ²⁺	ECEC
	g L ⁻¹	(KCl)	mg kg ⁻¹				%			cmol _c kg ⁻¹			
HAZY	3.03 ^c	0.26 ^a	0.13 ^c	0.22 ^b	0.41 ^{abc}	0.07 ^b	0.002 ^{de}	0.003 ^{def}	0.012 ^a	0.78 ^a	0.10 ^b	0.41 ^a	3.84 ^a
W	(1070)	(5.55)	(1.31)	(3.74)	(36.45)	(0.47)	(0.70)	(1.20)	(38.00)	(4.98)	(0.25)	(1.58)	(6901.9)
GABA	3.06 ^c	0.25 ^{abc}	0.13 ^c	0.31 ^a	0.43 ^a	0.06 ^b	0.003 ^{bcd}	0.005 ^{bcd}	0.012 ^a	0.73 ^a	0.09 ^b	0.49 ^a	3.83 ^a
	(1150)	(4.76)	(1.26)	(9.39)	(49.55)	(0.44)	(1.20)	(2.06)	(37.00)	(4.33)	(0.23)	(2.11)	(6736.2)
NHLAN	3.05 ^c	0.42 ^{bc}	0.19 ^{bc}	0.28 ^a	0.38 ^{bcd}	0.12 ^a	0.05 ^a	0.006 ^a	0.012 ^a	0.59 ^b	0.16 ^a	0.45 ^a	3.72 ^{ab}
G	(1121)	(4.55)	(2.88)	(7.51)	(28.48)	(1.08)	(1.86)	(3.20)	(35.88)	(3.00)	(0.45)	(1.89)	(5431.3)
CAST	3.09 ^b	0.25 ^{ab}	0.39 ^a	0.16 ^c	0.35 ^{cde}	0.09 ^{ab}	0.003 ^{de}	0.003 ^{ef}	0.013 ^b	0.56 ^{bc}	0.07 ^{bcd}	0.16 ^{bc}	3.52 ^{cde}
	(1240)	(5.20)	(28.47)	(1.70)	(16.53)	(0.69)	(0.70)	(1.20)	(22.50)	(2.60)	(0.18)	(0.45)	(3274.2)
NK	3.12 ^b	0.26 ^a	0.19 ^{bc}	0.10 ^d	0.33 ^{de}	0.07 ^b	0.003 ^{de}	0.004 ^{def}	0.011 ^d	0.55 ^{bc}	0.09 ^{bc}	0.19 ^{bc}	3.53 ^{cd}
	(1310)	(5.58)	(3.94)	(0.77)	(13.57)	(0.54)	(0.77)	(1.32)	(12.33)	(2.68)	(0.22)	(0.55)	(3536.2)
BUSH	3.11 ^b	0.26 ^a	0.28 ^b	0.14 ^c	0.42 ^{ab}	0.09 ^{ab}	0.002 ^e	0.003 ^{ef}	0.012 ^{cd}	0.54 ^{bc}	0.05 ^d	0.18 ^{bc}	3.50 ^{cde}
	(1280)	(5.51)	7.10)	(1.43)	(43.02)	(0.70)	(0.60)	(1.03)	(14.00)	(2.47)	(0.11)	(0.52)	(3137.5)
ZULU	3.05 ^c	0.24 ^c	0.10 ^c	0.13 ^c	0.27 ^f	0.09 ^{ab}	0.004 ^{ab}	0.006 ^{ab}	0.0126 ^{bc}	0.51 ^{bc}	0.08 ^{bc}	0.44 ^a	3.64 ^{bc}
	(1125)	(4.41)	(0.78)	(1.29)	(6.01)	(0.73)	(1.65)	(2.84)	(17.25)	(2.23)	(0.21)	(1.78)	(4340.5)
MKHL	3.11 ^b	0.25 ^{abc}	0.08 ^c	0.12 ^{cd}	0.32 ^e	0.12 ^a	0.004 ^{abc}	0.006 ^{abc}	0.010 ^{de}	0.46 ^{bc}	0.05 ^d	0.25 ^b	3.45 ^{de}
U	(1275)	(4.85)	(0.63)	(1.06)	(11.77)	(1.10)	(1.55)	(2.67)	(11.00)	(1.90)	(0.13)	(0.78)	(2846.7)
HLAM	3.15 ^a	0.25 ^{abc}	0.22 ^{bc}	0.08 ^{de}	0.32 ^{ef}	0.06 ^b	0.002 ^e	0.003 ^f	0.010 ^{de}	0.41 ^{cd}	0.05 ^{cd}	0.12 ^{bc}	3.32 ^{ef}
	(1400)	(5.12)	3.57)	(0.61)	(10.71)	(0.43)	(0.50)	(0.86)	(10.00)	(1.59)	(0.13)	(0.31)	(2078.6)
BF	3.16 ^a	0.25 ^{abc}	0.05 ^c	0.06 ^e	0.43 ^a	0.09 ^{ab}	0.003 ^{cde}	0.004 ^{cde}	0.009 ^e	0.30 ^d	0.04 ^d	0.09 ^c	3.136 ^f
	(1455)	(4.84)	(0.31)	(0.38)	(48.12)	(0.65)	(0.95)	(1.63)	(7.50)	(0.99)	(0.10)	(0.23)	(1344)
UMP	3.05 ^c	0.24 ^c	0.28 ^b	0.12 ^{cd}	0.42 ^{ab}	0.09 ^{ab}	0.002 ^{de}	0.006 ^{ab}	0.0126 ^{bc}	0.55 ^{bc}	0.09 ^b	0.09 ^c	3.64 ^{bc}
	(1125)	(4.41)	(7.10)	(1.06)	(43.02)	(0.69)	(0.70)	(0.86)	(17.25)	(2.68)	(0.23)	(0.23)	(4340.5)
F-value	22.08	3.08	11.61	58.41	9.31	3.25	10.13	10.94	20.27	6.61	21.99	11.44	11.81
P-value	0.0000	0.0187*	0.0000*	0.0000*	0.0000*	0.0146	0.0000*	0.0000*	0.0000*	0.0003*	0.0000*	0.0000*	0.0000*
	**		*	*	*	*	*	*	*	*	*	*	*
LSD _{0.05}	0.019	0.009	0.104	0.025	0.039	0.026	0.007	0.008	0.001	0.100	0.021	0.0979	0.1258

^x Column means that are followed by the same letter are not statistically different at $P \geq 0.05$, according to Fisher's least significant difference (LSD). Values in brackets are untransformed means; **Highly significant ($P \leq 0.01$), * significant ($P \leq 0.05$). GAB = Gabaza, ZULU = University of Zululand, HLAM = Hlamalani, CAST = Casteel, BUSH = Bushbuckridge, BF = Boschfontein, NHLANG = Nhlanguyuke, UMP = University of Mpumalanga, NK = Nkomazi, HAZYW = Hazyview, and MKHLU = Mkhuhlu)

4.3.3. Correlation between sample sites and soil nutrients

The principal components of all sample sites explained 46.95 % of the cumulative variability of the measured traits with principal component one (PC1) accounting for 29.8 % and the second principal component accounting for 17.15 % of the total variation (Figure 4.2). MKHULU 1 was separated from PCA1 with variation being a result of soil nutrients. The correlation matrix indicated that NHLANGE 3A and NHLANGE 3B were highly positively correlated. Moreover, NHLANGE 4B was positively (strongly) correlated to NKOMAZI 1b and BUSH 2. HAZYVIEW 1a and NKOMAZI 1a were highly correlated (Figure 4.2).

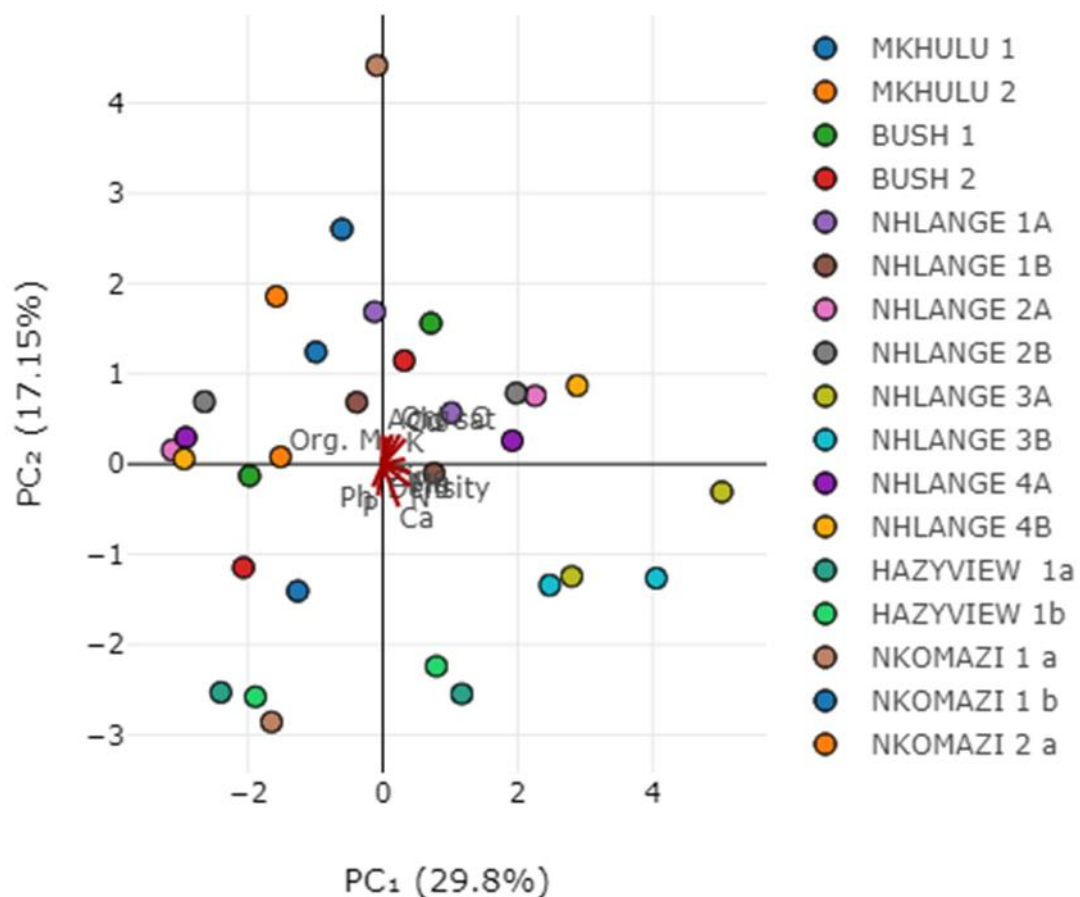


Figure 4.2: Correlation between soil nutrient and sample locations in Mpumalanga, KwaZulu-Natal, and Limpopo province.

