Selection of Efficient Indigenous Rhizobia Inoculants for the Production of Selected Tropical Legumes in Limpopo Province (South Africa)

by

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Abstract

Tropical legumes are important food crops for human and animal nutrition as well as the improvement of soil fertility. In Southern Africa, tropical legumes are cultivated mostly by smallholder farmers partly because of their ability to thrive in poor soils and adverse weather conditions. Tropical legumes are useful in these cropping systems because of their ability to fix nitrogen (N) thus minimizing the necessity for chemical nitrogenous fertilizers. Soil rhizobia (as bio-inoculants) can enhance the productivity of these legumes through the improvement of soil fertility. However, both the compatibility and competency of individual rhizobial strains is important for attaining optimum crop productivity. Therefore, it is imperative to identify the best efficient rhizobial strain x legume genotype combinations for successful nodulation and optimum legume yield for the benefit of resource-limited farmers in smallholder farming systems and end-users. The aim of this study was to improve the productivity of tropical legumes. The specific objectives of the study were to (i) determine efficient rhizobial strains that combine with tropical legume species to produce optimum crop productivity and (ii) determine efficient tropical legume species x rhizobial genus combinations that produce optimum crop productivity.

The study consisted of two experiments both of which were carried out at the Agricultural Research Council (ARC), Plant Health and Protection (Pretoria) greenhouse and laboratory facilities (25° 61’ 547” S, 28° 36’ 435” E). The conditions in the greenhouse were set at a 14 h day temperature of 28° C and 10 h night temperature of 15 °C. In the first experiment, four tropical legumes (pigeonpea; soybean; tepary bean; bambara groundnut) were used in the study. At planting, each legume species was inoculated separately with each of 15 rhizobial strains and allowed to grow for six weeks after which a range of N fixation variables were evaluated. The experiment was laid out in a split plot design with legume species as the main factor and rhizobial strain as the sub-factor. The quantitative data sets on leaf color score (LCS), nodule dry weight (NDW), shoot dry weight (SDW) and root dry weight (RDW) were subjected to standard analysis of variance (ANOVA) procedures and GGE biplot analysis. In the second experiment, three tropical legumes and four rhizobial strains (each from a distinct genus) which were selected from experiment 1 were used. A split-split plot design with the rhizobial strain as the main factor, legume species as the sub-factor and legume variety as sub-sub factor with two replications was used and the data sets of the N fixation variables were analyzed following the same procedure as described above.

The results showed that there was variation in legume species x rhizobial strain compatibility and the pattern of root nodulation varied within the legume species depending on the specific strain used. Tepary bean nodulated poorly with most of the rhizobial strains. There were

significant differences among the legume species for all the traits that were measured. However, there were no significant ($P < 0.05$) differences among the rhizobial strains for NDW, RDW and SDW. The highest NDW (0.05 g) and SDW (0.42 g) were attained by bambara groundnut. The mean LCS for the trial was (19.26) while the highest (23.65) and lowest (14.23) LCS were associated with the rhizobial strains *Bradyrhizobium elkanii* (R4) and *Phyllobacterium leguminum* (R11), respectively. Among the *Paraburkholderia* species, the rhizobial strain *Paraburkholderia phenolyruptix* (R1) was associated with the highest (22.08) LCS. The results also revealed that the rhizobial strains *Rhizobium leucaenae* (R8) and *Rhizobium* sp (R6) induced the highest (20.89) and lowest (15.24) LCS, respectively. However, the rhizobial strain *Bradyrhizobium elkanii* (R14) which attained a relatively high LCS, was associated with the heaviest NDW among all the strains.In contrast, there were detectable nodules associated with four rhizobial strains. Two distinct rhizobial species, namely *Paraburkholderia* sp (R2) (designated N362) and *Bradyhizobium lupini* (R5) were associated with the heaviest shoots across the legume species. In contrast, the control strain achieved the lowest (0.25 g) SDW. In addition, all the three strains from the genus *Paraburkholderia* and the single strain from the genus *Phyllobacterium* as well as all the five from the genus *Bradyrhizobium* (R4; R5; R12; R13 and R14) were associated with significantly ($P< 0.05$) heavier SDW than the control. The results also revealed highly significant ($P < 0.01$) positive correlations between the LCS and NDW. However, the LCS was negatively but significantly ($P < 0.05$) correlated with the RDW. In addition, there were highly significant positive correlations between the SDW and each of NDW and RDW.

The variety x legume species interaction was highly significant ($P < 0.001$) for all the attributes that were measured except for NDW. Inoculation with *Bradyrhizobium* sp (33a-PP4) showed varietal differences in the pattern of N fixation indicators in bambara groundnut and pigeonpea. Nonetheless, some of the soybean varieties formed no nodules after inoculation with the *Paraburkholderia* sp and *Phyllobacterium leguminum* strains. Similarly, soybean responded poorly to inoculation with *Rhizobium* sp. (34a-PP5) and *Bradyrhizobium* sp (33a-PP4). In pigeonpea, all the four varieties that were used in the study showed similar LCS values. The SDW was used for determining the ideal rhizobial genus and legume species by applying the GGE biplot method in which the legume species and rhizobial strains were coded as environment scores and genotypes scores, respectively.

The GGE biplot analysis indicated that 60.0% of the rhizobial strains were distributed in the top left quadrant but none in the bottom right quadrant. The rhizobial strains ‘R2’ (*Rhizobium* sp; 34a2-PP5) and ‘R1’ (*Paraburkholderia* sp; N362), were positioned far away from the origin suggesting that they uniquely influenced the legume species. The biplot analysis also revealed

that the legume species (coded as environment scores), particularly 'E1' (pigeonpea) and 'E2' (soybean), were separated by acute angles between them and grouped in the same top right quadrant. In contrast, the remainder of the pairs of legume species were separated by obtuse angles with each suggesting that they were negatively related to each other in terms of the SDW trait. The legume species 'E3' (tepary bean) showed the shortest absolute projection suggesting that it was the most stable in performance across the rhizobial strains. In determining the 'which-won where', the biplot analysis explained 95.23% total variation of which PC1 and PC2 accounted for 84.41% and 10.82%, respectively. The results also revealed that among the rhizobial strains (depicted as genotypes) on the vertices of the polygon 'R2' (*Rhizobium* sp; 34a2-PP5) and 'R4' (*Bradyrhizobium elkanii*; 33a-PP4), performed best with pigeonpea (E1) and soybean (E2), respectively (Fig. 10). Bambara groundnut (E4') showed the longest vector, suggesting that it had a high discriminating ability. The rhizobial strain 'R2' (*Rhizobium* sp; 34a2-PP5) was positioned in the innermost concentric circle, thus representing the ideal and most stable strain for SDW among the strains.

The study findings provided new information in the patterns of N fixation among a set of tropical legumes that were inoculated with distinct rhizobial genera. The information will be useful in future for formulating bio-inoculants that may improve legume productivity in Limpopo Province (South Africa) where all the legume species that were used in this study are cultivated. The validation of the symbiotic efficiency of *Bradyrhizobium elkanii* (33a-PP4) and *Phyllobacterium leguminum*; 31b-PP4) with more diverse bambara groundnut germplasm will be merited.

Key words: biplot analysis; genus; inoculant; legume species; productivity; symbiotic efficiency.

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Dedication

This work is dedicated to myself for doing all this hard work, for believing in myself and for not quitting.

Declaration

I, Nemaembeni, Phethani Muofhe, hereby declare that this dissertation, for the Master of Science in Agriculture (Crop Science) in the Department of Plant and Soil Sciences at the University of Venda, hereby submitted by me, has not previously been submitted for a degree at this or any other university. It is my own work in design and execution. All reference material contained therein has been duly acknowledged.

Student: Nemaembeni, P.M.

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Table of Contents

List of Abbreviations

ARC = Agricultural Research Council
ATP = adenosine triphosphate
cm = centimeter
DNA = deoxyribonucleic acid
FT-IR = fourier-transformed infrared spectroscopy
g = gram
GGE = genotype main effect plus genotype by environment interaction
ha = hectare
kg = kilogram
L = litre
LCS = leaf colour score
LS = legume species
LSD = least significant difference
MALDI-TOF MS = matrix-assisted laser desorption ionization-time-of-flight mass spectrometry
µL = micro litre
ml = millilitre
MLEE = multilocus enzyme electrophoresis
MLSA = multilocus sequence analysis
MLST = multilocus sequence typing
N = nitrogen
NDW = nodule dry weight
PCR = polymerase chain reaction
PHT = plant height
RDW = root dry weight
RFLP = restriction fragment length polymorphism
RI = rhizobial strain
rpm = revolutions per minute
SDW = shoot dry weight
TPF = targeted pcr fingerprinting
TY = tryptone yeast extract
VAR = variety
YMA = yeast mannitol agar

YMCR = yeast mannitol congo red

16 rRNA = 16s ribosomal ribonucleic acid

List of Figures

List of Tables

List of Appendices

1.0 CHAPTER ONE: INTRODUCTION

1.1 Introduction

Tropical legumes are crops which belong to the family Leguminosae and are characterized by the production of pods that contain seeds (Fig. 1.1). Tropical legumes include several species such as cowpea (*Vigna unguiculata*) and pigeonpea (*Cajanus cajan*) among others (Table 1.1). In general, tropical legumes are adapted to the hot climates that are prevalent in the tropics partly because of their root architecture that enables them to acquire nutrients and moisture from deeper soil layers (Stagnari *et al.,* 2017). In addition, their physiological traits such as chlorophyll content improves intercepted radiation and hence increases stomatal conductance. Furthermore, their tolerance to elevated temperatures helps in their adaptation to harsh tropical conditions (Pirasteh-Anosheh *et al.,* 2016).

Tropical legumes are important food crops for human and animal nutrition as well as the improvement of soil fertility (Ramakrishna *et al.,* 2000). They provide essential nutrients such as protein, complex carbohydrates, dietary fiber, minerals and vitamins to the human diet (Cakir *et al.,* 2019). Consumption of tropical legumes has also been related to a variety of positive health outcomes including the reduction of diseases such as cardiovascular diseases, diabetes, cancer, and obesity (Arnoldi *et al.,* 2015). This is attributed to the antioxidant and phytochemicals found in legume seeds. Legume oligosaccharides exhibit prebiotic properties that stimulate the growth of probiotics improving colon health thus lowering the risk of colon cancer (Maphosa and Jideani, 2017). Furthermore, because legumes have a low glycemic index, they release sugar into the bloodstream more slowly resulting in a lower after-meal peak in blood sugar levels making them ideal for diabetic patients and those at risk of developing diabetes (Wang *et al.,* 2014).

In Southern Africa, tropical legumes are cultivated mostly by smallholder farmers partly because of their ability to thrive in poor soils and adverse weather conditions as well as their resistance to diseases and pests (Kudapa *et al.,* 2013; Tadele and Bartels, 2019). Tropical legumes are useful in these cropping systems because of their ability to fix atmospheric nitrogen (N_2) thus minimizing the necessity for chemical nitrogenous fertilizers (Gwata *et al.,* 2004; Nedumaran *et al.,* 2015) consequently promoting a healthy environment. Therefore, they fit well in rotations (or inter-crops) with cereals where they contribute to the improvement of crop productivity and food security.

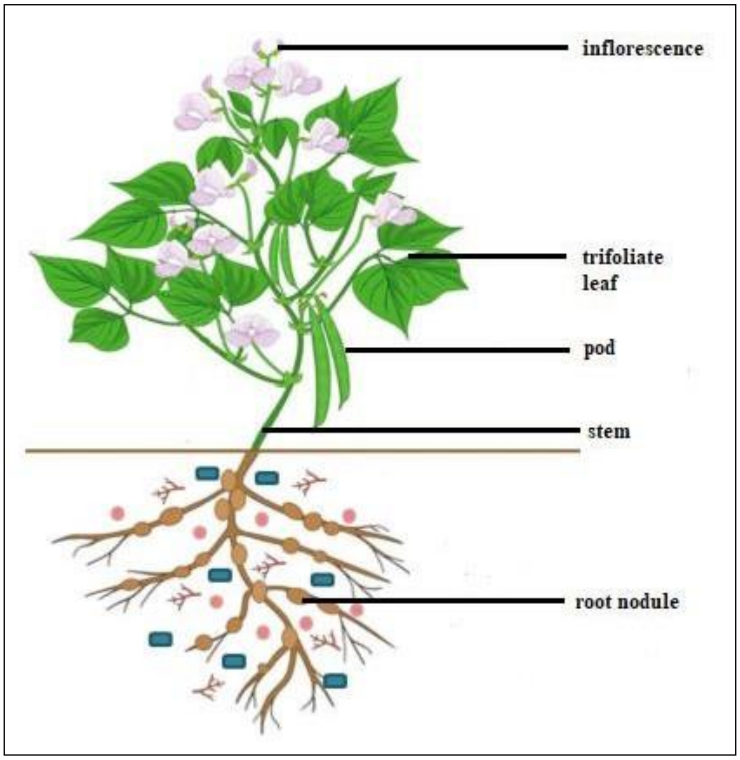

Figure 1.1 A schematic diagram of a legume plant (Adapted from: Sindhu et al., 2020).

