CHARACTERISATION OF BACTERIAL SYMBIONTS IN SELECTED DROUGHT-TOLERANT LEGUMES FOR BIOFERTILISERS DEVELOPMENT FOR USE IN NAMIBIA

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ABSTRACT

Namibia is a semi-arid country with approximately 1% of arable land. Crop cultivation is profoundly challenged by nutrient-poor sandy soils combined with low water retention. To meet the increasing food demand, farmers resort to applying synthetic fertilisers and pesticides despite their environmental consequences. However, there is increasing evidence that arid or semi-arid plant microbiomes offer an unexploited reservoir that is pivotal to plant health, growth, and development. Plant growth-promoting bacteria (PGPB) are of increased interest as they offer sustainable alternatives to environmentally unfriendly and unsustainable chemical fertilisers. The present study aimed to isolate, identify, and characterise plant-associated bacteria from five drought-tolerant legumes grown in Namibia. Identification was done using 16S rRNA sequencing and bioinformatics. Plant growth-promoting (PGP) abilities were characterised based on exopolysaccharide production, antifungal activity, indole acetic acid production, phosphate solubilization, siderophore production, and bacterial nitrogen fixation. Using 16S Illumina metagenomic sequencing, this study characterised the plant microbiomes of the nodules, roots, rhizosphere, and seeds. Isolates identified from the roots and rhizosphere were from the Proteobacteria (72%), Actinomycetota (15%), Bacteroidetes (5%) and Firmicutes (8%) phyla and included known plant growth-promoting species such as *Stenotrophomonas pavanii, Streptomyces murinus,* and *Enterobacter cloacae*. Nodule endophytes were mostly from the phylum Firmicutes (88%). The identified genera include *Bacillus, Priestia, Paenibacillus, Gottfriedia, Neobacillus, Lysinibacillus, Fictibacillus*, and *Brevibacillus.* Characterisation found that rhizobacteria expressed more plant growth-promoting traits compared to root endophytes. Siderophore production was observed

in most root endophytes and rhizobacteria. The following isolates, CRhi10, CRhi15, MBRhi17, HR5, RMBRhi4, RMBRhi1 and IPCRhi7 from the legume root endospheres and rhizospheres showed the most potential as plant growth promoters. A total of 34 nodule endophytes tested positive for at least one plant growth-promoting trait. Isolates MB1, MB3.1, H14, M25-11, M8-16.1 and M8-16.2 showed the most potential as plant-growth promoters. CRhi15 (*S. maltophilia*), HR5 (*E. mori*), H14 (*P. aryabhattai*), M25-11 (*L. boronitolerans*), and M8-16.1 (*Bacillus sp.*) were selected and assessed for their ability to induce drought tolerance on *Vigna unguiculata* seeds in potted trials. The inoculants were also combined and assessed in a consortium. Drought tolerance was observed to be highest with *S. maltophilia* (CRhi15), *Bacillus sp.* (M8-16.1) and *E. mori* (HR5). The average root length under drought stress was 37.5 cm, 51.8 cm, and 33.7 cm respectively while the average shoot length was 63.1 cm, 80.6 cm, and 75.3 cm. Microbiome analysis of the root, rhizosphere and seed microbiomes found important plant growth-promoting genera. These include *Bacillus, Mesorhizobium*, *Pseudomonas,* and *Bradyrhizobium*. The nodule microbiome was predominantly *Bradyrhizobium.* The relative abundance of the nitrogen-fixing *Bradyrhizobium* genus was determined in four drought-tolerant legume species- *Vigna aconitifolia* (mothbean), *V. unguiculata* (cowpea), *Lablab purpureus* (dolichos), and *Macrotyloma uniflorum* (horsegram). Both culture-dependent and independent methods revealed that these PGP bacteria can promote plant growth under drought, nutrient and biotic stress conditions. Therefore, *S. maltophilia* (CRhi15), *Bacillus sp.* (M8-16.1) and *E. mori* (HR5) may be further explored in field trials in efforts to develop commercial biofertilisers.

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DEDICATION

I dedicate this work to my mother, Mary Nyaradzayi Mataranyika, who has been the prime example of determination and hard work, and to my grandmother, Esther Susan Zvafadza Mvere Kutya Soko, I hope I have, even to the slightest bit, made you proud.

DECLARATIONS

I, Paidamoyo Natasha Mataranyika, hereby declare that this study is my own work and is a true reflection of my research, and that this work, or any part thereof has not been submitted for a degree at any other institution.

Paidamoyo N. Mataranyika	_____	October, 2023
Name of Student	Signature	Date

ABBREVIATIONS AND ACRONYMS

ACC:	1-aminocyclopropane-1-carboxylate
ADP:	Adenosine diphosphate
ARA:	Acetylene reduction assay
ASV:	Amplicon sequence variant
ATP:	Adenosine triphosphate
BLAST:	Basic Local Alignment Search Tool
BNF:	Biological nitrogen fixation
CAP:	Canonical analysis of principal coordinates
DNA:	Deoxyribonucleic acid
DYMV:	Dolichos yellow mosaic virus
FAO:	Food and Agriculture Organization
FLNF:	Free-living nitrogen fixing
HPLC:	High-performance liquid chromatography
IAA:	Indole acetic acid
IWMI:	International Water Management Institute
LB:	Luria-Bertani
mRNA:	Messenger RNA
NBRIP:	National Botanical Research Institute's phosphate medium
NCBI:	National Center for Biotechnology Information
OTU:	Operational taxonomic unit
PERMANOVA:	Permutational multivariate analysis of variance
PCA:	Principal component analysis
PCoA:	Principal coordinate analysis

PCR:	Polymerase chain reaction
PGP:	Plant growth promoting/ promotion
PGPB:	Plant growth-promoting bacteria
PGPR:	Plant growth-promoting rhizobacteria
RNA:	Ribonucleic acid
rRNA:	Ribosomal RNA
ROS:	Reactive oxygen species
SEA:	Soil extract agar
SOC:	Soil organic carbon
TSA:	Tryptic soy agar
WC:	Water crowding
WS:	Water scarcity
WSI:	Water stress indices
YEM:	Yeast extract mannitol medium

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LIST OF PUBLICATIONS/CONFERENCE PROCEEDINGS

List of publications

1. **Mataranyika PN** and Chimwamurombe PM (2021). Factors influencing dryland agricultural productivity. *Journal of Arid Environments* 189, 104489. DOI: 10.1016/j.jaridenv.2021.104489
2. **Mataranyika PN**, Chimwamurombe PM, Venturi V and Uzabakiriho, JD (2022). Bacterial bioinoculants adapted for sustainable plant health and soil fertility enhancement in Namibia. *Front. Sustain. Food Syst.* 6:1002797. DOI: 10.3389/fsufs.2022.1002797
3. **Mataranyika PN**, Venturi V, Bez C, Chimwamurombe PM, Uzabakiriho, JD. Rhizospheric, seed, and root endophytic associated bacteria of drought tolerant legumes grown in arid soils of Namibia. *Submitted to Microbiological Research.*
4. **Mataranyika PN**, Bez C, Mengoni A, Vaccaro F, Olanrewaju OS, Chimwamurombe PM, Uzabakiriho JD, Venturi V. *Bradyrhizobium sp. nov*., and *Rhizobium sp. nov*. and plant growth promoting bacteria from drought tolerant legumes grown in Namibia. *In preparation.*

Conference Proceedings

1. Presented at the University of Namibia Research Seminar (Plant microbiome for sustainable agriculture), University of Namibia, Windhoek, Namibia. April 2022

   Title: Illumina sequencing of rhizospheric, seed, and root endophytic associated bacteria of drought tolerant legumes in Namibia.