4.3.4. Soil enzyme activities

Soil enzyme activities N-cycling and P-cycling (acid phosphatase, β -glucosidase, β -glycosaminidase, and alkaline phosphatase) were statistically not significant ($P \geq 0.05$) among different locations. Hlamalani had the highest nitrate reductase of $19710 \text{ nmolh}^{-1} \text{ g}^{-1}$, followed by Hazyview and Nkomazi of $18176.67 \text{ nmolh}^{-1} \text{ g}^{-1}$. whereas Bushbuckridge had the lowest Nitrate reductase of $6243.33 \text{ nmolh}^{-1} \text{ g}^{-1}$.

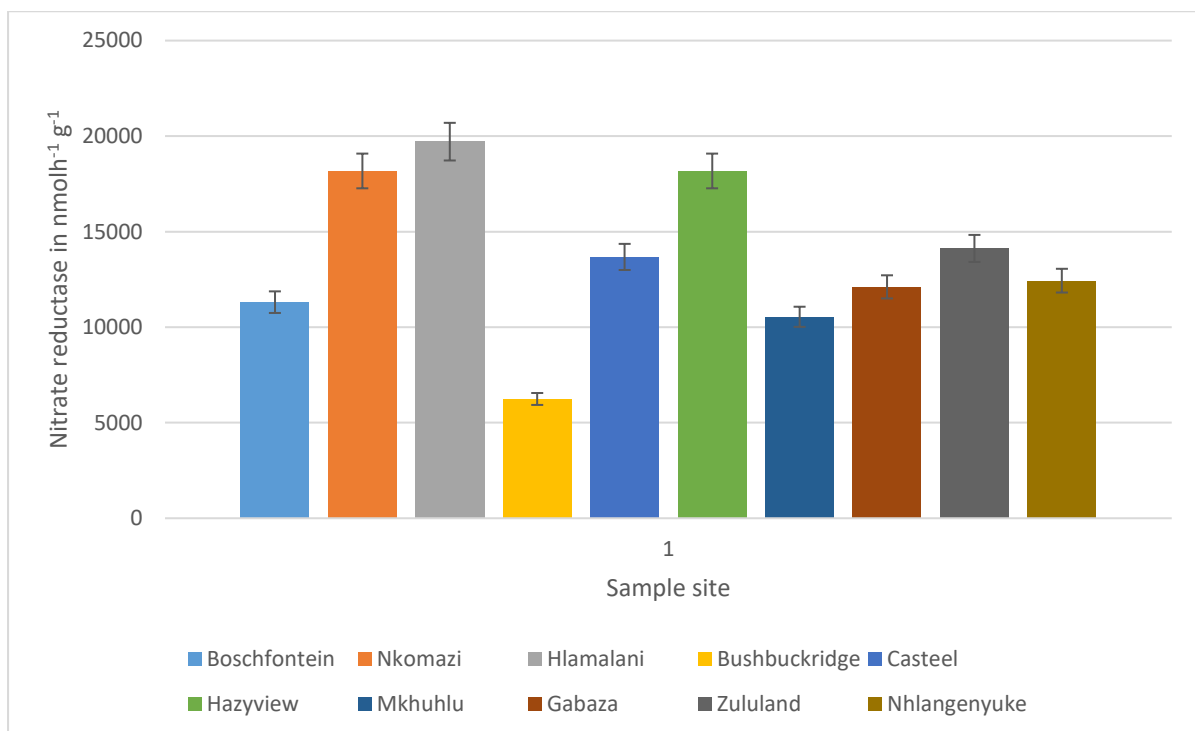


Figure 4.3: Soil nitrate reductase enzyme activities in $\text{nmolh}^{-1} \text{ g}^{-1}$.

4.4. Discussion

The study assessed soil nutrient status and enzyme activities of Bambara groundnut rhizosphere soil in Limpopo, KwaZulu-Natal, and Mpumalanga Province. The three selected provinces and their sample location presented different clay content in each soil which influences the diversity of bacterial isolates. In this study soil with low and high clay content were less diverse. The distribution and genetic diversity of rhizobacteria consider the specific soil parameters. The

soil pH and calcium concentration significantly drove the ecological distribution of the isolate's communities among the different studied soil (Florent, Cauchie, Herold, Jacquet & Ogorzaly, 2022). Interestingly, the distribution and genetic diversity of the community in the different soil types were strongly influenced by abiotic stress such as pH and calcium concentration. The pH of the University of Zululand and Mpumalanga was rated strongly acidic, followed by Nhlanguyuke, Gabaza, Boschfontein, and Casteel rated slightly acidic and Bushbuckridge, Hazyview, Nkomazi rated very slightly acidic. The findings of this study revealed that strongly acidic areas had a high level of bacterial diversity compared to slightly acidic areas. These findings contradict with Rousk *et al.* (2010) who indicated that low pH obstructs the development of microbial communities which results in the observed low values of diversity and richness in the soils. The pH of the soil was found to exert variation or even contrasting effects on bacterial communities, The variation in bacteria diversity was highlighted especially for the studied site, these sites did not only display an acidic pH (≤ 5.5) with low Ca^{2+} availability except for Hazyview with moderate Ca^{2+} . Moreover, Ca^{2+} is extremely related to the soil pH. Indeed, soils that are acidic display low Ca^{2+} availability and are high in Zinc (Zn), Manganese (Mn), and copper (Cu). Micronutrients such as Zn, Mn, and Cu were found to shape bacterial communities independent of the pH (Whalen, Smith, Grandy & Frey, 2018). On the contrary university of Zululand and Mpumalanga with low pH and Ca^{2+} concentrations showed higher bacterial diversity. Furthermore, low (acidic) pH soil decreases K^+ , Ca^{2+} , and Mg^{2+} and hinders the development of abundance communities (Rousk *et al.*, 2010) which results in the observed low values of richness and diversity in our soils. However, in this study, the level of P was not different in all soil sample sites which means that P didn't have an influence on the diversity and distribution of isolates in the different localities. Additionally, a complementary effect of magnesium concentration solely on the bacterial distribution was detected. Magnesium was low in Casteel, Nkomazi, Bushbuckridge, Mkhuhlu, Hlamalani,

Boschfontein, and the University of Mpumalanga and moderate in Hazyview, Gabaza, Nhlangenyuke, and the University of Zululand. High level of Mg^{2+} significantly increases soil pH and improve soil acidity, carbon, calcium, Mg^{2+} content, and nitrogen, also increasing soil bacterial diversity mostly from genera belonging to nitrogen fixation and phosphorus mineralization groups such as *Sphingomonas* and *Rhizomicrobium* (Wenhao *et al.*, 2023). Nitrogen levels in the soil were low from all studied sites. Nitrogen is referred to as the most essential nutrient for plant growth and development (Pasley *et al.*, 2019). However, some types of bacteria such as *Actinobacteria* are limited by N content, and their relative abundance increases in response to an increase in total N content (Zhou *et al.*, 2017). In the present study, nitrogen content increased in response to the decrease in pH value for the University of Zululand and Mpumalanga. This might be due to the presence of bacteria not influenced by the pH. High N levels in the soil inhibit the growth of soil rhizobia (Dabessa *et al.*, 2018), nodule formation, and limit N-fixation (Dabessa *et al.*, 2018; Weisany, Raei & Allahverdipoor, 2013). Nase activity, and reduce infection threads (Saturno *et al.*, 2017; Guan *et al.*, 2013; Liu, Wu, Baddeley & Watson, 2009).

The results showed that N rates decreased the N-fixing bacteria diversity, N-fixation efficiency, and *nifH* gene copies in the Bambara groundnut rhizosphere soil. This means that the high level of diversity in Zululand and Mpumalanga might be due to low N in the soil which stimulates rhizobacteria found in the soil to convert atmospheric N into ammonia for plant growth and development. Soil pH has been regarded as the key driver in determining the assembly of the bacteria community. However, recent studies have demonstrated that the compositions of soil bacterial communities were driven by a myriad of soil abiotic traits, such as organic matter contents, forms, and contents of soil nutrients (Tian *et al.*, 2018). A study on pears demonstrates that the pear production of high-yielding soils exhibited higher organic matter contents and harboured bacterial communities with greater diversity (Wang *et al.*, 2022). In this present

study, NHLANGE 3A and NHLANGE 3B showed positive correlation. NHLANGE 4B was positively (strongly) correlated to NKOMAZI 1b and BUSH 2. The positive correlation between NHLANGE 4B, NKOMAZI 1b, and BUSH 2 indicated in this present study could thus be attributed to the enhancement of functioning and the increase in biological nitrogen fixation (Bhattacharya, Sood & Citovsky, 2010).

