Salinity, pH tolerance, and efficiency of indigenous nitrogen-fixing bacteria on nodulation and growth of *Vigna subterranea* (L. Verdc.)

Gugu Cynthia Maseko

201971593

https://orcid.org/ 0000-0002-3469-5184

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

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

Co-Supervisor: Ms. E. Kola (University of Mpumalanga)

School of Agricultural Sciences

Faculty of Agriculture and Natural Sciences

2026

UNIVERSITY OF MPUMALANGA

TABLE OF CONTENTS

DECLARATION vi

DEDICATION vii

ACKNOWLEDGEMENTS viii

LIST OF ABBREVIATIONS x

LIST OF TABLES xi

LIST OF FIGURES xi

LIST OF APPENDICES xiv

PUBLICATIONS GENERATED FROM THIS DISSERTATION xvi

ABSTRACT xvii

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background 1

1.2 Problem statement 3

1.3 Justification 4

1.4 Purpose of the study 4

1.4.1 Aim 4

1.4.2 Objectives 5

1.4.3 Hypotheses 5

1.5 Reliability, validity, and objectivity 5

1.6 Bias 6

1.7 General overview of the thesis layout 7

1.8 References 8

CHAPTER TWO

LITERATURE REVIEW

2.1 Importance of Bambara Groundnut 11

2.1.1 Significance of Bambara groundnut 11

2.1.2 Agronomic significance 11

2.1.3 Nutritional significance 12

2.1.4 Medicinal significance 13

2.1.5 Economic significance 14

2.2 Sead coat varieties and compounds of Bambara groundnut 15

2.3 Production of Bambara groundnut 17

2.4 Factors affecting Bambara groundnut production 18

2.4.1 Biotic factors 18

2.4.2 Abiotic factors 21

2.5 Biological nitrogen fixation 24

2.5.1 Nitrogen-fixing bacteria's contribution to agriculture 24

2.5.2 Factors affecting the performance of nitrogen-fixing bacteria 24

2.6 Bambara groundnut production in South Africa 26

2.6.1 Constraints in Bambara groundnut production in SA 26

2.6.2 Opportunities in Bambara groundnut production in SA 27

2.7 References 27

CHAPTER THREE

INHIBITORY POTENTIAL OF BAMBARA GROUNDNUT SEEDCOAT EXTRACTS ON INDIGENOUS NITROGEN FIXING BACTERIAL ISOLATES

3.1 Introduction 39

3.2 Materials and Methods 40

3.2.1 Study location 40

3.2.2 Seed coat extracts preparation 40
3.2.3 Preparation of nitrogen-fixing bacteria 42
3.2.4 Antimicrobial assay of Bambara groundnut seed coat extracts 43
3.2.5 Data collection 45
3.2.6 Statistical analysis 45
3.3 Results 45
3.4 Discussion 49
3.5 Conclusion 50
3.6 References 51

CHAPTER FOUR

EVALUATION OF INDIGENOUS BAMBARA GROUNDNUT NITROGEN-FIXING BACTERIA ISOLATES PH AND SALINITY TOLERANCE

4.1 Introduction 54
4.2 Materials and Methods 55
4.2.1 Study site 55
4.2.2 Experimental treatments and design 55
4.2.3 Experimental procedure 55
4.2.4 Data collection 56
4.3 Results 57
4.3.1 Bacteria tolerance to pH 58
4.3.2 Salinity tolerance of bacterial strains 60
4.4 Discussion 64
4.4.1 Bacteria tolerance to low pH 64
4.4.2 Salinity tolerance of bacterial isolates 66
4.4.3 Modelling pH and salinity levels on bacterial growth 67

4.5 Conclusion 68

4.6 References 69

CHAPTER FIVE

EFFICIENCY OF INDIGENOUS NITROGEN-FIXING BACTERIAL ISOLATES ON GROWTH AND NODULATION OF RETAINED BAMBARA GROUNDNUT VARIETIES

5.1 Introduction 74

5.2 Materials and methods 75

5.2.1 Study site 75

5.2.2 Experimental treatments and design 75

5.2.3 Procedure 77

5.2.4 Data collection 77

5.2.5 Data analysis 80

5.3 Results 80

5.3.1 Plant growth variables 80

5.3.2 Nodulation variables 84

5.4 Discussion 88

5.4.1 Plant growth variables 88

5.4.2 Nodulation variables 89

5.5 Conclusion 92

5.6 References 92

CHAPTER SIX

SUMMARY, SIGNIFICANCE OF FINDINGS, FUTURE RESEARCH, CONCLUSION, AND RECOMMENDATION

6.1 Summary 97

6.2 Significance of findings 98
6.3 Future research 99
6.4 Conclusion 100
6.5 Recommendation 101
6.6 References 102
APPENDICES 104

DECLARATION

I, Gugu Cynthia Maseko, student number 201971593, hereby declare that the dissertation titled 'Salinity, pH tolerance, and efficiency of indigenous nitrogen-fixing bacteria on nodulation and growth of *Vigna subterranea*' submitted for the Master of Science in Agriculture is my own work and has not previously been submitted for assessment or completion of any postgraduate qualification to another university or another qualification. The study was granted ethical clearance by the University of Mpumalanga Faculty Research and Innovation Committee and given the protocol reference number: UMP/Maseko/201971593/MSC/2024. The materials and information used from any other sources have been fully acknowledged.

03/11/2025

Maseko, GC. (Ms) Date

Supervisor(s):

29/04/2026

Dube, ZP. (Dr) Date

Kola, E. (Ms) Date

DEDICATION

To my loving, supportive, and caring mother, Ms Zanele Nelsiwe Maseko.

ACKNOWLEDGEMENTS

Firstly, I would like to express my utmost gratitude and thanks to the LORD, God Almighty, for His love, grace, presence, and the strength He has given me throughout this academic journey. I would not have finished this dissertation without His grace in my life.

My most sincere and heartfelt gratitude to my supervisor, Dr. Zakheleni Dube, for his unwavering, timeless, and tireless guidance, patience, encouragement, and valuable suggestions throughout my study. He has contributed greatly to my academic success, providing me with a substantial amount of support and assistance. This includes ensuring that delayed materials are delivered and demonstrating how to conduct research. I am grateful for all the life lessons and fatherly advice he has shared with me while conducting this research. Special thanks to my Co-Supervisor, Ms Elizabeth Kola, for her support, expertise, kindness, guidance, and always being available for consultation.

I am extremely grateful to the National Research Foundation (NRF) for financial assistance towards my master's degree studies. A special thanks also goes to Ms. Rivoningo Ubisi from Crop Protection, for her support and sharing the indigenous Bambara groundnut nitrogen-fixing bacteria isolates identified from her study, which were a crucial element in this research.

I further extend my gratitude to the University of Mpumalanga Farm manager Mr. Petros Mtsweni and the farm workers for all the assistance and support they provided during the time of my research at the University farm. I am equally thankful to the Botany Research and Chemistry laboratories for granting me access to use their equipment and providing experimental support. Special thanks go to Ms. Sinorita Chauke and Ms. Sandra Mokhwanatsi for their assistance. Lastly, I would like to thank my family members for all their prayers and support in my studies, especially my sister, Agreement Lubisi, my mother, Zanele Maseko, all the church members, and pastors. I recognize Ms. Thobile Mkhwanazi, Ms. Siphesihle

Mthombeni, and Mr. Moses Timana, my fellow Master's and PhD candidates, whom I consider as my sisters and brother. The greenhouse termination team (Mr Musa Nkosi, Mr Nhlakanipho Nkuna, Mr Celuxolo Magagula, and Ms Nothando Madlala), their support, encouragement, and tireless help throughout my studies made my work lighter. May the LORD bless you all.

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BGN	Bambara groundnut
BNF	Biological nitrogen fixation
CFU	Colony-forming units
CRD	Completely randomized design
DAFF	Department of Agriculture, Forestry and Fisheries
HCl	Hydrochloric acid
KNO_3	Potassium nitrate
NRF	National Research Foundation
NaOH	Sodium hydroxide
RE	Relative nitrogen fixing effectiveness
RCBD	Randomized completely blocked design
R^2	Coefficient of determination
UMP	University of Mpumalanga
YEM	Yeast Mannitol media

LIST OF TABLES

Table 2.1: Phytochemicals of various Bambara groundnut seed coats.....16

Table 3.1: Morphological characteristics of the three Bambara groundnut seed landraces 41

Table 3.2: Antibiotics for gram-positive and gram-negative nitrogen-fixing bacteria isolates 44

Table 3.3: Antibacterial effect of Bambara groundnut seedcoat extracts on growth of indigenous nitrogen-fixing bacteria isolates 47

Table 4.1: Responses of indigenous Bambara groundnut bacteria isolates to pH 58

Table 4.2: The effect of pH on the absorbance of indigenous Bambara groundnut nitrogen-fixing bacteria isolates 59

Table 4.3: Indigenous Bambara groundnut bacteria isolate radial growth 60

Table 4.4: Impact of salinity levels on the radial growth of indigenous Bambara groundnut nitrogen-fixing bacteria isolates.....61

Table 4.5: Optimum level and coefficient correlation of indigenous Bambara groundnut nitrogen-fixing bacteria.....64

Table 5.1: Different coloured seed coats of Bambara groundnut seed varieties 76

Table 5.2: Effect of varieties on plant height (PH) number of leaves (NL), number of runners (NR), chlorophyll content (CC), stem diameter (SD), fresh root mass (FRM), and dry root mass (DRM) for retained Bambara groundnut 82

Table 5.3: Effect of Indigenous Bambara groundnut bacterial isolates on stem diameter (SD) 83

Table 5.4: Effect of varieties on nodule position (NP) and Plant growth vigor (PGV) for retained Bambara groundnut.....84

Table 5.5: Effect of Indigenous bacterial strains on the number of nodules (NN), active nodules (AN), nodulation colour and abundant score (NCAS), nodulation effectiveness score (NES), and relative nitrogen fixing effectiveness (RE) for retained Bambara groundnut varieties 86

LIST OF FIGURES

Figure 2.1: Bruchids damage on Bambara groundnut seeds.....19

Figure 2.2: Aphids (A) and beetles (B) on Bambara groundnut plant.....20

Figure 2.3: Red spider mites on Bambara groundnut. 21

Figure 3.1: Measurement of the zone of inhibition; +ve stands for positive control, E for ethanol extract, W for water extract, and A for acetone extract.. 45

Figure 4.1: Bacterial isolate inoculated at the center of a YEM media plate. 57

Figure 4.2: Impact of pH levels on the absorbance of indigenous Bambara groundnut nitrogen-fixing bacteria isolates. 63

Figure 4.3: Impact of salinity levels on the radial growth of indigenous Bambara groundnut nitrogen-fixing bacteria isolates.....64

Figure 5.1: Bambara groundnut seedlings after thinning.....77

LIST OF APPENDICES

Appendix 3.1: Shapiro-Wilk normality test for the zone of inhibition 104

Appendix 3.2: Analysis of variance for the zone of inhibition 104

Appendix 4.1: Salinity levels of sodium chloride 105

Appendix 4.2: Salinity levels of calcium chloride 105

Appendix 4.3: Salinity level ratios of sodium chloride and calcium chloride levels 106

Appendix 4.4: Shapiro-Wilk normality test for absorbance and radial growth 106

Appendix 4.5: Analysis of variance for absorbance 107

Appendix 4.6: Analysis of variance for radial growth 107

Appendix 5.1: Shapiro-Wilk normality test for plant growth variables 108

Appendix 5.2: Shapiro-Wilk test for nodulation variables 108

Appendix 5.3: Analysis of variance for plant height 109

Appendix 5.4: Analysis of variance for the number of leaves 109

Appendix 5.5: Analysis of variance for number of runners 110

Appendix 5.6: Analysis of variance for length of the longest runner 110

Appendix 5.7: Analysis of variance for chlorophyll content 111

Appendix 5.8: Analysis of variance for stem diameter 111

Appendix 5.9: Analysis of variance for fresh shoot mass 112

Appendix 5.10: Analysis of variance for dry shoot mass 112

Appendix 5.11: Analysis of variance for fresh root mass 113

Appendix 5.12: Analysis of variance for dry root mass 113

Appendix 5.13: Analysis of variance for relative nitrogen fixation effectiveness 114

Appendix 5.14: Analysis of variance for nodule position 114

Appendix 5.15: Analysis of variance for number of nodules 115

Appendix 5.16: Analysis of variance for active nodules 115

Appendix 5.17: Analysis of variance for non-active nodules.....116

Appendix 5.18: Analysis of variance for nodule colour and abundance score.....116

Appendix 5.19: Analysis of variance for plant growth vigor 117

Appendix 5.20: Analysis of variance for nodulation effectiveness score.....117

PUBLICATIONS GENERATED FROM THIS DISSERTATION

1. Oral Conference presentations

i. **Maseko, G**., Kola, E., Dube, Z., Mkhwanazi, TP. and Ubisi, R. 2023. Compatibility of commercial nitrogen-fixing bacteria and local *Vigna subterranea* (L. Verdc.) cultivars. ARC-TSC 2nd research symposium. 16 March 2023. University of Mpumalanga conference and wellness center.

ii. **Maseko, G**., Mthombeni, S., Mkhwanazi, T., Ubisi, R., Dube, Z., Kola, E. 2024. Evaluation of indigenous nitrogen-fixing bacteria from Bambara groundnut for tolerance to pH and saline conditions. One Health Student International Conference 4-6 December 2024. Bucharest, Romania.

ABSTRACT

Bambara groundnut (*Vigna subterranea* L. Verdc.) is an underutilized and highly nutritious crop with a variety of seed coat colours, influenced by secondary metabolites known to possess antimicrobial properties. Despite its potential, production remains low due to ecological stresses, such as extreme temperatures, drought, and poor rainfall, as well as plant genotype selection and seed coat properties, all of which contribute to soil salinity and pH fluctuations that limit plant growth and beneficial microorganisms. The study hence had three objectives (i) to assess the survival of indigenous nitrogen-fixing bacteria on Bambara groundnut seed coat extracts, (ii) their tolerance to low pH leve and high soil salinity levels, and (iii) the efficiency of these bacteria in promoting nodulation and growth of Bambara groundnut. Objective 1, a 3×10×3 factorial experiment in a CRD with 3 replications was used to test three Bambara groundnut (BR-W, B-W, and B-B) variety seed coats extracted using water, ethanol, and acetone, on 10 Bambara groundnut extracted bacterial strains (*Proteus columbae*, *Mammaliicoccus sciuri*, *Bacillus pumilus* P12, *Stenotrophomonas pavanii* , *Bacillus pumilus* P4, *Kaistella daneshvariae*, *Stenotrophomonas maltophilia*, *Cellulosimicrobium cullulans*, *Sphingobacterium faecium*, and *Enterobacter asburiae)* under laboratory conditions. The inhibitory effects of the three Bambara groundnut seed coat extracts varied with bacteria. *Proteus columbae*, *B. pumilus* P12, *B. pumilus* P4, and *S. pavanii* were inhibited by all three extracts, whereas the growth of *M. sciuri*, *S. maltophila*, *E. asburiae*, and *C. cullulans* was inhibited by water and acetone extracts only, with ethanol extracts having no negative effects on the four. The growth of *S. faecium* was inhibited by water and ethanol extracts only, while acetone extracts had no negative effect, and *K. daneshvariae* growth was inhibited by water extracts only. A laboratory experiment was established to assess the tolerance of the same bacterial isolates to pH levels (3.5, 4.5, 5.5) with a media pH of 6.9 as the control, and salinity levels (2–128 mM/m³ of NaCl + $CaCl_2$). *Cellulosimicrobium cellulans*, *S. pavanii*, and *P.*

columbae had the highest absorbance, ranging from 0.519 to 0.69, while *S. faecium*, *M. sciuri,* and *B. pumilus* P4 had the lowest absorbance values, ranging from 0.286 to 0.398 under various pH levels. The salinity levels of 0 mM exhibited the highest radial growth, followed by 4 mM and 8 mM, ranging from 11.833 mm to 12.222 mm, whereas the salinity levels of 128 mM, 64 mM, and 32 mM showed the lowest radial growth, ranging from 7.6 to 11.133 mm for all bacterial isolates. Three Bambara varieties inoculated with the ten aforementioned bacterial strains, compared to uninoculated and KNO_3-fertilized controls under greenhouse conditions. *Mammaliicoccus sciuri*, *K. daneshvariae*, and *S. maltophila* had the highest stem diameters, while *S. maltophila*, *B. pumilus* P4, and *P. columbae* produced the highest nodule colour and abundance score. All bacterial isolates had high relative nitrogen fixing effectiveness (>80), with *K. daneshvariae*, *M. sciuri*, and *S. faecium* treated plants demonstrating the highest. In conclusion, the Bambara groundnut seed coats exhibited notable antimicrobial activity against certain indigenous nitrogen-fixing bacterial strains, highlighting the potential influence of seedcoat compounds on beneficial soil microorganisms. The isolates demonstrated tolerance to acidic conditions ($pH \geq 4.12$) but were unable to withstand salinity levels above 4.24 mM/m^3. Despite these stress limitations, all isolates showed high relative nitrogen-fixing effectiveness, indicating strong potential for biofertilizer development in acidic and mildly saline soils. Notably, Mammaliicoccus sciuri, Kaistella daneshvariae, Stenotrophomonas maltophilia, Bacillus pumilus P4, and Proteus columbae enhanced Bambara groundnut growth and are promising candidates for an inoculant.

Keywords: Antimicrobial properties, Bambara groundnut, nitrogen-fixing bacteria, pH, salinity

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background

Bambara groundnut (*Vigna subterranea* (L.) Verdc.) is an underutilized, indigenous crop that is deemed as the third most common legume after cowpea (*Vigna unguiculata* L. Walp.) and groundnuts (*Arachis hypogaea* L.) in Africa (Khan, Rafii, Ramlee, Jusoh, & Al-Mamun, 2021; Ibny, Jaiswal, Mohammed, & Dakora, 2019). In South Africa, the crop is primarily produced in the provinces of Northwest, KwaZulu-Natal, Mpumalanga, Limpopo, and Gauteng (Majola, Gerrano, & Shimelis, 2021). Bambara groundnut has a protein content of 17-25%, lower than that of cowpea (*Vigna unguiculata* L.), groundnut (*Arachis hypogaea* L.), and soybean (*Glycine max* L.), which are 17.4-31.7%%, 25-30%, and 44-48%%, respectively (Mashiloane, Mlambo, Mhlongo, Dibakoane, & Mnisi, 2025; Abady, Shimelis, Janila, & Mashilo, 2019). It is, however, rich in minerals, amino acids, carbohydrates, fats, and fiber, which has led to the crop being labelled an indigenous key solution to food insecurity in Africa (Olanrewaju, Oyatomi, Babalola, & Abberton, 2022).

Fortunately, the production of Bambara groundnut is favored by its tolerance to salt stress, poor soil fertility, and drought, which is achieved through the formation of effective root nodules with compatible soil nitrogen-fixing bacteria (Zenabou *et al*., 2022). The symbiotic relationship between nitrogen-fixing bacteria and Bambara groundnut not only increases plant growth and productivity but also improves soil fertility, a crucial factor for sustainable agricultural production (Ubisi *et al*., 2023). In addition, this symbiotic association is both agronomically and economically important because of its substantial contribution to the nitrogen balance in

land-based ecosystems and to crop productivity. (Fwanyanga, Horn, Sibanda, & Reinhold-Hurek, 2022).

Because to colonial-era land dispossession and settlement patterns, many communal farmers who rely on Bambara groundnut cultivation are located in areas with degraded soils, characterized by very low natural populations of beneficial microorganisms and poor nutritional status (Khan *et al*., 2021). Climate change, as one of the constraints, has also exacerbated the problem, as increased temperatures and drought stress have resulted in challenges of even higher soil salinity and fluctuating soil pH (Torabian, Farhangi-Abriz, & Denton, 2019). Soils with high salinity and fluctuating pH affect the metabolic activity, growth, and survival of nitrogen-fixing bacteria (Torabian *et al*., 2019). Studies have also reported that high salinity levels in the soil result in soil rhizobia forming cells that do not permit growth and development of their host; however, some *Rhizobium* strains can tolerate saline soils and fix the atmospheric nitrogen (Etesami & Adl, 2020).

Rhizobia inocula have since been advocated for use as bio-fertilizers to improve soils with low fertility and have been reported as a sustainable approach for growing Bambara groundnut (Nelwamondo, 2020). They are also seen as a more cost-effective, safer, and efficient method for enhancing nitrogen fixation and grain legume crop production (Fwanyanga *et al*., 2022). Forming and utilizing inoculants based on indigenous strains is advisable as they possess superior traits of competitiveness in nodule infection and occupancy (Sanchez-Navarro *et al*., 2020). Indigenous bacterial strains possess a higher biological nitrogen fixation ability in the field due to their genetic adaptations to the local conditions (Ibny *et al*., 2019). Thus, this study seeks to evaluate the survival of indigenous nitrogen-fixing bacteria isolates on Bambara groundnut seed coat extracts, their tolerance to high salinity and low pH, and efficiency in

nodulation and growth of retained Bambara groundnut in the development of an inoculum that will enhance Bambara groundnut production.

1.2 Problem statement

Despite its potential contribution to soil fertility, symbiotic nitrogen fixation is mainly affected by climatic conditions such as prolonged drought, poor rainfall, and extreme temperatures (Fwanyanga *et al*., 2022). These conditions affect the population of *Rhizobium* species in the soil, resulting in reduced nodulation and Bambara groundnut yields (Fwanyanga *et al*., 2022). Prolonged droughts and extreme temperatures are often associated with increased soil salinity levels and fluctuating pH, which results in reduced plant growth and beneficial microorganisms (Tan *et al*., 2020). Furthermore, Sellars and Nunes (2021) reported that salinity and pH tolerance effects vary within rhizobial nitrogen-fixing bacteria. Therefore, identifying indigenous strains with enhanced tolerance to these abiotic stresses is essential for developing effective bioinoculants suited to challenging agroecological environments.

Furthermore, Bambara groundnut exhibits a variety of seed coat colours, such as red, cream/black-eye, black, cream/brow-eye, cream/ no-eye, brown, and speckled/flecked/ spotted, which are influenced by secondary metabolites (Adedayo *et al*., 2021). These metabolites are known to possess biological activities, including antioxidant, detoxification, and antimicrobial properties (Adedayo *et al*., 2021). However, the antimicrobial potential of these seed coat extracts, particularly concerning their effect on indigenous nitrogen-fixing bacteria, remains largely unexplored (Palamae *et al*., 2024). Hence, this study seeks to evaluate the salt and pH tolerance of indigenous nitrogen-fixing Bambara groundnut bacterial isolates and their nitrogen-fixing efficiency on selected retained Bambara groundnut varieties. Additionally, it

aims to investigate the inhibitory potential of Bambara groundnut seed coat extracts on the indigenous nitrogen-fixing bacterial isolates.

1.3 Justification

This study will advance microbial ecological knowledge by characterizing the stress tolerance and symbiotic efficiency of indigenous nitrogen-fixing bacteria associated with Bambara groundnut, thereby clarifying mechanisms of microbial adaptation and functional resilience under salinity and acidic stress conditions. It also contributes to climate-resilient agriculture by identifying locally adapted bacterial strains with potential application as bioinoculants to enhance biological nitrogen fixation, crop productivity, and sustainability in marginal environments. Furthermore, this study will aid in filling the knowledge gap that exists among farmers about the symbiotic relationship between indigenous nitrogen-fixing bacteria and Bambara groundnut (Tan *et al.*, 2020). It will also potentially increase Bambara groundnut and other agricultural crop yield levels for communal farmers by optimising on the rhizobacterial strains to Bambara groundnut variety (Nelwamondo, 2020). Improving Bambara groundnut production will potentially increase the income of small-scale producers, improving livelihoods (Majola *et al.,*2021). The information presented in the study will guide the sustainable production of Bambara groundnut in pursuit of food and nutrition security (Majola *et al*., 2021).

1.4 Purpose of the study

1.4.1 Aim

Evaluation of the survival of indigenous nitrogen-fixing bacteria on seed coat extracts, their tolerance to soil salinity, pH, and efficiency on retained Bambara groundnut in pursuit of producing high-quality nitrogen-fixing bacterial inoculants.

1.4.2 Objectives

i. To assess the inhibitory potential of Bambara groundnut seedcoat extracts on indigenous nitrogen-fixing bacterial isolates.
ii. To evaluate whether indigenous nitrogen-fixing bacteria will tolerate low pH and high saline conditions.
iii. To determine whether the indigenous nitrogen-fixing bacteria isolates will increase nodulation and growth of retained Bambara groundnut varieties.

1.4.3 Hypotheses

i. Bambara groundnut seed coat exhibits inhibitory effects on the growth of indigenous nitrogen-fixing bacteria isolates.
ii. Indigenous nitrogen-fixing bacteria will tolerate low pH and high saline conditions.
iii. Indigenous nitrogen-fixing bacteria isolates will increase nodulation and growth of retained Bambara groundnut varieties.

1.5 Reliability, validity, and objectivity

Reliability is the degree to which results remain consistent over time and accurately reflect the studied group (Golafshani, 2003). It is the idea of replicability or repeatability of observations (Golafshani, 2003). Reliability was ensured in the present study by having three or more replications of the treatments and three independent experiments in achieving Objectives 1 and 2. Additionally, two independent experiments were conducted to achieve Objective 3.