2. Presented at the Next Einstein Forum's Botswana Ambassador Conference, University of Botswana, Gaborone, Botswana. June 2022.

   Title: Isolation and identification of rhizosphere and root endosphere-associated bacteria of *Vigna radiata, Vigna aconitifolia, Vigna unguiculata, Lablab purpureus* and *Macrotyloma uniflorum.*

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CHAPTER 1

Introduction

1.1. Background of the study

Namibia is a country located in southwestern Africa with a climate that ranges from semi-arid to arid. It lies between the Namib Desert on the west and the Kalahari Desert on the east. The country is usually dry during the greater part of the year only receiving between 50 mm (in the south and along the western coast) and 700 mm (in the north-eastern region) of rainfall. The Kavango regions (East and West) receive approximately 527 mm a year. It is also prone to frequent droughts which affect agricultural productivity (Strohbach & Petersen, 2007; Braker *et al*., 2015; Watanabe *et al*., 2019). This productivity also heavily depends on rainfall (Muhoko, de Wassseige & de Cauwer, 2020). Given that approximately 70% of the Namibian population relies on the productivity of subsistence farmers, there is a need to develop sustainable practices that cater to both the environment and farmers (Braker *et al*., 2015).

Climate change continues to negatively impact dryland regions. The agricultural sector, as a result, suffers many losses which affect food security. Namibia is not exempted from these effects as it is a vulnerable region due to the arid to semi-arid climates (Reid *et al*., 2008). As such, with limited arable land (approximately 1%), Namibia is susceptible to food shortages and food insecurity, and there is, therefore, a need to find ways around these agricultural constraints (Wingate *et al*., 2016; Food and Agriculture Organization, 2022). The inclusion of sustainable soil remedial

practices in agriculture would sufficiently help mitigate these challenges (Luchen *et al*., 2018). Furthermore, the use of functional nitrogen-fixing symbioses would actively improve crop production (Finkel *et*., 2017).

These remedial efforts may be employed using environmentally sustainable biofertilisers. It has been documented that the interactions between plants and their PGP microbiomes have the potential to exert plant growth and manage soil and plant health. This increases the agricultural productivity of important crops including legumes. Such bacteria can be used as biological inputs for crop production (Turner, James & Poole, 2013). Therefore, the use of chemical-based fertilisers which often result in environmental degradation will be eliminated.

The majority of soils in Namibia are typically sandy soils with poor water and mineral retention capacity (Strohbach & Petersen, 2007; Watanabe *et al*., 2019). The soils in the Kavango regions are predominantly arenosol. They exhibit poor nutritional quality and mineral retention (Strohbach & Petersen, 2007; Strohbach, 2013) These soils are porous and offer limited support for crop production due to their low fertility and poor water retention properties. As a result, subsistence farmers often resort to using small patches of land to grow crops (Mendelsohn, 2009). Furthermore, observations have noted that crop production in the Kavango area often leads to low yield and further deterioration of crop yields over time (Strohbach, 2013).

Furthermore, mineral nitrogen deficiency is an important limiting factor for plant growth, functional nitrogen-fixing symbiosis would actively improve crop production (Wingate *et al.*, 2016; Finkel *et al.*, 2017). Overuse has also led to the low organic content of carbon and nitrogen in the soils. Consequently, this negatively affects soil

fertility and crop production (Braker *et al*., 2015). A combination of these different factors contributes to the state of agricultural productivity in the Kavango East region.

These challenges, though important to agricultural productivity, are not unique to Namibia. Over the past decades, there has been an increase in dryland agriculture and subsequently the need for sustainable agricultural practices. These needs are further intensified as the global population continues to grow (Lugtenberg *et al*., 2013). Therefore, research efforts have been aimed at developing methods and products which not only improve agricultural productivity but also have minimal environmental impacts. This has led to increased research in plant-microbe interactions. It has been documented that the interactions between plants and their PGP microbiome have the potential to exert plant growth and manage soil and plant health. This increases the agricultural productivity of important crops including legumes. PGPB in legumes, for example, can fix nitrogen or carry out other plant growth activities (Khandare *et al*., 2020). Therefore, such bacteria can be used as biological input for agricultural improvement (Turner, James & Poole, 2013).

A better understanding of the plant-microbe interactions in the Namibian context will provide possible sustainable solutions to tackling crop stresses and improving crop production in drylands (Turner, James & Poole, 2013). Therefore, this study aimed to isolate, assess, and analyse PGPB in five legume species known to be highly nutritious and well adapted to arid climates and nutritionally poor soils. These legumes are dolichos [*Lablab purpureus* (L.) Sweet var. Lignosus Prain], horsegram (*Macrotyloma uniflorum* Var. Madhu), mung bean [*Vigna radiata* (L.) R. Wilczek var. radiata], moth bean [*Vigna aconitifolia* (Jacq.) Marechal], and cowpea (*Vigna unguiculata* L. Walp).

1.2. Study area

Kavango East region (Figure 1.1) is part of the former Kavango region of North-eastern Namibia. The Kavango East region experiences a hot semi-arid climate. Temperatures average around 23°C with normal temperatures exceeding 30°C most of the year and a minimum of 10°C in the winter seasons. Annual rainfall ranges between 500 mm and 700 mm (Muhoko, de Wassseige & de Cauwer, 2020). The soil type is dominantly arenosol, however, small patches of calcisol and solonetz are present in the Kavango East region.

They are low-nutrient soils with organic carbon typically around 0.4% while nitrogen ranges between 0.03% and 0.16%. The pH ranges between 5.5 and 7.5 with the lower end of the spectrum observed more often (Grönemeyer *et al.*, 2012). This study focused on the Bagani settlement area (18°7′S 21°37′E) in the Kavango East region. The physicochemical composition of the soils in Bagani, Kavango East as described by Horn, Ghebrehiwot and Shimelis, (2016) are shown in Table 1.1.

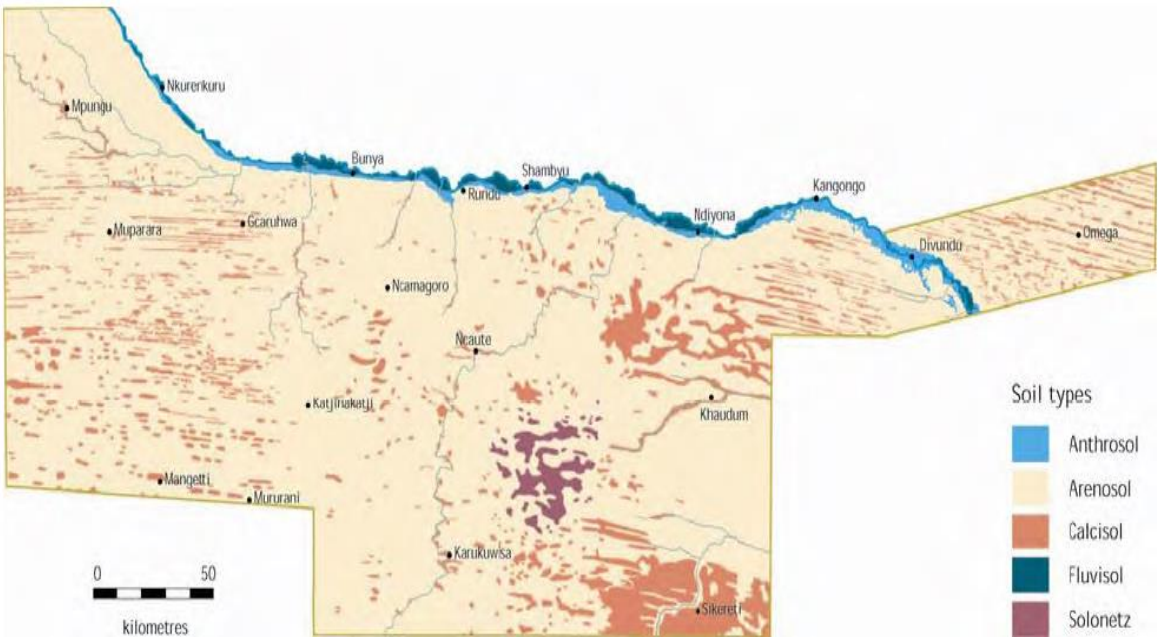

Figure 1.1: The soil profile of the Kavango regions. Adapted from Land use in Kavango: past, present, and future (Mendelsohn, 2009).