Table 1.1 Examples of tropical legumes

Common name	**Scientific name**	**Origin**	**Genome size and pollination**	**Reference**
Bambara groundnut	*Vigna subterranea*	Africa	Diploid; 2n = 2x = 22; mostly self pollinated	Majola *et al*., 2021
Cowpea	*Vigna unguiculata*	Africa	Diploid; 2n = 2x = 22; self pollinated	Boukar *et al*., 2019
Horse gram	*Macrotyloma uniflorum*	India and Africa	Diploid; 2n = 2x = 20; self pollinated	Kaldate *et al*., 2017
Lablab	*Lablab purpureus*	Africa and India	Diploid; 2n = 2x = 22; **s**elf pollinated	Maass *et* al., 2015; She and Jiang, 2015
Pigeonpea	*Cajanus cajan*	Asia (India-Pakistan region)	Diploid; 2n = 2x = 22; mostly cross pollinated	Fuller and Harvey, 2006
Soybean	*Glycine max*	China	Polyploid; 2n = 40; **s**elf pollinated	Qiu and Chang, 2010; Findley *et al*., 2010
Tepary bean	*Phaseolus acutifolius*	USA and Mexico	Diploid; 2n = 2x = 22; mostly self pollinated	Bhardwaj, 2013; Mhlaba *et al*., 2018
Velvet bean	*Mucuna pruriens*	Eastern India and China	Diploid; 2n = 2x = 22; self pollinated	Suryawanshi *et al*., 2020

Although tropical legumes are excellent food security crops for resource poor farmers, their productivity in smallholder farming systems is far below their potential partly due to decreasing nitrogen fixation and soil fertility (Ronner *et al.,* 2016; Allito *et al.,* 2015). Soil microorganisms (as bio-inoculants) can enhance the productivity of these legumes through the improvement of soil fertility and represent an ecologically safe and effective means of realizing optimum legume production. Specifically, soil rhizobia which are ubiquitous in African soils have demonstrated improved symbiotic efficiency in various tropical legumes (Bopape *et al.,* 2021; Desta *et al.,* 2015; Jida and Assefa, 2014). Nonetheless, both the compatibility and competency of individual rhizobial strains is important for attaining optimum crop productivity. Therefore, it is imperative to identify the best efficient rhizobial strain x legume genotype combinations for successful nodulation and optimum legume yield.

1.2 Problem statement

Most smallholder farmers in the Limpopo Province (South Africa) grow tropical legumes without the necessary production inputs, resulting in depressed grain yields. This low productivity is partly attributed to poor root nodulation due to various symbiotic factors in the soil. Therefore, it is necessary to identify optimum legume genotype x rhizobial strain to improve the productivity of the legumes particularly in the smallholder cropping systems. Currently, there is insufficient information on efficient rhizobia strain x tropical legume combinations that produce optimum grain yields in smallholder production systems particularly in the Limpopo Province (South Africa).

1.3 Rationale of the study

The identification of indigenous rhizobial strains that are compatible with tropical legumes and produce high grain yields will benefit resource-limited farmers in smallholder farming systems. Previous research showed that inoculating some specific legumes with strains from the genus *Bradyrhizobium* and *Rhizobium* can improve productivity. However, inoculating legumes with strains of the genus *Paraburkholderia* has not been adequately investigated. Selection of the most effective indigenous rhizobial strains that are compatible with tropical legumes will contribute to the improvement of crop productivity, hence food and nutrition security, particularly in resource-poor smallholder communities.

1.4 Aim and objectives of the study

The aim of this study was to improve the productivity of selected tropical legumes. The specific objectives of the study were to:

(i) determine efficient rhizobial strains that combine with tropical legume species to produce optimum crop productivity

(ii) determine efficient tropical legume species x rhizobia genus combinations that produce optimum crop productivity.

1.5 Hypotheses

The study tested the following hypotheses:

(i) there were no efficient rhizobial strains that combine with tropical legume species to produce optimum crop productivity

(ii) there were no efficient tropical legume species x rhizobia genus combinations that produce optimum crop productivity.

2.0 CHAPTER TWO: LITERATURE REVIEW

2.1 Characteristics of tropical legumes

Tropical legumes are crops of the Leguminosae family adapted to warm regions. They are characterized by compound trifoliate leaves separated into leaflets, pods and their ability to form nodules with soil rhizobia (de Faria *et al.,* 1989). Some examples of tropical legumes that are cultivated by smallholder farmers in South Africa include bambara groundnut (*Vigna subterranaea*), pigeonpea (*Cajanus cajan*), tepary bean (*Phaseolus acutifolius*) and tropical soybean (*Glycine max*). Often, these tropical legumes vary significantly in phenotypic characters such as plant height, root morphology, as well as root nodulation and nitrogen (N_2) fixation variables. Nonetheless, typically, the tropical legumes develop extensive and long root systems that help draw nutrients and moisture from the deep soil layers (Kebede, 2021).

2.2 Uses and importance

Tropical legumes are multipurpose crops consumed as dry grains or green vegetables by humans with the crop residue being utilized as livestock feed and soil fertility restorer. The grains of these legumes possess high quality protein, amino acids, complex carbohydrates, dietary fiber, vitamins, minerals and beneficial bioactive compounds to human diet (Akande *et al.,* 2009; Dinghani *et al.,* 2018; Anjulo *et al.,* 2021). Moreover, owing to their bioactive compounds, legumes help lower blood cholesterol (Bazzano *et al.,* 2011) and moderate insulin levels (Hosseinpour-Niazi *et al.,* 2014) making them advantageous for consumption by diabetic individuals. Their antioxidant and anticarcinogenic properties also help minimize the risks of cancer and heart attacks (Zhu *et al.,* 2015; Bazzano *et al.,* 2001).

Grower’s value tropical legumes partly because of their adaption to heat, drought (Rao *et al.,* 2013) and their ability to boost soil fertility through nitrogen fixation thus reducing the necessity for costly chemical nitrogenous fertilizers (Adeleke and Haruna, 2012; Njira *et al.,* 2017). Their ability to fix nitrogen makes them suitable for intercropping and crop rotations. Also, they are useful as cover crops for preventing soil erosion, suppressing weeds and conserving soil moisture (Matusso *et al.,* 2014; Lamessa *et al.,* 2016). In addition, legumes generate income for smallholder farmers who trade the grain in local and international markets.

2.3 Cultivation

In southern Africa, tropical legumes are cultivated by both small- and large-scale growers for local consumption and export. They are often grown in rotation or intercrop with other crops partly due to limited land availability (Degaga and Angusu, 2017). In some cases, tropical legumes are cultivated as sole crops to provide insurance against total cereal crop failure due to drought (Matusso *et al.,* 2014). In general, they require conventional tillage before planting at the beginning of the rainy season in November/December of each year. Growers in South Africa frequently rotate these legumes with cereal crops (Lengwati *et al.,* 2020). Planting is often done by hand, with spacing between rows ranging from 40 to 90 cm and between plants in a row ranging from 5 to 30 cm depending on the legume species. At maturity, the legume leaves dry down and may not drop off completely. Tepary bean matures early followed by bambara groundnut and cowpea and pigeon pea being the latest to mature (Jiri and Mafongoya, 2016) and the pods are harvested by hand and mechanically using combine harvesters for instance, soybean.

The productivity of these legumes relies largely on their compatibility with local (indigenous) soil rhizobia to fix atmospheric nitrogen in the root nodules (Ojo *et al.,* 2015). However, the grain yield in some of the tropical legumes often improves when commercial inoculants are applied at planting (Sivparsad *et al.,* 2021; Shisanya, 2005; Emmanuel *et al.,* 2021; Mathimaran *et al.,* 2020; Agba *et al.,* 2013). The positive response to seed inoculation in soybean, for instance was attributed to the ineffective low populations of indigenous rhizobia in the soil (Ulzen *et al.,* 2016). In bambra groundnut, the grain yield increased by 72.71% after inoculation (Gomoung *et al.,* 2017). In contrast, some tropical legumes such as cowpea show no significant yield improvement despite seed inoculation due to several factors including lack of effective nodulation or compatibility (Mathu *et al.,* 2012; Ulzen *et al.,* 2016) as well edaphic conditions (Ferreira *et al.,* 2013). Therefore, there is merit in investigating the symbiotic effectiveness of specific tropical legumes with individual (distinct) rhizobial strains from a variety of genera. Such information will be useful in the development of commercial inoculants for tropical legumes.

2.4 Production constraints

Environmental stress factors such as soil, pH, moisture, temperature as well as plant nutrient deficiency constrain the development of root nodules in tropical legumes. Increased aluminum and manganese toxicity as well as low levels of calcium, molybdenum and phosphorus due to soil acidity (Hungria and Vargas, 2000) limit the secretion of plant flavonoids which inhibits rhizobia from inducing root hair curling (Miransari *et al.,* 2006). In turn, this impairs rhizobia attachment to the host root hairs (Vargas and Graham, 1988) ultimately reducing nodulation. In depleted soil moisture situations, the movement and replication of rhizobia is hindered, leading to reduced infection thread formation and nodule development (Griffith and Roughley, 2008). Furthermore, moisture stress decreases nitrogenase activity, photosynthates supply to the root nodules and nodule respiration thus inhibiting nodulation (Khanna-Chopra *et al.,* 1984; Adjetey and Nxumalo, 2017). Low temperatures (<20° C) delay nodulation (Peltzer *et al.,* 2002; Lira Junior *et al.,* 2005) due to the dissimilation of sugars in the nodule (Hansen, 2017). Phosphorus, potassium, calcium, and sulfur deficiencies also constrain root development and nodulation due to decreased leghemoglobin content in nodules and lower ATP concentration required for nitrogenase activity (Dabessa *et al.,* 2018).

Nodulation is also affected by the genotype of the host plant. Elbanna *et al.,* (2009) reported absence of nodules in two snap bean genotypes (Paulista and Xera) and this was attributed to the lack of compatibility between the indigenous rhizobia and the genotypes thus constraining nodule initiation and development. Moji *et al.,* (2020) also found similar results regarding the lack of nodules in some bambara groundnut genotypes. Disease-resistant genotypes also impair the ability of the crop to associate with beneficial rhizobia, resulting in a lack of root colonization and nodulation (Plett *et al.,* 2016; Plett *et al.,* 2021). In addition, because non-promiscuous soybean genotypes are strain specific, they can only form effective nodules when inoculated with *Bradyrhizobium japonicum* type. Gwata *et al.,* (2004) found that non-promiscuous soybean genotypes produced smaller, lighter nodules than promiscuous types.

2.5 Classification of rhizobia

Rhizobia are soil bacteria responsible for nodule development on the roots of the host legume (Ramirez *et al.,* 2019). Rhizobia include the genera *Rhizobium, Bradyrhizobium, Azorhizobium, Sinorhizobium, Allorhizobium, Methylobacterium, Phyllobacterium, Ochrobactrum, Herbaspirillum, Burkholdera, Cuprivaidus* and *Devosia* (De Meyer *et al.,* 2011; Ramirez *et al.,* 2019; Ikbal *et al.,* 2020). Rhizobia are classified depending on the host plant with which they establish a symbiotic relationship and their growth. For instance, *Rhizobium* are classified as fast growers (Adjei *et al.,* 2002) whilst *Bradyrhizobium* and *Phyllobacterium* and *Paraburkholderia* as slow growers (Rojas *et al.,* 2001; Cooper and Scherer, 2012). From a nitrogen fixation viewpoint, it is unclear which genera are most efficient among common tropical legumes particularly in view of the various factors that impact the fixation process.

A range of techniques are used for the classification of rhizobia. The genome-based approaches are important for identifying rhizobia as alternative (or complementary) or alternative tools for the phenotypical methods. Some of the molecular methods use primers that target specific sequences, such as the 16S ribosomal RNA (rRNA) or *rec*A gene as well as multi-locus sequence analysis among others (Table 2.1). The 16S rRNA analysis technique is often in combination with culturing methods that involve selection of suitable nutrient media for rhizobia such as yeast mannitol agar (YMA) or yeast sucrose agar or YMA + bromothymol blue or YMA + Congo red medium (Begom *et al.,* 2021).

2.6 Nitrogen fixation

2.6.1 Variation in nitrogen fixation capacity in legumes

The symbiotic relationship between the legume and soil rhizobia enables the host plant to fix a substantial amount of nitrogen to meet its own requirements for optimum plant growth and utilization by other crops in rotations. Nevertheless, there is considerable variation in biological nitrogen fixation potential amongst legumes based on rhizobial strain, genotype and environmental factors (Allito *et al.,* 2015). In the set of tropical legumes that were selected for this study, soybean showed the highest amount of N fixation (Table 2.2).

Table 2.1 Examples of techniques that are used for the classification of rhizobia.