Validity is the extent to which a scientific claim is accurate (Baumgartner, Oh, Chung, & Hales, 2002). According to Sürücü and Maslakçi (2020), validity measures how well a measuring instrument fulfills its intended purpose and is concerned with whether the instrument measures the behavior or quality it is designed to assess. Furthermore, empirical research experiments

are repeated in place or time to broaden the validity of the findings that can be made from them (Little & Hills, 1981). This study ensured validity by following established methodologies from literature.

Objectivity guarantees that research findings are not influenced, that affirmative findings are not false positives, and that other scientists can independently replicate the study's findings (Van Dongen & Sikorski, 2021). According to Eisner (1992), objectivity aims to minimize or eliminate biases, prejudices, or subjective assessments as much as possible or practical by relying on validated evidence. This ensures that fair evaluations, decisions, and conclusions are made (Eisner, 1992). The study maintained objectivity by randomly assigning treatments to experimental units, discussing the findings based on empirical data, displaying the results through statistical analysis, and comparing the findings with those from other studies (Little & Hills, 1981).

1.6 Bias

A form of systematic error that has the potential to affect scientific investigations and impact measurement procedures is defined as bias (Krishna, Maithreyi, & Surapaneni, 2010). Bias exists across research designs and cannot be easily eliminated; it can occur at any point during the research process, impacting the reliability and validity of study findings, and misinterpreting data can have significant practical consequences (Smith & Noble, 2014). Bias was minimised in this research by increasing replications and randomization to ensure that the experimental error of each experiment was kept low.

1.7 General overview of the thesis layout

This dissertation is made up of six chapters. Chapter one outlines the background of the study, the research problem, justification, and purpose of the study in the form of aim, objectives, and hypotheses. Chapter two discusses the literature review relative to the agronomic, nutritional, medicinal, and economic significance of Bambara groundnut. It also details the biotic and abiotic factors affecting Bambara groundnut production, including but not limited to soil pH and salinity. Furthermore, this chapter covers Bambara groundnut's production constraints and opportunities in South Africa. The next three chapters, 3, 4, and 5 addresses each experimental objective postulated. Chapter three reports findings on the inhibitory potential of Bambara groundnut seedcoat extracts on indigenous nitrogen-fixing bacteria isolates, with Chapter four reporting findings on the ability of indigenous nitrogen-fixing bacteria isolates to tolerate pH and saline conditions. Chapter five describes and discusses findings on the efficiency of indigenous nitrogen-fixing bacteria isolates on the growth and nodulation of retained Bambara groundnut varieties. The last chapter, Chapter six summarizes the findings of the whole study, stating the significance of the findings, the future potential of indigenous nitrogen-fixing bacteria, and Bambara groundnut production. It also presents general conclusions and recommendations based on the results of the series of experiments in Chapters three, four, and five. The Harvard referencing style, with a few adjustments, was used to reference the literature and work done, whereby sources (journal articles, books, book chapters) with fewer than six authors' names are firstly written in full in text and written as *et al* when referenced the second time in each chapter. Sources that have six or more authors are written in *et al*. Dissertation and thesis work are referenced in the form of a book, Harvard referencing style, with the university being used as the publisher and the city in which the university is located as the publisher's city. Reference list was arranged alphabetically first and then chronologically for similar authored articles.

1.8 References

Abady, S., Shimelis, H., Janila, P. and Mashilo, J. 2019. Groundnut (*Arachis hypogaea* L.) improvement in sub-Saharan Africa: A review. *Acta Agriculturae Scandinavica, Section B—Soil & Plant Science*, 69(6):528-545.

Adedayo, B.C., Anyasi, T.A., Taylor, M.J., Rautenbauch, F., Le Roes-Hill, M. and Jideani, V.A. 2021. Phytochemical composition and antioxidant properties of methanolic extracts of whole and dehulled Bambara groundnut (*Vigna subterranea*) seeds. *Scientific Reports,* 11(1): 1-11.

Baumgartner, T.A., Oh, S., Chung, H. and Hales, D. 2002. Objectivity, reliability, and validity for a revised push-up test protocol. *Measurement in Physical Education and Exercise Science*, 6(4): 225-242.

Eisner, E. 1992. Objectivity in educational research. *Curriculum Inquiry*, 22(1): 9-15.

Etesami, H. and Adl, S. 2020. Can interaction between silicon and non–rhizobial bacteria help in improving nodulation and nitrogen fixation in salinity–stressed legumes? A review. *Rhizosphere*, 15(1): 1-20.

Fwanyanga, F.M., Horn, L.N., Sibanda, T. and Reinhold-Hurek, B. 2022. Prospects of rhizobial inoculant technology on Bambara groundnut crop production and growth. *Frontiers in Agronomy,* 4: 1-7.

Golafshani, N. 2003. Understanding reliability and validity in qualitative research. *The Qualitative Report*, 8(4): 597-607.

Ibny, F., Jaiswal, S., Mohammed, M. and Dakora, F. 2019. Symbiotic effectiveness and ecologically adaptive traits of indigenous rhizobial symbionts of Bambara groundnut (*Vigna subterranea* L. Verdc.) in Africa and their relationship with phylogeny. *Scientific Reports*, 9(1): 1-17.

Khan, M.M.H., Rafii, M.Y., Ramlee, S.I., Jusoh, M. and Al-Mamun, M. 2021. Bambara groundnut (*Vigna subterranea* L. Verdc.): A crop for the new millennium, its genetic diversity, and improvements to mitigate future food and nutritional challenges. *Sustainability*, 13(10): 1-27.

Krishna, R., Maithreyi, R. and Surapaneni, K.M. 2010. Research bias: A review for medical students. *Journal of Clinical and Diagnostic Research*, 4(2): 2320-2324.

Little, T.M. and Hills, F.J. 1981. *Statistical Methods in Agricultural Research*. Davis: University of California.

Majola, N., Gerrano, A. and Shimelis, H. 2021. Bambara groundnut (*Vigna subterranea* L. Verdc.) production, utilization, and genetic improvement in Sub-Saharan Africa. *Agronomy*, 11(7): 1-16.Mashiloane, T., Mlambo, V., Mhlongo, G., Dibakoane, S.R. and Mnisi, C.M. 2025. Cowpeas vs. soybeans: Can valorization bridge the nutritional gap for sustainable animal feeding systems in the Global South?. *Frontiers in Sustainable Food Systems*, 9: 1-17.

Nelwamondo, A. 2020. Assessment of co-inoculation of *Bradyrhizobium japonicum* and *Bacillus subtitles* on yield and metabolic profile of Bambara groundnut and cowpea under glasshouse conditions. *Institutional Repository*, 1(1): 1-80.Olanrewaju, O.S., Oyatomi, O., Babalola, O.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.

Palamae, S., Suyapoh, W., Boonrat, O., Zhang, B., Amin, M., Buatong, J. and Benjakul, S. 2024. Activity of Bambara groundnut seed coat extract against *Shewanella* species: Efficacy and mechanisms of action. *Foods*, 13(21): 1-16.

Sanchez-Navarro, V., Zornoza, R., Faz, Á., Egea-Gilabert, C., Ros, M., Pascual, J.A. and Fernández, J.A. 2020. Inoculation with different nitrogen-fixing bacteria and arbuscular

mycorrhiza affects grain protein content and nodule bacterial communities of a fava bean crop. *Agronomy*, 10(6): 1-15.

Sellars, S. and Nunes, V. 2021. Synthetic nitrogen fertilizer in the U.S. *Farmdoc Daily*, 11(24): 1-5.

Smith, J. and Noble, H. 2014. Bias in research. *Evidence-Based Nursing*, 17(4): 100-101.

Sürücü, L. and Maslakci, A. 2020. Validity and reliability in quantitative research. *Business and Management Studies: An International Journal,* 8(3): 2694-2726.

Tan, X.L., Azam-Ali, S., Goh, E.V., Mustafa, M., Chai, H.H., Ho, W.K., 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: 1-16.

Torabian, S., Farhangi-Abriz, S. and Denton, M. 2019. Do tillage systems influence nitrogen fixation in legumes? A review. *Soil and Tillage Research*, 185(1): 1-9.

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.

Van Dongen, N. and Sikorski, M. 2021. Objectivity for the research worker. *European Journal for Philosophy of Science*, 11(3): 1-25.

Zenabou, N., Firmin, S.L., Laurette, N.N., Daniel, W.F., Laurianne, T.N. and Martin, B.J. 2022. Nodulation potential of Bambara groundnut (*Vigna subterranea* L.) in Yaounde (Centre Region of Cameroon). *American Journal of Food and Nutrition*, 10(1): 34-39.

CHAPTER TWO

LITERATURE REVIEW

2.1 Importance of Bambara Groundnut

2.1.1 Significance of Bambara groundnut

Bambara groundnut (BGN) is utilized by different cultural groups to produce a wide range of food products in areas where the crop is cultivated (Esan, Oke, & Ogunbode, 2023). It is consumed by smallholder communities in South Africa as both grain and vegetable crops (Cook, 2017). Furthermore, traditional and local markets sell it as boiled (Jideani & Jideani, 2021). Bambara plants are utilized as animal feed in the tropics and the seeds are used to extract oil, protein supplements, and can be milled into powder and paste for making cake (Esan *et al*., 2023). Moreover, freshly harvested pods are eaten as snacks after being grilled or boiled (Muhammad *et al*., 2020). Chelangat, Muturi, Gichimu, Gitari, and Mukono (2023) reported that the legume seeds are high in calories and their flour can be utilized to make a thick porridge. According to Ogban, Oparaeke, Odey, and Udeme (2021), steamed BGN pudding called Okpa, a Nigerian dish made from the flour and red palm oil, plays a significant role in contributing to school children's dietary vitamin A and dietary protein intake. Hence, it is important to know the agronomic, nutritional, medicinal, and economic significance of BGN.

2.1.2 Agronomic significance

Vigna subterranea L. Verdc. is resistant to harsh environmental factors such as drought and its productivity can improve in areas with poor soil and water stress as compared to other legumes, making it a climate-smart crop (Ajilogba, Olanrewaju, & Babalola, 2022; Soumare, Diedhiou, & Kane, 2021). Similarly, physiological studies have revealed good recovery characteristics of BGN, whereby its yield is reported to be greater than chickpea and similar to groundnut varieties during comparable drought stress conditions (Tan *et al*., 2020). Kunene, Gerrano, and

Odindo (2024) reported that the legume landraces have revealed a remarkable ability to survive in the poorest environments by growing without irrigation, fertilizers, disease, and pest control. Considering all these factors, BGN can play a significant role in reducing poverty and malnutrition in the time of global warming and food security threats worldwide, especially in areas with extreme water problems (Ramatsetse, Ramashia, & Mashau, 2023).

According to Ajilogba *et al*. (2022), farmers with limited resources, particularly those who cannot afford to purchase synthetic fertilizers for production improvement, are encouraged to produce BGN in place of other legumes as they can grow well in poor soils. More importantly, BGN can fix atmospheric nitrogen through a symbiotic relationship with the soil nitrogen-fixing bacteria (Sarkar *et al*., 2023). This symbiotic association significantly improves soil fertility through crop rotation and intercropping farming systems with crops such as cassava (*Manihot esculenta* Crantz), maize (*Zea mays* L.), sorghum (*Sorghum bicolor* (L.) Moench), yam (*Dioscorea* spp.), millet (*Panicum miliaceum* L.), and peanuts (*Arachis hypogaea* L.) (El Bilali *et al*., 2024). El Bilali *et al*. (2024) also reported that a great number of farmers still decide to produce BGN as a monoculture, despite it being well-suited for intercropping systems.

2.1.3 Nutritional significance

In terms of nutritional value, BGN is a highly nutritious legume with an ideal balance of essential and nonessential amino acids, carbohydrates, protein, and oil (Mohammed, Shimelis, Laing, & Usman, 2023). Bambara groundnut grains consist of 6.3% fiber, 6.6% fats, 56.5% carbohydrates, and 20.6% protein, making it a comprehensive meal (Ibny, Jaiswal, Mohammed, & Dakora, 2019). It is also used as a crucial protein source for human

consumption, particularly for vegetarians and low-income households that cannot afford protein from meat-based sources (Maphosa, Jideani, & Maphosa, 2022).

According to Esan *et al*. (2023), Bambara groundnut also contains micronutrients like Na (2.9–12.0 mg/100 g), Fe (5.1–9.2 mg/100 g), Ca (95.8–99 mg/100 g), K (11.45–19.35 mg/100 g) and have about 33.31% essential amino acids and 66.69% nonessential amino acids. Mbuma, Labuschagne, Siwale, and Hugo (2022) reported that the *V. subterranea* seed also contains high levels of amino acids; tryptophan (0.7 g/100 g), methionine (2.0 g/100 g), and lysine (3.0 g/100g), distinguishing them from other leguminous crops. For instance, chickpea (*Cicer arietinum* L.) has lower levels of amino acids; tryptophan (0.2 g/100 g), methionine (0.3 g/100 g), and lysine (1.3 g/100 g) also cowpea contains tryptophan (0.3g/100 g), methionine (0.3 g/100 g), and lysine (1.6 g/100 g) (Mbuma *et al*., 2022). Bambara groundnut seeds have a higher gross energy value than cowpea, lentils (*Lens culinaris* Medik.), and pigeon peas (*Cajanus cajan* (L.) Huth) (Muhammad *et al.*, 2020). Therefore, the diverse nutritional composition of BGN suggests that it can meet the dietary requirements of people globally (Ramatsetse *et al*., 2023).

2.1.4 Medicinal significance

Bambara groundnut leaf extracts have been utilized in traditional medicine to treat wounds and abscesses in animals (Agajie, 2021). Additionally, the sap from the leaves can be used to treat epilepsy by applying it to the eyes (Khan, Rafii, Ramlee, Jusoh, & Al-Mamun, 2021). Moreover, they are also used in combination with *Lantana trifolia* L. leaf extracts as an insecticide in livestock to treat various diseases in Nigeria (Okafor, Jideani, Meyer, & Le Roes-Hill, 2022). In Senegal, the Bambara groundnut seeds are used to treat cataracts by grinding them and mixing the powder with water, whereas its roots have been used as an aphrodisiac

(Ajilogba *et al*., 2022). According to Harris, Jideani, and Le Roes-Hill (2018), black landraces of the BGN are used to treat impotence and have been utilized in South Africa to control nausea in pregnant women by having them chew and swallow the seeds. The pounded seeds of the legume are also utilized to treat skin rashes in Ghana and to treat diarrhea in children when combined with guinea fowl meat (Khan *et al*., 2021).

According to El Bilali *et al*. (2024), BGN possesses antioxidant and antimicrobial properties attributed to its phytochemicals, including phytic acids, tannins, and flavonoids, which can impede microbial growth and extend the shelf life of food products. These compounds provide health benefits by helping to prevent conditions such as cancer, diabetes, heart disease, stroke, cardiovascular diseases, Alzheimer's, and atherosclerosis (Ramatsetse *et al*., 2023). Furthermore, Uro-Chukwu *et al*. (2024) reported that, in addition to their antioxidant properties, BGN phytochemicals also regulate hormone metabolism, strengthen the immune system, and may play a role in enzyme detoxification. Jideani and Jideani (2021) stated that, although indigenous consumers of *Vigna subterranea* have reported medicinal benefits obtained from the plant, such knowledge is at risk of being lost due to urbanization and the changing lifestyles of younger generations.

2.1.5 Economic significance

Vigna subterranea has significant economic value for both subsistence farmers and those engaged in industrial or commercial production (Ibrahim *et al*., 2018). Bambara groundnut is an important source of income and protein for smallholder producers (Alomari, Hasan, Al-Najim, & Saadi, 2024). However, its production has remained low due to the continued reliance on traditional variety and storage methods (Gerrano, Eifediyi, Labuschagne, Ogedegbe, & Hassen, 2021). Despite these limitations, indigenous crops such as, BGN are recognized for

their low input requirements, providing them an economic advantage over commercial crops such as rice (*Oryza sativa* L.), maize, wheat (*Triticum aestivum* L.), and soybean (Nhamo *et al*., 2022). Furthermore, BGN is well-suited for production in marginal soils where other legumes cannot be produced (Onuche, Ibitoye, & Anthony, 2020). Boulay, Khan, and Morrissey (2021) reported that the socioeconomic literature on the BGN is scarce, an issue that crop scientists promoting the crop's potential recognize as a limitation.

2.2 Seed coat varieties and compounds of Bambara groundnut

Bambara groundnut is a herbaceous plant with slightly oval or round, wrinkled pods, early pods have a yellow-green color, and mature pods are yellow to purple (Mathonsi *et al*., 2025). It consists of various seed coat colours, such as red, black, cream with a black eye, cream with brown eye, cream with no eye, dark brown, brown with a black eye, and speckled/ flecked/ spotted, which are influenced by secondary metabolites (Table 2.1) (Adedayo *et al*., 2021). Besides their medicinal benefits, these phytochemicals, such as condensed tannins, saponins, flavonoids, and phytic acid, possess some anti-pest factors (Chelangat *et al*., 2023). According to Hussain *et al*. (2019), phytochemicals such as saponins have a significant role in plant defense mechanisms. The presence of phytochemicals such as flavonoids, phytic acids, and tannins in Bambara groundnut has antimicrobial properties that can impede microbial growth (El Bilali *et al*.,2024). Moreover, in legume–rhizosphere systems, this antimicrobial activity contributes to host-driven microbial selection, in which sensitive or incompatible microorganisms are suppressed while tolerant, adapted rhizobia persist (Shumilina *et al*., 2023). This selective pressure may enhance symbiotic specificity by favoring nitrogen-fixing strains capable of tolerating the host's phytochemical environment (Jaiswal & Dakora, 2024). However, El Bilali *et al*. (2024) reported that the impact of these compounds is strongly concentration-dependent, with high localized concentrations immediately after seed imbibition

having inhibitory effects, particularly under in vitro conditions where dilution and microbial degradation are absent.

Chelangat *et al*. (2023) also reported that the testa colour of BGN has been linked to tannin concentration and can also be used as a phenotypic marker for drought tolerance characteristics. Dark seed coat colours are reported to germinate faster than light varieties under drought conditions because of the presence of tannins. Additionally, Puozaa, Jaiswal, and Dakora (2021) reported that black landraces have higher concentrations of flavonoids and anthocyanins, followed by red and cream landraces. According to Chinnapun and Sakorn (2022), BGN seeds have been discovered to be made up of various flavonoid glycoside compounds such as quercetin hexoside, catechin hexoside, quercitrin, quercetin 3-galactoside 7-rhamnoside, and iso-quercitrin (Table 2.1).

Table 2.1: Phytochemicals of various Bambara groundnut seed coats

Seed coat colour	Phytochemicals	Reference
Red/ Dark red	• Anthocyanins (0.033 mg/g) • Flavanol (1.41 mg CE/g) • Flavonol (10.24 mg QE/g) • Tannin (chlorogenic acid and ellagic acid)	Uba, Oselebe, Tesfaye, Agbo, & Abtew, 2022; Adedayo *et al*., 2021.
Black	• Anthocyanin (0.145 mg/g) • Flavanol (2.00 mg CE/g) • Flavonol (7.74 mg QE/g)	Adedayo *et al*., 2021.
Brown	• Anthocyanin (0.032 mg/g) • Flavanol (1.41 mg CE/g)	Adedayo *et al*., 2021; Udeh, Nyila, & Kanu, 2020.

	• Flavonoids (rutin and myricetin) (6.33 mg QE/g) • Tannins	
Brown-eye	• Anthocyanin (0.033 mg/g) • Flavanol (0.33 mg CE/g) • Flavonoids (5.68 mg QE/g) • Tannins	Adedayo *et al*., 2021.
Black-eye	• Anthocyanin (0.032 mg/g) • Flavanol (0.36 mg CE/g) • Flavonoids (rutin and myricetin) (5.73 mg QE/g) • Tannins	Adedayo *et al*., 2021.
Mixture	• Anthocyanin (0.038 mg/g) • Flavanol (1.04 mg CE/g) • Flavonoids (7.92 mg QE/g) • Tannins	Adedayo *et al*., 2021.
White and cream	• Anthocyanins (0.008 Abs. g DM^{-1}) • Flavonoids (1.02 Abs. g DM^{-1}) • Tannin (low concentration)	Puozaa *et al*., 2021.

2.3 Production of Bambara groundnut

Bambara groundnut is grown across 0.25 million hectares worldwide, with Sub-Saharan Africa (SSA) being the main producer of the crop. Smaller quantities are also produced in Australia, the United States, and South Asia (El Bilali *et al*., 2024). In Africa, BGN production is reported to be approximately 0.3 million tons annually, with an average yield of 0.85 t/ha, although the

yield potential is reportedly over 3 t/ha (Tan *et al*., 2020). Mayes *et al*. (2019) reported that the BGN yield of 0.85 t/ha was reported to be equivalent to other legumes, even though the yield varies with location and landrace. According to Hillocks, Bennett, and Mponda (2012) Nigeria, Burkina Faso, and Niger were the largest producers of BGN in Africa, with a mean production of 0.1 million tons, 44 712 tons, and 30 000 tons, respectively. Recently, Cameroon, Burkina Faso, and Niger have been the largest producers of BGN, producing 74% of the world's production (Olanrewaju, Oyatomi, Babalola, & Abberton, 2022).

Based on recent estimates, the area and production under BGN have shown growth from 1999 to 2022, while productivity has remained stagnant or even declined during the same period (Saini *et al*., 2024). Saini *et al*. (2024) also reported that the global production of BGN in 2022 was 0.25 million tons, produced on 0.40 million hectares, resulting in a yield of 0.61 t/ha. According to Esan *et al*. (2023), all the pre-field, field, and post-field activities of BGN are frequently performed by women and are also being produced at a subsistence level to meet the family's immediate needs, which has led to the crop being regarded as a "women's crop" and underutilized.

2.4 Factors affecting Bambara groundnut production

2.4.1 Biotic factors

Insect pest: *Vigna subterranea* is less affected by field insect pests compared to other leguminous crops, such as cowpea (Abdullahi, Shuâ, Musa, Gambo, & Adamu, 2021). Moreover, cowpea weevil bruchids (*Callosobruchus maculatus* Boh) are the most destructive insect pest of Bambara groundnut, have been reported as the primary insect pest in postharvest, resulting in a 99% yield loss during grain storage (Figure 2.1) (Majola, Gerrano & Shimelis, 2021). Studies have also reported BGN field loss caused by leafhoppers (*Empoasca facialis*

and *Hilda patuelis*), whereas aphids have led to 65% of yield loss during high rainfall conditions (Majola *et al*., 2021). According to Tlankka, Mbega, and Ndakidemi (2020), aphids infest different growth stages of the plant, forming colonies in pods, leaves, and stems (Figure 2.2). Heavily infested plants may wilt and become yellow or die due to the removal of plant sap.

Figure 2.1: Weevil bruchids damage on Bambara groundnut seeds (Goudoum & Tnkeu, 2023).

In addition, Groundnut jassid/leafhopper (*Empoasca kerri* Pruthi), termites (Isoptera), and bruchid beetle (*Bruchus rufimanus* Boh.) are other insect pests of BGN. Termites attack the crop's pods together with the bruchid beetle that damages undeveloped pods (Olanrewaju *et al*., 2022). Brown leaf beetles (*Ootheca bennigseni* Sahlberg. and *Ootheca mutabilis* Weise.) are foliage beetles that make holes in leaves, feed on seedlings and root tissue, mainly feeding on flowers during the seedling stage, causing crop loss for BGN, cowpea, and common beans (Tlankka *et al*., 2020). Moreover, Tlankka *et al*. (2020) also reported that the magnitude of these foliage beetles on Bambara groundnut in Tanzania resulted in an 18 to 31% yield loss in common beans.

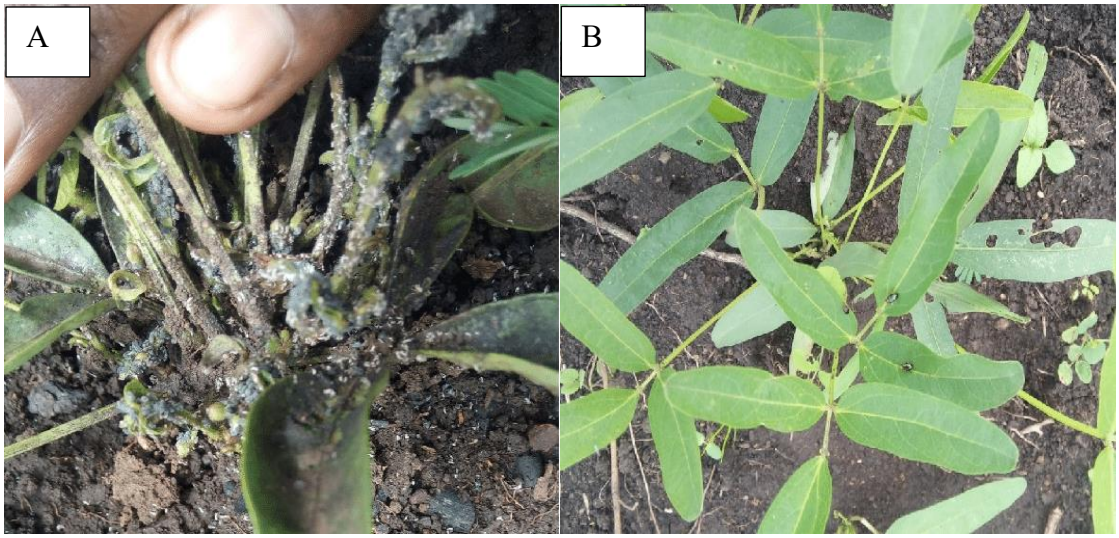

Figure 2.2: Aphids (A) and beetles (B) on Bambara groundnut plant (Tlankka *et al*., 2020).