Table 1.1: Physicochemical properties of the soil in Bagani, Kavango East.

Parameter	Value
Soil pH	7.50
Total nitrogen (%)	0.06
Organic carbon (%)	0.48
Phosphorus (ppm[1])	58.20
Potassium (me[2] %)	0.90
Calcium (me[2] %)	1.30
Magnesium (me[2] %)	1.70
Manganese (me[2] %)	0.18
Copper (ppm[1])	0.60
Iron (ppm[1])	0.70
Zinc (ppm[1])	0.50
Sodium (%)	0.09
EC[3] (mS/cm)	0.18

[1]ppm- part per million
[2]me- milliequivalent
[3]EC- electrical conductivity

1.3. Problem statement

The problem faced by subsistence farmers in northern Namibia is usually frequent droughts and poor soils which limit agricultural production. This adversely affects food security in the regions. This problem affects Namibia where only 1% of the land is arable (Food and Agriculture Organization, 2022) while over 70% of the population relies on subsistence farming (Braker *et al*., 2015). Furthermore, approximately 24% of children in Namibia under the age of 5 years are categorised as malnourished.

Undernutrition, however, dominates the number of cases reported. It is attributed to, among other socioeconomic factors, food insecurity (Mataranyika *et al*., 2020). In addition, the nitrogen deficient soils common in Kavango East and other parts of Namibia limit adequate agricultural production. To improve crop production and quality, subsistence farmers often resort to using chemical fertilisers which are both expensive and detrimental to the environment (Abah *et al*., 2014). Therefore, there is a crucial need to address the state of malnutrition and the negative environmental impact of chemical fertilisers in Namibia.

1.4. Research aim

This study sought to identify and characterise the PGP properties of bacterial symbionts associated with five drought-tolerant legumes. This study also aimed to present the microbiomes associated with the roots, root nodules, rhizosphere, and seeds of the legumes. Lastly, the study aimed to assess the bacterial isolates as bioinoculants within the context of the Namibian soil profile and climate.

1.5. Research objectives

a. To isolate and identify PGPB from roots, rhizosphere, and root nodules of horsegram (*M. uniflorum*), mung bean (*V. radiata*), cowpea (*V. unguiculata*), dolichos (*L. purpureus*) and two accessions of moth bean (*V. aconitifolia*) obtained from the National Bureau of Plant Genetic Resources (NBPGR), India in July 2019.
b. To determine the drought stress tolerance abilities and PGP traits of the isolated bacteria, particularly siderophore production, phosphate solubilization, indole acetic acid (IAA) production, exopolysaccharide (EPS) production, nitrogen fixation and antifungal activity against *Fusarium graminearum.*

c. To carry out microbiome analysis of microbial communities from the rhizosphere, roots, root nodules and seeds from the five species of legume.
d. To investigate enhanced plant growth abilities of isolated bacterial strains as bioinoculants assessing crop yield and stress tolerance in locally grown legumes.

1.6. Research questions

a. What PGPB are present in the roots, root nodules and rhizosphere of horsegram (*M. uniflorum*), mung bean (*V. radiata*), moth bean (*V. aconitifolia*), cowpea (*V. unguiculata*) and dolichos (*L. purpureus*)?
b. What PGP properties do these isolated microbes possess?
c. How do these PGPB perform in pot trials on the growth of cowpea plants under drought stress conditions?

1.7. Significance of the study

The current state of low agricultural lands (1% of the land is arable) and food insecurity (over 70% of rural populations rely on subsistence farming) are not without solutions (Braker *et al*., 2015; Food and Agriculture Organization, 2022). In Namibia, the isolation and characterisation of plant growth-promoting bacteria (PGPB) associated with the legume species dolichos [*Lablab purpureus* (L.) Sweet var. Lignosus Prain], horsegram (*Macrotyloma uniflorum* Var. Madhu), mung bean [*Vigna radiata* (L.) R. Wilczek var. radiata], moth bean [*Vigna aconitifolia* (Jacq.) Marechal], and cowpea (*Vigna unguiculata* L. Walp) have not been done. Particularly with Namibian soil profiles in mind. In addition, there is limited literature regarding the plant-microbe associations of these legumes, particularly in the African context. This research will

identify PGPB and provide possible alternatives to environmentally harmful chemical fertilisers. This will assist in reducing the use of expensive environmentally harmful chemical fertilizers particularly by subsistence farmers in Namibia. Therefore, it can be hypothesized that PGP bacterial inoculants will reduce plant susceptibility to drought stress, increase crop yield and mitigate food insecurity in the region. Furthermore, the functionality of the findings of this study will be available to agricultural sectors.

1.8. Limitations of the study

The major limitation faced in this study was the inadequate availability of equipment and resources to carry out studies on the bacterial microbiomes. The design of the project also anticipated the availability and access to fields for field trials. However, field studies could not be done due to limited access and funds. In addition, the COVID-19 pandemic significantly impacted availability of funds, cost of consumables and timely delivery of consumables.

1.9. Delimitations of the study

This study focused on the microbial PGP traits within the roots, root nodules and rhizosphere of five legume species (*M. uniflorum*, *V. radiata*, *V. aconitifolia*, *V. unguiculata* and *L. purpureus*) grown in soil obtained from Bagani, Kavango East region. Their ability to potentially increase yield as biofertilisers were assessed. Plant growth promotion assessment was limited to six known traits to inform towards stress tolerance. These are siderophore production, phosphate solubilization, IAA production, EPS production, nitrogen fixation and antifungal activity against *F.*

graminearum. Microbiome analysis was carried out on root endophytes, nodule endophytes, seed endophytes and rhizobacteria.

1.10. Research Ethics

The seeds were imported from the National Bureau of Plant Genetic Resources, India through the facilitation of a Material Transfer Agreement (MTA). In addition, Namibia was issued with a phytosanitary certificate by the Indian National Bureau of Plant Genetic Resources body. Ethical clearance was obtained from the University of Namibia Research Ethics Committee (UREC) and research permission was obtained from the Centre for Postgraduate Studies (CPS), University of Namibia to carry the research.

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CHAPTER 2

Literature review

2.1. Introduction

The world's growing population is estimated to reach 8.3 billion by 2025. This increase is directly linked to an increase in demand for food supply which will see food demand increasing by close to 100% more to feed the total global population by 2050 (Lugtenberg *et al*., 2013). However, food security is increasingly threatened by reduced arable lands, pests, diseases (Ezemenaka & Ekumaoko, 2018), socioeconomic instability (Li *et al*., 2018), wars (Couttenier & Soubeyran, 2014), and climate change (Cowie *et al*., 2011). Furthermore, conventional agricultural systems have been relying on the injudicious use of herbicides, fertilizers, and pesticides to obtain higher yields from food crops. These have contributed to land pollution and degradation (Akhtar-Schuster *et al*., 2017; Liu, Carvalhais, *et al*., 2017).