Abbreviation	**Bacterial species**	**Reference**
16S rRNA	*Rhizobium leguminosarum*	Drouin et al., (1996); Bopape et al., (2020)
	Rhizobium tropici	
	Bacillus licheniform	
RFLP	*Rhizobium galegae*	Kaija lainen and Lindstrom (1989)
MLEE	*Mesorhizobium amorphae*	Wang et al., (1999)
MLSA	*Agrobacterium tumefaciens*	Lu et al., (2011)
	Bradyrhizobium elkanii	
	Ensifer adhaerens	
	Rhizobium multihospitium	
MLST	*Sinorhizobium meliloti*	Berkum et al., (2006)
FT-IR	*Corynebacterium glutamicum*	Oberreuter et al., (2002)
MALDI-TOF MS	*Rhizobium radiobacter*	Ferreira et al ., (2011)
	Rhizobium leguminosarum	
	Ensifer fredii	
TPF	*Rhizobium fredii*	Perret and Broughton, (1998)

16SrRNA = 16S ribosomal RNA; RFLP = restriction fragment length polymorphism; MLEE = multi-locus enzyme electrophoresis; MLSA = multi-locus sequence analysis; MLST = multi-locus sequence typing; FT-IR = fourier-transformed infrared spectroscopy; MALDI-TOF MS = matrix-assisted laser desorption ionization-time-of-flight mass spectrometry; TPF = targeted PCR fingerprinting.

Table 2.2 Examples of the estimated quantity of nitrogen fixed by selected tropical legumes.

Legume species	**Quantity of N fixed (kg N ha^{-1})**	**Reference**
Bambara groundnut (*Vigna subterranea*)	32-81	Musa et al., (2016)
Pigeonpea (*Cajanus cajan*)	92.9	Njira et al., (2017)
Soybean (*Glycine max*)	118	Yang et al., (2010)
Tepary bean (*Phaseolus acutifolius*)	24-60	Shisanya, (2002)

2.6.2 Factors influencing biological nitrogen fixation

Biological nitrogen fixation is an efficient way of ensuring adequate nitrogen availability for legume development while also maintaining soil health. Several factors such as legume species, soil nutrient availability, temperature, pH and rhizobia strains which influence nodulation also affect the biological nitrogen fixation process. Havlin *et al.,* (2014) reported that perennial legumes fix the greatest amount of nitrogen when compared to annual legumes. This occurs because they live longer in the field.

Studies show that tropical legumes tend to nodulate more under relatively high temperatures (30ºC ± 5ºC). In general, high root temperatures increase root hair infection, nodulation and biological nitrogen fixation (Hungria and Franco, 1993). Deak *et al.,* (2019) indicated that high temperatures (20 - 30ºC) stimulate the development of greater numbers of new roots which in turn enhances the roots susceptibility to infectivity by rhizobia and consequently increases the capacity of nitrogen fixation. *Calopogonium mucunoides* nodulated and fixed nitrogen efficiently at pH 4.0, which indicated that the rhizobia were acid tolerant. (Ferreira *et al.,* 2016). Water availability increased the population levels of rhizobia in the soil, thus resulting in good nodulation and fixation (Deak *et al.,* 2019). For good nodulation, rhizobial inoculants must be highly effective and competitive for nodule occupancy in the background of local rhizobia that show high competitiveness with low nitrogen fixation capacity. Arora *et al.,* (2018) found that rapid growing rhizobial strains were more competitive than slow growers because they are tolerant to high levels of salinity and thus, stimulated nodulation in pigeon pea.

2.6.3 Cross compatibility of rhizobia

Cross compatibility occurs when a single rhizobia species nodulates multiple legumes (Somasergaran and Hoben, 1994). Nonetheless, the ability to nodulate extends within inoculation groups (Ali *et al.,* 2010). Forty rhizobial strains from *Arachis hypogaea*, *Crotalaria juncea*, *Dolichos lablab*, *Phaseolus mungo*, *Sesbania aculeata*, *Vigna radiata* and *Vigna unguiculata* formed nodules in *Cajan cajanus* (Arora *et al.,* 2018). Hassen *et al.,* (2022) demonstrated that rhizobial strains from peanut, silverleaf desmodium and cluster bean formed effective nodules on bambara groundnut indicating nitrogen fixation potential. Pulver *et al.,* (1985) affirmed the ability of promiscuous soybean to cross-nodulate with cowpea strains. Therefore, it is important to determine effective rhizobia capable of nodulating a diverse range of host plants as some strains exhibit nodulation specificity though other strains nodulate a wide range of host legumes (Pueppke and Broughton, 1999). Thus, selecting indigenous rhizobia inoculants with a broad host range is critical for developing commercial inoculants particularly for soils lacking effective indigenous rhizobia.

2.7 Summary of the literature review

This literature review indicated the following key points:

(i) there is a wide range of rhizobia genera that symbiotically interact with both wild and domesticated legumes

(ii) several environmental and genetic factors of both symbionts influence biological nitrogen fixation in tropical legumes

(iii) there is considerable variation in biological N fixation potential amongst tropical legumes depending on the rhizobial strain, genotype and environmental factors

(iv) in smallholder cropping systems, legumes are often rotated or intercropped with cereal crops to reduce the cost of nitrogenous chemical fertilizers

(v) the productivity of some tropical legumes can be improved through inoculation with compatible and effective rhizobia

(vi) the presence of high populations of rhizobia in the soil does not necessarily imply high N fixation capacity since some rhizobial strains are host specific and may not be effective.

3.0 CHAPTER THREE: MATERIALS AND METHODS

3.1 Study location

This study consisted of two experiments both of which were carried out at the Agricultural Research Council (ARC), Plant Health and Protection (Pretoria) greenhouse and laboratory facilities (25° 61’ 547” S, 28° 36’ 435” E). The conditions in the greenhouse were set at a 14 h day temperature of 28° C and 10 h night temperature of 15° C.

3.2 Experiment 1: *Determination of efficient rhizobial strains that combine with tropical legume species to produce optimum crop productivity.*

3.2.1 Genetic material

3.2.1.1 Legume crops

Four tropical legumes were used in the study (Table 3.1). The seed of the legume species varied markedly in size and testa color (Fig. 3.1).

Table 3.1 Tropical legumes that were used in the study.

Tropical legume species			**Duration to maturity (days)**	**Flower colour**	**Notes**
Common name	**Scientific name**	**Code**			
Pigeonpea	*Cajanus cajan*	L1	130 ± 20	Yellow	Medium-duration type; medium seed
Soybean	*Glycine max*	L2	110 ± 10	White	Early - medium duration type; large seed
Tepary bean	*Phaseolus acutifolius*	L3	60 ± 5	Purple	Early duration type; large seed
Bambara groundnut	*Vigna subterranea*	L4	120 ± 10	Yellow	Early - medium duration type; large seed

Fig. 3.1 The seed of the tropical legume species that were used in the study. (L1 = legume 1 (pigeonpea); L2 = legume 2 (soybean); L3 = legume 3 (tepary bean); L4 = legume 4 (bambara groundnut).

3.2.1.2 Rhizobial strains, validation and inoculum preparation

Fourteen rhizobial strains which were previously strains from pigeonpea root nodules were used in this study (Bopape *et al.,* 2021) (Table 3.2). In this study, the strains were also referred to as rhizobial isolates. There was at least one strain representing each of four distinct genera namely *Bradyrhizobium, Paraburkholderia, Phyllobacterium* and *Rhizobium* (Table 3.2).

For the inoculum preparation, each rhizobial strain was revived from frozen cultures stored at -70 ^{o}C by streaking on Yeast Mannitol Congo Red (YMCR) Agar (Vincent, 1970) before transferring to agar plates and incubated at 28 ^{o}C for 3 – 4 days. Pieces of agar supporting rhizobial colonies were excised from the sub-culturing dishes and then placed in bottles containing sterile distilled water (18.0 ml) (Gwata *et al.,* 2003). To disperse the rhizobial cells, each bottle was vigorously shaken on a vortex shaker.

Table 3.2 The rhizobial strains that were used in this study.

Rhizobial isolate		**Genus**	**Species**
Code	**Designation**		
R1	30a2-PP3	*Paraburkholderia*	*Paraburkholderia phenolyruptix*
R2	N362	*Paraburkholderia*	*Paraburkholderia* sp
R3	KB15	*Paraburkholderia*	*Paraburkholderia* sp
R4	19b-PP5	*Bradyrhizobium*	*Bradyrhizobium elkanii*
R5	32a1-PP2	*Bradyrhizobium*	*Bradyrhizobium lupini*
R6	32b2-PP5	*Rhizobium*	*Rhizobium* sp
R7	10a-PP3	*Rhizobium*	*Rhizobium tropici*
R8	39a3-PP3	*Rhizobium*	*Rhizobium leucaenae*
R9	31b1-PP5	*Rhizobium*	*Rhizobium celliloscum*
R10	34a2-PP5	*Rhizobium*	*Rhizobium* sp
R11	31b-PP4	*Phyllobacterium*	*Phyllobacterium leguminum*
R12	33a-PP4	*Bradyrhizobium*	*Bradyrhizobium elkanii*
R13	27b2-PP5	*Bradyrhizobium*	*Bradyrhizobium japonicum*
R14	11b2-PP5	*Bradyrhizobium*	*Bradyrhizobium elkanii*
R15	Control (distilled water)	_	_

3.2.2 Trial establishment

Three seeds of each legume species were planted in a plastic pot (15 cm in diameter with 2.0 L holding capacity) filled with 1.65 kg sterile river sand saturated with Hoaglandsolution. Three holes (approx. 3.0 cm deep) were punched into each pot for planting the seed. Ineach pot, the seed was inoculated separately with the specific rhizobial strain by saturation with 2.0 ml of each inoculum and covered carefully with sand immediately after inoculation. After planting, the pots were irrigated with 80 ml of water.

3.2.3 Trial management and measurements

The plants in each pot were irrigated with distilled water periodically as necessary during the entire growth period. There were no insect pest or weeds that were observed in the trial. At 7 weeks after germination, the leaf colour score (LCS), from the middle of at least two fully expanded leaves per plant, was measured using a chlorophyll meter (Minolta Chlorophyll Meter Spad-502, Minolta Co., Ltd., Tokyo, Japan) (Gwata *et al.,* 2003; Wang *et al.,* 2020). Thereafter, each plant was harvested and gently washed with tap water before detaching the nodules carefully from the roots. Similarly, the shoot was separated from the roots for each plant prior to oven drying all the harvested plant parts at 70 ^{o}C for 48 h followed by weighing.

3.2.4 Experimental design and data analysis

The experiment was laid out in a split plot design with legume species as the main factor and rhizobial strain as the sub-factor. Each treatment was replicated twice resulting in a total of 120 pots. The data sets on LCS, nodule dry weight (NDW), shoot dry weight (SDW) and root dry weight (RDW) were subjected to the analysis of variance (ANOVA) and Pearson's correlation analysis using R-programming language software (4.2.1) followed by mean separation using LSD test at the 5.0% probability level. To understand further the relationship between the host plants and the microsymbionts, a GGE biplot analysis was performed (Bosi *et al.,* 2022; Mohammadi *et al.,* 2010; Gauch *et al.,* 2008; Yan and Kang, 2002).

3.3 Experiment 2: ***Determination of efficient tropical legume species x rhizobia genus combinations that produce optimum crop productivity.***

3.3.1 Genetic materials

3.3.1.1 Tropical legume, rhizobial strains and inoculum preparation

Three tropical legumes were used in the study (Table 3.3). Each legume consisted of four distinct varieties (Fig. 3.1). The seed varied markedly in both testa color and size. Four rhizobial strains which were selected from experiment 1 were used in this study (Table 3.4).

3.3.1.2 Validation of rhizobial strains

Prior to inoculum preparation, the genus and species of each of the four rhizobial strains was validated using polymerase chain reaction (PCR). Each strain was separately grown on Yeast Mannitol Agar (YMA). The pure cultures were transferred into an Erlenmeyer flask containing 100.0 ml Tryptone Yeast Extract (TY) broth incubated at 28 ^{o}C on a rotary shaker at 150 rpm for 24-48 hours.

There is variation in the degree of differences in the genera and species of bacteria that can be visualized through molecular techniques (Mohania *et al.,* 2008). The 16S rRNA gene can be used for bacterial classification (Menna *et al.,* 2006; Hakim *et al.,* 2020; Castellano-Hinojosa *et al.,* 2022). Therefore, the 16S rRNA was important in confirming the genus of each of the four rhizobial strains that were used in the study. The genomic DNA was extracted from the 2.0 ml TY culture suspension of each strain using the WIZARD® Genomic DNA Purification kit (Promega, Maddison, WI, USA) based on the manufacturer's instruction and the extracted DNA was amplified using universal primers for 16S rRNA (Beukes *et al.,* 2013). The PCR amplification reaction was performed using standard procedures in a volume containing 40 µl buffer, 16 µl MgCl2, 8 µl dNTP, 1.6 µl Taq DNA polymerase, 20 µl each of forward and reverse primers as well as 5 µl of template DNA. The amplifications were performed in an Eppendorf Master Cycler Gradient device and visualized by electrophoresis on 1% agarose gel in TBE buffer containing 0.5 mg/ml ethidium bromide. The inoculum was prepared following the same procedure as described for experiment 1 (Section 3.2.1.2) above.