Soil extracellular enzyme activities provide information about soil fertility, soil quality, and soil production status (Vyas & Gulat, 2009). Moreover, plays a role in the conservation and recycling of key nutrients in nutrient-limited soils (Kutschera, 2007). Soil enzyme activities are dependent on several factors including soil properties, soil microbe interactions, and the presence of activators or inhibitors (Nannipieri, Giagnoni, Landi & Renella, 2011). In this present study soil enzyme activities N-cycling and P-cycling which include acid phosphatase, β -glucosidase, β -glycosaminidase, and alkaline phosphatase were not different, which means they didn't have an influence on the diversity and distribution of isolates in the different localities. Hlamalani soil had the highest nitrate reductase enzyme activities and the lowest was Bushbuckridge. Furthermore, the differences in nitrate reductase enzyme activity level, diversity, and distribution of rhizobia isolates in the soils could be attributed to the variations in the physicochemical properties of the soils (Puozaa, Jaiswal & Dakora, 2019). The variation in soil pH showed no effect on soil enzyme activities. Parkin, Sexstone and Tiedje (1985) stated that acidic soil from Bavarian Forest did not contain a nitrate reductase microbial population adapted to low pH levels, instead the diversity exhibited an increase in nitrate reductase activity when the pH of the soil rises.

4.5. Conclusion

The soil properties evaluated in the study, including soil density, clay content, pH, total nitrogen, exchangeable bases (Ca^{2+} , K^{+} , and Mg^{2+}), soil micronutrients (Mn, Cu, Zn), effective cation exchange capacity (ECEC), organic carbon (OC), and organic matter (OM), indicate

varying soil fertility across the study sites. Boschfontein and Hlamalani displayed higher soil density values, suggesting compact soils with potential limitations for root penetration and water capacity. Hazyview and Gabaza had the highest clay content, which could influence water retention and nutrient availability. The chemical properties of the soils revealed a range of pH values from strongly acidic to slightly acidic, with the University of Zululand and Mpumalanga having the most acidic soils. Total nitrogen levels were classified as very low to low across all study sites, indicating a need for nitrogen supplementation. Exchangeable bases were generally low, with Mg^{2+} content classified as low in most sites, while K^+ and Ca^{2+} levels were moderate in a few locations. Micronutrient levels varied significantly, with Mn levels being particularly high in Gabaza and Boschfontein. However, Zn and Cu contents were low in most sites, indicating possible deficiencies that could hinder crop growth. The organic carbon and organic matter contents were very low across all sites, further suggesting poor soil fertility and a need for organic amendments to improve soil structure and nutrient availability.

4.6. Recommendations

Nutrient Management: low nitrogen, potassium, and magnesium levels across the study sites, it is advisable to implement a nutrient management plan that includes the application of appropriate fertilizers to improve soil fertility and support plant growth. **Soil pH Adjustment:** The strongly acidic soils at the University of Zululand and Mpumalanga should be amended with lime to raise the pH to a more suitable range for most crops, thereby improving nutrient availability and reducing toxicity risks. **Micronutrient Supplementation:** For areas with low levels of essential micronutrients like Zn and Cu, targeted supplementation through foliar sprays or soil application is recommended to correct deficiencies and enhance crop yield and quality. Implementing these recommendations will improve soil fertility, support sustainable agricultural productivity, and promote long-term soil health in the study areas.

4.7. References

- Ameen, A., Han, L. and Xie, G. H. 2019. Dynamics of soil moisture, pH, organic carbon, and nitrogen under switchgrass cropping in a semiarid sandy wasteland. *Communications in Soil Science and Plant Analysis*, 50: 922-933.
- Aprile, F. and Lorandi, R. 2012. Evaluation of cation exchange capacity (CEC) in tropical soils using four different analytical methods. *Journal of Agricultural Science*, 4 (6): 278.
- Bhattacharya, A., Sood, P. and Citovsky, V. 2010. The roles of plant phenolics in defence and communication during *Agrobacterium* and *Rhizobium* infection. *Molecular Plant Pathology*, 11: 705-719.
- Billah, M., Khan, M., Bano, A., Hassan, T. U., Munir, A. and Gurmani, A. R. 2019. Phosphorus and phosphate solubilizing bacteria: keys for sustainable agriculture. *Geomicrobiology Journal*, 36: 904-916.
- Bruckner, A., Wright, J., Kampichler, C., Bauer, R. and Kandeler, E. 1995. A method of preparing mesocosms for assessing complex biotic processes in soils. *Biology and Fertility of Soils*, 19: 257-262.
- Dabessa, A., Abebe, Z. and Bekele, S. 2018. Limitations and strategies to enhance biological nitrogen fixation in sub-humid tropics of Western Ethiopia. *Journal of Agricultural Biotechnology and Sustainable Development*, 10: 122-131.
- Florent, P., Cauchie, H. M., Herold, M., Jacquet, S. and Ogorzaly, L. 2022. Soil pH, calcium content and bacteria as major factors responsible for the distribution of the known fraction of the DNA bacteriophage populations in soils of Luxembourg. *Microorganisms*, 10: 1458.
- Gavrić, S., Larm, T., Österlund, H., Marsalek, J., Wahlsten, A. and Viklander, M. 2019. Measurement and conceptual modelling of retention of metals (Cu, pb, Zn) in soils of three grass swales. *Journal of Hydrology*, 574: 1053-1061.

- Guan, D., Stacey, N., Liu, C., Wen, J., Mysore, K. S., Torres-Jerez, I., Vernié, T., Tadege, M., Zhou, C., Wang, Z., Udvardi, M. K., Oldroyd, G. E. D. and Murray, J. D. 2013. Rhizobial infection is associated with the development of peripheral vasculature in nodules of *Medicago truncatula*. *Plant Physiology*, 162: 107-115.
- Hunter, A. 1974. *Tentative ISFEI soil extraction procedure*. International Soil Fertility and Improvement Project. N.C. State University, Raleigh, NC.
- Jackson, C. R., Tyler, H. L. and Millar, J. J. 2013. Determination of microbial extracellular enzyme activity in waters, soils, and sediments using high throughput microplate assays. *Journal of Visualized Experiments*, 80: 3-9.
- Jaiswal, S. K. and Dakora, F. D. 2019. Widespread distribution of highly adapted *Bradyrhizobium* species nodulating diverse legumes in Africa. *Frontiers in Microbiology*, 10: 310.
- Kutschera, U. 2007. Plant-associated methylobacteria as co-evolved phytosymbionts: A hypothesis. *Plant Signaling and Behavior*, 2: 74-78.
- Lindsay, W. L. and Norvell, W. A. 1978. Development of DTPA soil test for Zn, Fe, Mn, and Cu. *Soil Science Society of American Journal*, 42: 421- 428.
- Liu, Y., Wu, L., Baddeley, J. A. and Watson, C. A. 2009. Models of biological nitrogen fixation of legumes. *Sustainable Agriculture*, 2: 883-905.
- Landon, J. R. 1991. *Booker Tropical Soil Manual, Handbook for Soil Survey and Agricultural Land Evaluation in Tropics and Sub-tropics*. London: Longman Scientific and Technical Publishers.
- Maier, R. M. and Pepper, I. L. 2009. Earth Environments. In: Maier, R. M., Pepper, I. L. and Gerba, C. P. eds. 2009. *Environmental microbiology*. New York: Elsevier Inc.
- Martínez-Hidalgo, P. and Hirsch, A. M. 2017. The nodule microbiome: N₂-fixing rhizobia do not live alone. *Phytobiomes*, 1: 70-82.

- Metson, A. J. 1961. *Method of chemical analysis for soil survey samples*. Wellington: Govt printer.
- Mmbaga, G. W., Mtei, K. M. and Ndakidemi, P. A. 2014. Extrapolations on the use of rhizobium inoculants supplemented with phosphorus (P) and potassium (K) on growth and nutrition of legumes. *Journal of Agricultural Science*, 05: 1207-1226.
- Mubaiwa, J., Fogliano, V., Chidewe, C. and 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.
- Murphy, J. and Riley, J. R. 1962. A modified single solution method for the determination of phosphate in natural waters. *Analytica Chimica Acta*, 27: 31-36.
- Nannipieri, P., Giagnoni, L., Landi, L. and Renella, G. 2011. Role of phosphatase enzymes in soil. In: Bunemann, E. K., eds. *Phosphorus in Action, Soil Biology*. Heidelberg: Springer-Verlag Berlin.
- Nelson, D. W. and Sommers, L. E. 1996. Total carbon, organic carbon, and organic matter. p. 961-1010. In: Sparks, D. L., Page, A. L., Helmke, P. A., Loeppert, R. H., Soltanpour, P. N., Tabatabai, M. A., Johnston, C. T. and Sumner, M. E. eds. *Methods of soil analysis*. Madison: Wiley research.
- Pasley, H. R., Cairns, J. E., Camberato, J. J. and Vyn, T. J. 2019. Nitrogen fertilizer rate increases plant uptake and soil availability of essential nutrients in continuous maize production in Kenya and Zimbabwe. *Nutrient Cycling in Agroecosystems*, 115: 373-389.
- Parihar, C. M., Jat, H. S., Singh, Y., Jat, S. L., Kakraliya, S. K. and Nayak, H. S. 2020. Precision Nutrient Management for Higher Nutrient Use Efficiency and Farm Profitability in