Over the past three decades, increasing detrimental effects of climate change have affected agricultural productivity around the world. A rise in drought occurrences has also contributed to a worsening state of water resources affecting agricultural production. Drought stress, therefore, plays a crucial role as a limiting factor in the state of food security across the world (Omar *et al*., 2021). Though not the only challenge, it contributes to diminished plant growth, quality, and yield (Botai *et al*., 2019). Production of cereals has decreased by up to 10% while crop production may significantly reduce in more than 50% of arable lands worldwide due to drought events (Ngumbi & Kloepper, 2016).

These factors in varying degrees all contribute to reduced crop yields and hampered access to food for many people around the world. Through scientific advancement, however, crop yield and soil quality may be improved through the use of plant growth-promoting (PGP) microbes or biofertilisers (Jilani *et al*., 2007; Khosro & Yousef, 2012; Olanrewaju, Glick & Babalola, 2017).

Legumes have been a crucial point of discussion concerning the extraction and use of PGP microbes. In addition to the beneficial plant-microbe interactions harnessed by legumes, they are also a top pick due to their nutritional benefits which include high protein content, nutritionally beneficial antioxidants and micronutrients (Caprioli *et al*., 2016; Bahroun *et al*., 2018). Plant-microbe interactions in chickpea (*Cicer arietinum*), common bean (*Phaseolus vulgaris L*), and Bambara groundnut (*Vigna subterranean*), for example, have been studied extensively. They have been found to possess beneficial microbes that may be used in the improvement of soils (Remans *et al*., 2008; Ajilogba & Babalola, 2020; Swarnalakshmi *et al*., 2020).

Plant-microbe interactions with particular attention to nitrogen-fixing bacteria are the main focus as nitrogen is one of the essential components in agriculture (Oldroyd & Dixon, 2014). This is of particular importance because high nitrogen content in soil contributes to an increase in seed yield and a higher quality of protein. Biological nitrogen fixation has been noted to be carried out by legumes with or without microbial assistance. It is closely linked to bacterial symbionts in nodules. However, it has been noted that nitrogen fertilisers decrease biological nitrogen fixation (Finkel *et al*., 2017; Tamagno *et al*., 2018). Rhizobia typically fix nitrogen but it has been noted that non-rhizobial bacteria such as *Bacillus subtilis* can also fix nitrogen (Dudeja & Giri, 2014; Martínez-Hidalgo & Hirsch, 2017). In addition to fixing nitrogen, plant endophytes

can improve general plant health and may help mitigate some abiotic and biotic stresses.

Furthermore, there is a rising concern about the harmful effects of chemical fertilisers also referred to as inorganic fertilizers. The most essential chemicals in crop production are phosphorus and nitrogen. Though nitrogen is not a rare element, it often requires supplementation in the form of nitrogen-based fertilizers (Oldroyd & Dixon, 2014). The effects of chemical pollution from inappropriate use and overuse of fertilisers negatively affect humans often causing chronic illnesses (Wimalawansa & Wimalawansa, 2014). Soils accumulate trace elements which may be taken up by plants and enter the food chain. In addition, the eutrophication of reservoirs and large water bodies is also of great concern. This is due to the accumulation of minerals in the physical environment. This has led to the restricted use of phosphorus-based fertilisers in many developed countries (Jiao *et al*., 2012; Wimalawansa & Wimalawansa, 2015).

Therefore, this literature review unpacked horsegram, mung bean, moth bean, cowpea and dolichos as nutritional sources. In addition, it also presented available knowledge around their beneficial plant-microbe associations. This review also sought to point out existing gaps in the literature about the plant-microbe associations with these legumes in Namibia.

2.2. Nutritional value of the selected legumes

Legumes are rich in protein and fibre and offer great nutritional support, particularly as a protein source. Legumes also offer high protein value and low-fat food in the form of seeds. In addition, they extend non-nutritional benefits such as anticancer phenolic compounds, and minerals and reduce the risk of diabetes and cardiovascular diseases

(Miedzianka *et al*., 2017). Different legumes contain valuable nutrients in varying compositions. This section details the nutrient composition of legumes such as pigeon pea horsegram, mung bean, cowpea and dolichos. It also presents some known medicinal uses of these legumes.

Cajanus cajan (L.) Millspaugh (pigeon pea) is a good example of these highly functional legumes. This is due to its durability as a crop whose functionality and value are increased by its nutritional content. This is shown by the medicinal properties of the leaves and stems in addition to the nutrient composition. The inedible parts of the plant may be used to treat bronchitis, measles, malaria and hepatitis (Ayenan *et al*., 2017; Syed & Wu, 2018). Furthermore, it has been observed to yield satisfactory output results even in dry conditions and low-quality soils. As a food crop, it may be incorporated into diets as flour, in bread or nutrition bars in addition to being eaten as seeds. Previous studies have found the protein content within pigeon pea seeds ranging between 19.3 and 25.5%. Mature seeds may contain approximately 63 g/100 g of carbohydrates and 15 g/100 g of fibre with suitable concentrations of iron, calcium and manganese among other essential micronutrients (Chaudhari *et al*., 2017; Obala *et al*., 2018).

Therefore, pigeon pea may be used as a reference crop. Its ability to grow in arid conditions while still being highly nutritious is a desirable trait in crops. These traits, however, are not unique to pigeon pea. Many drought-tolerant nutritious legumes are commonly cultivated and consumed in arid regions of the world. The legumes, horsegram, mung bean, moth bean, cowpea and dolichos, as with pigeon pea, are of particular interest due to both their high nutritional composition and drought tolerance abilities. These legumes are discussed in detail below.

The protein content of several dolichos (*Lablab purpureus*) accessions is similar to that of pigeon pea ranging between 18.8 and 24.5%. However, analysis of dry weight has shown higher protein concentrations of approximately 27% (Pranesh & Ramesh, 2019; Purwanti, Prihanta & Fauzi, 2019). *L. purpureus* has a high carbohydrate content constituting 54-63% of the total seed mass (Hossain *et al*., 2016). Compared to dolichos and pigeon pea, horsegram (*Macrotyloma uniflorum*) beans contain lower concentrations of protein with an average amount of 20.8%. The mineral content of horsegram bean ranges between 210.3-290.0 mg/100 g for calcium and 8.1-10.0 mg/100 g for minerals in 5 of the most common varieties consumed (Bhartiya *et al*., 2017; Patil & Kasturiba, 2019). However, horsegram also contains anti-nutrient compounds such as tannins and phytates that limit nutrient quality (Fuller & Murphy, 2018).

Species within the *Vigna* genus also offer competitive nutritional values. The protein content of mung bean (*V. radiata*) ranges between 21.0-23.3% with wild mung bean varieties containing most essential amino acids (Yi-Shen, Shuai & Fitzgerald, 2018). Some varieties in Australia have been noted to contain up to 30.1% of protein with carbohydrate content ranging between 45.5 and 53.5%. The protein content of moth bean (*V. aconitifolia*) typically ranges between 18.9-26.1%, however, a study on moth bean seeds from Nigeria recorded crude protein content of approximately 14.1% (Bhardwaj & Hamama, 2016; Opara, Egbuonu & Obike, 2017; Badami, Kasturiba & Ag, 2019). Compared to the other two species in the *Vigna* genus, cowpea (*V. unguiculata*) seeds recorded the highest protein content with ranges from 203 to 394 g/kg and with an approximate average content of 24.3% (Chikwendu, 2015; Gonçalves *et al*., 2016), while fat and carbohydrate content averaged 1.2% and 54.0%

respectively. Table 2.2.1 below summarises the nutritional composition of the 6 legumes. The data available represents approximate average values based on literature.

Table 2.1: Nutritional composition of pigeon pea, horsegram, mung bean, moth bean, cowpea and dolichos.