3.3.2 Trial establishment

Two seeds of each legume variety were planted in a plastic pot as described above(Section 3.3.1). Similarly, in each pot, the seed was inoculated separately with the specific rhizobial strain by saturation with 2.0 ml of each inoculum and covered carefully with sand immediately afterinoculation and irrigated with 80 ml of water thereafter.

3.3.3 Trial management and measurements

The trial was managed as described above for experiment 1. Similarly, no insect pest or weeds were observed in the trial. Four variables of nitrogen fixation that contribute to crop productivity namely leaf colour score, nodule dry weight (NDW), shoot dry weight (SDW) and root dry weight (RDW) were measured following the same procedure as described above (section 3.2.3). At harvesting, the height of each plant was measured from the base to the tip of the stem using a ruler.

Table 3.3 Tropical legumes that were used in this study. (L1-V1 = legume 1 – variety 1).

Tropical legume				**Flower colour**	**Seed size classification**
Common name	**Scientific name**	**Variety Designation**	**Code**		
Bambara groundnut	*Vigna subterranea*	WHT	L1-V1	Yellow	Large
		BLK	L1-V2	Yellow	Small
		RED	L1-V3	Yellow	Large
		LBR	L1-V4	Yellow	Medium
Soybean	*Glycine max*	4LF	L2-V1	White	Medium
		P-15	L2-V2	White	Small
		STG	L2-V3	Purple	Medium
		UNK	L2-V4	Purple	Medium
Pigeonpea	*Cajanus cajan*	DC	L3-V1	Red	Medium
		ENT-3	L3-V2	Yellow	Small
		SST	L3-V3	Yellow	Small
		UG-22	L3-V4	Yellow	Medium

Figure 3.1 Distinct seed of the tropical legumes that were used in the study.

Table 3.4 Rhizobial strains that were used in the study.

Entry (Previous Entry)	Code of rhizobial strain	Genus	Species
1 (2)	N362	*Paraburkholderia*	*Paraburkholderia sp*
2 (10)	34a2-PP5	*Rhizobium*	*Rhizobium sp*
3 (11)	31b-PP4	*Phyllobacterium*	*Phyllobacterium leguminum*
4 (12)	33a-PP4	*Bradyrhizobium*	*Bradyrhizobium elkanii*
5 (15)	Control (distilled water)	_	_

3.3.4 Experimental design and data analysis

The experiment was laid out in a split-split plot design with the rhizobial strain as the main factor, legume species as the sub-factor and legume variety as sub-sub factor. Each treatment was replicated twice resulting in a total of 120 pots. The data sets of all the nitrogen fixation variables were analyzed following the same procedure as described in experiment 1 (Section 3.2.4).

4.0 CHAPTER FOUR: RESULTS

4.1 Experiment 1

4.1.1 Effects of rhizobial strains on the productivity of tropical legume species

The results of this study showed that there was variation in legume species x rhizobial strain compatibility. The pattern of root nodulation varied within the legume species depending on the specific strain used. There were no nodules on the uninoculated (control) plants (Fig. 4.1). Tepary bean nodulated poorly with most of the rhizobial strains.

There were significant differences among the legume species (LS) for all the traits that were studied (Table 4.1). In addition, the rhizobial strain (isolate) (RI) and LS x RI interaction were highly significant ($P < 0.01$). However, there were no significant differences among the rhizobial strains for nodule dry weight (NDW), root dry weight (RDW) and shoot dry weight (SDW). The leaf color score (LCS) ranged between 14.23 – 23.63. (Table 4.2). The highest NDW (0.05 g) which was attained by bambara groundnut (LS-4) was more than two-fold higher than the trial mean (0.02 g).Overall, tepary bean nodulated poorly. The lowest RDW (0.15 g) which was observed for pigeonpea (LS-1) was only 41.67% and 46.87% in comparison with the mean RDW observed for bambara groundnut (LS-4) and soybean (LS-2), respectively. In addition, bambara groundnut attained the highest SDW (0.42 g) among the four legume species (Fig. 4.2).

Fig. 4.1 Uninoculated (control) plants bambara groundnut (left), soybean (middle) and pigeonpea (right) formed no root nodules

Table 4.1 Mean squares for nitrogen fixation attributes among four tropical legume species and fifteen rhizobial strains. (LCS = leaf colour score; NDW = nodule dry weight; RDW = root dry weight; SDW = shoot dry weight).

Source	df	LCS	NDW	RDW	SDW
Replication (R)	1	0.3500	0.0003	0.0006	0.0029
Legume species (LS)	3	453.2200 *	0.0179 *	0.2612 *	0.4265 ***
Rhizobial isolate (RI)	14	70.5600 ***	0.0044	0.0186	0.0040
LS x RI	42	63.9300 ***	0.0047	0.0129	0.0058
Mean		19.26	0.02	0.27	0.29
R^2 (%)		4.79	1.06	19.00	22.20
C.V. (%)		23.4	16.67	37.61	14.78

***; **; * Significant at the 0.1%; 1.0% and 5.0% probability level, respectively.

Table 4.2 Mean values of four tropical legume species (LS-1 = pigeonpea; LS-2 = soybean; LS-3 = tepary bean; LS-4 = bambara groundnut; LCS = leaf colour score; NDW = nodule dry weight; RDW = root dry weight; SDW = shoot dry weight).

Legume species	**LCS**	**NDW (g)**	**RDW (g)**	**SDW (g)**
LS-1	23.65 a	0.02 b	0.15 c	0.19 c
LS-4	20.10 ab	0.05 a	0.36 a	0.42 a
LS-2	19.18 b	0.01 b	0.32 a	0.36 b
LS-3	14.23 c	0.00 b	0.24 b	0.18 c

Means followed by the same letter in a column are not significantly different (P ≤ 0.05) (LSD test).

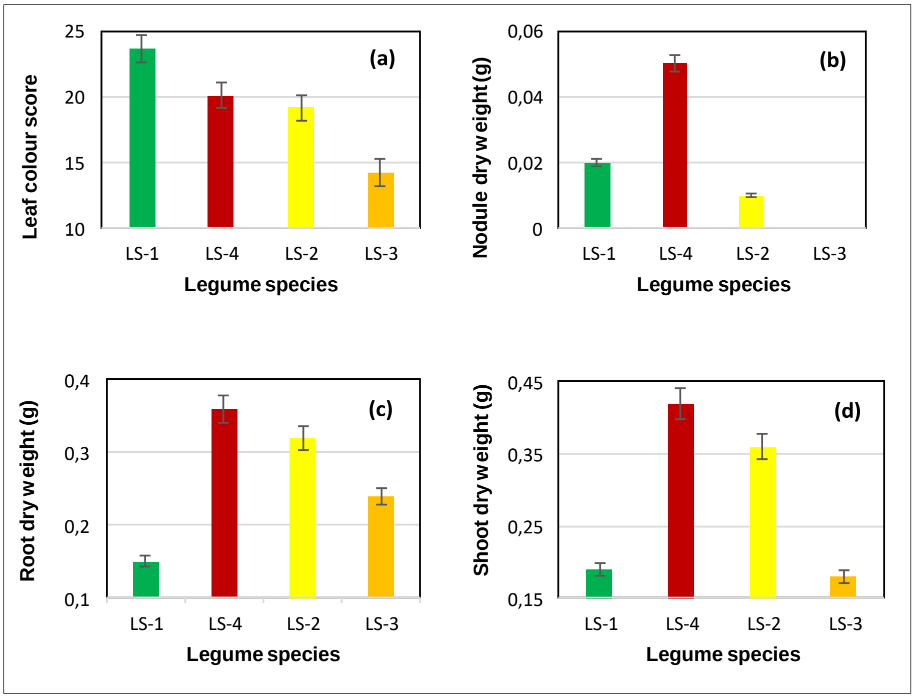

Fig. 4.2 Variation in (a) leaf colour score (b) nodule dry weight (c) root dry weight (d) and shoot dry among four distinct legume species (LS-1 = pigeonpea; LS-2 = soybean; LS-3 = tepary bean; LS-4 = bambara groundnut).

The mean LCS for the trial was (19.26) while the highest (23.65) and lowest (14.23) LCS were associated with the rhizobial strains *Bradyrhizobium elkanii* (R4) and *Phyllobacterium leguminum* (R11), respectively (Fig. 4.3). Among the *Paraburkholderia* species, the rhizobial strain *Paraburkholderia phenolyruptix* (R1) was associated with the highest (22.08) LCS (Fig. 4.3). The results also revealed that the *Rhizobia*, the rhizobial strains *Rhizobium leucaenae* (R8) and *Rhizobium* sp (R6) induced the highest (20.89) and lowest (15.24) LCS, respectively, among all the five bacterial species from the genus *Rhizobium* that were used in the study. However, the rhizobial strain *Bradyrhizobium elkanii* (R14) which attained a relatively high LCS, was associated with the heaviest NDW among all the strains (Fig. 4.3). In contrast, there were detectable nodules associated with four rhizobial strains, namely *Paraburkholderia* sp (R2), *Rhizobium celliloscum* (R9), *Rhizobium* sp (R10) (designated '34a2 (pp5)') and *Rhizobium* sp (R11) (designated '31b (pp4)'). Interestingly, all the four strains also attained significantly lower LCS than the control. The highest (0.40 g) RDW, which was significantly ($P<0.05$) higher than the control, was associated with the rhizobial strain *Bradyhizobium lupini* (R5) suggesting that the effects of the strains were variable across the N fixation traits thus making it difficult to identify the best strain for all legume species and all the traits of interest. Two distinct rhizobial species, namely *Paraburkholderia* sp (R2) (designated 'N362') and *Bradyhizobium lupini* (R5) were associated with the heaviest shoots across the legume species (Fig. 4.3). In contrast, the control strain achieved the lowest (0.25 g) SDW. In addition, all the three strains from the genus *Paraburkholderia* and the single strain from the genus *Phyllobacterium* as well as all the five from the genus *Bradyrhizobium* (R4; R5; R12; R13 and R14) were associated with significantly ($P< 0.05$) heavier SDW than the control.

4.1.2 Correlations between nitrogen fixation traits

There were highly significant ($P < 0.01$) positive correlations between the LCS and NDW (Table 4.3). However, the LCS was negatively but significantly ($P < 0.05$) correlated with the RDW. In addition, there were highly significant positive correlations between the SDW and each of NDW and RDW (Table 4.3). The coefficient of determination (R^2) which was estimated in the linear relationship between RDW and SDW was markedly high (>60.0 %) (Fig. 4.4) indicating that the association between the genes for root and shoot weight among the legume species that were used in this study was notable.

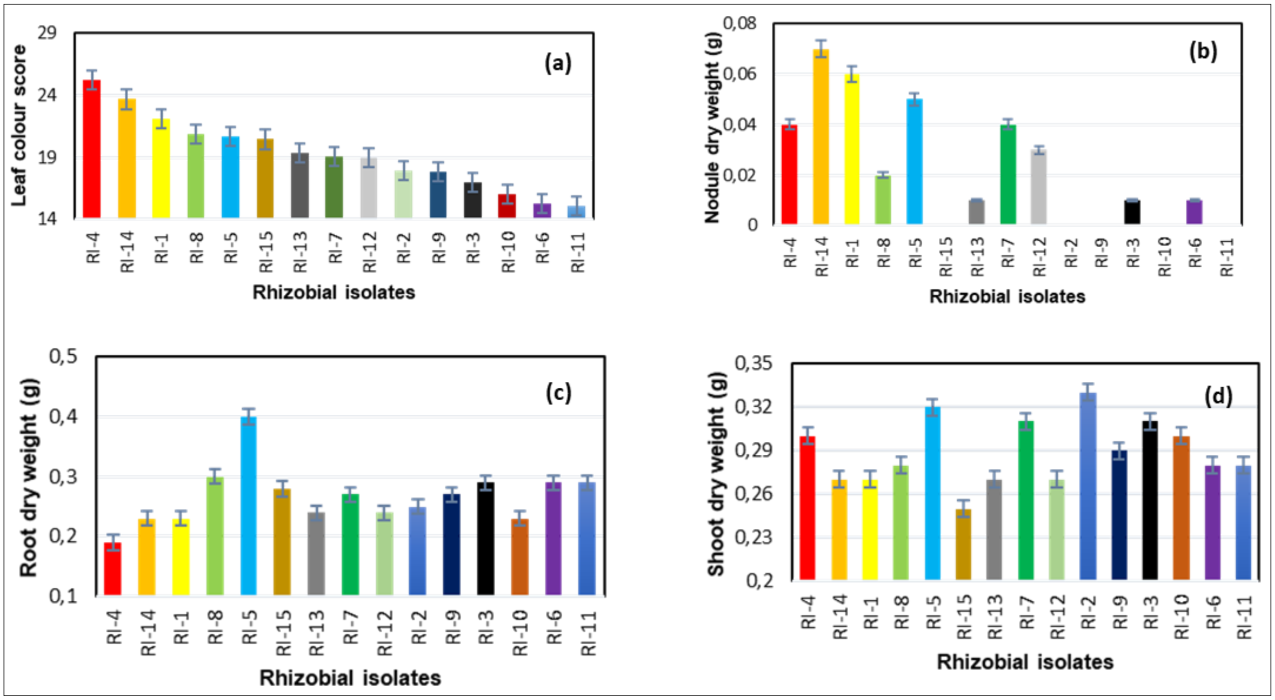

Fig. 4.3 Variation in (a) leaf colour score (b) nodule dry weight (c) root dry weight and shoot dry weight (d) among fifteen rhizobial strains.