- Irrigated Cereal-based Cropping Systems. *Indian Journal of Fertilisers*, 16(10): 1000-1014.
- Parkin, T. B., Sexstone, A. J. and Tiedje, J. M. 1985. Adaptation of denitrifying populations to low soil pH. *Applied Environment Microbiology*, 49: 1053-1056.
- Pontigo, S., Ulloa, M., Godoy, K., Nikolic, N., Nikolic, M., Mora, M. S. I. L. and Cartes, P. 2018. Phosphorus efficiency modulates phenol metabolism in wheat genotypes. *Journal of Soil Science and Plant Nutrition*, 18: 904-920.
- Puozaa, D. K., Jaiswal, S. K. and Dakora, F. D. 2019. Phylogeny and distribution of *Bradyrhizobium* symbionts nodulating cowpea (*Vigna unguiculata* L. Walp) and their association with the physicochemical properties of acidic African soils. *Systematic and Applied Microbiology*, 42: 403-414.
- Rousk, J., Bååth, E., Brookes, P., Lauber, C. L., Lozupone, C., Caporaso, J. G., Knight, R. and Fierer, N. 2010. Soil bacterial and fungal communities across a pH gradient in an arable soil. *The ISME Journal*, 4: 1340-1351.
- Sanderson, S. C., Diefenbach, M. A., Zinberg, R., Horowitz, C. R., Smirnoff, M., Zweig, M., Streicher, S., Wag Jabs, E. and Richardon, L. D. 2013. Willingness to participate in genomics research and desire for personal results among underrepresented minority patients: a structured interview study. *Journal of Community Genet*, 4: 469-482.
- Saturno, D. F., Cerezini, P., Moreira da Silva, P., Oliveira, A. B., de Oliveira, M. C. N., de Hungria, M. 2017. Mineral nitrogen impairs the biological nitrogen fixation in soybean of determinate and indeterminate growth types. *Journal of Plant Nutrition*, 40: 1690-1701.
- Sprent J. I., Odee D. W. and Dakora F. T. 2010. African legumes: A vital but under-utilized resource. *Journal of Experimental Botany*, 61(5): 1257-1265.

- Tian, J., He, N., Hale, L., Niu, S., Yu, G., Liu, Y., Blagodatskaya, E., Kuzyakov, Y., Gao, Q. and Zhou, J. 2018. Soil organic matter availability and climate drive latitudinal patterns in bacterial diversity from tropical to cold temperate forests, *Functional Ecology*, 32: 61-70.
- Veres, Z., Kotroczó, Z., Fekete, I., Tóth, J. A., Lajtha, K., Townsend, K. and Tóthmérész, K. 2015. Soil extracellular enzyme activities are sensitive indicators of detrital inputs and carbon availability. *Applied Soil Ecology*, 92: 18-23.
- Vyas, P. and Gulati, A. 2009. Organic acid production in vitro and plant growth promotion in maize under controlled environment by phosphate-solubilizing fluorescent *Pseudomonas*. *BMC Microbiology*, 9: 174.
- Wang, L., Ye, X., Hu, H., Du, J., Xi, Y., Shen, Z., Lin, J. and Chen, D. 2022. Soil bacterial communities triggered by organic matter inputs associates with a high-yielding pear production. *Soils*, 8 (1): 337-348.
- Weisany, W., Raei, Y. and Allahverdipoor, K. H. 2013. Role of some of mineral nutrients in biological nitrogen fixation. *Bulletin of Environment, Pharmacology and Life Sciences*, 2: 77-84.
- Wenhao, Y., Zongjun, J., Aolin, W., Dongdong, H., Christopher, R., Yanhua, C., Chengcong, C., Huihuang, W., Muhammad, A. M. and Liangquan W. 2023. Inconsistent responses of soil bacterial and fungal community's diversity and network to magnesium fertilization in tea (*Camellia sinensis*) plantation soils. *Applied Soil Ecology*, 191: 105055.
- Whalen, E. D., Smith, R. G., Grandy, A. S. and Frey, S. D. 2018. Manganese limitation as a mechanism for reduced decomposition in soils under atmospheric nitrogen deposition. *Soil Biology and Biochemistry*, 127: 252-263.

- Youssef, R. A., El-Azab, M. E., Mahdy, H. A., Essa, E. M. and Mohammed, K. A. 2017. Effect of salicylic acid on growth, yield, nutritional status, and physiological properties of sunflower plant under salinity stress. *International Journal of Pharmaceutical and Phytopharmacological Research*, 7: 54-58.
- Zhou, J., Jiang, X., Wei, D., Zhao, B., Ma, M., Chen, S., Cao, F., Shen, D., Guan, D. and Li, J. 2017. Consistent effects of nitrogen fertilization on soil bacterial communities in black soils for two crop seasons in China. *Science Reports*, 7: 3267.
- Zungu, N. S., Egbewale, S. O., Olaniran, A. O., Pérez-Fernández, M. and Magadlela, A. 2020. Soil nutrition, microbial composition and associated soil enzyme activities in KwaZulu-Natal grasslands and savannah ecosystems soils. *Applied Soil Ecology*, 155: 103663.

CHAPTER FIVE

STUDY SUMMARY, SIGNIFICANCE OF FINDINGS, FUTURE RESEARCH AND CONCLUSION

5.1. Summary

The present study focused on characterizing naturally occurring rhizobia associated with Bambara groundnut root and rhizosphere soil, analyzing physicochemical properties of the rhizosphere as a potential contributor to rhizobia diversity and quantify enzymatic activity in N-cycling and P-cycling which will result in improving the livelihood of farmers in Mpumalanga, Kwazulu-Natal, Limpopo province. A total of 209 rhizobia isolates were observed in the Mpumalanga, KwaZulu-Natal, and Limpopo provinces using the morphological characterization method. From the 209 rhizobia isolates, 43 were identified as different isolates based on their morphology. Isolate's morphological characteristics were based on colour, elevation, shape, surface, and margins. The colour of the isolates colony varied from yellow, bright yellow, golden yellow, cream, cream white, white, opaque, red, orange, bright orange, and brown; Colony surface was either rough or smooth; margins varied from entire, irregular, lobate, undulate, serrated, curled, or filamentous; the shape was round, irregular, filamentous, punctiform, rhizoid, and curled. Zero phosphate solubilization isolates were obtained in this present study. From the 209 isolates in this present study, 186 were N-cycling bacteria and 23 were not N-cycling bacteria. Furthermore, a total of 153 isolates were selected for molecular identification which classified isolates belonging to 12 genera, *Enterobacter*, *Leucobacter*, *Bacillus*, *Spingobacterium*, *Lysinibacillus*, *Stenotrophomonas*, *Cellulosimicrobium*, *Kaistella*, *Neorhizobium*, *Proteus*, *Micrococcus*, and *Mammalicoccus*. *Enterobacter asburiae* and *Leucobacter chromiiresistens* species were found in six sample sites. Mpumalanga province had a greater number of isolates followed by KwaZulu-Natal

province and Limpopo province had the lowest. High species diversity (H') and (D) was observed at the University of Zululand in KwaZulu-Natal province and the lowest was Nhlangenyuke also in KwaZulu-Natal. Consequently, Casteel species were more evenly distributed compared to all sample sites and less species evenness was observed at Boschfontein and Hazyview, Mpumalanga province. In Mpumalanga province, the University of Mpumalanga had the highest isolate number, with more species richness and the highest diversity index (H') and the lowest species evenness (J) while Casteel had the highest diversity (D) and the highest evenness (J). University of Zululand in KwaZulu-Natal province had a higher diversity index (H') and (D) and higher species evenness (J) compared to Nhlangenyuke. The pH value of all sampled sites was acidic, with the University of Zululand and Mpumalanga having the lowest pH and high number of isolates and species richness within the provinces. The soil housed a wide range of soil enzymes which are significant in the cycling of nutrients. Moreover, the identified soil enzymes Phosphorus and nitrogen cycling activities which include β -glucosaminidase, β -glucosidase, acid phosphatase, and alkaline phosphatase were not different. Nitrate reductase enzyme activities were high at Hlamalani and low at Bushbuckridge.

5.2. Significance of findings

The study identified a diverse range of rhizobia isolates in the rhizosphere soil of Bambara groundnut across the Mpumalanga, KwaZulu-Natal, and Limpopo provinces, with a significant proportion of these isolates involved in nitrogen cycling. Moreover, rhizobia isolated from Bambara groundnut root nodules has great potential to enhance nitrogen-fixing abilities in agricultural soil, at the same time improving soil fertility, boosting the income of smallholder farmers, and improving the yield of Bambara groundnuts. Moreover, rhizobia isolates obtained in the three provinces can be further tested to be used as possible inoculum.

5.3. Future research

Isolates from the rhizosphere soil of Bambara groundnut in KwaZulu-Natal were found to be more diverse compared to those from Mpumalanga and Limpopo provinces, while nearly all isolates from Limpopo province were capable of nitrogen cycling.. More research studies that will look into the diversity of rhizobia bacteria in the different localities of Bambara groundnut in each province can be done to find the most effective rhizobia that can be used as a promising inoculum for sustainable agriculture.

5.4. Conclusions

In conclusion, a high number of rhizobia isolates were mainly found in Mpumalanga followed by KwaZulu-Natal, and the least was Limpopo province. The three different provinces Mpumalanga, KwaZulu-Natal, and Limpopo province had diverse soil microbial compositions with KwaZulu-Natal province the highest and Limpopo province the lowest. About 89 % of isolates from the root nodules tested positive for nitrogen cycling and 11 % tested negative, while all isolates obtained from nodules in all three provinces tested negative for phosphate solubilization. Physico-chemical soil properties of all the sample sites were found to be low in essential nutrients. Moreover, all soil sample sites were acidic with the University of Zululand and Mpumalanga being the most acidic soil and with a greater number of isolates and species richness compared to all sample sites. Moreover, Hlamalani had the highest nitrate reductase enzyme activities and Bushbuckridge the lowest.