Legume	Protein (%)	Fats (%)	Carbohydrates (%)	Ash (%)	Reference
C. cajan	22.1	1.5	62.8	-*	Obala *et al.*, 2018; Syed & Wu, 2018
L. purpureus	21.7	0.5	58	3.9	Hossain *et al.*, 2016; Pranesh & Ramesh, 2019; Purwanti, Prihanta & Fauzi, 2019
M. uniflorum	20.5	1.4	53.6	-*	Bhartiya *et al.*, 2017; Fuller & Murphy, 2018; Patil & Kasturiba, 2019
V. radiata	26.9	1.9	49	3.2	Skylas, Blanchard & Quail, 2017; Yi-Shen, Shuai & Fitzgerald, 2018
V. aconitifolia	21.9	3.5	66.4	2.81	Bhardwaj *et al.*, 2016; Opara, Egbuonu & Obike, 2017; Badami, Kasturiba & Ag, 2019
V. unguiculata	24.3	2.1	59.1	0.96	Chikwendu, 2015; Gonçalves *et al.*, 2016

*Average ash content values could not be obtained

2.3. Stresses affecting agricultural productivity

Various forms of stress affect agricultural production across the world. These may be abiotic or biotic stresses. Abiotic stresses are defined as pressures that arise from the

environment. These include drought, extremes of temperatures (which include freezing), abnormal salt levels and nutrient abnormalities (Suzuki *et al*., 2014; Enebe & Babalola, 2018). Abiotic stresses may also influence the extent to which biotic stresses affect plants. These effects may cause oxidative damage to plant cells, increasing susceptibility to pathogenic infections and pests. A combination of both types of stresses increases the potential threat to crop yield (Haggag *et al*., 2015; Pandey *et al*., 2017).

Biotic stresses on the other hand arise from biological organisms such as pests or pathogenic microorganisms (Suzuki *et al*., 2014; Hashem, Tabassum & Fathi Abd_Allah, 2019). Microbial infections in plants may be fungal or bacterial causing a wide range of diseases that affect yield and crop quality (Chauhan, Yogindran & Rajam, 2017). Together, biotic and abiotic stresses are important factors in agriculture. As such, stress tolerance among legumes is a desirable trait for crop production.

2.3.1. Abiotic stresses

Drought

Drought is often defined as an extended period with reduced or sub-optimum rainfall (Long, 2021). It is further defined based on the environmental impact and influence which encompass meteorological, agricultural, and hydrological aspects (Esfahanian *et al*., 2017). A drought event can cause devastating damage to the agriculture sector. The United States of America records losses between €5,000,000 and €7,000,000 per year while the average loss in Europe approximates €3 billion per year over the past 30 years. The effects in Africa are equally devastating. A report published in 2018 by the Food and Agriculture Organization (FAO), estimated that in Africa, losses due to drought reached an upward of €9 billion between 2005 and 2015 (Food and

Agriculture Organization of the United Nations, 2018). Droughts between 2010 and 2011 in the Great Horn of Africa resulted in almost 260,000 deaths (Ahmadalipour *et al*., 2019). Therefore, drought and high temperatures occurring simultaneously, for example, are even more destructive to crop production and subsequently crop yields (Pandey *et al*., 2017). A brief overview of drought periods in some parts of Africa is shown in Table 2.

The morphological effects that droughts have on plants are the main causes of the reduced productivity of crops. These effects often present as reduced germination rate and seedling growth. Stunted plant growth is also often observed with decreased leaf, root and overall plant size (Hanaka *et al*., 2021). The plant-water potential is a parameter measured as a reflection of water energy in plants and is negatively affected by droughts. Drought stress reduces plant water potential which affects the transport of nutrients from the soil to the leaves. Plant fresh weight and biological processes such as photosynthesis which rely on water availability and nutrient transportation are also negatively affected by water stress (Ngumbi & Kloepper, 2016). Furthermore, drought stress negatively affects the biochemical processes that function intending to protect the plant. Due to the reduced availability of water, free radicals are stimulated which target antioxidants and reactive oxygen species (ROS). This results in protein and nucleic acid degradation, and the weakening of membranes (Vurukonda *et al*., 2016).

Future occurrences of droughts are progressively made probable by increased deforestation, soil quality degradation, growing water demand and climate change often characterised by global warming (Gebremeskel Haile *et al*., 2019). The dangers of drought, therefore, cannot be ignored. The reduction in crop yield results in a

cascade of effects which include food insecurity, socio-economic downfall, displacement of populations and deaths (Botai *et al*., 2019). It is, therefore, important to find ways to reduce the effects of drought. This is done essentially by preparing for it and putting in place mitigating measures to reduce the effects.

As previously mentioned, drought issues reflect larger climate issues. Efforts to reduce the impact of droughts may range from enforcing effective water use strategies or improving agricultural technologies that help mitigate the effect of droughts. In addition, microbes may be used to reinforce the natural plant defence mechanisms against droughts. Endophytes such as bacteria may be used to induce drought tolerance. The endophytes either trigger the plant's stress response system or secrete biochemicals which counter the effects of stress (Lata *et al*., 2018).

Bacteria with PGP properties may be used to improve drought tolerance in crops. The genus *Pseudomonas* has some species that have been found to actively improve drought tolerance by improving germination rates under water stress (García-fraile, Menéndez & Rivas, 2015). *Rhizobium* species also offer drought tolerance support to plants by preventing the accumulation of ROS (Hanaka *et al*., 2021).

Table 2.2: Significant drought periods in some African regions.

Region	**Period**	**Major Effects**	**Reference**
South Africa	2015-2016	Reduced harvest of grain crops, grain shortages, increased unemployment	Baudoin *et al.*, 2017; Botai *et al.*, 2019
East Africa (Horn of Africa)	2010-2011	Food insecurity results in malnutrition, epidemics,	Haile *et al.*, 2019

		famines, and high mortalities	
Southern Africa (Limpopo River basin)	1991–1992, 1994–1995 (severe drought), 2003–2004, 2005–2006	Food insecurity	Trambauer *et al.*, 2014
Namibia	2014-2015	Reduced grazing lands, food insecurity, water shortages	Schnegg & Bollig, 2016
Zimbabwe	1994-1995 (severe drought), 2001-2002	Food insecurity, soil degradation, the decline in the stock exchange market	Centre for Development Research and Information in Southern Africa (CEDRISA), 2009

Molecular analyses of plants that have been observed to grow in arid climates have highlighted the ways plants tolerate abiotic stress such as drought. Physiological and morphological mechanisms employed by plants to better survive drought periods include a fast-growing penetrating root system, wide canopy and trailing growth pattern (Tiwari *et al*., 2018). The unique molecular markers responsible for this ability point towards drought tolerance. Drought tolerance, therefore, is a desirable characteristic in agriculture as it allows plants to survive and/or complete life cycles despite a reduction in available water (Tardieu, Simonneau & Muller, 2018).

Advances in biotechnology have allowed for the development of techniques aimed at reducing the impact of droughts and efficiently predicting drought tolerance in seeds. Prior to planting, seeds may be tested for drought susceptibility scores that essentially provide information on the ability of the seedlings to withstand drought as the plant grows. Alternatively, molecular markers may be used to identify genetic diversity linked to drought tolerance (Sarkar *et al*., 2017; Ajayi, Gbadamosi & Olumekun, 2018; Iseki *et al*., 2018).

A similar method has been used to improve seed stress tolerance via marker-assisted selection. This has been used in improving drought tolerance in cowpea varieties in Africa using single nucleotide polymorphism (SNP) markers. A study done in Burkina Faso utilised drought-tolerant genes from two lines observed to remain green during times of low water availability (Benoit *et al*., 2016). Some common legumes known to tolerate drought include *L. purpureus, C. cajan* and species in the genus *Vigna* (Robotham & Chapman, 2017; Sarkar *et al*., 2017; Iseki *et al*., 2018).