Table 4.3 Coefficient of correlation (*r*) among nitrogen fixation attributes among four legume species. (LCS = leaf colour score; NDW = nodule dry weight; RDW = root dry weight; SDW = shoot dry weight).

	LCS	**NDW**	**RDW**	**SDW**
LCS	1.0000			
NDW	0.4068 **	1.0000		
RDW	-0.1918 *	0.0447	1.0000	
SDW	0.1345 *	0.2711 **	0.6163 **	1.0000

**; * Significant at the 1.0% and 5.0% probability level, respectively.

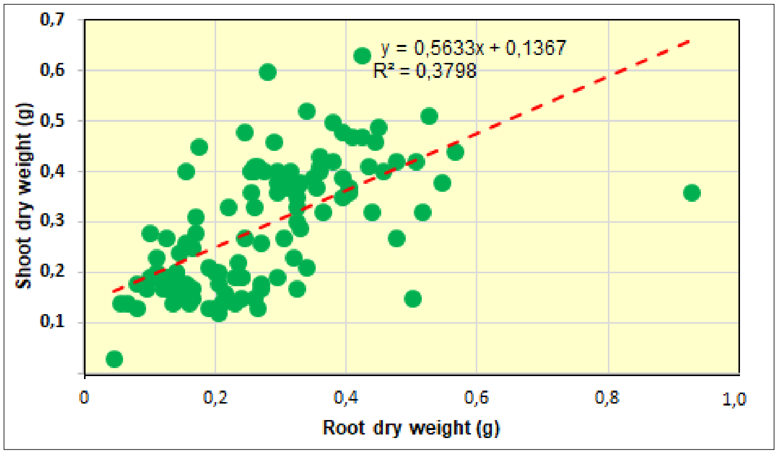

Fig. 4.4 Relationship between shoot dry weight (g) and root dry weight (g) among four legumes that were each inoculated separately with individual rhizobial strains.

4.2 Experiment 2

4.2.1 Inoculum production and validation of rhizobial genera

All the colonies did not absorb the Congo red dye on the YMA-CR agar plates indicating that they were rhizobia. A confirmatory test of all the rhizobial colonies showed growth on YM-CR without absorbing the Congo red (Appendix 4.1). The pure cultures of strains showed a variety of colony morphologies and shapes. (Appendix 4.1). The PCR amplification of the 16S rDNA region produced a marker band of approximately 1500 bp (Appendix 4.2).

4.2.2 Tropical legume species x rhizobial genera interactions

The best strain from each of the four rhizobial genera, in terms of SDW (since this trait showed at least significant ($P<0.01$) correlation with each of LCS, NDW and RDW (see Section 4.1.2) was used in this second component of the study to standardize the comparison between genera as well as determine varietal responses to inoculation with the specified strains.

The results showed highly significant ($P < 0.001$) differences among the legume species for all the nodulation attributes that were evaluated (Table 4.3). Similarly, there were highly significant ($P<0.001$) differences among the varieties of the legume species for plant height (PHT). The variety x rhizobial strain interactions for all the nodulation attributes were not significant ($P < 0.05$). In contrast, the variety x legume species interactions were highly significant ($P < 0.001$) for all the attributes that were measured except for NDW which also showed a high coefficient of variation likely due to the difficulties associated with recovering tiny nodules (Table 4.3). In some cases, there were clear varietal differences in response to inoculation with the same rhizobial strain. For instance, inoculation with *Bradyrhizobium* sp (33a-PP4) showed varietal differences in the pattern of nodulation in bambara groundnut (Fig. 4.5) and pigeonpea (Fig. 4.6). Nonetheless, some of the soybean varieties formed no nodules after inoculation with the *Paraburkholderia* sp and *Phyllobacterium leguminum* (Fig. 4.7). Similarly, soybean responded poorly to inoculation with *Rhizobium* sp. (34a-PP5) and *Bradyrhizobium* sp (33a-PP4) (Fig. 4.8).

Table 4.3 Mean squares for nitrogen fixation attributes among four tropical legume species. (LCS = leaf colour score; NDW = nodule dry weight; PHT = plant height (cm); RDW = root dry weight; SDW = shoot dry weight).

Source	df	LCS	NDW	PHT	RDW	SDW
Replication (R)	1	484.8000	0.0000	20.9300	0.0375	0.0094
Rhizobial isolate (RI)	4	34.2000	0.0086 ***	37.5700 *	0.0547 **	0.1208
Legume species (LS)	2	4184.3000 ***	0.0053 ***	522.3700 ***	0.3441 ***	1.5677 ***
RI x LS	8	59.7000	0.0024 ***	18.0700	0.0366	0.1217 *
Variety (VAR)	3	535.2000 *	0.0009	52.9000 ***	0.0496 **	0.1205 **
VAR x RI	12	156.8000	0.0004	3.6500	0.0044	0.0252
VAR x LS	6	1038.2000 ***	0.0007	82.3400 ***	0.1213 ***	0.1404 ***
VAR x RI x LS	24	135.5000	0.0007 *	8.0900	0.0089	0.0196
Mean		30.25	0.01	8.46	0.20	0.32
R^2 (%)		5.92	2.64	8.23	25.71	36.67
C.V. (%)		25.51	92.72	25.52	25.80	71.88

***; **; * Significant at the 0.1%; 1.0% and 5.0% probability level, respectively.

Fig. 4.5 Variation in the effects of inoculation between rhizobial genera in bambara groundnut varieties that were inoculated with *Bradyrhizobium elkanii* (33a-PP4) (top left), *Phyllobacterium leguminum* (31b-PP4) (top right); *Paraburkholderia* sp (strain 'N362') (bottom left) and *Rhizobium* sp (32b-PP5)) (bottom right).

Fig. 4.6 Variation in the effects of inoculation with the rhizobial strain *Bradyrhizobium* sp (33a-PP4) among pigeonpea plants of variety 1 (far left), variety 2 (second from left) and variety 3 third from left). Nodules were absent from the uninoculated plants (far right).

Fig. 4.7 Poor nodulation in soybean varieties (first three from left) after inoculation with *Phyllobacterium leguminum* (top) and *Paraburkholderia* sp ('N362') resulting in chlorotic leaves as in the uninoculated (control) plants (far right).

Fig. 4.8 Absence of root nodules in soybean plants that were inoculated with a rhizobial strain *Rhizobium* sp. (34a-PP5) (left) and *Bradyrhizobium* sp (33a-PP4) (right).

The results also revealed that the rhizobial strain 'R2' (*Rhizobium* sp (34a2-PP5R3) produced the highest (31.55) LCS although there were significant differences in this attribute among the strains (Table 4.4). The NDW ranged between 0.0 – 0.04 g but the SDW ranged between 0.21 – 0.38 g (Table 4.4). On average, the uninoculated plants (control plants that were treated with distilled water) achieved the shortest height (6.93 cm) and the lightest RDW (0.14 g). In addition, bambara groundnut (legume 1) outperformed the other two legumes in all the attributes that were measured (Table 4.4). However, there were significant ($P < 0.05$) varietal differences within each legume species for some of the attributes (Table 4.5). For instance, the NDW for the bambara groundnut variety 'BLK' was three-fold heavier than that for variety 'WHT' (Table 4.5). Similarly, in soybean, the variety 'STG' differed significantly ($P < 0.05$) from the remainder of the varieties in PHT, RDW and SDW. In pigeonpea, all the four varieties that were used in the study showed similar LCS values but differed significantly ($P < 0.05$) in the remainder of the attributes (Table 4.5).

Table 4.4 Mean values of three tropical legume species and five rhizobial strains (L1 = legume 1 (bambara groundnut); L2 = legume 2 (soybean); L3 = legume 3 (pigeonpea); LCS = leaf colour score; NDW = nodule dry weight; PHT = plant height (cm); RDW = root dry weight; SDW = shoot dry weight; R1 = *Paraburkholderia* sp (N362); R2 = *Rhizobium* sp (34a2-PP5R3 = *Phyllobacterium leguminum* (31b-PP4); R4 = *Bradyrhizobium elkanii* (33a-PP4); R5 = control (distilled water).

Rhizobial isolate	**LCS**	**NDW (g)**	**PHT (cm)**	**RDW (g)**	**SDW (g)**
R2	31.55 a	0.00 c	8.86 ab	0.26 a	0.37 a
R4	31.21 a	0.04 a	9.98 a	0.18 bc	0.38 a
R5	30.34 a	0.00 c	6.93 c	0.14 c	0.21 a
R1	29.46 a	0.00 c	7.44 bc	0.24 a	0.28 a
R3	28.69 a	0.02 b	9.10 ab	0.19 b	0.36 a
Legume species					
L1	40.89 a	0.02 a	12.08 a	0.28 a	0.54 a
L3	29.38 b	0.02 a	8.45 b	0.10 b	0.15 c
L2	20.46 c	0.00 b	4.85 c	0.23 a	0.27 b

Means followed by the same letter in a column are not significantly different ($P < 0.05$) (LSD test).

Table 4.5 Mean values of the four varieties in each of three tropical legume species that were inoculated separately with each of five rhizobial strains.

Variety	LCS	NDW (g)	PHT (cm)	RDW (g)	SDW (g)
Bambara groundnut (Legume species 1)					
BLK (V2)	42.42 a	0.03 a	12.06 a	0.25 a	0.53 a
LBR (V4)	41.60 a	0.02 ab	11.37 a	0.33 a	0.55 a
WHT (V1)	40.34 a	0.01 b	12.59 a	0.26 a	0.51 a
RED (V3)	39.18 a	0.02 ab	12.30 a	0.26 a	0.57 a
Soybean (Legume species 2)					
STG (V3)	30.74 a	0.01 a	6.35 b	0.38 a	0.46 a
4LF (V1)	25.84 a	0.00 a	7.14 a	0.24 b	0.31 b
P-15 (V2)	25.36 a	0.00 a	5.92 c	0.28 b	0.31 b
UNK (V4)	0.00 b	0.00 a	0.00 d	0.00 c	0.00 c
Pigeonpea (Legume species 3)					
UG-22 (V4)	35.21 a	0.03 a	9.43 c	0.13 a	0.20 a
SST (V3)	34.17 a	0.02 ab	10.22 b	0.11 ab	0.18 a
ENT-3 (V2)	30.66 a	0.01 ab	10.58 a	0.08 bc	0.13 b
DC (V1)	17.48 a	0.00 b	3.58 d	0.05 c	0.09 c

Means followed by the same letter in a column are not significantly different ($P < 0.05$) (LSD test).

4.3 Determination of the ideal rhizobial genus and legume species

In this component of the analysis, only the single best performing rhizobial strain from each rhizobial genus (except for the default only available rhizobial strain from the genus *Phyllobacterium*) was utilized. Because there was a consistently significant ($P<0.05$) correlation between SDW and each of LCS, NDW and RDW (see Section 4.1.2), the SDW was used to determine the ideal rhizobial genus and legume species. Similar graphical outputs were observed for the other three variables (LCS, NDW and RDW) (Appendix 4.3 – Appendix 4.7). To accomplish this objective, the GGE biplot method was utilized (Yan and Hunt, 1998; Yan and Rajcan, 2002; Yan and Wu, 2008; Aruna *et al.,* 2011). The legume species and rhizobial strains were coded as environment scores and genotypes scores, respectively.

4.3.1 The GGE biplot analysis for the relationship between the rhizobial genera and the legume species

The GGE biplot analysis indicated that 60.0% of the rhizobial strains were distributed in the top left quadrant but none in the bottom right quadrant (Fig. 4.9). The rhizobial strains ‘R2’, and ‘R1’were positioned far away from the origin suggesting that they uniquely influenced the legume species. The biplot analysis also revealed that the legume species (coded as environment scores), particularly ‘E1’ (pigeonpea) and ‘E2’ (soybean), were separated by acute angles between them and grouped in the same top right quadrant (Fig. 4.9). In contrast, the remainder of the pairs of legume species were separated by obtuse angles with each suggesting that they were negatively related to each other in terms of the SDW trait. The legume species ‘E3’ (tepary bean) showed the shortest absolute projection suggesting that it was the most stable in performance across the rhizobial strains.

