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A Study of the Genetics of Root Nodulation in Pigeonpea (*Cajanus cajan*) Using Indigenous Rhizobia

By

Phalane Francina Lebogang

(Student number: 11512441)

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Department of Plant and Soil Sciences

Faculty of Science, Engineering and Agriculture

University of Venda

South Africa

Supervisor:	Prof. E.T. Gwata
Co-supervisor:	Prof. G.R.A. Mchau
Co-supervisor:	Prof. A. I. Hassen

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Abstract

Pigeonpea (*Cajanus cajan)* is an important grain legume, which is grown in many African countries largely for human and animal consumption. Leguminous crops such as pigeonpea fix atmospheric nitrogen (N) symbiotically in the root nodules thus elimination of the need for expensive chemical nitrogenous fertilizers. The determination of host plant x microsymbiont combinations that produce optimum crop productivity is important in the pigeonpea value chain. In addition, the characterization of both symbionts is necessary for exploiting the N fixation in tropical legumes including pigeonpea. There is a dearth of information regarding the agronomic performance of pigeonpea and other common legume species such as Bambara groundnut (*Vigna subterranea*), soybean (*Glycine max*) and tepary (*Phaseolus acutifolius*) that are cultivated in South Africa. Therefore, the aim of this study was to evaluate the symbiotic effectiveness of indigenous rhizobia on pigeonpea.. The specific objectives of the study were to: (i) collect rhizobial strains that are associated with root nodulation in pigeonpea from diverse locations across South Africa (ii) perform molecular characterization of the rhizobia that are associated with root nodulation in pigeonpea from diverse locations in South Africa (iii) to sequence the whole genome of a selected rhizobial strain derived from pigeonpea and determine its molecular characteristics and (iv) determine the effectiveness of the rhizobial strains with pigeonpea and other common tropical legume species.

In the first objective of the study, forty soil samples were collected from diverse locations across the country and used for inoculating separately the seed of each of five randomly selected pigeonpea genotypes. The pigeonpea plants were raised in a N-depleted growth medium in the greenhouse. A split-plot experimental design with two replications was used in the study. After six weeks of growth, the plants were harvested to isolate rhizobia from the root nodules. Several morphological characteristics of the rhizobial colonies including shape and growth habit (type) were determined. In addition, a range of N fixation variables of the host pigeonpea plants was measured including the nodule dry weight (NDW) and shoot dry weight (SDW) per plant. A variety of the colony morphologies ranging from tiny to medium as well as cream white and large, watery oval colonies was observed. Two hundred and eighty putative pigeonpea rhizobial strains were obtained from the root nodules of the plants. Based on their morphological characteristics on YMA-CR, nutrient agar and peptone glucose agar, the isolates were deposited into the South African Rhizobium Culture Collection gene bank. There was >40.0 % difference in the number of nodules between 'Genotype-5' and 'Genotype-4' but the difference in NDW between the two genotypes was >80.0 %. In contrast, the heaviest dry shoots (0.4513 g) that were attained by

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'Genotype-3', weighed 52.0% more than the lightest dry shoots that were observed for 'Genotype-4'. The results indicated that the soil samples contained diverse rhizobial isolates with distinct morphological characteristics and significant differential N fixation ability of the pigeonpea genotypes suggesting that there was a potential to select for optimum host genotype x rhizobial strain combinations for N fixation in this legume species.

In the second objective of the study aimed at the molecular characterization of the rhizobial strains derived from pigeonpea, two housekeeping bacterial genes (namely 16S rRNA and *recA*) were used to identify each rhizobial strain to the species level. In addition, the phylogenetic relationships among these rhizobial strains were determined. The results showed that 56 strains were confirmed as rhizobia and deposited into the national rhizobia collection bank. Two primers successfully amplified both the *Rhizobium* strain (30bp3) as well as several *Bradyrhizobium* strains (16a2p3, 15bp3, 11a2p3, 13bp3, 33ap4 and 19a1p3). Two novel genera of rhizobia (*Phyllobacterium* and *Paraburkholderia*), were associated with root nodulation in pigeonpea. There was a considerable variation in the size of the sequences of both the 16S rRNA and *recA* genes among the rhizobial isolates. The sequences of the 16S rRNA genes across the four genera averaged 1015.73 bp. The 16S rRNA *Rhizobium* phylogenetic tree showed that the rhizobial isolates obtained from pigeonpea were dispersed in six different clusters and grouped with several type species of the genus *Rhizobium* including *R. tropici* with a high (77.0%) similarity grouping. The 16S rRNA based phylogenetic tree showed that the novel *Paraburkholderia* rhizobial isolate '30a2p3', grouped with several type strains including *P. rhizosphaerae* (with a similarity grouping >50%) but in the *recA* based phylogenetic tree, the same isolate was grouped in Cluster IV with 93.0% similarity grouping. The study concluded that the sequences of the two genes (16S rRNA and *rec*A) of the isolates from pigeonpea could provide sufficient phylogenetic information about the isolates up to the species level and confirmed that this legume is promiscuous in diverse soils from South Africa.

The objective focusing on the whole genome sequencing of a selected rhizobial strain derived from pigeonpea to determine its molecular characteristics selected the rhizobial strain 10ap3 (SARCC-755) (that was originally derived from pigeonpea in the trapping experiments). Upon DNA from the strain, the DNA libraries were prepared using the Nextera protocol (Illumina, USA) and paired-end (300bp x 2) sequenced on a MiSeq (Illumina) sequencer at the Biotechnology Platform, Agricultural Research Council-Onderstepoort Campus (Pretoria, South Africa). The genome was a large circular chromosome (6,297,373 bp) and containing the overall G + C content of 60.0%. The total number of genes in the genome of the strain was 6,013 of

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which 99.13% were coding sequences. However, only 5,833 of the genes were associated with proteins that could be assigned to specific functions. Several important genes that were found on the genome, included the genes for N metabolism, stress response, phosphorus metabolism and iron acquisition as well as adenosine monophosphate nucleoside for purine conversion. The nodulation gene (*nolR*), which functions as a DNA binding transcription factor was located on contig 12. Precursor genes for purine synthesis, for instance, inosine-5-monophosphate and adenylosuccinate, which are also responsible for nodule formation, were also present on the genome. The results showed that the genome of this strain (*Rhizobium tropici* SARCC-755) does not contain common *nod* and *nif* genes suggesting that an alternative pathway involving a purine derivative was involved in its symbiotic association with pigeonpea. The genome also possessed some genes that are associated with abiotic stresses and mineral nutrient acquisition thus making it a candidate for future formulation of commercial inoculants especially when considering its high symbiotic efficiency with pigeonpea.

In the fourth study objective, which focused (i) on determining the relative performance of individual tropical legume species when inoculated separately with each of the specific rhizobial strains that were previously derived from pigeonpea, (ii) quantify the magnitude of the effects of interactions between the host tropical legume species x rhizobial strain on a range of N fixation variables and (iii) identify the winning (superior) rhizobial strains with the specific test legume species. Thirty-six rhizobial strains which were previously isolated from pigeonpea root nodules were used in the study. There was at least one strain representing each of four distinct rhizobial genera namely *Bradyrhizobium, Paraburkholderia, Phyllobacterium* and *Rhizobium.* The experiment was laid out as a split plot design with legume species as the main factor and rhizobial strain as the sub-factor. Each treatment was replicated twice. The data sets of several N fixation variables including NDW and SDW were measured and subjected to the analysis of variance and Pearson's correlation analysis using SAS statistical software (version 9.3) followed by mean separation using LSD test at the 5.0% probability level. Further analysis using the GGE biplot model was carried out to understand better the relationship between the host plants and the microsymbionts.

For objective (i) of the fourth study, three healthy seeds of each legume species were planted in a plastic pot filled with 1.65 kg sterile river sand saturated with Hoagland solution. At seven weeks after germination, 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° C for 48 h followed by

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weighing to determine the dry weights. The results showed marked variability in the responses of the legume species to inoculations with individual rhizobial strains. Tepary bean showed poor nodulation as indicated by the chlorotic plants which contrasted sharply with those of Bambara groundnut. Pigeonpea responded differentially to each individual rhizobial strain resulting in marked differences in the nodule load per plant. Some rhizobial strains, (for instance, *Rhizobium* strain '26a-PP3') induced profuse nodulation in Bambara groundnut but not in the other legume species.

The principal component (PC) analysis showed that the first two principal components accounted for 78.74% of the total variation. Four N fixation variables, including the NDW and SDW, were moderately associated with PC1. The GGE biplot of the rhizobial strain x legume species interaction for NDW explained 82.44% of the total variation. For NDW, the environments represented by E3 (soybean) and E4 (pigeonpea) were positively correlated since their vectors were separated by an acute angle. However, E1 (Bambara groundnut) and E3 (soybean) were negatively correlated since they were characterized by an obtuse angle between them for the SDW. The 'which-won where' biplot for NDW explained 82.44% of the total variation of which PC1 and PC2 accounted for 50.40% and 32.04% of the total variation, respectively. Two rhizobial strains on the vertices of the polygon (*Rhizobium* sp. 36a-PP5) and (*Rhizobium* sp. 26a2-PP5) performed best with Bambara groundnut (E1) and soybean (E3), respectively. E3 (soybean) consisted of the longest vector line suggesting that it possessed a high discriminating ability. Two rhizobial strains, namely, (*Rhizobium* sp.; 33a-PP2) and (*Rhizobium multihospitium*; 37a-PP4) were identified as ideal for RDW and SDW, respectively. The biplot analysis also revealed that for SDW, E1 (Bambara groundnut) and E4 (pigeonpea), in that sequence, were plotted closet to the epicentre. The GGE biplot analysis also revealed that both pigeonpea and Bambara groundnut provided the most ideal symbiotic activity for NDW but tepary bean lacked the discriminatory ability for NDW. Further testing and validation of the symbiotic activities of the rhizobial strains identified in this study in field trials on diverse legume species and in multiple agro-ecological locations is recommended. It will also be desirable to identify new bio-inoculants for improving tepary bean productivity.

Key words: genome; legume productivity; symbiont; phylogeny; rhizobial strain.

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Dedication

This dissertation is dedicated to my family.

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Declaration

I, .. Phalane, F.L. .. hereby declare that this dissertation, for the Doctor of Philosophy in Agriculture 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, design and execution. All reference material contained therein has been duly acknowledged.

Student: Phalane, F.L.

Signature: ………………..… Date: ….17/07/2023 …...

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List of Abbreviations

AMP = adenosine monophosphate

ANOVA = analysis of variance

atpD = adenosine triphosphate D

Blastn = basic local alignment search tool nucleotides

bp = base pair

BUR = burkholderia primers for *recA* gene

^{o}C = degrees celcius

CDSs = coding sequences

cm = centimetre

df = degrees of freedom

DNA = deoxyribose nuclease acid

dnaK = protein coding gene

dNTPs = deoxynucleoside triphosphates

F = forward

Fe^{2+} = ferrous ions

g = grams

G + C = guanine plus cytosine

GGE = genotype plus genotype x environment

GPA = glucose peptone agar

glnII = glutamine synthetase isoform II

GTR+I+G = general time reversible with proportion invariable site and gamma distribution

gyrB = gyrase subunit B

h = hour

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Hsp70 = 70 kilodalton heat shock protein

ICRISAT = International Crops Research Institute for the Semi-Arid Tropics

Kb = kilobase

kg/ha = kilogram per hectare

LCOs = lipo-chito-oligosaccharides

LD = long duration

L-jar = leonard jar

MAFFT = multiple alignment using fast fourier transforform

Mb = megabase or million bases

MD = medium duration

$MgCl_2$ = magnesium chloride

m/v = mass per volume

mRNA = messenger RNA

µl = microliter

µM = micrometre

Min = minutes

Million t = million tons

mM = millimetre

MP = Mpumalanga

N = nitrogen

NA = nutrient agar

Nfixation = nitrogen fixation

NCBI = National Centre for Biotechnology Information

ncRNAs = non-coding Ribosomal Nucleic Acids

NDW = nodule dry weight

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NFW	= nodule fresh weight
NGS	= next generation sequencing
ng/µl	= nanogram per microliter
Nif	= nitrogen fixation
Nod	= nodulation
nolR	= nodulation gene R
ORFs	= open reading frames
P	= probability
PC	= principal component
PCA	= principal component analysis
PCR	= polymerase chain reaction
PG	= pigeonpea genotype
PGA	= peptone glucose agar
PGPR	= plant growth promoting rhizobacteria
PP	= pigeonpea
rpoB	= plastid-encoded RNA polymerase
PhyML	= phylogenetic estimation using maximum likelihood
PurL	= purine L
R	= Rhizobium
RDW	= root dry weight
recA	= recombinase A
rep	= replication
RFW	= root fresh weight
rRNA	= ribosomal RNA
RNAs	= Ribosomal Nucleic Acids

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SA	= South Africaof
SARCC	= South African Rhizobium Culture Collection
SAS	= statistical analysis software
SD	= short duration
SDW	= shoot dry weight
Sec	= seconds
SFW	= shoot fresh weight
SSC	= soil sample code
t/ha	= tons per hectare
tRNA	= transfer RNA
TY	= tryptone yeast
TYE	= tryptone yeast extract
USA	= United States of America
v/v	= volume per volume
WGS	= whole genome sequencing
YMA	= yeast mannitol agar
YMA-CR	= yeast-mannitol agar containing Congo red

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List of Research Outputs from this Study

1. **Bopape FL**, Beukes CW, Kopotsa K, Hassen AI, Steenkamp ET, Gwata ET. (2022). Symbiotic performance and phylogenetic characterization of pigeonpea (*Cajanus cajan* L. Millsp.) rhizobia occurring in South African soils. Agriculture. *In Review*.

2. **Bopape FL**, Gwata ET, Hassen AI, Zhou MM (2021). Symbiotic efficiency of pigeonpea (*Cajanus cajan*) with different sources of nitrogen. *Plant Genetic Resources*. 19(4): 1-4. doi:10.1017/S1479262121000356.

3. **Bopape FL**, Hassen AI, Swanevelder ZH, Gwata ET (2020). Draft genome sequence of *Rhizobium tropici* SARCC-755, a free-living *Rhizobium* that nodulated and promoted growth in pigeonpea [*Cajanus cajan* (L.) Millsp.] *Microbiology Resource Announcements*. 9: 1-2. doi.org/10.1128/MRA.01122-19.

4. Doctoral Candidate, Ms **Francina Bopape** discovers unusual Bio-Inoculant (2020). *Nendila*. https://www.univen.ac.za/news/doctoral-candidate-ms-**francina-bopape**-discovers-unusual-bio-inoculant/ (Accessed 24 November, 2022).

5. Doctoral student from Univen discovers unusual bio-inoculant. *Limpopo Mirror*. https://limpopomirror.co.za/articles/news/52125/2020-03-16/doctoral-student-from-univen-discovers-unusual-bio-inoculant (Accessed 24 November, 2022).

Conferences/ Symposia

1. **Bopape FL**, Gwata ET, Hassen IA (2021). Oral presentation" Symbiotic efficiency of pigeonpea (*Cajanus cajan*) with different sources of nitrogen" at ARC-PHP Online Webinar for Celebrating Woman in Science, 27 August 2021.

2. **Bopape FL**, Hassen IA, Gwata ET (2019). Poster presentation "Diverse group of rhizobia associate with root nodulation of pigeonpea in South Africa" at the 45th joint congress of the Southern African Association of Botanists, African Mycological Association and the Southern African Society for Systematic Biology held at University of Johannesburg, (South Africa), 08-11 January 2019.

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3. **Bopape FL** (2018). Oral presentation: "Diversity of rhizobia associated with root nodulation in pigeonpea (*Cajanus cajan*) from South Africa" at the joint SANBI Biodiversity Information Management and Foundational Biodiversity information programme forum held at Cape St. Francis Resort, Eastern Cape, (South Africa), 13-16 August 2018.

4. **Bopape FL** (2017). Oral presentation "The isolation of indigenous rhizobia from diverse locations across South Africa that are associated with root nodulation in pigeonpea" at the joint SANBI and Foundational Biodiversity Information Programme for Postgradraduate Student Forum held at Salt Rock and Beach Hotel, Durban, (South Africa), 14-17 August 2017.

5. **Bopape FL** (2017). Oral presentation "The isolation of indigenous rhizobia from diverse locations across South Africa that are associated with root nodulation in pigeonpea" at the PPRI Student Seminar, ARC-Roodeplaat, Pretoria (South Africa), May 2017.

6. **Bopape FL (**March, 2020) was interviewed by Berry FM and Malamulele FM in March 2020, about the discovery of an unusual rhizobial strain that is used as a bio-inoculant for beans to improve yields.

Award

1. **Bopape FL** (2016). Summer Research Internship Program (SRIP) Internship by University of Virginia through, University of Venda and DST between 27 May-5 August 2016.

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1.0 Chapter One: General Introduction

1.1 Introduction

Pigeonpea (*Cajanus cajan*) is a grain crop, which originated in the Indian sub-continent more than 3500 years ago (Patel *et al.,* 2010; Bohra *et al.,* 2012). It is an important crop in the tropics and subtropics. Currently, it is cultivated in many parts of the world including Africa and Asia as a source of food and family income. In the African continent, the crop is produced mainly in East Africa but recently, significant production was reported in Malawi, Mozambique, Tanzania, Kenya and Uganda (Jones *et al*., 2002; Høgh-Jensen *et al.,* 2007; Ayenan et al., 2017). In South Africa, pigeonpea is cultivated as a minor crop in home gardens or backyards mostly in Mpumalanga, KwaZulu-Natal and Limpopo provinces as a food source (Nndwambi, 2015; Hluyako *et al*., 2017).

The crop has multiple diverse uses that include animal feeding and as a source of food for humans. In some Asian countries, the crop is also used for medicinal purposes, as its roots, leaves and flowers are used to treat diseases of several organs like liver, skin and kidney (Yohane 2020). Pigeonpea also contributes to soil health and fertility through biological nitrogen fixation and improves the quality of the soil (Mapfumo et al, 1999; Hillocks *et al*., 2000). The crop is highly tolerant to drought than most legumes (Naylor *et al*., 2004; Emefiene *et al*., 2013). The grains of pigeonpea are rich in nutrients. The grains of pigeonpea are traded in both informal and formal markets, to generate household income (Gwata and Shimelis, 2013).

The productivity of pigeonpea is generally low particularly in the smallholder cropping systems. For instance, in Tanzania, a low average yield (0.4 t/ha) was reported by Myaka *et al*., (2006), while in southern Africa 3.0t/ha was obtained (Gwata and Siambi 2009). In addition, a wide variation in the N-fixation capacity (40-235 kg/ha) of the crop was reported (Mapfumo *et al*., 1999; Peoples *et al*., 1995; Myaka *et al*., 2006). It is possible this variation could be attributed partly to environmental factors as well as genetic factors (Saxena, 2008; Silim *et al*., 2007; Kumar *et al*., 2009). The knowledge of the genetic components of effective root nodulation (which results in nitrogen fixation) will assist in the improvement of pigeonpea growth as well as production.

The symbiotic relationship between the host plant genotype and the rhizobia is still poorly understood for pigeonpea. In addition, the fixation potential between rhizobia, which is compatible with pigeonpea and other tropical legumes such as Bambara groundnut (*Vigna subterranean*), chickpea (*Cicer arietinum*), tropical soybean (*Glycine max*), and tepary bean (*Phaseolus acutifolius*) is poorly understood. A recent study in India found that about 40.0% of rhizobial isolates collected from diverse legumes were compatible with pigeonpea (Arora et

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al., 2018). In South Africa, most of the soil samples that were collected from diverse locations and used for inoculating pigeonpea seed at planting, showed the presence of rhizobia that were compatible with pigeonpea (Bopape et al., 2021).Even though pigeonpea shows symbiotic efficiency with rhizobia, it is necessary to identify more efficient rhizobial strains in order to achieve optimum grain yields.

In South Africa, there are no commercial inoculants available for the crop. Therefore, it is necessary to identify optimum genotype x rhizobial strain combinations that produce optimum plant productivity. Characterization of rhizobial strains that combine efficiently with pigeonpea can contribute to the genetic improvement of the crop for the benefit of growers and end users.

1.2 Rationale of the study

Pigeonpea is important particularly in smallholder cropping systems in Africa. The collection of soil samples from diverse locations across South Africa for inoculating pigeonpea will enable trapping of rhizobial isolates in the root nodules and their subsequent purification, characterization and exploitation. In addition, this approach will provide useful information regarding the potential to select for optimum host x microsymbiont combinations for the future expansion of the pigeonpea production area in South Africa. Currently, there are no commercial inoculants available for the crop in the country. Therefore, it is necessary to identify indigenous rhizobial isolates that are compatible with the crop and have potential as commercial inoculants. The molecular and microbiological profiling of the rhizobial isolates that combine efficiently with pigeonpea can contribute to the selection and future development of commercial inoculants for pigeonpea and other tropical legumes in the region and beyond. Moreover, determination of the genetic relationships among the isolates will potentially contribute to the future designing of efficient inoculants for utilization in the cultivation of common tropical legumes that are produced in South Africa.

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1.3 Aim and objectives of the study

The aim of the study was to evaluate the symbiotic effectiveness of indigenous rhizobia on pigeonpea. The specific objectives of the study were to:

(i) collect and evaluate the abundance and variability of rhizobia in the soil from diverse locations across South Africa on pigeonpea genotypes
(ii) determine the genetic relationships among the indigenous rhizobial isolates from multiple genera that are associated with root nodulation in pigeonpea

(iii) identify and sequence the genome of an indigenous rhizobial isolate that was compatible with pigeonpea
(iv) determine the symbiotic effectiveness of the rhizobial isolates with pigeonpea and other common tropical legumes that are cultivated in South Africa.

1.4 Hypotheses

The study tested the following hypotheses:

(i) the soil samples that were collected from diverse locations across South Africa were similar in symbiotic effectiveness among pigeonpea genotypes
(ii) the indigenous rhizobial isolates from multiple genera that are associated with root nodulation in pigeonpea were genetically related
(iii) the sequence and size of the genome of the indigenous rhizobial isolate compatible with pigeonpea was similar to the genome of rhizobia from other legumes
(iv) there was similarity in the symbiotic effectiveness of the rhizobial strains among pigeonpea and other common tropical legumes that are cultivated in South Africa.

1.5 Structure of the thesis

The first chapter of this thesis introduces the background information regarding the genetics of nitrogen fixation in pigeonpea and presents the problem statement, rationale of the study and the study objectives. The second chapter reviews the literature that is relevant to the research topic. Chapter Three presents the results of the first study objective, which focused on collecting soil samples from across South Africa and use them for inoculating pigeonpea genotypes, in order to determine the abundance and the nodulation abilities of the rhizobia found in the soils. Chapter Four presents the results of the genetic relationships among the rhizobial isolates from the first study objective. In chapter Five, the results of the genome sequence and size of the selected indigenous rhizobial isolate that was compatible with pigeonpea, are presented and discussed. Chapter Six discusses the symbiotic

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effectiveness of a set of rhizobial isolates among common tropical legumes that are cultivated in South Africa. The final chapter summarizes the key findings of the study and assesses their implications.

References

Amarteifio JO, Muthali C, Karikari SK, Morake TK (2002). The composition of pigeonpea (*Cajanus cajan* (L) Millsp.) grown in Botswana. *Plant Foods for Human Nutrition*. 57: 173-177.

Arora NK, Khare E, Singh S, Tewari S (2018). Phenetic, genetic diversity and symbiotic compatibility of rhizobial strains nodulating pigeonpea in Northern India. *Biotechnology*. 8(52): 1-11. doi: org/10.1007/s13205-017-1074-1.

Ayenan MAT, Ofori K, Ahoton LE, Danguah A (2017). Pigeonpea [(*Cajanus cajan* (L.) Millsp)] production system, farmers' preferred traits and implications for variety development and introduction in Benin. *Agriculture and Food Security.* 6(48): 1-11.

Bohra A, Saxena RK, Gnanesh BN, Saxena K, Byregowda M, Rathore A, KaviKashor PB, Cook DR, Varshney RK (2012). An intra-specific consensus genetic map of pigeonpea (*Cajanus cajan* (L) Millspaugh) derived from six mapping populations. *Theoretical and Applied Genetics*. 125(6): 1325-1338.

Bopape FL, Gwata ET, Hassen AI, Zhou MM (2021). Symbiotic efficiency of pigeonpea (*Cajanus cajan*) with different sources of nitrogen. *Plant Genetic Resources*. 19(4): 1-4. doi:10.1017/S1479262121000356.

Emefiene ME, Salaudeen AB, Yaroson AY (2013). The use of pigeonpea (*Cajanus cajan*) for drought mitigation in Nigeria. *International Letters of Natural Sciences.* 1: 6-16.

Faris DG, Singh U (1990). Pigeonpea: nutrition and products. *In*: Nene YL, Hall SD, Sheila VK. (eds). The Pigeonpea. Wallingford, UK. CAB International, Wallingford. pp 401-434.

Gwata ET, Silim SN, Mgonja M (2006). Impact of a new source of resistance to *Fusarium* wilt in pigeonpea. *Journal of Phytopathology*. 154: 62-64.

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Gwata ET, Siambi M (2009). Genetic enhancement of pigeonpea for high latitude areas in Southern Africa. *African Journal of Biotechnology.* 8(18): 4413-4417.

Gwata ET, Shimelis H (2013). Evaluation of pigeonpea germplasm for important agronomic traits in Southern Africa. *In:* Goyal A, Asif M. (eds). *Agricultural and Biological Sciences - Crop Sciences*. Goyal A, Asif M. (eds). pp. 1-15.

Hillocks RJ, Minja E, Nahdy MS, Subrahmanyam P (2000). Diseases and pests of pigeonpea in eastern Africa. *International Journal of Pest Management.* 46: 7-18.

Høgh-Jensen H, Myaka FA, Sakala WD, Kamalongo D, Ngwira A, Vesterager JM, Odgaard R, Adu-gyamfi JJ (2007). Yields and qualities of pigeonpea varieties grown under smallholder farmer's conditions in eastern and southern Africa. *African Journal of Agric*ultural *Research*. 2: 269-278.

Jones RB, Freeman HA, Lo Monaco G (2002). Improving the access of small farmers in Eastern and Southern Africa to global pigeonpea markets. *Agricultural Research and Extension Network.* 120: 1-11.

Kumar CVS, Sreelakshmi C, Shivani D, Suresh M (2009). Gene effects for yield contributing characters in pigeonpea (*Cajanus cajan* L. Millsp) by generation mean analysis. *Journal of Research ANGRAU.* 37(3&4): 71-76.

Mapfumo P, Giller KE, Mpepereki S, Mafongoya PL (1999). Dinitrogen fixation by pigeonpea of different maturity types on granitic sandy soils in Zimbabwe. *Symbiosis.* 27: 305-318.

Myaka FA, Sakala WD, Adu-Gyamfi JJ, Kamalongo D, Ngwira A, Odgaard R, Nielsen NE, Høgh-Jensen H (2006). Yields and accumulations of N and P in farmer-managed maize-pigeonpea intercrops in semi-arid Africa. *Plant and Soil*. 285: 207-220.

Naylor RL, Falcol WP, Goodman RM, Jahn MM, Sengooba T, Tefera H, Nelson RJ (2004). Biotechnology in the developing world: A case for increased investments in orphan crops. *Food Policy*. 29: 15-44.

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Nndwambi FH (2015). Evaluation of dryland maize / pigeonpea intercropping under variable phosphorus application rates. MSc desitation, University of Limpopo. pp 7-13.

Odeny DA (2007). The potential of pigeonpea (*Cajanus cajan* (L.) Millsp.) in Africa. *Natural Resources Forum.* 31: 297-305.

Patel KJ, Singh AK, Nareshkumar G, Archana G (2010). Organic-acid-producing, phytate-mineralizing rhizobacteria and their effect on growth on pigeonpea (*Cajanus cajan*). *Applied Soil Ecology.* 44(3): 252-261.

Peoples MB, Herridge DF, Ladha JK (1995). Biological nitrogen fixation: An efficient source of nitrogen for sustainable agricultural production? *Plant and Soil.* 174: 3-28.

Rao SC, Coleman SW, Mayeux HS (2002). Forage production and nutritive value of selected pigeonpea ecotypes in the southern Great Plains. *Crop Sciences.* 42: 1259-1263.

Saxena KB (2008). Genetic improvement of pigeonpea-a review. *Tropical Plant Biology.* 1: 159-178.

Silim SN, Gwata ET, Coeb R, Omanga PA (2007). Response of pigeonpea genotypes of different maturity duration to temperature and photoperiod in Kenya. *African Crop Science Journal.* 15(2): 73-81.

Singha B, Das P, Mazumder PB (2015). Morphological and biochemical characterization of rhizobia isolated from root nodule of *Crotalaria junceae* L. grown in Assam. *International Journal of Sciences and Research.* (4)4: 1928-1931.

Sprent JI (2009). Legume nodulation: A global perspective. Wiley Blackwell. pp 1-200.

Yohane EN 2020. Genetic improvement of pigeonpea [*Cajanus cajan* (L.) Millspaugh] for yield, earliness and resistance to *Fusarium* wilt (*Fusarium udum* Butler) in Malawi. PhD thesis, University of KwaZulu-Natal. pp 79-153.

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2.0 Chapter Two: Literature Review

2.1 General background

This chapter reviews the relevant aspects of root nodulation in pigeonpea (*Cajanus cajan*). The aspects will cover interaction of soil rhizobia with pigeonpea and techniques used to identify and characterize root nodulating rhizobia. In addition, this chapter will review the compatibility of indigenous soil rhizobia with pigeonpea and other tropical legumes as well as the determination of rhizobial strain x legume genotype combinations for optimum productivity.

In leguminous plants, root nodulation is the formation of nodules on roots. It is important to distinguish between the root nodules and those that are formed on other plant organs such as leaves (Pinto-Carbo et al., 2018; Yang and Hu 2018). The root nodules are circular structures that are induced by the presence of soil and bacteria in the rhizosphere. The rhizobia (soil bacteria) utilize the nitrogenase enzyme (among many other enzymes) to catalyse the conversion of atmospheric nitrogen (N) to ammonia for use by the host plant (Ladha et al., 2022; Signorelli et al., 2020; Wagner 2011). This process is also referred to as biological nitrogen fixation or simply as N fixation (Graham and Vance, 2000). This symbiotic association benefits both the host plant and the microsymbiont (Tariq et al., 2017; Overstreet and Lotz 2016). For example, in soybean (*Glycine max*), the plant benefits from the synthesis of nitrogenous compounds in the nodule such as allantoin and allantoic acids (Yamashita et al., 2019; Streeter 1979) which are subsequently translocated and catabolized in the rest of the plant organs (for instance, stems, leaves, and seeds), thus providing the N requirement for the plant and reducing the need to apply chemical nitrogenous fertilizers which are generally expensive and maybe detrimental to the environment. Because N is the most limiting element in agricultural production, the N fixation process provides an alternative cheap (or affordable) source of organic N for the growth and development of legumes.

Apart from N fixation, the legumes, including pigeonpea, are important sources of carbohydrates, fibres, minerals, proteins and vitamins in human food (Emily et al., 2017) and livestock feeds (Jezierny et al., 2010; Thomas and Sumberg, 1995; Gatel, 1994). Some of the legumes are also used in making a wide range of pharmaceutical and industrial products (Benevides et al., 2018 Emily et al., 2017; Morris 2003). Moreover, the grain of legumes is traded in markets, thus generating household income (Oppewal and da Cruz 2017; Gwata and Shimelis 2010).

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2.2 Pigeonpea origin and uses

Pigeonpea is a grain legume, which originated from the Indian sub-continent more than 3500 years ago (Bohra et al., 2012; Patel et al., 2010). It is believed that, likely, colonial railway workers and storekeepers brought pigeonpea to the coastal areas in east Africa in the 19th century (Hillocks et al., 2000). Currently, it is cultivated in many parts of the world including southern Africa (Ayenan et al., 2017; Fossou et al., 2016).

Pigeonpea belongs to the subtribe *Cajaninae* of the tribe *Phaseoleae* that contains several other common legumes such as soybean, common bean (*Phaseolus vulgaris*) and mung bean (*Vigna radiata*) (Young et al., 2003). It has a perennial growth habit and considered as versatile partly because of its multiple uses (Table 2.1). The grain of pigeonpea provides the main source of proteins, vitamins, minerals as well as starch, fats and fibre (Ayenan et al., 2017). In addition, the grain is traded formally and informally, thus generating income for households particularly in the smallholder sector (Shiferaw et al., 2008). The crop has additional multiple benefits to cropping systems particularly the improvement of soil structure and soil fertility (Araujo et al., 2015; Mapfumo et al, 1999). It is also highly tolerant to drought (Araujo et al., 2015; Gwata and Siambi, 2009). It can also reduce soil erosion partly because of its dense and deep root system (Melchora and Fe, 2022).

2.3 Pigeonpea production

The major pigeonpea producing countries are India and Myanmar (Pais and Bansal, 2019). The production in Africa is dominated by countries in the eastern region (Fig. 2.1). However, the average yield in the east Africa region is relatively low (Table 2.2). Pigeonpea grain yields >2.0 t/ha in Africa were reported previously (Gwata, et al., 2006; Silim et al., 2005). In west Africa (for example in Benin, Nigeria and Ghana), pigeonpea is produced as a minor crop (Ayenan et al., 2017; Dansi et al., 2012; Adjei-Nsiah 2012). In South Africa, pigeonpea is cultivated as a minor crop mostly in Mpumalanga Province, Limpopo Province and the coastal regions of KwaZulu-Natal Province (Musokwa and Mafongoya 2020). However, the crop has considerable potential in the rest of the country (Bopape et al., 2021; Mogashoa and Gwata, 2009).

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Table 2.1 Examples of the multiple uses of pigeonpea.

Component of pigeonpea plant	General use	Example	Reference
1. Grain	Food	(i) human food; market / export	Ayenan et al., 2017; Odeny 2007; Daniel and Ong 1990
		(ii) animal feeds / livestock feeding	Pat et al., 2011; Wallis et al., 1986
		(iii) green pods as vegetables	Pat et al., 2011
2. Stem	Fuel and shelter	(i) fire wood	Ayenan et al., 2017; Daniel and Ong 1990; Wallis et al., 1986
		(ii)building structures / thatching and fencing	Daniel and Ong, 1990
		(iii) hedge; basket making	Upadhyaya, 2011; Daniel and Ong 1990; Wallis et al., 1986
	Medicinal purpose	(i) malaria and diziness	Ayenan et al., 2017; Pat et al., 2011
		(ii) eye infection and measles	Pat et al., 2011
3. Leaves	Fertilizer	(i) organic manure (crop residue)	Pat et al., 2011; Daniel and Ong 1990; Wallis et al., 1986
	Medicinal	(i) gingivitis; fever; dizziness; eye infection; measles; snake bite; ulcer; malaria	Ayenan et al., 2017,Pat et al., 2011
	Fodder / forage crop	(i) dried hay / straw for livestock feeding	Pat et al., 2011; Daniel and Ong 1990; Wallis et al., 1986
4. Roots	Medicinal (phenolic compound)	(i) anti-inflammation; antibacterial; antioxidant; anticarcinogenic; antidiabetic	Pat et al., 2011
		(ii) measles	Pat et al., 2011
		(iii) smallpox; chicken pox; fever	Pat et al., 2011
5. Whole plant	Forage crop	(i) livestock grazing	Pat et al., 2011
	Cover crop	(i) manage soil erosion; improve soil health / physical and chemical parameters	Pat et al., 2011; Daniel and Ong 1990
		(ii) manage water weeds, pests and diseases	Pat et al., 2011
	Medicinal	(i) diabetes; skin irritation; hepatitis; constipation	Ayenan et al., 2017, Pat et al., 2011
		(ii) bladder stones	Ayenan et al., 2017, Pat et al., 2011

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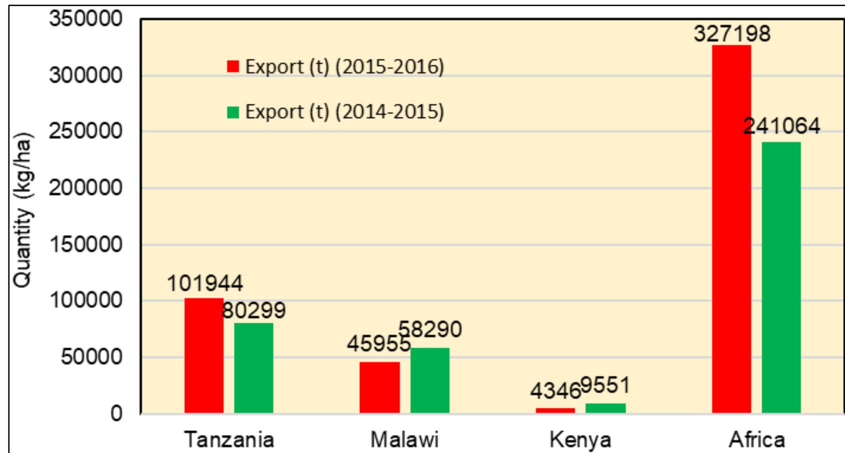

Fig. 2.1 The production of pigeonpea in Africa (*Adapted from*: Pais and Bansal, 2019).

Table 2.2 The production of pigeonpea from three east African countries during the 2012/2013 season.

Country	Production (million t)		Average grain yield (kg/ha)
	Total	% (Africa)	
Tanzania	0.2	4.5	855.0
Malawi	0.3	6.8	1 268.0
Kenya	0.2	4.5	749.0
Africa	4.4		718.0

Adapted from: Pais and Bansal, 2019.

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2.4 Agronomy and performance of pigeonpea

There are three types of pigeonpea based on the duration to maturity. The first group consists of short-duration (SD) pigeonpea genotypes that require approximately 90 – 110 days to mature. The second and third groups consist of medium duration (MD) and long duration (LD) types, which mature in approximately 150 days and 180 days, respectively (Gwata and Siambi, 2009). The seed is planted at about 3.0 cm depth and spaced at 0.5 m (inter-row) x 0.15 m (intra-row) (for SD types) or at 1.5 m (inter-row) x 0. 5 m (intra-row) (for MD types) or at 1.7 m (inter-row) x 0. 6 m (intra-row) (for LD types). In general, smallholder farmers use no fertilizer for pigeonpea. On average, the crop requires weeding at least twice where herbicides are not in use. Where chemical fertilizer is available, growers apply compound fertilizer with both phosphorus and magnesium depending on the soil pH and fertility and (Pal et al., 2011).

The agronomic performance of pigeonpea is affected by both abiotic and biotic factors. For instance, terminal season drought reduced severely the grain yield and quality of MD pigeonpea genotypes at Chitedze (Malawi) (Gwata and Siambi, 2009). In addition, some pigeonpea genotypes are susceptible to low winter temperatures (Gwata, 2010) and soil pH (Amijee and Giller,1998). Several factors also influence the process of N fixation (Table 2.3). In general, pigeonpea is compatible with indigenous soil rhizobial strains that occur naturally in the African soils and are generally classified as the cowpea miscellany types (Bopape et al., 2021; Abaidoo, 1999). Some indigenous soil rhizobia are competitive and effective on pigeonpea, rendering seed inoculation unnecessary in cropping systems (Khurana and Dudeja 1994). In South Africa, pigeonpea is currently cultivated without seed inoculation by smallholder growers since there are no commercial inoculations for the crop.

The root nodules in pigeonpea are formed in a similar process as in other legumes, which were described previously (Wang et al., 2018; Oldroyd et al., 2011; Jones et al., 2007). The subsequent N fixation in the root nodules of the crop is influenced by a wide range of factors including rhizospheric nutrient dynamics (Makoi and Ndakidemi, 2011) and flavonoid accumulation (Antunes et al., 2006), among others (Table 2.3). Moreover, the diversity of the rhizobia that are associated with N fixation in pigeonpea in many production regions including South Africa, has not been determined adequately. The information is critical for expanding the crop to new production areas and for selecting rhizobial strains that produce optimum N fixation with pigeonpea.

One of the major diseases that plagued pigeonpea particularly in east Africa was Fusarium wilt (Gwata et al., 2006). In addition, damage caused by insect pests such as the pod borer (*Helicoverpa armigera*) is well documented (Babariya et al., 2010; Hillocks et al., 2000). In a survey of insect pests that affect pigeonpea, blister beetles and pod suckers were reported (Kunjeku and Gwata, 2011). The improved genotypes with resistance (or tolerance)

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to various stresses tend to perform significantly better than the unimproved landraces (Gwata and Silim, 2009).