Water scarcity

The increase in frequency and intensity of droughts present another complication. As the state of land degradation increases, an already existing problem presents itself as only worsening- water scarcity. Water scarcity refers to a state in which water demand exceeds availability in this case with respect to natural reservoirs (El Kharraz *et al*., 2012). Depleting water availability is one of the leading indications of land degradation (Prăvălie, 2016; Pacheco *et al*., 2018). As such agricultural water availability is threatened by the reduction of surface and groundwater due to diminishing biomass (Akhtar-Schuster *et al*., 2017). This results in less water available for both domestic and agricultural use.

Water scarcity may occur as a combination of poor management of resources, inaccessibility (as in absence of clean safe water) and droughts (Mukheibir, 2010; El Kharraz *et al*., 2012; Vallino, Ridolfi & Laio, 2020). Another major contributing factor to water scarcity is climate change (Schewe *et al*., 2014). In 2012, approximately 1 billion people (Bogardi *et al*., 2012) did not have access to safe water, this number has since increased to 2.3 billion (Rosa *et al*., 2020). Additionally, 4 billion people are estimated to be affected by water scarcity every month (Mekonnen & Hoekstra, 2016). Therefore, access to sufficient safe water is an essential aspect as a means to reduce disease prevalence and social ills including poverty (Mukheibir, 2010).

The greatest amount of water is utilized at a large scale in industrial settings and agricultural irrigation. The utilized resource is usually drawn from rivers, lakes and underground water reserves (Schewe *et al*., 2014). Water scarcity poses a great threat to the agricultural sector which relies heavily on the availability of sufficient water for production (Falkenmark, 2013). Food production, and consequently food security, is heavily reliant on the availability of water (Fereres, Orgaz & Gonzalez-Dugo, 2011; Assouline *et al*., 2015). Though not directly accessible to plants, the amount of rainfall influences vegetative cover over land as rainwater is accessible through soil moisture (Ibrahim *et al*., 2015). Water scarcity leads to increased plant stress which results in several environmental implications including increased salt concentration and soil erosion (Lanfredi *et al*., 2015). With increasing populations, land degradation and continuous climate change, there is an urgent need to improve water management systems in order to better improve the state of water scarcity (Bogardi *et al*., 2012). Figure 2.1 below illustrates the threat level of water scarcity across the globe.

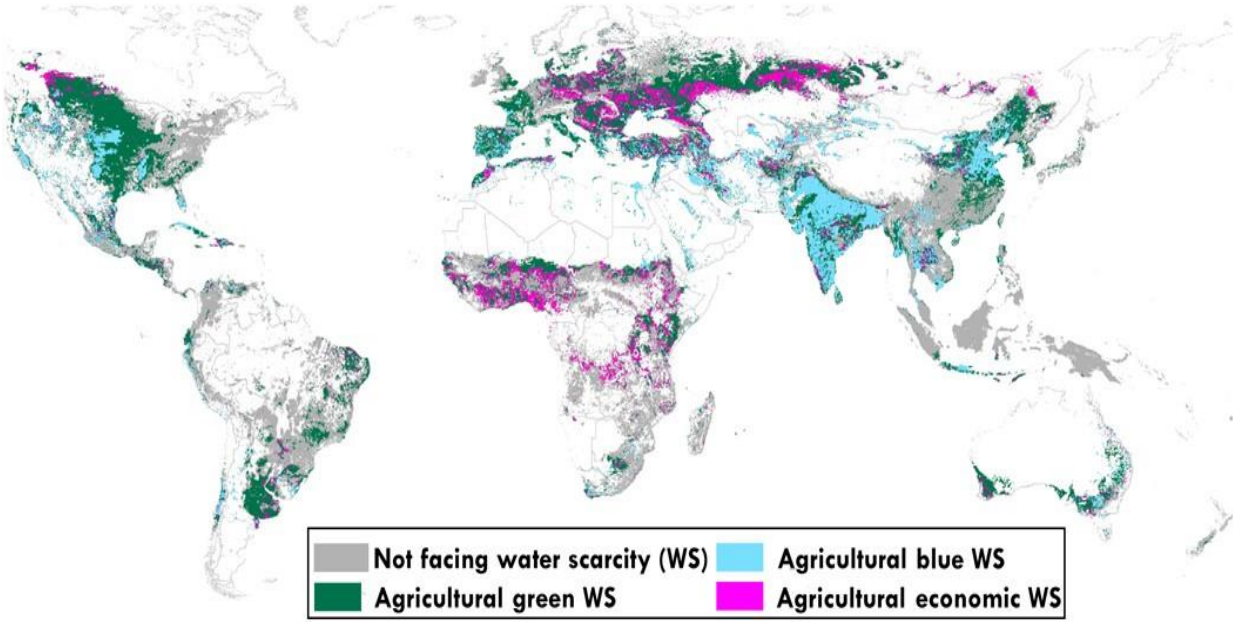

Figure 2.1: Global distribution of threat of water availability. **WS**: Water scarcity. **Agricultural blue water scarcity:** Arises when irrigation is unsustainable and renewable freshwater availability is insufficient to sustainably meet crop water requirements. **Agricultural green water scarcity:** Arises when root soil moisture is insufficient to sustain unstressed crop production and irrigation is needed to boost yields. **Agricultural economic water scarcity:** Arises when there is green water scarcity i.e., there is renewable blue water to irrigate but a lack of economic or institutional capacity. Adapted from Rosa *et al.* (2020).

Common indicators used to assess water scarcity include the Falkenmark indicator (which compares the amount of available water against the number of people who use it), the criticality ratio (measures water used compared to amount available in the resources) and the International Water Management Institute (IWMI) indicator, (a complex system that assesses the physical and economic variabilities affecting water availability within a country) (Liu, Yang, *et al*., 2017). Water crowding (WC) and water stress indices (WSI) may also be used to assess water scarcity in a region. These indices are capable of indicating increases and decreases in water scarcity (Gosling & Arnell, 2016).

The importance of water availability in agriculture cannot be ignored. Therefore, measures need to be put in place to address water scarcity challenges. This includes effective water management in high-risk areas and enforcing efficient water use

practices in agricultural production (Mekonnen & Hoekstra, 2016). These measures all seek to reduce water scarcity, improve access to safe water, and sufficient agricultural water.

Soil degradation

Land or soil degradation is the reduction in the operational abilities of soils based on biological activity and productivity (Nijbroek *et al*., 2018). Despite this, more than a third of the world's population (35%) lives in desert regions often referred to as drylands. These areas are prone to low rainfall and progressing land degradation (Middleton & Sternberg, 2013). This is seen by a progressive loss in biological activity which affects crop productivity resulting in economic losses. Human activity (Lanfredi *et al*., 2015) and climate change (Cowie *et al*., 2011) are the greatest influencers of land degradation. This impacts many regions of the world with Africa being the most affected.

Globally, land degradation is worst in arid, semi-arid and dryland areas. These include the Mediterranean basin (Lanfredi *et al*., 2015), some parts of Africa, China (Prăvălie *et al*., 2019) and central Asia (Vicente-Serrano *et al*., 2015). Land degradation in Africa greatly affects the horn of Africa (Ethiopia, Eritrea and Somalia) (Prăvălie *et al*., 2019). The Namibian eastern region of Omaheke is largely sandy soils ranging from loamy sand, sandy loam to sandy. The region is well-drained with signs of vegetative strain (Strohbach & Kutuahuripa, 2014). Though the Kavango regions get more rainfall compared to the Omaheke region, the soils are largely sandy. Some areas have high salinity and pH with reduced support for vegetation (Strohbach, 2013). Land degradation in Namibia is attributed to over-use with overgrazing being the primary malefactor (Coetzee *et al*., 2014) and failure to replenish soils as needed (Sitienei *et*

al., 2017). This contributes to the ever-reducing arable land of Namibia. Therefore, to improve soil quality, it is worthwhile to employ conservation agriculture together with biofertilisers (Lugtenberg *et al*., 2013; Jat *et al*., 2018; Matse *et al*., 2020).