5.5. Recommendation

Bambara groundnut root nodules used in this study were collected in a few farms due to financial constraints farms in Mpumalanga, two farms in KwaZulu-Natal province, and one farm in Limpopo province. Therefore, the study recommends that more samples can be

collected from the different farms, in Mpumalanga, KwaZulu-Natal, and Limpopo province since Bambara groundnut is mostly planted in the three provinces to avoid biases. In KwaZulu-Natal province soil was collected and used to plant Bambara groundnut seeds in a controlled environment, the greenhouse, this might have resulted in a few isolates being found. Therefore, the study recommends that Bambara groundnut root nodules be collected in each area for a study. The present study also showed that non-rhizobia bacteria are more diverse compared to the rhizobia bacteria. Therefore, it is recommended that more studies can be done on the non-rhizobia isolates.

APPENDICES

Appendix 3.1: Morphological features of 209 root nodule bacteria colonies isolated from Bambara groundnut.

Province	Location	Isolates name	Colour	Elevation	Shape	Surface	Margin	Probable organisms
Mpumalanga	Boschfontein	BF2P6G1	White	Flat	Irregular	Rough and wrinkled	undulate	<i>Bacillus licheniformis</i>
		BF2P3P	White	Flat	Irregular	Rough and wrinkled	undulate	<i>Bacillus licheniformis</i>
		BF1P11P	Opaque	Flat	Filamentous	Rough	Filamentous	<i>Bacillus pumilus</i>
		BF1P3P	Opaque	Flat	Filamentous	Rough	Filamentous	<i>Bacillus pumilus</i>
		BF1P3G	Opaque	Flat	Filamentous	Rough	Filamentous	<i>Bacillus pumilus</i>
		BF2P11G	Opaque	Flat	Filamentous	Rough	Filamentous	<i>Bacillus pumilus</i>
		BF2P3G	Opaque	Flat	Filamentous	Rough	Filamentous	<i>Bacillus pumilus</i>
		BF1P4G	Opaque	Flat	Filamentous	Rough	Filamentous	<i>Bacillus pumilus</i>
		BF1P12P	Opaque	Flat	Filamentous	Rough	Filamentous	<i>Bacillus pumilus</i>

		BF1P8G	Grey white	Convex	Round	Smooth	Entire	<i>Enterobacter absuriae</i>
		BF1P4G2	White	Flat	Filamentous	Rough	Filamentous	unidentified
		BF1P9G	White	Flat	Round	Smooth	Entire	<i>Stenotrophomonas pavanii</i>
		BF1P12PA	White	Flat	Round	Smooth	Entire	<i>Stenotrophomonas pavanii</i>
		BF1P13G	Cream white	Flat	Round	Smooth	Entire	<i>Stenotrophomonas geniculata</i>
		BF2P9G	Cream white	Flat	Round	Smooth	Entire	<i>Stenotrophomonas lactitubi</i>
	Hlamalani	HLAM6B1	White	Flat	Irregular	Rough and wrinkled	undulate	<i>Bacillus licheniformis</i>
		HLAM1B2	Opaque	Flat	Filamentous	Rough	Filamentous	<i>Bacillus pumilus</i>
		HLAM1A4	Opaque	Flat	Filamentous	Rough	Filamentous	<i>Bacillus pumilus</i>
		HLAM2B2	Cream	Flat	Irregular	Smooth	Irregular	unidentified

		HLAM3B4	White brown	Flat	Round	Smooth	Entire	<i>Leucobacter chromiiresistens</i>
		HLAM3B3	White brown	Flat	Round	Smooth	Entire	<i>Leucobacter chromiiresistens</i>
		HLAM3B1	White brown	Flat	Round	Smooth	Entire	<i>Leucobacter chromiiresistens</i>
		HLAM3B5	White	Flat	Round	Smooth	Entire	<i>Stenotrophomonas pavanii</i>
		HLAM3B2	Slightly yellow	Convex	Round	Smooth	Entire	<i>Sphingobacterium faecium</i>
		HLAM2A1	Orange	Flat	Punctiform	Smooth	Entire	unidentified
	Casteel	CAST4B2	White	Flat	Irregular	Rough and wrinkled	undulate	<i>Bacillus licheniformis</i>
		CAST2B1	Grey white	Convex	Round	Smooth	Entire	<i>Enterobacter asburiae</i>

		CAST4B1	Grey white	Convex	Round	Smooth	Entire	<i>Enterobacter asburiae</i>
		CAST3A1	White brown	Flat	Round	Smooth	Entire	<i>Leucobacter chromiirensistens</i>
		CAST1B2	White	Flat	Round	Smooth	Entire	<i>Stenotrophomonas pavanii</i>
	Nkomazi	NKP8W	Opaque	Flat	Filamentous	Rough	Filamentous	<i>Bacillus pumilus</i>
		NKP1W1	Opaque	Flat	Filamentous	Rough	Filamentous	<i>Bacillus pumilus</i>
		NKP6G	Opaque	Flat	Filamentous	Rough	Filamentous	<i>Bacillus pumilus</i>
		NK10WA	Creamy white	Flat	Irregular	Smooth	Lobate	unidentified
		NKP4G	Grey white	Convex	Round	Smooth	Entire	<i>Enterobacter absuriae</i>
		NKP10G	Grey white	Convex	Round	Smooth	Entire	<i>Enterobacter absuriae</i>

		NKP5G	Grey white	Convex	Round	Smooth	Entire	<i>Enterobacter absuriae</i>
		NKP11G	White	Flat	Filamentous	Rough	Filamentous	unidentified
		NKP3G	Yellow	Round	Raised	Smooth	Entire	<i>Micrococcus yunnanensis</i>
		NKP5P	Grey white	Convex	Round	Smooth	Entire	<i>Enterobacter asburiae</i>
		NKP65G	Yellow to orange	Raised	Round	Smooth	Entire	<i>Stenotrophomonas maltophilia</i>
		NKP4P	Cream white	Flat	Round	Smooth	Entire	<i>Stenotrophomonas lactitubi</i>
		NKF10WB	Cream white	Flat	Round	Smooth	Entire	<i>Stenotrophomonas lactitubi</i>
		NKP12F	Yellow to orange	Raised	Round	Smooth	Entire	<i>Stenotrophomonas maltophilia</i>
		UMPP2PB6	Opaque	Flat	Filamentous	Rough	Filamentous	<i>Bacillus pumilus</i>

	University of Mpumalanga	UMPP9G5	Opaque	Flat	Filamentous	Rough	Filamentous	<i>Bacillus pumilus</i>
		UMPP2PB3	Cream white	Flat	Irregular	Smooth	Lobate	unidentified
		UMPBG1B2	Cream	Flat	Irregular	Smooth	Undulate	unidentified
		UMPBG1A	Cream	Flat	Irregular	Rough	Irregular	unidentified
		UMPBG9A3	Cream white	Flat	Filamentous	Rough	Filamentous	unidentified
		UMPBG6A2	Yellow	Flat	Filamentous	Smooth	Filamentous	unidentified
		UMPBG4B4	Grey white	Convex	Round	Smooth	Entire	<i>Enterobacter asburiae</i>
		UMP1P3PB5	Grey white	Convex	Round	Smooth	Entire	<i>Enterobacter asburiae</i>
		UMPBG9A2	Grey white	Convex	Round	Smooth	Entire	<i>Enterobacter asburiae</i>
		UMPP2PB7	Grey white	Convex	Round	Smooth	Entire	<i>Enterobacter asburiae</i>

		UMP1P3PB4	White	Flat	Filamentous	Smooth	Filamentous	unidentified
		UMPP2PA3	Cream white	Flat	Filamentous	Smooth	Filamentous	unidentified
		UMPP6PB1	White brown	Flat	Round	Smooth	Entire	<i>Leucobacter chromiiresistens</i>
		UMPP7GA2	White brown	Flat	Round	Smooth	Entire	<i>Leucobacter chromiiresistens</i>
		UMPBGPA3	White brown	Flat	Round	Smooth	Entire	<i>Leucobacter chromiiresistens</i>
		UMPP9GA2	White	Flat	Round	Smooth	Entire	<i>Stenotrophomonas pavanii</i>
		UMPBG5A2	White	Flat	Round	Smooth	Entire	<i>Stenotrophomonas pavanii</i>
		UMPP9G4	Yellow to orange	Raised	Round	Smooth	Entire	<i>Stenotrophomonas maltophilia</i>

		UMP1P3PB3	Yellow	Round	Raised	Smooth	Entire	<i>Micrococcus yunnanensis</i>
		UMPP2PB2	Cream white	Flat	Round	Smooth	Entire	<i>Stenotrophomonas lactitubi</i>
		UMPP2PB5	Cream white	Flat	Round	Smooth	Entire	<i>Stenotrophomonas lactitubi</i>
		UMPP9PA	Cream white	Flat	Round	Smooth	Entire	<i>Stenotrophomonas lactitubi</i>
		UMPP7GA3	Cream white	Flat	Round	Smooth	Entire	<i>Stenotrophomonas geniculata</i>
		UMPP9G3	Slightly yellow	Convex	Round	Smooth	Entire	<i>Sphingobacterium faecium</i>
		UMPBG1B1	Slightly yellow	Convex	Round	Smooth	Entire	<i>Sphingobacterium faecium</i>
		UMPBG4A5	Slightly yellow	Convex	Round	Smooth	Entire	<i>Sphingobacterium faecium</i>