Nematodes and Red Spider mites: Root-knot nematodes (*Meloidogyne javanica* and *M. incognita*) are plant-parasitic nematodes that attack the roots of BGN in the soil (Valombola, 2020). Furthermore, symptoms of nematode infection include reduced root system, chlorosis, extreme root galling, stunting, reduced pod yield, and plant density injury. Of the two nematode species, *M. javanica* is reported to cause the most severe damage, especially in sandy soils (Ayeni, Claudius-Cole, Abberton, & Coyne, 2022).

Furthermore, spider mites (*Tetranychus urticae* Koch.) are pests that feed on the undersides of leaves by extracting plant sap, resulting in the development of brown spots and webs on the leaves (Figure 2.3) (Ghosh & Hasan, 2021). According to Tlankka *et al*. (2020), spider mites affect various crops, such as common beans, where high infestations are influenced by factors like high temperatures, long sunshine hours, low relative humidity, and drought in plants, resulting in the drying of leaves, which can cause the entire plant to wilt.

Figure 2.3: Red spider mites on Bambara groundnut (Tlankka *et al*., 2020).

Diseases: Fungal diseases affect BGN seed germination, physiological processes, crop development, and seed quality, in some cases resulting in the production of mycotoxins that are harmful to livestock and humans (Maphosa *et al*., 2022). Fungal diseases reported on BGN includes fusarium wilt (*Fusarium oxypolygoni* (Fop), powdery mildew (*Erysiphe polyoni* DC.), and leaf spot (*Cercospora* spp.) (Tlankka *et al*., 2020). According to Nafula, Masinde, Otaye, and Wafula (2021), fusarium wilt attacks all stages of BGN development. *Cercospora* leaf spots appear as reddish-brown, round spots on the leaves and wounds on the pods, petioles, and stems, with severe attacks causing the plant's leaves to fall, and the plant can die (Hlanga, 2021). Hlanga (2021) further reported that fusarium wilt causes vascular discoloration, necrosis, yellowing, and wilting, whereby BGN plants affected by the disease become short and die.

2.4.2 Abiotic factors

Drought: Bambara groundnut has been reported to be drought tolerant, a defense mechanism, and the ability to utilize water in low quantities, which are key traits that allow the crop to survive in harsh water stress environments (Feldman, Ho, Massawe, & Mayes, 2019;

Mabhaudhi, Chibarabada, Chimonyo, & Modi, 2018). However, Agyeman *et al*. (2023) and Majola *et al*. (2021) reported that drought stress on BGN causes lower grain yield and reduced dry pod matter if it occurs during the flowering period of the legume. Olanrewaju *et al.* (2022) stated that BGN productivity could be hindered by limited rainfall, despite being regarded as a drought-resistant crop. According to Kunene, Gerrano, and Odindo (2025), insufficient water still has significant effects on BGN growth and yield, with different varieties responding differently. Moreover, drought can decrease nutrient uptake and tolerance of the legume crop to pests and diseases (Olanrewaju *et al.*, 2022). Additionally, limited studies have indicated that, although field drought conditions reduce seed yield in BGN, they have no effect on the nutritional quality of the seed (Tan *et al*., 2020).

Extreme temperatures: Bambara groundnut is a frost-sensitive plant, where warm temperatures enhance plant growth, with an optimum temperature range of 30 to 35 °C for germination and an average daily temperature of 27 °C (Maphosa *et al*., 2022; Gerrano *et al*., 2021). However, conditions above 30 °C result in heat stress during seed germination, while extreme rainfall leads to drastic yield reduction, especially during BGN harvest time (Majola *et al*., 2021). Khan *et al*. (2021) reported that high temperatures cause leaves to die and decrease the crop's yield biomass.

Soil nutrient limitations: Bambara groundnut is often cultivated on nutrient-depleted soils in sub-Saharan Africa, where poor soil fertility can constrain growth, nodulation, and yield (Khan *et al*., 2021). Among macronutrients, phosphorus (P) is the most critical limiting factor, as it is essential for ATP production, nodule formation, and the energy-demanding process of biological nitrogen fixation (BNF) (Sokoine University of Agriculture, 2025; Jaiswal & Dakora, 2024). Furthermore, Phosphorus deficiency reduces nodule number and mass, limits nitrogenase activity, and delays flowering, ultimately reducing grain yield. While BGN can fix atmospheric nitrogen, soils are often low in total nitrogen, which can limit early plant growth

and the effectiveness of rhizobial symbiosis before BNF establishment (Springer Nature, 2026). Other nutrients such as potassium (K), calcium (Ca), sulphur (S), iron (Fe), zinc (Zn), boron (B), and molybdenum (Mo) also influence BGN productivity, nodulation, and nodule function. Molybdenum and iron are particularly significant for nitrogenase function and leghemoglobin synthesis, in which the presence of leghaemoglobin in nodules results in a strong pinkish colour, which is an indication of active nitrogen fixation (Zeng *et al*., 2022; Mweetwa *et al*., 2016). According to Saleem *et al*. (2023), K, Ca, and S contribute to enzyme activation, root hair formation, and amino acid synthesis, respectively, while Zinc and boron, although secondary, affect reproductive development and nodule initiation. Baseline soil analyses from several BGN production regions consistently show deficiencies in P, N, K, S, Mg, and Zn, indicating multiple nutrient limitations in smallholder cropping systems (Sokoine University of Agriculture, 2025). Consequently, the management of these nutrients is essential for optimizing growth, symbiotic nitrogen fixation, and yield in Bambara groundnut.

2.4.3 Socioeconomic factors: Bambara groundnut is a minor legume grown by female subsistence farmers in sub-Saharan Africa with limited research support on agronomic management methods, breeding, and seed systems (Suhairi, Jahanshiri, & Nizar, 2018; Cook, 2017). Moreover, BGN farmers have limited access to funding for expanding the production of the leguminous crop through the utilization of developed seed varieties, crop inputs (fertilizers, crop protection resources, irrigation systems, and effective harvesting systems), and post-harvest storage facilities (Majola *et al*., 2021). Majola *et al*. (2021) further reported that small-scale farmers also lack access to regional markets for the economic grains produced from legume crop production. According to Khan, Rafii, Ramlee, Jusoh, and Al-Mamun (2024), *Vigna subterranea* has not received sufficient financial support from non-governmental and governmental organizations for its advancement and has gained limited attention from the

international research community altogether, whereas other important leguminous crops, such as groundnuts and soybeans, have received support.

2.5 Biological nitrogen fixation

2.5.1 Nitrogen-fixing bacteria's contribution to agriculture

Biological nitrogen fixation enables legumes to enhance soil quality and reduce the use of synthetic nitrogen fertilizers in subsequent crops (Goswami *et al*., 2022). It has been estimated that leguminous crops can fulfill 80 to 90% of their nitrogen requirements during one crop season from biological nitrogen fixation and transfer 0 to 70% of the biological fixed nitrogen to successive crops (Goswami *et al*., 2022). According to Bashandy, Abd-Alla, and Bagy (2019), biological nitrogen fixation is the primary source of nitrogen in terrestrial environments. Symbiotic nitrogen fixation produces a total of 100 million metric tons of nitrogen for terrestrial ecosystems (Dash & Deole, 2019). Ayangbenro, Adem, and Babalola (2023) reported that a beneficial phytomicrobiome confers benefits to the plant, such as water retention, production of growth-stimulating hormones, and pathogen resistance.

2.5.2 Factors affecting the performance of nitrogen-fixing bacteria

The key factors that significantly limit symbiotic nitrogen fixation in legume-rhizobia interactions are changes in temperature, soil pH, and soil salinity (Seidu, Githiri, Wesonga, & Ngumi, 2025).

Soil pH: An acidic pH reduces the persistence of *Rhizobium* strains in the soil, nodulation, and nitrogen fixation of legume crops (Torabian, Farhangi-Abriz & Denton, 2019). According to Ferreira *et al*. (2016), fluctuations in pH affect the growth, metabolic activity, and survival of nitrogen-fixing bacteria. Ferreira *et al*. (2016) also reported that nitrogen fixation can be enhanced under acidic conditions but decreased significantly when the pH shifts to neutral

(7.0). Torabian *et al*. (2019) reported that rhizobial species can survive well under pH values ranging from 4.5 to 5.0 and optimize nitrogen fixation. Sellars and Nunes (2021) reported that salinity and pH tolerance vary within rhizobial nitrogen-fixing bacteria. Currently, there are no studies that link the interaction of pH with Bambara groundnut growth and development.

Soil salinity: Excess salts reduce soil water potential and induce ionic stress by accumulating Na^+ and Cl^- ions, leading to impaired nutrient uptake and restricted root development (Shahid *et al.*, 202). Salinity in soils with a greater pH value prevents legume crops, such as cowpea and lentils, from thriving (Etesami & Adl, 2020). It also affects nitrogen-fixing bacteria by inhibiting their growth, metabolic activity, and symbiotic (Igiehon & Babalola, 2018). Moreover, high soil salinity causes nitrogen-fixing bacteria to form cells that inhibit the growth and development of their host (Torabian *et al*., 2019). Furthermore, salinity disrupts the early stages of symbiosis by inhibiting root hair elongation and curling, processes that are essential for rhizobial attachment and infection thread formation (Kirova & Kocheva, 2021). Consequently, it reduces rhizobia activity in legumes, resulting in low nitrogen yields. However, some *Rhizobium* strains can resist salty soils and fix atmospheric nitrogen (Etesami and Adl, 2020). Kirova and Kocheva (2021) also reported that some bacterial strains are inhibited at electrical conductivity levels corresponding to 100 mM NaCl, whereas others can survive in saline soils. Strains such as *Sinorhizobium meliloti* can tolerate higher salinity levels, maintaining growth at 300-700 mM NaCl. Just like with pH, there are limited reports linking salinity with the interaction of rhizobia and Bambara groundnut

Climate change: Climate change is associated with various conditions, such as drought. It is reported that *Rhizobium* species are able to thrive in dry environments, but their diversity is significantly lower under these conditions (Igiehon & Babalola, 2018). In addition, McCulloch,

Piotto, and Porder (2021) reported that an increase in drought severity and frequency reduces symbiotic nitrogen fixation activity.

Temperature: According to Rousk, Sorensen, and Michelsen (2018), extreme temperatures can cause the reduction of available moisture for the nitrogen fixing bacteria and result in reduced nitrogen fixation rates. This leads to low nitrogen input through nitrogen fixation pathways (Plunkett, Knutson & Barney, 2020). Plunkett *et al*. (2020) further reported that biological nitrogen fixation levels increase at 24 °C and 26 °C, resulting in an increased threshold of ammonium yields, but are reduced when temperatures are above 30 °C.

2.6 Bambara groundnut production in South Africa

2.6.1 Constraints in Bambara groundnut production in SA

Bambara groundnut in South Africa is primarily produced by small-scale farmers who plant locally adapted retained seed varieties with low yield potential, highlighting the need to develop modern and improved landraces to increase crop production and enter the market value chain (Hlanga, Modi, & Mathew, 2022). Kunene *et al*. (2024) reported that there has never been an understandable breeding program in South Africa for BGN. Furthermore, BGN is not commercially produced in the country, with a plot size that ranges from 300 to 2500 m² per farmer (DAFF, 2016). This is mostly due to the fact that *V. subterranea* was previously neglected by the national research institutes, resulting in limited knowledge about the optimum agronomic practices for the crop (DAFF, 2016). According to Nkambule, Workneh, Kassim, Sibanda, and Lagerwall (2022), Bambara groundnut is a labour-intensive crop, from land preparation to crop establishment, crop protection, and harvesting, due to the lack of appropriate equipment. Shelling process in South Africa is not mechanized but carried out manually, involving the use of stones and sticks to break the kernels.

2.6.2 Opportunities in Bambara groundnut production in SA

In South Africa, BGN is primarily produced in rural and tropical areas of Mpumalanga, Limpopo, KwaZulu-Natal, Northwest, and Gauteng provinces (Baloyi & Swanepoel, 2023). In Limpopo province, the crop is cultivated in Capricorn, Waterberg, Vhembe, and Mopani districts (Cook, 2017). The crop is broadly grown in the Ehlanzeni district of Mpumalanga and produced in Nkandla, Makhati, Greytown, Msinga, Kosibaai, and Nguthuthu areas in KwaZulu-Natal province (DAFF, 2016). Ilembe and Umzinyathi are districts where the crop is grown on a minor scale (DAFF, 2016). According to Nelwamondo (2020), BGN is recognized by different names in South Africa, Ditloo in Sepedi, Izindlubu in isiZulu, Indlubu in Isidebele, Jugo in isiXhosa, Phonda in Venda, and Jugoboon in Afrikaans. Minnaar-Ontong, Gerrano, and Labuschagne (2021) reported that BGN is used as food and for enhancing soil fertility due to its ability to fix nitrogen in the soil. Additionally, the legume is grown solely or intercropped with melons, maize, and cowpea. Bambara groundnut is regarded as the third most important crop after maize and groundnut in the middle and lowveld areas of Mpumalanga (Cook, 2017). Nkambule, Workneh, Sibanda, Alaika, and Lagerwall (2023) reported that BGN is mainly produced for selling to the local market and for household consumption by subsistence farmers.

2.7 References

Abdullahi, N., Shuâ, A.B., Musa, I., Gambo, B.Z. and Adamu, Z.A. 2021. Effect of Cowpea seed beetle (*Callosobruchus maculatus*) infestation on selected landraces of Bambara groundnut during storage. *Bioremediation Science and Technology Research*, 9(2): 31-34.

Adedayo, B.C., Anyasi, T.A., Taylor, M.J., Rautenbauch, F., Le Roes-Hill, M. and Jideani, V.A. 2021. Phytochemical composition and antioxidant properties of methanolic extracts

of whole and dehulled Bambara groundnut (*Vigna subterranea*) seeds. *Scientific Reports,* 11(1): 1-11.

Agajie, M. 2021. Bioprospecting potential of Bambara groundnut (*Vigna subterranea* [L.] Verdc.) for access and benefit sharing. *South Asian Research Journal Agriculture and Fisheries*, 3(6): 88-92.

Agyeman, K., Frimpong, F., Amankwaa-Yeboah, P., Osei-Bonsu, I., Yeboah, S., Keteku, A., Marno, P., Brempong, M.B., Danquah, E.O., Adjei, A.E. and Quaye, M.O. 2023. Physiological resilience of Bambara groundnut (*Vigna subterranea* L. Verdc) genotypes to intermittent periods of drought stress at different growth stages. *Agricultural Sciences*, 14(11): 573-1592.

Ajilogba, C.F., Olanrewaju, O.S. and Babalola, O.O. 2022. Improving Bambara groundnut production: Insight into the role of omics and beneficial bacteria. *Frontiers in Plant Science*, 13: 1-17.

Alomari, S.M., Hasan, R.S., Al-Najim, A.N. and Saadi, A.M. 2024. Nitrogen-fixing bacteria and their applications in the environment: A review. *International Journal of Medical and All Body Health Research*, 4(5): 178-187.

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): 1-8.

Ayeni, B.P., Claudius-Cole, A.O., Abberton, M. and Coyne, D. 2022. Host status of Bambara groundnut accessions to *Meloidogyne incognita* using conventional screenhouse and novel seedling pouch methods. *Nematology*, 24(5): 543-558.

Baloyi, N.H. and Swanepoel, P.A. 2023. Variability assessment of Bambara groundnut (*Vigna subterranea* (L) Verdc) landraces based on the qualitative trait seed coat colour. *South African Journal of Botany*, 163: 165-171.

Bashandy, S.R., Abd-Alla, M.H. and Bagy, M.M.K. 2019. Biological nitrogen fixation and biofertilizers as ideal potential solutions for sustainable agriculture. In: Islam, S.U. ed. *Integrating green chemistry and sustainable engineering*. Hoboken: John Wiley & Sons, 343-396.

Boulay, B., Khan, R. and Morrissey, O. 2021. Under-utilised crops and rural livelihoods: Bambara groundnut in Tanzania. *Oxford Development Studies*, 49(1): 88-103.

Chelangat, M., Muturi, P., Gichimu, B., Gitari, J. and Mukono, S. 2023. Nutritional and phytochemical composition of Bambara groundnut (*Vigna subterranea* [L.] Verdc) landraces in Kenya. *International Journal of Agronomy*, 2023(1): 1-11.

Chinnapun, D. and Sakorn, N. 2022. Structural characterization and antioxidant and anti-inflammatory activities of new chemical constituent from the seeds of Bambara groundnut (*Vigna subterranea* (L.) Verdc.). *CyTA-Journal of Food*, 20(1): .93-101.

Cook, D. 2017. *Small scale farmer utilization and perception of Bambara group in South Africa: A case study in a semi-arid region of Limpopo*. Cape Town: University of Cape Town.

Dash, D. and Deole, S. 2019. Review on the role of biological nitrogen fixation in the environmental Terms. *International Journal of Current Microbiology and Applied Sciences*, 8(8): 2660-2665.

Department of Agriculture, Forestry, and Forestry and Fisheries (DAFF). 2016. Production guideline for Bambara groundnuts (*Vigna subterranean*). Pretoria: National Department of Agriculture and Agricultural Research Council Grain Crops Institute. [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.

El Bilali, H., Kiebre, Z., Nanema, R.K., Dan Guimbo, I., Rokka, V.M., Gonnella, M., Tietiambou, S.R.F., Dambo, L., Nanema, J., Grazioli, F. and Acasto, F. 2024. Mapping research on Bambara groundnut (*Vigna subterranea* (L.) Verdc.) in Africa: Bibliometric, geographical, and topical perspectives. *Agriculture*, 14(9): 1-33.

Esan, V.I., Oke, G.O. and Ogunbode, T.O. 2023. Genetic variation and characterization of Bambara groundnut [*Vigna subterranea* (L.) Verdc.] accessions under multi-environments considering yield and yield components performance. *Scientific Reports*, 13(1): 1-19.

Etesami, H. and Adl, S. 2020. Can interaction between silicon and non–rhizobial bacteria help in improving nodulation and nitrogen fixation in salinity–stressed legumes? A review. *Rhizosphere*, 15(1): 1-20.

Feldman, A., Ho, W.K., Massawe, F. and Mayes, S. 2019. Bambara groundnut is a climate-resilient crop: How could a drought-tolerant and nutritious legume improve community resilience in the face of climate change? In: Sarkar, A., Sensarma, S. and Vanloon, G. eds. *Sustainable solutions for food security: Combating climate change by adaptation*. Cham: Springer, 51-167.

Ferreira, T.C., Aguilar, J.V., Souza, L.A., Justino, G.C., Aguiar, L.F. and Camargos, L.S. 2016. pH effects on nodulation and biological nitrogen fixation in *Calopogonium mucunoides*. *Brazilian Journal of Botany*, 39: 1015-1020.

Gerrano, A.S., Eifediyi, E.K., Labuschagne, M., Ogedegbe, F.O. and Hassen, A.I. 2021. Production practices of Bambara groundnut. In: Oyeyinka, S.S. and Ade-Omowaye, B.I. eds. *Food and potential industrial applications of Bambara groundnut*. Cham: Springer, 7-25.

Ghosh, S.K. and Hasan, W. 2021. Red spider mite (*Tetranychus urticae* Koch) infestation on brinjal/eggplant (*Solanum melongena* L.) and environmental sustainability. In: Hasan,

W., Rohman, M.D., Mehjabeen. and Panja, P. eds. *Hi-Tech Crop Production and Pest Management*. New Delhi: Biotech Books, 299-312.

Goswami, R., Ray, K., Ghosh, S., Dutta, S., Mandal, S., Nayak, J., Chakraborty, S. and Banerjee, H. 2022. Multiple pathways of legume-based systems towards environmental, social, and economic sustainability in smallholder systems. In: Meena, R.S. and Kumar, S. eds. *Advances in legumes for Sustainable intensification*. Cambridge: Academic Press, 657-678.

Goudoum, A. and Tinkeu, L.S.N. 2023. Susceptibility of three varieties of Bambara groundnut (*Vigna subterranea* L.) towards *Callosobruchus maculatus* according to their nutritional and antinutrient contents. *Moroccan Journal of Agricultural Sciences*, 4(1): 28-34.

Harris, T., Jideani, V. and Le Roes-Hill, M. 2018. Flavonoids and tannin composition of Bambara groundnut (*Vigna subterranea*) of Mpumalanga, South Africa. *Heliyon*, 4(9): 1-12.

Hillocks, R.J., Bennett, C. and Mponda, O.M. 2012. Bambara nut: A review of utilisation, market potential and crop improvement. *African Crop Science Journal*, 20(1): 1-16.

Hlanga, N., Modi, A.T. and Mathew, I. 2022. Assessment of genetic diversity among Bambara groundnut lines using SSR markers. *Research Square*, 1-12.

Hlanga, N.C. 2021. *Agro-morphological, nutritional and genetic diversity analyses of bambara groundnut (Vigna subterranea (L.) Verdc)*. Pietermaritzburg: University of KwaZulu-Natal.

Hussain, M., Debnath, B., Qasim, M., Bamisile, B.S., Islam, W., Hameed, M.S., Wang, L. and Qiu, D. 2019. Role of saponins in plant defense against specialist herbivores. *Molecules*, 24(11): 1-22.

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): 1-17.

Ibrahim, A.R., Dansi, A., Salifou, M., Ousmane, A., Alzouma, A. and Alou, W. 2018. Farmers' practices, utilization, conservation and marketing of Bambara groundnut (*Vigna subterranea* (L.) Verdc.) in Dosso Region, Western Niger. *Genetic Resources and Crop Evolution*, 65(7): 1907-1914.

Igiehon, N. and Babalola, O. 2018. Rhizosphere microbiome modulators: Contributions of nitrogen fixing bacteria towards sustainable agriculture. *International Journal of Environmental Research and Public Health*, 15(4): 1-25.

Jaiswal, S.K. and Dakora, F.D. 2024. Seed-coat pigmentation plays a crucial role in partner selection and N2 fixation in legume-root–microbe associations in African soils. *Plants*, *13*(11): 1-12.

Jideani, V.A. and Jideani, A.I. 2021. Ethnonutritional and ethnomedicinal uses of Bambara Groundnut. In: Jideani, V.A. and Jideani, A.I. eds. *Bambara groundnut: Utilization and future prospects*. Cham: Springer, 49-60.

Khan, M.M.H., Rafii, M.Y., Ramlee, S.I., Jusoh, M. and Al-Mamun, M. 2024. Bambara groundnut (*Vigna subterranea* L.): A climate-resilient crop to address food and nutritional security. *Journal of Underutilised Crops Research*, 3(1): 1-12.

Khan, M.M.H., Rafii, M.Y., Ramlee, S.I., Jusoh, M. and Al-Mamun, M. 2021. Bambara groundnut (*Vigna subterranea* L. Verdc): A crop for the new millennium, its genetic diversity, and improvements to mitigate future food and nutritional challenges. *Sustainability*, 13(10): 1-27.

Kunene, S., Gerrano, A.S. and Odindo, A.O. 2024. Stability and performance of Bambara groundnut (*Vigna subterranea* (L.) Verdc.) genotypes in different South African environments. *South African Journal of Plant and Soil*, 41: 1-12.

Kunene, S., Gerrano, A.S. and Odindo, A.O. 2025. Drought tolerance assessment of Bambara groundnut (*Vigna subterranea* L. Verdc.) accessions based on morphological and physiological traits. *South African Journal of Botany*, 184: 880-894.

Mabhaudhi, T., Chibarabada, T.P., Chimonyo, V.G.P. and Modi, A.T. 2018. Modelling climate change impact: A case of Bambara groundnut (*Vigna subterranea*). *Physics and Chemistry of the Earth*, 105: 25-31.

Majola, N.G., Gerrano, A.S. and Shimelis, H. 2021. Bambara groundnut (*Vigna subterranea* [L.] Verdc.) production, utilisation and genetic improvement in Sub-Saharan Africa. *Agronomy,* 11(7): 1-16.

Maphosa, Y., Jideani, V.A. and Maphosa, L. 2022. Bambara groundnut production, grain composition and nutritional value: Opportunities for improvements. *The Journal of Agricultural Science*, 160(6): 448-458.

Mathonsi, L., Nkosi, S., Gebashe, F., Gupta, S., Kulkarni, M. and Van Staden, J. 2025. Effect of smoke-water (SW) and smoke bioactive compound karrikinolide (KAR1) on seed germination, plumule and radicle growth of *Vigna subterranea* (L.) Verdc. (Bambara groundnut)–An underutilized African legume crop. *South African Journal of Botany*, 185: 516-525.

Mayes, S., Ho, W.K., Chai, H.H., Gao, X., Kundy, A.C., Mateva, K.I., Zahrulakmal, M., Hahiree, M.K.I.M., Kendabie, P., Licea, L.C. and Massawe, F. 2019. Bambara groundnut: An exemplar underutilised legume for resilience under climate change. *Planta*, 250: 803-820.