Table 2.3 Examples of factors that influence nitrogen fixation in pigeonpea and other legumes

	Factor	Effect	Reference
1	Flavonoid accumulation / nod gene inducers	Activates transcription of nodulation genes; flavonoid nod-gene inducers are specific for each legume rhizobium interaction their production is influenced by nod factors	Mohammadi et al., 2012; Antunes et al., 2006
2	Moisture / drought	Reduces development of root hair, affect the site of entry by rhizobia into the legume, which results in poor nodulation or lack of nodulation.	Rupela and Rao, 1987
3	Organic matter / soil nutrients / soil fertility	Excess soil nitrate availability supresses N fixation	Mohammadi et al., 2012; Makoi and Ndakidemi, 2011
4	Salinity	Reduces development of root hair, affect the site of entry by rhizobia into the legume, which results in poor nodulation or lack of nodulation.	Rupela and Rao, 1987
5	Soil pH	Low soil pH may limits plant growth, nodulation and N fixation; rhizobia that tolerate acidity fix inadequate N; reduces the uptake of nutrients from the soil; optimum recommended for plant growth and N fixation the recommendedis between 6.0 - 7.0.	Mohammadi et al., 2012; Amijee and Giller,1998
6	Temperature	Extreme soil temperatures reduce development of root hair, affect the site of entry by rhizobia into the legume, resulting in poor nodulation or lack of nodulation; survival and availability of rhizobia in the soil and N fixation; symbiotic relationship requires optimum temperature.	Rupela and Rao, 1987; Mohammadi et al., 2012

2.5 Uses and performance of similar common tropical legumes in South Africa

The common tropical legumes such as Bambara groundnut (*Vigna subterranean*), soybean (*Gycine max*) and tepary bean (*Phaseolus acutifolius*), common bean (*Phaseolus vulgaris*) and cowpea (*Vigna unguiculata*) are well adapted to most soil and climatic conditions. They are utilized mainly for human food in a similar manner to pigeonpea. For example, the dry grain of cowpea as well as the tender leaves can be cooked for human consumption while the remaining haulms used for animal feeds. They are rich in micronutrients, vitamins and antioxidants (Semba et al., 2021; Ohanenye et al., 2020; Çakir et al., 2019).

The tropical legumes are also frequently intercropped with maize, sorghum or millet and they can produce good grain yields under drought conditions partly due to their long tap roots as well as their abilities to employ various drought tolerance mechanisms (Khatun et al., 2021; Pnueli et al., 2002). The legumes also provide raw materials for several industrial products. For instance, soybean is useful in the production of cooking oil, soy oil cake, soy meal and biodiesel (Cao et al. 2005; Johnson and Myers1995). Therefore, these common legumes have many beneficial uses for both growers and end-users.

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In terms of symbiotic performance, most *Vigna* species nodulate with indigenous slow-growing *Bradyrhizobium* that are present in most African soils (Fadimata 2021; Nyaga and Njeru 2020; Sprent et al., 2010). In Ethiopia, *Bradyrhizobium* and *Rhizobium* strains were isolated from pigeonpea (Degefu et al., 2018). The response of tepary bean to the various rhizobial genera is not adequately documented. More importantly, the evidence of significant improvement in their grain yield or general productivity in response to inoculation is still fragmented. This is partly due to the multiple factors that influence N fixation. Moreover, these tropical legumes are cultivated largely by subsistence smallholder growers especially in Africa. In some cases, the farmers are constrained by lack of storage infrastructure, cost and technical competence to apply bio-inoculants correctly (Mpepereki et al., 2000).

2.6 Rhizobia which associate with pigeonpea and other tropical legumes

The soil bacteria that nodulate legumes and fix N belong to *Proteobacteria* which is found in the domain Eubacteria and is divided into the five sub-groups, namely, α-, *β*-, *γ*-, δ- and ε-*Proteobacteria* (Zakhia and de Lajudie, 2001; Jordan, 1984) based on the highly conserved small subunit 16S ribosomal RNA (16S rRNA) gene sequence analyses. Only *α*- and *β*-*Proteobacteria* are capable of nodulation and nitrogen fixation (Chen et al., 2003a).

Pigeonpea can promiscuously associate with natural soil rhizobia from a diverse range of genera such as *Bradyrhizobium* and *Rhizobium* that commonly nodulate pigeonpea (Table 2.4) (Bopape et al., 2021; Degefu et al., 2018; Rufini et al., 2016; Ramsubhag et al., 2002). Similarly, Bambara groundnut is nodulated with a wide of range of *Rhizobium* sp. strains (Hassen et al., 2022). When these rhizobia are applied as bio-fertilizers (or inoculants) to seed before or during planting, they form nodules on the plant roots and subsequently fix N. However, the rhizobia can fail to compete for nodule occupancy against indigenous and natural rhizobia thus leading to low crop yields. The ability to form and develop effective nodules in the presence of other strains determines the nodulation competitiveness of each rhizobial strain (Onishchuk et al., 2017; Yates et al., 2011). Superior strains under controlled conditions can be selected as potential bio-inoculants but failure to out-compete natural rhizobia as well as the inability to remain in the fields for several seasons due to the transfer of symbiotic genes to the indigenous strains, can limit the use of commercial inoculants (Mendoza-Suarez et al., 2021). Currently, the rhizobia that are used for inoculant production in South Africa include *Bradyrhizobium japonicum* (for soybean) (Table 2.5). These commercial inoculants were developed mainly from only three rhizobial genera implying that the strains from the remainder of the genera are either not compatible or they show no significant impact on the N fixation of the common tropical legumes. In addition, it is logical to conclude that there is variation among tropical legumes in response to rhizobial strains from

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distinct genera. Moreover, there are no reports of N fixation of the common tropical legumes resulting from inoculation with *Paraburkholderia* or *Phyllobactrium*.

Table 2.4 Summary characteristics of rhizobial genera.

	Genus	Summary characteristics and important notes	References
1	*Bradyrhizobium*	Slow-growing; nodulates soybean, cowpea,lima bean, peanuts; most dominant genus in African soil; colonies produce an alkaline reaction in mineral salts medium containing mannitol.	Delamuta et al., 2013; Xu et al, 1995; Duran et al., 2014; Steenkamp et al., 2008; Ormeno-Orrillo et al., 2019; Jordan 1984).
2	*Rhizobium*	Fast-growing; nodulates bean, clover and pea; production of acid in mineral salts-mannitol medium; contains >60 species.	Amat et al., 2020; Fred *et al*., 1932; Young, 1996, Willems, 2006
3	*Mesorhizobium*	Species in this genus were previously members of the *Rhizobium* genus; separated from the fast-growing rhizobia based on the location of symbiotic genes, 16S rRNA phylogeny and DNA homology checks.	Van Berkum and Eardley, 1998; Jarvis et al., 1997
4	*Sinorhizobium*	Fast-growing; nodulates *Medicago* spp. including lucerne and soybean; considered synonymous with the genus Ensife; contains 11 species, with S. meliloti as the main type strain.	Chen et al., 1988; Martens et al., 2007;de Lajudie et al. 1994
5	*Azorhizobium*	Associate with *Sesbania rostrata* forming nodules on their stem and roots; intermediate to fast-growing; closely related to genus *Xanthobacter* and *Bradyrhizobium*.	Lee et al., 2008; Rinaudo et al., 1991; Dreyfus et al., 1988
6	*Allorhizobium*	*Allorhizobium undicola* only nitrogen fixing rhizobia from this genus. Effective on tropical *Neptunia natans* that was discovered in Senegal. Closest relative of *Allorhizobium* genus is *Agrobacterium vitis*.	de Lajudie et al., 1998
7	*Phyllobacterium*	Intermediate in growth; contains five species capable of nodulation; originally isolated from root nodules of *Brassica napus* in France; *P. trifolii* associates with plants of the genus *Trifolium pratense* and *Lupinus albus.*	Mantelin *et al.,* 2006; Valverde et al., 2005; Young et al., 2001
8	*Onchrobactrum*	From the family *Brucellaceae;* genus Ochrobactrum has 11 species; *Ochrobactrum lupini* and *Ochrobactrum cytisi* were the first species from this genus; *O. lupini* originally isolated from *Lupinus albus* plant nodules in Argentina; *O. cytisi* was isolated from *Cytisus scoparius* root nodules in Spain.	Zurdo-pineiro et al., 2007; Trujillo et al., 2005; Holmes et al., 1988
9	*Methylobacterium*	Pink-pigmented facultative methylotrophic bacteria; *M. nodulans* is the only species from this genus which is facultative methylotropic, unpigmented; originally isolated from *Crotalaria* spp.; *M. nodulans* utilizes methanol and formate as sole carbon source.; other strains were isolated from water and leaf surfaces.	Green, 1992; Sy et al., 2001; Jourand et al., 2004; Holland, 1997
10	*Devosia*	Belongs to the family *Hyphomicrobiaceae*; soil and water bacteria; *D. neptuniae* is the first Devosia species found to fix N in symbiosis with the aquatic legume *Neptunia natans* from India.	Nakagawa et al., 1996; Rivas et al., 2003
11	*Paraburkholderia*	Beneficial root nodulating *Burkholderia* that nodulate mimosoid legumes. *Burkholderia aspalathi* was isolated in South Africa from the plant *Aspalathus abietina.*	Moulin et al., 2001; Sy et al., 2001; Perin et al., 2006; Mavengere et al., 2014.
12	*Cupriavidis*	*Cupriavidus taiwanensis* (Chen *et al.,* 2003b) is the only effective rhizobial symbiont of *Mimosa pigra* and *Mimosa pudica.*	Vandamme and Coenye, 2004; Barrett and Parker, 2006; Chen et al., 2003b.
13	*Hebarspirillum*	*Herbaspirillum lusitanum* originally isolated from the root nodules of *Phaseolus vulgaris* in Portugal; only diazotrophic species within the genus *Herbaspirillum* with the ability to form symbiotic associations with *Oryza officicalis*. Induce the formation of indeterminate nodules on most Mimosa species.	Valverde et al., 2003; Yabuuchi et al., 1995
14	*Pararhizobium*	The genus is adjacent to *Rhizobium; type* species is *Pararhizobium giardinii;* isolated initially from *Phaseolus vulgaris* (Common bean).	Mousavi et al., 2015; Amarger et al., 1997
15	*Neorhizobium*	Isolated from dryland agricultural soil with no record of cultivation of legumes.	Soenes et al., 2019; Lindstrom and Mousavi 2019
16	*Shinella*	Identified as related to *Rhizobiaceae* group using 16S rRNA sequence analysis.	Lee et al., 2011
17	*Aminobacter*	*Aminobacter* are legume nodulating bacteria for the plant Anthyllis vulneraria*;* contains *nodA* sequences related to *Mesorhizobium.*	Maynaud et al., 2012
18	*Microvirga*	Isolated from root nodules of *Retama sphaerocarpa* in Morocco.	Mouad et al., 2020

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Table 2.5 Examples of legumes for which commercial inoculants exist in South Africa.

Legume crop		Rhizobial species
1	Common bean (*Phaseolus vulgaris*)	*Rhizobium tropici*
2	Clover (*Trifolium repens*)	*Rhizobium leguminosarum biovar trifolii*
3	Lupin (*Lupinus angustifolius*)	*Bradyrhizobium sp.*
4	Lurcerne (*Medicago sativa*)	*Sinorhizobium meliloti*
5	Peanut (*Arachis hypogaea*)	*Bradyrhizobium sp.*
6	Soybean (*Glycine max*)	*Bradyrhizobium japonicum*
7	Vetch (*Vicia sativa*)	*Rhizobium leguminosarum biovar viciae*

Adapted from: Microbial Biological Fertilizers International Group, 2022.

2.7 Characterization of rhizobia

Characterization of rhizobia based on phenotypic methods generally requires the use of live cultures. The techniques include morphological and biochemical characters. Morphological techniques include the characteristics of bacterial colonies when grown on agar media, while biochemical features include traits such as growth temperature, optimum pH, substrate utilization, salt concentration and activities of various enzymes. The ability of the strain to tolerate different stress conditions such as growth at different pH, temperatures, salts and tolerance to drought conditions, as other rhizobia contain traits that assist in tolerance to abiotic stresses (Gopalakrishnan et al., 2015). The phenotypic test includes ability of strains to utilize antibiotics (Florentino *et al.*, 2015), utilization of carbohydrates (Ceapa *et al.*, 2015) and host specificity. In a study involving trapping indigenous rhizobial strains from root nodules of pigeonpea plants grown in Ethiopia, a wide phenotypic diversity among the strains was reported (Degefu et al., 2018). The phenotypic results suggested that the soil samples contained diverse rhizobial strains with distinct morphological characteristics. Generally, phenotypic methods are labor intensive, time consuming and allow only a limited number of samples tested at a time. The phenotypic methods are important as the first levels of initial characterization and grouping of rhizobia. Nevertheless, phenotypic characterization is useful when used in combination with molecular characterization (Van Rossum *et al.*, 1995).

Recently, the molecular characterization of rhizobia used the sequencing of 16S rRNA gene, which is highly conserved across bacterial species (Janda and Abbott, 2007; Clarridge, 2004). In addition, the housekeeping genes inclduding *recA, gyrB* and *rpoB* were also sequenced to provide the species information of rhizobial strains (Fossou et al., 2020; Rocha et al., 2005; Mahenthiralingam et al., 2000). In other studies, sequences of N fixation (*nif*) and

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nodulation (*nod*) genes were used (Thies *et al*., 2001). The DNA-based approaches are relatively easy to apply and resolve even closely related species (Rai *et al*., 2012). However, studies aimed at the elucidation of the whole genome in an organism such as a rhizobial strain, currently employ the next generation sequencing (NGS) method which enables fast, accurate molecular characterization of the organism of interest (Gautam et al., 2019; Kwong et al., 2015). The whole genome sequencing (WGS) method is a powerful tool for genomics research and it can generate large volumes of data that require powerful algorithms for accurately determining the intrinsic genetic information such as the structural or copy number variations as well as their breakpoints and sizes (Kasugi et al., 2019). Such information is useful in understanding the functional properties of rhizobial genes. Because of the phylogenetic diversity of both the rhizobia and host legume genotypes, there is no documented evidence of rhizobial strains that can form symbiosis with all the legumes and vice versa. In most cases, there is specificity, which occurs at both the species and genotypic levels (Wang et al., 2018, Wang et al., 2012) at different stages. In addition, incompatibility can occur at a late stage of nodule development, resulting in significant reduction in the efficiency of the N fixation in a host plant x rhizobial strain combination (Yang et al., 2017; Wang et al., 2017, 2012). Therefore, it is necessary to develop tools for the genetic manipulation of the host or bacteria to improve N fixation efficiencies. In this regard, knowledge of the genetic and molecular basis of the N fixation is essential. Specifically, the genome sizes of the different rhizobial species may provide some additional insights into the variation in symbiotic efficiencies of host genotype x rhizobial strain interactions.

2.8 Selection of superior rhizobial strains

The variation in interaction between the host plant genotype x rhizobial strain is often limited by the differential responses among legumes species, to inoculation with specific strains. A similar problem is frequently encountered in selecting superior crop genotypes from multiple environmental trials in which the genotype x environment interactions are often significant (Yan and Tinker, 2006; Yan et al., 2002). Consequently, plant breeders employ useful tools such as the genotype plus genotype x environment (GGE) methods to identify the ideal and stable genotypes in specific environments (Yan and Tinker 2006). The GGE biplot analysis enables a graphical visualization of the genotypic performances in specific environments as well as their stability over the environments (Yan and Tinker 2005). In determining the 'which-won where', the GGE biplot is made of an irregular polygon consisting of a set of lines drawn from the origin to dissect perpendicularly each side of the polygon and dividing it into sectors as well as determining the winning genotypes (or rhizobial strains in this case) for each sector (Yan et al. 2007). The ideal rhizobial strain should have the power to

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discriminate the tropical legumes main effects and is defined by an arrow pointing to it while concentric circles enable easy visualization of the distances between the legume species (i.e. the environments) (Yan and Wu, 2008). Therefore, this approach can be useful in selecting the ideal rhizobial strain over different legume species.

2.9 Summary of literature review election of superior rhizobial strains

This literature review indicated the following main points:

(i) in some parts of South Africa, pigeonpea is cultivated mainly by smallholder growers with no seed inoculation

(ii) based on duration to maturity, three main classes (short duration, medium duration and long duration) of pigeonpea are recognized

(iii) pigeonpea is a multi-purpose grain legume that can tolerate soil moisture stress

(iv) a wide range of rhizobial genera symbiotically interacts with common tropical legumes including pigeonpea

(v) N fixation in pigeonpea and other legumes is influenced by several environmental and genetic factors of both symbionts

(vi) in smallholder cropping systems, pigeonpea is often intercropped with cereal crops to reduce the need for chemical nitrogenous fertilizers

(vii) the genetic and phylogenetic characteristics of the rhizobial genera that are associated with root nodulation in pigeonpea have not been determined adequately

(viii) modern molecular characterization of rhizobia uses at least the 16S rRNA and recA house-keeping genes

(ix) potential of the rhizobial strains that are associated with root nodulation in pigeonpea among other common tropical legumes has not been investigated adequately.

References

Abaidoo RC, Dashiell KE, Sanginga N, Keyser HH, Singleton PW (1999). Time course of dinitrogen fixation of promiscuous soybean cultivars measured by the isotope dilution method. *Biology and Fertility of Soils*. 30: 187-192.

Adjei-Nsiah S (2012). Role of pigeonpea cultivation on soil fertility and farming system sustainability in Ghana. *International Journal of Agronomy*. 2012: 1-8. doi.org/10.1155/2012/702506.

© University of Venda

Amarger N, Macheret V, Laguerre G (1997). *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii* sp. nov., from *Phaseolus vulgaris* nodules. *International Journal of Systematic Bacteriology*. 47: 996-1006.

Amat D, Thakur JK, Mandal A, Sahu A, Reddy KKK (2020). Production and utilization of legume inoculants (*Rhizobium*) in India. *Harit Dhara*. 3(2): 28-31.

Amijee F, Giller KE (1998). Environmental constraints to nodulation and nitrogen fixation of *Phaseolus vulgaris* L. in Tanzania. I. A survey of soil fertility, root nodulation and multi-locational responses to *Rhizobium* inoculation. *African Crop Science Journal.* 6(2): 159-169.

Antunes PM, de Varennes A, Rajcan I, Goss MJ (2006). Accumulation of specific flavonoids in soybean (*Glycine max* (L.) Merr) as a function of the early tripartite symbiosis with arbuscular mycorrhyizal fungi and *Bradyrhizobium japonicum* (Kirchner) Jordan. *Soil Biology Biochemistry*. 38: 1234-1242.

Araujo J, Alcántara CAD, Velázquez E, Urban B, Gonzalez-Andrès FG (2015). *Bradyrhizobium yuanmingense* related strains form nitrogen-fixing symbiosis with *Cajanus cajan* L. in Dominican Republic and are efficient biofertilizers to replace N fertilization. *Scientia Horticulturae*. 192: 421-428.

Ayenan MAT, Ofori K, Ahoton LE, Danguah A (2017). Pigeonpea [(*Cajanus cajan* (L.) Millsp)] production systems, farmers' preferred traits and implications for variety development and introduction in Benin. *Agriculture and Food Security.* 6 (48): 1-11.

Babariya PM, Kabaria BB, Patel VN, Joshi MD (2010). Chemical control of gram pod borer, *Helicoverpa armigera* Hubner infesting pigeonpea. *Legume Research*. 33: 224-226.

Barrett CF, Parker MA (2006). Coexistence of *Burkholderia*, *Cupriavidus* and *Rhizobium* sp. nodule bacteria on two *Mimosa* spp. in Costa Rica. *Applied and Environmental Microbiology*. 72: 1198-1206.

Benevides CMJ, Trindade BA, Lopes MV (2018). Potentialities of legumes in the pharmaceutical industry. *Journal of Analytical and Pharmaceutical Research.* 7(3): 369-373. doi: 10.15406/japlr.2018.07.00253.

© University of Venda

Bohra A, Saxena RK, Gnanesh BN, Saxena K, Byregowda M, Rathore A, KaviKashor PB, Cook DR, Varshney RK (2012). An intra-specific consensus genetic map of pigeonpea (*Cajanus cajan* (L) Millspaugh) derived from six mapping populations. *Theoretical and Applied Genetics*. 125(6): 1325-1338. doi: 10.1007/s00122-012-1916-5.

Bopape FL, Gwata ET, Hassen AI, Zhou MM (2021). Symbiotic efficiency of pigeonpea (*Cajanus cajan*) with different sources of nitrogen. *Plant Genetic Resources.* 19(4): 1-4. doi:10.1017/S1479262121000356.

Cao W, Han H, Zhang J (2005). Preparation of biodiesel from soybean oil using supercritical methanol and co-solvent. *Fuel*. 84(4): 347-351.

Çakir Ö, Uçarli C, Tarhan Ç, Pekmez M, Turgut-Kara N (2019). Nutritional and health benefits of legumes and their distinctive genomic properties. *Food Science and Technology*. 39: 1-12.

Ceapa C, Lambert J, Van Limpt K, Wels M, Smokvina T, Knol J, Kleerebezem M (2015). Correlation of *Lactobacillus rhamnosus* genotypes and carbohydrates utilization signatures determined by phenotype profiling. *Applied Environmental Microbiology.* 81(16): 5458-5470.

Chen WX, Yan GH, Li JL (1988). Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. *International Journal of Systematic Bacteriol*ogy. 38: 392-397.

Chen WM, Moulin L, Bontemps C, Vandamme GB, Masson CB (2003a). Legume symbiotic nitrogen-fixation by *β-Proteobacteria* is widespread in nature. *Journal of Bacteriology.* 185: 7266-7272.

Chen WM, James EK, Prescott AR, Kieraus M, Sprent JI (2003b). Nodulation of *Mimosa* spp. by β-proteobacterium *Ralstonia taiwanensis*. *Molecular Plant Microbe Interactions Journal*. 16: 1051-1061.

Clarridge III JE (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews*. 17 (4): 840-862.

Daniel JN, Ong CK (1990). Perennial pigeonpea multi-purpose species for agroforestry systems. *Agroforestry Systems*. 10: 113-129.

© University of Venda

Dansi A, Vodouhe R, Azokpota P, Yedomonhan H, Assogba P, Adjatin A, LOko YL, Dossou-Aminon I, Akpagana K (2012). Diversity of the neglected and underutilized crops species of importance in Benin. *The Scientific World Journal*. 2012: 1-19. doi.org/10.1100/2012/932947.

Degefu T, Wolde-meskel E, Adem M, Fikre A, Amede T, Ijiewo C (2018). Morphophysiological diversity of rhizobia nodulating pigeonpea (*Cajanus cajan* L. Millsp.) growing in Ethiopia. *African Journal of Biotechnology*. 17(6):167-177.

De Lajudie P, Willems A, Pot B, Dewettinck D, Maestrojuan G, Neyru M, Collins MD, Dreyfus B, Kersters K, Gillis M (1994). Polyphasic taxonomy of rhizobia: Emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp., nov., and *Sinorhizobium teranga* sp. nov. *International Journal of Systematic Bacteriol*ogy. 44: 715-733.

De Lajudie P, Laurent-Fulele E, Willems A, Torck U, Coopman R, Collins MD, Dreyfus B, Kersters K, Gillis M (1998b). *Allorhizobium undicola* gen. nov., nitrogen-fixing bacteria that efficiently nodulate *Neptunia natans* in Senegal. *International Journal of Systematic Bacteriology*. 48: 1277-1283.

Delamuta JRM, Ribeiro RA, Ormeño-Orrillo E, Melo IS, Martínez-Romero E, Hungria M (2013). Polyphasic evidence supportingthe reclassification of *Bradyrhizobium japonicum* Group IA strains as *Bradyrhizobium diazoefficiens* sp. nov*. International Journal Systematic Evolutionary Microbiology.* 63(9): 3342–3351. doi: 10.1099/ijs.0.049130-0.

Durán D, Rey L, Mayo J, Zúñiga-Dávila D, Imperial J, Ruiz-Argüeso T, Martínez-Romero E, Ormeño-Orrillo E (2014). *Bradyrhizobium paxllaeri* sp. nov. and *Bradyrhizobium icense* sp. nov., nitrogen-fixing rhizobial symbionts of Lima bean (*Phaseolus lunatus* L.) in Peru. International *Journal of Systematic and Evolutionary Microbiology* 64: 2072-2078. doi: 10.1099/ijs.0.060426-0.

Dreyfus B, Garcia JL, Gillis M. 1(988). Characterization of *Azorhizobium caulinodans* gen. nov. sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rastrata*. *International Journal of Systematic Bacteriol*ogy. 38: 89-98.

© University of Venda

Emily MT, Padhi D, Ramdath D (2017). A review of the relationship between pulse consumption and reduction of cardiovascular disease risk factors. *Journal of Functional Foods*. 38: 635–643. doi.org/10.1016/j.jff.2017.03.043.

Florentino LA, Guimaräes AA, da Silva JS, Rangel WM, Jaramillo PMD, de Souza Moreira FM (2015). Genetic and phenotypic diversity of *Azorhizobium doebereinerae* strains. *Centro Cientifico Conhecer-Goianoia.* 11(21): 1129-1139.

Fossou RK, Ziegler D, Zeze A, Barja F, Perret X (2016). Two major clades of bradyrhizobia dominate symbiotic interactions with pigeonpea in fields of Côte d'Ivoire. *Frontiers in Microbiol*ogy. 7(1793): 1-11.

Fossou RK, Pothier JF, Zézé, A, Perret X (2020). *Bradyrhizobium ivorense* sp. nov. as a potential local bioinoculant for *Cajanus cajan* cultures in Côte d'Ivoire. *International Journal of Systematic Evolutionary Microbiology.* 70, 1421–1430. doi: 10.1099/ijsem.0.003931.

Fred EB, Baldwin IL, McCoy E (1932). Root nodule bacteria and leguminous plants. University of Wisconsin studies in Science.

Gatel F (1994). Protein quality of legume seeds for non-ruminant animals: a literature review. *Animal Feed Science and Technology*. 45(3-4): 317-348.

Gautam SS, Rajendra KC, Leong KWC, Mac Aogáin M, O'Toole RF (2019). Step-by step beginner's protocol for whole genome sequencing of human bacterial pathogens. *Journal of Biological Methods*. 6(1): 1-9. e110. doi:10.14440/jbm.2019.276.

Gopalakrishnan S, Sathya A, Vijayabharathi R, Varshney RK, Laxmipathi Gowda CL, Krishnamurthy L (2015). Plant growth promoting rhizobia: challenges and opportunities. *Biotechnology*. 5:355-377.

Graham PH, Vance CP (2000). Nitrogen fixation in perspective: an overview of research and extension needs. *Field Crops Research*. 65: 93–106.

Green P (1992). The genus *Methylobacterium*. *In*: The prokaryotes, Second edition. Balows A, Truper HG, Dworkin M, Harder W, Schleifer KH (eds). Springer-Verlag, New York. pp 2342-2349.

© University of Venda

Gwata ET, Shimelis H (2010). Evaluation of pigeonpea germplasm for important agronomic traits in Southern Africa. *In*: Crop Production, Goyal A, Asif M (eds). pp 1-15. doi:org/10.5772/56094.

Gwata ET, Siambi M (2009). Genetic enhancement of pigeonpea for latitude areas in southern Africa. *African Journal of Biotechnology*. 8: 4413-4417.

Gwata ET, Silim SN (2009). Utilization of landraces for the genetic enhancement of pigeonpea in Eastern and Southern Africa. *Journal of Food, Agriculture and Environment. 7*: 803-806.

Gwata ET, Silim SN, Mgonja M (2006). Impact of a new source of resistance to Fusarium wilt in pigeonpea. *Journal of Phytopathology*. 154: 62-64.

Hassen AI, van Vuuren A, Bopape FL, Gerrano AS (2022). Nodulation compatibility and symbiotic performance of rhizobia spp. with different landraces of Bambara groundnut (*Vigna subterranea* (L.) Verdc.) collections. *Research Square*. pp 1-23. doi:org/10.21203/rs.3.rs-1233082/v1.

Hillocks RJ, Minja E, Nahdy MS, Subrahmanyam P (2000). Diseases and pests of pigeonpea in eastern Africa. *International Journal of Pest Management*. 46: 7-18. doi: 10.1080/096708700227534.

Hluyako LL, Odindo AO, Mafongoya P, Sithole NJ, Magwaza LS (2017). Characterisation of pigeonpea (*Cajanus cajan*) landraces grown in two climatic zones in KwaZulu-Natal province, South Africa. *South African Journal of Plant and Soil*. 3(3): 191-199.

Holland MA (1997). *Methylobacterium* and plants. *Recent Research Development and Plant Physiolology.* 1: 207-212.

Holmes B, Popoff M, Kiredjian M, Kersters K (1988). *Ochrobactrum anthropi* gen. nov., sp. nov. from human clinical specimen and previously known as group Vd. *International Journal of Systematic Bacteriology.* 38: 406-416.

Janda JM, Abott SL (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, pitfalls. *Journal of Clinical Micobiology*. 45(9): 2761-2764.

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Jarvis BDW, van Berkum P, Chen WX, Nour SM, Fernandez MP, Cleyet-Marel JC, Gillis M (1997). Transfer of *Rhizobium loti*, *Rhizobium haukii, Rhizobium ciceri, Rhizobium meditteraneum* and *Rhizobium tianshanense* to *Mesorhizobium* gen. nov. *International Journal of Systematic Bacteriol*ogy. 47: 895-898.

Jezierny D, Mosenthin R, Bauer E (2010). The use of grain legumes as a protein source in pig nutrition: a review. *Animal Feed Science and Technology*.157: 111-128.

Johnson LA, Myers DJ (1995). Industrial uses for soybeans. *In*: *Practical handbook of soybean processing and utilization*. AOCS Press. pp. 380-427.

Jones KM, Kobayashi H, Davies BW, Taga ME, Walker GC (2007). How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. *Nature Reviews Microbiology*. 5: 619-633. doi: 10.1038/nrmicro1705.

Jordan DC (1984). Family III *Rhizobiaceae* Conn 1938. *In*: Krieg NR, Holt JG (eds) Bergey's Manual of Systematic Bacteriology. William and Wilkins, Baltimore. pp 234-243.

Jourand P, Giraud E, Bena G, Sy A, Willems A, Gillis M, Dreyfus B, de Lajudie P (2004). *Methylobacterium nodulans* sp. nov., for a group of aerobic, facultative methylotrophic, legume root-nodule-forming and nitrogen-fixing bacteria. *International Journal of Systematic and Evolutionary Microbiology*. 54: 2269-2273.

Kasugi S, Momozawa Y, Liu X, Terao C, Kubo M, Kamatani Y (2019). Comprehensive evaluation of structural variation detection algorithms for whole genome sequencing. *Genome Biology*. 20(117): 1-18. doi:org/10.1186/s13059-019-1720-5.

Khatun M, Sumi S, Farzana ME, Mominul Islam AKM,Parvez Anwar MD, Shah F, Rahul D, Aminul Islam AKM (2021). Drought stress in grain legumes: Effects, tolerance mechanisms and management." *Agronomy*.11: 2374.

Khurana AL, Dudeja SS (1994). On-farm experience in the use of rhizobia inoculants on pigeonpea in India. In: Linking Biological Nitrogen Fixation Research in Asia: report of a meeting of the Asia Working Group on Biological Nitrogen Fixation in Legumes, 6-8 Dec, ICRISATA Asia Center, India. (Rupela OP, Kumar Rao JVOK, Wani SP, Johansen C (eds.) Patancheru 502 324, Andhra Pradesh, India: International Crops Research Institute for the Semi-Arid Tropics. pp 36-41.

© University of Venda

Kunjeku EC, Gwata ET (2011). A preliminary survey of insect species occurring at the reproductive stage in pigeonpea grown in Limpopo (South Africa)." *Journal of Food, Agriculture and Environment*. 9: 988-991.

Kwong JC, McCallum N, Sintchenko V and Howden BP (2015). Whole genome sequencing in clinical and public health microbiology. *Pathology*. 47(3): 199-210.

Ladha JK, Peoples MB, Reddy PM, Biswas JC, Bennett ML, Krupnik TJ (2022). Biological nitrogen fixation and prospects for ecological intensification in cereal-based cropping systems. *Field Crops Research*. 283 (10854): 1-34. doi:org/10.1016/j.fcr.2022.108541.

Lee KB, Backer P, Aono T, Liu CT, Suzuki S, Suzuki T, Kanelo T, Yamada M, Tabata S etal., (2008).The genome of the versatile nitrogen fixer *Azorhizobium caulinodons* ORS571. *BMC Genomics*. 9:271-285.

Lee M, Woo SG, Ten LN (2011). *Shinella daejeonensis* sp. nov., a nitrate-reducing bacterium isolated from sludge of leachate treatment plant. *International Journal of Systematic and Evolutionary Microbiology*. 61: 2123-2128.

Lindstrom K and Mousavi SA (2019). Effectiveness of nitrogen fixation in rhizobia. *Microbial Biotechnology*. 13: 1314-1335.

Mahenthiralingam E, Bischof J, Byrne SK, Radomski C, Davies JE, Av-Gay Y, Vandamme P (2000). DNA-Based diagnostic approaches for identification of *Burkholderia cepacia complex*, *Burkholderia vietnamiensis*, *Burkholderia multivorans*, *Burkholderia stabilis*, and *Burkholderia cepacia* genomovars I and III. *Journal of Clinical Microbiology*. 38:3165-3173.

Makoi JHJR, Ndakidemi PA (2011). Changes in plant growth, nutrient dynamics and accumulation of flavonoids and anthocyanins by manipulating the cropping systems involving legumes and cereals. *Australian Journal of Agricultural Engineering*. 2(3): 56-65.

Mantelin S, Fisher-Le Saux M, Zakhia F, Bena G, Bonneau S, Jeder H, de Lajudie P, Cleyet-Marel JC (2006). Emended description of the genus *Phyllobacterium* and description of four novel species associated with plant roots: *Phyllobacterium bourgognense* sp. nov., *Phyllobacterium ifriqiyense* sp. nov., *Phyllobacterium leguminum* sp. nov. and *Phyllobacterium*

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brassicacearum sp. nov. *International Journal of Systematic and Evolutionary Microbiology.* 56: 827-839.

Mapfumo P, Giller KE, Mpepereki S, Mafongoya PL (1999). Dinitrogen fixation by pigeonpea of different maturity types on granitic sandy soils in Zimbabwe. *Symbiosis.* 27(3-4): 305-318.

Martens M, Delaere M, Coopman R, de Vos P, Gillis M, Willems A (2007). Multilocus sequence analysis of *Ensifer* and related taxa. *International Journal of Syst*ematic and *Evolutionary Microbiol*ogy. 57: 489-503.

Mavengere NR, Ellis AG, Le Roux JJ (2014). *Burkholderia aspalathi* sp. nov., isolated from root nodules of the South African legume *Aspalathus abietina* Thunb. *International Journal of Systematic and Evolutionary Microbiology*. 64: 1906-1912.

Maynaud G, Willems A, Soussou S, Vidal C, Maure L, Moulin L, Cleyet-Marel JC, Brunel B (2012). Molecular and phenotypic characterization of strains nodulating *Anthyllis vulneraria* in mine tailings, and proposal of *Aminobacter anthyllidis* sp. nov., the first definition of *Aminobacter* as legume-nodulating bacteria. 35(2): 65-72.

Melchora VA, Fe BP (2022). Runoff and Soil Loss on Different Cropping Systems with Pigeonpea as Hedgerow, Philippines. *In*: Presto PB, Amata AJP, Caning MCM, Cervantes CN, Lirag MTB (eds). CBSUA Journal of Research. pp 37-43. doi: 10.13140/RG.2.2.33601.63842.

Mendoza-Suárez M, Andersen SU, Poole PS, Sánchez-Caῃizares C (2021). Competition, nodule occupancy, and persistence of inoculant strains: Key factors in the *Rhizobium*-Legume Symbioses. *Frontiers in Plant Science*. 12 (690567): 1-26.

Microbial Biological Fertilizers International Group (2022). *Harnessing nature's power*. https://mbfi.co.za/ (Accessed 12/11/2022).

Mogashoa KE, Gwata ET (2009). Preliminary indicators of adaptation"of pigeonpea (*Cajanus cajan* (L.) Millsp.) to Limpopo (South Africa). *In*: Proceeding African Crop Science, 2009 Conference,Cape Town, RSA, pp 141.

Mohammadi K, Sohrabi Y (2012). Bacterial biofertilizers for sustainable crop production: A review. *Journal Agricultural and Biological Science.* 7(5): 307-316.

© University of Venda

Morris JB (2003). Bio-functional legumes with nutraceutical, pharmaceutical, and industrial uses. *Economic Botany*. 57: 254-261.

Mouad L, Hanane L, Omar B, Meryeme B, Hanaa A, Bedmar EJ, Mustapha MEI (2020). Nodulation of *Retama* species by members of the genus *Microvirga* in Morocco. *Symbiosis.* 82: 249-259.

Moulin L, Munive A, Dreyfus B, Boivin-Masson C (2001). Nodulation of legumes by members of the *β*-subclass of *Proteobacteria*. *Nature*. 411: 948-950.

Mousavi SA (2016). Revised taxonomy of the family *Rhizobiaceae*, and phylogeny of mesorhizobia nodulating *Glycyrrhiza* spp. PhD thesis, University of Helsinki, Finland.

Mousavi SA, Willems A, Nesme X, de Lajudie P, Lindström K (2015). "Revised phylogeny of *Rhizobiaceae*: proposal of the delineation of *Pararhizobium* gen. nov., and 13 new species combinations" *Systematic and Applied Microbiology*. 38(2): 84-90. doi:10.1016/j.syapm.2014.12.003. PMID 25595870.

Mpepereki S, Javaheri F, Davis P, Giller KE (2000). Soyabeans and sustainable agriculture: promiscuous soyabeans in southern Africa. *Field Crops Research*. *65*(2-3): 137-149.

Musokwa M, Mafongoya P (2021). Pigeonpea yield and water use efficiency: a savior under climate change-induced water stress. *Agronomy*. 11(5): 1-14.

Nakagawa Y, Sakane T, Yokota A (1996). Transfer of "*Pseudomonas riboflavina*" (Foster 1944) a gram-negative, motile rod with long-chain 3-hydroxy fatty acids, to *Devosia riboflavina* gen. nov., sp. nov., nom. rev. *International Journal of Systematic Bacteriology*. 46: 16-22.

Nyaga JW, Njeru EM (2020). Potential of native rhizobia to improve cowpea growth and production in semiarid regions of Kenya. *Frontiers in Agronomy*. 28: 1-13.

Odeny DA (2007). The potential of pigeonpea (*Cajanus cajan* (L.) Millsp.) in Africa. *Natural Resources Forum.* 31: 297-305.

© University of Venda

Ohanenye IC, Tsopmo A, Ejike CE, Udenigwe CC (2020). Germination as a bioprocess for enhancing the quality and nutritional prospects of legume proteins. *Trends in Food Science and Technology*. *101*: 213-222.

Oldroyd GE, Murray JD, Poole PS, Downie JA (2011). The rules of engagement in the legume-rhizobial symbiosis. *Annual Review Genetics*. 45: 119–144. doi: 10.1146/annurev-genet-110410-132549.

Onishchuk OP, Vorobyov NI, Provorov NA (2017). Nodulation competitiveness of nodule bacteria: genetic control and adaptive significance: review. *Applied Biochemistry and Microbiology*. 53: 131-139. doi: 10.1134/S0003683817020132.

Oppewal J, Da Cruz A (2017). The pigeonpea value chain in Mozambique. Final Report. F-36409-MOZ-1. *International Growth Centre*. pp 1-55.

Ormeño-Orrillo E, Martínez-Romero E (2019). A genomotaxonomy view of the *Bradyrhizobium* genus. *Frontiers in Microbiology*. 10(1334): 1-13.

Overstreet RM, Lotz JM (2016). Host- symbiont relationships: understanding the change from guest to pest. *In*: Hurst CH (ed). The Rasputin effect when commensals and symbionts become plastic, Advances in environmental microbiology, Springer. pp 27-63.

Pais J, Bansal V (2019). Pigeonpea smallholder production. *In*: The Global Economy of Pulses. Rawal V, Navarro DK (eds). Rome, FAO. pp 101-111.

Pal D, Mishra P, Sachan N, Ghosh AK (2011). Biological activities and medicinal properties of *Cajanus cajan* (L) Millsp. *Journal of Advanced Pharmaceutical Technology Research*. 2(4): 207-214.

Patel KJ, Singh AK, Nareshkumar G, Archana G (2010). Organic-acid- producing, phytate-mineralizing rhizobacteria and their effect on the growth of pigeonpea (*Cajanus cajan*). *Applied Soil Ecology*. 44(3): 252-261.

Ng CP, Kirkness EF (2010). Whole genome sequencing. *Methods in Molecular Biology*. 628: 215-26. doi:10.1007/978-1-60327-367-1_12. PMID: 20238084.

© University of Venda

Payne GW, Vandamme P, Morgan SH, Lipuma JJ, Coenye T, Weightman AJ, Jones TH, Mahenthiralingam E (2005). Development of a *recA* gene-based identification approach for the entire *Burkholderia* genus. *Applied and Environmental Microbiology.* 71: 3917-3927.