		UMPP1P3PB2	Slightly yellow	Convex	Round	Smooth	Entire	<i>Sphingobacterium faecium</i>
		UMPP7GA1	Yellow white	Convex	Round	Smooth	Entire	<i>Neorhizobium petrolearium</i>
		UMP1P3PB2	Yellow white	Convex	Round	Smooth	Entire	<i>Kaistella daneshvariae</i>
		UMPBG4A1	Bright yellow	Convex	Filamentous	Smooth	Filamentous	<i>Cellulosimicrobium cellulans</i>
		UMPP9GA1	Bright yellow	Convex	Filamentous	Smooth	Filamentous	<i>Cellulosimicrobium cellulans</i>
		UMPP2PA2	Cream white	Flat	Punctiform	Smooth	Entire	<i>Lysinibacillus sphaericus</i>
		UMPBG8B	Cream	Flat	Punctiform	Smooth	Entire	<i>Lysinibacillus sphaericus</i>
		UMPP4GB	Cream	Flat	Punctiform	Smooth	Entire	<i>Lysinibacillus sphaericus</i>

		UMPP3PB3	Yellow	Flat	Rhizoid	Smooth	Irregular	<i>Proteus columbae</i>
		UMPBG4B	Cream	Flat	Rhizoid	Smooth	Irregular	unidentified
		UMPP2PA1	Cream	Flat	Rhizoid	Smooth	Irregular	unidentified
		UMPP9PB	White	Raised	Round	Smooth	Serrated	unidentified
		UMPPBG4A4	Cream	Crateriform	Round	Rough	Entire	unidentified
	Bushbuckridge	BUSHP1A	Cream white	Flat	Round	Smooth	Entire	<i>Stenotrophomonas geniculata</i>
		BUSHP2B1	Cream white	Flat	Round	Smooth	Entire	<i>Stenotrophomonas lactitubi</i>
		BUSHP2B3	Cream white	Flat	Round	Smooth	Entire	<i>Stenotrophomonas lactitubi</i>
		BUSHPA2	Yellow orange	Raised	Round	Smooth	Entire	<i>Stenotrophomonas maltophilia</i>
		BUSHPA7	White	Flat	Irregular	Rough and wrinkled	undulate	<i>Bacillus licheniformis</i>

		BUSHPA9	White	Flat	Irregular	Rough and wrinkled	undulate	<i>Bacillus licheniformis</i>
		BUSHPA1	Slightly yellow	Convex	Round	Smooth	Entire	<i>Sphingobacterium faecium</i>
		BUSHPAP1	Cream white	Flat	Punctiform	Smooth	Entire	<i>Lysinibacillus sphaericus</i>
		BUSHPA3	Cream white	Flat	Punctiform	Smooth	Entire	<i>Lysinibacillus sphaericus</i>
		BUSHP1P4	Cream white	Flat	Punctiform	Smooth	Entire	<i>Lysinibacillus sphaericus</i>
		BUSHP1P5	Cream white	Flat	Punctiform	Smooth	Entire	<i>Lysinibacillus sphaericus</i>
		BUSHP2P1	Cream	Flat	Rhizoid	Smooth	Irregular	unidentified
	Hazyview	HAZYW1B1	Yellow	Flat	Irregular	Smooth	Lobate	unidentified
		HAZYW4B	Yellow orange	Raised	Round	Smooth	Entire	<i>Stenotrophomonas maltophilia</i>

		HAZYW4A	Yellow orange	Raised	Round	Smooth	Entire	<i>Stenotrophomonas maltophilia</i>
		HAZYW4B1	Cream	Flat	Rhizoid	Smooth	Irregular	unidentified
		HAZYW2B	Cream	Flat	Rhizoid	Smooth	Irregular	unidentified
		HAZYW2B1	Cream	Flat	Rhizoid	Smooth	Irregular	unidentified
	Mkhuhlu	MKHLUP1A1	White	Flat	Irregular	Rough and wrinkled	undulate	<i>Bacillus licheniformis</i>
		MKHLUP2A2	White	Flat	Irregular	Rough and wrinkled	undulate	<i>Bacillus licheniformis</i>
		MKHLUP2A1	Cream white	Flat	Round	Smooth	Entire	<i>Stenotrophomonas lactitubi</i>
		MKHLUP2A3	Cream white	Flat	Round	Smooth	Entire	<i>Stenotrophomonas lactitubi</i>
		MKHLUP2B1	Cream	Flat	Rhizoid	Smooth	Irregular	unidentified
	KwaZulu-Natal	ZULU30A4	Opaque	Flat	Filamentous	Rough	Filamentous	<i>Bacillus pumilus</i>
		ZULU9A3	Opaque	Flat	Filamentous	Rough	Filamentous	<i>Bacillus pumilus</i>

		ZULU12A3	Opaque	Flat	Filamentous	Rough	Filamentous	<i>Bacillus pumilus</i>
		ZULU27B5	Opaque	Flat	Filamentous	Rough	Filamentous	<i>Bacillus pumilus</i>
		ZULU8A2	Cream white	Flat	Irregular	Smooth	Lobate	unidentified
		ZULU18A2	Cream white	Flat	Irregular	Smooth	Lobate	unidentified
		ZULU2B1	Cream white	Flat	Irregular	Smooth	Lobate	unidentified
		ZULU276	White yellow	Flat	Irregular	Smooth	Undulate	unidentified
		ZULU9A9	Cream	Flat	Irregular	Smooth	Undulate	unidentified
		ZULU10B1	Cream	Flat	Irregular	Smooth	Irregular	unidentified
		ZULU28A3	Orange	Flat	Filamentous	Smooth	Filamentous	unidentified
		ZULU9A1	Grey white	Convex	Round	Smooth	Entire	<i>Enterobacter asburiae</i>

		ZULU9A8	Grey white	Convex	Round	Smooth	Entire	<i>Enterobacter asburiae</i>
		ZULU18B4	Grey white	Convex	Round	Smooth	Entire	<i>Enterobacter asburiae</i>
		ZULU30A3	Grey white	Convex	Round	Smooth	Entire	<i>Enterobacter asburiae</i>
		ZULU4B4	Grey white	Convex	Round	Smooth	Entire	<i>Enterobacter asburiae</i>
		ZULU11A2	Grey white	Convex	Round	Smooth	Entire	<i>Enterobacter asburiae</i>
		ZULU27A5	Grey white	Convex	Round	Smooth	Entire	<i>Enterobacter asburiae</i>
		ZULU11A3	Orange	Flat	Filamentous	Smooth	Filamentous	unidentified
		ZULU9B8	Orange	Flat	Filamentous	Smooth	Filamentous	unidentified
		ZULU27A1	White brown	Flat	Round	Smooth	Entire	<i>Leucobacter chromiiresistens</i>

		ZULU18B2	White brown	Flat	Round	Smooth	Entire	<i>Leucobacter chromiiresistens</i>
		ZULU2A5	White brown	Flat	Round	Smooth	Entire	<i>Leucobacter chromiiresistens</i>
		ZULU9B3	White brown	Flat	Round	Smooth	Entire	<i>Leucobacter chromiiresistens</i>
		ZULU11A1	White brown	Flat	Round	Smooth	Entire	<i>Leucobacter chromiiresistens</i>
		ZULU20A4	White brown	Flat	Round	Smooth	Entire	<i>Leucobacter chromiiresistens</i>
		ZULU30A2	White brown	Flat	Round	Smooth	Entire	<i>Leucobacter chromiiresistens</i>
		ZULU20A3	White brown	Flat	Round	Smooth	Entire	<i>Leucobacter chromiiresistens</i>
		ZULU12B4	White	Flat	Round	Smooth	Entire	<i>Stenotrophomonas pavanii</i>

		ZULU32B1	White	Flat	Round	Smooth	Entire	<i>Stenotrophomonas pavanii</i>
		ZULU9B5	Cream white	Flat	Round	Smooth	Entire	<i>Stenotrophomonas lactitubi</i>
		ZULU2A1	Yellow orange	Raised	Round	Smooth	Entire	<i>Stenotrophomonas maltophilia</i>
		ZULU9A6	Cream white	Flat	Round	Smooth	Entire	<i>Stenotrophomonas lactitubi</i>
		ZULU9B8	Yellow orange	Raised	Round	Smooth	Entire	<i>Stenotrophomonas maltophilia</i>
		ZULU27A7	Slightly yellow	Convex	Round	Smooth	Entire	<i>Sphingobacterium faecium</i>
		ZULU30A5	Slightly yellow	Convex	Round	Smooth	Entire	<i>Sphingobacterium faecium</i>
		ZULU4B3	Slightly yellow	Convex	Round	Smooth	Entire	<i>Sphingobacterium faecium</i>

		ZULU32B2	Slightly yellow	Convex	Round	Smooth	Entire	<i>Sphingobacterium faecium</i>
		ZULU27A3	Slightly yellow	Convex	Round	Smooth	Entire	<i>Sphingobacterium faecium</i>
		ZULU9B4	Yellow white	Convex	Round	Smooth	Entire	<i>Kaistella daneshvariae</i>
		ZULU24A1	Yellow white	Convex	Round	Smooth	Entire	<i>Kaistella daneshvariae</i>
		ZULU9B2	Yellow white	Convex	Round	Smooth	Entire	<i>Neorhizobium petrolearium</i>
		ZULU9B1	Yellow white	Convex	Round	Smooth	Entire	<i>Neorhizobium petrolearium</i>
		ZULU27B2	Yellow white	Convex	Round	Smooth	Entire	<i>Kaistella daneshvariae</i>
		ZULU24A3	Orange	Flat	Round	Smooth	Entire	unidentified
		ZULU27A4	Orange	Flat	Round	Smooth	Entire	unidentified