Mbuma, N.W., Labuschagne, M., Siwale, J. and Hugo, A. 2022. Diversity in seed protein content, selected minerals, oil content and fatty acid composition of the Southern African Bambara groundnut germplasm collection. *Journal of Food Composition and Analysis*, 109: 1-11.

McCulloch, L.A., Piotto, D. and Porder, S. 2021. Drought and soil nutrients effects on symbiotic nitrogen fixation in seedlings from eight Neotropical legume species. *Biotropica*, 53(2): 703-713.

Minnaar-Ontong, A., Gerrano, A.S. and Labuschagne, M.T. 2021. Assessment of genetic diversity and structure of Bambara groundnut [*Vigna subterranea* (L.) verdc.] landraces in South Africa. *Scientific Reports*, 11(1): 1-9.

Mohammed, M.S., Shimelis, H.A., Laing, M.D. and Usman, A. 2023. Progress and opportunities on Bambara groundnut (*Vigna subterranea* [L.] Verdc.): genetic improvement and product development. In: Farooq, M. and Siddique, K.H. eds. *Neglected and underutilized crops: Future smart food*. Amsterdam: Elsevier, 617-645.

Muhammad, I., Rafii, M., Ramlee, S., Nazli, M., Harun, A., Oladosu, Y., Musa, I., Arolu, F., Chukwu, S., Sani Haliru, B., Silas Akos, I., Halidu, J. and Arolu. 2020. Exploration of Bambara groundnut (*Vigna subterranea* (L.) Verdc.), an underutilized crop, to aid global food security: Varietal improvement, genetic diversity and processing. *Agronomy*, 10(6): 1-20.

Nafula, W.C., Masinde, I.T., Otaye, D.O. and Wafula, W.V. 2021. Incidence and severity of Fusarium wilt on Bambara nut (*Vigna subterranea* L.) landraces in Western Kenya. *African Journal of Biological Sciences*, 3(1): 67-75.

Nelwamondo, A. 2020. Assessment of co-inoculation of *Bradyrhizobium japonicum* and *Bacillus subtitles* on yield and metabolic profile of Bambara groundnut and cowpea under glasshouse conditions. *Institutional Repository*, 1(1): 1-80.

Nhamo, L., Paterson, G., van der Walt, M., Moeletsi, M., Modi, A., Kunz, R., Chimonyo, V., Masupha, T., Mpandeli, S., Liphadzi, S. and Molwantwa, J. 2022. Optimal production areas of underutilized indigenous crops and their role under climate change: Focus on Bambara groundnut. *Frontiers in Sustainable Food Systems*, 6: 1-13.

Nkambule, S., Workneh, T., Kassim, A., Sibanda, S. and Lagerwall, G. 2022. Development and testing of a vertical-spikes shelling machine for Bambara groundnuts. *African Journal of Agricultural Research*, 18(12): 1013-1022.

Nkambule, S., Workneh, T., Sibanda, S., Alaika, K. and Lagerwall, G. 2023. The effect of moisture contents on the physical properties of both Bambara groundnut seeds and pods in South Africa. *African Journal of Food, Agriculture, Nutrition and Development*, 23(6): 23817-23834.

Ogban, E.I., Oparaeke, A.M., Odey, P.O. and Udeme U.O. 2021. Insecticidal evaluation of some tropical leaf powders for the protection of stored Bambara groundnut against *Callosobruchus subinnotatus* (Pic) (*Coleoptera: Chrysomelidae*). *Journal of Entomology and Zoology Studies*, 9(6): 197-202.

Okafor, J.N., Jideani, V.A., Meyer, M. and Le Roes-Hill, M. 2022. Bioactive components in Bambara groundnut (*Vigna subterraenea* (L.) Verdc) as a potential source of nutraceutical ingredients. *Heliyon*, 8(3): 1-11.

Olanrewaju, O.S., Oyatomi, O., Babalola, O.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.

Onuche, U., Ibitoye, S.J. and Anthony, T. 2020. Profitability and efficiency of Bambara groundnut production in Nigeria: A case study. *Review of Agricultural and Applied Economics*, 23(2): 92-101.

Plunkett, M., Knutson, C. and Barney, B. 2020. Key factors affecting ammonium production by an *Azotobacter vinelandii* strain deregulated for biological nitrogen fixation. *Microbial Cell Factories*, 19(1): 1-12.

Puozaa, D.K., Jaiswal, S.K. and Dakora, F.D. 2021. Black seedcoat pigmentation is a marker for enhanced nodulation and N2 fixation in Bambara groundnut (*Vigna subterranea* L. Verdc.) landraces. *Frontiers in Agronomy*, 3:1-15.

Ramatsetse, K.E., Ramashia, S.E. and Mashau, M.E. 2023. A review on health benefits, antimicrobial and antioxidant properties of Bambara groundnut (*Vigna subterranean*). *International Journal of Food Properties*, 26(1): 91-107.

Rousk, K., Sorensen, P.L. and Michelsen, A. 2018. What drives biological nitrogen fixation in high arctic tundra: Moisture or temperature? *Ecosphere*, 9(2): 1-12.

Saini, H.K., Sharma, A., Vaidya, D., Kaushal, M., Anand, A., Verma, R. and Sharma, A. 2024. Bambara groundnut (*Vigna subterranean* L.): An underutilized source of plant protein. *South African Journal of Botany*, 174: 495-509.

Saleem, S., Mushtaq, N.U., Rasool, A., Shah, W.H., Tahir, I. and Rehman, R.U. 2023. Plant nutrition and soil fertility: physiological and molecular avenues for crop improvement. In: Aftab, T. and Hakeem, K.R. eds. *Sustainable plant nutrition: Molecular interventions and advancements for crop improvement*. Academic Press, 23-49.

Sarkar, A., Fwanyanga, F.M., Horn, L.N., Welzel, S., Diederichs, M., Kerk, L.J., Zimmermann, M. and Reinhold-Hurek, B. 2023. Towards inoculant development for Bambara groundnut (*Vigna subterranean* (L.) Verdc) pulse crop production in Namibia. *Frontiers in Plant Science*, 14: 1-16.

Seidu, O.A., Githiri, S.M., Wesonga, J.M. and Ngumi, V.W. 2025. Isolation, screening and in-vitro characterization of plant growth-promoting *Bradyrhizobium* isolates from the

nodules of Bambara groundnut (*Vigna subterranean*) for potential use as bioinoculants. *Frontiers in Sustainable Food Systems*, 8: 1-14.

Sellars, S. and Nunes, V. 2021. Synthetic nitrogen fertilizer in the U.S. *Farmdoc Daily*, 11(24): 1-5.

Shahid, M.A., Sarkhosh, A., Khan, N., Balal, R.M., Ali, S., Rossi, L., Gómez, C., Mattson, N., Nasim, W. and Garcia-Sanchez, F. 2020. Insights into the physiological and biochemical impacts of salt stress on plant growth and development. *Agronomy*, 10(7): 1-34.

Shumilina, J., Soboleva, A., Abakumov, E., Shtark, O.Y., Zhukov, V.A. and Frolov, A. 2023. Signaling in legume–Rhizobia symbiosis. *International journal of molecular sciences*, 24(24): 1-25.

Sokoine University of Agriculture. 2025. Optimizing P and K for Improving Biological Nitrogen Fixation and Productivity of Bambara Groundnut (*Vigna subterranea)* in South-Eastern Tanzania, Sokoine University of Agriculture, July 2025. Available at: https://agris.fao.org/search/en/providers/125429/records/6851871253e52c13fc771c05 (Accessed: 1 March 2026).

Soumare, A., Diedhiou, A. and Kane, A. 2022. Bambara groundnut: A neglected and underutilized climate-resilient crop with great potential to alleviate food insecurity in sub-Saharan Africa. *Journal of Crop Improvement*, 36(5): 747-767.

Suhairi, T., Jahanshiri, E. and Nizar, N. 2018. Multicriteria land suitability assessment for growing underutilised crop, Bambara groundnut in Peninsular Malaysia. IOP Conference Series: *Earth and Environmental Science*, 169(1): 1-10.

Tan, X.L., Azam-Ali, S., Goh, E.V., Mustafa, M., Chai, H.H., Ho, W.K., 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: 1-6.

Tlankka, N., Mbega, E. and Ndakidemi, P. 2020. Potential of indigenous pesticidal plants in the control of field and post-harvest arthropod pests in Bambara groundnuts (*Vigna subterranea* (L.) Verdc.) in Africa: A review. *American Journal of Plant Science*, 11: 745-772.

Torabian, S., Farhangi-Abriz, S. and Denton, M. 2019. Do tillage systems influence nitrogen fixation in legumes? A review. *Soil and Tillage Research*, 185(1): 1-9.

Uba, C.U., Oselebe, H.O., Tesfaye, A.A., Agbo, C.U. and Abtew, W.G. 2022. Comparative analysis of variation in African Bambara groundnut [*Vigna subterranea* (L) Verdc.] landraces assessed through seed traits. *South African Journal of Botany*, 150: 48-57.

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.

Uro-Chukwu, H.C., Ezekwe, A.S., Okorie, U., Eleazu, C.O., Uro-Chukwu, F.C.U., Ajah, D.O.J., Nwokpoku, B.I. and Okari, K.E.N.P. 2024. Hypoglycemic and antioxidant effects of high concentration of Bambara groundnut (*Vigna Subterranean*) flour fed on Streptozotocin-induced diabetic rats. *International Journal of Basic and Clinical Toxicology*, 3: 10-20.

Valombola, J.S. 2020. *Assessment of farmer preferences, constraints and angro-morphological variation among Bambara groundnut (Vigna subterranea (L.) Verdc.) accessions as a basis for seed yield selection index*. Windhoek: University of Namibia.

Zeng, Q., Ding, X., Wang, J., Han, X., Iqbal, H.M. and Bilal, M. 2022. Insight into soil nitrogen and phosphorus availability and agricultural sustainability by plant growth-promoting rhizobacteria. *Environmental Science and Pollution Research*, 29(30): 45089-45106.

CHAPTER THREE

INHIBITORY POTENTIAL OF BAMBARA GROUNDNUT SEEDCOAT EXTRACTS ON INDIGENOUS NITROGEN FIXING BACTERIAL ISOLATES

3.1 Introduction

Bambara groundnut (*Vigna subterranea*) is known for its ability to tolerate drought and low soil fertility, producing high yields in low-input agricultural systems due to its capacity to form effective root nodules with compatible soil nitrogen-fixing bacteria (Mamoudou & Mune, 2024; Mayes *et al*., 2019). However, the relationship between Bambara groundnuts and nitrogen-fixing bacteria is not always straightforward but is influenced by various factors, including the seed coat properties (Fwanyanga, Horn, Sibanda, & Reinhold-Hurek, 2022).

Seed coat compounds, displayed through color variations, are believed to play a crucial role in the successful establishment of the symbiotic relationship (Puozaa, Jaiswal, & Dakora, 2021). According to Fwanyanga *et al*. (2022), variations in seed coat colour among Bambara groundnut genotypes may influence the choice of microsymbiont partners the plant makes for successful symbiosis. Bambara groundnut seed coat colour differences are primarily influenced by the levels of secondary metabolites contained; the most common of these secondary metabolites are anthocyanins and flavonoids such as rutin, kaempferol, quercetin, catechin, and epigallocatechin (Palamae *et al*., 2024; Zenabou *et al*., 2022). Additionally, these phytochemicals in Bambara groundnut possess antimicrobial properties that can impede microbial growth (El Bilali *et al*., 2024). In legume systems, flavonoids are particularly important as they can influence nodulation signaling pathways by interacting with microbial signal transduction systems and affecting nod gene expression in compatible symbionts (Dong & Song, 2020).

Although biological nitrogen fixation (BNF) occurs several days after germination, bacterial populations in the spermosphere encounter seed-derived metabolites well before root emergence, which can alter early microbial colonization and competitive dynamics (Saccaram *et al.*, 2025). According to Hassen, Muema, Diale, Mpai, and Bopape (2025), water-soluble seed coat metabolites from legumes can inhibit or delay bacterial growth, alter chemotaxis, and interfere with signaling molecules such as Nod factors, which are critical for nodule initiation. Although it is acknowledged that seed coat compounds affect the bacterial symbionts, empirical evidence for this relationship remains limited (Palamae *et al*., 2024). Hence, the objective of this study was to evaluate the inhibitory potential of Bambara groundnut seedcoat extracts on indigenous nitrogen-fixing bacterial isolates.

3.2 Materials and Methods

3.2.1 Study location

The study was conducted under controlled laboratory conditions at the University of Mpumalanga (25º27′06.18″S and 30º58′5.21″E), Mbombela campus, South Africa. The temperature was set at 25± 2ºC.

3.2.2 Seed coat extracts preparation

Three Bambara groundnut (BGN) varieties characterized based on their seed coat and eye colour: BR-W, B-W and B-B seeds, were separately soaked in distilled water for 48 hours to allow for the removal of the seed coats (Table 3.1). The removed seed coats were dried for 3 days at room temperature before being ground into a fine powder using a kitchen blender (Summit Pro blender, model BBS1200, China) to pass through a 1 mm sieve. The powder was kept in brown paper bags for further study (Abd-ElGawad *et al*., 2020). Water, ethanol, and acetone seed coat extracts were prepared by separately adding 20 grams of powdered BGN

seed coat powder in 200 ml of distilled water, 99.9% ethanol, and 99.9% acetone. The suspensions were left in an orbital shaker (Labotec, model 207, South Africa) for 24 hours at 67 rpm before being filtered through a Whatman filter No. 1 paper. The ethanol and acetone extract filtrates were collected in a 100ml conical flask and subjected to solvent removal using a rotary vacuum evaporator (HemTron, Wiggens Strike 280, China) set at 56ºC for acetone and 78.37ºC for ethanol. This process resulted in the complete evaporation of the liquid solvent, leaving behind a concentrated crude extract in semi-solid or solid form (Ragunathan, Pandurangan, & Ramakrishnan, 2019). The resultant extracts were further air dried in a fume hood to ensure complete removal of residual solvent. The water filtrates were freeze-dried to obtain the extract used for antimicrobial study (Ragunathan *et al*., 2019). Acetone was used as a universal reconstitution solvent to dissolve crude extracts under sterile conditions, preparing the desired dilutions before antimicrobial testing (Famuyide, Aro, Fasina, Eloff, & McGaw, 2019).

Table 3.1: Morphological characteristics of the three Bambara groundnut seed landraces

Bambara groundnut image	Seed coat colour	Hilum colour	Landrace code/ name
	Burgundy red	White	'BR-W'

	Brown	White	‘B-W’
	Brown with black colour around the hilum	White	‘B-B’

3.2.3 Preparation of nitrogen-fixing bacteria

Ten indigenous Bambara groundnut nitrogen-fixing bacteria isolates consisting of four gram-positive strains, *Mammaliicoccus sciuri*, *Bacillus pumilus* P12, *Stenotrophomonas pavanii, Bacillus pumilus* P4, and *Cellulosimicrobium cellulans*, and two gram-negative strains, *Proteus columbae*, *Sphingobacterium faecium, Kaistella daneshvariae*, *Stenotrophomonas maltophilia,* and *Enterobacter asburiae* (Ubisi, 2024). All isolates were obtained from the University of Mpumalanga Biology Laboratory. They were originally isolated from Bambara groundnut roots collected in Mpumalanga, Limpopo, and KwaZulu-Natal provinces (Ubisi, 2024). Following isolation, the strains were characterized morphologically and screened for plant growth-promoting traits, including nitrogen cycling capacity and phosphate solubilization. Molecular identification was performed through bacterial DNA sequencing before being used in this study (Ubisi, 2024). The bacterial isolates were preserved in a 20 % glycerol solution at – 80 °C . For experimental use, fresh cultures were prepared by subculturing

stock bacterial cultures into newly prepared nutrient agar and incubated at 30 °C for seven days.

3.2.4 Antimicrobial assay of Bambara groundnut seed coat extracts

Antimicrobial assessment of Bambara groundnut seed coat extracts were conducted using the agar disc diffusion method (Othman *et al*., 2011). Treatments were arranged in a 3×10×3 factorial arrangement laid out in a completely randomized design (CRD) with 3 replications. The first factor consisted of three Bambara groundnut varieties as stated above (Table 3.1), while the second factor consisted of the ten indigenous nitrogen-fixing Bambara groundnut bacteria strains (*Proteus columbae*, *Mammaliicoccus sciuri*, *Bacillus pumilus* P12, *Stenotrophomonas pavanii*, *Bacillus pumilus* P4, *Kaistella daneshvariae*, *Stenotrophomonas maltophilia, Cellulosimicrobium cellulans, Sphingobacterium faecium,* and *Enterobacter asburiae*). The third factor consisted of three extracts: ethanol, acetone, and water. Penicillin and Ciprofloxacin (6µl disc-1) were used as positive controls for gram-positive and gram-negative bacteria, respectively, (Table 3.2), while acetone solvent and distilled water (6µl disc-1) were negative controls (Pandey *et al*., 2011). Indigenous Bambara groundnut nitrogen-fixing bacteria isolates grown for seven days were seeded on Mueller-Hinton agar plates. Plates were individually divided into quadrats, one quadrant was allocated to controls, and the other three were then allocated to treatments (ethanol, water, and acetone extracts) (Figure 3.1). While plates for the two negative controls were split in half and labelled accordingly. A 40 µl of each previously prepared extract was impregnated onto a 6 mm sterilized Whatman No. 1 filter paper disc and allowed to dry for one minute. The impregnated discs were then placed on seeded agar plates using sterile forceps. The plates were incubated for 24 hours at 37°C for antibacterial activity (Figure 3.1). Three independent experiments were performed to validate the results.

Table 3.2: Antibiotics for gram-positive and gram-negative nitrogen-fixing bacteria isolates

Antibiotics	Nitrogen-fixing bacterial isolates	Isolate codes according to location	Gram-stain
Ciprofloxacin	*Sphingobacterium faecium*	Zulu 32 B3	Gram-negative
	Enterobacter asburiae	UMPBG4 B4	Gram-negative
Penicillin	*Proteus columbae*	Zulu 18 B1	Gram-positive
	Mammaliicoccus sciuri	Zulu 16 A1	Gram-positive
	Bacillus pumilus P12	BF1 P12 P	Gram-positive
	Bacillus pumilus P4	BF1 P4 G	Gram-positive
	Stenotrophomonus pavanii	Cast1 B2	Gram-positive
	Kaistella daneshvariae	UMP1 P3 PB2	Gram-positive
	Stenotrophomonus maltophila	UMPP9 G4	Gram-positive
	Cellulosimicrobium cullulans	Nhlange22 B2	Gram-positive

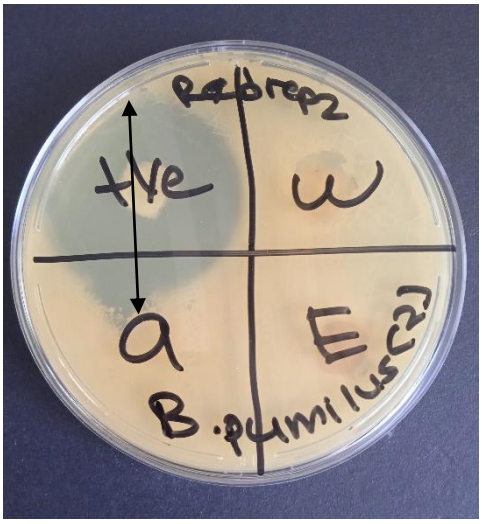

Figure 3.1: Measurement of the zone of inhibition; +ve stands for positive control, E for ethanol extract, W for water extract, A for acetone extract, and the arrows indicate the zone of inhibition. Photo captured by Gugu Maseko in 2025.

3.2.5 Data collection

A zone of inhibition was identified as a distinct, circular area around the center of the discs where no visible bacterial growth was observed. Antimicrobial activity was evaluated by measuring the diameter of the zone of inhibition in millimeters edge-to-edge across the zone of inhibition over the center of the discs in each agar plate 30 cm using a ruler (Figure 3.1) (Arif *et al*., 2022). The extract was considered inhibitory if its zone of inhibition was greater than that of a negative control.

3.2.6 Statistical analysis

Data collected were tested for normality using the Shapiro-Wilk normality test and if not normally distributed, transformed using the $Log_{10}(x+1)$ formula (Shapiro & Wilk, 1965). The data were then subjected to factorial Analysis of Variance (ANOVA) using Statistix 10 software (Gomez & Gomez, 1984). Where significant effects were detected ($P \leq 0.05$), Fisher's Least Significant Differences (LSD) test at 5 % was used for mean separation.

3.3 Results

Shapiro-Wilk normality test indicated that the zone of inhibition variable was not normally distributed ($P \leq 0.05$), hence transformed accordingly (Appendix 3.1). The three independent experiments were statistically similar; hence, the data were pooled and reanalyzed as a single set. The second-order interaction of variety, bacteria and extract was not significant ($P > 0.05$) for zone of inhibition, whereas only bacteria and extract first-order interaction was highly

significant ($P \leq 0.01$) (Appendix 3.2). When factors were considered separately, bacteria and extracts were significant ($P \leq 0.05$), whereas variety was not (Appendix 3.2).

The inhibitory effects of the three extracts varied with bacteria (Table 3.3). *Proteus columbae*, *B. pumilus* P12, *B. pumilus* P4, and *S. pavanii* were significantly inhibited by all three (water, ethanol, and acetone) Bambara groundnut seed coat extracts (Table 3.3). Whereas the growth of *M. sciuri*, *S. maltophila*, *E. asburiae*, and *C. cullulans* was inhibited by water and acetone extracts only, with ethanol extracts having no effect on the four (Table 3.3). The growth of *S. faecium* was inhibited by water and ethanol extracts only, while acetone extracts had no effect (Table 3.3). *Kaistella daneshvariae* growth was inhibited by water extracts only (Table 3.3). The negative control (pure distilled water) did not inhibit the growth of any of the tested indigenous Bambara groundnut nitrogen-fixing bacteria isolates (Table 3.3).

Water extracts had the highest zone of inhibition on *B. pumilus* P12, *B. pumilus* P4, and *S. pavanii*, whereas the lowest zone of inhibition for the same was observed on *S. faecium*, *C. cullulans*, *E.* asburiae, and *K. daneshvariae* (Table 3.3). Ethanol extracts had the highest zone of inhibition on *P. columbae*, *B. pumilus* P12, and *B. pumilus* P4, while the smallest zone of inhibition was observed on *M. sciuri*¸ *K. daneshvariae*, *S. maltophila*¸ *E. asburiae*, and *C. cullulans* (Table 3.3). With acetone extracts, the highest zone of inhibition was recorded on *B. pumilus* P12, *C. cullulans*, and *M. sciuri*, while its lowest zone of inhibition was observed on *S. faecium*, *K. daneshvariae*, and *S. pavanii* (Table 3.3).

Table 3.3: Antibacterial effect of Bambara groundnut seedcoat extracts on growth of indigenous nitrogen-fixing bacteria isolates

	Extracts				
Bacteria	Antibiotics (mm)	Water (mm)	Ethanol (mm)	Acetone (mm)	Negative control Water (mm)
Sphingobacterium faecium	1.21^{c} (27.6)	0.09^{ghij} (1.0)	0.05^{hij} (0.7)	0.00^{j} (0.0)	0.00^{j} (0.0)
Proteus columbae	1.40^{b} (26.9)	0.15^{fghi} (1.8)	0.14^{f-j} (1.7)	0.09^{f-j} (1.2)	0.00^{j} (0.0)
Mammaliicoccus sciuri	1.41^{b} (28.3)	0.24^{ef} (3.7)	0.00^{j} (0.0)	0.10^{f-j} (1.5)	0.00^{j} (0.0)
Bacillus pumilus P12	1.53^{ab} (33.9)	0.45^{d} (7.4)	0.13^{f-j}(1.6)	0.21^{efg} (2.9)	0.00^{j} (0.0)
Bacillus pumilus P4	1.56^{a} (35.9)	0.45^{d} (7.3)	0.10^{f-j} (1.5)	0.05^{hij} (0.6)	0.00^{j} (0.0)
Stenotrophomonas pavanii	1.55^{ab} (34.8)	0.32^{de} (5.8)	0.09^{f-j} (1.2)	0.04^{hij} (0.5)	0.00^{j} (0.0)
Kaistella daneshvariae	1.53^{ab} (33.4)	0.09^{f-j} (1.1)	0.00^{j} (0.0)	0.00^{j} (0.0)	0.00^{j} (0.0)

Stenotrophomonas maltophilia	1.50^{ab} (30.9)	0.19^{e-h} (2.7)	0.00^{j} (0.0)	0.03^{ij} (0.2)	0.00^{j} (0.0)
Enterobacter asburiae	1.12^{c} (23.9)	0.09^{f-j} (1.1)	0.00^{j} (0.0)	0.04^{hij} (0.5)	0.00^{j} (0.0)
Cellulosimicrobium cellulans	1.59^{a} (37.8)	0.11^{f-j} (1.2)	0.00^{j} (0.0)	0.10^{f-j} (1.7)	0.00^{j} (0.0)
P-value			0.00*		
F-value			2.31		
$LSD_{0.05}$			0.15		

* Highly significant at $P \leq 0.01$; Means followed by the same letters are not statistically different $P \leq 0.05$; Values not in brackets are transformed $[Log_{10}(x+1)]$ means.