Perin L, Martinez-Aguilar L, Castro-Gonzalez R, Estrada-de los Santos P, Cabellos-Avelar T, Guedes HV, Reis VM, Caballero-Mellado J (2006). Diazotrophic *Burkholderia* species associated with field-grown maize and sugar cane. *Applied Environmental. Microbiology*. 72: 3103-3110.

Pinto-Carbo M, Gademann K, Eberl L, Carlier A (2018). Leaf nodule symbiosis: function and transmission of obligate bacterial endophytes. *Current Opinions in Plant Biology*. 44: 23-31.

Pnueli L, Hallak-Herr E, Rozenberg M, Cohen M, Goloubinoff P, Kaplan A, Mittler R (2002). Molecular and biochemical mechanisms associated with dormancy and drought tolerance in the desert legume *Retama raetam*. *The Plant Journal*. 31(3): 319-330.

Ramsubhag A, Umaharan P, Donawa A (2002). Partial 16S rRNA gene sequence diversity and numerical taxonomy of slow growing pigeonpea (*Cajanus cajan* L. Millsp) nodulating rhizobia. *FEMS Microbiology Letters*. 216(2): 139-144.

Rai R, Dash PK, Mohapatra T, Singh A (2012). Phenotypic and molecular characterization of indigenous rhizobia nodulating chickpea in India. *Indian Journal Experimental Biology.* 50: 340-350.

Rinaudo G, Orenga S, Fernandez MP, Meugnier H, Bardin R (1991). DNA homologies among members of the genus *Azorhizobium* and other stem- and root-nodulating bacteria isolated from the tropical legume *Sesbania rostrata*. *International Journal of Systematic Bacteriol*ogy. 41(1): 114-120.

Rocha EPC, Cornet E, Michel B (2005). Comparative and evolutionary analysis of the bacterial homologous recombination systems. *PLoS Genetics*. 1(2): 0247-0259.

Rivas R, Willems A, Subba-Rao NS, Mateos PF, Dazzo FB, Kroppenstedt RM, Martinez-Molina E, Gillis M, Velázquez E (2003). Description of *Devosia neptunae* sp. nov. that nodulates and fixes nitrogen in symbiosis with *Neptunia natans*, an aquatic legume from India. *Systematic and Appl*ied *Microbiology*. 26: 47-53.

© University of Venda

Rufini M, Oliveira DP, Trochmann A, Soares BL, de Andrade MJB, de Souza Moreira FM (2016). *Bradyrhizobium* spp. Strains in symbiosis with pigeonpea cv. Fava-larga under greenhouse and field conditions. *Revista Brasileira Ciência do Solo*. 40: 1-14.

Sahoo BK, Senapati B (2000). Determination of economic thresholds for pod borer complex in pigeonpea. *Indian Plant Protection*. 28: 176-179.

Semba RD, Ramsing R, Rahman N, Kraemer K, Bloem MW (2021). Legumes as a sustainable source of protein in human diets. *Global Food Security*. 28(100520): 1-4. doi: 10.1016/j.gfs.2021.100520.

Shiferaw B, Okello J, Muricho G, Omiti J, Silim SN, Jones R (2008). Unlocking the potential of high value legumes in the semi-arid regions: analyses of the pigeon pea value chains in Kenya. International Crops Research Institute for the Semi-Arid Tropics. *Research Report* 1. pp 1-57. doi: 10.13140/RG.2.2.29121.45921.

Singnorelli S, Sainz M, Rosa ST, Monza J (2020). The role of nitric oxide in nitrogen fixation by legumes. *Frontiers in Plant Science*. 521(11): 1-14.

Silim SN, Gwata ET, Mligo JK., Siambi M, Karuru O, King SB, Omanga P (2005). Registration of pigeonpea cultivar 'ICEAP 00040'. *Crop Science*. 45(6): 2647-2648.

Soenens A, Gomila M, Imperial J (2019). *Neorhizobium tomejilense* sp. nov., first non-symbiotic *Neorhizobium* species isolated from a dryland agricultural soil in southern Spain. 42(2): 128-134.

Sprent JI, Odee DW, Dakora FD (2010). African Legumes: A vital but under-utilized resource. *Journal of Experimental Botany*. 61: 1257-1265. doi:10.1093/jxb/erp342.

Steenkamp ET, Stepkowski T, Przymusiak A, Botha WJ, Law IJ (2008). Cowpea and peanut in southern Africa are nodulated by diverse *Bradyrhizobium* strains harboring nodulation genes that belong to the large pantropical clade common in Africa. *Molecular Phylogenetics and Evolution*. 48(3): 1131–1144. doi:10.1016/j.ympev.2008.04.032.

Streeter JG (1979). Allantoin and allantoic acid in tissues and stem exudate from field-grown soybean plants. *Plant Physiology*. 63: 478-480.

© University of Venda

Sy A, Giraud E, Jourand P, Garcia N, Willems A, de Lajudie P, Prin Y, Neyra M, Gillis M, Boivin-Masson C, Dreyfus B (2001). Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. *Journal of Bacteriology.* 183: 214-220.

Tariq M, Hameed S, Khan HU, Munir MI, Nushin F, Jamil S, Nawaz S, Zafar M (2017). Role of microsymbionts in plant microbe symbiosis. *Journal of Applied Microbiology and Biochemistry*. 1(2): 1-5.

Thies JE, Holmes EM, Vachot A (2001). Application of molecular techniques to studies in *Rhizobium* ecology. *Australian Journal of Experimental Agriculture*. 41: 299-319.

Thomas D and Sumberg JE (1995). A review of the evaluation and use of tropical forage legumes in sub-Saharan Africa. *Agriculture Ecosystems and Environment.* 54(3): 151–163. doi.org/10.1016/0167-8809(95)00584-F.

Trujillo ME, Willems A, Abril A, Planchuelo AM, Rivas R, Ludena D, Mateos PF, Martinez-Molina E, Velazquez E (2005). Nodulation of *Lupinus albus* by strains of *Ochrobactetrum lupine* sp. nov. *Applied Environmental Microbiology*. 71: 1318-1327.

Upadhyaya HD, Reddy KN, Sharma S, Varshney RK, Bhattacharjee R, Singh S, Gowda CLL (2011). Pigeonpea composite collection and identification of germplasm for use in crop improvement programmes. *Plant Genetic Resources: Characterization and Utilization.* 9(1): 97-108. doi: 10.1017/S1479262110000419.

Valverde A, Velazquez E, Gutierrez C, Cervantes E, Ventosa A, Iqual JM (2003). *Herbaspirillum lusitanum* sp. nov., a novel nitrogen-fixing bacterium associated with root nodules of *Phaseolus vulgaris*. *International Journal of Systematic Evolutionary Microbiol*ogy. 53 : 1979-1983.

Valverde A, Velazquez E, Fernandez-Santos F, Vizcaino N, Rivas R, Mateos PF, Martinez-Molina E, Igual JM, Willems A. (2005). *Phyllobacterium trifolii* sp. nov., nodulating *Trifolium* and *Lupinus* in Spanish soils. *International Journal of Systematic and Evolutionary Microbiology.* 55: 1985-1989.

Van Berkum P, Eardly BD (1998). Molecular evolutionary systematics of the *Rhizobiaceae*. *In*: The *Rhizobiaceae*: Molecular Biology of model plant-associated bacteria. Spainck HP (ed). Dordrecht, Kluwer Academic Publishers. pp 1-24.

© University of Venda

Vandamme P, Coenye T (2004). Diversity and significance of *Burkholderia* species occupying diverse ecological niches. *Environmental Microbiology.* 5: 719-729.

Van Rossum D, Schuurmans FP, Gillis M, Muyotcha A, Van Verseveld HW, Stouthamer AH, Boogerd FC (1995). Genetic and phenetic analysis of *Bradyrhizobium* strains nodulating peanut (*Arachis hypogaea* L.) roots. *Applied Environmental Microbiology*. 61(4): 1599-1609.

Wagner SC (2011). Biological nitrogen fixation. *Nature*. 3: 15.

Wallis ES, Faris DG, Elliot R, Byth DE (1986). Varietal improvement of pigeonpea for smallholder livestock production systems. *In*: Proceedings of the Crop-livestock systems research workshop. 7-11 July 1986, Kon Kaen, Thailand. pp 1-19.

Wang D, Yang S, Tang F, Zhu H (2012). Symbiosis specificity in the legume–rhizobial mutualism. *Cell Microbiology*. 14: 334–342. doi: 10.1111/j.1462-5822.2011.01736.

Wang Q, Yang S, Liu J., Terecskei K, Ábrahám E, Gombár A, Domonkos Á, Szűcs A., Körmöczi P, Wang T, Fodor L. (2017). Host-secreted antimicrobial peptide enforces symbiotic selectivity in *Medicago truncatula*. *PNAS.* 114(26): 6854–6859. doi: 10.1073/pnas. 1700715114.

Wang Q, Liu J, Zhu H (2018). Genetic and Molecular mechanism underlying symbiotic specificity in legume-rhizobium interactions. *Frontiers in Plant Science*. 9(313): 1-8.

Willems A (2006). The taxonomy of rhizobia: an overview. *Plant and Soil.* 287: 3-14.

Xu LM, Ge C, Cui Z, Li J, Fan H (1995). *Bradyrhizobium liaoningense* sp. nov., isolated from the root nodules of soybeans. *International Journal Systematic Bacteriology*. 45: 706–711. doi: 10.1099/00207713-45-4-706

Yabuuchi E, Kosako Y, Yano I, Hotta H, Nishiuchi Y (1995). Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov.: proposal of *Ralstonia picketii* (Ralston, Palleroni and Doudoroff 1973) comb. nov., *Ralstonia solanacearum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. nov. *Microbiology and Immunology*. 39: 897-904.

© University of Venda

Yamashita N, Tanabata S, Ohtake N, Sueyoshi K, Sato T, Higuchi K, Saito A, Ohyama T (2019). Effects of different chemical forms of nitrogen on the quick and reversible inhibition of soybean nodule growth and nitrogen fixation activity. *Frontiers in Plant Science*. 10(131): 1-18. doi.org/10.3389/fpls.2019.00131.

Yan W, Wu HX (2008). Application of GGE biplot analysis to evaluate genotype (G), environment (E) and G x E interaction on Pinus radiate: A case study. *New Zealand Journal of Forestry Science*. 38(1): 132-142.

Yan W, Kang MS, Ma B, Woods S, Cornelius PL (2007). GGE biplot vs. AMMI analysis of genotype–by-environment data. *Crop Science*. 47: 643-655.

Yan W, Tinker NA (2006). Biplot analysis of multi-environment trial data: Principles and applications. *Canadian Journal of Plant Science*. 86(3): 623–645.

Yan W, Tinker N (2005). An integrated biplot analysis system for displaying, interpreting and exploring the genotype x environment interaction. *Crop Science*. 45: 1004-1016.

Yan W (2002). Singular-value partitioning in biplot analysis of multi-environment trial data. *Agronomy Journal*. 94: 990-996.

Yang S, Wang Q, Fedorova E, Liu J, Qin Q, Zheng Q, (2017). Microsymbiont discrimination mediated by a host-secreted peptide in *Medicago truncatula*. *PNAS*. 114(26): 6848-6853. doi: 10.1073/pnas.1700460114.

Yang CJ, Hu JM (2018). Bacterial leaf nodule symbiosis in flowering plants. *Symbiosis* 2:1-24.

Yates RJ, Howieson JG, Reeve WG, O'Hara GW (2011). A reappraisal of the biology and terminology describing rhizobial strains success in nodule occupancy of legumes in agriculture. *Plant and Soil*. 348: 255-267. doi: 10.1007/s11104-011-0971-z.

Young JPW (1996). Phylogeny and taxonomy of rhizobia. *Plant and Soil*. 186: 45-52.

Young J M, Kuykendall LD, Martínez-Romero E, Kerr A, Sawada H (2001). A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium undicola* de Lajudie *et al*., 1998 as

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new combinations: *Rhizobium radiobacter*, *R. rhizogenes*, *R. rubi*, *R. undicola* and *R. vitis*. *International Journal of Systematic Evolutionary Microbiology.* 51: 89-103

Young ND, Mudge J, Ellis TN (2003). Legume genomes: More than peas in a pod. *Current Opinion in Plant Biology*. 6: 199–204.

Zakhia F, de Lajudie P (2001). Taxonomy of rhizobia. *Agronomie.* 21(6-7): 569-576.

Zurdo-pineiro JL, Rivas R, Trujillo ME, Viscaino N, Carrasco JA, Chamber M, Palomares A, Mateos PF, Martinez-Molina E, Velazquez E (2007). *Ochrobactetrum cytisi* sp. nov., isolated from nodules of *Cytiasus scoparius* in Spain. *International Journal of Systematic and Evolutionary Microbiology*. 57: 794-798.

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3.0 Chapter Three: Evaluation of Root Nodulation in Pigeonpea, Isolation and Morphological Characterization of indigenous Soil Rhizobia from Diverse Soil

Abstract: Pigeonpea (*Cajanus cajan)* is an important grain legume, which is grown in many African countries and used largely for human consumption. It contributes to the improvement of soil fertility through biological nitrogen fixation. In South Africa, it is cultivated mainly in the eastern-coastal and the north-eastern regions. The compatibility of the crop with soil bacteria from the remainder of the country has not been investigated adequately. This information could be useful in pigeonpea breeding programs that are aimed at expanding the crop to new regions in the country, thus improving the production of the crop. Therefore, the objectives of this study were to (i) collect and characterize the morphological diversity of rhizobia that are associated with pigeonpea root nodulation (ii) evaluate the nodulation parameters in the host pigeonpea plant and (iii) identify the optimum host genotype x rhizobial isolate combinations. Forty soil samples were collected from diverse locations across the country and used for inoculating separately the seed of each of five randomly selected pigeonpea genotypes. The pigeonpea plants were raised a nitrogen-depleted growth medium in the greenhouse. A split-plot experimental design with two replications was used in the study. After six weeks of growth, the plants were harvested to isolate rhizobia from the root nodules by picking morphologically distinct single colonies and re-streaking on yeast-mannitol agar containing Congo red (YMA-CR) plates to obtain pure cultures. Several morphological characteristics of the rhizobial colonies including shape and growth habit (type) were determined. In addition, a range of N fixation variables of the host pigeonpea plants were measured including the number of nodules per plant, nodule dry weight (NDW) and shoot dry weight (SDW). A variety of the colony morphologies ranging from tiny to medium as well as cream white and large, watery oval colonies was observed. Two hundred and eighty potential new pigeonpea rhizobial isolates were obtained from the root nodules of the plants. Based on their morphological characteristics on YMA-CR, nutrient agar and peptone glucose agar, the isolates were deposited into the national gene bank. In terms of the root nodulation parameters among the host plants, there was >40.0 % difference in the number of nodules between 'Genotype-5' and 'Genotype-4' but the difference in NDW between the two genotypes was >80.0 %. In contrast, the heaviest dry shoots (0.45 g) that were attained by 'Genotype-3', weighed 52.0% more than the lightest dry shoots that were observed for 'Genotype-4'. The principal component analysis showed that the first principal component was dominated by three root nodulation traits including RDW and accounted for 77.51 % of the total variation. The results indicated that the soil samples contained diverse rhizobial isolates with distinct morphological characteristics and significant differential N fixation ability of the pigeonpea genotypes suggesting that there was potential to select for optimum host genotype x rhizobial isolate combinations for N fixation in the species.

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Key words: bacterial cultures; dry weight; nodule; peptone glucose agar; soil sample.

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3.1 Introduction

Pigeonpea (*Cajanus cajan)* is an important grain legume, which is grown in many African countries and used largely for human consumption. The leaves, flowers, pods and seeds are used as fodder for livestock. The grains are rich in proteins and can be used as green peas or dried peas or ground into flour. The crop contributes to the improvement of soil fertility through biological nitrogen (N) fixation as it forms root nodules in symbiosis with soil bacteria known as rhizobia.

Pigeonpea originated in the Indian sub-continent about 3500 years ago (Patel *et al.,* 2010; Bohra *et al*., 2012). Currently, it is cultivated in many parts of the world including Africa and Asia. In Africa, the crop is produced mainly in east Africa including Malawi and Mozambique (Jones *et al*., 2002; Høgh-Jensen *et al.,* 2007). In South Africa, pigeonpea is cultivated as a minor crop mostly in the eastern-coastal and the north-eastern regions i.e. Mpumalanga and KwaZulu-Natal provinces. The productivity of pigeonpea is generally low in the smallholder cropping systems. For instance, in Tanzania, a low average yield (0.4 t/ha) was reported (Myaka *et al*., 2006). In addition, a wide variation in the N-fixation capacity (40-235 kg/ha) of the crop was reported by (Mapfumo *et al*., 1999; Peoples *et al*., 1995). This variation could be attributed partly to environmental factors as well as genetic factors (Saxena, 2008; Silim *et al*., 2006; Kumar *et al*., 2009).

The symbiotic relationship between the host plant genotype and the rhizobia in pigeonpea is poorly understood. For instance, it is unclear if pigeonpea can grow well in the rest of the regions in South Africa particularly in terms of effective nodulation with the indigenous rhizobia. Currently, there are no commercial bio-inoculants to enhance root nodulation and N fixation. The soils from these areas that have no history of pigeonpea cultivation in the country have not been evaluated adequately for both the presence and diversity of compatible rhizobia. In a previous study that was aimed at isolating rhizobia from

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pigeonpea, individual soil samples were used successfully for inoculating the seed at planting (Bopape et al., 2020). In another study that was conducted in different soil types in Zimbabwe, the variation in root nodulation of pigeonpea was attributed to a wide range of factors including soil pH, organic matter content and distribution of the competitive and effective indigenous rhizobia (Mapfumo et al., 2000). Therefore, there is merit in evaluating the response of pigeonpea to inoculation with soils from diverse agro-ecologies in South Africa. The cultivation of legumes in soils that have no sufficient populations of effective native rhizobia could require seed inoculation at planting to enhance productivity. However, inconsistencies due to differences in host specificity of strains within legume species and cross compatibility can occur. Nevertheless, the cross compatibility provides the foundation for selecting suitable and effective rhizobial strains for pigeonpea. In general, the crop nodulates with the cowpea miscellany group of rhizobia and is promiscuous. Determination of optimum host genotype x rhizobial strain could be useful particularly in terms of expanding the crop to new areas as well as enhancing the productivity of the crop. Therefore, the objectives of this study were to (i) collect and characterize the morphological diversity of rhizobia that are associated with pigeonpea root nodulation (ii) evaluate the nodulation parameters in the host pigeonpea plant and (iii) identify the optimum host genotype x rhizobia combinations.

3.2 Materials and methods

3.2.1 Collection of soil samples

Forty soil samples were collected from diverse locations across South Africa. Each soil sample (approximately 200.0g) was collected from a depth of 10-20 cm from virgin land (with no history of pigeonpea production) during spring and summer season of year 2016-2017. The soil collection locations were distributed across the South Africa transect covering the nine Provinces (Fig. 3.1). Only the soil pH and cation exchange capacity of the soil samples were determined because of the limited quantity of soil per sample (Ross and Ketterings, 1995) (Appendix 3.1).

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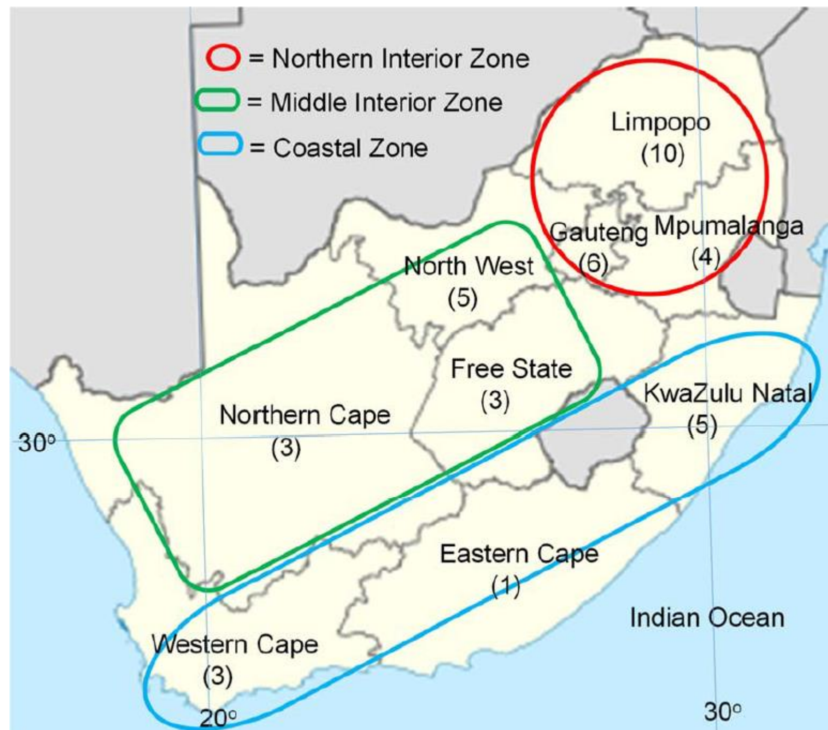

Fig. 3.1 Approximate demarcations of the three broad agro-ecological zones across South Africa from which soil samples were collected.

3.2.2 Trial establishment

Five randomly selected pigeonpea genotypes (consisting of three local landraces and two improved types), were used for trapping the rhizobia. The improved genotypes were exotic and originated from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT). The three landraces were originally collected from Mpumalanga Province (South Africa). The seed characteristics of the genotypes differed markedly (Table 3.1). More than one genotype was used in the trapping experiment to increase the chances of nodulation, hence trapping the rhizobia since rhizobial isolates (or strains) can be host specific (Sharma et al., 1993).

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Table 3.1 Characteristics of the pigeonpea germplasm that was use in the study

Genotype		Origin	Seed clolor	Notes
Code	Designation			
Genotype-1	PP1-3018	MP	Grey	Unimproved landrace
Genotype-2	PP2-3014	MP	Brown (speckled)	Unimproved landrace
Genotype-3	PP3-3016	MP	Black	Unimproved landrace
Genotype-4	PP4-3012	ICRISAT	White/ Cream	Exotic improved
Genotype-5	PP5-3021	ICRISAT	White/ Cream	Exotic improved

ICRISAT= International Crops Research Institute for the Semi-Arid Tropics, MP = Mpumalanga, PP = pigeonpea

At the time of planting, three seeds of each genotype were planted in a Leonard jar containing quartz sand and Hoagland's solution, which is free of N (Law et al., 2007; Hassen et al., 2014) (Appendix 3.2). Prior to planting, the seeds of each genotype were sterilized by immersion in 3% sodium hypochlorite for 3 minutes followed by rinsing with sterile distilled water. The seeds in each jar were inoculated separately with soil (approximately 3.0 g) from a specific location as represented by the soil sample code (Appendix 3.1). The pigeonpea seedlings in the greenhouse were raised under the light and temperature regime set at 14h daylight at a temperature of 28°C and 10h night at a temperature of 15-20°C (Phalane, 2008).

3.2.3 Colony growth and characterization

After six weeks of growth, the plants were harvested to isolate rhizobia from the root nodules. The nodules from each plant were detached and surface-sterilized for 2-3 min in 3.5% (m/v) sodium hypochlorite followed by washing in five changes of sterile distilled water. The isolations were performed by squashing individual nodules in a drop of sterile distilled water with sterile forceps and streaking the nodule extracts onto yeast mannitol congo red (YM-CR) agar plates with the inoculation loop (Somasagaren & Hoben, 1994; Phalane 2008; Howieson and Dilworth, 2016). The agar plates were incubated at 28°C until there was sufficient bacterial growth to form colonies. The colonies of the cultures that developed were purified by picking morphologically distinct single colonies and re-streaking on YM-CR agar plates to obtain pure cultures.

After an incubation of 3 to 7 days at 28°C on YM-CR agar plates, individual colonies were classified based on their size, colour, shape and elevation. A preliminary characterization

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to genus level included isolates that could grow after 3 days (classified as fast-growers or *Rhizobium*) and 5 days (classified as slow-growers or *Bradyrhizobium)*. Two other rhizobia confirmatory tests included culturing the isolates on glucose peptone agar (GPA) medium and nutrient agar were carried out (Singha et al., 2015). GPA is used to confirm rhizobial purity and differentiate rhizobia from other contaminating bacterial microbes and is routinely combined with Bromo-thymol in the isolation of pure rhizobial colonies. Rhizobia show no growth or very poor growth on GPA within 24 hours and cause very little change in pH when incubated at 25-30°C (Singha et al., 2015). Any heavy growth is indicative of bacterial contamination and changes the colour purple to yellow. The isolates were cultured on GPA agar plates and incubated at 28°C for 48 hours. (Most rhizobia have limited growth on nutrient agar in 24 hours such that anything that grows within this time range is considered as a contaminant). Rhizobia growing on YM-CR normally produce white / cream colonies, whereas most contaminants strongly absorb the Congo red dye. The bacterial isolates were streaked on YM-CR plates and incubated at 28°C for 3-7 days. The cultures that were purified on YM-CR by re-streaking single colonies were stored at -70^{0}C in sterile 20% (v/v) glycerol as a cryoprotectant.

3.2.4 Measurement of nitrogen fixation variables

The roots of each harvested plant (at six weeks after germination) was carefully rinsed free of the soils and placed in a labelled plastic bag. Several N fixation variables including nodule number (NN), nodule dry weight (NDW), root dry weight (RDW) and shoot dry weight (SDW) per plant were measured. Each plant was separated (or cut) into three parts, namely, the nodules, roots and shoots. After counting the nodules to obtain the NN, they were transferred to labelled brown paper bags. Similarly, the roots and the shoots of each plant were placed into labelled brown bags and oven-dried for 2 days at 80 ^{o}C and weighed thereafter.

3.2.5 Experimental design and data analysis

The experiment was laid out as split plot design replicated twice, with the pigeonpea genotype as the main-plot and the soil sample (inoculant) as the sub-plot. For each genotype, a control (uninoculated) was included resulting in 205 genotype x inoculant treatment combinations per replication. The quantitative data sets were subjected to analysis of variance using the PROC Mixed (SAS Institute, 2013) procedure to separate the fixed effects from the random effects followed by mean separation using Fischer's least significance difference procedure at the 5.0% probability level.

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3.3 Results

3.3.1 Effects of soil samples on root nodulation

Overall, the soil samples indicated that they contained rhizobia which was compatible with pigeonpea particularly as indicated by the green leaves among the inoculated plants in the study (Fig. 3.1). The uninoculated plants (controls) produced chlorotic leaves (Fig. 3.2). The inoculated plants produced root nodules predominantly around the crown area while in contrast, the controls developed no root nodules (Fig. 3. 3). In addition, the root nodules varied in size from small to large from one soil sample x plant genotype treatment combination to another as well as within the plant (Fig. 3. 4). The functional (effective) root nodules showed pinkish-red nodular tissue (Fig. 3.5).

Fig. 3.2 Inoculated pigeonpea plants growing in L-jars in the greenhouse showing green leaves as an indicator of effective nitrogen fixation in the root nodules.

Fig. 3.3 The uninoculated (control) pigeonpea plants (red circles) showing chlorotic leaves.

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Fig. 3.4 Uninoculated plants produced no root nodules (left) while the inoculated plants developed root nodules (right) predominantly around the crown area.

Fig. 3.5 Variation in nodule size ranging from small (green circle) to large (red circle) within the root system of a pigeonpea plant that was inoculated with a soil sample.

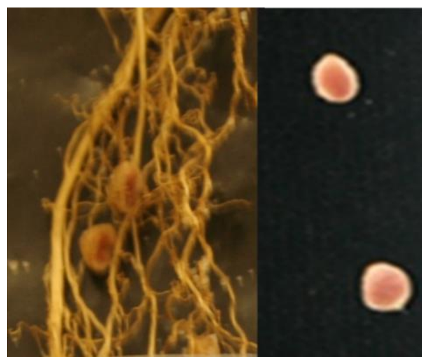

Fig. 3.6 Pinkish-red colour of the internal nodular tissue among the nodules produced by pigeonpea plants that were inoculated with soil samples.

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3.3.2 Colony and isolate characterization

The soil bacteria that were obtained from the root nodules of the inoculated pigeonpea plants produced a variety of the colony morphologies ranging from tiny to medium as well as cream white and large, watery colonies were observed (Fig. 3.6). Most of the colonies consisted of gram-negative rods with a few that were gram-positive with spiral rods. Similarly, most of the isolates were fast-growers, while some were slow to intermediate in their growth rate. On average, the colonies diameter was 2-3mm after 3 days of incubation for the fast-growing bacteria but less than 0.5mm after 5-7 days for the intermediate or slow growing bacteria. Some of the colonies from the slow bacteria group had a long thread-like producing mucous. In addition, 159 and 121 colonies were classified as acidic and alkaline, respectively.

The colonies that resembled rhizobia did not absorb the congo red dye on the YMA-CR agar plates. There were two hundred and eighty potential new (putative) pigeonpea rhizobial isolates that were obtained from the root nodules of the plants that were inoculated with the soil samples (Table 3.2). Seven soil samples produced no rhizobial isolates (Table 3.3). In addition, the pigeonpea 'Genotype-5' (PP5-3021) produced the highest number of putative rhizobial colonies (Fig. 3.7). A confirmatory test of all selected putative rhizobial colonies was performed by culturing them on PGA and NA (Fig. 3.8). Most of the isolates showed growth on YM-CR without absorbing the Congo red dye and there was little growth on NA (Fig. 3.9). Based on their growth pattern and morphological characteristics on media YMA-CR, NA and PGA the confirmed and purified rhizobial isolates where deposited into the national gene bank, the South African Rhizobium Culture Collection in 20% glycerol for further studies.

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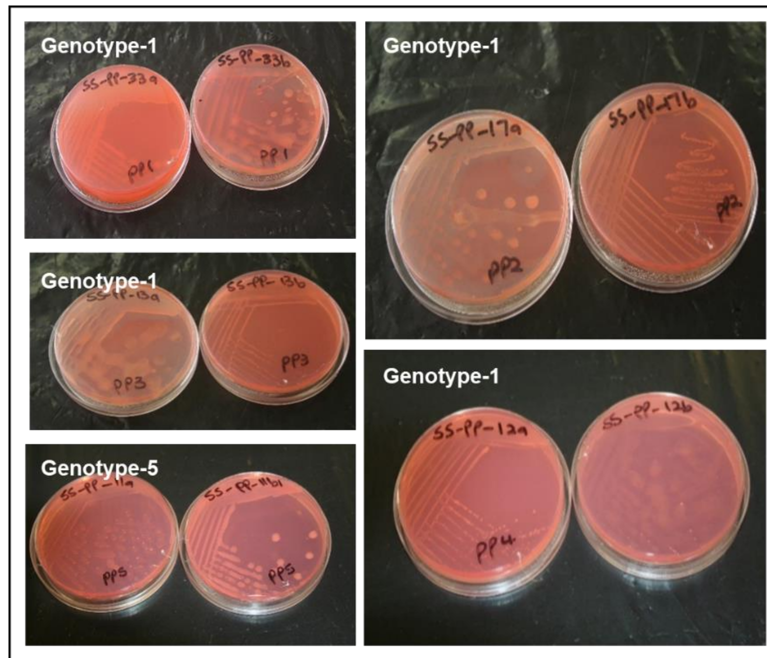

Fig. 3.7 Examples of variations in the morphology of pure cultures among rhizobial isolates (growing on yeast mannitol agar-congo red) that were derived from individual soil samples and isolated from the root nodules of different pigeonpea genotypes.

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Table 3.2 The distribution of confirmed rhizobial isolates derived from the root nodules of five distinct pigeonpea genotypes which were inoculated with individual soil samples collected from various locations across South Africa.

Soil sample number	Number of initial purported	Number of isolates per pigeonpea genotype					Final number of confirmed rhizobia isolates
		Genotype-1	Genotype-2	Genotype-3	Genotype-4	Genotype-5	
1	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0
4	4	0	2	0	0	2	0
5	12	4	2	3	2	1	2
6	8	1	2	2	1	2	1
7	11	3	2	3	2	1	2
8	12	3	2	3	2	2	2
9	7	2	2	2	1	0	0
10	8	1	2	1	2	2	1
11	9	2	2	3	2	0	2
12	6	1	1	2	2	0	0
13	9	1	2	2	2	2	2
14	3	1	1	0	1	0	2
15	9	2	1	2	3	1	4
16	13	3	2	4	2	2	2
17	11	1	2	3	2	3	1
18	10	2	3	2	1	2	3
19	7	1		3	1	2	2
20	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0
22	4	1	1	0	0	2	1
23	2	0	0	0	0	2	1
24	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0
26	11	1	1	2	3	4	2
27	10	1	1	4	2	2	2
28	0	0	0	0	0	0	0
29	3	1	2	0	0	0	3
30	13	2	2	3	3	3	3
31	12	1	3	3	2	3	5
32	12	1	3	1	3	4	4
33	9	2	1	2	2	2	2
34	3	0	0	1	0	2	1
35	4	1	1	1	0	1	2
36	3	0	0	2	1	0	2
37	12	3	2	2	3	2	1
38	5	2	2	1	0	4	1
39	9	0	2	5	0	2	1
40	7	1	1	1	3	1	0
Total	258	45	50	63	48	56	57

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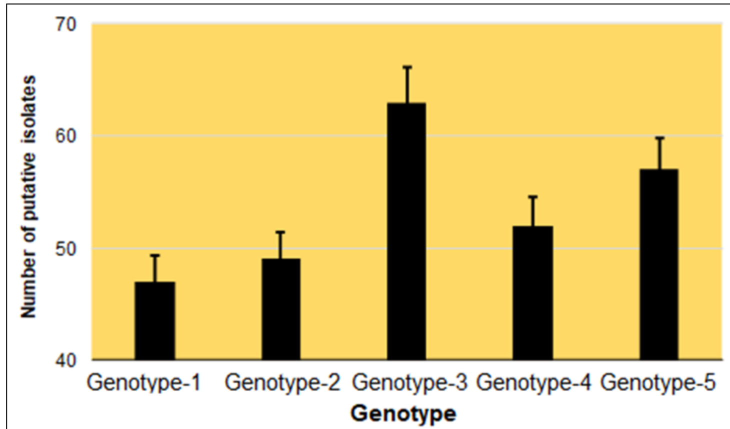

Fig. 3.8 Approximate distribution of putative rhizobial colonies derived from the root nodules of five distinct pigeonpea genotypes which were inoculated with individual soil samples collected from various locations across South Africa.

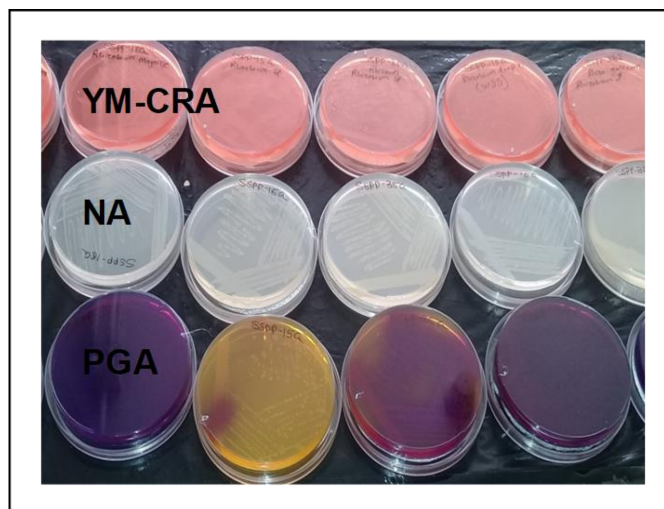

Fig. 3.9 Rhizobial isolates from pigeonpea root nodules that were cultured on three media yeast mannitol Congo red agar (YM-CR), nutrient agar (NA) and peptone glucose agar (PGA).

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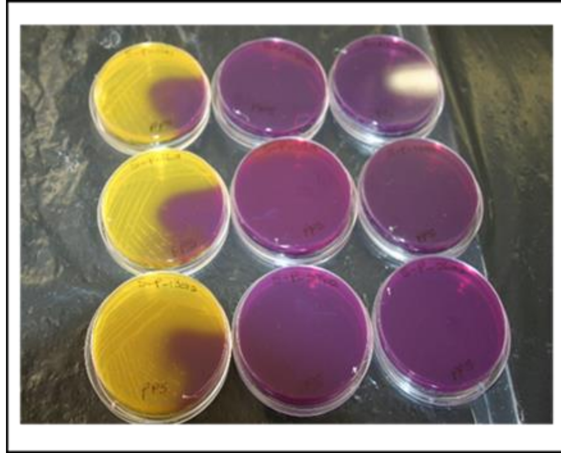

Fig. 3.10 Rhizobia showed limited or no growth on peptone glucose agar (purple colour of the growth medium does not change to yellow in the absence of bacterial growth).

3.3.3 Characterization of the host plant genotypes and soil samples inoculants

There were statistically significant differences in all the seven root nodulation traits among the five pigeonpea genotypes that were inoculated with individual soil samples at the time of planting (Table 3.3). In addition, the genotype x isolate interaction effects were highly significant ($P < 0.01$) for NN, RFW, SDW and significant ($P < 0.05$) for the remaining N fixation parameters. ‘Genotype-5’ attained the highest NN (6.62) while ‘Genotype-4’ achieved the lowest (4.16) (Table 3.4). ‘Genotype-1’ and ‘Genotype-3’ were similar for most of the root nodulation traits. The mean NN per plant among the pigeonpea genotypes was 5.22 (Table 3.5). However, both soil sample-10 (23.27) and sample-33 (21.90) produced more than four-fold the mean NN with relatively high NFW and NDW. In addition, the highest (0.31g) RDW was observed for soil sample-33 while the control (uninoculated soil sample-41) produced the lightest (0.13g) roots (Table 3.5). At least 11 soil samples produced significantly ($P < 0.05$) higher SDW in comparison with the trial mean. However, on average, the SFW was at least three-fold higher than the SDW among the pigeonpea genotypes.

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Table 3.3 The mean squares for root nodulation traits among five pigeonpea genotypes that were inoculated with soil samples collected from various locations across South Africa. (NN = nodule number; NFW = nodule fresh weight; NDW = nodule dry weight; RDW = root dry weight; RFW = root fresh weight; SFW = shoot fresh weight; SDW = shoot dry weight).

Source	df	NN	NFW	NDW	RFW	RDW	SFW	SDW
Replication (R)	2	52.99	6.88**	5.05**	0.19	0.31	0.02	0.00
Genotype (G)	4	105.53**	3.46**	8.66**	29.23**	0.43**	0.15**	0.01**
Isolate (I)	40	556.16**	4.91**	1.34**	2.95**	0.37**	0.04**	0.01**
G * I	160	40.11**	0.61*	0.86*	1.21**	0.04*	0.02*	0.00**

***; **; * = significant at the 0.001, 0.01 and 0.05 probability level, respectively.

Table 3.4 The means of root nodulation traits among five pigeonpea genotypes that were inoculated with soil samples collected from various locations across South Africa. (NN = nodule number; NFW = nodule fresh weight; NDW = nodule dry weight; RDW = root dry weight; RFW = root fresh weight; SFW = shoot fresh weight; SDW = shoot dry weight).

Genotype	NN	NFW	NDW	RFW	RDW	SFW	SDW
Genotype-5	6.62 a	0.27 cb	0.04 ab	0.21 a	1.89 a	0.38 a	1.60 ab
Genotype-2	6.03 a	0.19 c	0.04 ab	0.14 b	1.41 dc	0.39 a	1.50 ab
Genotype-3	5.11 ab	1.39 a	0.05 a	0.19 a	1.85 ab	0.45 a	1.68 a
Genotype-1	4.94 ab	0.35 b	0.03 b	0.18 a	1.59 bc	0.40 a	1.51 ab
Genotype-4	4.16 b	0.11 c	0.01 c	0.11 b	1.23 d	0.29 b	1.27 b

In each, column, mean values that are followed by different small letters are significantly ($P < 0.05$) different from each other as determined by the Least Significant Difference ($LSD_{0.05}$).

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Table 3.5 Performance of soil samples that were used for inoculating five pigeonpea genotypes (SSC = soil sample code; NN = nodule number; NFW = nodule fresh weight; NDW = nodule dry weight; RDW = root dry weight; RFW = root fresh weight; SFW = shoot fresh weight; SDW = shoot dry weight).