In determining the ‘which-won where’, the biplot is made of an irregular polygon with a set of lines drawn from the origin to dissect perpendicularly each side of the polygon and divide the polygon into sectors as well as determining the winning rhizobial strains (or the rhizobial genera in this case) for each sector (Yan *et al.,* 2007). The results showed that ‘which-won-where’, the biplot explained 95.23% total variation of which PC1 and PC2 accounted for 84.41% and 10.82%, respectively (Fig. 4.10). The results also revealed that among the rhizobial strains (depicted as genotypes) on the vertices of the polygon ‘R2’ (*Rhizobium* sp (34a2-PP5) and ‘R4’ (*Bradyrhizobium elkanii* (33a-PP4), performed best with pigeonpea (E1) and soybean (E2), respectively (Fig. 10). Because the GGE biplot analysis uses vector lines that are drawn from the origin of the biplot to each test environment marker to measure the discriminative power of the environment, (coded as environment scores), ‘E4’ showed the longest vector, suggesting that it had a high discriminating ability.

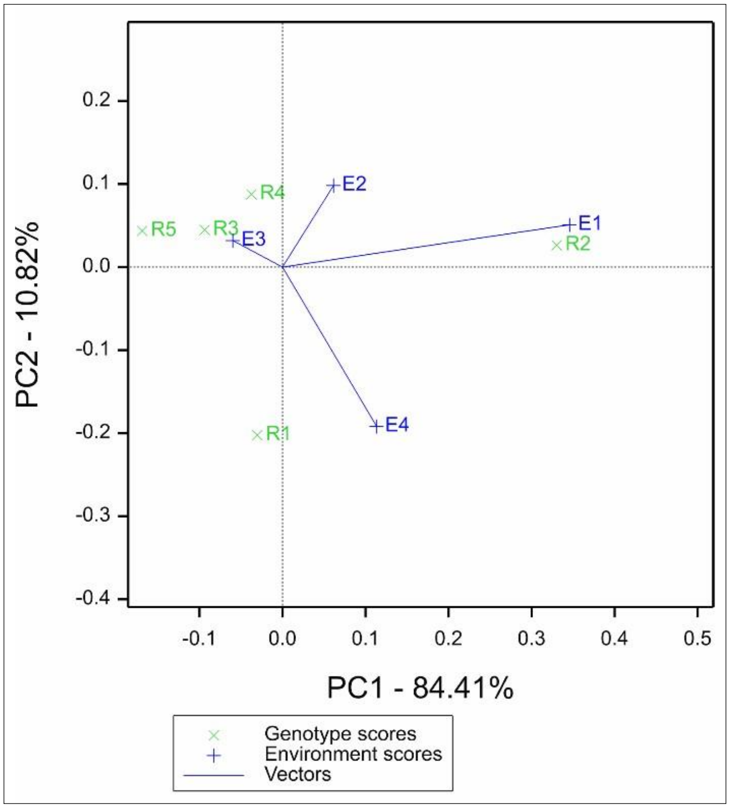

Fig. 4.9 The vector view of the GGE biplot for shoot dry weight showing the representativeness and discrimination power of the four legume species (coded as environment scores) against the five rhizobial strains (coded as genotype scores). (E1 = pigeonpea; E2 = soybean; E3 = tepary bean; E4 = bambara groundnut; R1 = *Paraburkholderia* sp (N362); R2 = *Rhizobium* sp (34a2-PP5); R3 = *Phyllobacterium leguminum* (31b-PP4); R4 = *Bradyrhizobium elkanii* (33a-PP4); R5 = control (distilled water).

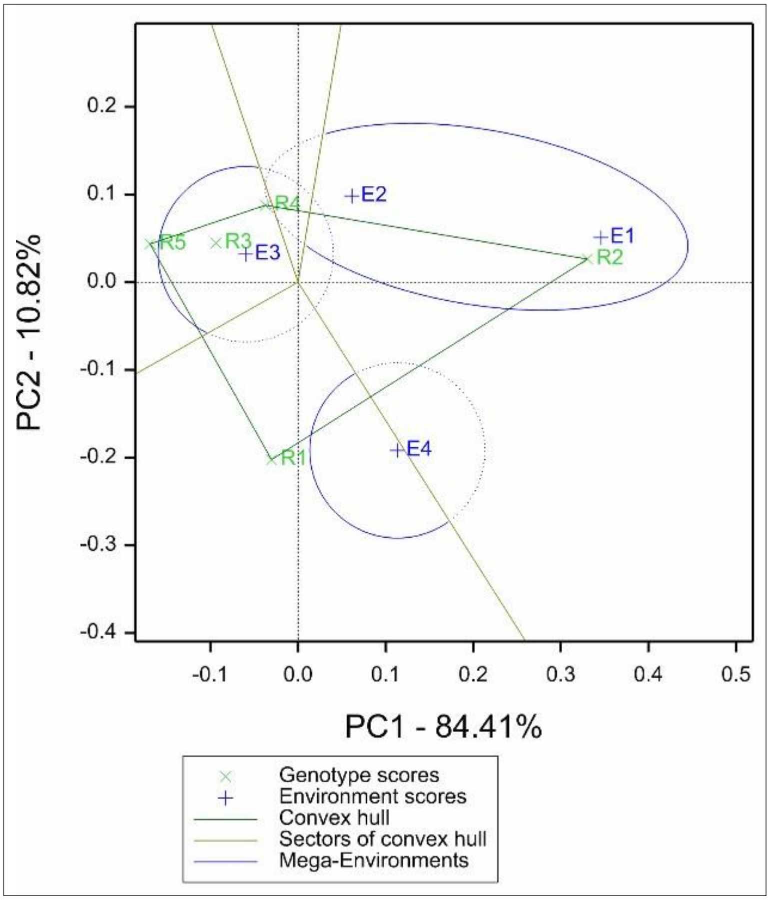

Fig. 4.10 A “which-won-were” pattern of GGE biplot polygon view for shoot dry weight between the rhizobial strains (coded as genotype scores) and the legumes species (coded as environment scores). (E1 = pigeonpea; E2 = soybean; E3 = tepary bean; E4 = bambara groundnut; R1 = *Paraburkholderia* sp (N362); R2 = *Rhizobium* sp (34a2-PP5); R3 = *Phyllobacterium leguminum* (31b-PP4); R4 = *Bradyrhizobium elkanii* (33a-PP4); R5 = control (distilled water).

4.3.2 The GGE biplot analysis for the ideal rhizobial strain and legume species

The ideal rhizobial strain (coded as a genotype score in this study), is the one with the highest mean performance and stability; hence performs best across all the test legume species (i.e. environments) (Kaya et al., 2006). The strain is identified by its location in the innermost concentric circle, indicated by an arrowhead in the biplot (Yan and Tinker (2006). The rhizobial strain ‘R2’ (*Rhizobium* sp; 34a2-PP5) was positioned in the innermost concentric circle, thus representing the ideal strain for SDW among the strains (Fig.4.11). The concentric circles were drawn to enable easy visualization of the distances between all the genotypes under evaluation. In contrast, as expected, the rhizobial strains ‘R5’ (control) was positioned furthest from the ideal strain, indicating low stability.

The ideal legume species (coded as the environment) should have the power to discriminate the rhizobial strains (coded as genotypes) main effects and is defined by an arrow pointing to it while concentric circles are drawn to aid easy visualization of the distances between the legume species (i.e. the environments) (Yan and Wu, 2008). The legume species (or environment) which is positioned closest to the ideal one is also usually desirable. The GGE biplot comparing the legume species (coded as environmental scores) relative to an ideal environment (centre of the concentric circles) for SDW showed that ‘E1’ (pigeonpea) (followed by ‘E4’) was positioned closest to the epicenter of the concentric circles, thus providing the ideal production conditions for SDW (Fig 4.12). This legume species was most powerful in the discrimination of the rhizobial strains (coded as genotypes) (Kaya *et al.,* 2006). In contrast, the poor environment provided by ‘E3’ (tepary bean) which was positioned farthest from the epicenter, represented the least discriminatory legume species.

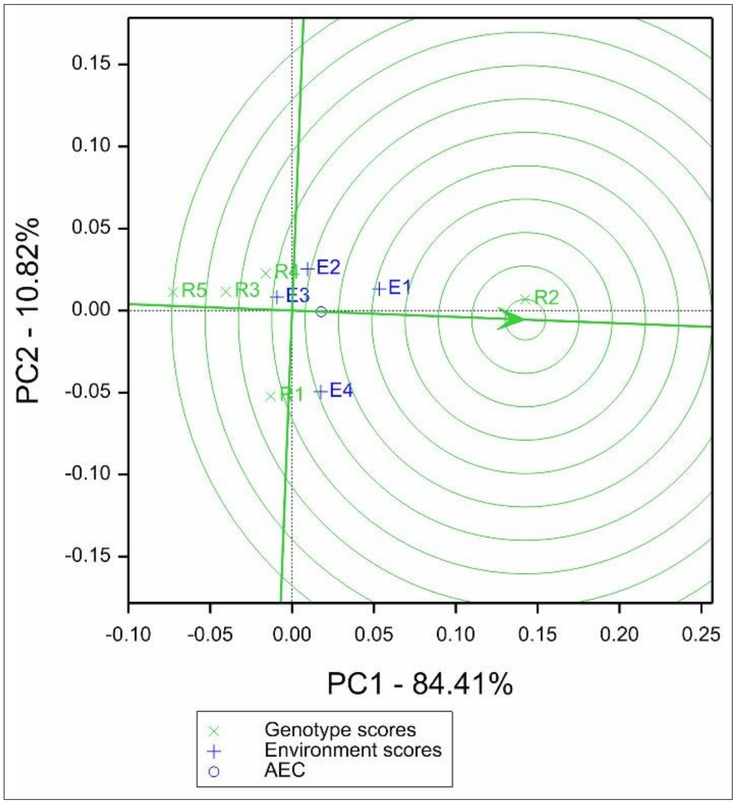

Fig. 4.11 A comparison view of GGE biplot showing an ideal rhizobial strain for shoot dry weight among four distinct legumes. (Rhizobial strains and legume species are coded as genotype scores and environmental scores, respectively). (E1 = pigeonpea; E2 = soybean; E3 = tepary bean; E4 = bambara groundnut; R1 = *Paraburkholderia* sp (N362); R2 = *Rhizobium* sp (34a2-PP5); R3 = *Phyllobacterium leguminum* (31b-PP4); R4 = *Bradyrhizobium elkanii* (33a-PP4); R5 = control (distilled water).

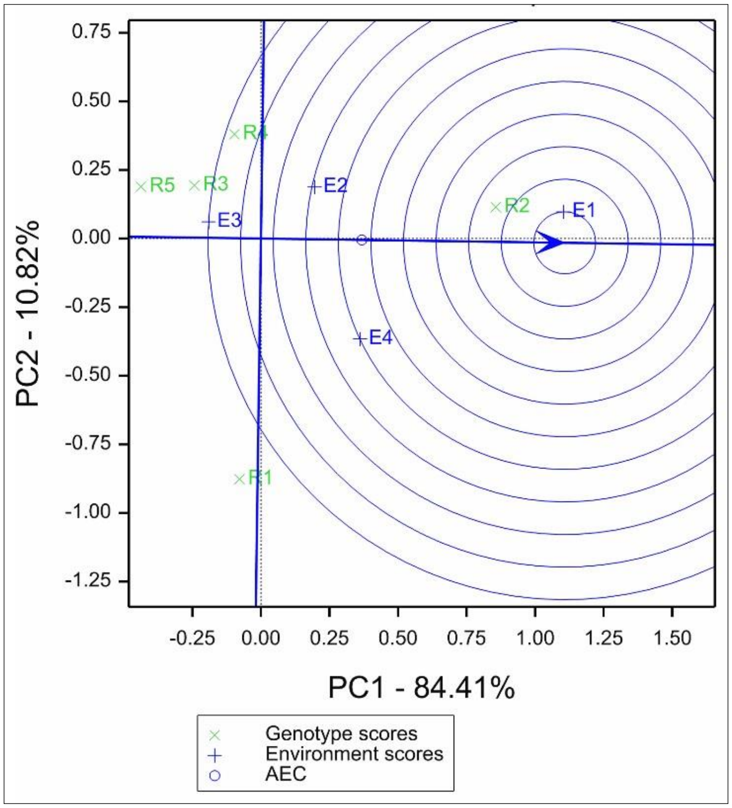

Fig. 4.12 The average-environment coordination (AEC) view comparison biplot comparing the legume species (coded as environmental scores) relative to an ideal environment (centre of the concentric circles) for shoot dry weight. (The rhizobial strains are coded as genotype scores). (E1 = pigeonpea; E2 = soybean; E3 = tepary bean; E4 = bambara groundnut; R1 = *Paraburkholderia* sp (N362); R2 = *Rhizobium* sp (34a2-PP5); R3 = *Phyllobacterium leguminum* (31b-PP4); R4 = *Bradyrhizobium elkanii* (33a-PP4); R5 = control (distilled water).