3.4 Discussion

According to El Bilali *et al*. (2024), the presence of phytochemicals such as flavonoids, phytic acids, and tannins in Bambara groundnut has antimicrobial properties that are capable of impeding microbial growth. Based on the results of this study, the antimicrobial activity of Bambara groundnut seed coat extracts varied depending on the solvent used and the bacterial strains tested, suggesting differences in metabolite solubility and strain-specific susceptibility (Ekanayake *et al*., 2025). Among the extracts, the water extract exhibited the highest zones of inhibition against *B. pumilus* P12, *B. pumilus* P4, and *S. pavanii*, while it demonstrated the lowest inhibition against *S. faecium*, *C. cellulans*, and *K. daneshvariae*. This may indicate that highly polar compounds, such as water-soluble phenolics, contribute significantly to antimicrobial effects (Žagar, Frlan, & Kočevar Glavač, 2024). Furthermore, the ethanol extract showed the highest inhibition zones against *P. columbae*, *B. pumilus* P12, and *B. pumilus* P4, with the lowest activity recorded against *M. sciuri*, *K. daneshvariae*, and *S. maltophilia*. Similarly, the acetone extract demonstrated the greatest inhibition against *B. pumilus* P12, *C. cellulans*, and *M. sciuri*, while the lowest activity was seen against *S. faecium*, *K. daneshvariae*, and *S. pavanii*. These findings suggest that semi-polar phytochemicals also play an important role and demonstrate that solvent polarity influences the spectrum of extracted metabolites and, consequently, antimicrobial performance (Assouguem, Annemer, Mohammed, & Lazraq, 2025).

Overall, these findings indicate that Bambara groundnut seed coat extracts, irrespective of the solvent, exhibited greater inhibitory effects against Gram-positive bacteria (*B. pumilus* P12 and P4, *C. cellulans, M. sciuri*) than against Gram-negative strains such as *S. faecium*. Currently, there is limited reference studies that focused on seed coat extracts on nitrogen fixing bacteria but findings of Ibrahim and Kebede (2020), reported that Gram-positive bacteria are more

susceptible to plant extracts, while Gram-negative bacteria are generally more resistant due to the presence of lipopolysaccharides in their external membrane that act as a barrier to many antimicrobial agents. However, contrary findings have been reported in other studies. For instance, Palamae *et al*. (2024) found that red and brown Bambara groundnut hulls exhibited greater inhibition against Gram-negative bacteria *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* ATCC 700603, compared to Gram-positive bacteria *Staphylococcus aureus* ATCC 33591. Furthermore, Udeh, Nyila, and Kanu (2020) reported that the antimicrobial potential of Bambara groundnut extracts was primarily associated with high phenolic content, especially tannins and flavonoids found in brown and red hull varieties. This discrepancy may be attributed to differences in phytochemical composition, extraction methods, or strain-specific susceptibility, suggesting the need for further research into the bioactive compounds responsible for antimicrobial activity in Bambara groundnut seed coats.

3.5 Conclusion

The study was based on the hypothesis that Bambara groundnut seed coat extracts exhibit inhibitory effects on the growth of indigenous nitrogen-fixing bacterial isolates. The results revealed that water, ethanol, and acetone extracts of Bambara groundnut seed coats significantly inhibited the growth of *P. columbae*, *B. pumilus* P12, *B. pumilus* P4, and *S. pavanii*. Therefore, the hypothesis is accepted, as the findings confirm that Bambara groundnut seed coats have notable antimicrobial properties, especially against some indigenous nitrogen-fixing bacterial strains. The observed antimicrobial activity raises important considerations with regard to the potential interference of seed coat compounds with beneficial soil microorganisms, especially those involved in biological nitrogen fixation. These findings reveal the need for further research into the specific bioactive compounds responsible for antimicrobial activity effects and their potential impact on the symbiotic relationship between

Bambara groundnut and nitrogen-fixing bacteria. Such insights are important for optimizing Bambara groundnut production within sustainable agricultural systems and for guiding the development of compatible inoculants.

3.6 References

Abd-ElGawad, A.M., El Gendy, A.E.N.G., Assaeed, A.M., Al-Rowaily, S.L., Omer, E.A., Dar, B.A., Al-Taisan, W.A.A. and Elshamy, A.I. 2020. Essential oil enriched with oxygenated constituents from invasive plant *Argemone ochroleuca* exhibited potent phytotoxic effects. *Plants*, 9(8): 998.

Assouguem, A., Annemer, S., Mohammed, K. and Lazraq, A., 2025. Innovative approaches in the extraction, identification, and application of secondary metabolites from plants. *Phyton*, 94(6): 1631-668.

Dong, W. and Song, Y. 2020. The significance of flavonoids in the process of biological nitrogen fixation. *International journal of molecular sciences*, 21(16): 1-18.

Ekanayake, C.P., Johnson, J., Batley, R.J., du Preez, R., Trotter, T., Nastasi, J.R., Strappe, P., Broszczak, D. and Naiker, M. 2025. Targeted metabolomic analysis reveals solvent-dependent phenolic variation and associated antioxidant and antibacterial activity in Coriander seeds (*Coriandrum sativum* L.). *Molecules*, 30(22): 1-22.

El Bilali, H., Kiebre, Z., Nanema, R.K., Dan Guimbo, I., Rokka, V.M., Gonnella, M., Tietiambou, S.R.F., Dambo, L., Nanema, J., Grazioli, F. and Acasto, F. 2024. Mapping research on Bambara groundnut (*Vigna subterranea* (L.) Verdc.) in Africa: Bibliometric, geographical, and topical perspectives. *Agriculture*, 14(9): 1-33.

Famuyide, I.M., Aro, A.O., Fasina, F.O., Eloff, J.N. and McGaw, L.J. 2019. Antibacterial and antibiofilm activity of acetone leaf extracts of nine under-investigated South African

Eugenia and Syzygium (Myrtaceae) species and their selectivity indices. *BMC complementary and alternative medicine*, 19(1): 1-13.

Fwanyanga, F.M., Horn, L.N., Sibanda, T. and Reinhold-Hurek, B. 2022. Prospects of rhizobial inoculant technology on Bambara groundnut crop production and growth. *Frontiers in Agronomy,* 4: 1-7.

Gomez, K.A. and Gomez, A.A. 1984. *Statistics Procedure for Agricultural Research*. 2nd ed. Philippines: John Wily and Sons.

Ibrahim, N. and Kebede, A. 2020. In vitro antibacterial activities of methanol and aqueous leave extracts of selected medicinal plants against human pathogenic bacteria. *Saudi Journal of Biological Sciences*, 27(9): 2261-2268.

Mamoudou, H. and Mune, M.A.M. 2024. Investigating Bambara bean (*Vigna subterranea* (Verdc.) L.) protein and hydrolysates: A comprehensive analysis of biological and biochemical properties. *Applied Food Research*, 4(2): 1-18.

Mayes, S., Ho, W.K., Chai, H.H., Gao, X., Kundy, A.C., Mateva, K.I., Zahrulakmal, M., Hahiree, M.K.I.M., Kendabie, P., Licea, L.C. and Massawe, F. 2019. Bambara groundnut: An exemplar underutilised legume for resilience under climate change. *Planta*, 250: 803-820.

Othman, M., San Loh, H., Wiart, C., Khoo, T.J., Lim, K.H. and Ting, K.N. 2011. Optimal methods for evaluating antimicrobial activities from plant extracts. Journal of Microbiological Methods, 84(2): 161-166.

Palamae, S., Suyapoh, W., Boonrat, O., Zhang, B., Amin, M., Buatong, J. and Benjakul, S. 2024. Activity of Bambara groundnut seed coat extract against Shewanella species: efficacy and mechanisms of action. *Foods*, 13(21):1-16.

Pandey, P., Mehta, A. and Hajra, S. 2011. Evaluation of antimicrobial activity of *Ruta graveolens* stem extracts by disc diffusion method. *Journal of Phytology*, 3(3): 92-95.

Puozaa, D.K., Jaiswal, S.K. and Dakora, F.D. 2021. Black seedcoat pigmentation is a marker for enhanced nodulation and N2 fixation in Bambara groundnut (*Vigna subterranea* L. Verdc.) landraces. *Frontiers in Agronomy*, 3:1-15.

Ragunathan, V., Pandurangan, J. and Ramakrishnan, T. 2019. Gas chromatography-mass spectrometry analysis of methanol extracts from marine red seaweed Gracilaria corticata. *Pharmacognosy Journal*, 11(3): 547-554.Saccaram, C., Simonin, M., Boutet, S., Brosse, C., Peng, S., François, T., Collet, B., Perreau, F., Sourdeval, D., Marais, C. and Barret, M. 2025. Elucidating the interplay between metabolites and microorganisms in the spermosphere of common bean (*Phaseolus vulgaris* L.) seeds. *Msystems*, 10(8): 120.

Shapiro, S.S. and Wilk, M.B. 1965. An analysis of variance test for normality (complete samples). *Biometrika*, 52(3-4): 1-12.

Ubisi, R. 2024. *Diversity of rhizobacteria associated with communal produced Vigna subterranea in Mpumalanga, KwaZulu-Natal, and Limpopo province, South Africa*. Mbombela: University of Mpumalanga.

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.

Žagar, T., Frlan, R. and Kočevar Glavač, N. 2024. Using subcritical water to obtain polyphenol-rich extracts with antimicrobial properties. *Antibiotics*, 13(4), 1-22.

Zenabou, N., Firmin, S.L., Laurette, N.N., Daniel, W.F., Laurianne, T.N. and Martin, B.J. 2022. Nodulation potential of Bambara groundnut (*Vigna subterranea* L.) in Yaounde (Centre Region of Cameroon). *American Journal of Food and Nutrition*, 10(1): 34-39.

CHAPTER FOUR

EVALUATION OF INDIGENOUS BAMBARA GROUNDNUT NITROGEN-FIXING BACTERIA ISOLATES PH AND SALINITY TOLERANCE

4.1 Introduction

Bambara groundnuts are mostly produced in areas with poor soil, low rainfall, and high temperatures (Pasipanodya *et al*., 2025). According to Owaresat *et al*. (2023), these environmental stresses pose a significant threat to both agriculture and symbiotic nitrogen fixation, affecting the survival, growth, and metabolic activities of symbiotic bacteria and plants, as well as their ability to enter into symbiotic interactions. Prolonged droughts and extreme temperatures are often associated with fluctuating pH and increased salinity levels in the soil, resulting in reduced plant growth and beneficial microorganisms (Tan *et al*., 2020). Benmoussa *et al*. (2022) reported that salt stress strongly inhibits biological nitrogen fixation, which then reduces plant growth and biomass production in grain. Although this is true, salinity and pH tolerance are reported to vary with rhizobial nitrogen-fixing bacteria (Sellars & Nunes, 2021). Elsiddig and Elsheikh (1998) report that the fast-growing *Rhizobium* strains are more salt-tolerant than the slow-growing strains of *Bradyrhizobium*. The effects of salt stress also varied with crop, as *Bradyrhizobium* strains grown on soyabeans showed some tolerance to salinity (Nitawaki, Kitabayashi, Mason, Yamamoto, & Saeki, 2021). Similar variations in salt stress sensitivities of rhizobial microbes have also been reported among species of the same genus (Kirova & Kocheva, 2021). Currently, there are no studies on the effects of salinity and pH on indigenous rhizobial strains of Bambara groundnuts.

With worldwide estimates indicating that 50% of arable land will be saline by 2050 (Khondoker, Mandal, Gurav, & Hwang, 2023). There is a need for an understanding of the effects of salinity and pH on the nitrogen-fixing bacteria of Bambara groundnuts, for effective

maximization and improved productivity of the crop. Therefore, identifying indigenous strains with enhanced tolerance to these abiotic stresses is crucial for developing effective bioinoculants suitable for challenging agroecological environments. Hence, the study aimed to assess the tolerance of indigenous nitrogen-fixing bacteria to low pH and high saline conditions.

4.2 Materials and Methods

4.2.1 Study site

As previously described (Chapter 3).

4.2.2 Experimental treatments and design

Tolerance to pH levels: The experiments were arranged in a 10 × 3 factorial arrangement in a completely randomized design with three replications; the first factor was the ten indigenous BGN nitrogen-fixing bacteria isolates, as previously described (Chapter 3). The second factor consisted of three pH levels: 3.5, 4.5, and 5.5, with nutrient broth at pH 6.9 as the control.

Tolerance to salinity levels: The experiments were arranged in a 10 × 8 factorial arrangement in a completely randomized design with three replications. The first factor consisted of 10 Bambara groundnut nitrogen-fixing bacterial isolates, while the second factor consisted of eight salinity levels, namely 2, 4, 8, 16, 32, 64, and 128 NaCl + CaCl2 mM/m^3 at a 3:1 ratio.

4.2.3 Experimental procedure

Tolerance to pH levels: The ability of the 10 indigenous Bambara groundnut bacterial strains to tolerate low pH was tested by inoculating them in 15 mL nutrient broth tubes, with pH levels of 3.5, 4.5, and 5.5 adjusted using hydrochloric acid (HCl) or sodium hydroxide (NaOH). Hydrochloric acid was used to decrease pH, while NaOH was used to increase pH. Nutrient

broth media tubes with a pH of 6.9 without adjusted pH was used as control. All the tubes were incubated at 28 °C for 5 days and kept at 120 rev. min^{-1} in an incubator shaker (Labotec, model 353, South Africa) (Laurette *et al*., 2015). Three independent experiments were conducted to validate the experiment.

Tolerance to salinity levels: The experiment was established by impregnating the Yeast Mannitol (YEM) media with the described salts, with the control consisting of YEM medium without salts. The individual bacterial isolates were then separately grown on the impregnated YEM media plates placed in an incubator set at 30°C for 7 days. However, an experiment was terminated if one bacterial isolate reaches Petri dish edge before 7 days. Three independent experiments were conducted separately.

4.2.4 Data collection

The bacterial response to pH was determined by measuring absorbance at 470nm using an E-SP1100-UV-P spectrophotometer. Higher absorbance indicated higher bacterial growth. Seven days after incubation of bacteria in different saline conditions, the radial growth of indigenous Bambara groundnut bacteria colony were measured horizontally using a 300 mm ruler from the center to the colony edge (Figure 4.1). The isolates with a colony radius equal to or greater than the control were considered tolerant to respective salt levels. Microsoft Window 11 Excel was used to generate polynomial graphs with quadratic equation $Y = ax^2 + bx + c$, where Y= absorbance/radial growth values, x = pH/ salinity levels, and a, b, and c being constants. Optimal values were calculated after plotting the polynomial graphs and using the formular $Y = \frac{-b}{2a}$ to determine the optimum level. For salinity the concentration variable was first expressed in a geometric progression of $a_0=0$, $a_n=2^n$ for ≥1 series were n = term number in a

graph for even x-axis values. E.g. Concentration 64 was considered as the 6^{th} term of progression expressed as $2^6 = 64$.

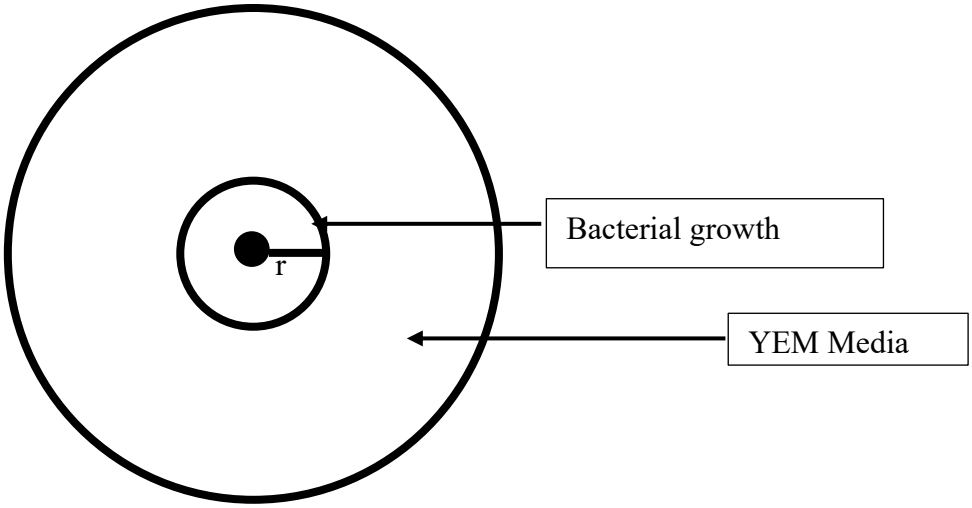

Figure 4.1: Bacterial isolate inoculated at the center of a YEM media plate.

4.2.5 Data analysis

The pH and salinity tolerance data were collected and analyzed as previously discussed (Chapter 3).

4.3 Results

The three independent experiments for both pH and salinity tolerance were not statistically different ($P > 0.05$); hence, the data were pooled separately and reanalyzed. The Shapiro-Wilk normality test for the pooled data revealed that the absorbance and radial growth variables were not normally distributed ($P \leq 0.05$); therefore, they were transformed accordingly (Appendix 4.4).

4.3.1 Bacteria tolerance to pH

The interaction between bacteria and pH on absorbance was not statistically significant ($P > 0.05$) (Appendix 4.5). However, when the factors were considered individually, both bacteria and pH had a highly ($P \leq 0.01$) significant effect on absorbance (Appendix 4.5).

Cellulosimicrobium cellulans, *S. pavanii*, and *P. columbae* had the highest absorbance, ranging from 0.519 to 0.691 (absorbance) AU (Table 4.1), indicating their ability to grow better across varying pH levels. In contrast, *S. faecium*, *M. sciuri*, and *B. pumilus* P4 had the lowest absorbance values, ranging from 0.286 to 0.398 (Table 4.1), indicating that these bacteria did not grow well across varying pH levels (Table 4.1).

Table 4.1: Responses of indigenous Bambara groundnut bacteria isolates to pH

Bacteria	Absorbance (AU)
Cellulosimicrobium cellulans	0.203^{a} (0.691)
Stenotrophomonas pavanii	0.176^{ab} (0.544)
Proteus columbae	0.170^{ab} (0.519)
Stenotrophomonas maltophilia	0.160^{b} (0.494)
Enterobacter asburiae	0.148^{b} (0.436)
Mammaliicoccus sciuri	0.145^{b} (0.341)
Bacillus pumilus P4	0.141^{bc} (0.398)

Kaistella daneshvariae	0.139[bc] (0.441)
Bacillus pumilus P12	0.139[bc] (0.404)
Sphingobacterium faecium	0.101[c] (0.286)
P-value	0.001*
F-value	3.310
$LSD_{0.05}$	0.042

* Highly significant at $P \leq 0.01$; Means followed by the same letters are not statistically different $P \leq 0.05$; Values not found in brackets are transformed [$Log_{10}(x+1)$] means.

Response of absorbance to pH was level depended, with lower pH levels having lower absorbance when compared to higher pH levels (Table 4.2). The control treatment at pH 6.9 had the highest absorbance, followed by pH 5.5 and 3.5, ranging from 0.331 to 0.789, while the pH level of 4.5 had the lowest absorbance of 0.321, though not different from 3.5 (Table 4.2).

Table 4.2: The effect of pH on the absorbance of indigenous Bambara groundnut nitrogen-fixing bacteria isolates

pH levels	Absorbance (AU)
3.5	0.116[bc] (0.331)
4.5	0.113[c] (0.321)
5.5	0.141[b] (0.421)

6.9	0.239[a] (0.789)
P-value	0.000*
F-value	39.370
$LSD_{0.05}$	0.101

* Highly significant at $P \leq 0.01$; Means followed by the same letters are not statistically different $P \leq 0.05$; Values not found in brackets are transformed [$Log_{10}(x+1)$] means.

4.3.2 Salinity tolerance of bacterial strains

The interaction between salinity and bacteria on radial growth was not statistically significant ($P > 0.05$). However, when the factors were considered separately, bacteria and salinity treatments were highly significant ($P \leq 0.01$) (Appendix 4.6).

Bacillus pumilus P12 had the highest radial growth, followed by *B. pumilus* P4 and *S. maltophila*, ranging from 10.653 to 21.889 mm (Table 4.3). While *K. daneshvariae* had the lowest radial growth followed by *P. columbae* and *C. cullulans*, ranging from 5.917 to 8.903 mm (Table 4.3).

Table 4.3: Indigenous Bambara groundnut bacteria isolate radial growth

Bacteria	Radial growth (mm)
Bacillus pumilus P12	1.259[a] (21.889)
Bacillus pumilus P4	1.011[b] (15.431)
Sphingobacterium faecium	0.871[bc] (8.903)

Stenotrophomonas maltophilia	0.874^{bc} (10.653)
Stenotrophomonas pavanii	0.819^{cd} (9.556)
Enterobacter asburiae	0.792^{cde} (9.069)
Mammaliicoccus sciuri	0.771^{cde} (9.542)
Cellulosimicrobium cellulans	0.759^{cde} (8.903)
Proteus columbae	0.701^{de} (7.056)
Kaistella daneshvariae	0.631^{e} (5.917)
P-value	0.000*
F-value	10.290
$LSD_{0.05}$	0.154

*Highly Significant at $P \leq 0.01$; Means followed by the same letters are not statistically different $P \leq 0.05$; Values not found in brackets are transformed [$Log_{10}(x+1)$] means.

Salinity, just like pH, was concentration-dependent; unlike pH, lower salinity levels had higher bacterial growth relative to lower salinity levels (Table 4.4). The salinity levels of 0 mM exhibited the highest radial growth, followed by 4 mM and 8 mM, ranging from 11.833 mm to 12.222 mm, whereas the salinity levels of 128 mM, 64 mM, and 32 mM showed the lowest radial growth, ranging from 7.6 to 11.133 mm (Table 4.4).

Table 4.4: Impact of salinity levels on the radial growth of indigenous Bambara groundnut nitrogen-fixing bacteria isolates

Salinity levels (mM/m^3)	Radial growth (mm)
0	0.918^{a} (11.589)
2	0.879^{ab} (11.500)
4	0.868^{ab} (12.222)
8	0.893^{a} (12.022)
16	0.891^{ab} (11.833)
32	0.917^{a} (11.133)
64	0.691^{c} (7.600)
128	0.756^{bc} (7.633)
P-value	0.006*
F-value	2.810
$LSD_{0.05}$	0.137

*Highly significant at $P \leq 0.01$; Means followed by the same letters are not statistically different $P \leq 0.05$; Values not found in brackets are transformed [$Log_{10}(x+1)$] means.

4.3.3 Modelling pH and salinity levels on bacterial growth

The quadratic relationships between pH and salinity and bacterial growth exhibited ranges that could stimulate, saturate, or inhibit growth (Figures 4.2 and 4.3). Opposite quadratic curves were observed for the two environmental stresses when plotted against bacterial growth; pH exhibited a U-shaped curve, while salinity showed an inverse U-shaped curve (Figures 4.2 and 4.3).

At low pH, bacterial growth decreased with an increase in pH up to a level of 4.12 and increased with an increase in pH, while salinity increased bacterial growth with an increase in salinity at lower levels up to 4.24 mM/m3 and increased with an increase in salinity at higher levels (Table 4.5). The bacterial growth relationship with pH and salinity was explained by 99.9% and 87.9 % of the derived model (Figures 4.2 and 4.3). The lowest pH that could be tolerated by the bacteria was calculated to be 4.12, while the highest salinity that could be tolerated by the bacteria was determined to be 4.24 mM/m³ (Table 4.5).

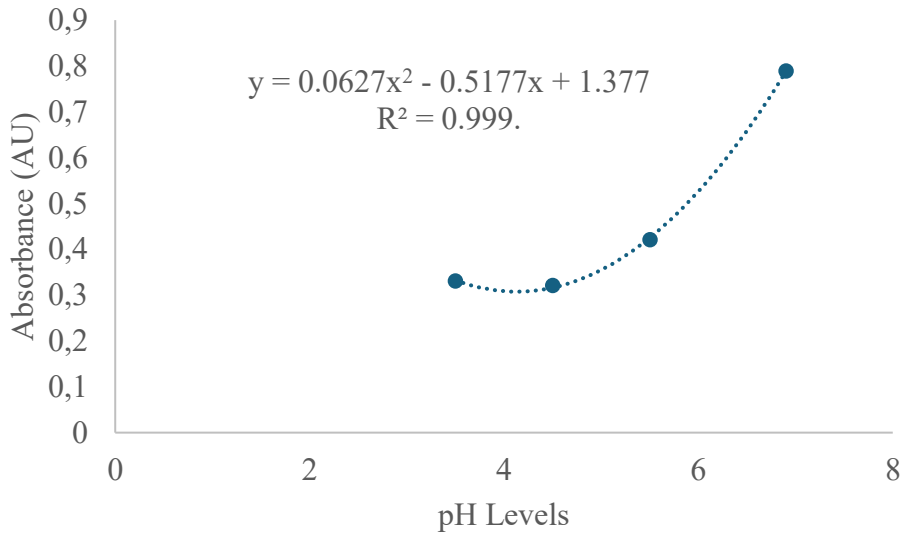

Figure 4.2: Impact of pH levels on the absorbance of indigenous Bambara groundnut nitrogen-fixing bacteria isolates.