SSC	NN	NFW	NDW	RDW	RFW	SDW	SFW
10	23.27	1.39	0.11	0.23	1.86	0.56	1.91
33	21.90	1.18	0.09	0.31	2.34	0.77	2.59
16	18.30	1.13	0.08	0.22	1.85	0.63	2.49
32	16.10	0.86	0.08	0.19	1.43	0.66	2.38
17	11.60	1.02	0.06	0.22	1.67	0.54	2.25
37	11.40	1.05	0.05	0.17	1.63	0.48	1.97
11	10.90	0.36	0.06	0.12	1.11	0.53	1.98
18	10.50	0.69	0.06	0.14	1.31	0.52	1.88
30	10.23	1.05	0.07	0.19	1.89	0.57	2.29
39	9.50	0.89	0.04	0.22	1.89	0.51	2.07
6	8.86	1.03	0.08	0.26	1.95	0.64	2.63
15	8.00	0.94	0.05	0.11	1.12	0.40	1.68
8	7.13	0.43	0.06	0.20	1.55	0.59	2.21
7	7.07	0.69	0.06	0.26	1.77	0.56	2.23
5	6.93	1.33	0.06	0.21	1.65	0.43	1.88
9	6.00	0.93	0.07	0.22	1.18	0.55	2.12
31	5.57	0.85	0.04	0.11	1.09	0.43	1.67
26	4.46	0.49	0.07	0.19	1.91	0.48	1.92
27	4.36	0.62	0.04	0.19	1.39	0.38	1.58
12	3.39	0.32	0.02	0.15	1.66	0.26	1.01
38	2.40	0.36	0.02	0.16	1.81	0.32	1.39
13	1.67	0.11	0.02	0.15	1.64	0.34	1.30
19	1.00	0.02	0.01	0.12	1.59	0.22	0.90
36	0.71	0.67	0.01	0.15	1.62	0.27	1.08
14	0.57	0.01	0.01	0.16	1.68	0.27	1.19
34	0.50	0.02	0.01	0.09	1.04	0.19	0.77
40	0.47	0.21	0.00	0.17	1.89	0.27	1.17
35	0.39	0.01	0.00	0.13	1.75	0.33	1.05
29	0.29	0.01	0.00	0.12	1.51	0.25	0.99
22	0.25	0.02	0.01	0.08	1.24	0.17	0.80
28	0.23	0.02	0.00	0.12	1.32	0.22	0.89
4	0.14	0.02	0.01	0.15	1.99	0.27	0.02
24	0.00	0.00	0.00	0.13	1.57	0.21	0.79
25	0.00	0.00	0.00	0.12	1.56	0.21	0.89
1	0.00	0.00	0.00	0.15	1.78	0.22	0.92
2	0.00	0.00	0.00	0.13	1.55	0.28	1.11
20	0.00	0.00	0.00	0.14	1.73	0.24	1.02
21	0.00	0.00	0.00	0.14	1.27	0.22	1.04
3	0.00	0.00	0.00	0.16	1.74	0.24	1.03
23	0.00	0.00	0.00	0.15	1.83	0.23	0.87
41 (Control)	0.00	0.00	0.00	0.13	1.40	0.19	0.90
Mean	5.22	0.46	0.03	0.16	1.60	0.381	1.48
C.V.(%)	37.05	26.07	23.47	25.01	38.52	28.79	33.81
$LSD_{0.05}$	3.38	0.36	0.03	0.08	0.58	0.14	0.49

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3.3.4 Relationship between the root nodulation traits

There was a highly significant (P <0 .01) positive correlation between the NN and all the other N fixation variables except for RFW (Table 3.6). In addition, there was a highly significant (P < 0.01) positive correlation between the RFW and RDW. However, the positive correlation between RFW and both the NFW and NDW was not significant. The SDW showed a highly significant (P < 0.01) positive correlation with the RDW but not with the RFW (Table 3.6). The SDW also showed a highly significant (P < 0.01) positive correlation with each of the variables except for RFW.

Table 3.6 Coefficients of correlation (*r*) between the root nodulation traits among the pigeonpea genotypes. (NN=nodule number; NFW=nodule fresh weight; NDW= nodule dry weight; RFW=root fresh weight; RDW= root dry weight; SFW= shoot fresh weight; SDW=shoot dry weight).

	NN	NFW	NDW	RDW	RFW	SDW	SFW
NN	1.0000						
NFW	0.8101***	1.0000					
NDW	0.8908***	0.8543***	1.0000				
RDW	0.6438***	0.6758***	0.7136***	1.0000			
RFW	0.2867	0.2313	0.2232	0.6743***	1.0000		
SDW	0.8565***	0.7803***	0.9250***	0.7830***	0.2722	1.0000	
SFW	0.7796***	0.7996***	0.8843***	0.7353***	0.1975	0.9451***	1.0000

3.4 Discussion

The results of this study confirmed that the indigenous soil samples from South Africa contain rhizobia compatible with pigeonpea. There was evidence of variable but effective N fixation among the pigeonpea genotypes that were used in this study. Both the pinkish-red nodular interior tissue and green leaves of the inoculated plants clearly indicated successful N fixation. The findings have the potential to enhance and expand the pigeonpea production and cultivation by smallholder farmers to new areas in the country. Currently, the production of pigeonpea is limited in South Africa and confined mainly to the eastern coastal belt encompassing parts of KwaZulu as well as the inland areas of Mpumalanga and Limpopo Provinces. The limitations to cultivation of pigeonpea are partly due to insufficient information regarding the compatibility of the crop with indigenous soil rhizobia in those areas that have no history of cultivating the crop.

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The results also indicated that the soil samples contained diverse rhizobial isolates with distinct morphological characteristics and significant differential N fixation ability on the pigeonpea genotypes, suggesting that there was potential to select for optimum host genotype x rhizobial isolate combinations for N fixation in the species. In a study involving trapping indigenous rhizobial isolates from root nodules of pigeonpea plants grown in Ethiopia, a wide phenotypic diversity among the isolates was reported (Degefu et al., 2018). In common bean (*Phaseolus vulgaris* L.) inoculation with diverse native rhizobial isolates from Kenyan soil resulted in a significant increase in the shoot dry weight (Kawaka et al., 2014). Moreover, indigenous soils have been used to trap isolates of rhizobia for various legumes (Ouma et al., 2016; Hassen et al., 2012). Other studies conducted in Zimbabwe, Ethiopia and Kenya, demonstrated that indigenous rhizobia (miscellany cowpea rhizobia) occur naturally in the soils (Mpepereki et al., 1996; Kebeke et al., 2021; Nyaga et al., 2020). Therefore, the results that were observed in this study agree with the findings of other researchers who investigated the symbiotic efficiency of indigenous (native) soil rhizobia on legumes.

The diverse colony morphologies ranging from tiny to large sizes that were observed in this study indicated that the soil samples were diverse and varied from each other. This suggested that different bacterial isolates, most probably from distinct genera of rhizobia, were present in the soil samples. This was partly confirmed by the selection media that were used in the study. The bacterial colonies which did not absorb the congo red dye in the YM-CR (putative rhizobial isolates) showed a quantitative variation across the genotypes. This suggested that there was merit in using multiple host plant genotypes to optimize the chances of trapping the isolates. Furthermore, nodules from other leguminous species that are found in South Africa such as *Lebeckia* sp (Phalane et al., 2008), tea rooibos (*Aspalathus linearies*) (Hassen et al., 2012) and sweet thorn (*Vachelia karoo*) (Beukes et al., 2019) have also produced diverse rhizobia. In addition, the growth rate of the bacterial colonies suggested a high probability of the occurrence of multiple genera of the rhizobia that were compatible with pigeonpea. The fast-growing rhizobia are usually associated with the symbiosis of acidic N but the slow-growing ones are associated with the synthesis of alkaline N compounds (Sprent, 2009). Out of the 280 putative isolates, only 57 were confirmed as rhizobia and deposited into the South African Rhizobium Culture Collection (SARCC) using 20% cryoprotectant glycerol for long term preservation and future exploitation. Specifically, molecular characterization of the isolates will provide more information about their phylogenetic relationships as reported in other studies (Arora et al., 2018; Kawaka et al., 2018; Hassen et al., 2014; Guant et al., 2001). In addition, the isolates could be used in future studies aimed at the selection and development of commercial inoculants for field legumes.

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The significant differences that were observed among the host plant genotypes in terms of the seven root nodulation traits indicated that there was potential to select for optimum host genotype x soil sample. In addition, significant interaction between the host plant genotype x soil sample also indicated the differential impact of the soil samples on pigeonpea suggesting that there will be a need to choose the best genotypes for specific production areas. Moreover, the agronomic characteristics of the genotypes that were used in this study varied markedly across the diverse soil samples. Even though both 'Genotype-4' and 'Genotype-5' were improved types, the latter performed relatively better in terms of N fixation as indicated by the NDW and SDW. A relatively high NN that was observed for 'Genotype-5' must be approached with caution partly because of the complexity and unreliability of this variable as an indicator of N_2 fixation. The nodule sizes per plant vary considerably from tiny to large nodules thus making it subjective and difficult to determine the minimum threshold size of nodules that can be counted in such studies. In addition, tropical legumes such as pigeonpea form determinate nodules which senesce and cease to function at some point. Moreover, the number of the nodules per plant is sensitive to environmental factors such as N availability (Streeter, 1988; Cabeza et al., 2014). According to Schwember et al., (2019), there is no unifying theory that accounts for all of the aspects that are involved in the regulation of N fixation.

The results of this study also showed that four soil samples (10, 16, 32 and 33) were superior, in terms of their impact on the host plant genotypes, particularly when considering the NDW and SDW that have been used widely as reliable indicators of N fixation (Bopape et al., 2021; Nyaga et al., 2020; Ouma et al., 2016; Hassen et al., 2014; Hassen et al., 2021). Nonetheless, the remainder of the N fixation indicators that were used in the study were still useful in providing additional information about the symbiotic efficiency of pigeonpea. They have also been used variably by other researchers, albeit, with mixed conclusions (Hassen et al., 2014; Hardarson and Danso, 1993). Moreover, this study found a positive relationship between SDW and SFW for instance, implying that it is difficult to separate the reliability of such traits in determining their usefulness as indicators of N fixation.

3.5 Conclusions

The results of this study were useful in determining the symbiotic potential of indigenous rhizobial populations with pigeonpea. This information will assist in determining accurately the need to inoculate the crop as well as the nodulation patterns. Diverse rhizobia were isolated successfully from the soil samples that were collected from different locations across the South Africa transect and used for inoculating the seed of the host plant genotypes at planting in a N_2 free growth medium. The rhizobial isolates were confirmed using

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morphological and biochemical characters and deposited into the national rhizobia gene bank for further exploitation and molecular characterization.

In future, there will be merit also in validating the impact of the isolates on a field basis to determine the grain yield of the crop. This could lead to the expansion of the production area under pigeonpea in South Africa and hence an improvement in the quantity of pigeonpea production in the country as well as food security. In addition, it will be useful to investigate the impact of the isolates on other tropical legumes with the aim of developing efficient commercial inoculants for a range of tropical legumes other than just pigeonpea.

References

Arora NK, Khare E, Singh S, Tewari S (2018). Phenetic, genetic diversity and symbiotic compatibility of rhizobial strains nodulating pigeonpea in Northern India. *Biotechnology*. 8 (52): 1-11.

Beukes CW, Boshoff FS, Phalane FL, Hassen AI, Le Roux MM, Stępkowski T, Venter SN, Steenkamp ET (2019). Both alpha-and beta-rhizobia occupy the root nodules of *Vachellia karroo* in South Africa. *Frontiers in Microbiology*. 10(1195): 1-15. doi:10.3389/fmicb.2019.01195.

Bohra A, Saxena RK, Gnanesh BN, Saxena K, Byregowda M, Rathore A, KaviKashor PB, Cook DR, Varshney, RK (2012). An intra-specific consensus genetic map of pigeonpea (*Cajanus cajan* (L) Millspaugh) derived from six mapping populations. *Theological Applied Genetics*. 125(6): 1325-1338.

Bopape FL, Hassen AI, Swanevelder ZW, Gwata ET (2020). Draft genome sequence of *Rhizobium tropici* SARCC-755 a free-living *Rhizobium* that nodulated and promoted growth in pigeonpea [*Cajanus cajan* (L.) Millsp.]. *Microbiology Resource Announcements*. 9(2): 1-2, e01122-19. doi:10.1128/MRA.01187-19. doi: 10.1128/MRA.01122-19.

Bopape FL, Gwata ET, Hassen AI, Zhou MM (2021). Symbiotic efficiency of pigeonpea (*Cajanus cajan*) with different sources of nitrogen. *Plant Genetic Resources*. 19(4): 1-4. doi:10.1017/S1479262121000356.

Cabeza RA, Koester B, Liese R, Lingner A, Baumgarten V, Dirks J, Salinas-Riester G, Pommerenke C, Dittert K, Schulze J (2014). An RNA sequencing transcriptome analysis

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reveals novel insights into molecular aspects of the nitrate impact on the nodule activity of *Medicago truncatula*. *Plant Physiology*. 164: 400-411. doi:10.1104/pp.113.228312.

Degefu T, Wolde-meskel E, Adem M, Fikre A, Amede T, Ijiewo C (2018). Morphophysiological diversity of rhizobia nodulating pigeonpea (*Cajanus cajan* L. Millsp.) growing in Ethiopia. *African Journal of Biotechnology*. 17(6): 167-177.

Gaunt MW, Turner SL, Rigottier-Gois L, Lloyd-Macgilp SA, Young JPW (2001). Phylogenies of *atpD* and *recA* support the small subunit rRNA-based classification of rhizobia. *International Journal of Systematic Evolutionary Microbiology* 51: 2037-2048.

Hardarson G, Danso SKA (1993). Methods for measuring biological nitrogen fixation in grain legumes. *Plant and Soil*. 152: 19-23.

Hassen AI, Bopape FL, Habig JH, Lamprecht SC (2012). Nodulation of rooibos (*Aspalathus linearis* Burm. f.), an indigenous South African legume, by members of both α and β-proteobacteria. *Biology and Fertility of Soils*. 48: 295-303.

Hassen AI, Bopape FL, Trytsman M (2014). Nodulation study and characterization of rhizobial microsymbionts of forage and pasture legumes in South Africa. *World Journal of Agricultural Research.* 2(3): 93-100.

Howieson JG, Dilworth MJ (2016). Working with rhizobia manual. *Australian Centre for International Agriculture*, Canberra.

Høgh-Jensen H, Myaka FA, Sakala WD, Kamalongo D, Ngwira A, Vesterager JM, Odgaard R, Adu-gyamfi JJ (2007). Yields and qualities of pigeonpea varieties grown under smallholder farmer's conditions in eastern and southern Africa. *African Journal of Agric*ultural *Res*easrch. 2: 269-278.

Jolliffe IT, Cadima J (2016). Principal Component Analysis: A Review and Recent Developments. *Philosophical Transactions Royal Society A.* 374: 1-10. doi:org/10.1098/rsta.2015.0202.

© University of Venda

Jones RB, Freeman HA and Lo Monaco G (2002). Improving the access of small farmers in Eastern and Southern Africa to global pigeonpea markets. *Agricultural Research and Extension Network.* 120: 1-11.

Kawaka F, Makonde H, Dida M, Opala P, Ombori O, Maingi J, Muoma J (2018). Genetic diversity of symbiotic bacteria nodulating common bean (*Phaseolus vulgaris*) in western Kenya. *PLOS One*. pp 1-13. doi:org/10.1371/journal.pone.0207403.

Kebeke E, Amsalu B, Argaw A, Tamiru S (2021). Abundance of native rhizobia nodulating cowpea in major production areas of Ethiopia as influenced by cropping history and soil properties. *Sustainable Environment.* 7(1): 1-13. doi:org/10.1080/27658511.2021.1889084.

Kumar CVS, Sreelakshmi C, Shivani D, Suresh M (2009). Gene effects for yield contributing characters in pigeonpea (*Cajanus cajan* L. Millsp) by generation mean analysis. *Journal of Research ANGRAU.* 37(3&4): 71-76.

Law IJ, Botha WJ, Majaule UC, Phalane FL (2007) Symbiotic and genomic diversity of “Cowpea” bradyrhizobia from soils in Botswana and South Africa. *Biology and Fertility of Soil*. 43: 653-663.

Mapfumo P, Giller KE, Mpepereki S, Mafongoya PL (1999). Dinitrogen fixation by pigeonpea of different maturity types on granitic sandy soils in Zimbabwe. *Symbiosis.* 27: 305-318.

Mapfumo P, Mpepereki S, Mafongoya P (2000). Pigeonpea rhizobia prevalence and crop response to inoculation in Zimbabwean smallholder-managed soils. *Experimental Agriculture*. 36(4): 423-434. doi:10.1017/S0014479700001009.

Mpepereki S, Wollum II AG , Makonese F (1996). Diversity in symbiotic specificity of cowpea rhizobia indigenous to Zimbabwean soils. *Plant and Soil.* 186: 167-171.

Myaka FA, Sakala WD, Adu-Gyamfi JJ, Kamalongo D, Ngwira A, Odgaard R, Nielsen NE, Høgh-Jensen H (2006). Yields and accumulations of N and P in farmer-managed maize-pigeonpea intercrops in semi-arid Africa. *Plant and Soil*. 285: 207-220.

© University of Venda

Nyaga JW, Njeru EM (2020). Potential of native rhizobia to improve cowpea growth and production in semiarid regions of Kenya. *Agronomy*. 2: 1-13. doi:10.3389/fagro.2020.606293.

Ouma EW, Asango AM, Maingi J, Njeru EM (2016). Elucidating the potential of native rhizobial isolates to improve biological nitrogen fixation and growth of common bean and soybean in sallholder farming systems of Kenya. *International Journal of Agronomy*. 2016: 1-8. doi:org/10.1155/2016/4569241.

Patel KJ, Singh AK, Nareshkumar G, Archana G (2010). Organic-acid-producing, phytate-mineralizing rhizobacteria and their effect on growth on pigeonpea (*Cajanus cajan*). *Applied Soil Ecology.* 44(3): 252-261.

Peoples MB, Herridge DF, Ladha JK (1995). Biological nitrogen fixation: An efficient source of nitrogen for sustainable agricultural production? *Plant and Soil.* 174: 3-28.

Phalane FL (2008). The diversity of root nodule bacteria associated with *Lebeckia* species in South Africa. MSc Thesis, University of Pretoria.

Ross DS and Ketterings Q. (1995). Recommended methods for determining soil cation exchange capacity. *Recommended soil testing procedures for the northeastern United States*. 493(101): 62.

Saxena KB (2008). Genetic improvement of pigeonpea-a review. *Tropical Plant Biology.* 1:159-178.

Silim SN, Gwata ET, Coeb R, Omanga PA (2007). Response of pigeonpea genotypes of different maturity duration to temperature and photoperiod in Kenya. *African Crop Science Journal.* 15(2): 73-81.

Singha B, Das P, Mazumder PB (2015). Morphological and biochemical characterization of rhizobia isolated from root nodules of *Crotalaria junceae* L. Grown in Assam. *International Journal Scientific Research*. 4 (4): 1928-1931.

SAS Institute (2003). SAS/STAT guide for personal computers, *SAS Institute,* Cary.

Schwember AR, Schulze J, Del Pozo A, Cabeza RA (2019). Regulation of Symbiotic Nitrogen fixation in legume root nodules. *Plants*. 8(333): 1-18. doi:10.3390/plants8090333.

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Somasegaran P, Hoben HJ (1994). Hand book for Rhizobia. Springer-Verlag, NewYork.

Sprent JI (2009). Legume nodulation: A global perspective. Wiley Blackwell. pp 1-200.

Streeter J (1988). Inhibition of legume nodule formation and N2 fixation by nitrate. *Plant Science*. 7: 1-13. doi:10.1080/07352688809382257.

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4.0 Chapter Four: Determination of the Genetic Relationships of Rhizobial Strains that Originated from Soil Samples which were Collected from Diverse Locations in South Africa and used for Inoculating Pigeonpea

Abstract: Leguminous crops such as pigeonpea (*Cajanus cajan*) can fix atmospheric nitrogen symbiotically in the root nodules thus eliminating the need for expensive chemical nitrogenous fertilizers in legume production particularly in the smallholder systems in Africa. The knowledge about the host plant x microsymbiont interaction is critical for the identification and selection of combinations that produce optimum crop productivity in such systems. However, the molecular characteristics and genetic relationships of the rhizobial isolates compatible with pigeonpea from South African soils for the present study, were unknown. Therefore, this study was designed to characterize, identify (using both 16S rRNA and *recA* genes) and determine the phylogenetic relationships of the indigenous rhizobia isolates from South Africa that are associated with effective root nodulation and nitrogen fixation in pigeonpea. The results showed that 56 isolates were confirmed as rhizobia. The primer set TSrecAf5 ('5 CAC TGC MYT GCG TAT YGT CGA AGG 3') and TSrecAr3 ('5 GAT CTT CAT SCG GAT CTG GTT GATG 3') successfully amplified both the *Rhizobium* strain (30bp3) as well as several *Bradyrhizobium* strains (16a2p3, 15bp3, 11a2p3, 13bp3, 33ap4 and 19a1p3). Two novel genera of rhizobia (*Phyllobacterium* and *Paraburkholderia*), were associated with root nodulation in pigeonpea. There was considerable variation in the size of the sequences of both the 16S rRNA and *recA* genes among the rhizobial isolates. The sequences of the 16S rRNA genes across the four genera averaged 1015.73 bp. The longest (1470 bp) and shortest (403 bp) sequences were observed for the 16S rRNA gene (genus *Rhizobium*, isolate '5b2p1') and *recA* gene (genus *Bradyrhizobium*, isolate '27b2p5'), respectively. The 16S rRNA *Rhizobium* phylogenetic tree showed that the rhizobial isolates obtained from pigeonpea were dispersed in six different clusters and grouped with several type species of the genus *Rhizobium* including *R. tropici* with a high (77.0%) similarity grouping. The 16S rRNA based phylogenetic tree showed that the novel *Paraburkholderia* rhizobial isolate '30a2p3', grouped with several type strains including *P. rhizosphaerae* (with a similarity grouping >50%) but in the *recA* based phylogenetic tree, the same isolate was grouped in Cluster IV with 93.0% similarity grouping. The study concluded that the sequences of the two genes (16S rRNA and *rec*A) of the isolates from pigeonpea could provide sufficient phylogenetic information about the isolates up to the species level and confirmed that this legume is promiscuous in diverse soils from South Africa.

Key words: genera; phylogeny; reference strain; rhizobia; sequences

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One publication emanated from this chapter as follows:

Bopape FL, Beukes CW, Kopotsa K, Hassen AI, Steenkamp ET, Gwata ET. (2022). (2022). Symbiotic performance and phylogenetic characterization of pigeonpea (*Cajanus cajan* L. Millsp.) rhizobia occurring in South African soils. *Agriculture. In Review*.

4.1 Introduction

Leguminous crops such as pigeonpea (*Cajanus cajan*) can promiscuously associate with naturally occurring rhizobia to fix atmospheric nitrogen in the root nodules (Bopape et al., 2021). This process of biological nitrogen fixation results in the reduction and elimination of using expensive chemical nitrogenous fertilizers in legume production particularly in the smallholder systems in Africa. In South Africa, pigeonpea is cultivated as a minor crop such that there is limited information in literature on studies aimed at the various production aspects of this crop including agronomic performance, response to rhizobia as well as genetic improvement.

The knowledge about the host plant x microsymbiont interaction is critical for the identification and selection of combinations that produce optimum productivity of the crop. The promiscuity of pigeonpea root nodulation with indigenous rhizobia implies that the crop responds differentially to specific genera of rhizobia (Bopape et al., 2021). Previous taxonomic studies showed that rhizobia generally fall into 12 genera namely *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium, Sinorhizobium, Azorhizobium, Allorhizobium*, *Phyllobacterium, Onchrobactrum, Methylobacterium, Devosia* (*Alphaproteobacteria*), *Paraburkholderia* (previously *Burkholderia*) and *Cupriavidis* (*Betaproteobacteria*) (Laguerre et al., 2001; Hassen et al., 2014; Wier et al., 2016). Nonetheless, the taxonomy of the rhizobia is continually changing with new species identified and characterized. To date the latest updated taxonomy of rhizobia contain 18 genera, with the addition of *Pararhizobium*, *Neorhizobium*, *Shinella*, *Aminobacter*, *Methylobacterium*, *Microvirga* (Mousavi, 2016; de Lajudie et al., 2019; Lindstrom and Mousavi 2019)

Rhizobia applied as bio-fertilizers (or inoculants) to seed before planting play a vital role in sustainable agriculture. However, the rhizobia may fail to compete for nodule occupancy against indigenous and natural rhizobia. This may be due to inferior nitrogen-fixing abilities from the applied inoculants, which leads to low crop yields. The isolates with excellent performance in controlled conditions are selected as inoculants for field trial. Nonetheless, the lack of the ability to out compete natural rhizobia and remain in the fields after several agricultural crop rotations due to the transfer of symbiotic genes from the inoculant strains to

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the indigenous population, may limit the suitability of commercial inoculants. The knowledge gathered from glasshouse and field trials may allow for the selection and exploitation of the effective indigenous rhizobial strains (Mendoza-Suarez et al., 2021). The order of competitive nodulation for different rhizobial species is also not yet clear (Ji et al., 2017)

The ability to form nodules in the presence of other strains determines the nodulation competitiveness of rhizobial strains (Yates et al., 2011; Onishchuk et al., 2017). Rhizobial competitiveness has a vital practical implication for agriculture, because differences in nitrogen fixation efficiency between strains can be large (Irisarri et al., 2019). However, currently, there is dearth of information regarding the specific rhizobial genera that associate optimally with pigeonpea. The majority of the indigenous rhizobia are diverse and interact promiscuously with different legumes such as pigeonpea and tropical legumes. These rhizobia form part of the Cowpea Miscellany Group (CMG) and ubiquitous in African soils (Thies et al. 1991, Abaidoo et al. 1999; Saxena 2008). The four legumes in the CMG includes cowpea (*Vigna unguiculata*), siratro (*Macropitilium atropurpureum*), lima bean (*Phaseolus lunatus*) and peanut (*Arachis hypogaea*) that nodulate with *Bradyrhizobium* (Thies et al., 1991; Abaidoo et al., 1999; Saxena 2008). These hosts nodulate with a wide range of rhizobia but do so with poor efficiency (Thies et al., 1991).

The characterization of rhizobia with phenotypic methods generally requires the use of live cultures as well as determining ability of isolates to utilize antibiotics, growth at different media, pH and temperatures (Florentino et al., 2015) and sometimes utilization of carbohydrates (Ceapa et al., 2015). These phenotypic methods are generally labour intensive, time consuming and only a limited number of samples can be evaluated at a time. Recent studies aimed at the characterization of rhizobia utilized sequencing the 16S rRNA gene, which is highly conserved across bacterial species (Clarridge 2004; Janda and Abbott 2007). In addition, the *recA* gene that plays an important role in homologous DNA repair is also used for the confirmation of 16S rRNA sequencing identification (Mahenthiralingam et al., 2000; Rocha et al., 2005).

The molecular characteristics and genetic relationships of the rhizobial isolates compatible with pigeonpea from South African soils as reported in the previous Chapter, were unknown. Therefore, this study was designed to characterize and identify (using both 16S rRNA and *recA* genes) as well as determine the phylogenetic relationships of the indigenous rhizobia from South Africa that are associated with effective root nodulation and nitrogen fixation in pigeonpea.

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4.2 Materials and methods

4.2.1 DNA extraction and primer selection

The purified cultures were grown in Tryptone Yeast Extract (TYE) broth for 24 to 48 hours at 28 °C and 150 rpm on a rotary shaker. DNA was extracted from each of the 280 putative bacterial isolates originating from pigeonpea nodules using the WIZARD genomic DNA purification kit per the manufacturer's instructions (Promega, USA). The 16S rRNA PCR amplification was performed with the primers, 27F (5' AGA GTT TGA TCC TGG CTC AG 3') and 1485R (5' TAC CTT GTT ACG ACT TCA CCC CA 3') (Lane, 1991).

4.2.2 16S rRNA PCR amplification and sequencing

There is variation in the degree of differences in the families, genera and species of bacteria. The 16S rRNA can be used for bacterial classification. Therefore, the 16S rRNA was important to confirm the genus. The 16S rRNA PCR amplification for each isolate was performed with the primers, 27F (5' AGA GTT TGA TCC TGG CTC AG 3') and 1485R (5' TAC CTT GTT ACG ACT TCA CCC CA 3') (Lane, 1991). The total volume of each PCR reaction was 50 µl containing 5 ng/µl DNA, 25 mM $MgCl_2$, 2.5 mM dNTPs, 10 µM of each primer and 0.1 U/µl Super-Therm Taq polymerase and 50-100ng genomic DNA (Southern Cross Biotechnology, Cape Town, SA). The amplification was carried out in an Eppendorf Master Cycler Gradient apparatus (Applied Biosystems, USA) using the following PCR protocol: an initial denaturation at 94 °C for 2 min (modified to 94 °C for 4 min) followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 62 °C for 1 min (modified to annealing at 55 °C for 30 sec) then extension at 72°C for 1 min, followed by a final extension step at 72 °C for 7 min (modified to 5 min) for the 16S gene. Successful amplification was verified on horizontal 1% agarose gel electrophoresis. The PCR products were purified with Exonuclease 1 and FASTAP alkaline phosphatase (Themo Scientific).

The PCR products were sequenced in both directions using the same primers together with ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit on an ABI3100 Automated Capillary DNA sequencer (Applied Biosystems). All raw sequence files were inspected and edited, using Chromas Lite version 2.0 (Technelysium, Australia) and Bio Edit version 5.0.9 (Hillocks et al. 2000). The consensus 16S rRNA sequences for each isolate was compared to all publicly available sequences in the GenBank database of the National Centre for Biotechnology Information (NCBI) using the blastn software thereby identifying the genus to which each isolate was related using percent similarity (Altschul et al., 1990; Benson et al., 2015; Leray et al., 2019). The genus identity was then considered when deciding on which *recA* primers and protocol to follow for each set of isolates. Only 56 isolates were identified as rhizobia and analyzed further using primers for the *recA* gene which mediates the repair

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pathway in *Bacteroides* by encoding the bacterioferritin co-migratory protein (which is responsible for conferring protection from oxygen radicals and the enzyme glutamine synthetase III) (Konola et al., 1994).

4.2.3 *recA* PCR primer selection, amplification and sequencing

Based on the 16S rRNA sequencing results, amplification and sequencing of the *recA* locus was done for strains, which were assigned to rhizobial genera based upon 16S rRNA sequence similarity. The *recA* primer sets that were used in this study recA63F ('5 ATC GAG CGG TCG TTC GGC AAG GG 3') and recA504R ('5 TTG CGC AGC GCC TGG CTC AT 3') (Gaunt et al., 2001), which could successfully amplify this locus for strains belonging to multiple genera including *Rhizobium*, *Bradyrhizobium*, *Paraburkholderia* as well as *Phyllobacterium*. This primer set could however not amplify all putative rhizobial strains and therefore included another set: recA6F ('5 CGK CTS GTA GAG GAY AAA TCG GTG GA 3') and recA555R ('5-CGR ATC TGG TTG ATG AAG ATC ACC AT 3') (Gaunt et al., 2001). Primer set, recA6F and recA555R, amplified only five isolates 29ap1, 36ap3, 17a1p3, 34a2p5, 14a1p5 from the genus *Rhizobium*. Each *recA* PCR reaction included the same reagents (except for primers) and reagent volumes as used for the 16S rRNA reactions. PCR cycle conditions for both *recA* primer sets remained the same (Gaunt et al., 2001).

A third primer set, TSrecAf5 ('5 CAC TGC MYT GCG TAT YGT CGA AGG 3') and TSrecAr3 ('5 GAT CTT CAT SCG GAT CTG GTT GATG 3'), was used to amplify this locus in the remaining strains (since the initial amplification with the primer pairs recA6F/recA555R and recA63F/recA504R was unsuccessful) (Beukes et al. 2016). However, for these PCR reactions, the Fast Start High-Fidelity *Taq* and its $MgCl_2$ and buffer (Roche Diagnostics) were used. The cycling conditions were similar to those that were used previously by Beukes et al. (2016) except for the annealing temperature of 58 °C. The *recA* PCR products were cleaned, sequenced and the resulting sequences inspected and edited as described for the 16S rRNA (above). Homology searches were performed for the consensus sequences using *blastn* on the GenBank database National Centre for Biotechnology Information (NCBI) data library (http://blast.ncbi.nlm.nih.gov/) using *blastn* (Benson et al., 2015).

4.2.4 Phylogenetic dataset analyses and construction

A separate dataset was constructed for each of the four genera as well as for the individual loci. The type strain information necessary to construct the datasets was obtained from the List of Prokaryotic Names with Standing in Nomenclature (LPSN; https://www.bacterio.net/; Euzéby, 1997; Parte, 2018, Parte 2020) and included in multiple

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sequence alignment files together with the pigeonpea rhizobial sequences generated in this study. The accession numbers of the type strains are included in the phylogenies in brackets next to each type strain.

Sanger sequencing of the forward and reverse sequences was conducted, with editing in Chromas Lite, followed thereafter by creating the full-length consensus sequences for each isolate in Bio Edit and adding the full-length sequences to generate a multiple sequence alignment of the specific gene in the respective BioEdit file. This was followed by aligning these sequences using the Q-INS-i strategy in Multiple Alignment with Fast Fourier Transform (MAFFT version 7) for the 16Sr RNA and manually aligning the *recA* datasets. The 16S rRNA datasets were aligned online using MAFFT version 7 after which they were loaded on Jmodel test 2.1.7. The Jmodel computes the likelihood analysis scores and processes the Akaike information criterion as well as select the best model and parameters for the datasets. A text file was generated containing model instructions for use on PhyML 3.0 online. For *Paraburkholderia rec*A datasets, the model GTR+I+G was used with the proportion invariable site of 0.5030 and gamma shape of 0.8300 (Table 4.1)

The PhyML 3.0 (converted) dataset, was submitted online for analysis and produced a nerwick file that generates the maximum likelihood analysis using 1000 replicates to obtain bootstrap support values. The nerwick file also contains the generated phylogenetic tree for each genus as a nerwick tree, which was constructed, viewed on Mega 7 and edited.

Table 4.1 The settings in the online PhyML 3.0 software for the rhizobial genera

Genus	Dataset	Model	Proportion invariable site value	Gamma shape value	Bootstrap value
Paraburkholderia	*recA*	GTR+I+G	0.5030	0.8300	1000
	16S	GTR+I+G	0.8070	0.5430	1000
Bradyrhizobium	*recA*	GTR+I+G	0.4986	0.6930	1000
	16S	GTR+I+G	0.5800	0.7800	1000
Rhizobium	*recA*	GTR+I+G	0.4310	0.8190	1000
	16S	GTR+I+G	0.5900	0.3500	1000

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4.3 Results

4.3.1 DNA extraction and primer selection

DNA was extracted from 280 putative isolates trapped from pigeonpea. The 16S rRNA primers, 27F (5’ AGA GTT TGA TCC TGG CTC AG 3’) and 1485R (5’ TAC CTT GTT ACG ACT TCA CCC CA 3’) was used for the primary identification on these isolates to genus level (Lane, 1991). The primers for 16S rRNA (27F and 1485R) could amplify all the confirmed rhizobial isolates (see below) while the remainder of the putative isolates that were not amplified were likely related to be plant growth promoting rhizobacteria such as *Bacillus*, *Azospirillum*, *Pseudomonas, Paenibacillus* and *Agrobacterium*.

4.3.2 16S rRNA PCR amplification and sequencing

The 16S rRNA PCR products were sequenced in both directions using the same 16S rRNA primers resulting in 56 rhizobial isolates of which the majority (>70.0%) belonged to the genus *Rhizobium* (Table 4.2). The remainder belonged to three other genera, namely *Paraburkholderia*, *Phyllobacterium* and *Bradyrhizobium* (Fig. 4.1). However, there was a single rhizobial isolate that was identified for each of the two genera (*Paraburkholderia* and *Phyllobacterium*) while the remainder (14) were identified as *Bradyrhizobium*.

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Table 4.2 The rhizobial isolates that were amplified using the 16S rRNA primers

Entry #	Isolate	Genus	Accession number	Sequence size (bp)
1	10ap3	*Rhizobium*	OK376715	1393
2	30bp3	*Rhizobium*	OK384644	1391
3	37ap4	*Rhizobium*	OP013029	931
4	29a1p2	*Rhizobium*	OK384642	771
5	23ap5	*Rhizobium*	OK384609	1397
6	18ap3	*Rhizobium*	OK384614	921
7	17ap1	*Rhizobium*	OK377268	1386
8	29ap1	*Rhizobium*	OK384643	805
9	39a3p3	*Rhizobium*	OP013028	794
10	17a1p3	*Rhizobium*	OK377065	920
11	5b2p1	*Rhizobium*	OK376606	1470
12	35ap5	*Rhizobium*	OK384675	1394
13	35ap3	*Rhizobium*	OK384671	854
14	35bp1	*Rhizobium*	OK384700	1389
15	32b2p5	*Rhizobium*	OK384673	1392
16	32b1p5	*Rhizobium*	OK384666	1395
17	26a2p5	*Rhizobium*	OK384615	1393
18	15ap1	*Rhizobium*	OK377037	1393
19	31b1p5	*Rhizobium*	OK384652	1396
20	13b1p4	*Rhizobium*	OP013027	807
21	14a1p5	*Rhizobium*	OP013030	814
22	8b2p1	*Rhizobium*	OK376646	1395
23	31b1p3	*Rhizobium*	OK384650	1397
24	31b2p3	*Rhizobium*	OK384662	1398
25	16a2p1	*Rhizobium*	OK377114	878
26	26bp3	*Rhizobium*	OK384638	1387
27	36ap5	*Rhizobium*	OK384704	853
28	22ap5	*Rhizobium*	OK384604	655
29	5ap4	*Bradyrhizobium*	OP010913	808
30	8a2p3	*Bradyrhizobium*	OK392643	1391
31	27b2p5	*Bradyrhizobium*	OK393634	1390
32	11a2p3	*Bradyrhizobium*	OP010914	921
33	7a2p3	*Bradyrhizobium*	OK392633	827
34	15bp3	*Bradyrhizobium*	OP010916	947
35	19bp5	*Bradyrhizobium*	OK393635	1401
36	15bp5	*Bradyrhizobium*	OP010918	926
37	33ap4	*Bradyrhizobium*	OK393623	854
28	11b2p5	*Bradyrhizobium*	OP010915	810
39	13bp3	*Bradyrhizobium*	OK392642	502
40	19a1p3	*Bradyrhizobium*	OP010917	745
41	30a2p3	*Paraburkholderia*	OK393667	1434
42	31bp4	*Phyllobacterium*	-	798
43	32a1p2	*Rhizobium*	-	967
44	29a2p2	*Rhizobium*	-	435
45	15ap3	*Rhizobium*	-	888
46	16a2p3	*Bradyrhizobium*	-	827
47	6bp3	*Bradyrhizobium*	-	806
48	18bp5	*Rhizobium*	-	551
49	38a1p5	*Rhizobium*	-	1046
50	34a2p5	*Rhizobium*	-	563
51	27ap1	*Rhizobium*	-	966
52	32bp2	*Rhizobium*	-	688
53	31b3p3	*Rhizobium*	-	711
54	30b2p2	*Rhizobium*	-	705
55	14bp5	*Rhizobium*	-	679
56	22ap1	*Rhizobium*	-	926

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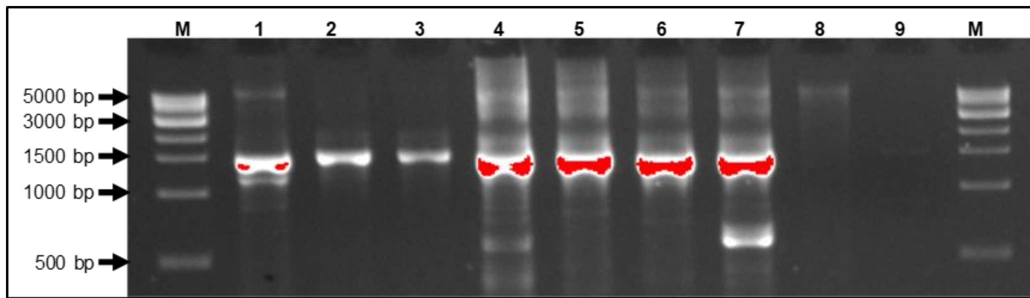

Fig.4.1 The gel of 16S rRNA PCR gel. M = 1kb DNA ladder / marker; lane 1 = *Paraburkholderia* sp (isolate '30a2p3'); lane 2 = *Rhizobium* sp (isolate '34a2p5'); lane 3 = *Phyllobacterium* sp (isolate '31bp4'); lane 4 = *Bradyrhizobium* sp (isolate '33ap4'); lane 5 = (*Bradyrhizobium* sp (isolate '11a2p3'); lane 6 = *Bradyrhizobium* sp (isolate '19bp5'); lane 7 = (*Bradyrhizobium* sp (isolate '15bp5'); lane 8 = *Rhizobium* sp (isolate '37ap4'); lane 9 = *Rhizobium* sp (isolate '36ap3').

4.3.3 *recA* PCR primer selection, amplification and sequencing

The primer set TSrecAf5 ('5 CAC TGC MYT GCG TAT YGT CGA AGG 3') and TSrecAr3 ('5 GAT CTT CAT SCG GAT CTG GTT GATG 3') successfully amplifying *Phyllobacterium* strain (31bp4), *Rhizobium* strain (30bp3) as well as *Bradyrhizobium* strains (16a2p3, 15bp3, 11a2p3, 13bp3, 33ap4 and 19a1p3) (Beukes et al., 2016). In addition, the *recA* primer also amplified the *Phyllobacterium* isolate (Fig. 4.2).