The physical state of the soils in which crops are grown plays a crucial role in the growth abilities and, therefore, may contribute to the stress experienced by the crops. Poor or bad soils are described as soils with low or unbalanced nutrients as needed by crops. They are also described based on poor water retention capacity which affects water uptake by crops (Luchen *et al.*, 2018). Furthermore, the presence of certain compounds in excess is also considered detrimental to crop production. This is often exemplified by soils with high saline concentrations (Mukhtar *et al*., 2016).

To improve saline soils for crop production, saline-tolerant crops or halophilic bacteria may be used to reduce the amount of salt in the soils or improve salt tolerance of crops (Mukhtar *et al*., 2016). The impact of high salinity in soils is discussed in detail below. Furthermore, the use of integrated farming methods like intercropping (Gan *et al*., 2015; Sitienei *et al*., 2017) and a combination of organic and chemical fertilisers (Yoshinori *et al*., 2016; Watanabe *et al*., 2019) may improve soils damaged over time. More sustainable biofertilisers may also be used to solubilise essential minerals into organic forms that may be taken up by plants (Bhattacharyya & Jha, 2012; Puri, Padda & Chanway, 2020).

Soil quality

The definition of soil quality centres around the ability of soil to function as a part of the ecosystem to sustain biological activity and productivity. This includes maintaining environmental standards and promoting both human and animal health (Doran & Parkin, 1994; Bünemann *et al*., 2018). Therefore, the nutrient composition

of soil plays a direct role in crop production essentially affecting quality and yield. The soil quality or the composition of nutrients and soil organic carbon (SOC) in soils is greatly influenced by the amount of organic material in the soil (Nghalipo *et al*., 2019). Research has shown that land tended with organic material such as manure, often has higher SOC compared to soils treated with chemical fertilisers (Hu, Sørensen & Olesen, 2018). Losses due to decrease in soil quality have been mentioned in the section under land degradation.

However, there are methods often employed to relieve the stress on soil quality particularly with respect to nutrition. The use of organic fertilisers such as cattle manure has been found to increase SOC by up to 61%. Subsistence farmers in Namibia, for example, tend to use cattle manure as organic fertilisers as substitutions for the more expensive chemical fertilisers. (Watanabe *et al*., 2019). Furthermore, the use of conservation agricultural techniques that aim to improve yields and soil quality by retaining crop residue and practising crop rotation may also be considered (Araya *et al*., 2016). Crop rotation in particular has been widely used as a means to improve nutrients in the soil particularly soil nitrogen (Yu, Xue & Yang, 2014). Despite the positive effect these techniques have on the soil, they are often limited and often fail to fully restore soil quality and nutrients. Coupled with soil degradation, soil quality continues to worsen (Feng & Fu, 2013; Prăvălie *et al*., 2019).

This inherent problem of worsening soil quality has led to the popularisation of chemical fertilisers. They have been found to increase productivity and to some extent soil quality. As a result, some publications have advocated for the use of chemical fertilisers to restore soil quality and improve yields (Jones *et al*., 2013). However, they

have only intensified the problem by exacerbating soil degradation and environmental pollution (Chaudhary *et al*., 2020).

Decreased amounts of essential compounds like nitrogen and carbon negatively affect the overall growth and quality of crops (Sithole, Pérez-Fernández & Magadlela, 2019). Nutrient deficiencies in plants may be due to compounds present in forms that are not biologically available for use by plants or simply reduced amounts of the particular nutrients. This translates to plants being nutrient stressed (Saharan & Nehra, 2011). The bioavailability of phosphorus and sulphur is often limited as these compounds are mostly in inorganic forms that cannot be taken up by plants (Bhattacharyya & Jha, 2012). In addition, shortcomings such as nitrogen deficiency also cause equally distressing effects on crop quality (Oldroyd & Dixon, 2014). The resultant mineral deficiency in the plants negatively affects growth of plants. This often leads to the overuse of phosphate fertilisers which have detrimental effects on the environment (Jiao *et al*., 2012).

Soil pollution

The presence of salts and heavy metals in soils inhibits the successful growth of crops. Yields have been observed to drop by up to 50% in soils with high amounts of salts (Shrivastava & Kumar, 2015) and devastating effects on crop production and human health (Ahemad, 2015; Tirry *et al*., 2018). Salt stress refers to osmotic stress applied to plants that grow in soils with excessive amounts (conductivity that exceeds 20 mM) of salts such as carbonates, sulphate ions and sodium (Numan *et al*., 2018). High salt concentrations disturb nutrient uptake and utilisation. This negatively affects biochemical processes within the plant due to disrupted ion exchange activities

resulting in stunted growth and eventually reduced crop yield (Li & Jiang, 2017; Sapre, Gontia-mishra & Tiwari, 2018).

Contamination of soils by heavy metals is often found around abandoned mines and industrial sites which use heavy metals in their chemicals. Furthermore, inappropriate and continuous use of chemical fertilisers often results in cumulative deposit of heavy metals and soils (Abah *et al*., 2014; Zhang & Wang, 2020). Some of the most common heavy metals often found in polluted soils include chromium (Tirry *et al*., 2018), arsenic and mercury (Franchi *et al*., 2017). The use of chemical fertilisers and fungicides is also a major source of heavy metal contamination as they tend to leach into the soil and water (Tirry *et al*., 2018). In addition, heavy metals tend to be assimilated into crops as free metal ions, bound to soil organic matter and may be taken up as oxides or carbonates (Franchi *et al*., 2017). This may result in DNA damage due to free radicals and consequentially a reduction in seed germination (Ahemad, 2015). Figure 2.2 below shows the effects of heavy metals on plants and the potential of PGPB.

Plants have developed methods to tolerate saline soils through hormone regulation and metabolic regulations (Numan *et al*., 2018). However, microbes may be used to alleviate the effects of heightened salinity and metal pollution in soils. Additional tolerance and resistance may be employed by the action of PGPB. Salt-loving (halophilic) and salt-tolerant (halotolerant) PGPB when applied to soils can improve salt tolerance of plants (Orhan, 2016). Strains from *P. mendocina, P. simiae*, *Bacillus polymyxa* and *Mycobacterium phlei* have been found to actively reduce salt stress on plants due to their PGP activities (Shrivastava & Kumar, 2015; Kumari *et al*., 2016). Moreover, a combination of PGPB and mycorrhiza creates a mutually beneficial

symbiotic interaction between them that offers increased action against salt tolerance (Shrivastava & Kumar, 2015; Moreira *et al*., 2020).

Some PGPB can mobilise heavy metal contaminants in soils reducing the impact on plants in a process known as phytoremediation. These bacteria are also often referred to as heavy metal tolerant bacteria. (El-Meihy *et al*., 2019). PGPB achieve this through the solubilisation of metal minerals, rhizospheric pH adjustments, increased root surface area and increased root exudates discharge (Ahemad, 2015; Tirry *et al*., 2018).

Given the worsening state of soil quality in dryland areas in Africa, the threat to soil quality is ever increasing. As such, subsistence farmers are particularly vulnerable to the resulting effects due to limited resources. Therefore, there might be need to assess soils being utilised by subsistence farmers in more rural locations for their SOC quality, nutrient quality, and pollution levels. A comprehensive study in this respect will be able to fill the existing gap in knowledge of the soil quality status of the Kavango region in Namibia (Prudat, Bloemertz & Kuhn, 2018).