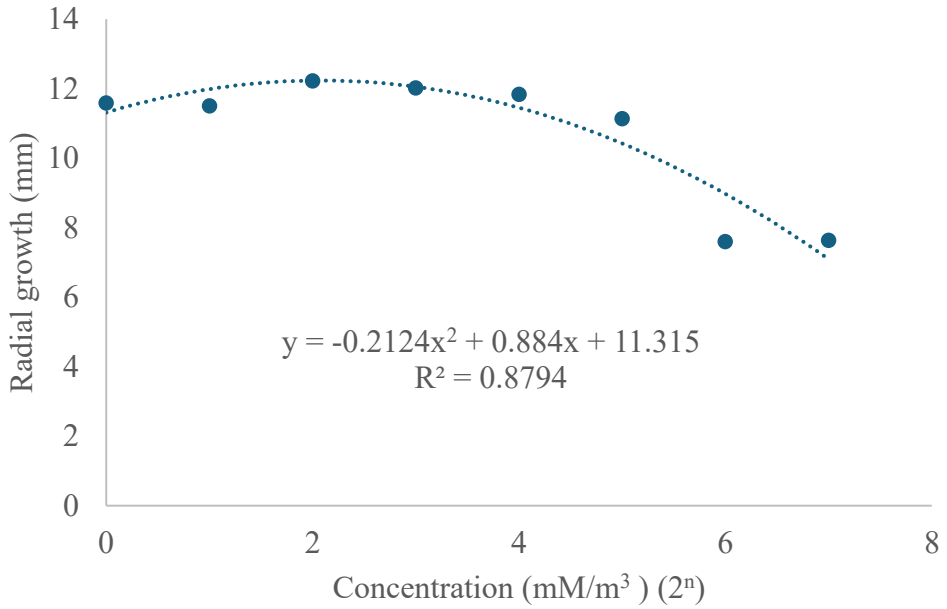

Figure 4.3: Impact of salinity levels on the radial growth of indigenous Bambara groundnut nitrogen-fixing bacteria isolates.

Table 4.5: Optimum level and coefficient correlation of indigenous Bambara groundnut nitrogen-fixing bacteria

Treatments	Optimum level	R^2 (%)	Equation
pH	4.12	0.9997	$Y=0.0627x^2 + 0.5177x + 1.377$
Salinity	4.24	0.8794	$Y= -0.2124x^2 +0.884x + 11.315$

4.4 Discussion

4.4.1 Bacteria tolerance to low pH

One of the major factors that affects the efficiency of symbiosis between plants and *Rhizobia* is the pH of the soil in which they interact (Nohwar, Khandare, & Desai, 2019). Acidic environments significantly limit the survival of rhizobia, inhibiting their ability to bind to root hairs and form nodules, which then reduces nitrogen fixation (Yeremko, Czopek, Staniak,

Marenych, & Hanhur, 2025). According to Msimbira and Smith (2020), pH variation in the environment has a direct impact on plant growth and the availability of nutrients. Moreover, important nutrients like magnesium and calcium are less available in acidic soils, while poisonous ions such as aluminum and magnesium become more soluble (Vista, Gaihre & Dahal, 2024).

Ferreira *et al*. (2016) observed in their study that fluctuations in pH affect the growth, metabolic activity, and survival of nitrogen-fixing bacteria, whereby the number of nodules and nitrogen fixation were enhanced during acidic conditions, but nitrogenase activity was greatly decreased when the pH was neutral (7.0). While pH tolerance varies within rhizobial nitrogen-fixing bacteria (Sellars & Nunes, 2021). Some can survive and fix nitrogen at pH values as low as 4.5 to 5.0 (Torabian, Farhangi-Abriz, & Denton, 2019). In general, increased absorbance indicates a higher bacterial population, suggesting favorable growth conditions at a specific pH. Based on the results of the study, the response of absorbance to pH was level depended, with lower pH levels having lower absorbance when compared to higher pH levels. The control treatment at pH 6.9 had the highest absorbance, followed by pH 5.5 and 3.5, ranging from 0.331 to 0.789, while the pH level of 4.5 had the lowest absorbance of 0.321, though not different from 3.5, as reported by Torabian *et al*. (2019). Similarly, a study by Zoundji *et al*. (2022) found that all Bambara groundnut-nodulating rhizobia grew well within a pH range of 5.6 to 7.5.

In contrast, Arsita, Karim, Hala, Iriany, and Jumadi (2020) reported that most isolates survived at pH levels of 4.6 and 8, except for four isolates (BKr4, BKn1, TTg1, and TTg2). In the current study, *C. cullulans*, *S. pavanii*, and *P. columbae* had the highest absorbance, ranging from 0.519 to 0.69, indicating their ability to grow better across varying pH levels. However, *S. faecium*, *M. sciuri*, and *B. pumilus* P4 had the lowest absorbance values, ranging from 0.286 to 0.398, indicating that these bacteria does not grow well across varying pH levels.

4.4.2 Salinity tolerance of bacterial isolates

Salinity is one of the key issues affecting soil fertility and agricultural productivity (Nitawaki, Kitabayashi, Mason, Yamamoto, & Saeki, 2021). High soil salinity extremely decreases crop yield and productivity. It negatively affects the growth, nodulation, nitrogen fixation, and survival of legume-*Rhizobium* symbiosis (Kirova & Kocheva, 2021). Benmoussa *et al*. (2022) also reported that salt stress strongly inhibits biological nitrogen fixation, which then reduces plant growth and biomass production in grain. The salt stress sensitivity of rhizobial strains varies significantly among them, and some rhizobial strains can survive in saline soils (Ilangumaran, Schwinghamer, & Smith, 2021). Moreover, salinity-tolerant *Rhizobium* species have the potential to regulate ethylene synthesis in plants and improve yield in legumes in both semi-arid and arid regions (Ali *et al*., 2023).

The results of the study revealed that the radial growth of indigenous BGN nitrogen-fixing bacterial isolates increased at low salinity levels and decreased as salinity levels increased. Similarly, Seidu, Githiri, Wesonga, and Ngumi (2025) reported that an increase in NaCl concentration led to a decrease in the NaCl tolerance of the isolates. Moreover, based on the results of this study, the salinity levels of 0 mM exhibited the highest radial growth, followed by 4 mM and 8 mM, ranging from 11.833 mm to 12.222 mm, whereas the salinity levels of 128 mM, 64 mM, and 32 mM showed the lowest radial growth, ranging from 7.6 to 11.133 mm. These findings align with those of Seidu *et al*. (2025), who observed in their study that Bambara groundnut *Bradyrhizobium* isolates grew at 1% NaCl, 3% NaCl, and 5% NaCl but did not thrive in the presence of an increased salt concentration of 10% NaCl. Correspondingly, Zoundji *et al.* (2022) reported in their study that many of their presumptive rhizobia isolates survived at NaCl concentrations between 1% and 7%, while isolate growth decreased when the

NaCl concentration was above 7%. In contrast, Owaresat *et al*. (2023) reported that some symbiotic rhizobia, such as *Sinorhizobium meliloti* and *Rhizobium leguminosarum,* can be tolerant to 300 mM and 700 mM NaCl.

The results of this study revealed that *B. pumilus* P12 had the highest radial growth, followed by *B. pumilus* P4 and *S. maltophila*, ranging from 10.653 to 21.889 mm. While *K. daneshvariae* had the lowest radial growth, followed by *P. columbae* and *C. cullulans*, ranging from 5.917 to 8.903 mm. Additionally, Dong *et al*. (2017) reported that the growth of rhizobia is affected by NaCl treatment, and different rhizobial strains reveal significant variation in salt tolerance capability. Generally, fast-growing rhizobia are more salt-tolerant than slow-growing rhizobia, having a slower growth rate (Chen, 2024; Zhang, Singh, Guo, Shang, & Peng, 2020).

4.4.3 Modelling pH and salinity levels on bacterial growth

Coefficient of determination (R^2) quantified the proportion of variance explained by a statistical model (Nakagawa, Johnson, & Schielzeth, 2017). According to Chicco, Warrens, and Jurman (2021), the original formulation of R^2 aims to measure the proportion of variance in the dependent variable that is explained by the independent variables. The results of the study revealed opposite quadratic curves for the two variables; pH and salinity, when plotted against bacterial growth, with pH exhibiting a U-shaped curve, while salinity showed an inverse U-shaped curve.

According to Kirova and Kocheva (2021), rhizobia are generally more salt-tolerant than their associated host plant, but the functional symbiosis is even more sensitive to salinity than either of the partners. Based on the results of the study, bacterial growth of tested isolates decreased with an increase in pH up to a level of 4.12 and increased with an increase in pH, while salinity

increased bacterial growth with an increase in salinity at lower levels up to 4.24 mM/m^3 and decreased with an increase in salinity at higher levels. Similarly, Mataboge, Mohammed, and Dakora (2025) reported that 31 soybean isolates tested in their study revealed different growth rates to different salinity concentrations and pH levels, with 16 isolates showing the ability to grow at 5 % NaCl, and isolates TUTGMGH3, TUTGMGH4, and TUTGMGH9 showing positive growth in acidic medium, having absorbances that range from 0.479 to 0.697.

According to Azari, Nabizadeh, Mahvi, and Nasseri (2022), the value of coefficient determination (R^2) that is closer to 1 or 100 % indicates a better fit of the model to the observed data, meaning that the predictions of the model are closer to the actual measurements. However, a value of 0 or 0 % indicates that the model does not explain the variability in the dependent variable any better than simply using the average of the observed data (Chicco *et al*., 2021). Based on the results of the study, the bacterial growth relationship with pH and salinity was explained by 99.9 % and 87.9 % of the derived model, indicating high model reliability and a strong relationship. Azari *et al*. (2022) and Logue and Manandhar (2018) reported that R^2 values of 0.999 (9.99 %) and 1.00 (100 %) show the goodness of fit of the regression and a strong relationship. The lowest pH that could be tolerated by the bacteria was calculated to be 4.12, while the highest salinity that could be tolerated by the bacteria was determined to be 4.24 mM/m^3.

4.5 Conclusion

Identifying indigenous strains with enhanced tolerance to low pH and high salinity stresses is crucial for developing effective bioinoculants suitable for challenging agroecological environments. Among the tested isolates, *C. cullulans*, *S. pavanii*, and *P. columbae* outperformed and thrived at pH levels of 4.5, 5.5, and 6.9. While most Bambara groundnut

bacteria isolates could not tolerate extremely high salinity levels, *B. pumilus* P12, *B. pumilus* P4, and *S. maltophila* demonstrated superior tolerance to such salinity concentrations. The bacteria of Bambara groundnut had a general tolerance of 4.12 and higher pH, while salinity higher than 4.24 mM/m^3 could not be tolerated. The results indicate that selected indigenous nitrogen-fixing bacterial isolates exhibit substantial tolerance to acidic conditions, with several strains thriving at pH 4.5. However, tolerance to high salinity was not universal, as only a few isolates demonstrated resilience under elevated salt concentrations. Therefore, the hypothesis is partially supported, with tolerance being dependent on strained identity and stress intensity. However, the findings still show the potential of these indigenous Bambara groundnut bacteria strains for use in biofertilizer development under acidic and moderately saline soil conditions.

4.6 References

Adjei, J.A., Aserse, A.A., Yli-Halla, M., Ahiabor, B.D., Abaidoo, R.C. and Lindstrom, K. 2022. Phylogenetically diverse *Bradyrhizobium* genospecies nodulate Bambara groundnut (*Vigna subterranea* L. Verdc) and soybean (*Glycine max* L. Merril) in the northern savanna zones of Ghana. *FEMS Microbiology Ecology*, 98(5): 1-17.

Arsita, R., Karim, H., Hala, Y., Iriany, N. and Jumadi, O. 2020. Isolation and identification of nitrogen-fixing bacteria in the corn rhizosphere *(Zea mays* L.) originating from Jeneponto Regency, South Sulawesi. *In IOP Conference Series: Earth and Environmental Science*, 484(1): 1-8.

Azari, A., Nabizadeh, R., Mahvi, A.H. and Nasseri, S. 2022. Integrated Fuzzy AHP-TOPSIS for selecting the best color removal process using carbon-based adsorbent materials: Multi-criteria decision making vs. systematic review approaches and modeling of textile wastewater treatment in real conditions. *International Journal of Environmental Analytical Chemistry*, 102(18): 7329-7344.

Benmoussa, S., Nouairi, I., Rajhi, I., Rezgui, S., Manai, K., Taamali, W., Abbes, Z., Zribi, K., Brouquisse, R. and Mhadhbi, H. 2022. Growth performance and nitrogen fixing efficiency of faba bean (*Vicia faba* L.) genotypes in symbiosis with rhizobia under combined salinity and hypoxia stresses. *Agronomy*, 12(3): 1-18.

Chen, P.Y. 2024. *Symbiotic effectiveness, competitiveness and salt tolerance of lucerne rhizobia*. Melbourne: RMIT University.

Chicco, D., Warrens, M.J. and Jurman, G. 2021. The coefficient of determination R-squared is more informative than SMAPE, MAE, MAPE, MSE and RMSE in regression analysis evaluation. *PeerJ Computer Science*, 7: 1-24.

Dong, R., Zhang, J., Huan, H., Bai, C., Chen, Z. and Liu, G. 2017. High salt tolerance of a *Bradyrhizobium* strain and its promotion of the growth of *Stylosanthes guianensis*. *International Journal of Molecular Sciences*, 18(8): 1-17.

Ferreira, T.C., Aguilar, J.V., Souza, L.A., Justino, G.C., Aguiar, L.F. and Camargos, L.S. 2016. pH effects on nodulation and biological nitrogen fixation in *Calopogonium mucunoides*. *Brazilian Journal of Botany*, 39: 1015-1020.

Fwanyanga, F.M., Horn, L.N., Sibanda, T. and Reinhold-Hurek, B. 2022. Prospects of rhizobial inoculant technology on Bambara groundnut crop production and growth. *Frontiers in Agronomy,* 4: 1-7.

Ilangumaran, G., Schwinghamer, T.D. and Smith, D.L. 2021. Rhizobacteria from root nodules of an indigenous legume enhance salinity stress tolerance in soybean. *Frontiers in Sustainable Food Systems,* 4: 1-18.

Khondoker, M., Mandal, S., Gurav, R. and Hwang, S. 2023. Freshwater shortage, salinity increase, and global food production: A need for sustainable irrigation water desalination—A scoping review. *Earth*, 4(2): 223-240.

Kirova, E. and Kocheva, K. 2021. Physiological effects of salinity on nitrogen fixation in legumes–a review. *Journal of Plant Nutrition,* 44(17): 2653-2662.

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 Microbiological Research*, 3(2): 65-75.

Logue, B.A. and Manandhar, E. 2018. Percent residual accuracy for quantifying goodness-of-fit of linear calibration curves. *Talanta*, 189: 527-533.

Mataboge, M.T., Mohammed, M. and Dakora, F.D. 2025. Symbiotic N2 fixation, leaf photosynthesis, and abiotic stress tolerance of native rhizobia isolated from soybean nodules at Da, Upper West Region, Ghana. *Microorganisms*, 13(4): 1-23.

Msimbira, L.A. and Smith, D.L. 2020. The roles of plant growth promoting microbes in enhancing plant tolerance to acidity and alkalinity stresses. *Frontiers in Sustainable Food Systems*, 4: 1-14.

Nakagawa, S., Johnson, P.C. and Schielzeth, H. 2017. The coefficient of determination R^2 and intra-class correlation coefficient from generalized linear mixed-effects models revisited and expanded. *Journal of the Royal Society Interface*, 14(134): 1-11.

Nitawaki, Y., Kitabayashi, H., Mason, M.L.T., Yamamoto, A. and Saeki, Y. 2021. Effect of salt stress on soybean growth and nodulation under inoculation with soybean rhizobia. *Soil Science and Plant Nutrition*, 67(2): 103-113.

Nohwar, N., Khandare, R.V. and Desai, N.S. 2019. Isolation and characterization of salinity tolerant nitrogen fixing bacteria from *Sesbania sesban* (L) root nodules. *Biocatalysis and Agricultural Biotechnology*, 21: 1-6.

Owaresat, J.K., Siam, M.A., Dey, D., Jabed, S., Badsha, F., Islam, M.R. and Kabir, M.S. 2023. Factors impacting rhizobium-legume symbiotic nitrogen fixation with the physiological

and genetic responses to overcome the adverse conditions: A review. *Agricultural Reviews*, 44(1): 22-30.

Pasipanodya, J.T., Horn, L.N., Togarepi, C., Sibiya, J., Musango, R. and Achigan-Dako, E.G. 2025. Exploring farmer perspectives on Bambara groundnut [*Vigna subterranea* (L.) Verdc]: production constraints, preferred varietal traits and implications for varietal development in northern Namibia. *Frontiers in Sustainable Food Systems*, 9: 1-14.

Seidu, O.A., Githiri, S.M., Wesonga, J.M. and Ngumi, V.W. 2025. Isolation, screening and in-vitro characterization of plant growth-promoting Bradyrhizobium isolates from the nodules of Bambara groundnut (*Vigna subterranean*) for potential use as bioinoculants. *Frontiers in Sustainable Food Systems*, 8: 1-14.

Sellars, S. and Nunes, V. 2021. Synthetic Nitrogen Fertilizer in the U.S. *Farmdoc Daily*, 11(24): 1-5.

Tan, X.L., Azam-Ali, S., Goh, E.V., Mustafa, M., Chai, H.H., Ho, W.K., 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: 1-16.

Torabian, S., Farhangi-Abriz, S. and Denton, M. 2019. Do tillage systems influence nitrogen fixation in legumes? A review. *Soil and Tillage Research*, 185(1): 1-9.

Vista, S.P., Gaihre, Y.K. and Dahal, K.R. 2024. Plant nutrient availability in acid soil and management strategies. In: Rahman, M.M., Biswas, C. and Meena, R.S. eds. *Climate change and soil-water-plant Nexus*: *Agriculture and environment*. Singapore: Springer Nature, 331-353.

Yeremko, L., Czopek, K., Staniak, M., Marenych, M. and Hanhur, V. 2025. Role of environmental factors in legume-rhizobium symbiosis: A review. *Biomolecules*, 15(1): 1-17.

Zhang, J., Singh, D., Guo, C., Shang, Y. and Peng, S. 2020. Rhizobia at extremes of acidity, alkalinity, salinity, and temperature. In Singh, R., Geetanjali Manchanda, I. Maurya, and Yunlin Wei. eds. *Microbial versatility in varied environments: Microbes in Sensitive Environments*. Singapore: Springer Singapore, 51-65.

Zoundji, M.C.C., Ahoglé, A.M.A., Akplo, T.M., Gangnon, S.O., Montéiro, D., Zanvo, Y., Alladassi, F.K. and Houngnandan, P. 2022. Resistance to abiotic stress and effectiveness of native rhizobia on Bambara groundnut [*Vigna subterranea* (L.) Verdc.] in Benin. *Open Journal of Soil Science*, 12(6): 193-215.

CHAPTER FIVE

EFFICIENCY OF INDIGENOUS NITROGEN-FIXING BACTERIAL ISOLATES ON GROWTH AND NODULATION OF RETAINED BAMBARA GROUNDNUT VARIETIES

5.1 Introduction

Vigna subterranea (L.) commonly referred to as Bambara groundnut, can fix 4 to 200kg of nitrogen per hectare through forming effective root nodules with compatible soil rhizobia (Hassen, Bopape, van Vuuren, Gerrano, & Morey, 2023; Ibny, Jaiswal, Mohammed, & Dakora, 2019). This symbiotic relationship enables Bambara groundnut to be produced under unfavourable environmental conditions, such as drought and nutrient-poor soil, producing high yields under these conditions, making it a climate-smart crop (Tan *et al*., 2020). The symbiotic relationship that Bambara groundnut and other legumes have with nitrogen-fixing root nodule bacteria is of economic and agronomic importance due to its nitrogen contribution to the total balance of nitrogen in the terrestrial ecosystems (Ubisi *et al*., 2023).

Although it contributes to soil wellbeing, symbiotic nitrogen-fixation is affected by climatic conditions such as extreme temperatures, poor rain, and prolonged drought, which affect the population of rhizobium in the soil, therefore reducing nodulation and yields of locally cultivated Bambara groundnut (Fwanyanga, 2023). These conditions are often associated with fluctuating pH and increased salinity levels in the soil (Tan *et al*., 2020). Benmoussa *et al*. (2022) also reported that salt stress strongly inhibits biological nitrogen fixation, which then reduces plant growth and biomass production in grain. Although this is true, salinity and pH tolerance vary within rhizobial nitrogen-fixing bacteria (Chapter 4). Indigenous Bambara groundnut nitrogen-fixing bacteria strains are able to tolerate a low pH range of 4.5 to 6.9 and low salinity levels but cannot tolerate extremely high salinity levels such as 64mM to 128mM

(Chapter 4). The effects of seedcoat extracts, pH and salinity were not influenced by the variety of the Bambara groundnut (Chapter 3 and 4)

Bitire *et al* (2023) reported that the prolonged utilization of agrochemicals has been proven to affect soil structure and fertility, diversity of beneficial microorganisms, and water holding capacity of the soil. Moreover, indigenous rhizobia can develop strategies for survival under ecologically severe conditions, besides their ability to occasionally reveal elevated levels of nitrogen fixation and plant growth promotion compared to introduced commercial strains (Ibny *et al*., 2019). Hence, it is important to study the nitrogen-fixing potential of indigenous strains and use them in developing native inoculum strains to improve the production of Bambara groundnut and other agricultural crops. Therefore, the objective of the study was to determine whether the indigenous nitrogen-fixing bacteria isolates would increase nodulation and growth of retained Bambara groundnut varieties.

5.2 Materials and methods

5.2.1 Study site

The study was conducted at the University of Mpumalanga (UMP) farm (25º27′06.18″S and 30º58′5.21″E), Mbombela campus, South Africa, under greenhouse conditions. The temperature and relative humidity were set at 25 ± 3 ºC and 70 ± 10%, respectively.

5.2.2 Experimental treatments and design

Treatments were arranged in a 10 × 3 factorial arrangement in a randomized complete block design (RCBD) replicated 5 times. The first factor consisted of the 10 different indigenous Bambara groundnut bacteria isolates previously described (Chapter 3), whereas the second factor consisted of retained Bambara groundnut varieties (Black, Red, and Cream-white eye)

(Table 5.1). Uninoculated and unfertilized plants were included as a negative control, whereas plants that were uninoculated and fertilized with potassium nitrate (KNO_3) were considered as positive controls.

Table 5.1: Different coloured seed coats of Bambara groundnut seed varieties

Bambara groundnut image	Seed coat colour	Hilum colour	Variety code/ name
	Dark purple	White	'DP-W'
	Cream white	White	'CW-W'
	Burgundy red	White	'BR-W'

5.2.3 Procedure

Sandy and loam soil mixture (3:1 ratio) collected from the University of Mpumalanga farm was pasteurized at 100 °C for 4 hours and used to fill 180 plastic pots that were 20 cm in diameter. Bambara groundnut seeds were sterilized using 70% ethanol for 30 seconds and rinsed with distilled water 3 times. Four surface-sterilized seeds of each variety were then sown in each 20 cm plastic pot. Seven days after sowing, the seedlings were thinned to one per pot (Figure 5.1). The seedlings were then inoculated with 3 mL of each Bambara groundnut bacterial isolate strain containing 1×10^7 colony-forming units (cfu) (Amani *et al*., 2020). Additionally, the positive control treatments received 13% KNO_3 as a nitrogen source, and 300 mL of distilled water was used to irrigate the plants twice a week. Pest and disease scouting was done daily.

Figure 5.1: Bambara groundnut seedlings after thinning. Photo by Gugu Maseko, 2024.

5.2.4 Data collection

Plant growth variables: Forty-five days after sowing, the plants were harvested and data collected included plant height measured from the crown to the end of the flag leaf using a

ruler. The number of runners and number of leaves were counted and the length of the longest runner was measured using a 30 cm ruler. Chlorophyll content was measured from the two most mature leaves using a chlorophyll content meter (Opti-Sciences, CCM-200 PLUS, Tyngsboro, Massachusetts). Shoots were cut at the soil surface level, and the stem diameter was measured 5 cm from the cut end using a digital Vernier Caliper (RS Components, RS PRO 200mm, South Africa). Fresh shoot mass was taken before oven-drying them at 65 °C for 72 hours. Dry shoots were weighed to obtain dry shoot mass. Plant roots were carefully removed from the pots, rinsed in water to remove soil particles, and blotted dry using a paper towel. Fresh roots were weighed and later over-dried at 65 °C for 72 hours to obtain the dry root mass. Plant growth vigour score as a measure of plant nitrogen availability levels was evaluated based on the following 1-5 scoring system (Sanni & Adenuni, 2020):

- Vigorous and green plants 5
- Relatively small and green plants 4
- Less green and small plants 3
- Slightly chlorotic plants 2
- Extremely chlorotic plants 1

Nodulation variables: Before the roots were oven-dried, the number of nodules per root system were counted and nodule colour and abundance were assessed, nodules that had a pinkish or red colour inside when cut in half were recorded as active nodules. Nodules that had a lightly pink or white with green colour when cut open were recorded as less active and those that were brown or had a greyish green colour were recorded as inactive nodules (Pommeresche & Hansen, 2017). Nodule colour and abundance scores were determined based on the following 0-5 score (Mweetwa, Chilombo, & Gondwe, 2016):

- More than five clusters of pink pigmented nodules 5

- Four to five clusters of mostly pink nodules 4
- Three to four clusters of less pink nodules 3
- Some pink or whitish with green area 2
- Less than three clusters of nodules or green or white nodules 1
- No nodules or white or green nodules 0

The position of nodules on roots in reference to root crown and lateral root was recorded and scored as follows:

- Both lateral and crown nodulation 3
- Mostly crown nodulation only 2
- Mostly lateral nodulation only 1

The plant growth vigour, nodule colour and abundance score, and nodule position scores were considered in determining the nitrogen fixation potential and nodulation efficiency, which was rated using the following total scores:

- 11 – 13 score indicates nodulation effectiveness (various nodules with excellent nitrogen fixing potential).
- 7 – 10 score indicates fewer effective nodulation (nodules with limited nitrogen fixing potential).
- 1 – 6 score indicates poor nodulation (nodules with little nitrogen-fixation potential).