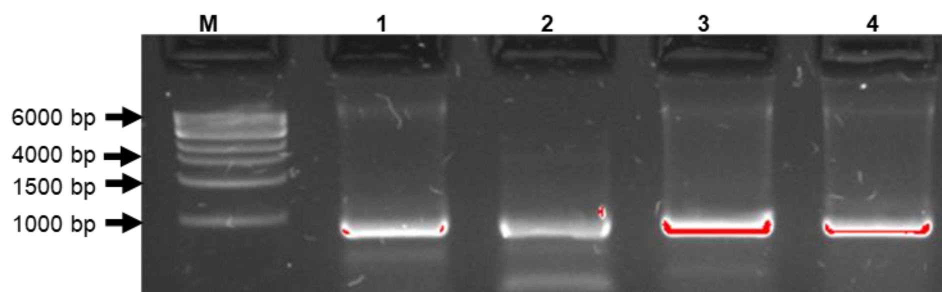

Figure 4.2 The gel of *recA* PCR amplified with primers 63F and 504R. M=1kb DNA ladder / marker, lane 1 = *Paraburkholderia* sp (isolate '30a2p3'); lane 2 = *Rhizobium* sp (isolate '34a2p5'); lane 3 = *Phyllobacterium* sp (isolate '31bp4'); lane 4 = *Bradyrhizobium* sp (isolate '33ap4').

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4.3.4 Variation in gene sequence size

There was considerable variation in the size of the sequences of both the 16S rRNA and *recA* genes among the rhizobial isolates. The longest (1470 bp) and shortest (403 bp) sequences were observed for the 16S rRNA gene (genus *Rhizobium*, isolate '5b2p1') and *recA* gene (genus *Bradyrhizobium*, isolate '27b2p5'), respectively. The sequences of the 16S rRNA genes across the four genera averaged 1015.73 bp (Fig. 4.3). In contrast, the sequences of the *recA* genes averaged 471.19 bp (Table 4.3). Overall, the size of the sequences for the 16S rRNA gene were double the size of the sequences of the *recA* gene (Table 4.3).

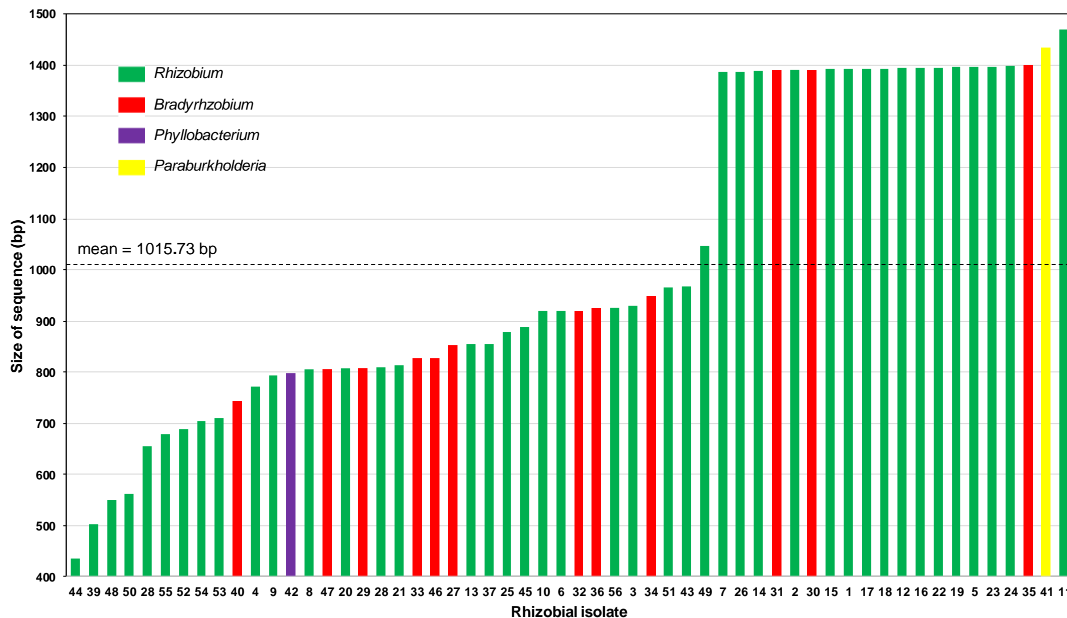

Fig.4.3 The distribution of the size of sequences of the 16 S rRNA gene across four genera of rhizobia.

Table 4.3 The size of the sequences for the16 S rRNA and *rec*A genes among the four genera of rhizobia that were used in the study.

Genus	Sequence size (bp)				
	n	16 S rRNA	n	*recA*	16 S rRNA / *rec*A
Rhizobium	40	1037.35	30	464.37	2.23
Bradyrhizobium	14	939.64	10	457.80	2.05
Paraburkholderia	1	1434.00	1	836.00	1.72
Phyllobacterium	1	798.00	1	445.00	1.79
Mean		1015.73		471.19	2.15

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4.3.5 Phylogenetic dataset analyses and construction

A separate phylogenetic diagram (or tree) for each of the three genera (*Rhizobium*, *Bradyrhizobium* and *Paraburkholderia*) using their respective sequence datasets was constructed. For the fourth genus only one isolate was identified as *Phyllobacterium* that was amplified successfully with *recA* primers (TSrecAf5 and TSrecAr3) designed for *Bradyrhizobium* and the 27F 16S rRNA primers. The blast result for this isolate ('31bp4') indicated that it was closely related to *Phyllobacterium* sp KW15 and *Phyllobacterium pellucidum* at 93% similarity. In addition, the EZ Biocloud showed that the isolate ('31bp4') was related to *Phyllobacterium* strain JMLL_s at 90.80% similarity grouping; hence, it was not included in a *Phyllobacterium* phylogeny.

4.3.5.1 Relationship of the isolates in *Rhizobium*

The 16S rRNA *Rhizobium* phylogenetic tree showed that the rhizobial isolates obtained from pigeonpea were dispersed in six different clusters (Table 4.4). The isolates grouped with several type species of the genus *Rhizobium* (Fig.4.4). Cluster I consisted of 5 isolates that grouped with the *Rhizobium* type strain *R. tropici* with a high (77.0%) similarity grouping (or bootstrap support). The second and third clusters consisted of three isolates while Cluster V contained the highest (8) number of the newly sequenced isolates including isolates '35ap3', '35bp1', '32b2p5', and '32b1p5' (Fig.4.4). The phylogenetic tree for the *recA* sequenced isolates consisted of five clusters over which 25 newly sequenced isolates were distributed (Fig. 4.5). Cluster V and Cluster III possessed the highest (7) and lowest (5) newly sequenced isolates, respectively (Table 4.4). In Cluster II, three isolates ('30bp3', '10ap3' and '32b2p'5) were grouped also with the type strain *R. tropici*.

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Table 4.4 Distribution of rhizobial strain (isolate) clusters for two genes of four distinct genera.

Genus	Cluster		16 S rRNA			*rec* A		
			Type / new strain	Number of new strains	Similarity grouping (%)	Type / new strain	Number of new strains	Similarity grouping (%)
Rhizobium	I	Start	*R. tropici* LMG 9503^T	5	77%	*R. leguminosarum* USDA 2370^T	5	71%
		End	***Rhizobium* sp. 23ap5**			***Rhizobium* sp. 31b2p3**		
	II	Start	***Rhizobium* sp. 18ap3**	3	96%	*R. halophytocola* YC6881^T	6	64%
		End	***Rhizobium* sp. 29ap1**			*R. flavescens* " FML-4^T		
	III	Start	*R. tumorigenes* 1078^T	3	<50%	***Rhizobium* sp. 17ap1**	3	<50%
		End	***Rhizobium* sp. 5b2p1**			*R. altiplani* BR 10423^T		
	IV	Start	*R. mayense* CCGE526^T	1	<50%	*R. yantingense* H66^T	4	<50%
		End	*R. vallis* CCBAU 65647^T			***Rhizobium* sp. 26a2p5**		
	V	Start	*R. metallidurans* ChimEc512^T	8	<50%	*R. rosettiformans* W3^T	7	<50%
		End	*R. populisoli* " XQZ8^T			*R. alvei* LMG 26895^T (KX938338)		
	VI	Start	*R. halophytocola* YC6881^T	7	<50%	N/A	N/A	N/A
		End	*R. terrae* NAU-18^T			N/A		
Bradyrhizobium	I	Start	*B. hipponense* aSej3^T	1	<50%	*B. agreste* CNPSo 4010^T HZZ13	0	N/A
		End	*B. huanghuaihaiense* CCBAU 233			*B. diversitatis* CNPSo 4019^T_H1B27		
	II	Start	*B. symbiodeficiens* 85S1MB^T	3	53%	*B. ingae* BR 10250^T	1	<50%
		End	***Bradyrhizobium* sp. 11a2p3**			*B. daqingense* CGMCC 1.10947^T_IQ17		
	III	Start	*B. agreste* CNPSo 4010^T	1	<50%	*B. stylosanthis* BR_446^T_A7X65	2	<50%
		End	"*B. centrolobii* "BR10245^T			*B. nanningense* CCBAU 53390^T_XH84		
	IV	Start	*B. algeriense* RST89^T	0	N/A	*B. hipponense* aSej3^T_FXV83	0	N/A
		End	*B. retamae* Ro19^T			*B. denitrificans* LMG 8443^T_HL671		
	V	Start	*B. tropiciagri* 6148^T	7	<50%	"*B.valentinum*" LmjM3^T_CP49	0	N/A
		End	***Bradyrhizobium* sp. 19a1p3**			*B. jicamae* PAC68^T_CQ12		
	VI	Start	N/A	N/A	N/A	*B. septentrionale* 1S1^T _HAP48	2	86%
		End	N/A			***Bradyrhizobium* sp. 13bp3**		
Paraburkholderia	I	Start	*P. heleia* SA41^T	0	N/A	*P. kirstenboschensis* Kb15^T	0	N/A
		End	*P. hiiakae* 12^T			*P. bryophila* LMG 23644^T		
	II	Start	*P. oxyphila* NBRC 105797^T	0	N/A	*P. terricola* LMG 20594^T	0	N/A
		End	*P. pallida* 7MH5^T			*P. phenazinium* LMG 2247^T		
	III	Start	*P. caballeronis* TNe-841^T	1	78%	"*P. telluris*" DHOC27^T	0	N/A
		End	*P. youngii* JPY169^T			"*P. gardini*" LMG 32171^T_R54767		
	IV	Start	*P. panacihumi* D-CY115^T	0	N/A	*P. humisilvae* Y-12^T	1	93%
		End	*P. pallidirosea* DHOK13^T			P. megapolitana LMG 23650^T		
	V	Start	*P. insulsa* PNG-April^T	0	N/A	*P.monticola* JC2948^T_CI15	0	N/A
		End	*P. strydomiana* WK1.1f^T			"*P. atlantica*" CNPSo 3155^T_GCT19		
	VI	Start	*P. caribensis* CIP 106784^T	0	N/A	*P. phosphatilytica* 7QSK02^T_DVS29	0	N/A
		End	"*P. hayleyella*" BhQS11^T			*P. silviterrae* 4M-K11^T_EYW47		

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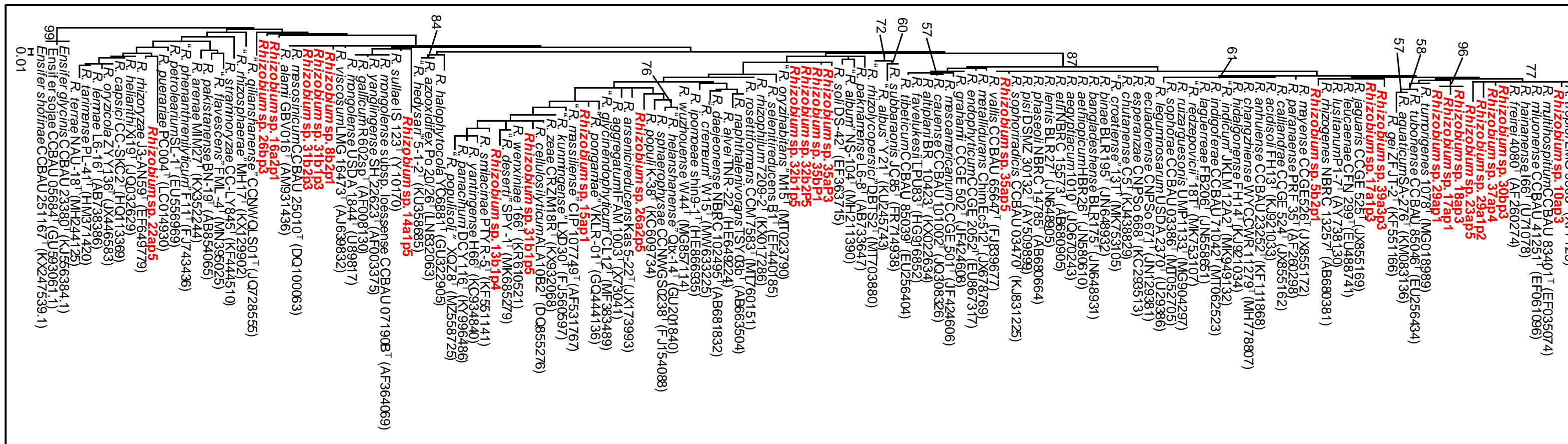

Fig. 4.4 A 16S rRNA maximum-likelihood phylogeny of *Rhizobium* isolates associated with pigeonpea. Bootstrap support for the groupings above ≥ 50% is indicated. Information regarding the type strain is provided while the gene bank accession number or locus tag for each species is indicated in brackets. Species names which appear in inverted commas (“..”) are combinations which have not yet been validly published. The scale bar corresponds to the number of nucleotide changes per site.

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Fig. 4.5 A *rec*A maximum-likelihood phylogeny of *Rhizobium* isolates associated with pigeonpea. Bootstrap support for the groupings above ≥ 50% is indicated. Information regarding the type strain is provided while the gene bank accession number or locus tag for each species is indicated in brackets. Species names which appear in inverted commas (“..”) are combinations which have not yet been validly published. The scale bar corresponds to the number of nucleotide changes per site.

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4.3.5.2 Relationship of the isolates in *Bradyrhizobium*

The 16S rRNA *Bradyrhizobium* phylogenetic tree carried only 12 new isolates (Table 4.4). Both the first and third clusters contained a single new isolate each while Cluster V carried the highest (7) new isolates which were grouped together with the reference strain *B. elkanii* (Fig. 4.6). Two isolates in Cluster II, namely '27b2p5' and '11a2p3', were grouped with the type strain *B. cajani* among others (Fig. 4.6). The *recA* phylogenetic relationship in the genus *Bradyrhizobium* indicated that of the five new isolates, there were two isolates in each of two clusters (II and VI) while the remaining isolate ('7a2p3') was grouped in Cluster II with the reference strain *B. arachidis* (Fig. 4.7).

4.3.5.3 Relationship of the isolates in *Paraburkholderia*

The16S rRNA *Paraburkholderia* rhizobial isolate '30a2p3' which was identified in this study, grouped with several type strains including *P. rhizosphaerae* among others with a similarity grouping >50% (Fig. 4.8). In the *recA* phylogenetic tree (Fig. 4.9), the same isolate was grouped in Cluster IV with 93.0% similarity grouping (Table 4.4). The isolate was also grouped with *P. rhizozphaerae* as in the previous (16S rRNA) phylogenetic test for this genus.

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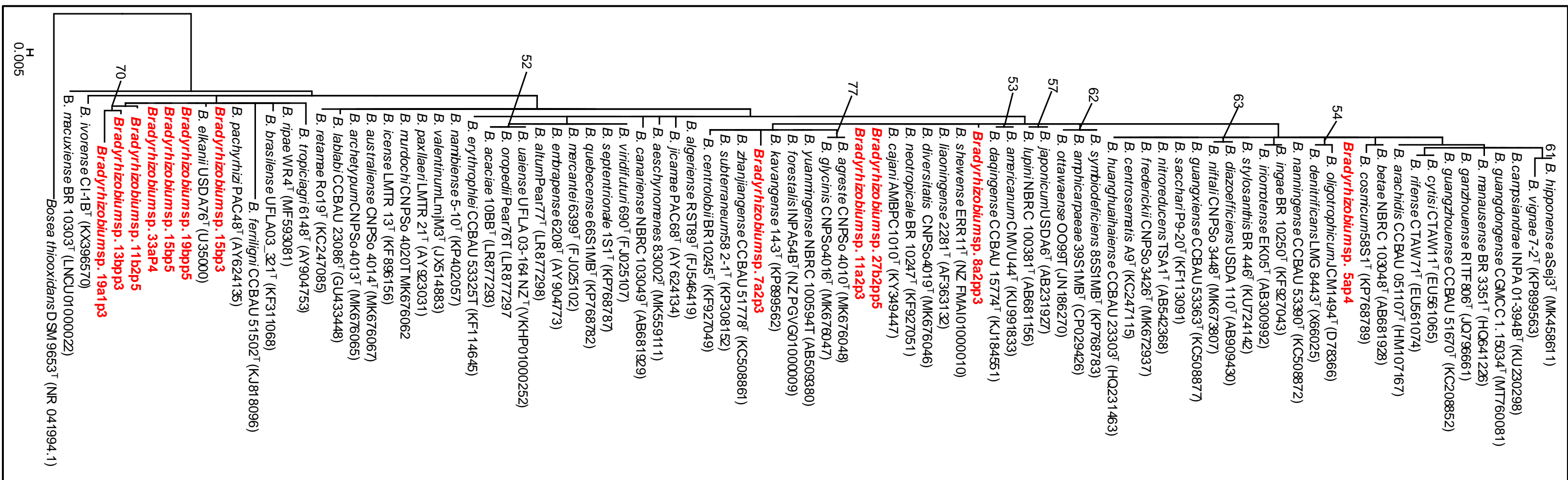

Fig. 4.6 A 16S rRNA maximum-likelihood phylogeny of *Bradyrhizobium* isolates associated with pigeonpea. Bootstrap support for the groupings above ≥ 50% is indicated. Information regarding the type strain is provided while the gene bank accession number or locus tag for each species is indicated in brackets. Species names which appear in inverted commas ("..") are combinations which have not yet been validly published. The scale bar corresponds to the number of nucleotide changes per site.

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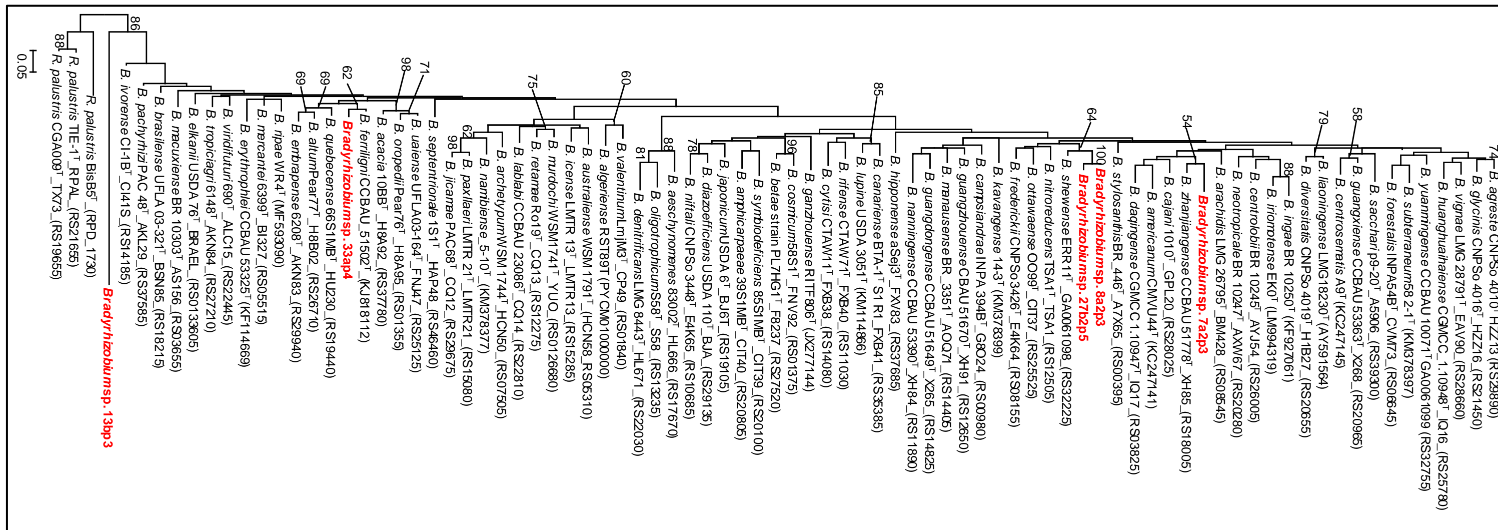

Fig. 4.7 A *rec*A maximum-likelihood phylogeny of *Bradyrhizobium* isolates associated with pigeonpea. Bootstrap support for the groupings above ≥ 50% is indicated. Information regarding the type strain is provided while the gene bank accession number or locus tag for each species is indicated in brackets. Species names which appear in inverted commas (“..”) are combinations which have not yet been validly published. The scale bar corresponds to the number of nucleotide changes per site.

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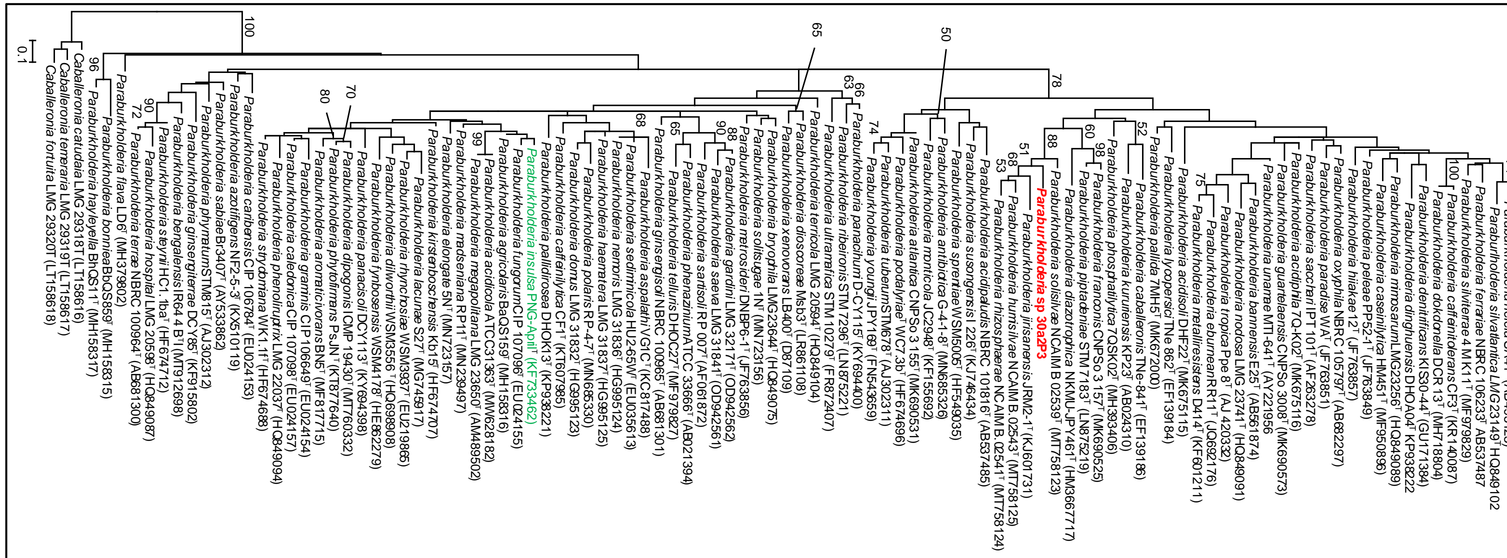

Fig. 4.8 A *16S* rRNA maximum-likelihood phylogeny of *Paraburkholderia* isolates associated with pigeonpea. Bootstrap support for the groupings above ≥ 50% is indicated. Information regarding the type strain is provided while the gene bank accession number or locus tag for each species is indicated in brackets. Species names which appear in inverted commas (“..”) are combinations which have not yet been validly published. The scale bar corresponds to the number of nucleotide changes per site.

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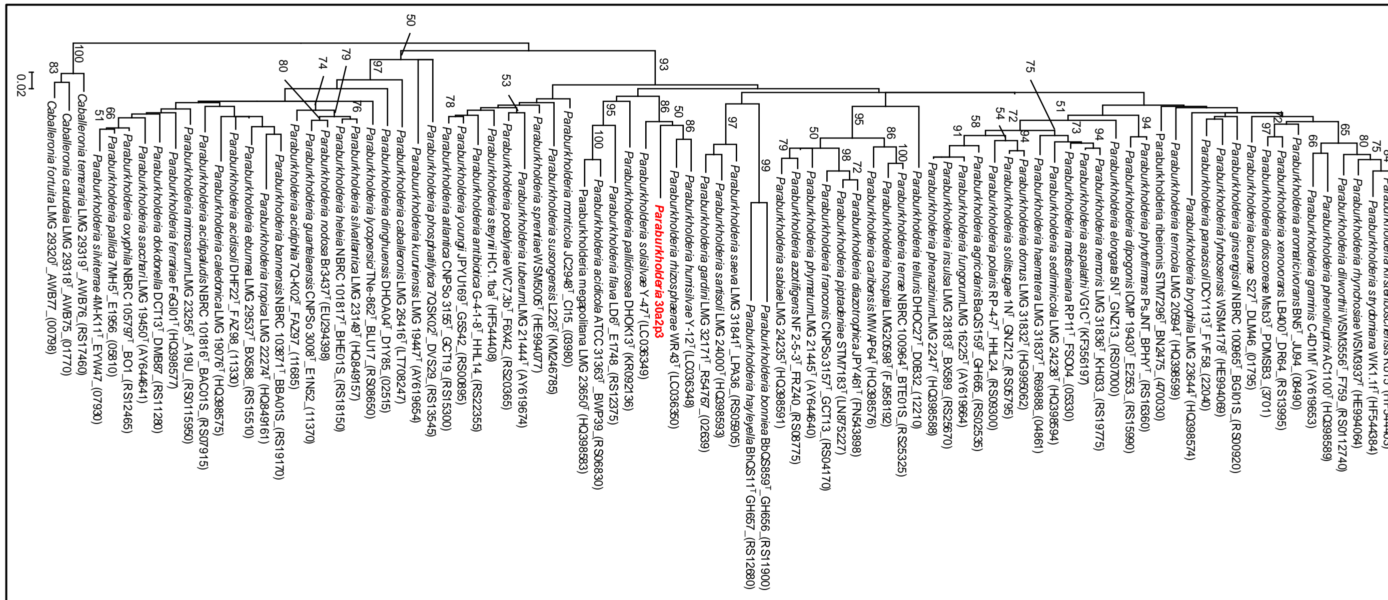

Fig. 4.9 A *rec*A maximum-likelihood phylogeny of *Paraburkholderia* isolates associated with pigeonpea. Bootstrap support for the groupings above ≥ 50% is indicated. Information regarding the type strain is provided while the gene bank accession number or locus tag for each species is indicated in brackets. Species names which appear in inverted commas ("..") are combinations which have not yet been validly published. The scale bar corresponds to the number of nucleotide changes per site.

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4.4 Discussion

The results revealed that diverse bacteria from both the α- and β-proteobacteria occupy the root nodules of pigeonpea. Among the 280 isolates collected from trapping experiments, only 56 were confirmed as rhizobia, with 55 isolates belonging to three α-rhizobial genera, *Rhizobium, Bradyrhizobium* and *Phyllobacterium* and one isolate identified as β-rhizobium from the genus *Paraburkholderia*. Pigeonpea can nodulate successfully a with a diverse group of rhizobia in South African soils. Moreover, other non-rhizobial bacteria that were also isolated and partially identified with 16S rRNA likely accounted for the remainder of the putative isolates. These isolates were mostly related to *Bacillus, Pseudomonas, Burkholderia, Paenibacillus* and *Agrobacterium*, which have no documented history of root nodulation (Mrabet et al., 2006). These genera are known as endophytes and plant growth promoting rhizobacteria (PGPR). The endophytes and PGPR were identified previous by other authors occupying plant tissues. The endophytes were also isolated from pigeonpea nodules that had been coinoculated with *Rhizobium* species and benefited the host plants as they assisted in plant growth promotion by increasing plant fresh weight, chlorophyll content and nodule number (Rajendran et al., 2008, Dhole 2016). Another recent study identified endophytes in nodules of groundnut improving plant growth (Dhole, 2016). A similar study involving coinoculation of *Burkholderia* and rhizobia on *Medicago sativa* increased the number of nodules and plant biomass of *Medicago sativa* (Hassen et al., 2021). Therefore, PGPR most probably contribute indirectly to the growth of the pigeonpea plants.

The *Rhizobium* and *Bradyrhizobium* genera appear to be common symbionts of pigeonpea as most of the isolates obtained from pigeonpea nodules were members of the α-rhizobial genera *Rhizobium* (39 isolates) and *Bradyrhizobium* (16 isolates). On the *Bradyrhizobium* phylogenetic tree of the current study, *B. elkanii* dominated the sample set, suggesting that the this genus commonly associates with pigeonpea, which is in agreement with previous observations that were reported elsewhere. For instance, the ability of nodulation of pigeonpea by indigenous isolates (mostly α-rhizobial bacteria) belonging to *B. japonicum* and *B. elkanii* in Ethiopia was reported (Degefu et al., 2018). In another study involving pigeonpea, the root nodulation was attributed to different subgroups of rhizobia (Mapfumo et al., 2000).

Perhaps more importantly was that in addition to these two genera that are commonly associated with root nodulation in pigeonpea, the current study discovered two new genera associating with pigeonpea, namely, *Phyllobacterium* and *Paraburkholderia* for the first time in South Africa. We can therefore deduce that pigeonpea is promiscuous and is nodulated by several genera of rhizobia. It will be interesting to evaluate the symbiotic effectiveness of the isolates of these new genera on other tropical legumes. Ideally, more than one isolate of each

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genus will be desirable in such future studies aimed at determining the symbiotic efficiency and development of commercial inoculants.

The results of the current study also demonstrated that molecular techniques or sequencing of the 16S rRNA provided more clarity on the identity of soil rhizobia in comparison with phenotypic methods that can differentiate between slow and fast growing isolates but no further capability to identify the genus or species of the isolate. Moreover, the molecular techniques could determine the phylogenetic relationships of the isolates identifying both their genera and the species. Furthermore, the techniques were also able to demonstrate considerable variation in the size of the sequences of both the 16 S rRNA and *rec*A genes among the rhizobial isolates and affirm that the size of the sequences of the former were larger than those of the latter. The differences in size between the two genes was consistent with previous reports. For instance, the fragment size for *Paraburkholderia rec*A gene was between 800 and 869bp using BUR1 and BUR2 primers for PCR amplification but *the alpha rhizobia (Bradyrhizobium, Rhizobium, Mesorhizobium and Sinorhizobium)*, about 600bp region of recA was amplified with *rec*A41F and *rec*A640R (Payne et al., 2005, Beukes et al., 2013, Beukes et al., 2016, Beukes et al., 2019). In a similar study, the PCR amplification for *rec*A using 63F and 504R produced about 441bp for rhizobial species (Guant et al., 2001), which was of the same size range obtained for the genera *Bradyrhizobium, Rhizobium and Phyllobacterium* in this study. The full length 16S rRNA gene (approx. 1400-1490bp) was obtainable when amplified with 16S-F primers and achievable also on the genera *Rhizobium, Bradyrhizobium, Mesorhizobium and Sinorhizobium* (Lane 1991; Beukes et al., 2019; Beukes et al., 2016). Nonetheless, one of the drawbacks in the molecular approach was associated with failure to amplify the DNA of some isolates or production of short sequences that could not be aligned appropriately for determining their phylogenetic positions. However, these limitations are clearly outweighed by the advantages of applying these molecular techniques which were utilized in this study. While only two genes per isolate were sequenced in this study, single gene sequences of 16S rDNA were used successfully to identify pigeonpea rhizobia belonging to two genera, namely *Agrobacterium* and *Rhizobium* (Singn et al., 2018). A wide diversity of rhizobia that associate with pigeonpea and other tropical legumes has the potential to enhance the development of biofertilizers (or commercial inoculants) that improve the crop productivity thus benefitting growers. In this regard, the molecular techniques in combination with the agronomic evaluation of the rhizobial isolates may present opportunities for accurate selection of rhizobial isolates that produce effective root nodulation of legumes for utilization particularly in agro-ecological environments where the indigenous rhizobial populations are depleted or fail to produce optimum symbiotic N fixation (Bopape et al., 2021).

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4.5 Conclusions and recommendations

The study concluded that the sequences of the two genes (16S rRNA and *rec*A) of the rhizobial isolates from pigeonpea could provide sufficient phylogenetic information about the isolates to the species level. The newly sequenced isolates from pigeonpea were phylogenetically grouped with several type strains such as *B. cajani*, *P. rhizozphaerae* and *R. tropicii.* There was considerable variation in size within each genus as well as between the genera for the two genes particularly among the isolates from the *Bradyrhizobium* and *Rhizobium* genera. Overall, the *rec*A genes were smaller that the 16S rRNA genes among the rhizobial isolates. Apart from the previously identified two genera of rhizobia (*Bradyrhizobium* and *Rhizobium*), the study discovered two new genera (*Paraburkholderia* and *Phyllobacterium*) that also associate with root nodulation in pigeonpea, thus confirming that this legume is promiscuous.

While the results of this study demonstrate the sizes of both the 16S rRNA and *rec*A genes among the rhizobial isolates that are associated with pigeonpea, the genome size of the isolates was unclear and beyond the scope of this Chapter. Therefore, there could be merit in sequencing the whole genome of the rhizobial isolates. In the same regard, a comparative study of the symbiotic efficiencies with other tropical legumes involving the four distinct genera that were reported in this study could provide opportunities for selecting the isolates that provide optimum productivity for legume growers particularly in agro-ecological environments where the indigenous rhizobial populations are depleted.

References

Abaidoo RC, Dashiell KE, Sanginga N, Keyser HH, Singleton PW (1999). Time course of dinitrogen fixation of promiscuous soybean cultivars measured by the isotope dilution method. *Biology and Fertility of Soils*. 30: 187-192.

Bopape FL, Gwata ET, Hassen AI, Zhou MM (2021). Symbiotic efficiency of pigeonpea (*Cajanus cajan*) with different sources of nitrogen. *Plant Genetic Resources*. 19(4)1-4.doi:10.1017/S1479262121000356

Ceapa C, Lambert J, Van Limpt K, Wels M, Smokvina T, Knol J, Kleerebezem M (2015). Correlation of *Lactobacillus rhamnosus* genotypes and carbohydrates utilization signatures determined by phenotype profiling. *Applied Environment and Microbiology.* 81 (16): 5458-5470.

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Clarridge III JE (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews*. 17 (4): 840-862.

Florentino LA, Guimaräes AA, da Silva JS, Rangel WWM, Jaramillo PMD, de Souza Moreira FM (2015). Genetic and phenotypic diversity of *Azorhizobium doebereinerae* strains. *Centro Cientifico Conhecer-Goianoia*. 11 (21): 1129-1139.

Hassen AI, Bopape FL, Trytsman M (2014). Nodulation study and characterization of rhizobial micro symbionts of forage and pasture legumes in South Africa*. World Journal of Agricultural Research.* 2 (3): 93-100.

Janda JM, Abott SL (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, pitfalls*. Journal of Clinical Microbiology.* 45 (9): 2761-2764

Ji ZJ, Yan H, Cui QC, Wang ET, Chen WF, Chen WX (2017). Competition between rhizobia under different environmental conditions affects the nodulation of a legume. *Systematic and Applied Microbiology*. 40: 114-119

Laguerre G, Nour SM, Macheret V, Sanjuan J, Droiun P, Amarger N (2001). Classification of rhizobia based on *nod*C and *nif*H gene analysis reveals a close phylogenetic relationship among *Phaseolus vulgaris* symbionts. *Journal of Microbiology*. 147: 981-993.

de Lajudie PM, Andrews M, Ardley J, Eardly B, Jumas-Bilak E, Kuzmanovic N, et al. (2019). Minimal standards for the description of new genera and species of rhizobia and agrobacteria. *International Journal Systematic Evolution Microbiology*. 69(7):1852-1863.doi: 10.1099/ijsem.0.003426.

Lindstrom K, Mousavi SA (2019). Effectiveness of nitrogen fixation in rhizobia. *Microbial Biotechnology.* 13: 1314-1335.

© University of Venda

Mahenthiralingam E, Bischof J, Byrne SK, Radomski C, Davies JE, Av-Gay Y, Vandamme P (2000). DNA-Based diagnostic approaches for identification of *Burkholderia cepacia* complex, *Burkholderia vietnamiensis, Burkholderia multivorans*, *Burkholderia stabilis* and *Burkholderia cepacia* genomovars I and III. *Journal of Clinical Microbiology*. 38:3165-3173.

Mendoza-Suárez M, Andersen SU, Poole PS, Sánchez-Caῆizares C (2021). Competition, nodule occupancy, and persistence of inoculant strains: Key factors in the Rhizobium-Legume Symbioses. *Frontiers in Plant Science*. 12 (690567): 1-26

Mousavi SA (2016). Revised taxonomy of the family *Rhizobiaceae*, and phylogeny of mesorhizobia nodulating *Glycyrrhiza* spp. *Dissertationes Schola Doctoralis Scientiae Circumiectalis, Alimentariae, Biologicae*

Onishchuk OP, Vorobyov NI, Provorov NA (2017). Nodulation competitiveness of nodule bacteria: genetic control and adaptive significance: review. *Applied Biochemistry Microbiology*. 53: 131-139. doi: 10.1134/S0003683817020132

Rocha EPC, Cornet E, Michel B (2005). Comparative and evolutionary analysis of the bacterial homologous recombination systems. *Genetics*. 1 (2): 0247-0259.

Saxena KB (2008). Genetic improvement of pigeonpea-a review. *Tropical Plant Biology.* 1: 159-178.

Thies JE, Bohlool BB, Singleton PW (1991). Subgroups of the Cowpea miscellany: Symbiotic Specificity within *Bradyrhizobium* spp. for *Vigna unguiculata*, *Phaseolus lunatus*, *Arachis hypogaea* and *Macroptilium atropurpureum*. *Applied Environmental Microbiology.* 57: 1540-1545.

Wier BS (2016). The current taxonomy of rhizobia. *NZ rhizobia website*. http://www.rhizobia.co.nz/taxonomy/rhizobia, accessed 02 June 2022

Yates RJ, Howieson JG,Reeve WG, O'Hara GW (2011). A reappraisal of the biology and terminology describing rhizobial strains success in nodule occupancy of legumes in agriculture. *Plant Soil*. 348: 255-267. doi: 10.1007/s11104-011-0971-z

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5.0 Chapter Five: Whole Genome Sequencing of a Rhizobial Strain Compatible with Pigeonpea

Abstract: The symbiotic fixation of atmospheric nitrogen (N) in root nodules of tropical legumes such as pigeonpea (*Cajanus cajan*) is a complex process, which is regulated by multiple genetic factors of both the host plant genotype and the micro-symbionts. The symbiotic process involves genes with various modes of action and accomplished only when both symbionts are compatible. Therefore, it is necessary to develop tools for the genetic manipulation of the host or bacteria in order to improve Nfixation efficiencies. This study focused on sequencing the whole genome of a selected rhizobial strain derived from pigeonpea and determine its genome size as well as identify some of the key genes in the strain. DNA was extracted from the rhizobial strain (or isolate) 10ap3 (SARCC-755) using Wizard genomic DNA extraction kit and DNA libraries were prepared using the Nextera protocol (Illumina, USA) and paired-end (300bp x 2) sequenced on a MiSeq (Illumina) sequencer at the Biotechnology Platform, Agricultural Research Council-Onderstepoort Campus, South Africa. The genome was a large circular chromosome (6 297 373 bp) and the overall G + C content of 60.0%. The total number of genes in *Rhizobium tropici* SARCC-755 genome was 6 013 of which 99.13% were coding sequences. However only 5 833 of the genes were associated with proteins that could be assigned to specific functions. Several important genes that were found on this genome, included the genes for nitrogen metabolism, stress response, phosphorus metabolism and iron acquisition and metabolism as well as adenosine monophosphate nucleoside for purine conversion, auxin biosynthesis genes for tryptophan synthase alpha and beta chains. The nodulation gene (*nolR*), which functions as a DNA binding transcription factor was located on contig 12. Precursor genes for purine synthesis inosine-5-monophosphate and adenylosuccinate, which are also responsible for nodule formation, were also present on the genome. The results showed that the genome of *Rhizobium tropici* SARCC-755 does not contain common *nod* and *nif* genes. The absence of the common *nodABC* nodulation genes suggested that an alternative pathway involving a purine derivative was involved in the symbiotic association with pigeonpea. The genome of the *R. tropici* SARCC-755 also possessed some genes that are associated with abiotic stresses and mineral nutrient acquisition thus making it a candidate for future formulation of commercial inoculants especially when considering its high symbiotic efficiency with pigeonpea.