4.3.3 The GGE biplot analysis of rhizobial strain stability

The ranking of rhizobial strains (coded as genotypes) based on their stability in SDW and stability over four distinct legume species (coded as environments) was determined drawing a line that has an arrow, passing through a small circle (mean of the legume species) and crossing the coordinate source (Fig. 4.13). The rhizobial strain to the right of this axis possessed heavier dry shoots while the line which is perpendicular to this 'arrowed' line and has two arrows is a measure of the stability of the strains. According to Yan *et al.,* (2000) any rhizobial strain closer to this line isconsidered as more stable. Therefore, the rhizobial strain 'R2' (Rhizobium sp (34a2 (pp5)) was the most stable in terms of SDW (Fig. 4.13). The control ('R5') was stable but weak in the SDW.

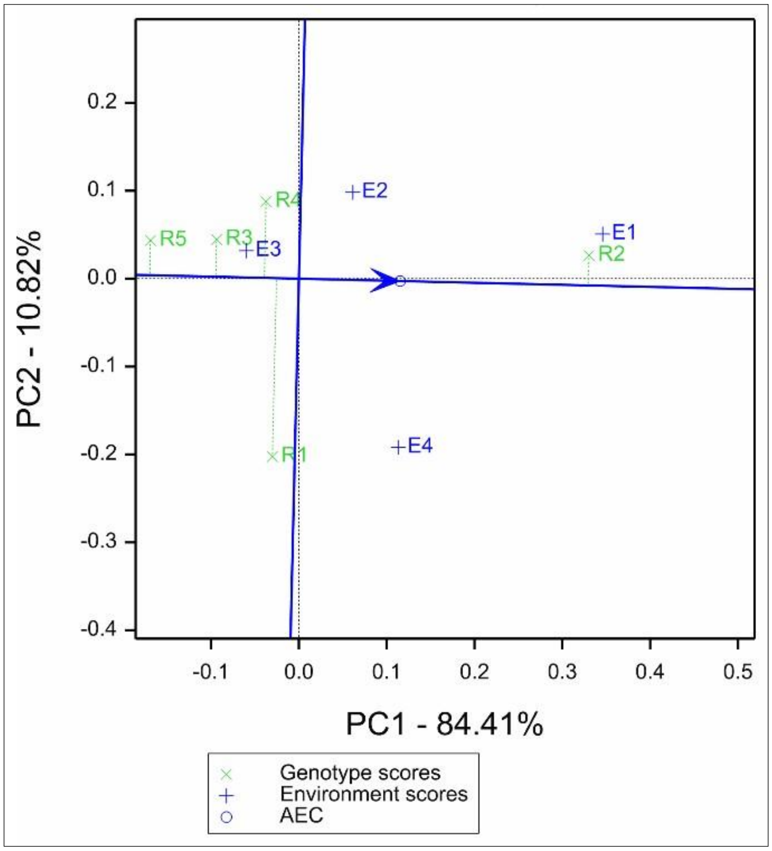

Fig. 4.13 The stability of rhizobial strains over the four distinct legume species. (E1 = pigeonpea; E2 = soybean; E3 = tepary bean; E4 = bambara groundnut; R1 = *Paraburkholderia* sp (N362); R2 = *Rhizobium* sp (34a2-PP5); R3 = *Phyllobacterium leguminum* (31b-PP4); R4 = *Bradyrhizobium elkanii* (33a-PP4); R5 = control (distilled water).

5.0 CHAPTER FIVE: DISCUSSION

The effects of both rhizobia inoculation and legume species were significant in nitrogen (N) fixation as indicated by the leaf colour score (LCS). The positive response to inoculation by the legume species could be attributed to improved biological N fixation by rhizobial strains which consequently increased leaf chlorophyll N content particularly in the form of nitrogenous compounds such as allantoin and allantoic acid (Senthilkumar *et al.,* 2021). Inoculating legumes such as soybean, tepary bean, pigeonpea and bambara groundnut with compatible rhizobia strain increased the chlorophyll content of the leaves (Sara *et al.,* 2013; Neelipally *et al.,* 2020). Tairo and Ndakidemi (2013) attributed an increase in chlorophyll content of inoculated soybean to adequate availability of N derived from the symbiotic process. In this study, pigeonpea (LS-1) produced the LCS following inoculation with *Bradyrhizobium elkanii* (19b pp5). This was consistent with observations that were reported in previous similar studies involving soybean (Gwata *et al.,* 2003; 2005), common bean (Sara *et al.,* 2013) and cowpea (Nyoki and Ndakidemi, 2014). Furthermore, the increased N availability in inoculated plants leads to an improvement in the efficiency of photosynthesis as well as the crop yield.

The results also showed that rhizobial strains had a positive effect on plant height compared to the uninoculated treatments. The significant increase in plant height observed could be attributed to efficient rhizobial strains to stimulate the formation of more nodules which provides legumes with more fixed nitrogen resulting in greater plant height in inoculated legumes. This was consistent with the observations that were reported for garden pea (*Pisum sativum*) (Kumar, 2011) and peanut (*Arachis hypogaea*) (Sajid *et al.,* 2010) in which the increment in plant height was attributed to inoculation.

The variation in root nodulation variables among the legumes depended on the crop species and environmental conditions (or more precisely, the rhizobial strain). Hassen *et al.,* (2022) also reported significant variation in number of nodules among different bambara groundnut landraces in response to inoculation with five different rhizobial strains. *Bradyrhizobium elkanii* (R14) demonstrated the greatest compatibility with bambara groundnut (LS-4) by eliciting the highest nodule dry weight. Similar findings in bambara groundnut which was inoculated with strains of *Bradyrhizobium* sp were reported recently (Hassen *et al.,* 2021). In addition, root nodulation was also achieved in the plants that were inoculated with the rhizobial strains *Paraburkholderia sp* (R2) and *Rhizobium* sp (R10). However, tepary bean, nodulated poorly with all the rhizobial strains which were tested. Probably, this was due to the limited number of strains that were used in the study. The significant increase in nodule dry weight increase in nodule dry weight (NDW) observed in this study was consistent with the findings that were reported for faba bean (Allito *et al.,* 2021) after seed inoculation with rhizobial strains. However, the four legume species that were used in this study showed variation in root dry weight possibly due to inherent genetic differences in capacity for lateral root proliferation (Zhang *et al.,* 2020; Samudin and Kuswantoro, 2018). In addition, the shoot dry weight (SDW) was similar between rhizobial strains in this study but there were differences between legume species suggesting the importance of genetic factors in the host plant. In comparison with the uninoculated plants, both bambara groundnut (LS-4) and soybean (LS-2) as well as pigeonpea (LS-1) showed increased shoot dry weight (SDW) after inoculation with each of the three *Paraburkholderia* rhizobial strains and the *Phyllobacterium leguminum* (R11) strain*.* This is the first report in which evidence of successful N fixation in field legumes was observed with these rhizobial species. In addition, these legumes demonstrated their promiscuous type of nodulation (Perret *et al.,* 2000) since they have been associated variably with other rhizobial genera including *Bradyrhizobia* (Fossou *et al.,* 2016; Rufini *et al.,* 2016; Araújo *et al.,* 2017; Sharma *et al.,* 2017) and *Rhizobium* (Degefu *et al.,* 2013; Coutinho *et al.,* 1999; Freiberg *et al.,* 1997). However, sporadic symbiotic activities with strains from the *Phyllobacterium* genus have been reported in lupin (Valverde *et al.,* 2005) and other legume species (Mantelin *et al.,* 2006; Sánchez *et al.,* 2014). Therefore, there will be merit in further investigation of the strains from these two genera as potential commercial bio-inoculants for these legumes. Of interest in future studies is the field performance of each of these rhizobial types. It is unclear if the profuse nodulation that was observed in bambara groundnut with *Bradyrhizobium elkanii* (33a-PP4) and *Phyllobacterium leguminum* (31b-pp4) can positively influence the grain yield for instance. The results showed that same strain was not compatible with soybean probably due to in ability to exchange molecular signals at the initiation of the nodulation process (Oldroyd, 2013; Hungria and Stacey, 1997; Bassam *et al.,* 1986). Therefore, bio-inoculants from this genus are likely to have limited usage in these legumes. In addition, the varietal differences in N fixation suggested the need to evaluate potential commercial bio-inoculants (Schütz *et al.,* 2018) to identify the optimum variety x rhizobial strain combinations for use in each legume. Perhaps a broad range strain (Lewin *et al.,* 1990; Pueppke and Broughton,1999) could be desirable especially in the smallholder cropping systems where multiple legumes are often cultivated (Mhango et al., 2013; Snapp and Silim, 2002; Sanginga, 2003).

The GGE biplot analysis revealed that pigeonpea and soybean were separated by acute angles between them and grouped in the same quadrant but the remainder of the legume species were separated by obtuse angles suggesting that they were negatively related to each other in terms of the SDW trait. In addition, tepary bean showed the shortest absolute projection suggesting that it was the most stable in performance and stability across the rhizobial strains at least for the shoot weight. These results affirmed the variability among the legumes in terms of their responses to inoculation with rhizobial strains. In determining the 'which-won-where', the results revealed that among the rhizobial strains (depicted as genotypes) on the vertices of the polygon 'R2' (*Rhizobium* sp.; 34a2-PP5) and 'R4' (*Bradyrhizobium elkanii*; 33a-PP4), performed best with pigeonpea (E1) and soybean (E2), respectively. The rhizobial strain 'R2' (*Rhizobium* sp; 34a2-PP5) was positioned in the innermost concentric circle, thus representing the ideal strain for SDW among the strains (Fig. 4.11). The biplot analysis also confirmed that as expected, the rhizobial strains 'R5' (control) was positioned furthest from the ideal strain, indicating low stability. The rhizobial strain 'R2' was also determined as the most stable in terms of SDW. Moreover, the biplot analysis identified the ideal legume species with the power to discriminate the main effects of the rhizobial strains. However, one of the limitations of this approach was that the ideal legume species was identified for a specific N fixation indicator such as SDW implying therefore, that it may not be the ideal one in terms of other indicators for N fixation. On the other hand, the study showed positive relationships between SDW and the other traits agreed with the observations that were reported in previous similar studies (Gwata *et al.,* 2004; Sinclair *et al.,* 1991).

In summary, the study findings provided new information in the patterns of N fixation among a set of tropical legumes that were inoculated with distinct rhizobial genera. The information will be useful for formulating commercial bio-inoculants that may improve legume productivity (Mabrouk *et al.,* 2018; Wang *et al.,* 2018; Ormeño-Orrillo *et al.,* 2012). Inconsistencies that may have occurred were likely due to the complexity of the N fixation process which, in some microsymbionts such as*Rhizobium leguminosarum*, require 593 genes for competitive nodulation and fixation (Wheatley *et al.,* 2020). In contrast, other rhizobial strains possess no common nodulation genes such as the nod*ABC* and most likely, use alternative biochemical pathways to initiate symbiosis (Young *et al.,*2006; Giraud *et al.,* 2007; Bopape *et al.,* 2021). Specifically, validation of the symbiotic efficiencyof *Bradyrhizobium elkanii* (33a-PP4) and *Phyllobacterium leguminum*; 31b-PP4) with more diverse bambara groundnut germplasm is recommended.

6.0 CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

The results of this study demonstrated that both the compatibility and efficiency of individual rhizobial strains is important for attaining optimum crop productivity. Overall, these results are similar to those from previous similar studies. Therefore, it is imperative to identify the best efficient rhizobial strain x legume genotype combinations for successful nodulation and optimum legume yield for the benefit of resource-limited farmers in smallholder farming systems and end-users.

The results also revealed variation in legume species x rhizobial strain compatibility and the pattern of nitrogen (N) fixation (as indicated by the fixation variables that were measured in this study) within the legume species, depending on the specific strain (source of N) used. There were significant differences among the legume species for all the traits that were measured. Tepary bean nodulated poorly with most of the rhizobial strains and soybean showed poor N fixation ability with specific rhizobial strains. Overall, bambara groundnut responded to inoculation with specific strains better than any of the other tropical legumes. Two distinct rhizobial strains, namely *Paraburkholderia* sp and *Bradyhizobium lupini* were associated with the heaviest shoots across the legume species.