Nodulation data were also used to calculate relative nitrogen fixing effectiveness (RE) using the following formula (Amani *et al*., 2020):

$$\text{RE}=\frac{\text{Inoculated plant dry matter}}{\text{N}-\text{Fertilized plant dry matter}}\times 100$$

Whereby, the dry shoot mass and dry root mass were added together to obtain the plant dry matter. The nitrogen-fixing effectiveness was classified as highly effective when RE > 80%, Effective at 50% < RE < 80%, lowly effective at 35% < RE < 50%, and ineffective when RE < 35%.

5.2.5 Data analysis

Collected data were assessed for normal distribution using the Shapiro-Wilk normality test and $Log_{10}(x+1)$ was used to transform data that was not normally distributed (Shapiro & Wilk, 1965). Relative nitrogen fixing effectiveness percentage was transformed using $arcsine\sqrt{(percentage\ effectiveness \div 100)}$ formula. The data were later subjected to Analysis of Variance (ANOVA) through Statistix 10 software (Gomez & Gomez, 1984). The Fisher's Least Significant Differences (LSD) at 5 % was used to obtain the treatment mean separation.

5.3 Results

Shapiro-Wilk normality test indicated that most plant growth variables, except for chlorophyll content and plant height, and all nodulation variables were not normally distributed ($P > 0.05$), ($P \leq 0.05$) (Appendix 5.1 and 5.2). Therefore, the variables that failed the normality test were transformed accordingly (Appendix 5.1 - 5.2).

5.3.1 Plant growth variables

The interaction between bacteria and varieties on all measured plant growth variables were not statistically significant ($P > 0.05$) (Appendix 5.3-5.12). However, when the factors were considered individually, bacterial treatments were statistically significant for stem diameter only ($P \leq 0.05$), whereas varieties had a highly ($P \leq 0.01$) significant effect on the number of runners, number of leaves, stem diameter, and dry root mass (Appendix 5.4, 5.5, 5.8, 5.12).

Varieties also had a significant effect on plant height, chlorophyll content, and fresh root mass (Appendix 5.3, 5.7, and 5.9). The treatment effects on the number of runners, stem diameter, and dry root mass were highly significant ($P \leq 0.01$), while plant height, chlorophyll content, number of leaves, and fresh root mass were significant ($P \leq 0.05$) (Appendix 5.3 -5.12). In contrast, the length of the longest runner, fresh shoot mass, and dry shoot mass were not significant ($P > 0.05$) (Appendix 5.3 -5.12). Among the three evaluated varieties, the cream-white eye variety had the highest plant height, number of leaves, and number of runners compared to the black and red varieties, with the black variety having the lowest plant height, number of leaves, and number of runners (Table 5.2). However, the black variety had the highest chlorophyll content, fresh root mass, and dry root mass compared to cream-white eye and red varieties, with the cream-white eye variety having the lowest chlorophyll content, fresh root mass, and dry root mass (Table 5.2). The red variety had the highest stem diameter compared to the black and cream-white eye varieties, with the cream-white eye variety having the lowest stem diameter (Table 5.2).

Table 5.2: Effect of varieties on plant height (PH), number of leaves (NL), number of runners (NR), chlorophyll content (CC), stem diameter (SD), fresh root mass (FRM), and dry root mass (DRM) for retained Bambara groundnut

Varieties	PH	NL	NR	CC	SD	FRM	DRM
Cream-white eye	31.502^{a}(31.502)	1.037^{a}(10.133)	0.654^{a}(3.733)	24.470^{b}(24.470)	0.256^{b}(0.862)	0.797^{b}(5.569)	0.134^{b} (0.370)
Red Variety	31.152^{a}(31.152)	1.010^{ab}(9.400)	0.628^{a}(3.561)	25.598^{ab}(25.598)	0.329^{a}(1.187)	0.816^{ab}(5.736)	0.147^{ab} (0.412)
Black Variety	29.985^{b}(29.985)	0.985^{b}(8.817)	0.529^{b}(2.753)	27.408^{a}(27.408)	0.326^{a}(1.177)	0.856^{a}(6.520)	0.162^{a} (0.465)
F-value	3.700	5.420	8.190	4.000	10.560	3.370	4.220
P-value	0.027*	0.005**	0.000**	0.021*	0.000**	0.037*	0.017**
$LSD_{0.05}$	1.154	0.032	0.065	2.073	0.036	0.046	0.019

**Highly significant at $P \leq 0.01$ and *Significant at $P \leq 0.05$; Means followed by the same letters are not statistically different $P \leq 0.05$; Values not in brackets are transformed [$Log_{10}(x+1)$] means.

The plants treated with potassium nitrate (KNO_3) had the highest stem diameter, followed by those inoculated with *M. sciuri*, *K. daneshvariae*, and *S. maltophila.* In contrast, plants treated with negative control, *P. columbae*, *B. pumilus* P4, and *S. pavanii* had the lowest stem diameter (Table 5.3).

Table 5.3: Effect of Indigenous Bambara groundnut bacterial isolates on stem diameter (SD)

Bacteria	SD
Positive control	0.356^{a} (1.320)
Negative control	0.251^{c} (0.840)
Mammaliicoccus sciuri	0.341^{ab} (1.267)
Stenotrophomonas maltophilia	0.332^{ab} (1.180)
Kaistella daneshvariae	0.332^{ab} (1.207)
Enterobacter asburiae	0.324^{ab} (1.153)
Cellulosimicrobium cellulans	0.323^{ab} (1.160)
Bacillus pumilus P12	0.294^{abc} (1.047)
Sphingobacterium faecium	0.281^{abc} (1.007)
Stenotrophomus pavanii	0.277^{bc} (0.953)
Bacillus pumilus P4	0.274^{bc} (0.920)
Proteus columbae	0.249^{c} (0.847)
F-valve	1.980
P-valve	0.035*
$LSD_{0.05}$	0.071

*Significant at $P \leq 0.05$; Means followed by the same letters are not statistically different $P \leq 0.05$; Values not in brackets are transformed [$Log_{10}(x+1)$] means.

5.3.2 Nodulation variables

The number of nodules, nodule position, active nodules, nodule colour and abundance score, plant growth vigour, and nodulation effectiveness score were highly significant ($P \leq 0.01$), except for non-active nodules and relative nitrogen fixing effectiveness ($P > 0.05$) (Appendix 5.12- 5.19). Moreover, the cream-white eye variety had the highest nodule position compared to the red and black varieties, with the red variety having the lowest nodule position (Table 5.4). Additionally, the black variety exhibited the highest plant growth vigour compared to the red and cream-white eye varieties, with the cream-white eye variety having the lowest plant growth vigour (Table 5.4).

Table 5.4: Effect of varieties on nodule position (NP) and plant growth vigor (PGV) for retained Bambara groundnut

Varieties	NP	PGV
Cream-white eye	0.538^{a}(2.513)	0.556^{b}(2.933)
Red Variety	0.488^{b}(2.109)	0.565^{b}(3.033)
Black Variety	0.496^{b}(2.187)	0.645^{a}(3.717)
F-value	6.340	4.430
P-value	0.002**	0.013**
$LSD_{0.05}$	0.029	0.065

**Highly significant at $P \leq 0.01$; Means followed by the same letters are not statistically different $P \leq 0.05$; Values not in brackets are transformed [$Log_{10}(x+1)$] means.

The plants that were uninoculated and unfertilized plants had the highest number of nodules, followed by those inoculated with *S. faecium*, *K. daneshvariae*, and *S. pavanii* whereas the ones uninoculated and fertilized had the lowest number of nodules, followed by those

inoculated with *S. maltophila*, *C. cullulans* and *B. pumilus* P12 (Table 5.5). Furthermore, uninoculated and unfertilized plants those inoculated with *P. columbae*, *M. sciuri*, and *K. daneshvariae* had the highest nodulation effectiveness score whereas the uninoculated and fertilized plants, those inoculated with *S. maltophila*, *S. faecium*, and *B. pumilus* P4 had the lowest nodulation effectiveness score (Table 5.5).

Additionally, uninoculated and unfertilized plants followed by those inoculated with *S. faecium*, *K. daneshvariae,* and *P. columbae* had the highest active nodules, whereas uninoculated and fertilized plants along with those inoculated with *Bacillus pumilus* P12, *C. cullulans*, and *S. maltophila* had the lowest active nodules (Table 5.5). However, *S. maltophila* inoculated followed by uninoculated and unfertilized plants, *B. pumilus* P4, and *P. columbae* had the highest nodule colour and abundance score, whereas the uninoculated and fertilized plants, those inoculated with *B. pumilus* P12, *C. cullulans*, and *E. asburiae* had the lowest nodule colour and abundance score (Table 5.5). Although the relative nitrogen-fixing effectiveness did not differ among plants inoculated with different nitrogen fixing bacteria, all plants exhibited very high relative nitrogen-fixing effectiveness (Table 5.5).

Table 5.5: Effect of Indigenous bacterial strains on the number of nodules (NN), active nodules (AN), nodulation colour and abundant score (NCAS), nodulation effectiveness score (NES), and relative nitrogen fixing effectiveness (RE) for retained Bambara groundnut varieties

Bacteria	NN	AN	NCAS	NES	RE
Positive control	0.964^{c}(8.371)	0.291^{d}(0.200)	0.222^{d} (0.933)	0.841^{b}(6.267)	2.004^{a} (100.000)
Negative control	1.512^{a} (36.000)	1.357^{a}(25.333)	0.773^{ab} (4.933)	1.075^{a}(11.000)	2.037^{a} (115.270)
Sphingobacterium faecium	1.485^{ab} (31.225)	1.360^{a}(23.673)	0.726^{abc}(4.667)	1.019^{a}(9.733)	2.026^{a} (114.330)
Proteus columbae	1.447^{ab} (28.600)	1.330^{ab}(22.200)	0.768^{abc}(4.867)	1.050^{a}(10.333)	2.016^{a} (113.470)
Mammaliicoccus sciuri	1.407^{ab} (29.333)	1.245^{abc}(19.267)	0.757^{abc}(4.733)	1.045^{a}(10.200)	2.047^{a} (117.470)
Bacillus pumilus P12	1.392^{ab} (27.133)	1.101^{c}(15.400)	0.657^{bc}(3.933)	1.024^{a}(9.867)	1.991^{a} (110.470)
Bacillus pumilus P4	1.392^{ab}(28.733)	1.252^{abc}(21.467)	0.773^{ab}(4.933)	1.027^{a}(9.800)	1.990^{a} (112.870)
Stenotrophomus pavanii	1.434^{ab} (29.776)	1.283^{abc}(20.487)	0.721^{abc}(4.600)	1.035^{a}(10.133)	1.969^{a} (99.470)
Kaistella daneshvariae	1.460^{ab} (31.000)	1.329^{ab}(22.367)	0.7463^{abc}(4.733)	1.031^{a}(10.200)	2.039^{a} (118.870)
Stenotrophomonas maltophilia	1.353^{b} (23.333)	1.271^{abc}(18.733)	0.778^{a}(5.000)	1.016^{a}(9.533)	1.991^{a} (102.330)
Enterobacter asburiae	1.386^{ab} (27.267)	1.224^{abc}(21.433)	0.6839^{abc}(4.267)	1.023^{a}(9.867)	1.991^{a} (103.400)
Cellulosimicrobium cellulans	1.356^{b} (24.667)	1.1498^{bc}(17.567)	0.654^{c}(4.000)	1.018^{a}(9.800)	2.006^{a} (110.330)

F-valve	4.720	10.680	13.720	5.050	0.280
P-valve	0.000**	0.000**	0.000**	0.000**	0.988^{ns}
$LSD_{0.05}$	0.153	0.190	0.116	0.072	0.065

**Highly Significant at $P \leq 0.01$ and *Not significant at $P > 0.05$; Means followed by the same letters are not statistically different $P \leq 0.05$;

Values not in brackets are transformed [$Log_{10}(x+1)$] means.

5.4 Discussion

5.4.1 Plant growth variables

Nitrogen is among the most important elements in agriculture, playing a key role in plant growth and development (Fathi, 2022). In soils low in nutrients, nitrogen and organic matter can be replenished via root nodule symbiosis with climate-adapted rhizobial symbionts, thereby supporting increased plant productivity (Sarkar *et al*., 2023). According to Mathenge (2017), the nitrogen-fixing potential of leguminous crops is likely to be greater when the soil mineral nitrogen levels are low compared to when nitrogen levels are high. Rhizobial bacteria enhance plant growth and productivity by producing different compounds (Ayangbenro, Adem, & Babalola, 2023).

According to Esan, Oke, and Ogunbode (2023), Bambara groundnut varieties exhibit variation in seed quality, pod characteristics, colour, texture, plant vigor, leaf shape, and both nutritional and anti-nutritional attributes. Based on the study findings, the cream-white eye variety showed the highest plant height, leaf number, and runner count, while the black variety recorded the lowest measurements among the tested varieties, including the red variety. In contrast, Nelwaondo (2020) observed that the brown and red landraces of BGN exhibited superior performance compared to the cream variety in all recorded growth traits. However, the black variety had the highest chlorophyll content, fresh root mass, and dry root mass, while the cream-white eye variety had the lowest chlorophyll content, fresh root mass, and dry root mass. Ajayi, Dianda, and Fagade (2024) reported that TVSU 1248 (Brown variety) had higher values of chlorophyll readings in the 2nd week than the TVSU 631 (Black variety) and the plant inoculated with rhizobia, which is different from the findings of the current study. Contrarily, Ubisi *et al*. (2023) reported that the black Bambara groundnut variety exhibited the highest chlorophyll content, whereas the red variety had the lowest, consistent with the findings of the

present study. The red variety had the highest stem diameter compared to the black and cream-white eye varieties, with the cream-white eye variety having the lowest stem diameter.

Furthermore, the positive control (uninoculated and fertilized plants) followed by *M. sciuri*, *K. daneshvariae*, and *S. maltophila* had the highest stem diameter, while *P. columbae*, *B. pumilus* P4, and *S. pavanii* had the lowest stem diameter. The superior performance of the positive control can be due to the immediate availability of mineral nitrogen and the absence of the metabolic costs associated with biological nitrogen fixation (Hasan, Uddin, Muda Mohamed, & Kee Zuan, 2018). Among the inoculated treatments, plants inoculated with *M. sciuri, K. daneshvariae, and S. maltophilia* demonstrated comparatively greater stem diameter, suggesting improved functional compatibility with Bambara groundnut and potentially higher nitrogen fixation efficiency or additional plant growth–promoting traits (Tang, Li, Wei, Huang, & Zeng, 2024). Such traits may include phytohormone production, enhanced nutrient solubilization, or improved stress tolerance (Tang *et al*., 2024). Similarly, Ajilogba, Babalola, Adebola, and Adeleke (2022) reported significant increases in vegetative growth parameters, including stems, leaves, shoots, and roots in Bambara groundnut following bacterial inoculation compared to the uninoculated control.

5.4.2 Nodulation variables

According to Hassen *et al*. (2023), the nodulation of various genotypes by a given rhizobium species is not consistent for all the landraces due to the presence of several genotypes of Bambara groundnut. Based on the findings of the study, the cream-white eye variety outperformed all the tested varieties in terms of the highest nodule position, with the red variety having the lowest nodule position. Additionally, the black variety had the highest plant growth

vigour compared to the red and cream-white eye varieties, with the cream-white eye variety having the lowest plant growth vigour.

The results of this study revealed that the negative control (uninoculated and unfertilized plants) outperformed all the indigenous Bambara groundnut bacteria isolates and the positive control (uninoculated and fertilized plants). The negative control (uninoculated and unfertilized plants) had the highest number of nodules, followed by *S. faecium*, *K. daneshvariae*, and *S. pavanii,* while the positive control (uninoculated and fertilized plants), followed by *S. maltophila*, *Cellulosimicrobium cellulans*, and *B. pumilus* P12 had the lowest number of nodules. Although the soil and seeds were sterilized, nodulation in the negative control may have occurred due to endophytic nitrogen-fixing bacteria residing within the seeds or root tissues, which can survive surface sterilization (Hassen, Muema, Diale, Mpai, & Bopape, 2025). Additionally, although the study was conducted under controlled greenhouse conditions to minimize contamination, the possibility of low-level bacterial contamination from the air or handling cannot be completely excluded (Kozdrój, Ropek, Frączek, Bulski, & Breza-Boruta, 2024). Contrary to these findings, Imoro, Pobee, and Akabanda (2023) reported that the Bambara groundnut strain 2CL+ treated with nitrogen produced a higher number of nodules, while the 2CL-N strain had a greater nodule dry weight in their study. According to Bitire *et al*. (2023), the variation in the nodule number, nodule weight, and nitrogen fixation observed in Bambara groundnut genotypes was due to the different genetic compositions and their responses to specific nitrogen-fixing bacterial strains.

According to Mweetwa *et al*. (2016), the presence of leghaemoglobin in the nodules results in a strong pinkish colour, which is an indication of active nitrogen fixation. Based on the results of the study, the negative control had the highest active nodules followed by *S. faecium*, *K.*

daneshvariae, and *P. columbae*, whereas the positive control (uninoculated and fertilized plants) had the lowest active nodules followed by *B. pumilus* P12, *C. cullulans*, and *S. maltophila*. However, *S. maltophila* followed by negative control (uninoculated and unfertilized plants), *B. pumilus* P4, and *P. columbae* had the highest nodule colour and abundance score, while the positive control, *B. pumilus* P12, *C. cullulans*, and *E. asburiae* had the lowest nodule colour and abundance score. Similarly, Oloyede, Ojo, and Taiwo (2024) observed that the effective nodules were pink in colour, indicating the presence of leghaemoglobin in greater concentrations.

Furthermore, the negative control (uninoculated and unfertilized plants), *P. columbae*, *M. sciuri*, and *K. daneshvariae* had the highest nodulation effectiveness score, while the positive control (uninoculated and fertilized plants), *S. maltophila*, *S. faecium*, and *B. pumilus* P4 had the lowest nodulation effectiveness score. These results suggest that nitrogen availability and plant-microbe compatibility played an important role in nodule formation, while the reduced nodulation in the positive control is likely attributable to nitrogen-induced suppression of nodulation, as legumes are known to downregulate symbiosis when mineral nitrogen is readily available (Lagunas *et al*., 2019). Moreover, an earlier report by Onyango *et al*. (2015) similarly found that *Rhizobium*, *Bradyrhizobium*, and *Burkholderia* species produced highly effective nodules in Bambara groundnuts with a nodule number that is above 20 per plant. Consistent with the findings of this study, Oloyede *et al*. (2024) reported that out of the 35 rhizobial isolates obtained in their study, only 15 rhizobial isolates successfully induced effective nodulation in Bambara groundnut plants.

Moreover, *K. daneshvariae* had the highest relative nitrogen-fixing effectiveness, followed by *M. sciuri*, negative control, and *S. faecium*, ranging from 114.330 % to 118.870 %. However,

S. pavanii had the lowest relative nitrogen-fixing effectiveness, followed by the positive control, *S. maltophila*, and *E. asburiae*, ranging from 99.470 % to 110.330 %. This can be due to the compatibility of the indigenous nitrogen-fixing bacteria isolates with Bambara groundnut varieties used in this study, revealing *M. sciuri* and *S. faecium* to be more compatible with Bambara groundnut than *S. maltophila*, and *E. asburiae* (Bitire *et al*., 2023; Allito, Ewusi-Mensah, Logah, & Hunegnaw, 2021).

5.5 Conclusion

The results revealed that plants inoculated with *M. sciuri*, *K. daneshvariae*, and *S. maltophila* had the highest stem diameters. *Stenotrophomonas maltophilia*, *B. pumilus* P4, and *P. columbae* enhanced Bambara groundnut nodulation by producing the highest nodule colour and abundance score. Even though all bacteria resulted in very high relative nitrogen-fixing effectiveness (>80%), *K. daneshvariae*, *M. sciuri*, and *S. faecium* inoculated plants demonstrated the highest levels.Moreover, the black variety demonstrated the highest plant growth vigour; chlorophyll content, fresh root mass, and dry root mass, while the red variety had the highest stem diameter. The cream-white eye variety outperformed all the tested varieties in terms of the highest nodule position and the highest plant height, as well as the number of leaves and runners. Although varietal differences were observed, the overall findings support the hypothesis that indigenous nitrogen-fixing bacterial isolates enhance Bambara groundnut nodulation and growth.

5.6 References

Ajayi, O.O., Dianda, M. and Fagade, O.E. 2024. Rhizobia inoculation's impact on the biomass and moisture content of leguminous Bambara groundnut (*Vigna subterranean* L. Verdc). *Discover Sustainability*, 5(1): 1-16.

Ajilogba, C.F., Babalola, O.O., Adebola, P. and Adeleke, R. 2022. Bambara groundnut rhizobacteria antimicrobial and biofertilization potential. *Frontiers in Plant Science*, 13: 1-16.

Allito, B.B., Ewusi-Mensah, N., Logah, V. and Hunegnaw, D.K. 2021. Legume-rhizobium specificity effect on nodulation, biomass production and partitioning of faba bean (*Vicia faba* L.). *Scientific Reports*, 11(1): 1-13.

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): 8218721.

Amani, K., Fondio, L., Ibrahim, K., N'Gbesso, M.F.D., Maxwell, B.G., Sanogo, T.A. and Filali-Maltouf, A. 2020. Response of indigenous Rhizobia to the inoculation of Soybean [*Glycine max* (L.) Merrill] varieties cultivated under controlled conditions in Côte d'Ivoire. *Advances in Microbiology*, 10(03): 110-122.

Bitire, T.D., Abberton, M., Tella, E.O., Edemodu, A., Oyatomi, O. and Babalola, O.O. 2023. Impact of nitrogen-fixation bacteria on nitrogen-fixation efficiency of Bambara groundnut [*Vigna subterranea* (L) Verdc] genotypes. *Frontiers in Microbiology*, 14: 1-11.

Bitire, T.D., Abberton, M., Tella, E.O., Edemodu, A., Oyatomi, O. and Babalola, O.O. 2023. Impact of nitrogen-fixation bacteria on nitrogen-fixation efficiency of Bambara groundnut [*Vigna subterranea* (L) Verdc] genotypes. *Frontiers in Microbiology*, 14: 1-11.

Benmoussa, S., Nouairi, I., Rajhi, I., Rezgui, S., Manai, K., Taamali, W., Abbes, Z., Zribi, K., Brouquisse, R. and Mhadhbi, H. 2022. Growth performance and nitrogen fixing efficiency of faba bean (*Vicia faba* L.) genotypes in symbiosis with rhizobia under combined salinity and hypoxia stresses. *Agronomy*, 12(3): 1-18.

Esan, V.I., Oke, G.O. and Ogunbode, T.O. 2023. Genetic variation and characterization of Bambara groundnut [*Vigna subterranea* (L.) verdc.] accessions under multi-environments considering yield and yield components performance. *Scientific Reports*, 13(1): 1-9.

Fathi, A. 2022. Role of nitrogen (N) in plant growth, photosynthesis pigments, and N use efficiency: A review. *Agrisost*, 28: 1-8.

Fwanyanga, F.M. 2023. *Rhizobia inoculant effects on Bambara groundnut growth and yield in Namibia*. Windhoek: University of Namibia.

Gomez, K.A. and Gomez, A.A. 1984. *Statistical procedures for agricultural research*. Canada: John Wiley & Sons.

Hasan, M., Uddin, M.K., Muda Mohamed, M.T. and Kee Zuan, A.T. 2018. Nitrogen and phosphorus management for Bambara groundnut (*Vigna subterranea*) production-A review. *Legume Research: An International Journal*, 41(4): 1- 483- 489).

Hassen, A.I., Muema, E.K., Diale, M.O., Mpai, T. and Bopape, F.L. 2025. Non-rhizobial endophytes (NREs) of the nodule microbiome have synergistic roles in beneficial tripartite plant–microbe interactions. *Microorganisms*, 13(3): 1- 28.

Hassen, A.I., Bopape, F.L., van Vuuren, A., Gerrano, A.S. and Morey, L. 2023. Symbiotic interaction of Bambara groundnut (*Vigna subterranea*) landraces with rhizobia spp. from other legume hosts reveals promiscuous nodulation. *South African Journal of Botany*, 160: 493-503.

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): 1-17.

Imoro, A.W.M., Pobee, J. and Akaband, F. 2023. Isolation of rhizobia from the nodules of Bambara groundnuts for inoculant production. *Journal of Agricultural Science*, 15: 47-54.