Key words: Rhizobia; whole genome; *nod* genes; *nif* genes; purine.

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One publication emanated from this chapter as follows:

Bopape FL, Hassen AI, Swanevelder ZH, Gwata ET (2020). Draft genome sequence of *Rhizobium tropici* SARCC-755, a free-living *Rhizobium* that nodulated and promoted growth in pigeonpea [*Cajanus cajan* (L.) Millsp.] *Microbiology Resource Announcements*. 9: 1-2. doi.org/10.1128/MRA.01122-19.

5.1 Introduction

The symbiotic fixation of atmospheric nitrogen (N) in root nodules of tropical legumes such as pigeonpea (*Cajanus cajan*) is a complex process, which is regulated by multiple genetic factors of both the host plant genotype and the micro-symbionts. The symbiotic process involves genes with various modes of action and accomplished only when both symbionts are compatible. The initial step requires a mutual exchange of molecular signals prior to the formation of both the root nodule and symbiosome within which the rhizobia differentiate further into N fixing bacteriods (Wang et al., 2018; Jones et al., 2007; Oldroyd et al., 2011).

Apart from the genes that are involved in each of these steps, three additional genes control the symbiotic fixation of N in the rhizobia namely, *nod, nif* and *fix* (Table 5.1). The *nod* and *nif* proteins are encoded by accessory genes of bacteria that are housed in genetic elements (for instance, plasmids and chromids) that can be transmitted from one generation to the other. All the three genes are transferable horizontally in high frequencies within the species of bacterial species and infrequently between genera (Remigi et al., 2016). *The nifHDK* genes code for proteins that are associated with fixing atmospheric N. The rhizobial signal molecules (or Nod factors) called lipo-chito-oligosaccharides (LCOs) are encoded by a unique set of rhizobial genes called nod genes which induce host responses resulting in nodule formation (Broughton et al., 2003). On the other hand, the host plants secrete a variety of substances, which include flavonoids, and phenolic compounds into the rhizosphere, in conjunction with the bacterial activator protein *NodD* followed by the expression of rhizobial nodulation genes *nod*, *nol* and *noe* (Broughton et al., 2003).

Because of the phylogenetic diversity of both the rhizobia and host legume genotypes, there is no documented evidence of rhizobial strains that can form symbiosis with all the legumes and vice versa. In most cases, there is specificity, which occurs at both the species and genotypic levels (Wang et al., 2018; Broughton et al., 2000; Perret et al., 2000; Wang et al., 2012) at different stages. In addition, incompatibility can occur at a late stage of nodule

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development, resulting in significant reduction in the efficiency of the N fixation in a host plant x rhizobial strain combination (Wang et al., 2012; 2017; Yang et al., 2017). Therefore, it is necessary to develop tools for the genetic manipulation of the host or bacteria i to improve N fixation efficiencies. In this regard, knowledge of the genetic and molecular basis of the genetic material is essential. Specifically, the genome sizes of the different rhizobial species may provide some additional insights into the variation in symbiotic efficiencies of host plant genotype x rhizobial strain combinations.

Currently, the methods for the identification and sequencing of rhizobial genes routinely use the 16S rRNA and *recA* housekeeping genes (Janda and Abbott 2007; Rocha et al., 2005; Clarridge 2004). In some cases, additional housekeeping genes such as *atpD* encoding ATP synthase subunit beta (Beukes et al., 2016; Gaunt et al., 2001), *glnII* encoding glutamine synthetase isoform II, *dnaK* encoding the Hsp70 chaperone, *gyrB* encoding DNA gyrase subunit B and *rpoB* encoding RNA polymerase beta subunit (Beukes et al., 2016; Stępkowski et al., 2005), have been used. However, studies aimed at the elucidation of the whole genome in an organism such as a rhizobial strain, currently employ the next generation sequencing (NGS) method which enables fast, accurate molecular characterization of the organism of interest (Gautam et al., 2019; Kwong et al., 2015; Ng and Kirkness, 2010). The whole genome sequencing (WGS) method is a powerful tool for genomics research which can generate large volumes of data that require powerful algorithms that accurately determine the intrinsic genetic information such as the structural or copy number variations as well as their breakpoints and sizes (Kasugi et al., 2019). Such information is useful in understanding the functional properties of rhizobial genes. Therefore, the aim of this study was to profile the genes that are associated with a typical rhizobial strain that showed superior symbiotic efficiency with pigeonpea. The specific objectives of the study were to (i) sequence the whole genome of a selected rhizobial strain derived from pigeonpea (ii) determine the genome size of the rhizobial strain and (iii) identify some of the key genes in the rhizobial strain.

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Table 5.1 A list of the common rhizobial *nod*, *nif* and *fix* genes (*Sources:* Laranjo et al., 2014; Lindstrom and Mousavi, 2019)

Genes	Function of gene product
Nodulation genes	
nodA	Acyltransferase
nodB	Chitooligosaccharide deacetylase
nodC	N-acetylglucosaminyltransferase
nodD	Transcriptional regulator of common *nod* genes
nodIJ	Nod factor transport
nodPQ	Synthesis of Nod factor substituents
nodX	Synthesis of Nod factor substituents
nofEF	Synthesis of Nod factor substituents
Other *nod* genes	Several functions in synthesis of Nod factors
nol genes	Several functions in synthesis of Nod factor substituents and secretion
noe genes	Synthesis of Nod factos substituents
Nitrogen fixation genes	
nifH	Dinitrogenase reductase (Fe protein)
nifD	α subunits of dinitrogenase (MoFe protein)
nifK	β subunits of dinitrogenase (MoFe protein)
nifA	Transcriptional regulator of the other *nif* genes
nifBEN	Biosynthesis of the Fe-Mo cofactor
fixABCX	Electron transport chain to nitrogenase
fixNOPQ	Cytochrome oxidase
fixLJ	Transcriptional regulators
fixK	Transcriptional regulator
fixGHIS	Copper uptake and metabolism
fdxN	Ferredoxin

5.2 Materials and methods

5.2.1 Rhizobial strain used in the study and DNA extraction

The strain 10ap3 (SARCC-755) was revived by streaking on Yeast Mannitol agar (YMA) with Congo red and incubated at 28oC for 3 days. For DNA extraction, a single pure colony of the bacterium 10ap3 (SARCC-755) grown on YM agar was transferred to Tryptone Yeast (TY) broth medium and incubated for 3 days on a rotary shaker (150 rpm) at 28°C. A total of 1.5 ml of the broth culture was used to extract DNA using the Wizard genomic DNA extraction kit according to the manufacturer's instruction (Promega, Madison, WI, USA) for use in NGS. DNA concentration to 0.2 ng/ul was quantified by adjusting and diluting with a required volume of distilled water. For Nextera XT tagment amplicon construction, in a PCR

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tube 5 ul tagmentation DNA buffer and 2.5 ul amplification tagmentation were mixed with 2.5 ul input DNA (0.2 ng/ul). The samples were transferred to thermocycler, programmed for one-step at 55ºC for 5 min, with heated lid, followed by hold at 10ºC for a 10-ul volume. This was followed by neutralize NTA by adding 2.5 ul neutralization tagmentation buffer and incubation for 5 min at room temperature (Gautam et al., 2019).

5.2.2 Generation of DNA libraries, sample preparation and normalization

PCR amplification and clean up of the PCR product was similar to the protocol by Gautam et al (2019). DNA libraries were prepared using the Nextera protocol (Illumina, USA) and paired-end (300bp x 2) sequenced on a MiSeq (Illumina) sequencer at the Biotechnology Platform, Agricultural Research Council-Onderstepoort Campus, South Africa (Fig. 5.1). The sample preparation process included the addition of adaptors to the end of the DNA fragments. The sequencing binding sites such as indices and regions complementary to the flow cell oligonucleotides were introduced to the fragments, which allow the DNA to bind to the flow cell. The fragments are amplified and purified (Kwong et al., 2015).

5.2.3 Cluster generation

The libraries or samples are loaded on to the flow cell and placed on the sequencer. The flow cell is a glass slide with lanes each quoted with lawn like structure which compose of the two oligonucleotides. The clusters of DNA fragments are amplified in a process called cluster generation that results in millions of copies of single stranded DNA, which occur automatically on most Illumina sequencing instruments. Cluster generation initiate with hybridization, which is enabled by any of the two oligonucleotides on the surfaces of the flow cell and the polymerase creates a complement of a hybridized fragment.

The double stranded molecule is denatured and original molecule washed away. The strands are clonally amplified by a bridge amplification method, as strands fold over and adapters hybridize to the second oligonucleotide on the flow cell after which the polymerases generate complementary strands, forming a double strand bridge. The bridge is denatured, resulting in two single strand copies attached to the flow cell. Then the process is repeated simultaneously for millions of times, resulting in clonal amplification of all fragments. After bridge amplification, the reverse strand is cleaved and washed off, leaving only the forward strand. In addition, the 3’ -end is blocked to prevent unwanted priming (Illumina Sequencing by Synthesis, 2016**)**.

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5.2.4 Whole genome sequencing, data generation using Illumina platform

Pooled libraries were prepared for loading on MiSeq (Illumina) sequencer (Gautam et al., 2019) at the Biotechnology Platform, Agricultural Research Council (Onderstepoort, South Africa) for sequencing (Fig. 5.1). The illumina sequencer is the standard that is used internationally for read depth and base calling accuracy, genome coverage, scalability and the range of sequencing application it delivers (Koboldt, 2020; Gautam et al., 2019). Bioinformatics analyses were conducted by first selecting the bioinformatics software for the analysis of the WGS data using CLC Genomic Workbench version 8.5.1 (Gautam et al., 2019)

The sequencing starts with the extension of the first sequence primer to produce the first read. Fluorescence tagged nucleotides compete for attachment to the growing chain as only one nucleotide base is incorporated at a time, based on the sequence of the template. Once all the nucleotides are added, clusters are excited by a light source and then a fluorescence signal is emitted. This is called sequencing by synthesis. The fluorescent signal indicates which nucleotide has been added and the terminator is cleaved so that the next base can bind (Kwong et al., 2015). The number of cycles determines the length of reads, while emission wavelength, with signal intensity determines the base call. For any given cluster, all identical strands are read simultaneously. Millions of clusters are sequenced at the same time in a parallel process. On completion of the first read, a repeat with the reverse primer starts after which the read product is washed away culminating the hybridizing process to the template. A subsequent read is generated in a similar manner as the first read. This process generates millions of reads (products) and the forward and reverse sequences are paired to form continuous sequences that can be aligned back to the reference genome for variant identification, or resolving of ambiguous alignment or comparison with database sequences to those in the database (Illumina Sequencing by Synthesis, 2016).

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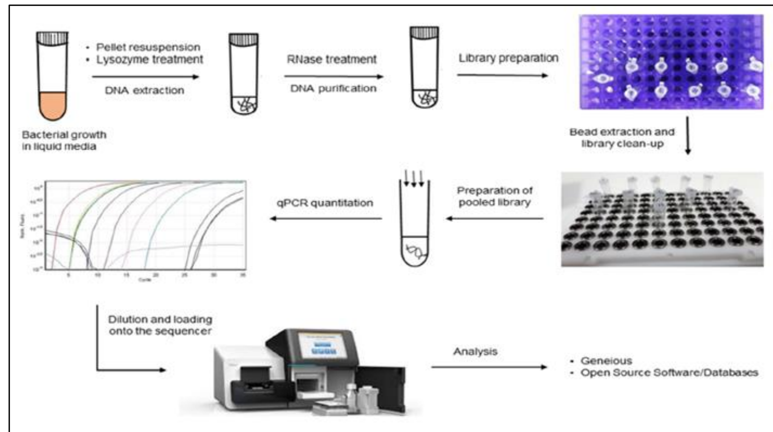

Fig. 5.1 Graphical summary of the process of obtaining whole genome sequence data from a bacterial culture (*Source:* Gautam et al., 2019).

5.2.5 Analysis of next generation sequencing (NGS) products

Once sequencing is complete, the software in the instrument identifies nucleotides in a process called base calling, and predicts the accuracy of those base callings. The tools in the software provide sequence alignment, variant calling, data visualization and interpretation (Ng and Kirkness 2010).

5.3 Results

5.3.1 Whole genome sequencing and size

The sequencing of the strain *Rhizobium tropici* SARCC-755 whole genome resulted in 10,364,436 raw sequence reads, which further resulted in 8,265,062 reads when trimming and adapter adding was complete. Upon successful sequencing of the whole genome of the strain, it was allocated the accession number VNIP00000000 and deposited into the DNA DataBank of Japan/European Nucleotide Archive/Gene bank at NCBI for further reference and uses (https://www.ncbi.nlm.nih.gov/search/all/?term=VNIP00000000, Bopape et al., 2020). The whole genome was a large chromosome with circular shape (Fig. 5.2) and the overall G + C content of 60.0%.

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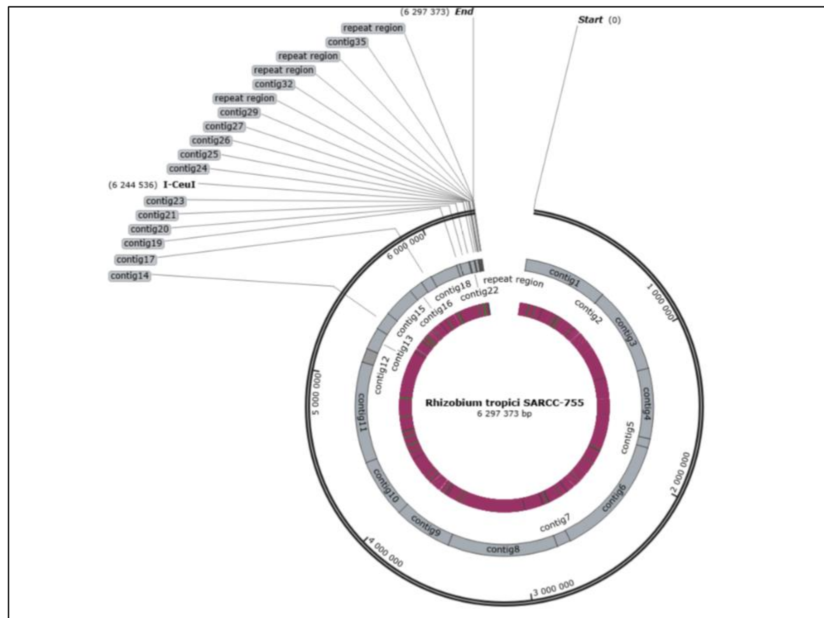

Fig. 5.2 Snap gene circular view of a 6.3 MB genome of *Rhizobium tropici* SARCC-755 showing all the contigs that resulted from the RAST annotation of the genome assembly.

The size of the whole genome of the SARCC-755 strain was 6 297 373 bp, which consisted of 36 contigs (including scaffolds), that were obtained after the assembly. The maximum scaffold size on the rhizobial strain's genome was 876 087 bp, consisting of several contigs each coding a protein. Furthermore, additional features were identified on the genome, including 45 tRNAs and 48 RNAs (Table 5.2). The number of contigs was 36 whereas the number of subsystems was 361 with an additional four ncRNAs, (Fig. 5.3).

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Table 5.2 The general features of *Rhizobium tropici* SARCC-755 genome.

Genome feature	*Rhizobium tropici* SARCC-755
Genome size (bp)	6 297 373
G + C content (%)	60
Ribosomal RNA operons	3
Transfer RNAs	45
Protein coding genes/ sequences	5 961
Assigned functionality (%)	5 833
Genes (total) (%)	6 013
CDSs (total) (%)	5 961
Genes (coding) (%)	5 833
CDSs (with protein) (%)	5 833
Number of plasmids	0
ncRNAs	4
Pseudogenes	128

bp = base pairs, CDS = coding sequences, G + C = guanine + cytosine, ncRNAs = non-coding Ribosomal Nucleic Acids, SARCC = South African *Rhizobium* Culture Collection

5.3.3 Key genes in the genome

The total number of genes in *Rhizobium tropici* SARCC-755 genome was 6 013 of which 99.13% were coding sequences (or protein-coding genes). However only 5 833 of the protein-coding genes had proteins that could be assigned to specific functions. The nodulation gene *nolR* (Fig. 5.4) was the only gene present on the genome, which functions as a DNA binding transcription factor. Several important genes that were found on this genome, included the genes for nitrogen metabolism, stress response, phosphorus metabolism and iron acquisition as well as some additional ones such as the siderophores aerobactin synthesis genes, inosine-5-monophosphate for purine conversion, adenosine monophosphate nucleoside for purine conversion, auxin biosynthesis genes for tryptophan synthase alpha and beta chains (Fig. 5.3 and Fig. 5.5). Precursor genes for purine synthesis inosine-5-monophosphate and adenylosuccinate were also present on the genome, which are also responsible for nodule formation (Fig. 5.5).

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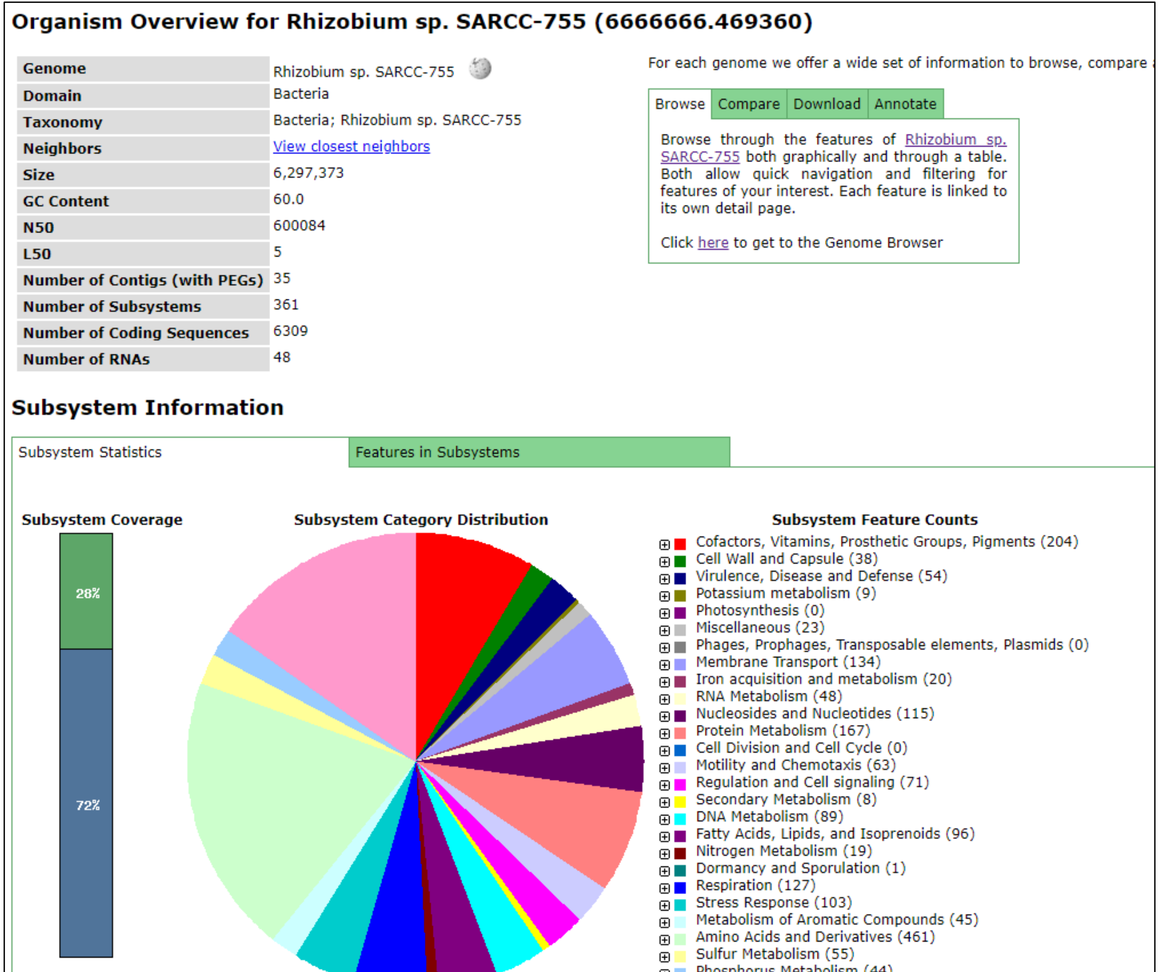

Fig. 5.3 Graphical representation of the functions coded on the whole genome of *Rhizobium tropici* (SARCC-755)

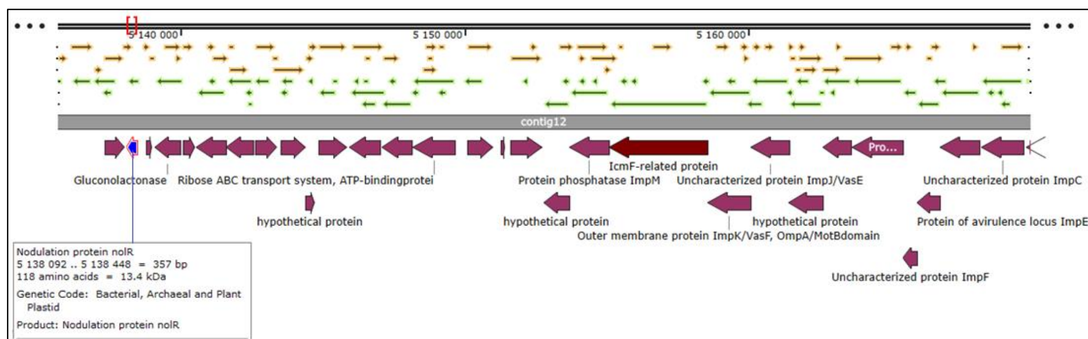

Fig. 5.4 Sections of the chromosomal region of *Rhizobium tropici* SARCC-755 showing Open reading frames (ORF), contigs and coding regions (CDs. The nodulation protein (***nolR***) located on contig 12.

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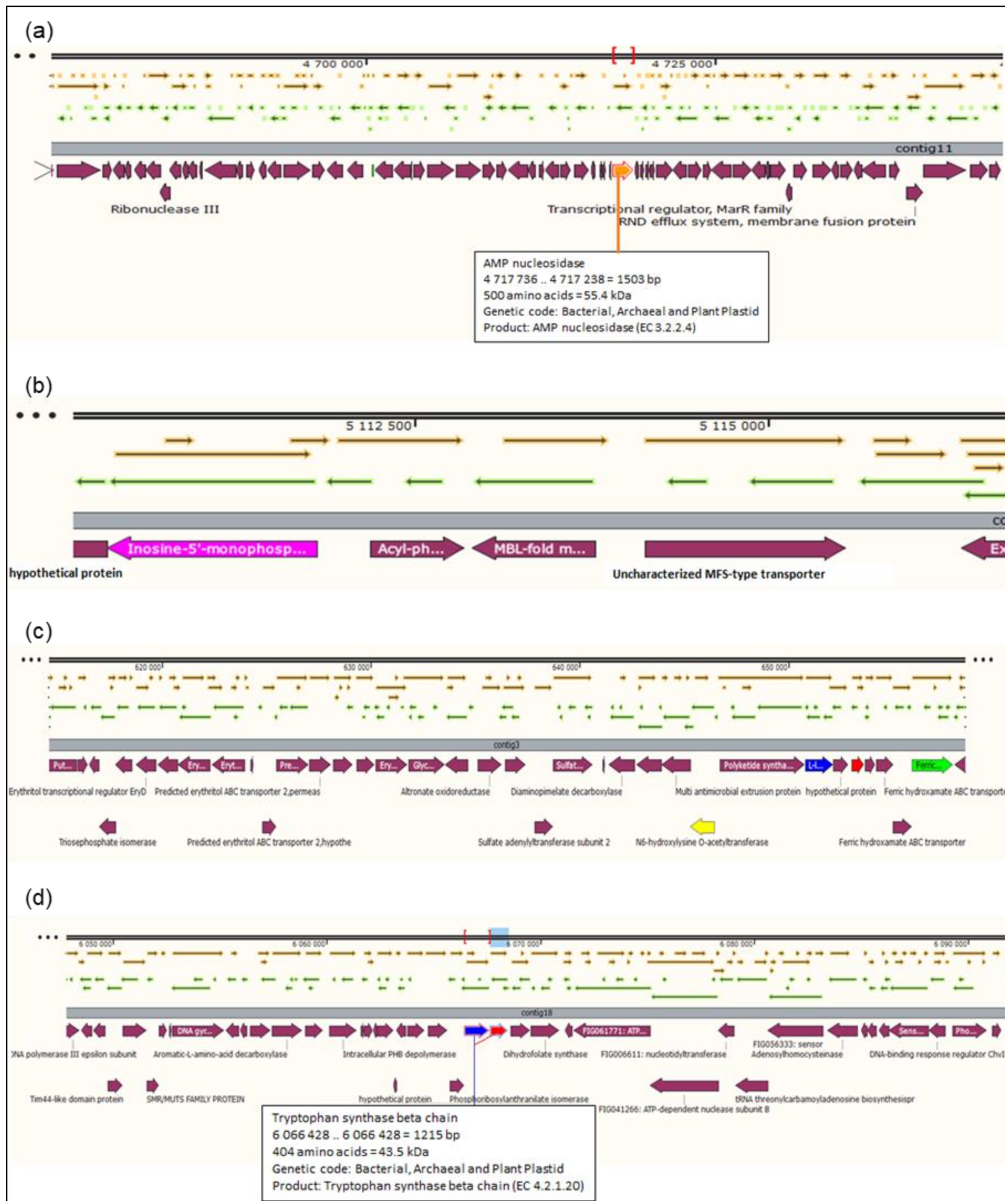

Fig. 5.5 Sections of the chromosomal region of *Rhizobium tropici* SARCC-755 showing open reading frames (ORFs), contigs and coding regions (CDs). Selected genes of interest are highlighted with different colours on the CDs just below the contigs. (a) adenosine monophosphate (AMP) nuclease, purine conversion located on contig 11 (b) inosine-5-monophosphate located on contig 11 (c) siderophores aerobactin synthesis genes located on contig 3 and (d) auxin biosynthesis genes, tryptophan synthase alpha and beta chains located on contig 18. (The chromosomal view was generated using Snap gene software, of Dotmatics, 2022 Insightful Science).

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5.4 Discussion

The results showed that the genome of *Rhizobium tropici* SARCC-755 does not contain common *nod* and *nif* genes. In comparison with similar studies, the full genomes of other strains of *R. tropici;* (for instance, CIAT899, CF286, PTD1, IGFRI Rhi19, YR635 and NFR14) indicated that strain CIAT899 contains most of the *nod* and *nif* genes (Ayala-Garcia et al., 2022). However, genomes of the remainder of the strains (CF286, PTD1, IGFRI Rhi19, YR635 and NFR14) shared only two common genes, namely the nod *nfeD* for amino deoxy chrorismate synthatase component I and *nif* gene *nifU* for multispecies family protein. In another similar study, involving photosynthetic *Bradyrhizobia* (strains BTAi1 and ORS278), the absence of *nod* genes was reported (Giraud et al., 2007). However, the *nifU* gene is constant in all *R. tropici* genomes. Therefore, it appears that some rhizobial genomes, including the *R. tropici* genome, lack the common *nod* and *nif* genes. Nonetheless, the genome of strain CIAT899 possessed at least 13 *nod* genes and 13 *nif* genes (Ormeño-Orrillo et al., 2012). Therefore, it is logical to deduce that in some legumes such as pigeonpea, canonical *nodABC* genes as well as typical lipo-chito-oligosaccharidic *Nod* factors (that are encoded by the *nodABC* genes) which subsequently bind to kinase-like receptors of the host plant are not necessary for symbiosis. This is partly because the *R. tropici* SARCC-755 demonstrated symbiotic efficiency with pigeonpea (Bopape et al., 2021). Secondly, some unique rhizobia can use alternative biochemical pathways to initiate symbiosis using purine derivatives to trigger the formation of nodules (Xie et al., 2009; Giraud et al., 2007).

The genome of strain SARCC-755 contained various genes and only one nodulation gene (*nolR*) that was located at contig 12 on the chromosome similar to *R. tropici* CIAT899, *Rhizobium* sp. PRF18 and *R. leguminosarum* (Ormeño-Orrillo et al., 2012). The genes that are required for purine biosynthesis that encode inosine-5-monophosphate, AMP and adenylosuccinate were also present on the chromosome of SARCC-755. The purine biosynthesis might be the alternative pathway used by SARCC-755 to initiate nodulation on pigeonpea as the common nod genes (*NodABC*) responsible for nodulation present on this chromosome (Bopape et al., 2020).

The genome size of *Rhizobium tropici* SARCC-755 (6.2 Mb) agreed with the findings that were reported previously for *R. tropici* CIAT899 (6.7 Mb) in a similar study (Ormeño-Orrillo et al., 2012). The overall G+C content in each of these genomes was approximately 60.0%, which was comparable with that for *R. leguminosarum viciae* strain 3841 at 61% (Bopape et al., 2020; Ormeño-Orrillo et al., 2012; Young et al., 2006). One of the possible explanations for this near-identical amount of G+C content is that the strains belong to the same genus (*Rhizobium*) and they are positioned next to each other on both the taxonomic (16S rRNA)

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and housekeeping (*recA*) phylogenies or are in the same cluster (Reeve et al., 2010) (see Chapter 4, section 4.2.6.1 of this study).

For the complete process of nitrogen fixation to occur, successful nodule formation by the rhizobia is necessary as the first step. There is a positive linear correlation between nodulation and the expression of the gene responsible for the purine synthesis. The amount of purine expressed determines the ability of the *Rhizobium* to form and occupy nodules. Other purine precursors such as inosine and AMP also promote nodulation (Xie et al., 2009), while adenine promotes strain competitive nodulation. This implies that the gene for purine biosynthesis pathway (*PurL*) can control competitive nodulation abilities of rhizobia by monitoring the accumulation of certain purine precursors. These same genes responsible for the purine pathway were also expressed in *Sinorhizobium fredii* during competitive nodulation (Xie et al., 2009). The presence of the genes that assist in oxidative stress and tolerance in the SARCC-755 genome, suggests that this strain can tolerate abiotic stresses such as soil moisture stress, high soil temperature and low soil pH, which are generally prevalent in most legume cropping systems in South Africa (Sindhu et a., 2020; Atieno and Lesueur 2019; Mabrouk et al., 2018; Grover et al., 2011). Likely, commercialization of such a rhizobial strain will benefit legume growers in situations where these abiotic stresses hamper optimum productivity of pigeonpea and similar tropical legumes. In future, there may be merit in evaluating the symbiotic efficiency of the SARCC-755 under a range of soil moisture stress conditions to determine the threshold at which the strain functions normally.

Apart from possessing the genes that are associated with abiotic stresses, the genome of SARCC-755 is equipped with a range of subsystem features that are responsible for various functions. The functions including iron acquisition and metabolism or siderophore aerobactin, which is responsible for iron chelator utilization protein and the ferric aerobactin ABC transporter (Delepelaire 2019; Brear et al., 2013; Geetha and Joshi 2013). Previous studies showed that iron is critical for the formation of bacterial biofilms since it controls surface activities and can stabilize the polysaccharide matrix (Chhibber et al., 2013). When there is iron deficiency, the hydrophobicity of the microbial surface diminishes significantly leading to alterations in the composition of the surface proteins, hence restrictions in the formation of biofilms (Simões et al., 2007). The siderophores are made up of low (600−1000 Da) molecular weight molecules that possess a high affinity for ferric ions (Fe^{3+}) but low affinity for ferrous ions (Fe^{2+}), that are produced by bacteria under iron deficiency conditions but their production is suppressed when iron is available. Moreover, siderophores can also complex with several other essential elements such as cobalt, manganese, molybdenum and nickel to avail these metals to rhizobia (or microbes) (Ahmed and Holmström, 2014). In general, iron in the rhizosphere is acquired by plants by acidification through proton extrusion or chelation by

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secreting complexing molecules (including siderophores, phenolics and carboxylic acids, among others) or the reduction process through secreting compounds that have reducing properties (or reductase activity) (Dubey and Maheshwari, 2011). Therefore, this rhizobial strain has potential as a bio-inoculant particularly in soils that are constrained by iron deficiency since it appears to have an ecological advantage for survival in the rhizosphere.

In comparison with the genomes of two other strains, namely, *B. japonicum* (USDA110) and *R. leguminosarum viciae* (strain 3841) that have one rRNA (Giraud et al., 2007) and three rRNAs (Young et al., 2006), respectively, the SARCC-755 genome also contained three rRNAs. However, the rRNA is a non-coding sequence, which is available and useful for cellular function. The RNA contains the genetic material and information that can be translated into proteins by ribosomes (Harrow et al., 2009). The SARCC-755 genome also revealed 45 tRNAs, which was comparable to the 52 tRNAs that were reported for *R. leguminosarum viciae* (Young et al., 2006). The role of these tRNAs is to transfer amino acids to the ribosome, which will produce peptides when instructed by mRNA.

5.5 Conclusions and recommendations

In conclusion, the successful sequencing of the *R. tropici* SARCC-755 genome revealed the absence of the common *nod*ABC nodulation genes suggesting that an alternative pathway involving a purine derivative was involved in the symbiotic association with pigeonpea. This observation was consistent with findings that were reported in other similar studies involving both the *Rhizobium* and *Bradyrhizobium* strains. The genome of the *R. tropici* SARCC-755 also possessed some genes that are associated with abiotic stresses and mineral nutrient acquisition thus making it a candidate for future formulation of commercial inoculants for pigeonpea. It will be interesting to perform the WGS of the novel rhizobial genera (*Paraburholderia* and *Phyllobacterium*) that were reported in this study (see Chapter 4 section 4.3.2). From a perspective of legume crop production, a comparison of the genome composition of these distinct genera might shed some light on the variation in symbiotic compatibility and efficiency with various legumes. Such new information might be useful in formulation of commercial inoculants in future.

References

Ayala-García P, Jimènez-Guerrero I; Jacott CN, Lȯpez-Baena J, Ollero FJ, Del Cerro P, Pèrez-Montaῃo F (2022). The *Rhizobium tropici* CIAT 899 *NodD2* protein promotes symbiosis

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and extends rhizobia nodulation range by constitutive nodulation factor synthesis. *Journal of Experimental Botany.* Pp 1-11. doi.org/10.1093/jxb/erac325

Ahmed E, Holmström SJM (2014). Siderophores in environmental research: roles and applications. *Microbial Biotechnology*. 7: 196-208.

Argaw A, Muleta D (2017). Effect of genotypes-*Rhizobium*-environment interaction on nodulation and productivity of common bean (*Phaseolus vulgaris* L.) in eastern Ethiopia. *Environmental Systems Research*. 6(14): 1-16.

Atieno M, Lesueur D (2019). Opportunities for improved legume inoculants: enhanced stress tolerance of rhizobia and benefits to agroecosystems. *Symbiosis*. 77: 191-205.

Beukes CW, Stępkowski T, Cłapa TC, Venter SN, Phalane FL, Le Roux MM, Steenkamp ET (2016). *Crotalarieae* and *Genisteae* of the South African great escarpment are nodulated by novel *Bradyrhizobium* species with unique and diverse symbiotic loci. *Molecular Phylogenetic and Evolution.* 100:206-218.

Bopape FL, Gwata ET, Hassen AI, Zhou MM (2021). Symbiotic efficiency of pigeonpea (*Cajanus cajan*) with different sources of nitrogen. *Plant Genetic Resources*. 19(4): 1-4.doi:10.1017/S1479262121000356.

Bopape FL, Hassen AI, Swanevelder ZH, Gwata ET (2020). Draft genome sequence of *Rhizobium tropici* SARCC-755, a free-living *Rhizobium* that nodulated and promoted growth in pigeonpea [*Cajanus cajan* (L.) Millsp.] *Microbiology Resource Announcements*. 9: 1-2 e01122-19. https://doi.org/10.1128/MRA.01122-19.

Brear EM, Day DA, Smith PMC (2013). Iron: an essential micronutrient for the legume-*Rhizobium* symbiosis. *Frontiers in Plant Science*. 4(359): 1-15.

Broughton WJ, Jabbouri S, Perret X (2000). Keys to symbiotic harmony. *Journal of Bacteriology*. 182: 5641–5652. doi: 10.1128/JB.182.20.5641-5652.2000.

© University of Venda

Broughton WJ, Zhang F, Perret X, Staehelin C (2003). Signal exchanged between legumes and *Rhizobium*: agricultural uses and perspectives. *Plant and Soil*. 252: 129-137.

Chhibber S, Nag D, Bansal S (2013). Inhibiting biofilm formation by *Klebsiella pneumoniae* B5055 using an iron antagonizing molecule and a bacteriophage. *BMC Microbiology* 13: 1-8.

Clarridge III JE (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews*. 17(4): 840-862.

Delepelaire P (2019). Bacterial ABC transporters of iron containing compounds. *Research in Microbiology*. 170: 345-357.

Desai A, Archana G. (2011). Role of Siderophores in Crop Improvement. *In*: D.K. Maheshwari (eds.), Bacteria in Agrobiology: Plant Nutrient Management, pp. 1-15 doi 10.1007/978-3-642-21061-7_1.

Gaunt MW, Turner SL, Rigottier-Gois L, Lloyd-Macgilp SA, Young JPW (2001) Phylogenies of *atpD* and *recA* support the small subunit rRNA-based classification of rhizobia. *International Journal of Systematic and Evolutionary Microbiology*. 51: 2037-2048.

Gautam SS, Rajendra KC, Leong KWC, Mac Aogáin M, O'Toole RF (2019). Step-by step beginner's protocol for whole genome sequencing of human bacterial pathogens. *Journal of Biological Methods*. 6(1): 1-9. e110. doi:10.14440/jbm.2019.276.

Geetha SJ, Joshi SJ (2013). Engineering rhizobial bio inoculants: A strategy to improve iron nutrition. *The Scientific World Journal*. Pp1-15.

Giraud E, Moulin L, Vallenet D, Barbe V, Cytryn E, Avarre JC, Jaubert M, Simon D, Cartieaux F, Prin Y, Bena G, Hannibal L, Fardoux J, Kojadinovic M, Vuillet L, Lajus A, Cruveiller S, Rouy Z, Mangenot S, Segurens B, Dossat C, Franck WL, Chang WS, Saunders E, Bruce D, Richardson P, Normand P, Dreyfus P, Pignol D, Stacey G, Emerich D, Verméglio A, Médigue

© University of Venda

C, Sadowsky M (2007). Legumes Symbioses: Absence of *Nod* genes in photosynthetic Bradyrhizobia. *Science*. 316: 1307-1312.

Grover M, Ali SkZ, Sandhya V, Rasul A, Venkateswarlu B (2011). Role of microorganisms in adaptation of agriculture crops to abiotic stresses. *World Journal of Microbiology and Biotechnology*. 17: 1231-1240.

Harrow J, Nagy A, Reymond A, Alioto T, Patty L, Antonarakis SE, Guigo R (2009). Identifying protein-coding genes in genomic sequences. *Genome Biology*. 10(201): 1-8.

Hassen AI, Khambani LS, Swanevelder ZH (2018). The Draft genome sequence of *Burkholderia* sp. strain Nafp2/4-1b confirms its ability to produce the plant growth-promoting traits pyoverdine siderophores, 1-aminocyclopropane-1-carboxylate deaminase, and the phytohormone auxin. Microbiology Resource Announcements. 7(23): 1-2.

Hirsch AM (1992). Developmental biology of legume nodulation. *New Phytologist.* 122: 211–237. doi: 10.1111/j.1469-8137.1992.tb04227.x.

Illumina Sequencing by Synthesis (2016). (https://www.youtube.com, Accessed 30 August 2022).

Janda JM, Abott SL (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, pitfalls*. Journal of Clinical Microbiology.* 45(9): 2761-2764.

Jones KM, Kobayashi H, Davies BW, Taga ME, Walker GC (2007). How rhizobial symbionts invade plants: the *Sinorhizobium – Medicago* model. *Nature Reviews Microbiology*. 5: 619–633. doi: 10.1038/nrmicro1705.

Koboldt DC 2020). Best practices for variant calling in clinical sequencing. *Genome Medicine*. 12 (91):1-13.

© University of Venda

Kosugi S, Momozawa Y, Liu X, Terao C, Kubo M, Kamatani Y (2019). Comprehensive evaluation of structural variation detection algorithms for whole genome sequencing. *Genome Biology*. 20(117): 1-18. https://doi.org/10.1186/s13059-019-1720-5.

Kwong JC, McCallum N, Sintchenko V and Howden BP (2015). Whole genome sequencing in clinical and public health microbiology. *Pathology*. 47(3): 199-210.

Lindstrom K, Mousavi SA (2019). Effectiveness of nitrogen fixation in rhizobia. *Microbial Biotechnology*. 13(5): 1314-1335.