		ZULU32B4	Orange	Flat	Round	Smooth	Entire	unidentified
		ZULU9A1A	Orange	Flat	Round	Smooth	Entire	unidentified
		ZULU9A2	Orange	Flat	Round	Smooth	Entire	unidentified
		ZULU8A3	Orange	Flat	Round	Smooth	Entire	unidentified
		ZULU8A1	Orange	Flat	Round	Smooth	Entire	unidentified
		ZULU9A5	Bright yellow	Convex	Filamentous	Smooth	Filamentous	<i>Cellulosimicrobium cellulans</i>
		ZULU24A5	Bright yellow	Convex	Filamentous	Smooth	Filamentous	<i>Cellulosimicrobium cellulans</i>
		ZULU18B5	Bright yellow	Convex	Filamentous	Smooth	Filamentous	<i>Cellulosimicrobium cellulans</i>
		ZULU27B4	Bright yellow	Convex	Filamentous	Smooth	Filamentous	<i>Cellulosimicrobium cellulans</i>
		ZULU32B3	Brown	Flat	Round	Smooth	Entire	unidentified
		ZULU18B1	Yellow	Flat	Rhizoid	Smooth	Irregular	<i>Proteus columbae</i>
		ZULU27A2	Orange	Flat	Punctiform	Smooth	Entire	unidentified

		ZULU16A1	White	Convex	Round	Smooth	Entire	<i>Mammaliicoccus sciuri</i>
		ZULU12B2	Cream	Flat	Punctiform	Rough	Entire	unidentified
		ZULU7B1	Cream	Flat	Rhizoid	Smooth	Irregular	unidentified
		ZULU27B1	Cream	Convex	Round	Smooth	Entire	<i>Sphingobacterium multivorum</i>
		ZULU27B9	White	Raised	Round	Smooth	Serrated	unidentified
		ZULU2A4	White	Raised	Round	Smooth	Serrated	unidentified
	Nhlangenyuke	NHLANG7A2	White	Flat	Irregular	Rough and wrinkled	undulate	<i>Bacillus licheniformis</i>
		NHLANGE2A2	Cream white	Flat	Irregular	Smooth	Lobate	unidentified
		NHLANGE22A	Cream white	Flat	Irregular	Smooth	Lobate	unidentified
		NHLANG7A1	White	Flat	Filamentous	Rough	Filamentous	unidentified
		NHLANG7A2A	White	Flat	Filamentous	Rough	Filamentous	unidentified

		NHLANGE2B2	Grey white	Convex	Round	Smooth	Entire	<i>Enterobacter asburiae</i>
		NHLANGE17B 1	Grey white	Convex	Round	Smooth	Entire	<i>Enterobacter asburiae</i>
		NHLANGE15B 1	Grey white	Convex	Round	Smooth	Entire	<i>Enterobacter asburiae</i>
		NHLANGE7B2	Cream white	Flat	Filamentous	Smooth	Filamentous	unidentified
		NHLANGE1B5	White brown	Flat	Round	Smooth	Entire	<i>Leucobacter chromiiresistens</i>
		NHLANGE5A1	White brown	Flat	Round	Smooth	Entire	<i>Leucobacter chromiiresistens</i>
		NHLANGE5A5	White brown	Flat	Round	Smooth	Entire	<i>Leucobacter chromiiresistens</i>
		NHLANG1B1	White	Flat	Round	Smooth	Entire	<i>Stenotrophomonas pavanii</i>

		NHLANGE17B 2	Cream white	Flat	Round	Smooth	Entire	<i>Stenotrophomonas geniculata</i>
		NHLANGE7B4	Yellow orange	Raised	Round	Smooth	Entire	<i>Stenotrophomonas maltophilia</i>
		NHLANGE17A 1	Slightly yellow	Convex	Round	Smooth	Entire	<i>Sphingobacterium faecium</i>
		NHLANGE6B	Yellow white	Convex	Round	Smooth	Entire	<i>Kaistella daneshvariae</i>
		NHLANGE2B1	Yellow white	Convex	Round	Smooth	Entire	<i>Neorhizobium petrolearium</i>
		NHLANGE2A1	Orange	Flat	Round	Smooth	Entire	unidentified
		NHLANGE7B3	Pink	Flat	Round	Smooth	Entire	unidentified
		NHLANGE14A	Cream white	Flat	Punctiform	Smooth	Entire	unidentified
		NHLANGE8B	Cream white	Flat	Punctiform	Smooth	Entire	<i>Lysinibacillus sphaericus</i>

		NHLANGE7B1	Cream white	Flat	Curled	Smooth	Curled	unidentified
Limpopo	Gabaza	GAB12B4	Cream white	Flat	Irregular	Rough	Lobate	unidentified
		GAB1B1	Grey white	Convex	Round	Smooth	Entire	<i>Enterobacter absuriae</i>
		GAB13B1	Yellow	Flat	Filamentous	Smooth	Filamentous	unidentified
		GAB2B1	White brown	Flat	Round	Smooth	Entire	<i>Leucobacter chromiirensistens</i>
		GAB6B2	White	Flat	Round	Smooth	Entire	<i>Stenotrophomonas pavanii</i>
		GAB7A1	White	Flat	Round	Smooth	Entire	<i>Stenotrophomonas pavanii</i>
		GAB10A1	Cream white	Flat	Punctiform	Rough	Lobate	<i>Lysinibacillus pakistanensis</i>

		GABA6B1	Cream white	Flat	Round	Smooth	Entire	<i>Stenotrophomonas lactitubi</i>
		GAB5A1	Yellow orange	Raised	Round	Smooth	Entire	<i>Stenotrophomonas maltophilia</i>
		GAB12B2	Slightly yellow	Convex	Round	Smooth	Entire	<i>Sphingobacterium faecium</i>
		GAB13A1	Slightly yellow	Convex	Round	Smooth	Entire	<i>Sphingobacterium faecium</i>
		GAB4B3	Bright yellow	Convex	Filamentous	Smooth	Filamentous	<i>Cellulosimicrobium cellulans</i>
		GAB1	Red	Flat	Round	Smooth	Entire	unidentified
		GAB12A	Cream	Flat	Punctiform	Smooth	Entire	<i>Lysinibacillus sphaericus</i>
		GAB11A	Cream	Flat	Punctiform	Smooth	Entire	<i>Lysinibacillus sphaericus</i>

Appendix 4.1: Summary of soil analysis result on Bambara groundnut rhizosphere soil.

Your sample ID	Lab number	Sample g/mL	density	P mg/L	K mg/L	Ca mg/L	Mg mg/L	Exch. Acidity cmol/L	Total Cations cmol/L	Acid sat. %	pH (KCl)	Zn mg/L	Mn mg/L	Cu mg/L	Mid-Infrared Estimates		
															Org. C %	N %	Clay %
MKHULU 1	F7427	1.28		4	63	502	126	0.05	3.75	1	4.89	0.8	15	1.3	1.8	0.15	9
MKHULU 2	F7428	1.27		4	64	468	115	0.06	3.51	2	4.80	0.8	15	1.4	1.3	0.13	13
BUSH 1	F7432	1.30		8	53	593	76	0.05	3.77	1	5.55	8.8	56	1.8	0.7	0.09	16
BUSH 2	F7433	1.28		8	61	684	88	0.03	4.32	1	5.46	9.5	55	1.9	0.5	0.09	12
NHLANGE 1A	F7434	1.19		8	189	530	179	0.06	4.66	1	4.52	2.2	15	5.6	1.8	0.12	24
NHLANGE 1B	F7435	1.17		7	192	560	221	0.05	5.15	1	4.52	7.9	13	4.4	1.4	0.09	25
NHLANGE 2A	F7436	1.09		6	148	585	224	0.18	5.32	3	4.36	2.9	21	8.6	1.8	0.09	40
NHLANGE 2B	F7437	1.10		6	152	581	208	0.16	5.16	3	4.35	2.4	23	9.6	1.7	0.10	37
NHLANGE 3A	F7438	1.06		4	193	980	417	0.05	8.86	0	5.03	1.8	60	9.5	2.6	0.20	43
NHLANGE 3B	F7439	1.05		5	192	983	428	0.06	8.98	1	5.04	2.4	70	8.8	2.5	0.17	43
NHLANGE 4A	F7440	1.16		6	251	543	174	0.15	4.93	3	4.28	4.1	23	9.8	1.2	0.07	34
NHLANGE 4B	F7441	1.15		6	246	572	172	0.16	5.06	3	4.28	2.4	24	10.6	1.9	0.12	41
HAZYVIEW 1a	F1049	1.07		7	105	1109	224	0.08	7.73	1	5.60	1.2	36	3.6	2.2	0.10	18
HAZYVIEW 1b	F1050	1.07		10	107	1027	186	0.11	7.04	2	5.50	1.6	42	4.4	1.6	0.07	17
NKOMAZI 1a	F1051	1.27		31	95	799	96	0.06	5.08	1	6.20	7.8	14	1.2	1.6	0.08	18
NKOMAZI 1b	F1052	1.26		27	82	876	103	0.08	5.51	1	6.33	6.9	14	1.0	1.2	0.08	16
NKOMAZI 2a	F1053	1.40		3	159	402	60	0.22	3.13	7	4.22	0.3	26	0.8	1.0	0.09	8