Kozdrój, J., Ropek, D.R., Frączek, K., Bulski, K. and Breza-Boruta, B. 2024. Microbial indoor air quality within greenhouses and polytunnels is crucial for sustainable horticulture (Malopolska Province, Poland conditions). *Sustainability*, 16(22): 1-22.

Lagunas, B., Achom, M., Bonyadi-Pour, R., Pardal, A.J., Richmond, B.L., Sergaki, C., Vázquez, S., Schäfer, P., Ott, S., Hammond, J. and Gifford, M.L. 2019. Regulation of resource partitioning coordinates nitrogen and rhizobia responses and autoregulation of nodulation in *Medicago truncatula*. *Molecular plant*, 12(6): 833-846.

Mathenge, C.N. 2017. *Soybean (Glycine max) response to rhizobia inoculation as influenced by soil nitrogen levels*. Nairobi: Kenyatta University.

Mweetwa, A.M., Chilombo, G. and Gondwe, B.M. 2016. Nodulation, nutrient uptake and yield of common bean inoculated with rhizobia *and trichoderma* in an acid soil. *Journal of Agricultural Science*, 8(12): 61-71.

Nelwamondo, A. 2020. Assessment of co-inoculation of *Bradyrhizobium japonicum* and *Bacillus subtitles* on yield and metabolic profile of Bambara groundnut and cowpea under glasshouse conditions. *Institutional Repository*, 1(1): 1- 80.

Oloyede, A.R., Ojo, A.E. and Taiwo, E.A. 2024. Genetic characterisation and symbiotic efficiency of rhizobia nodulating different landraces of Bambara groundnut (*Vigna subterranea* L. Verdc). *Nigerian Journal of Microbiology*, 38(1): 6987-6997.

Onyango, B., Anyango, B., Nyunja, R., Koech, P.K., 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(1): 1-10.

Pommeresche, R. and Hansen, S. 2017. Examining root nodule activity on legumes. Fertilcrop Technical Note.

Sarkar, A., Fwanyanga, F.M., Horn, L.N., Welzel, S., Diederichs, M., Kerk, L.J., Zimmermann, M. and Reinhold-Hurek, B. 2023. Towards inoculant development for Bambara groundnut (*Vigna subterranean* (L.) Verdc) pulse crop production in Namibia. *Frontiers in Plant Science*, 14: 1-16.

Sanni, K.O. and Adenubi, O.O. 2020. Influence of intra row spacing on weed suppression in cucumber (*Cucumis sativus*) production in humid rainforest agro-ecological zone of Lagos, Nigeria. *Global Journal of Ecology*, 5(1): 038-043.

Shapiro, S.S. and Wilk, M.B. 1965. An analysis of variance test for normality (complete samples). *Biometrika*, 52(3-4): 1-12.

Tan, X.L., Azam-Ali, S., Goh, E.V., Mustafa, M., Chai, H.H., Ho, W.K., 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: 1-16.

Tang, J., Li, W., Wei, T., Huang, R. and Zeng, Z. 2024. Patterns and mechanisms of legume responses to nitrogen enrichment: A global meta-analysis. *Plants*, 13(22): 1- 27.

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.

CHAPTER SIX

SUMMARY, SIGNIFICANCE OF FINDINGS, FUTURE RESEARCH, CONCLUSION, AND RECOMMENDATION

6.1 Summary

Bambara groundnut (*Vigna subterranea* (L.) Verdc.) is a nutrient-rich and drought-resilient legume considered the third most important leguminous crop in Africa, after cowpea and groundnut, with notable production in several South African provinces (Majola, Gerrano, & Shimelis, 2021). Its capacity to establish symbiotic associations with nitrogen-fixing bacteria enhances both crop productivity and soil fertility, making it a potential solution to food insecurity and sustainable agriculture (Olanrewaju, Oyatomi, Babalola, & Abberton, 2022). However, many communal farmers grow Bambara groundnut in degraded soils with low microbial diversity and fertility (Khan, Rafii, Ramlee, Jusoh, & Al-Mamun, 2021). Climate change has worsened these conditions by increasing soil salinity and altering pH levels, which negatively affect rhizobial activity, nodulation, and plant growth (Torabian, Farhangi-Abriz, & Denton, 2019). Rhizobial inoculants are promoted as sustainable biofertilizers, especially when derived from indigenous strains that are well adapted to local environmental conditions (Nelwamondo, 2020; Sanchez-Navarro *et al*., 2020).

Differently coloured Bambara groundnut seed coat extracts inhibited the growth of some indigenous nitrogen-fixing bacterial strains. This confirms that Bambara groundnut seed coats possess notable antimicrobial properties, particularly against certain indigenous nitrogen-fixing bacterial strains, highlighting the necessity to examine the chemical compounds present in each seed coat. The tolerance of Bambara groundnut indigenous nitrogen-fixing bacterial isolates to low pH levels was level-dependent, with lower pH levels exhibiting lower absorbance compared to higher pH levels. None of the indigenous bacterial isolates could

tolerate extremely high salinity levels, but isolates were tolerant and grew well in low salinity concentrations. This reveals the capability of these native BGN bacterial strains for use in biofertilizer development under acidic and mildly saline soil conditions.

Nitrogen-fixing bacteria isolates had different effects on the nodulation and growth of BGN, whereby plants inoculated with *Mammaliicoccus sciuri*, *Kaistella daneshvariae*, and *Stenotrophomonas maltophilia* had the highest stem diameters. *Stenotrophomonas maltophilia*, *Bacillus pumilus* P4, and *Proteus columbae* produced the highest nodule colour and abundance score. In contrast, *Kaistella daneshvariae*, *Mammaliicoccus sciuri*, the negative control, and *Sphingobacterium faecium* treated plants demonstrated the highest relative nitrogen-fixing effectiveness. However, the negative control outperformed all indigenous Bambara nitrogen-fixing isolated strains and the positive control in having the greatest number of nodules, active nodules, and nodulation effectiveness score. The black variety demonstrated the highest plant growth vigour; chlorophyll content, fresh root mass, and dry root mass, while the red variety had the highest stem diameter. The cream-white eye variety outperformed all tested varieties in terms of the highest number of leaves, the highest plant height, number of runners, and the highest nodule position.

6.2 Significance of findings

The tested Bambara groundnut seed coat extracts revealed that they possess antimicrobial properties that can negatively impact the symbiotic association between nitrogen-fixing bacteria and the crop, influencing the type of native bacterial strain that can successfully induce nodulation and fix nitrogen. *Cellulosimicrobium cellulans*, *Stenotrophomonas pavanii* , and *P. columbae* had the highest absorbance, ranging from 0.519 to 0.69, indicating their ability to grow better across varying pH levels. *Bacillus pumilus* P12, *Bacillus pumilus* P4, and *S.*

maltophila demonstrated superior tolerance to salinity concentrations, with the highest radial growth ranging from 10.653 to 21.889 mm. The bacteria of Bambara groundnut had a general tolerance of pH of 4.12 and higher, while salinity higher than 4.24 mM/m3 could not be tolerated. The information generated can be used to develop an inoculum for biofertilizer use in Bambara groundnut production, utilizing indigenous bacterial strains adapted to local environments and tolerant to acidic and mildly saline soil conditions.

Only *M. sciuri*, *K. daneshvariae*, and *S. maltophila* had the highest stem diameters, while *S. maltophila*, *B. pumilus* P4, and *P. columbae* produced the highest nodule colour and abundance score from all the tested isolates. *Kaistella daneshvariae*, *M. sciuri*, the negative control, and *S. faecium* treated plants demonstrated the highest relative nitrogen-fixing effectiveness. The results revealed that plant performance varied significantly among the black, red, and cream-white eye varieties. The cream-white eye variety showed superior vegetative growth traits such as plant height, number of leaves, and runners, while the black variety excelled in chlorophyll content, fresh and dry root mass, and plant growth vigour. The red variety demonstrated the greatest stem diameter, further emphasizing the phenotypic variability among the landraces.

6.3 Future research

The antimicrobial properties of BGN seed coat extract should be investigated further using all Bambara groundnut varieties to determine the effect that the untested varieties have on indigenous nitrogen-fixing bacteria. Future studies should focus on characterizing the bioactive compounds that contribute to antimicrobial activity and evaluating their impact on the symbiosis between nitrogen-fixing bacteria and BGN. This is essential for improving the effective use of Bambara groundnut in sustainable agriculture and inoculant development. There is a need to use the selected Bambara groundnut indigenous nitrogen-fixing bacterial

isolates that were tolerant to low pH, low salinity concentrations, and had efficient nitrogen fixation in forming inocula for Bambara groundnut to enhance plant productivity, biological nitrogen fixation, and soil health in sustainable agricultural systems. The type of organic carriers that can be utilized for developing biofertilizer inoculum and their effect on the survival of indigenous nitrogen-fixing bacteria isolates need to be investigated in field experiments. The selected bacterial isolates must be subjected to field trials across different provinces and agroecological zones to assess their consistency and effectiveness in improving Bambara groundnut performance.

6.4 Conclusion

The development of effective bioinoculants for challenging agroecological environments requires the identification of indigenous nitrogen-fixing bacterial strains that are tolerant to acidic and saline soil conditions while maintaining high nitrogen-fixing efficiency. This study aimed to evaluate the survival of indigenous nitrogen-fixing bacteria on seed coat extracts, their tolerance to soil salinity and pH, and their efficiency on retained Bambara groundnut in pursuit of producing high-quality nitrogen-fixing bacterial inoculants.

The results demonstrated that Bambara groundnut seed coat extracts possess antimicrobial properties. Despite this inhibitory effect, several isolates exhibited notable resilience under abiotic stress conditions. All evaluated isolates showed some degree of tolerance to acidic and saline environments, however, growth performance varied among strains. Notably, *C. cullulans*, *S. pavanii*, and *P. columbae* showed superior adaptability across varying pH levels. The findings therefore partially support the hypothesis that indigenous nitrogen-fixing bacteria are tolerant of low pH and high salinity, as tolerance to salinity was moderate and strain-dependent rather than universal.

Furthermore, *Mammaliicoccus sciuri*, *K. daneshvariae*, *S. maltophila*, *B. pumilus* P4, and *P. columbae* showed enhanced nodule performance, high relative nitrogen-fixing effectiveness, and significant improvement in the growth parameters of Bambara groundnut. These results indicate strong potential for selected indigenous isolates to be developed into bioinoculant formulations aimed at improving Bambara groundnut productivity under deficient soil conditions.

Overall, the study achieved its objectives by confirming the antimicrobial activity of BGN seed coat extracts, identifying bacterial isolates tolerant to low pH and moderate salinity, and selecting strains with high nitrogen-fixing effectiveness suitable for future bioinoculant development.

6.5 Recommendation

Studies can be conducted on the compatibility of Bambara groundnut seed coat extracts with beneficial rhizobial strains, due to the observed antimicrobial effects of the extracts, in order to improve bioinoculant formulations. *Cellulosimicrobium cellulans*, *S. pavanii*, and *P. columbae* thrived various pH levels, while *B. pumilus* P12, *Bacillus pumilus* P4, and *S. maltophila* were more tolerant to low salinity concentrations, with the lowest pH that could be tolerated by the bacteria calculated to be 4.12, while the highest salinity that could be tolerated by the bacteria was determined to be 4.24 mM/m^3. These isolates can be used in marginal soils where low pH and salinity limit crop productivity in order to improve it. Indigenous bacterial isolates *M. sciuri*, *K. daneshvariae*, *S. maltophila*, *B. pumilu*s P4, and *P. columbae* can be prioritized when developing locally adapted inoculants suited to low-input and environmentally stressed farming systems, as they were among the most effective in enhancing nodulation, nitrogen fixation, and plant growth parameters of Bambara groundnut. The cream-

white eye variety outperformed all the tested varieties in leaf production and is recommended for use where Bambara groundnut leaves are harvested for medicinal purposes, such as treating wounds and abscesses in animals. The black variety had the highest plant growth vigour, chlorophyll content, fresh root mass, and dry root mass, and may be suitable when focusing on the vegetative growth of the plant and improving soil health.

6.6 References

Khan, M.M.H., Rafii, M.Y., Ramlee, S.I., Jusoh, M. and Al-Mamun, M. 2021. Bambara groundnut (*Vigna subterranea* L. Verdc): A crop for the new millennium, its genetic diversity, and improvements to mitigate future food and nutritional challenges. *Sustainability*, 13(10): 1-27.

Majola, N.G., Gerrano, A.S., & Shimelis, H. 2021. Bambara Groundnut (*Vigna subterranea* [L.] Verdc.) production, utilisation and genetic improvement in Sub-Saharan Africa. *Agronomy,* 11(7): 1-16.

Nelwamondo, A. 2020. Assessment of co-inoculation of *Bradyrhizobium japonicum* and *Bacillus subtitles* on yield and metabolic profile of Bambara groundnut and cowpea under glasshouse conditions. *Institutional Repository*, 1(1): 1-80.

Olanrewaju, O.S., Oyatomi, O., Babalola, O.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.

Sanchez-Navarro, V., Zornoza, R., Faz, Á., Egea-Gilabert, C., Ros, M., Pascual, J.A. and Fernández, J.A. 2020. Inoculation with different nitrogen-fixing bacteria and arbuscular mycorrhiza affects grain protein content and nodule bacterial communities of a Fava Bean Crop. *Agronomy*, 10(6): 1-15.

Torabian, S., Farhangi-Abriz, S. and Denton, M. 2019. Do tillage systems influence nitrogen fixation in legumes? A review. *Soil and Tillage Research*, 185(1): 1-9.

Zenabou, N., Firmin, S.L., Laurette, N.N., Daniel, W.F., Laurianne, T.N. and Martin, B.J. 2022. Nodulation potential of Bambara groundnut (*Vigna subterranea* L.) in Yaounde (Centre Region of Cameroon). *American Journal of Food and Nutrition*, 10(1): 34-39.

APPENDICES

Appendix 3.1: Shapiro-Wilk normality test for the zone of inhibition

Variable	N	W	P
Zone of inhibition	1620	0.5232	0.0000

Appendix 3.2: Analysis of variance for the zone of inhibition

Source	DF	SS	MS	F	P
Replication	2	0.583	0.2915		
Variety (V)	2	0.328	0.1639	2.02	0.1327
Bacteria (B)	9	4.457	0.4952	6.11	0.0000
Extracts (E)	5	432.236	86.4472	1067.06	0.0000
Variety*Bacteria	18	0.595	0.0330	0.41	0.9867
Variety*Extracts	10	1.153	0.1153	1.42	0.1642
Bacteria*Extracts	45	8.430	0.1873	2.31	0.0000
Variety*Bacteria*Extracts	90	4.382	0.0487	0.60	0.9988
Error	1438	116.498	0.0810		
Total	1619	568.661			

Appendix 4.1: Salinity levels of Sodium chloride

NaCl in mM/m^3	Mass (g)
0	0
2	0.12
4	0.23
8	0.47
16	0.94
32	1.87
64	3.74
128	7.48

Appendix 4.2: Salinity levels of calcium chloride

$CaCl_2$ in mM/m^3	Mass (g)
0	0
0.67	0.10
1.33	0.20
2.67	0.39
5.33	0.78
10.67	1.57

21.33	3.14
42.67	6.27

Appendix 4.3: Salinity level ratios of sodium chloride and calcium chloride levels

Salinity levels	NaCl (g)	$CaCl_2$ (g)
0	0	0
1	0.12	0.10
2	0.23	0.20
3	0.47	0.39
4	0.94	0.78
5	1.87	1.57
6	3.74	3.14
7	7.48	6.27

Appendix 4.4: Shapiro-Wilk normality test for absorbance and radial growth

Variable	N	W	P
Absorbance	360	0.8519	0.0000
Radial growth	720	0.8262	0.0000

Appendix 4.5: Analysis of variance for absorbance

Source	DF	SS	MS	F	P
Replication	2	0.00610	0.00305		
Bacteria	9	0.24000	0.02667	3.31	0.0007
pH	3	0.95091	0.31697	39.37	0.0000
Bacteria*pH	27	0.26225	0.00971	1.21	0.2245
Error	318	2.56050	0.00805		
Total	359	4.01974			

Appendix 4.6: Analysis of variance for radial growth

Source	DF	SS	MS	F	P
Replication	2	0.027	0.01373		
Salinity levels	7	4.335	0.61932	2.81	0.0068
Bacteria	9	20.371	2.26340	10.29	0.0000
Salinity levels*Bacteria	63	11.582	0.18384	0.84	0.8124
Error	158	19.9137	0.12604		
Total	239	65.7328			

Appendix 5.1: Shapiro-Wilk normality test for plant growth variables

Variable	N	W	P
Plant height (PH)	180	0.9929	0.5335
Number of leaves (NL)	180	0.9748	0.0024
Number of runners (NR)	168	0.8037	0.0000
Length of the longest runner (LLR)	167	0.9174	0.0000
Chlorophyll content (CC)	179	0.9888	0.1687
Stem diameter (SD)	180	0.9815	0.0170
Fresh shoot mass (FSM)	179	0.9789	0.0081
Dry shoot mass (DSM)	180	0.9791	0.0082
Fresh root mass (FRM)	180	0.9769	0.0043
Dry root mass (DRM)	180	0.8455	0.0000

Appendix 5.2: Shapiro-Wilk test for nodulation variables

Variable	N	W	P
Nodule position (NP)	175	0.7426	0.0000
Number of nodules (NN)	175	0.9494	0.0000
Active nodules (AN)	169	0.9661	0.0004
Non active nodules (NA)	163	0.8702	0.0000

Nodule colour and abundance score (NCAS)	180	0.5082	0.0000
Plant growth vigor (PGV)	180	0.7639	0.0000
Nodulation effectiveness score (NES)	180	0.9308	0.0000
Relative effectiveness (RE)	180	0.9468	0.0000

Appendix 5.3: Analysis of variance for plant height

Source	DF	SS	MS	F	P
Replication	4	91.56	22.8906		
Varieties (V)	2	75.68	37.8389	3.7	0.0270
Bacteria (B)	11	44.66	4.0602	0.40	0.9551
Varieties*Bacteria	22	289.02	13.1374	1.29	0.1907
Error	140	1429.87	10.2134		
Total	179	1930.79			

Appendix 5.4: Analysis of variance for the number of leaves

Source	DF	SS	MS	F	P
Replication	4	0.06889	0.01722		
Varieties (V)	2	0.08251	0.04125	5.42	0.0054
Bacteria (B)	11	0.06731	0.00612	0.80	0.6366

Varieties*Bacteria	22	0.09321	0.00424	0.56	0.9449
Error	140	1.06649	0.00762		
Total	179	1.37841			

Appendix 5.5: Analysis of variance for number of runners

Source	DF	SS	MS	F	P
Replication	4	0.37881	0.09470		
Varieties (V)	2	0.48575	0.24288	8.19	0.0004
Bacteria (B)	11	0.14968	0.01361	0.46	0.9250
Varieties*Bacteria	22	0.53229	0.02420	0.82	0.7011
Error	128	3.79471	0.02965		
Total	167				

Appendix 5.6: Analysis of variance for length of the longest runner

Source	DF	SS	MS	F	P
Replication	4	0.7985	0.19962		
Varieties (V)	2	0.2237	0.11184	1.13	0.3262
Bacteria (B)	11	1.2095	0.10995	1.11	0.3578
Varieties*Bacteria	22	2.1698	0.09863	1.00	0.4733

Error	127	12.5663	0.09895		
Total	166				

Appendix 5.7: Analysis of variance for chlorophyll content

Source	DF	SS	MS	F	P
Replication	4	300.78	75.194		
Varieties (V)	2	263.62	131.809	4.00	0.0205
Bacteria (B)	11	561.06	51.006	1.55	0.1214
Varieties*Bacteria	22	676.47	30.749	0.93	0.5529
Error	139	4582.42	32.967		
Total	178				

Appendix 5.8: Analysis of variance for stem diameter

Source	DF	SS	MS	F	P
Replication	4	0.12577	0.03144		
Varieties (V)	2	0.20546	0.10273	10.56	0.0001
Bacteria (B)	11	0.21204	0.01928	1.98	0.0345
Varieties*Bacteria	22	0.27690	0.01259	1.29	0.1855

Error	140	1.36196	0.00973		
Total	179	2.18213			

Appendix 5.9: Analysis of variance for fresh shoot mass

Source	DF	SS	MS	F	P
Replication	4	0.21587	0.05397		
Varieties (V)	2	0.01126	0.00563	0.43	0.6503
Bacteria (B)	11	0.08270	0.00752	0.58	0.8455
Varieties*Bacteria	22	0.28504	0.01296	0.99	0.4767
Error	139	1.81299	0.01304		
Total	178				

Appendix 5.10: Analysis of variance for dry shoot mass

Source	DF	SS	MS	F	P
Replication	4	0.08911	0.02228		
Varieties (V)	2	0.00063	0.00031	0.06	0.9445
Bacteria (B)	11	0.04361	0.00396	0.72	0.7132
Varieties*Bacteria	22	0.12339	0.00561	1.03	0.4380

Error	140	0.76579	0.00547		
Total	179	1.02254			

Appendix 5.11: Analysis of variance for fresh root mass

Source	DF	SS	MS	F	P
Replication	4	0.22748	0.05687		
Varieties (V)	2	0.10905	0.05453	3.37	0.0372
Bacteria (B)	11	0.11056	0.01005	0.62	0.8083
Varieties*Bacteria	22	0.22185	0.01008	0.62	0.9016
Error	140	2.26482	0.0168		
Total	179	2.93376			

Appendix 5.12: Analysis of variance for dry root mass

Source	DF	SS	MS	F	P
Replication	4	0.03561	0.00890		
Varieties (V)	2	0.02284	0.1142	4.22	0.0166
Bacteria (B)	11	0.00566	0.00051	0.19	0.9979
Varieties*Bacteria	22	0.05532	0.00251	0.93	0.5563

Error	140	0.37581	0.00270		
Total	179				

Appendix 5.13: Analysis of variance for relative effectiveness

Source	DF	SS	MS	F	P
Replication	4	1.00176	0.25044		
Varieties (V)	2	0.16024	0.08012	2.55	0.0814
Bacteria (B)	11	0.09645	0.00877	0.28	0.9888
Varieties*Bacteria	22	0.29650	0.01348	0.43	0.9881
Error	140	4.39223	0.03137		
Total	179	5.94716			

Appendix 5.14: Analysis of variance for nodule position

Source	DF	SS	MS	F	P
Replication	4	0.04815	0.01204		
Varieties (V)	2	0.08148	0.04074	6.34	0.0023
Bacteria (B)	11	0.06591	0.00599	0.93	0.5114
Varieties*Bacteria	22	0.08258	0.00375	0.58	0.9284

Error	135	0.86737	0.00642		
Total	174				

Appendix 5.15: Analysis of variance for number of nodules

Source	DF	SS	MS	F	P
Replication	4	1.63468	0.40867		
Varieties (V)	2	0.14662	0.07331	1.63	0.2001
Bacteria (B)	11	2.33832	0.21257	4.72	0.0000
Varieties*Bacteria	22	0.86173	0.03917	0.87	0.6336
Error	135	6.07904	0.04503		
Total	174				

Appendix 5.16: Analysis of variance for active nodules

Source	DF	SS	MS	F	P
Replication	4	1.59325	0.39831		
Varieties (V)	2	0.13269	0.06635	0.96	0.3859
Bacteria (B)	11	8.12449	0.73859	10.68	0.0000
Varieties*Bacteria	22	1.42689	0.06486	0.94	0.5466

Error	129	8.92132	0.06916		
Total	168				

Appendix 5.17: Analysis of variance for non-active nodules

Source	DF	SS	MS	F	P
Replication	4	3.2374	0.80934		
Varieties (V)	2	0.0282	0.01409	0.13	0.8755
Bacteria (B)	11	0.9500	0.08636	0.82	0.6242
Varieties*Bacteria	22	2.8247	0.12839	1.21	0.2494
Error	123	13.0198	0.10585		
Total	162				

Appendix 5.18: Analysis of variance for nodule colour and abundance score

Source	DF	SS	MS	F	P
Replication	4	0.14364	0.03591		
Varieties (V)	2	0.00234	0.00117	0.05	0.9555
Bacteria (B)	11	3.87838	0.35258	13.72	0.0000
Varieties*Bacteria	22	0.48960	0.02225	0.87	0.6383

Error	140	3.59646	0.02569		
Total	179	8.11042			

Appendix 5.19: Analysis of variance for plant growth vigor

Source	DF	SS	MS	F	P
Replication	4	0.42187	0.10547		
Varieties (V)	2	0.28610	0.14305	4.43	0.0137
Bacteria (B)	11	0.49718	0.04520	1.40	0.1799
Varieties*Bacteria	22	0.45591	0.02072	0.64	0.8875
Error	140	4.52495	0.03232		
Total	179	6.18600			

Appendix 5.20: Analysis of variance for nodulation effectiveness score

Source	DF	SS	MS	F	P
Replication	4	0.04509	0.01127		
Varieties (V)	2	0.03827	0.01914	1.91	0.1519
Bacteria (B)	11	0.55694	0.05063	5.05	0.0000

Varieties*Bacteria	22	0.15979	0.00726	0.72	0.8080
Error	140	1.40273	0.01002		
Total	179	2.20283			