Mabrouk Y, Hemissi I, Salem IB, Mejri S, Saidi M, Belhadj O (2018). Potential of rhizobia in improving nitrogen fixation and yields of legumes.*e-book*. Pp107-117.

Nap JP, Bisseling, T (1990). Developmental biology of a plant-prokaryote symbiosis: the legume root nodule. *Science.* 250: 948–954. doi: 10.1126/science. 250.4983.948.

Oldroyd GE (2013). Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. *Nature Reviews Microbiology*. 11: 252–264. doi: 10.1038/nrmicro2990.

Oldroyd GE, Murray JD, Poole PS, Downie JA (2011). The rules of engagement in the legume-rhizobial symbiosis. *Annual Reviews Genetics*. 45, 119–144. doi: 10.1146/annurev-genet-110410-132549.

Ormeño-Orrillo E, Menna P, Almeida LGP, Ollero FJ, Nicolás MF, Rodrigues EP, Nakatani AS, Batista JSS, Chueire LMO, Souza RC, Vasconcelos ATR, Megías M, Hungria M, Martínez-Romero E (2012). Genomic basis of broad host range and environmental adaptability of *Rhizobium tropici* CIAT 899 and *Rhizobium* sp. PRF 81 which are used in inoculants for common bean (*Phaseolus vulgaris* L.). *BMC Genomics*. 13(735): 1-26.

Ng PC, Kirkness EF (2010). Whole genome sequencing. *Methods in Molecular Biology*. 628: 215-26. doi: 10.1007/978-1-60327-367-1_12. PMID: 20238084.

© University of Venda

Perret X, Staehelin C, Broughton WJ (2000). Molecular basis of symbiotic promiscuity. *Microbiology Molecular Biology Reviews*. 64: 180–201. doi: 10.1128/MMBR.64.1.180-201.2000.

Reeve W, O'Hara G, Chain P, Ardley J, Bräu L, Nandesena K, Tiwari R, Malfatti s, Kiss H, Lapidus A, Copeland A, Nolan M, Land M, Ivanova N, Mavromatis K, Markowitz V, Kyrpides N, Melino V, Denton M, Yates R, Howieson J (2010). Complete genome sequence of *Rhizobium leguminosarum* bv *trifolii* strain WSM2304, an effective microsymbiont of the South American clover *Trifolium polymorphum*. *Standards in Genomic Sciences* 2(1): 66-76. doi: 10.4056/sigs.44642.

Reeve W, O'Hara G, Chain P, Ardley J, Brau L, Nandesena K, Tiwari R, Copeland A, Nolan M, Han C, BrettinT, Land M, Ovchinikova G, Ivanova N, Mavromatis K, Markowitz V, Krypides N, Melino V, Denton M, Yates R, Howieson J (2010). Complete genome sequence of *Rhizobium leguminosarum bv. trifolii* strain WSM strain WSM1325, an effective microsymbionts of annual Mediterranean clovers. *Standards Genomics of Science* 2(3): 347-356. doi: 10.4056/sigs.852027.

Remigi P, Zhu J, Young JPW, Masson-Boivin, C (2016). Symbiosis within symbiosis: evolving nitrogen-fixing legume symbionts. *Trends in Microbiology*. 24: 63–75.

Rocha EPC, Cornet E, Michel B (2005). Comparative and evolutionary analysis of the bacterial homologous recombination systems. *Genetics*. 1(2): 0247-0259.

Simões LC, Simões M, Vieira MJ (2007). Biofilm interactions between distinct bacterial genera isolated from drinking water. *Applied and Environmental Microbiology*. 73(19): 6192-6200.
Sindhu S, Dahiya A, Gera R, Sindhu SS (2020). Mitigation of abiotic stress in legume-nodulating rhizobia for sustainable crop production. *Agricultural Research*. 9(4): 444-459.

Stepkowski T, Moulin L, Krzyzanska A, McInnes A, Law IJ, Howieson J (2005). European origin of *Bradyrhizobium* populations infecting lupins and serradella in soils of Western Australia and South Africa. *Applied Environmental Microbiology*. 71(11): 7041-7052.

© University of Venda

Wang D, Yang S, Tang F, Zhu H (2012). Symbiosis specificity in the legume–rhizobial mutualism. *Cell Microbiology*. 14: 334–342. doi: 10.1111/j.1462-5822.2011.01736.

Wang Q, Liu J, Zhu H (2018). Genetic and Molecular mechanism underlying symbiotic specificity in legume-rhizobium interactions. *Frontiers in Plant Science*. 9(313): 1-8.

Wang Q, Yang S, Liu J, Terecskei K, Ábrahám , Gombár A (2017). Host-secreted antimicrobial peptide enforces symbiotic selectivity in *Medicago truncatula*. *PNAS.* 114(26): 6854–6859. doi: 10.1073/pnas. 1700715114.

Xie B, Chen D, Cheng G (2009). Effect of the *purL* gene expression level on the competitive nodulation ability of *Sinorhizobium fredii*. *Current Microbiology* 59: 193-198.

Yang S, Wang Q, Fedorova E, Liu J, Qin Q, Zheng Q, (2017). Microsymbiont discrimination mediated by a host-secreted peptide in *Medicago truncatula*. *PNAS*. 114(26): 6848–6853. doi: 10.1073/pnas.1700460114.

Young JPW, Crossman LC, Johnston AW, Thomson NR, Ghazoui ZF, Hull KH, Wexler M, Curson ARJ, Todd JD, Poole PS, Mauchline TH, East AK, Quail MA (2006). The genome of *Rhizobium leguminosarum* has recognizable core and accessory components. *Genome Biology*. 7 (R34): 1- 20. doi: 10.1186/gb-2006-7-4-r34.

Zhong Y, Yang Y, Liu P, Xu R, Rensing C, Fu X, Liao H (2019). Genotype and *Rhizobium* inoculation modulate the assembly of soybean rhizobacterial communities. *Plant Cell Environmental.* 42: 2028-2044.

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6.0 Chapter Six: The Symbiotic Performance of Pigeonpea Rhizobial Strains with Selected Tropical Legumes

Abstract: Common tropical legumes, which include Bambara groundnut (*Vigna subterranaea*), tepary bean (*Phaseolus acutifolius*), tropical soybean (*Glycine max*) and pigeonpea (*Cajanus cajan*) are cultivated widely in Africa particularly by smallholder farmers mainly for human consumption. The smallholder farmers rely largely on the promiscuous type of root nodulation which is associated with these leguminous crops, hence no commercial inoculants are applied on the seed during planting. Therefore, this study was designed to (i) determine the relative performance of individual tropical legume species when inoculated separately with each of the specificc rhizobial strains that were previously derived from pigeonpea (ii) quantify the magnitude of the effects of interactions between the host tropical legume species x rhizobial strain on a range of N fixation variables and (iii) identify the winning (superior) rhizobial strains with the specific test legume species.

Thirty-six rhizobial strains which were previously isolated from pigeonpea root nodules were used in the study to inoculate separately the seed of each of four common tropical legumes which was planted in N depleted medium. There was at least one strain representing each of four distinct rhizobial genera namely *Bradyrhizobium, Paraburkholderia, Phyllobacterium* and *Rhizobium.* The experiment was laid out as a split plot design with legume species as the main factor and rhizobial strain as the sub-factor with two replications. The data sets on nodule number (NN), nodule fresh weight (NFW), nodule dry weight (NDW), shoot fresh weight (NFW), shoot dry weight (SDW) and root fresh weight (RFW) and root dry weight (RDW) were subjected to the analysis of variance and the GGE biplot model. At six weeks after germination, 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° C for 48 h followed by weighing to determine the dry weights. There was marked variability in the responses of the legume species to inoculations with individual rhizobial strains. Tepary bean showed poor nodulation as indicated by the chlorotic plants which contrasted sharply with those of Bambara groundnut. Pigeonpea responded differentially to each individual rhizobial strain resulting in marked differences in the nodule load per plant. Some rhizobial strains, (for instance, *Rhizobium* strain '26a-PP3') induced profuse nodulation in Bambara groundnut but not in the other legume species. On average, the rhizobial strain 'R24' (*Rhizobium multihospilium* '37a-PP4') induced significantly ($P < 0.05$) heavier nodules (0.127 g) than the trial mean (0.064 g) but a relatively moderate SDW (0.688 g).

The principal component (PC) analysis showed that the first two principal components accounted for 78.74% of the total variation. Four N fixation variable, including the NDW and

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SDW, were moderately associated with PC1. Both the RDW and RFW contributed negatively to PC1 but they were highly associated with PC2. The GGE biplot of the rhizobial strain x legume species interaction for NDW explained 82.44% of the total variation. For NDW, the environments represented by E3 (soybean) and E4 (pigeonpea) were positively correlated since their vectors were separated by an acute angle. However, E1 (Bambara groundnut) and E3 (soybean) were negatively correlated since they were characterized by an obtuse angle between them for the SDW. The 'which-won where' biplot for NDW explained 82.44% of the total variation of which PC1 and PC2 accounted for 50.40% and 32.04% of the total variation, respectively. Two rhizobial strains on the vertices of the polygon (*Rhizobium* sp. 36a-PP5) and (*Rhizobium* sp. 26a2-PP5) performed best with Bambara groundnut (E1) and soybean (E3), respectively. E3 (soybean) consisted of the longest vector line suggesting that it possessed a high discriminating ability. The biplot for SDW, explained 85.85% of the total variation of which PC1 and PC2 accounted for 18.16% and 67.69% of the total variation and two rhizobial strains, namely, (*Rhizobium* sp. 33a-PP2) and (*Rhizobium multihospitium* 37a-PP4) were identified as ideal for RDW and SDW, respectively. The biplot analysis also revealed that for SDW, E1 (Bambara groundnut) and E4 (pigeonpea), in that sequence, were plotted closet to the epicentre. The study found variation in the legume species x rhizobial strain interactions and identified two rhizobial strains which performed best in both the tropical soybean type and Bambara groundnut. The GGE biplot analysis also revealed that both pigeonpea and Bambara groundnut provided the most ideal symbiotic activity for NDW but tepary bean lacked the discriminatory ability for NDW. Further testing and validation of the symbiotic activities of the rhizobial strains identified in this study in field trials on diverse legume species and in multiple agro-ecological locations is recommended. It will also be desirable to identify new bio-inoculants for improving tepary bean productivity.

Key words: biplot analysis; bio-inoculant; legume species; productivity; rhizobial genera.

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6.1 Introduction

Common tropical legumes, which include Bambara groundnut (*Vigna subterranaea*), tepary bean (*Phaseolus acutifolius*), tropical soybean (*Glycine max*) and pigeonpea (*Cajanus cajan*) are cultivated widely in Africa particularly by smallholder farmers mainly for human consumption. The legumes also improve soil fertility through the nitrogen (N) fixation in the root nodules as well as addition of quality residual litter (Sakala et al., 2000). In some cases, the surplus grain of these legumes is traded in formal or informal markets, thus generating household income (Shiferaw et al., 2007). However, these legumes are often cultivated in mixed cropping systems partly because of the limited arable land for the smallholder growers (Godfray et al., 2010). In general, the smallholder growers rely on the promiscuous type of root nodulation which is associated with these leguminous crops, hence no commercial inoculants are applied on the seed during planting. Some previous studies reported that some tropical legumes positively respond to seed inoculation with rhizobia suggesting that growers can benefit from the application of such inoculants. In a study involving inoculation of pigeonpea with soil samples from across diverse locations in South Africa, pigeonpea showed differential N fixation ability, indicating that there was potential to select for optimum host x rhizobial strain combinations for the process as well as expand the production area of the crop in the country (Bopape et al., 2021). Similar variability in N fixation was reported also in Bambara groundnut (Bitire et al., 2022) and soybean (Mpepereki et al., 2000). In Kenya, tepary bean positively responded to inoculation with commercial inoculants (Shisanya, 2005).

The differential response of tropical legumes to inoculation with rhizobia is complex for several reasons. Firstly, to succeed, the microsymbiont needs to invade and persist in the environment of indigenous microbes and local abiotic factors that exist in variable rhizospheric conditions. The subsequent N fixation in the root nodules is influenced by a wide range of factors including soil pH (Alves et al., 2021), soil temperature (Alexandre and Oliveira, 2013), rhizospheric nutrient dynamics (Makoi and Ndakidemi, 2011; Divito and Sadras, 2014; Torabian et al., 2019), soil water content (Munjonji et al., 2018), and flavonoid accumulation (Antunes et al., 2006), among others. Secondly, the host plant genotype x rhizobial strain interactions involve multiple genes. For instance, the nitrogenase enzyme complex which is critical for nitrogen reduction consists of as many as 20 genes of which at least six (*nifB, nifD, nifE, nifH, nifK* and *nifN*) are critical for the enzyme to function (Dos Santos et al., 2012). Therefore, it is important to determine the optimum host legume species x rhizobial strain combination for use in cropping systems to benefit farmers and the future development of commercial rhizobial inoculants. However, one of the limitations in evaluations that involving large numbers of rhizobial strains is their inconsistent performance across the legume species and N fixation indicators leading to the complexities that are associated with genotype x

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environment interactions. A methodology such as the genotype plus genotype x environment (GGE) that can utilize principal components, can analyse data sets with two-way structures (Burgueno et al., 2008; Dardanelli et al., 2006; Casanoves et al., 2005; Sarmonte et al., 2005; Yan et al., 2007). In addition, the GGE biplot analysis can depict easy graphical representations of complex genotype x environment effects since the biplot can show two factors (G plus GE) that are important in genotype evaluation and are also sources of variation in multiple environment trials (Yan et al., 2000). Moreover, the GGE methodology can identify superior performing genotypes and test environments as well as the stability of genotypes across multiple environments (Yan et al., 2007; Amira et al., 2013; Atnaf et al., 2013). The GGE biplot method can also determine the interrelationships among traits and compare genotypes based on multiple traits. Therefore, the aim of this study was to evaluate the symbiotic performance of rhizobial strains with a range of host tropical legumes. The specific objectives were to (i) determine the relative performance of individual tropical legume species when inoculated separately with each of the rhizobial strains that were previously derived from pigeonpea (ii) quantify the magnitude of the effects of interactions between the host tropical legume species x rhizobial strain on a range of N fixation variables and (iii) identify the winning (superior) rhizobial strains with the specific test legume species (test environment).

6.2 Materials and Methods

6.2.1 Study location and tropical legume species

The study was carried out in a greenhouse at the Agricultural Research Council (ARC), Plant Health and Protection (Pretoria) (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. Four distinct tropical legumes that are cultivated in South Africa in varying proportions were used in the study (Table 6.1)

6.2.2 Rhizobial strains and inoculum preparation

Thirty-six rhizobial strains which were previously isolated from pigeonpea root nodules were used in this study (Bopape *et al*., 2021) (Table 3.2). There was at least one strain representing each of four distinct rhizobial genera namely *Bradyrhizobium, Paraburkholderia, Phyllobacterium* and *Rhizobium* (Table 3.2). For the inoculum preparation, each rhizobial strain was revived from frozen cultures that were stored at -70 ^{o}C by streaking on yeast mannitol congo red (YMCR) agar (Vincent, 1970) before transferring them to agar plates followed by incubation at 28 ^{o}C for 3 – 4 days. Pieces

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of agar supporting rhizobial colonies were excised from the sub-culturing Petri-dishes and then placed in bottles containing sterile distilled water (18.0 ml). To disperse the rhizobial cells, each bottle was vigorously shaken on a vortex shaker.

6.2.3 Trial establishment, management and measurements

Three healthy 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 Hoagland solution. Three holes (approx. 3.0 cm deep) were punched into each pot prior to planting the seed and inoculating separately with the specific rhizobial strain by saturating with 2.0 ml of each inoculum and covering the seed carefully with sand immediately thereafter. After planting, the pots were irrigated with 80 ml of water.

The seedlings in each pot were irrigated with distilled water periodically as necessary during the entire growth period. At 7 weeks after germination, 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° C for 48 h followed by weighing to determine the dry weights.

Table 6.1 The tropical legumes that were used in the study.

Tropical legume species		**Duration to maturity (days)**	**Flower colour**	**Notes**
Common name	**Scientific name**			
Bambara groundnut	*Vigna subterranea*	120 ± 10	Yellow	Early-medium duration type; large seed
Tepary bean	*Phaseolus acutifolius*	60 ± 5	Purple	Early duration type; large seed
Soybean	*Glycine max*	115 ±10	White	Medium duration, tropical type; large seed
Pigeonpea	*Cajanus cajan*	130 ± 20	Yellow	Medium duration type; medium seed

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Table 6.2 The rhizobial strains that were used in the study.

Rhizobial strain		Genus	Species
Designation	Code		
R1	5b2-PP1	*Rhizobium*	*Rhizobium leucaenae*
R2	16a2-PP1	*Rhizobium*	*Rhizobium* sp
R3	29a-PP1	*Rhizobium*	*Rhizobium* sp
R4	17a-PP1	*Rhizobium*	*Rhizobium multihospitium*
R5	8b2-PP1	*Rhizobium*	*Rhizobium alamii*
R6	33a-PP2	*Rhizobium*	*Rhizobium* sp
R7	29a2-PP2	*Rhizobium*	*Rhizobium tropici*
R8	29a1-PP1	*Rhizobium*	*Rhizobium* sp
R9	30b-PP3	*Rhizobium*	*Rhizobium multihospitium*
R10	31b1-PP3	*Rhizobium*	*Rhizobium* sp
R11	31b2-PP3	*Rhizobium*	*Rhizobium galicum*
R12	36a-PP3	*Rhizobium*	*Rhizobium* sp
R13	35a-PP3	*Rhizobium*	*Rhizobium* sp
R14	32b2-PP3	*Rhizobium*	*Rhizobium* sp
R15	39a3-PP3	*Rhizobium*	*Rhizobium leucaenae*
R16	15a-PP3	*Rhizobium*	*Rhizobium* sp
R17	10a-PP3	*Rhizobium*	*Rhizobium tropici*
R18	26b-PP3	*Rhizobium*	*Rhizobium* sp
R19	18a-PP3	*Rhizobium*	*Rhizobium mayense*
R20	17a1-PP3	*Rhizobium*	*Rhizobium tropici*
R21	13b1-PP4	*Rhizobium*	*Rhizobium galegae*
R22	37a-PP4	*Rhizobium*	*Rhizobium multihospitium*
R23	32a1-PP2	*Rhizobium*	*Rhizobium lupini*
R24	36a-PP5	*Rhizobium*	*Rhizobium* sp
R25	23a-PP5	*Rhizobium*	*Rhizobium* sp
R26	38a1-PP5	*Rhizobium*	*Rhizobium phaseoli*
R27	31b1-PP5	*Rhizobium*	*Rhizobium cellulosilyticum*
R28	26a2-PP5	*Rhizobium*	*Rhizobium* sp
R29	34a2-PP5	*Rhizobium*	*Rhizobium* sp
R30	14a1-PP5	*Rhizobium*	*Rhizobium alamii*
R31	LMG9503	*Rhizobium*	*Rhizobium tropici*
R32	LMG17827	*Rhizobium*	*Rhizobium etli*
R33	XS21	*Bradyrhizobium*	*Bradyrhizobium* sp
R34	WB 74	*Bradyrhizobium*	*Bradyrhizobium japonicum*
R35	Control (Water)	-	-

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6.2.4 Experimental design and data analysis

The experiment was laid out as a split plot design with legume species as the main factor and rhizobial strain as the sub-factor. Each treatment was replicated twice. The data sets on nodule number (NN), nodule fresh weight (NFW), nodule dry weight (NDW), shoot fresh weight (NFW), shoot dry weight (SDW) and root fresh weight (RFW) and root dry weight (RDW) were subjected to the analysis of variance (ANOVA) and Pearson's correlation analysis using SAS statistical software (version 9.3) followed by mean separation using LSD test at the 5.0% probability level. Further analysis using the GGE biplot model (Olivoto, 2019; Olivoto et al., 2019) was carried out to understand better the relationship between the host plants and the microsymbionts (Bosi *et al*., 2022; Gauch *et al*., 2008; Yan and Kang, 2002).

6.3 Results

6.3.1 Variation in the nitrogen fixation variables among the legumes

The results showed that marked variability in the responses of the legume species to inoculations with individual rhizobial strains (Fig. 6.2). The control (uninoculated) plants showed chlorotic (yellow) leaves deficient of chlorophyll and developed no detectable root nodules. In contrast, the plants that were compatible with individual rhizobial strains showed dark green leaves indicating the availability of N (Fig. 6.4). Tepary bean showed poor nodulation as indicated by the chlorotic plants which contrasted sharply with those of Bambara groundnut (Fig. 6.2). In some cases, there was a mixture of dark green and chlorotic plants with the legume species depending on its compatibility with the inoculant (Fig. 6.3). Some of the *Rhizobium* inoculants appeared to be more compatible than *Bradyrhizobium* strains with soybean suggesting that there was a differential response to the inoculum by the legume species (Fig. 6.4). Similarly, pigeonpea responded differently to each individual rhizobial strain resulting in marked differences in the nodule load per plant (Fig. 6.5). Some rhizobial strains, (for instance, *Rhizobium* strain '26a-PP3') induced profuse nodulation in Bambara groundnut but not in the other species (Fig. 6.6).

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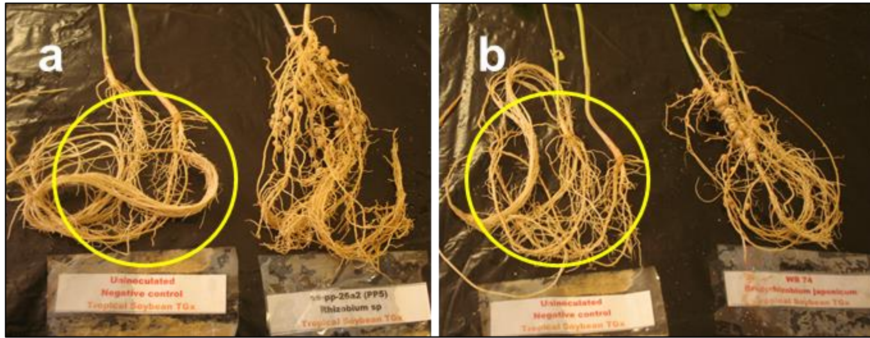

Fig. 6.1 Impact of inoculation with *Rhizobium* (left) and *Bradyrhizobium* (right) strains on root nodulation in a tropical soybean type; uninoculated plants formed no nodules (yellow circles).

Fig. 6.2 Contrast in response to inoculation with rhizobial strains in (a) tepary bean with chlorotic leaves and (b) Bambara groundnut with dark green leaves.

Fig. 6.3 Variation in leaf color score between (a) pigenopea and (b) soybean plants that were inoculated separately with specific rhizobial strains; within each legume species chlorotic (yellow) leaves and dark green leaves indicated effective and ineffective nitrogen fixation, respectively.

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Fig. 6.4 Variation in response to rhizobial inoculation among soybean plants (a) control; uninoculated (b) inoculated with *Bradyrhizobium japonicum* 'WB74' (c) inoculated with *Rhizobium* sp. '34a2-PP5' (d) *Rhizobium* sp. '26a2-PP5' and (e) inoculated with *Rhizobium* sp. '30b-PP3'.

Fig. 6.5 Variation in response to rhizobial inoculation among pigeonpea plants (a) inoculated with *Bradyrhizobium japonicum* 'XS21' (b) control; uninoculated (c) inoculated with *Rhizobium tropici* '10a-PP3'.

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Fig. 6.6 (a) Absence of root nodulation (b) profuse nodulation and (c) sparse nodulation among Bambara groundnut plants that received different inoculation treatments.

The results also revealed highly significant ($P < 0.001$) differences in all the N fixation parameters that were measured among the legume species (Table 6.3). Similarly, the rhizobial strains showed highly significant ($P < 0.001$) differences in all the parameters except for root dry weight (RDW) (Table 6.3). In addition, the LS x RS interactions were highly significant ($P < 0.001$) for all the N fixation variables except for the RDW suggesting that the rhizobial strains were unable to discriminate between the tropical legumes in terms of this variable. The highest (> 28.0) and lowest (< 3.0) number of nodules (NN) per plant among the legume species were observed for Bambara groundnut and tepary bean, respectively (Table 6.4). The SDW in Bambara groundnut was almost three-fold higher than in pigeonpea at the time of harvesting (Table 6.4). In general, Bambara groundnut appeared to exhibit a comparatively stronger compatibility with the rhizobial strains as indicated by the N fixation variables that were measured particularly the NN and NDW (Table 6.4). On average, the rhizobial strain 'R24' (*Rhizobium multihospilium*, '37a-PP4') induced significantly ($P < 0.05$) heavier nodules (0.127 g) than the trial mean (0.064 g) but a relatively moderate SDW (0.688 g) (Table 6.5).

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Table 6.3 Sum of squares for six nitrogen fixation variables among four tropical legume species that were inoculated separately with each of thirty-five rhizobial strains. (NN = number of nodules per plant; NDW = nodule dry weight per plant; RDW = root dry weight per plant; RFW = root fresh weight per plant; SDW = shoot dry weight per plant; SFW = shoot fresh weight per plant).

Source	df	Sum of Squares					
		NN	NDW	RDW	RFW	SDW	SFW
Replication	2	31.400	0.002	0.011	1.240	0.033	5.910
Legume species (LS)	3	14969.600***	0.255***	1.104***	81.991***	24.901***	613.610***
Error	6	108.300	0.002	0.014	1.102	0.056	1.060
Rhizobial strain (RS)	34	447.600***	0.013***	0.023	3.144***	0.136**	4.280***
LS x RS	102	354.800***	0.008***	0.023	2.184***	0.139***	3.400***
Error	272	51.900	0.002	0.017	1.752	0.069	1.570

***; **; * = significant at the 0.001, 0.01 and 0.05 probability level, respectively.

Table 6.4 Mean separation for six root nodulation traits among four tropical legume species that were inoculated separately with each of thirty-five rhizobial strains. (NN = number of nodules per plant; NDW = nodule dry weight (g) per plant; RDW = root dry weight (g) per plant; RFW = root fresh weight (g) per plant; SDW = shoot dry weight (g) per plant; SFW = shoot fresh weight (g) per plant).

Legume species	NN	NDW	RDW	RFW	SDW	SFW
Bambara groundnut	28.342 a	0.124 a	0.295 b	2.947 a	1.333 a	6.831 a
Tepary bean	2.952 c	0.008 d	0.138 c	1.926 b	0.185 d	1.324 d
Soybean	5.819 c	0.045 c	0.338 a	3.101 a	0.635 b	2.949 b
Pigeonpea	19.761 b	0.077 b	0.144 c	1.231 c	0.482 c	2.279 c

In each, column, mean values that are followed by different small letters are significantly ($P < 0.05$) different from each other as determined by the Least Significant Difference ($LSD_{0.05}$).

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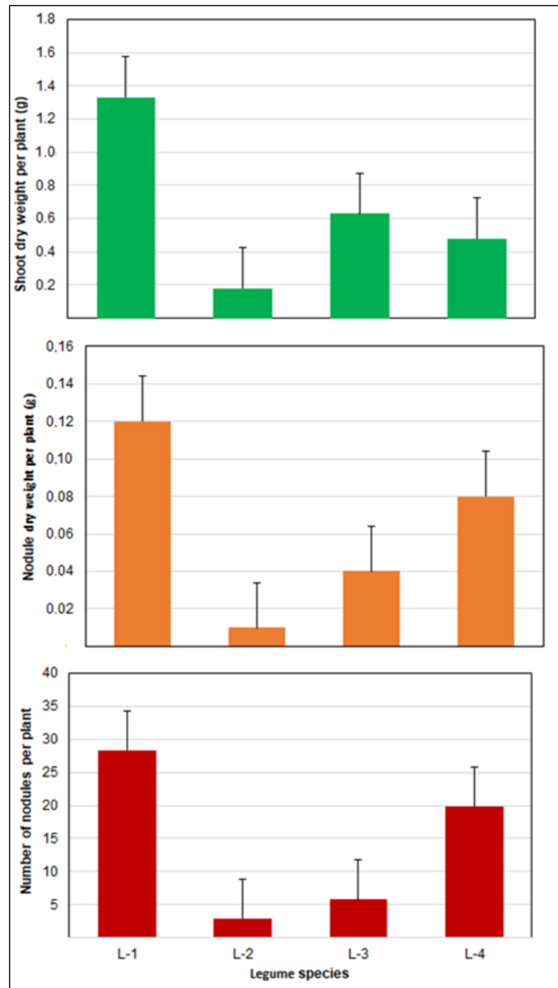

Fig. 6.7 The mean number of nodules (bottom), nodule dry weight (middle) and shoot dry weight per plant among four tropical legumes that were inoculated separately with specific rhizobial strains. (L-1 = Bambara groundnut; L-2 = tepary bean; L-3 = soybean; L-4 = pigeonpea).

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Table 6.5 Mean separation for six root nodulation traits among thirty-five rhizobial strains that were used for inoculating each of four tropical legume species. (NN = number of nodules per plant; NDW = nodule dry weight (g) per plant; RDW = root dry weight (g) per plant; RFW = root fresh weight (g) per plant; SDW = shoot dry weight (g) per plant; SFW = shoot fresh weight (g) per plant).

Rhizobial strain		NDW	NN	RDW	RFW	SDW	SFW
Designation	Code						
R24	36a-PP5	0.127	13.750	0.298	2.725	0.688	3.798
R28	26a2-PP5	0.125	21.500	0.225	1.675	0.649	3.743
R9	30b-PP3	0.105	17.250	0.219	2.039	0.697	3.676
R22	37a-PP4	0.102	16.416	0.259	2.211	0.831	4.147
R4	17a-PP1	0.099	23.333	0.228	1.807	0.661	3.668
R30	14a1-PP5	0.098	26.333	0.223	2.182	0.768	3.379
R19	18a-PP3	0.093	21.916	0.256	2.407	0.871	4.369
R23	32a1-PP2	0.093	17.833	0.195	2.061	0.899	4.520
R26	38a1-PP5	0.089	17.833	0.199	2.779	0.671	3.750
R16	15a-PP3	0.087	14.916	0.207	2.284	0.800	4.126
R14	32b2-PP3	0.077	17.500	0.240	2.402	0.648	3.461
R25	23a-PP5	0.076	23.583	0.236	2.299	0.723	3.503
R34	WB74-B	0.073	16.750	0.106	1.058	0.549	2.580
R15	39a3-PP3	0.072	14.750	0.219	2.25	0.536	2.724
R17	10a-PP3	0.071	11.500	0.268	2.469	0.631	2.915
R20	17a1-PP3	0.069	20.500	0.221	2.457	0.706	3.577
R5	8b2-PP1	0.069	12.333	0.229	3.002	0.659	3.598
R11	31b2-PP3	0.068	14.750	0.148	1.868	0.774	4.215
R29	34a2-PP5	0.065	19.833	0.177	2.238	0.627	3.445
R33	XS21-B	0.065	19.750	0.221	1.734	0.721	3.239
R13	35a-PP3	0.056	7.500	0.233	1.915	0.783	3.729
R21	13b1-PP4	0.054	9.667	0.208	2.335	0.669	3.367
R6	33a-PP2	0.051	12.417	0.385	3.527	0.668	3.399
R27	31b1-PP5	0.050	13.333	0.235	2.320	0.708	3.477
R10	31b1-PP3	0.047	12.417	0.204	2.982	0.630	3.639
R18	26b-PP3	0.041	8.833	0.202	1.987	0.497	2.733
R7	29a2-PP2	0.039	6.750	0.252	2.712	0.559	2.957
R31	LMG9503-R	0.036	11.75	0.259	2.318	0.525	2.787
R12	36a-PP3	0.032	15.000	0.228	1.803	0.652	3.352
R2	16a2-PP1	0.029	10.667	0.234	2.574	0.581	2.891
R1	5b2-PP1	0.025	9.583	0.228	2.799	0.565	2.926
R8	29a1-PP1	0.021	11.583	0.232	2.595	0.607	2.677
R3	29a-PP1	0.009	4.333	0.273	2.925	0.496	2.276
R32	LMG17827-R	0.001	1.5	0.252	2.720	0.465	2.454
R35	Control	0.000	0.000	0.203	1.083	0.548	2.017
Mean		0.064	14.219	0.229	2.301	0.659	3.346
Coefficient of Variation (%)		24.840	24.387	18.867	19.170	13.250	12.472
$LSD_{(0.05)}$		0.038	3.514	0.104	1.064	0.210	1.006

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6.3.2 Principal component analysis and principal component biplot

The principal component analysis showed that the first two principal components accounted for 79.74% of the total variation (Table 6.6). Four N fixation variable, including the NDW and SDW, were moderately associated with PC1. Both the RDW and RFW contributed negatively to PC1 but they were highly associated with PC2. In contrast, PC3 was dominated by SDW. However, PC4 was dominated by the RFW (Table 6.6). The results also revealed NDW dominated PC5. The biplot analysis indicated that only two rhizobial strains ('R27' and 'R13') were positioned close to the origin but five rhizobial strains ('R6', 'R23', 'R32', 'R34' and 'R35') were distinct and positioned far away from the origin (Fig. 6.8). The rhizobial strain 'R6' in the top left quadrant was associated and characterized by high RDW and RFW. However, the rhizobial strains that were highly associated with heavy nodules as well as shoot weight were in the top right quadrant (Fig. 6.8).

Table 6.6 Principal component analysis showing the eigenvector, eigenvalue and cumulative percentage of the first five principal component axes for six nitrogen fixation variables among legume species.

Nitrogen fixation variable	PC1	PC2	PC3	PC4	PC5
Number of nodules	0.47	- 0.05	- 0.59	0.21	- 0.61
Nodule dry weight	0.50	0.03	-0.41	- 0.12	0.69
Root dry weight	- 0.09	0.69	- 0.21	- 0.651	- 0.12
Root fresh weight	- 0.11	0.69	- 0.01	0.67	0.04
Shoot dry weight	0.49	0.11	0.534	- 0.22	- 0.32
Shoot fresh weight	0.51	0.18	0.39	0.15	0.18
Eigenvalue	3.14	1.65	0.54	0.39	0.23
Variability (%)	52.28	27.46	9.03	6.44	3.80
Cumulative (%)	52.28	79.74	88.77	95.21	99.02

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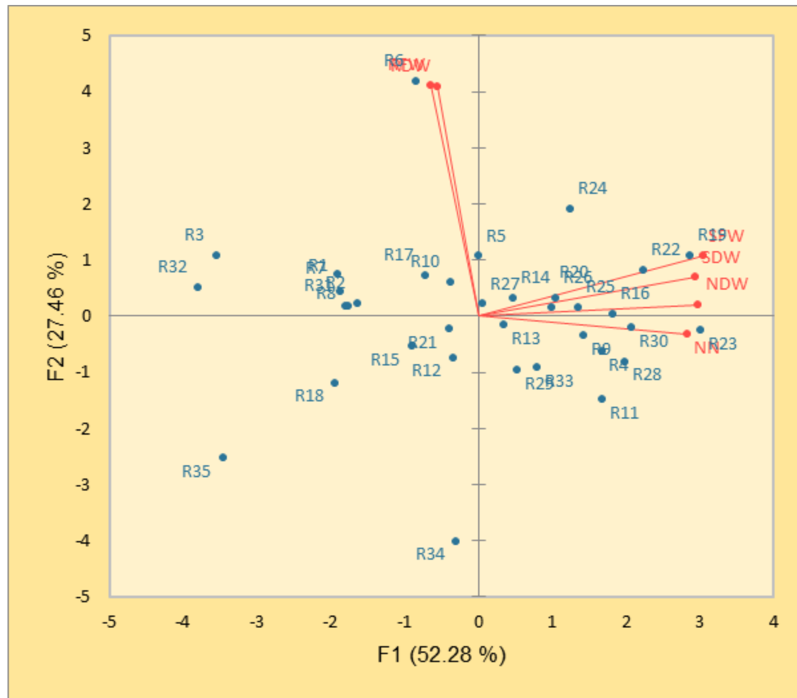

Fig. 6.8 Principal component score plot of PC1 and PC2 describing the variation among 35 rhizobial strains estimated using the data set of nitrogen fixation variables. after soil moisture stress. (NN = number of nodules per plant; NDW = nodule dry weight (g) per plant; RDW = root dry weight (g) per plant; RFW = root fresh weight (g) per plant; SDW = shoot dry weight (g) per plant; SFW = shoot fresh weight (g) per plant).

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6.3.3 Selection of rhizobial strains and tropical legumes

6.3.3.1 Relationships between the symbionts

The GGE biplot of the rhizobial strain x legume species interaction for NDW explained 82.44% of the total variation (Fig. 6.9a). The first principal component (PC1) explained 50.40% while the second principal component (PC2) explained 32.04% of the total variation for NDW. However, 75.99% and 85.85% of the total variation were attributed to the RDW (Fig. 6.9b) and SDW, respectively (Fig. 6.9c). For NDW, the environments represented by E3 (soybean) and E4 (pigeonpea) were positively correlated since the angle between their vectors was small (Fig. 6.9a). However, E1 (Bambara groundnut) and E3 (soybean) were negatively correlated since they were characterized by an obtuse angle between them for the SDW (Fig. 6.9c).

6.3.3.2 Determining the 'which-won-where' rhizobial strain

The biplot depicts an irregular polygon with a set of lines constructed from the origin so as to dissect perpendicularly each side of the polygon and dividing the polygon into sectors as well as determining the winning rhizobial strains (coded as genotype scores) for each sector (Yan et al., 2007). The 'which-won where' biplot for NDW explained 82.44% of the total variation of which PC1 and PC2 accounted for 50.40% and 32.04% of the total variation, respectively (Fig. 6.10a). The biplot produced three sectors and the rhizobial strains were clustered into all the sectors. The biplot revealed that for the NDW, the rhizobial strains (depicted as genotypes) on the vertices of the polygon 'R24' (*Rhizobium* sp. 36a-PP5) and 'R28' (*Rhizobium* sp. 26a2-PP5) performed best with Bambara groundnut (E1) and soybean (E3), respectively (Fig. 6.10a). Based on the vector lines drawn from the origin of the biplot to each test environment (i.e. test legume species) which measure the discriminative power of the environment, E3 (soybean) consisted of the longest vector line from the origin suggesting it possessed a high discriminating ability.

Similarly, the 'which-won where' biplot for RDW explained 75.99% of the total variation of which PC1 and PC2 accounted for 39.06% and 36.93% of the total variation, respectively (Fig. 6.10b). The biplot showed that for the RDW, the rhizobial strains on the vertices of the polygon 'R12' (*Rhizobium* sp. 36a-PP3) and 'R31' (*Rhizobium tropici*; LMG9503) performed best with Bambara groundnut (E1) and soybean (E3), respectively (Fig. 6.10b). Soybean (E3) showed the highest discriminating ability. The biplot for SDW, explained 85.85% of the total variation of which PC1 and PC2 accounted for 67.69% and 18.16% of the total variation, respectively (Fig. 6.10c). The rhizobial strains on the vertices of the polygon 'R13' (*Rhizobium* sp.; 35a-PP3) and 'R22' (*Rhizobium* sp.; 37a-PP4) performed best with Bambara groundnut

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(E1) and pigeonpea (E4), respectively (Fig. 6.10b). In addition, soybean (E3) showed the highest discriminating ability for the rhizobial strains in terms of SDW.

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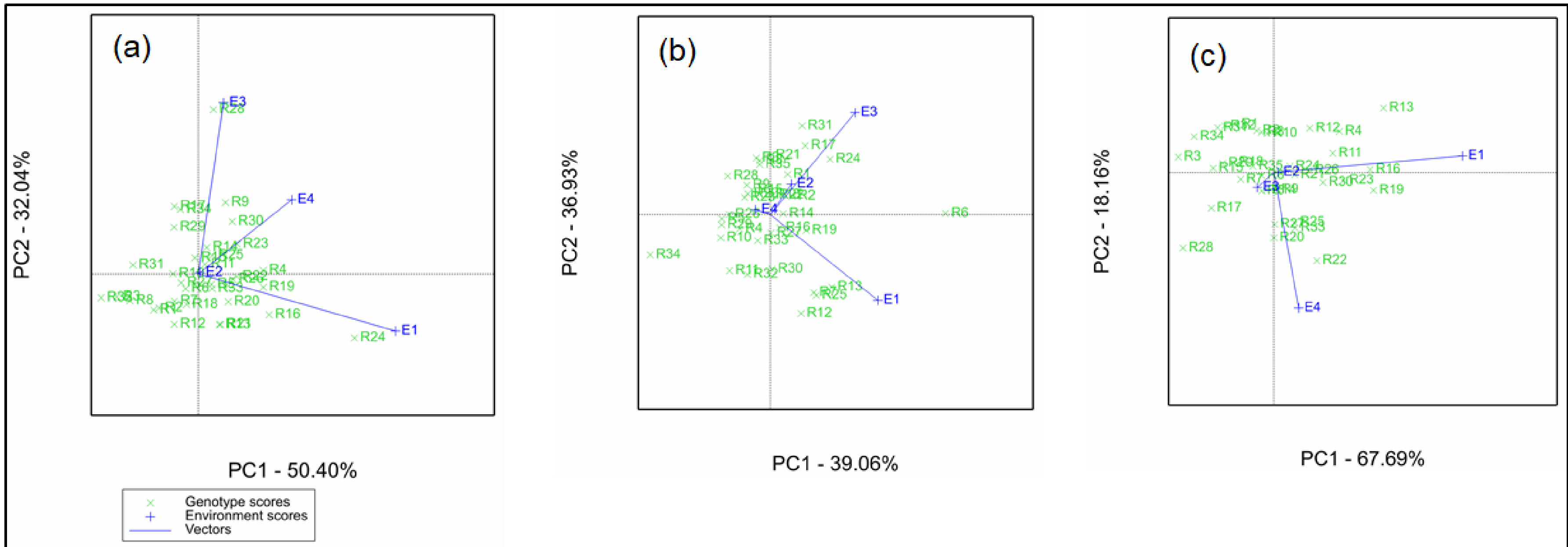

Fig. 6.9 GGE biplot analysis showing the rhizobial strains (coded as genotype scores) x tropical legumes (coded as environment scores) relationships for (a) nodule dry weight (b) root dry weight and (c) shoot dry weight; the designation of each rhizobial strain is stated in Table 6.2; E1 = Bambara groundnut; E2 = tepary bean; E3 = soybean; E4 = pigeonpea.