The results indicated highly significant ($P < 0.01$) positive correlations between the leaf color score and nodule dry weight of the tropical legumes. Similarly, there were highly significant positive correlations between the shoot dry weight and nodule dry weight. This information will be useful in future studies involving the evaluation of N fixation in these tropical legumes. A modified GGE biplot analysis was applied successfully to understand better the relationships between the symbionts for specific N fixation traits. The method was utilized to identify superior rhizobial strain (bio-inoculant) x legume species combinations that result in improved productivity of the host. In addition, the method identified the best rhizobial genus which can be exploited for potential commercial use. This novel information from this study will be useful in future for formulating bio-inoculants that may improve legume productivity in Limpopo Province (South Africa) where all the legume species that were used in this study are cultivated. The validation of the symbiotic efficiency of *Bradyrhizobium elkanii* (33a-PP4) and *Phyllobacterium leguminum*; 31b-PP4) with more diverse bambara groundnut germplasm is highly recommended. In addition, the application of bio-inoculants in the production of these tropical legumes is also recommended since the inoculation can provide affordable sources of N requirements in the smallholder cropping systems in Limpopo Province (South Africa) and other similar regions. It is recommended that further validation of the symbiotic efficiencies of the bio-inoculants that were identified in this study be conducted under field conditions in multiple agro-ecological environments. The inclusion of molecular techniques (with higher precisions and resolutions), in future studies aimed at understanding N fixation will be merited.

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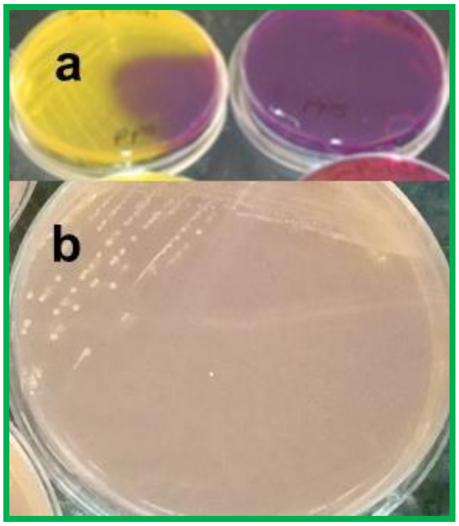
APPENDICES

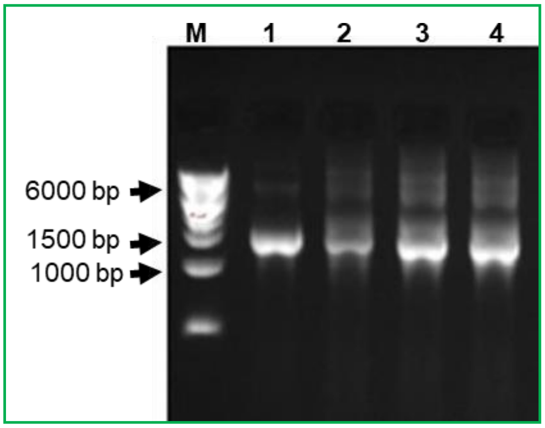
Appendix 4.1

(a) Rhizobial strains showing little or no growth on peptone glucose agar; the purple change to yellowish when bacteria are growing; (b) pure colonies of rhizobial strains cultured on yeast mannitol – congo red medium.



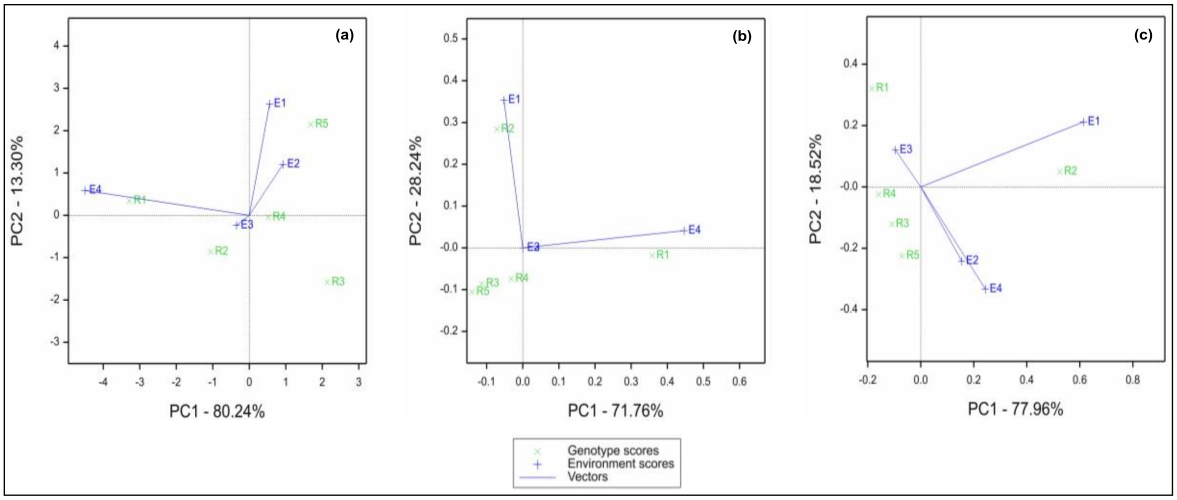
Appendix 4.2

The PCR gel of 16S rRNA for the rhizobial strains. M = 1kb DNA ladder / marker; lane 1 = *Paraburkholderia* sp (strain ‘N362’); lane 2 = *Rhizobium* sp (strain ‘34a2-PP5’); lane 3 = *Phyllobacterium* sp (strain ‘31b-PP4’); lane 4 = *Bradyrhizobium* sp (strain ‘33a-PP4’)



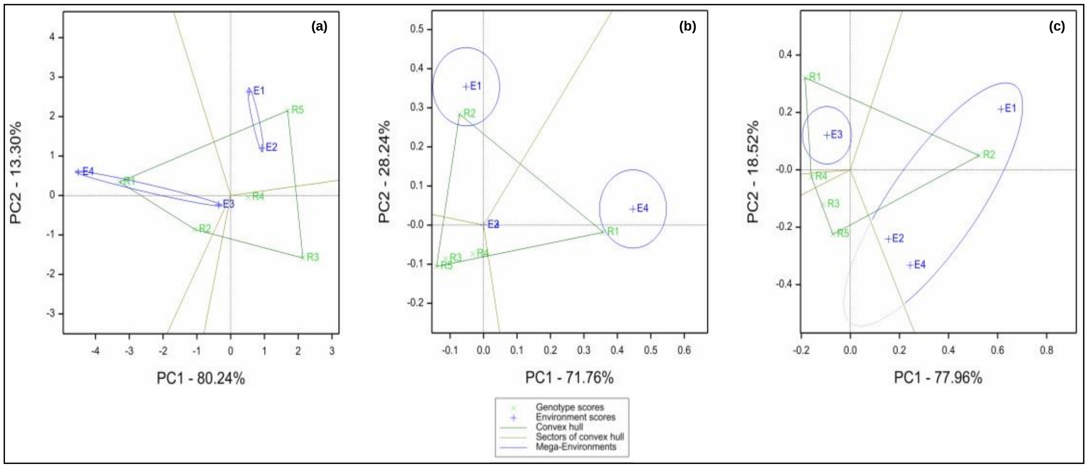
Appendix 4.3

The vector view of the GGE biplot for (a) leaf color score (b) nodule dry weight and (c) root dry weight showing the representativeness and discrimination power of four legume species against five rhizobial strains. (The legume species and rhizobial strains were coded as environment scores and genotype scores, respectively). (E1 = pigeonpea; E2 = soybean; E3 = tepary bean; E4 = bambara groundnut; R1 = *Paraburkholderia* sp (N362); R2 = *Rhizobium* sp (34a2-PP5); R3 = *Phyllobacterium leguminum* (31b-PP4); R4 = *Bradyrhizobium elkanii* (33a-PP4); R5 = control (distilled water).



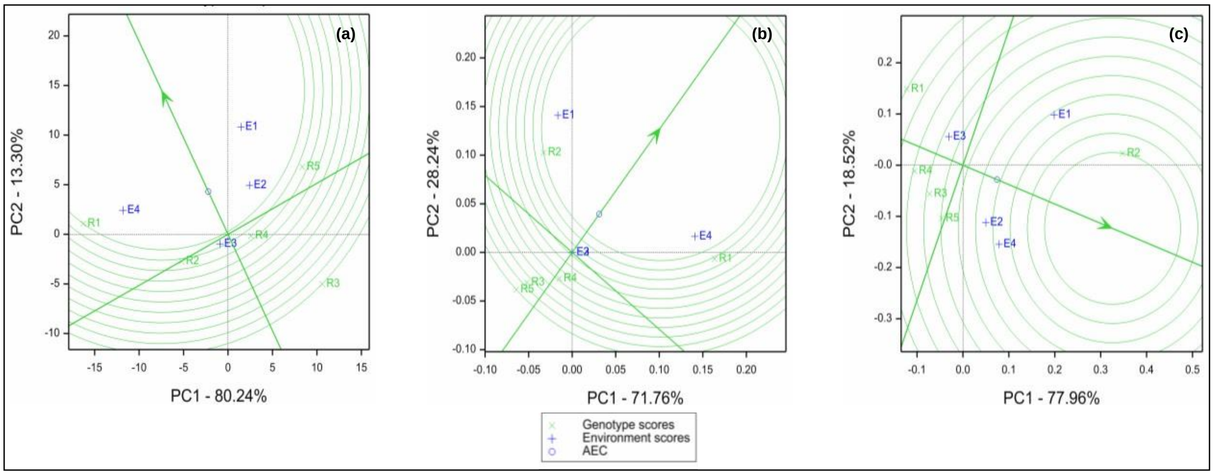
Appendix 4.4

A “which-won-were” pattern of GGE biplot polygon view for (a) leaf color score (b) nodule dry weight and (c) root dry weight among four legume species against five rhizobial strains. (The legume species and rhizobial strains were coded as environment scores and genotype scores, respectively). (E1 = pigeonpea; E2 = soybean; E3 = tepary bean; E4 = bambara groundnut; R1 = *Paraburkholderia* sp (N362); R2 = *Rhizobium* sp (34a2-PP5); R3 = *Phyllobacterium leguminum* (31b-PP4); R4 = *Bradyrhizobium elkanii* (33a-PP4); R5 = control (distilled water).



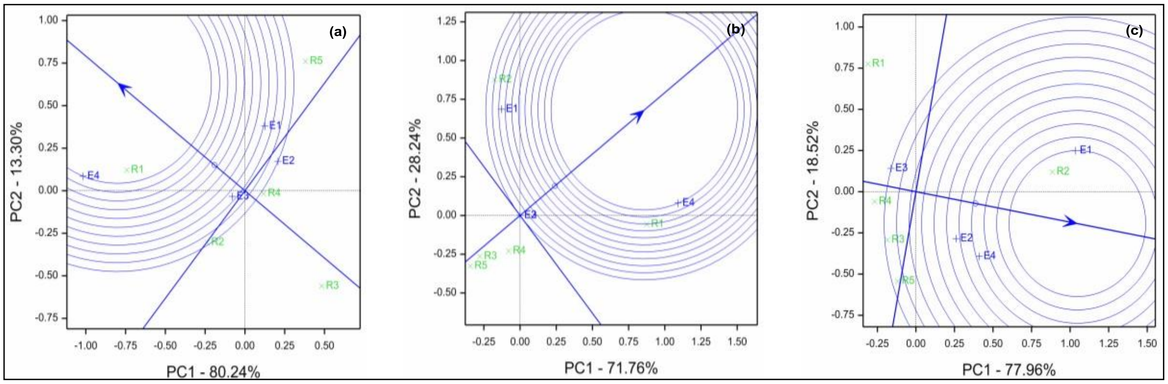
Appendix 4.5

A comparison view of GGE biplot showing an ideal rhizobial strain for (a) leaf color score (b) nodule dry weight and (c) root dry weight among four legume species against five rhizobial strains. (The legume species and rhizobial strains were coded as environment scores and genotype scores, respectively). (E1 = pigeonpea; E2 = soybean; E3 = tepary bean; E4 = bambara groundnut; R1 = *Paraburkholderia* sp (N362); R2 = *Rhizobium* sp (34a2-PP5); R3 = *Phyllobacterium leguminum* (31b-PP4); R4 = *Bradyrhizobium elkanii* (33a-PP4); R5 = control (distilled water).



Appendix 4.6

The average-environment coordination (AEC) view comparison biplot comparing the legume species relative to an ideal environment (centre of the concentric circles) for (a) leaf color score (b) nodule dry weight and (c) root dry weight among four legume species. (The legume species and rhizobial strains were coded as environment scores and genotype scores, respectively). (E1 = pigeonpea; E2 = soybean; E3 = tepary bean; E4 = bambara groundnut; R1 = *Paraburkholderia* sp (N362); R2 = *Rhizobium* sp (34a2-PP5); R3 = *Phyllobacterium leguminum* (31b-PP4); R4 = *Bradyrhizobium elkanii* (33a-PP4); R5 = control (distilled water).



Appendix 4.7

The stability of rhizobial strains over four distinct legume species for (a) leaf color score (b) nodule dry weight and (c) root dry weight among four legume species. (The legume species and rhizobial strains were coded as environment scores and genotype scores, respectively). E1 = pigeonpea; E2 = soybean; E3 = tepary bean; E4 = bambara groundnut; R1 = *Paraburkholderia* sp (N362); R2 = *Rhizobium* sp (34a2-PP5); R3 = *Phyllobacterium leguminum* (31b-PP4); R4 = *Bradyrhizobium elkanii* (33a-PP4); R5 = control (distilled water).