UNIVERSITY OF MPUMALANGA a	F1054	1.14	3	104	95	44	0.16	7.73	5.08	4.36	8.8	56	1.4	2.2	1.2	0.08
UNIVERSITY OF MPUMALANGA b	F1055	1.13	3	105	82	37	0.16	7.04	5.50	4.44	0.8	55	1.4	1.6	1.2	0.08
ZULULAND 1	F7418	1.14	6	91	513	240	0.16	4.93	3	4.36	0.7	7	1.4	0.9	0.10	7
ZULULAND 2	F7419	1.13	3	96	571	247	0.15	5.28	3	4.44	1.2	7	1.4	0.7	0.05	41
ZULULAND 3	F7420	1.10	3	93	473	212	0.15	4.49	3	4.33	0.8	8	1.8	0.7	0.05	35
ZULULAND 4	F7421	1.13	3	86	455	274	0.09	4.84	2	4.51	0.8	5	1.2	0.7	0.07	16
BOCHFON 1	F7422	1.45	3	57	306	44	0.06	2.09	3	4.83	0.4	72	0.6	0.9	0.09	14
BOCHFON 2	F7423	1.46	3	53	268	37	0.04	1.82	2	4.85	0.5	68	0.5	0.9	0.10	7
HLAMALANI 1a	F1055	1.40	2	71	442	50	0.05	2.85	2	5.08	5.5	14	0.8	0.5	0.05	10
HLAMALANI 2b	F1056	1.40	3	73	448	55	0.10	2.97	3	5.16	4.5	16	0.9	0.5	0.07	10
GAVAZA 1a	F1061	1.14	5	104	1001	306	0.08	7.86	1	4.78	1.3	54	10.4	1.3	0.05	39
GAVAZA 1b	F1062	1.16	5	105	993	283	0.08	7.63	1	4.79	1.6	60	11.2	1.1	0.05	35
CASTEEL 1a	F1057	1.24	12	80	619	73	0.07	3.96	2	5.20	28.7	19	2.1	0.5	0.05	20
CASTEEL 1b	F1058	1.24	14	95	672	61	0.06	4.16	1	5.20	41.9	22	2.1	0.9	0.12	25

Appendix 4.2: Shapiro-Wilt normal distribution test for soil properties and enzyme activities on Bambara groundnut rhizosphere soil.

Variables	N	W	P
Density	29	0.9206	0.0316
Phosphorus	29	0.1838	0.0000
Potassium	29	0.8872	0.0049
Calcium	29	0.9087	0.0159
Magnesium	29	0.9057	0.0135
Exchangeable acidity	29	0.8348	0.0004
Total cation (ECEC)	29	0.9369	0.0831
Acid saturation	29	0.7754	0.0000
pH	29	0.9145	0.0222
Zinc	29	0.5380	0.0000
Manganese	29	0.8641	0.0015
Copper	29	0.7750	0.0000
Organic carbon	29	0.9276	0.0477
Organic matter	29	0.9276	0.0013
Nitrogen	29	0.8617	0.0013
Clay	29	0.8889	0.0054
Acid	48	0.2310	0.0000
Alkaline	48	0.6832	0.0000
B-Glucosaminidase	48	0.8279	0.0000
Glucosidase	48	0.5806	0.0000

Appendix 4.3: Analysis of variance (ANOVA) for phosphorus on Bambara groundnut rhizosphere soil.

Source	DF	SS	MS	F	P
Treatment	9	4.5378	0.50420	0.30	0.9643
Error	19	31.5345	1.65971		
Total	28	36.0723			

Appendix 4.4: Analysis of variance (ANOVA) for exchangeable acidity ($\text{cmol}_c \text{kg}^{-1}$) on Bambara groundnut rhizosphere soil.

Source	DF	SS	MS	F	P
Treatment	9	0.003755	0.00004173	1.31	0.2947
Error	19	0.006049	0.00003184		
Total	28	0.009804			

Appendix 4.5: Analysis of variance (ANOVA) for acid saturation (%) on Bambara groundnut rhizosphere soil.

Source	DF	SS	MS	F	P
Treatment	9	0.001189	0.0001321	0.66	0.7359
Error	19	0.003818	0.0002009		
Total	28	0.005007			

Appendix 4.6: Analysis of variance (ANOVA) for manganese (mg kg⁻¹) on Bambara groundnut rhizosphere soil.

Source	DF	SS	MS	F	P
Treatment	9	0.08037	0.008930	9.31	0.0000
Error	19	0.01821	0.0009587		
Total	28	0.09858			

Appendix 4.7: Analysis of variance (ANOVA) for pH on Bambara groundnut rhizosphere soil.

Source	DF	SS	MS	F	P
Treatment	9	0.001566	0.0001740	3.08	0.0187
Error	19	0.001074	0.00005655		
Total	28	0.002641			

Appendix 4.8: Analysis of variance (ANOVA) for Zinc (mg kg⁻¹) on Bambara groundnut rhizosphere soil.

Source	DF	SS	MS	F	P
Treatment	9	0.20371	0.02263	9.12	0.0000
Error	19	0.04714	0.00248		
Total	28	0.25085			

Appendix 4.9: Analysis of variance (ANOVA) for copper (mg kg⁻¹) on Bambara groundnut rhizosphere soil.

Source	DF	SS	MS	F	P
Treatment	9	0.20926	0.02325	58.41	0.0000
Error	19	0.00756	0.00040		
Total	28	0.21682			

Appendix 4.10: Analysis of variance (ANOVA) for organic carbon (%) on Bambara groundnut rhizosphere soil.

Source	DF	SS	MS	F	P
Treatment	9	0.00003042	0.000003830	10.13	0.0000
Error	19	0.000006339	0.0000003337		
Total	28	0.00003676			

Appendix 4.11: Analysis of variance for organic matter (%) on Bambara groundnut rhizosphere soil.

Source	DF	SS	MS	F	P
Treatment	9	0.00004741	0.000005268	10.94	0.0000
Error	19	0.000009150	0.0000004816		
Total	28	0.00005656			

Appendix 4.12: analysis of variance (ANOVA) for nitrogen (mg kg^{-1}) on Bambara groundnut rhizosphere soil.

Source	DF	SS	MS	F	P
Treatment	9	0.01243	0.001382	3.25	0.0146
Error	19	0.00808	0.0004250		
Total	28	0.02051			

Appendix 4.13: Analysis of variance (ANOVA) for clay (%) on Bambara groundnut rhizosphere soil.

Source	DF	SS	MS	F	P
Treatment	9	0.0001518	0.00001687	20.27	0.0000
Error	19	0.00001581	0.0000008323		
Total	28	0.0001676			

Appendix 4.14: Analysis of variance (ANOVA) for soil density (g L^{-1}) on Bambara groundnut rhizosphere soil.

Source	DF	SS	MS	F	P
Treatment	9	0.04668	0.005186	20.08	0.0000
Error	19	0.00446	0.0002349		
Total	28	0.05114			

Appendix 4.15: Analysis of variance (ANOVA) for potassium ($\text{cmol}_c \text{ kg}^{-1}$) on Bambara groundnut rhizosphere soil.

Source	DF	SS	MS	F	P
Treatment	9	0.05306	0.005895	21.99	0.0000
Error	19	0.00509	0.0002681		
Total	28	0.05815			

Appendix 4.16: Analysis of variance (ANOVA) for calcium ($\text{cmol}_c \text{ kg}^{-1}$) on Bambara groundnut rhizosphere soil.

Source	DF	SS	MS	F	P
Treatment	9	0.36669	0.04074	6.61	0.0003
Error	19	0.11711	0.00616		
Total	28	0.48380			

Appendix 4.17: Analysis of variance (ANOVA) for magnesium ($\text{cmol}_c \text{ kg}^{-1}$) on Bambara groundnut rhizosphere soil.

Source	DF	SS	MS	F	P
Treatment	9	0.60085	0.06676	11.44	0.0000
Error	19	0.11091	0.00584		
Total	28	0.71176			

Appendix 4.18: Analysis of variance (ANOVA) for soil acid phosphatase enzyme activity $\text{nmol h}^{-1} \text{ g}^{-1}$ of Bambara groundnut rhizosphere soil.

Source	DF	SS	MS	F	P
Treatment	7	0.0002016	0.00002880	0.89	0.5225
Error	40	0.001292	0.00003231		
Total	47	0.001494			

Appendix 4.19: Analysis of variance (ANOVA) for soil Alkaline phosphatase enzyme activity in $\text{nmol h}^{-1} \text{ g}^{-1}$ of Bambara groundnut rhizosphere soil.

Source	DF	SS	MS	F	P
Treatment	7	0.000001671	0.0000002387	0.44	0.08724
Error	40	0.00002181	0.0000005451		
Total	47	0.00002348			

Appendix 4.20: Analysis of variance (ANOVA) for soil β -glucosaminidase enzyme activity in $\text{nmol h}^{-1} \text{ g}^{-1}$ of Bambara groundnut rhizosphere soil.

Source	DF	SS	MS	F	P
Treatment	7	0.000001671	0.0000002387	0.44	0.08724
Error	40	0.00002181	0.0000005451		
Total	47	0.00002348			

Appendix 4.21: Analysis of variance (ANOVA) for β -glucosidase soil enzyme activity in $\text{nmol h}^{-1} \text{ g}^{-1}$ of Bambara groundnut rhizosphere soil.

Source	DF	SS	MS	F	P
Treatment	7	0.000001676	0.0000002394	1.72	0.1314
Error	40	0.000005560	0.0000001390		
Total	47	0.000007236			