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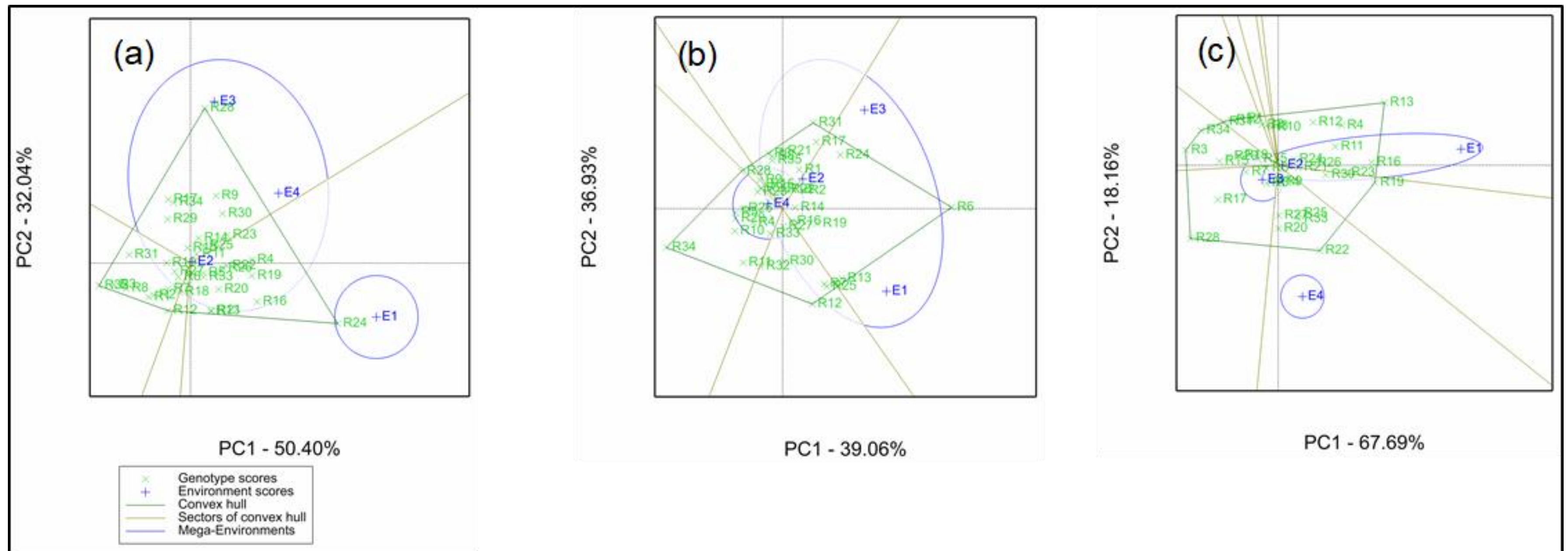

Fig. 6.10 GGE biplot analysis of 'which-won-where' among thirty-five rhizobial strains (coded as genotype scores) that were used for inoculating each of four tropical legume species (coded as environment scores) for (a) nodule dry weight (b) root dry weight and (c) shoot dry weight; the designation of each rhizobial strain is stated in Table 6.2; E1 = Bambara groundnut; E2 = tepary bean; E3 = soybean; E4 = pigeonpea.

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6.3.3.3 Comparison view of GGE biplot analysis for ideal rhizobial strain across the tropical legumes

The GGE biplot analysis can rank the performance of the rhizobial strains across the tropical legumes. In this regard, the ideal rhizobial strain (coded as a genotype score in this analysis), is positioned in the innermost concentric circle, indicated by an arrowhead in the biplot (Yan and Tinker, 2006). The rhizobial strain 'R30' (*Rhizobium alamii*; 14a1-PP5) was identified as ideal for NDW (Fig. 6.11a). In addition, the rhizobial strain 'R9' was also desirable due to its closeness to the arrowhead in the circle for NDW (Khan et al., 2021). Two rhizobial strains, namely, 'R6' (*Rhizobium* sp.; 33a-PP2) and 'R22' (*Rhizobium multihospitium*; 37a-PP4) were identified as ideal for RDW (Fig. 6.11b) and SDW (Fig. 6.11c), respectively. In contrast, the control (uninoculated) ('R35') and rhizobial strain 'R34' *(Bradyrhizobium japonicum*; WB74) were located furthest from the ideal strains, indicating low stability.

6.3.3.4 Ranking of the legume species

The GGE biplot analysis can also rank environments (i.e. legume species in this case) based on their effects on the genotypes (i.e. rhizobial strains in this case). The determination of the best (ideal) test legume species (environment) is critical for the selection of superior rhizobial strains. In this regard, ideal legume species should be able to distinguish between the rhizobial strains and all the other evaluated legume species (Oladosu et al., 2017). The legume species (environments) E4 (pigeonpea) and E1 (Bambara groundnut) were plotted closest to the epicentre of the concentric circles, in that order providing the most ideal symbiotic activity for NDW (Fig. 6.12a). However, E2 (tepary bean) was plotted farthest from the epicentre indicating that it was the worst discriminatory legume species for NDW. For RDW, the legume species E3 (soybean) and E2 (tepary bean) were plotted closest to the epicentre of the concentric circles, but E4 (pigeonpea) was positioned farthest from the epicentre (Fig. 6.12b). The biplot analysis also revealed that for SDW, E1 (Bambara groundnut) and E4 (pigeonpea), in that sequence, were plotted closet to the epicentre but E3 (soybean) was plotted farthest from the epicentre suggesting that it was the worst discriminatory ability for this N fixation indicator (Fig. 6.12c).

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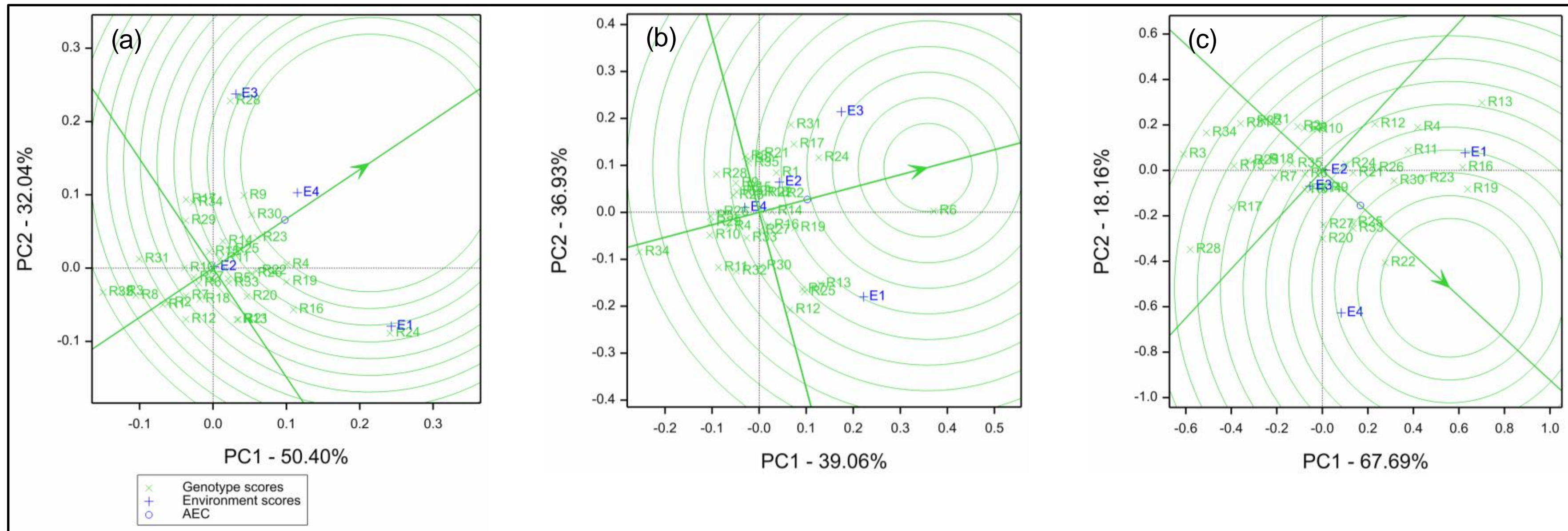

Fig. 6.11 GGE biplot analysis of relative rhizobial strain (coded as genotype score) performance over four distinct tropical legume species (coded as environment scores) for (a) nodule dry weight (b) root dry weight and (c) shoot dry weight; the designation of each rhizobial strain is stated in Table 6.2.

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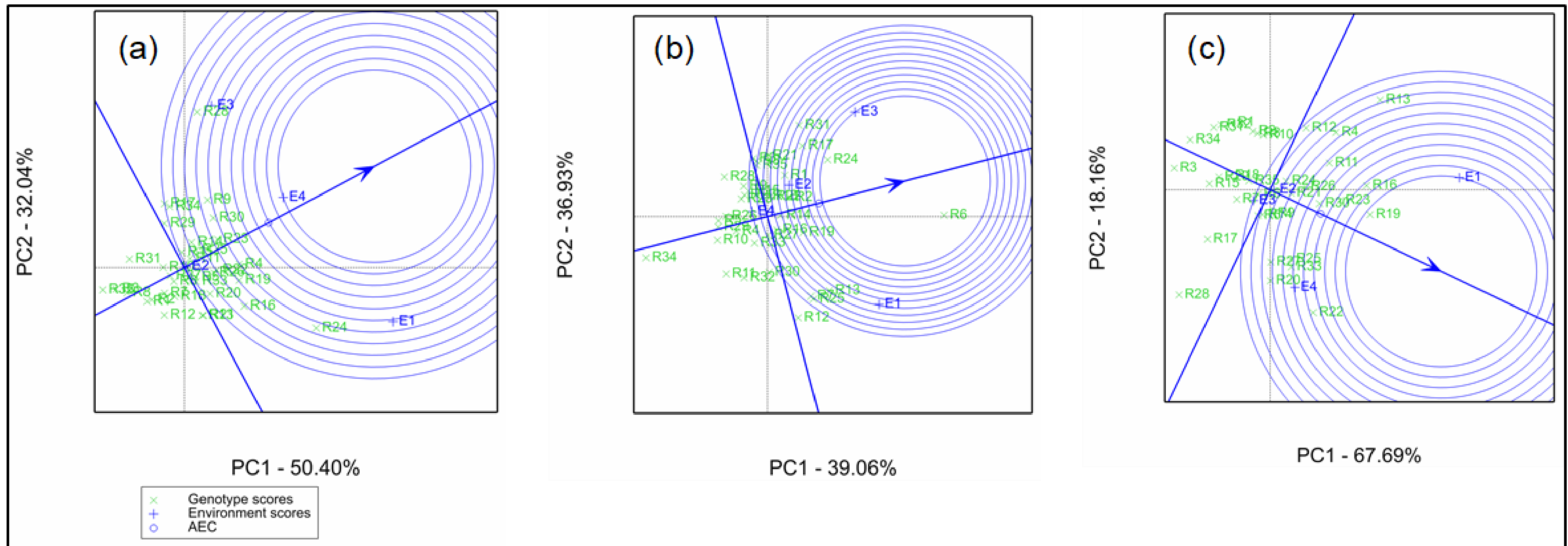

Fig. 6.12 GGE biplot analysis of relative performance of environments (individual tropical legume species) after inoculation separately with each of thirty-five rhizobial strains (genotypes) for (a) nodule dry weight (b) root dry weight and (c) shoot dry weight.

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6.4 Discussion

The results of this study demonstrated the complexity of selecting bio-inoculants for multiple tropical legume species. This is partly because neither of the symbionts is static in the interaction process leading to N fixation. Secondly, the N fixation variable of interest also determines the hierarchy of the legume species. For instance, the results showed the changing role of ideal legume species (environment) depending on the N fixation variable of interest. Specifically, pigeonpea (coded as E4) and Bambara groundnut (E1) were identified as the most ideal species for NDW. However, tepary bean, which had the weakest discriminatory ability for NDW, was the ideal (together with soybean) legume species for RDW. Furthermore, Bambara groundnut and pigeonpea, (in that sequence) were selected as the most ideal legeme species for SDW (Fig. 6.12c). These findings suggest the compatibility between the symbionts per se does not imply optimum N fixation. Secondly, this observation suggested that probably, the selection of potential bio-inoculants should include grain yield as, at least, one of the N fixation indicators particularly in legume improvement programs aimed at developing high yielding cultivars. In general, the bio-inoculant selection process needs to consider the N fixation variable of the end-use importance. Legume species can be improved genetically for fodder value for instance, hence the SDW may be more desirable than grain yield (Hintz and Albrecht, 1994; Açikgöz et al., 2013). Often, forage cultivars of legume species can have greater stem and leaf dry matter accumulation than the grain-types (Darmosarkoro et al., 2001). Overall, this variation in both the host legume and rhizobial strain underscored the need for simultaneous selection of both symbionts to attain optimum N fixation.

The variability in the performance of the rhizobial strains that was observed in this study was consistent with the findings that were reported in similar studies (Appunu et al., 2008; Vuong et al., 2017). However, the current study findings also demonstrated that some of the rhizobial strains derived originally from pigeonpea nodules, do not necessarily induce better N fixation in the original host than in different legume species. For example, the rhizobial strain 'R13' (Rhizobium sp. 35a-PP3) performed best with Bambara groundnut in terms of RDW. In a recent study involving soybean, a strong positive correlation between root architecture and SDW as well as other N fixation traits was reported (Yang et al., 2017). The root is an integral component of the N fixation process. Nonetheless, it must be pointed out that the choice of an ideal strain should be approached with caution for several reasons. For instance, in this study, there was no competition for nodule occupancy against native rhizobia with inferior N-fixing abilities that could potentially influence the fixation process negatively (Mendoza-Suárez et al., 2021). Secondly, as

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alluded to above, the N fixation variable for an ideal bio-inoculant needs to be defined since it is unlikely to find an inoculant ideal for all symbiotic situations.

The GGE biplot analysis confirmed the positive relationship between NDW and SDW among the legume species. This was consistent with reports from similar studies (Gwata et al., 2004; Sinclair et al., 1991). The biplot analysis also identified pigeonpea and Bambara groundnut as the ideal legume species with the power to discriminate between the rhizobial strains. However, one of the drawbacks in this approach was that the ideal legume species was identified for a specific N fixation trait such as SDW. An ideal legume species that can be identified based on multiple N fixation traits could be more desirable. Nonetheless, the positive correlations between SDW and NDW for instance, could be exploited to make valuable inferences about the whole N fixation process but bearing in mind its complex nature. Previous studies involving molecular analysis of the microsymbionts reported that some rhizobial strains lack common nodulation genes such as the *nod*ABC and most probably utilize alternative biochemical pathways to initiate symbiosis (Young et al., 2006; Giraud et al., 2007; Bopape et al., 2021). In contrast, Wheatley et al., (2020) reported that the rhizobial strain *Rhizobium leguminosarum*, requires 593 genes for competitive nodulation and fixation.

In summary, the findings of this study provided novel information in the pattern of N fixation in legume species that were each inoculated separately with distinct rhizobial strains. The information will be useful formulating bio-inoculants for improving legume productivity (Mabrouk et al., 2018; Wang et al., 2018; Ormeño-Orrillo et al., 2012). It is recommended to validate the symbiotic efficiencies of the rhizobial strains identified in this study on a field scale with a diverse range of legume species and in multiple agro-ecologies. In addition, the identification of new bio-inoculants for tepary bean which showed relatively poor performance in this study could be useful for improving the productivity of this legume species.

6.5 Conclusions and recommendations

The study found variation in the legume species x rhizobial strain interactions. However, the study identified two rhizobial strains (*Rhizobium* sp. 26a2-PP5 and 36a-PP5) which performed best in inducing NDW in tropical soybean type and Bambara groundnut. In addition, the rhizobial strains (*Rhizobium* sp., 35a-PP3 and *Rhizobium multihospitium*, 37a-PP4) also performed best in Bambara groundnut and pigeonpea. The GGE biplot analysis also revealed that both pigeonpea and Bambara groundnut provided the most ideal symbiotic activity for NDW but tepary bean

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lacked the discriminatory ability for NDW. In addition, the study revealed that Bambara groundnut and pigeonpea possessed the discriminatory ability for SDW.

Further testing and validation of the symbiotic activities of the rhizobial strains identified in this study in field trials on diverse legume species and in multiple agro-ecological locations is recommended. It will also be desirable to identify new bio-inoculants for improving tepary bean productivity. Probably, co-inoculation of these legume species rhizobial strains from more than one genus or growth promoting microorganisms (Hassen et al. 2016; 2021; Tchakounté, 2018).

References

Açikgöz E, Sincik M, Wietgrefe G, Sürmen M, Çeçen S, Yavuz T, Erdurmuş C, Göksoy AT (2013). Dry matter accumulation and forage quality characteristics of different soybean genotypes. *Turkish Journal of Agriculture and Forestry*. 37(1): 22-32.

Alexandre A, Oliveira S (2013). Response to temperature stress in rhizobia. *Critical Reviews in Microbiology*. 39: 219-228. doi: 10.3109/1040841X.2012.702097.

Alves LA, Ambrosini VG, de Oliveira Denardin LG, Flores JPM, Martins AP, Filippi D, Bremm C, de Faccio Carvalho PC, Farias GD, Ciampitti IA, Tiecher T (2021). Biological Nfixation by soybeans grown with or without liming on acid soils in a no-till integrated crop-livestock system. *Soil and Tillage Research*. 209(104923). doi: 10.1016/j.still.2020.104923.

Amijee F, Giller KE (1998). Environmental constraints to nodulation and nitrogen fixation of *Phaseoulus vulgaris* L in Tanzania. I. A survey of soil fertility, root nodulation and multi-locational responses to *Rhizobium* inoculation. *African Crop Science Journal*. 6(2): 159-169.

Antunes PM, de Varennes A, Rajcan I, Goss MJ (2006). Accumulation of specific flavonoids in soybean (*Glycine max* (L.) Merr.) as a function of the early tripartite symbiosis with arbuscular mycorrhizal fungi and *Bradyrhizobium japonicum* (Kirchner) Jordan. *Soil Biology and Biochemistry. 38: 1234–1242*.

© University of Venda

Appunu C, Sen D, Singh MK, Dhar B (2008). Variation in symbiotic performance of *Bradyrhizobium japonicum* strains and soybean cultivars under field conditions. *Journal of Central European Agriculture*. 9(1): 169-174.

Bitire TD, Abberton M, Oyatomi O, Babalola OO (2022). Effect of *Bradyrhizobium japonicum* Strains and Inorganic Nitrogen Fertilizer on the Growth and Yield of Bambara Groundnut (*Vigna subterranea (L.) Verdc)* Accessions. *Frontiers in Sustainable Food Sytems.* 6: 913239. doi: 10.3389/fsufs.2022.913239

Blanche SB, Myers GO (2006). Identifying discriminating locations for cultivar selection in Louisiana. *Crop Science*. 46: 946-949.

Burgueno J, Crossa J, Cornelius PL, Yang R (2008). Using factor analytic model for joining environments and genotype without cross-over genotype × environment interaction. *Crop Science*. 48: 1291-1305.

Casanoves F, Baldessari J, Balzarini M (2005). Evaluation of multi-environment trials of peanut cultivars. *Crop Science*. 45: 18-26.

Dardanelli JL, Balzarini M, Martinez MJ, Crruberti M, Resnik S, Ramunda SF, Herrero R Baigorri H (2006). Soybean maturity groups, environments, and their interaction define mega-environments for seed composition in Argentina. *Crop Science*. 46: 1939-1947.

Darmosarkoro W, Harbur MM, Buxton DR, Moore KJ, Devine TE, Anderson IC (2001). Growth, development, and yield of soybean lines developed for forage. *Agronomy Journal*. 93: 1028-1034.

© University of Venda

Divito GA, Sadras VO (2014). How do phosphorus, potassium and sulphur affect plant growth and biological nitrogen fixation in crop and pasture legumes? A meta-analysis. *Field Crops Research*.156: 161-171. doi: 10.1016/j.fcr.2013.11.004.

Dos Santos PC, Fang Z, Mason S, Setuba JC, Dixon R (2012). Distribution of nitrogen fixation and nitrogenase-like sequences amongst microbial genomes. *BMC Genomics* 13:162. doi:10.1186/1471-2164-13-162

Godfray HCJ, Beddington JR, Crute IR, Haddad L, Lawrence D, Muir JF. (2010). Food security: the challenge of feeding 9 billion people. *Science* 327: 812–818. doi: 10.1126/science.1185383

Hassen AI, Bopape FL, Sanger LK (2016). Microbial inoculants as agents of growth promotion and abiotic stress tolerance in plants. *In*: Microbial inoculants in sustainable agricultural productivity, Springer, New Delhi. pp 23-36.

Hassen AI, Khambani LS, Swanevelder ZH, Mtsweni NP, Bopape FL, van Vuuren A, van der Linde EJ, Morey L (2021). Elucidating key plant growth–promoting (PGPR) traits in *Burkholderia* sp. Nafp2/4-1b (=SARCC-3049) using gnotobiotic assays and whole-genome-sequence analysis. *Letters in Applied Microbiology*. 73: 658-671. doi:10.1111/lam.13556.

Hintz RW, Albrecht KA. (1994). Dry matter partitioning and forage nutritive value of soybean plant components. *Agronomy Journal*. 86: 59-62.

Khan MMH, Rafii MY, Ramlee SI, Mashitah J, Mamun MA (2021). AMMI and GGE biplot analysis for yield performance and stability assessment of selected Bambara groundnut (*Vigna subterranea* L. Verdc.) genotypes under the multi-environmental trials (METs). *Scientific Rep*orts.11: 22791. https://doi.org/10.1038/s41598-021-01411-2.

© University of Venda

Lock JM (1989). Legumes of Africa: a check-list. Royal Botanic Gardens.

Mabrouk Y, Hemissi I, Salem IB, Mejri S, Saidi M, Belhadj O (2018). Potential of rhizobia in improving nitrogen fixation and yields of legumes. *In*: Symbiosis. Rigobelo EC (ed). pp 1-15. doi:10.5772/intechopen.73495

Makoi JHJR, Ndakidemi PA, (2011). Changes in plant growth, nutrient dynamics and accumulation of flavonoids and anthocyanins by manipulating the cropping systems involving legumes and cereals - A review. *Australian Journal Agricultural Engineering*. 2(3): 56-65.

Mendoza-Suárez M, Andersen SU, Poole PS, Sánchez-Cañizares C (2021). Competition, Nodule Occupancy, and Persistence of Inoculant Strains: Key Factors in the *Rhizobium*-Legume Symbioses. *Frontiers in Plant Sciences*. 12: 690567. doi: 10.3389/fpls.2021.690567.

Munjonji L, Ayisi KK, Haesaert G, Boeckx P (2018). Screening cowpea genotypes for high biological nitrogen fixation and grain yield under drought conditions. *Agronomy Journal*. doi: 110: 1925-1935, 10.2134/agronj2017.01.0037.

Mpepereki S, Javaheri F, Davis P, Giller KE (2000). Soybeans and sustainable agriculture: promiscuous soybean in Southern Africa. *Field Crops Research*. 65: 137-149.

Oladosu Y, Rafii MY, Abdullah N, Magaji U, Miah G, Hussin G, Ramli A (2017). Genotype x environment interaction and stability analyses of yield and yield components of established and mutant rice genotypes tested in multiple locations in Malaysia, Acta Agriculturae Scandinavica, Section B. *Soil and Plant Science*. 67(7): 590-606. doi: 10.1080/09064710.2017.1321138.

© University of Venda

Olivoto T (2019). Analysing multi-environment trials using GGE. https://tiagoolivoto.github.io/metan/articles/vignettes_gge.html#the-gge-model, (Accessed on 19 June 2022).

Olivoto T, Lúcio AD, da Silva JA, Sari BG, Diel MI (2019). Mean Performance and Stability in Multi-Environment Trials II: Selection Based on Multiple Traits. *Agronomy Journal*. 111(6): 2961-2969. doi: https://doi.org/10.2134/agronj2019.03.0221.

Ormeno-Orrillo E, Menna P, Almeida LGP, Ollero FJ, Nicolas MF, Rodrigues EP, Nakatani AS, Batista JSS, Chueire LMO, Souza RC, Vasconcelos ATR, Megias M, Hungria M, Martinez-Romero E (2012). Genomic basis of broad host range and environmental adaptability of *Rhizobium tropici* CIAT 899 and *Rhizobium* sp. PRF 81 which are used in inoculants for common bean (*Phaseolus vulgaris* L.). *BMC Genomics*. 13(735): 1-26. doi:10.1186/1471-2164-13-735.

Sakala WD, Cadisch G, Giller KE (2000). Interactions between residues of maize and pigeonpea and mineral N fertilizers during decomposition and N mineralization. *Soil Biology and Biochemistry*. 32: 679-688.

SAS Institute (2009). Users Guide: the SAS Statistics System for Windows Version 9.3. Institute Inc., Cary, NC, USA.

Sarmonte SOP, Wilson LT, McClung AM, Medley JC (2005). Targeting cultivars onto rice growing environments using AMMI and SREG GGE biplot analyses. *Crop Science*. 45: 2414-2424.

Shiferaw B, Silim S, Muricho G, Audi P, Mligo J, Lyimo S, You L, Christiansen JL (2005). Assessment of the adoption and impact of improved pigeonpea varieties in Tanzania. *Journal of SAT Agricultural Research*. 3(1): 1-27.

© University of Venda

Shisanya CA (2005). Yield and Nitrogen Fixation Response by Drought Tolerant Tepary Bean (*Phaseolus acutifolius* A. Gray *var. latifolius*) in Sole and Maize Intercrop Systems in Semi-Arid Southeast Kenya . *Journal of Food Technology*. 3: 300-307.

Torabian S, Farhangi-Abriz S, Denton MD (2019). Do tillage systems influence nitrogen fixation in legumes? A review. *Soil Tillage Research*. 185: 113-121.

Vuong HB, Thrall PH, Barrett LG (2017). Host species and environmental variation can influence rhizobial community composition. *Journal of Ecology*. 105: 540–548. doi: 10.1111/1365-2745.12687.

Yang Y, Zhao Q, Li X, Ai W, Liu D, Qi W, Zhang M, Yang C, Liao H (2017). Characterization of Genetic Basis on Synergistic Interactions between Root Architecture and Biological Nitrogen Fixation in Soybean. *Frontiers in Plant Science.* 8: 1466. doi:10.3389/fpls.2017.01466

Yan W, Kang MS, Ma B, Woods S, Cornelius PL (2007). GGE biplot vs. AMMI analysis of genotype–by-environment data. *Crop Science*. 47: 643-655.

Yan W, Tinker N (2005). An integrated biplot analysis system for displaying, interpreting and exploring the genotype x environment interaction. *Crop Science*. 45: 1004-1016.

Yan W (2002). Singular-value partitioning in biplot analysis of multi-environment trial data. *Agronomy Journal*. 94: 990-996.

Yan W, Hunt LA, Sheng Q, Szlavnics Z (2000). Cultivar evaluation and mega-environments investigation based on GGE biplot. *Crop Science*. 40: 597-605.

© University of Venda

Tchakounté GVT, Berger B, Patz S, Fankem H, Ruppel, S (2018). Community structure and plant growth-promoting potential of cultivable bacteria isolated from Cameroon soil. *Microbiological Research*. 214: 47-59.

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7.0 Chapter Seven: Summary, Conclusions and Recommendations

Abstract: Pigeonpea (*Cajanus cajan*) is an important multi-purpose grain legume which is cultivated largely by smallholder growers. The aim of this study was to improve the productivity of pigeonpea. The study generated new information regarding the symbiotic efficiencies of pigeonpea with a broad range of rhizobial strains. The study findings and conclusions as well as their implications are summerized in this chapter.

Key words: genome; housekeeping gene; nitrogen fixation; rhizobial strain.

7.1 Introduction

Pigeonpea (*Cajanus cajan*) is an important multi-purpose grain legume which is cultivated in many parts of the world largely by smallholder growers. In South Africa, pigeonpea is cultivated as a minor crop on a limited hectarage mainly in the eastern coastal belt and the semi-arid north-eastern region. The crop can fix atmospheric nitrogen (N) symbiotically with soil bacteria that are called rhizobia. The aim of this study was to improve the productivity of pigeonpea. The specific objectives of the study were to:

(i) collect and evaluate the symbiotic effectiveness of soil samples from diverse locations across South Africa on pigeonpea genotypes
(ii) determine the genetic relationships among the indigenous rhizobial isolates (strains) from multiple genera that are associated with root nodulation in pigeonpea

(iii) identify and sequence the genome of an indigenous rhizobial strain that was compatible with pigeonpea
(iv) determine the symbiotic effectiveness of the rhizobial strains with pigeonpea and other common tropical legumes that are cultivated in South Africa.

7.2 Summary of the study findings and conclusions

The key study findings were as follows:

(i) forty soil samples that were collected from diverse locations across South Africa and used for inoculating pigeonpea, contained diverse rhizobial isolates (strains) with distinct morphological characteristics

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(ii) there were significant differences in the symbiotic efficiencies of the pigeonpea genotypes suggesting that there was potential to select for optimum host genotype x rhizobial strain combinations for N fixation in pigeonpea and expand the cultivation of the crop to new areas in South Africa (Bopape et al., 2021)

(iii) 57 rhizobial strains (out of 280 putative rhizobial strains) that were isolated from the pigeonpea were subsequently confirmed and deposited into the South African Rhizobium Culture Collection gene bank for future exploitation

(iv) rhizobial strains derived from pigeonpea were characterized using two housekeeping bacterial genes (namely 16S rRNA and *recA*) and identified to the species level (Bopape et al., 2022)

(v) the phylogenetic relationships among these rhizobial strains were determined

(vi) two novel genera of rhizobia (*Phyllobacterium* and *Paraburkholderia*), were associated with root nodulation in pigeonpea (Bopape et al., 2022)

(vii) the genome size of the rhizobial strain (10ap3 - SARCC-755) that was originally isolated from pigeonpea was determined and consisted of a large circular chromosome (6,297,373 bp) and containing the overall G + C content of 60.0%

(viii) the genome of the strain consisted of was 6,013 genes of which 5,833 were associated with proteins that could be assigned to specific functions

(ix) the important genes that were found on the genome of the strain, included the genes for N metabolism, stress response, phosphorus metabolism and iron acquisition, precursor genes for purine synthesis inosine-5-monophosphate and adenylosuccinate (which are also responsible for nodule formation) as well as adenosine monophosphate nucleoside for purine conversion

(x) the nodulation gene (*nolR*), which functions as a DNA binding transcription factor was located on contig 12 of the genome

(xi) the genome of the strain contained no common *nod* and *nif* genes suggesting that an alternative pathway involving a purine derivative was involved in its symbiotic association with pigeonpea (Bopape et al. 2020)

(xii) the genome also possessed some genes that are associated with abiotic stresses and mineral nutrient acquisition thus making it a candidate for future formulation of commercial bio-inoculants especially when considering its high symbiotic efficiency with pigeonpea.

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(xiii) rhizobial strains from all the four distinct genera (*Bradyrhizobium, Paraburkholderia, Phyllobacterium* and *Rhizobium*) could fix N with most of the tropical legumes used in the study

(xiv) two rhizobial strains, namely, *Rhizobium* sp. 36a-PP5 and *Rhizobium* sp. 26a2-PP5, performed best in terms of nodule dry weight with Bambara groundnut and soybean respectively

(xv) two rhizobial strains, namely, *Rhizobium* sp. 33a-PP2 and *Rhizobium multihospitium* 37a-PP4, were identified as ideal for root dry weight and shoot dry weight among the legume species.

7.3 Key recommendations

The study made the following key recommendations:

(i) further testing and validation of the symbiotic efficiencies of the rhizobial strains identified in this study in field trials with diverse legume species and in multiple agro-ecological locations; this could lead to the expansion of the pigeon production area in South Africa and hence an improvement in the quantity of pigeonpea productivity and production in the country as well as food security
(ii) exploit the identified rhizobial strains from this study in developing commercial bio-inoculants for pigeonpea and other common tropical legumes
(iii) determine new bio-inoculants for improving the nitrogen fixation and productivity of tepary bean which is cultivated in arid regions in Limpopo Province (South Africa).

References

Bopape FL, Beukes CW, Kopotsa K, Hassen AI, Steenkamp ET, Gwata ET. (2022). (2022). Symbiotic performance and phylogenetic characterization of pigeonpea (*Cajanus cajan* L. Millsp.) rhizobia occurring in South African soils. *Agriculture. In Review.*

Bopape FL, Gwata ET, Hassen AI, Zhou MM (2021). Symbiotic efficiency of pigeonpea (*Cajanus cajan*) with different sources of nitrogen. *Plant Genetic Resources.* 19(4): 1-4. doi:10.1017/S1479262121000356.

Bopape FL, Hassen AI, Swanevelder ZH, Gwata ET (2020). Draft genome sequence of *Rhizobium tropici* SARCC-755, a free-living *Rhizobium* that nodulated and promoted growth in

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pigeonpea [*Cajanus cajan* (L.) Millsp.] *Microbiology Resource Announcements*. 9: 1-2. doi.org/10.1128/MRA.01122-19.

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APPENDICES

Appendix 3.1

Origins of the soil samples that were used in the study.

Zone of Collection	Soil Sample Designation Code	Province of Origin	EC (mS/m)	Soil pH
1. Coastal (9)	1	Western Cape	212.0	7.1
	2	Western Cape	80.1	7.3
	3	Western Cape	37.4	7.6
	4	KwaZulu Natal	77.6	6.8
	5	KwaZulu Natal	32.6	6.9
	6	KwaZulu Natal	99.1	6.4
	7	KwaZulu Natal	71.6	6.6
	8	KwaZulu Natal	64.8	6.7
	25	Eastern Cape	18.0	7.8
2. Middle interior (11)	19	Free State	22.1	6.5
	20	Free State	56.3	6.0
	21	Free State	33.8	7.0
	22	Northern Cape	17.9	7.5
	23	Northern Cape	17.3	7.4
	24	Northern Cape	50.3	7.1
	26	North West	84.8	7.0
	27	North West	54.5	7.2
	28	North West	42.8	7.2
	29	North West	61.8	6.2
	30	North West	72.7	6.2
3. Northern interior (20)	9	Gauteng	148.6	6.5
	10	Gauteng	72.8	6.8
	11	Gauteng	45.9	6.9
	12	Gauteng	193.7	6.6
	13	Gauteng	37.5	7.6
	14	Gauteng	97.8	7.8
	15	Mpumalanga	49.3	6.5
	16	Mpumalanga	36.7	6.5
	17	Mpumalanga	101.1	7.5
	18	Mpumalanga	97.5	6.6
	31	Limpopo	106.4	6.2
	32	Limpopo	188.4	6.8
	33	Limpopo	100.1	6.4
	34	Limpopo	27.6	6.9
	35	Limpopo	47.2	7.6
	36	Limpopo	66.7	7.2
	37	Limpopo	26.5	6.2
	38	Limpopo	239.0	6.4
	39	Limpopo	57.5	6.9
	40	Limpopo	19.8	7.1

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Appendix 3.2

Composition of the Hoagland solution.

Chemical		Weight / Volume
KCl		100.0g
$Ca_3 (PO_4)_2$		100.0g
$CaSO_4.2H_2O$		100.0g
$MgSO_4.7H_2O$		156.0g
Nafe solution		400.0ml
Trace element		40.0ml
	Nafe solution	
	N_2EDTA	15.6g
	$FeSO_4.7H_2O$	15.0g
	Trace element solution	
	LiCl	0.2g
	$CuSO_4.5H_2O$	1.0g
	$ZnSO_4.7H_2O$	1.0g
	TiO_2	1.0g
	H_3BO_3	1.0g
	$AL_2(SO_4)_3$	1.0g
	$SnCl._2.2H_2O$	0.5g
	$MnCl_2.4H_2O$	7.0g

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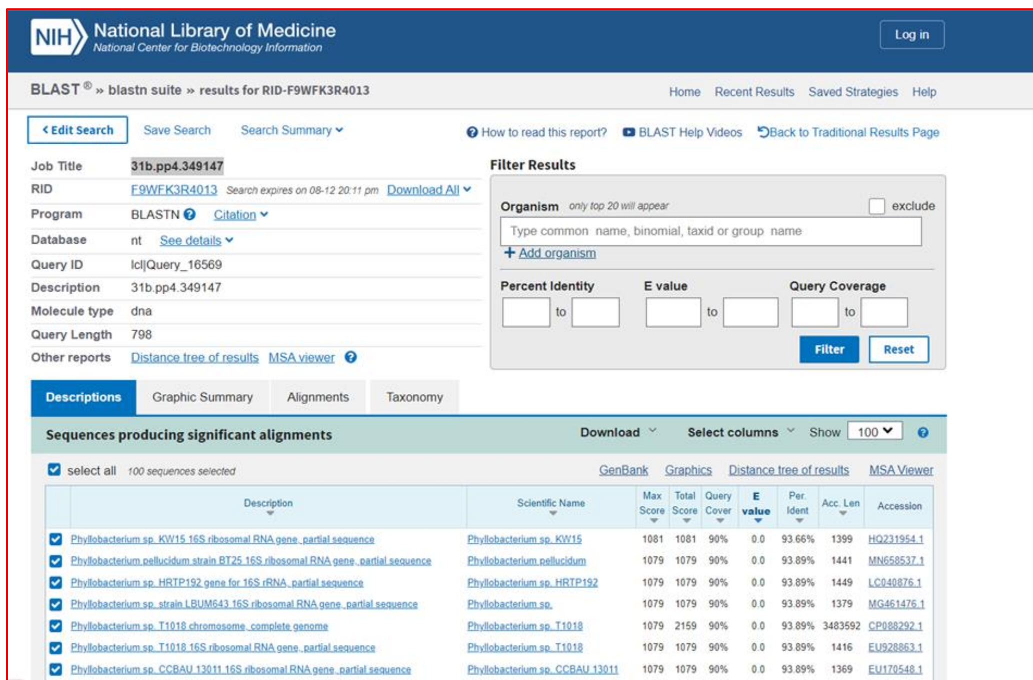
Appendix 4.1

The blast result for isolate '31bp4' indicating its close relationship to *Phyllobacterium* sp KW15 and *Phyllobacterium pellucidum* at 93% similarity grouping.



Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Phyllobacterium sp. KW15 16S ribosomal RNA gene, partial sequence	Phyllobacterium sp. KW15	1081	1081	90%	0.0	93.66%	1399	HQ231954.1
Phyllobacterium pellucidum strain BT25 16S ribosomal RNA gene, partial sequence	Phyllobacterium pellucidum	1079	1079	90%	0.0	93.89%	1441	MN658537.1
Phyllobacterium sp. HRTP192 gene for 16S rRNA, partial sequence	Phyllobacterium sp. HRTP192	1079	1079	90%	0.0	93.89%	1449	LC040876.1
Phyllobacterium sp. strain LBUM643 16S ribosomal RNA gene, partial sequence	Phyllobacterium sp.	1079	1079	90%	0.0	93.89%	1379	MG461476.1
Phyllobacterium sp. T1018 chromosome, complete genome	Phyllobacterium sp. T1018	1079	2159	90%	0.0	93.89%	3483592	CP088292.1
Phyllobacterium sp. T1018 16S ribosomal RNA gene, partial sequence	Phyllobacterium sp. T1018	1079	1079	90%	0.0	93.89%	1416	EU928863.1
Phyllobacterium sp. CCBAU 13011 16S ribosomal RNA gene, partial sequence	Phyllobacterium sp. CCBAU 13011	1079	1079	90%	0.0	93.89%	1369	EU170548.1

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