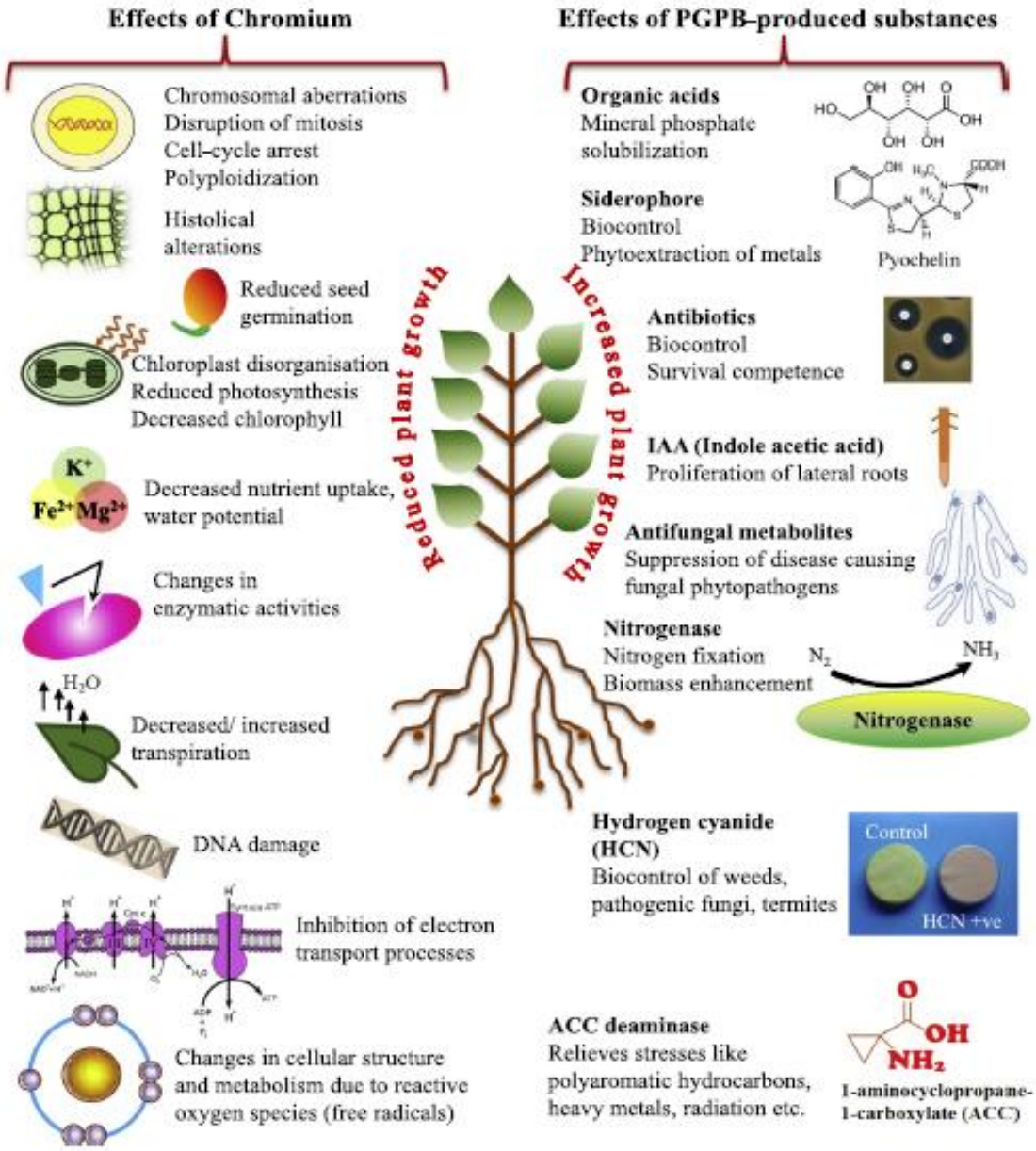

Figure 2.2: The effects of chromium contamination on plant growth and the counteractive effective of PGPB as presented by Ahemad (2015).

2.3.2. Biotic stresses

Plant diseases have the potential of reducing crop yield by 30 to 60% across the world. Fungal, bacterial, and viral infections contribute largely to losses caused by biotic stress. However, pests such as aphids also contribute to biotic stress-induced losses across the world (Chauhan, Yogindran & Rajam, 2017; Pandey *et al.*, 2018). In addition to this, grain or fruit quality is greatly compromised (Hussain, 2015).

Evidence has shown that many different biological agents contribute to biotic stress factors. This section explores some of those causative agents.

An example of biotic stress is brown apical necrosis, which is often seen in walnuts. It is caused by a combination of fungal and bacterial pathogens including *Fusarium* spp., *Alternaria* spp., *Cladosporium* spp., *Colletotrichum* spp. and *Xanthomonas arboricola* (Pandey *et al*., 2017). The disease itself presents with premature fruit drop and dark brown lesions in and on the fruit and is known to reduce yield by 20% (Akat, Özaktan & Yolageldi, 2016; Wang *et al*., 2019). Another example is seen by *Pyrenophora tritici-repentis* and *Septoria spp*. infections in wheat which cause rust and septoria complex respectively. *P. teres* infections in barley cause net blotch (Backes *et al*., 2021). Selective breeding and other biotechnology techniques such as improvement of soil microbiota could lessen the impact of biotic stresses in crops (Suzuki *et al*., 2014; Haggag *et al*., 2015).

Pathogenic infestations threaten crop yields (Suzuki *et al*., 2014). Biotic stress on plants raises a few concerns. Firstly, biotic stresses cause the greatest losses in agriculture (Backes *et al*., 2021). With respect to agricultural productivity, biotic stresses offer a significant problem in crop production. In northern Namibia, cowpea production is often constrained by parasitic weeds (*Alectra*) and pests such as leaf beetles, pod borers and bruchids. These biotic stresses affect close to 80% of all cowpea farmers (Horn, Shimelis & Laing, 2015). Furthermore, these interactions with pests have also been identified to be the cause of increased susceptibility to infections. Therefore, crops also tend to lose their competitive ability against invasive plants such as weeds (Haggag *et al*., 2015).

The most common method used to treat pest and microbial infections is the use of chemical inhibitors to prevent the continuation of biotic infestations. This method is not only expensive particularly for subsistence farmers, but also causes extreme damage to the environment (Haggag *et al*., 2015; Singh *et al*., 2015). Research has provided fewer damaging options which are available. Plants may be genetically enhanced in order to increase tolerance and resistance to biotic stresses (Suzuki *et al*., 2014; Singh *et al*., 2015). In addition to this, PGP microbes may be used to tackle biotic stresses. Bacteria do this by producing antibiotics that inhibit the growth of microbes such as fungi (Bahroun *et al*., 2018; Khan *et al*., 2020). The latter methods offer more environmentally friendly options. These are also cheaper compared to agrochemicals, particularly for subsistence farmers.

2.4. Plant microbiome

The plant microbiome plays a crucial role in the health of a plant. The presence of pathogenic bacteria or fungi affects the quality and productivity of the plants. On the other hand, a complex balance in the microbial communities is required to fully benefit the plant. This balance is found within or may be considered the plant microbiome. The plant microbiome constitutes both bacterial and fungal communities. These are found within the rhizosphere and endosphere (plant tissue). Performing metagenomic analysis will allow one to get a clear picture of the plant microbiome.

Metagenomic analysis is a technique employed to sequence and characterise microbial species from the environment. This is done without the need to culture the bacterial samples (Bragg & Tyson, 2014). It allows for the characterisation of microbes concerning their structure and functionality (Tamames & Puente-Sánchez, 2019), offering a way to identify and analyse functional genes in microbiomes. Metagenomics

also gives insight into biochemicals associated with microbes including enzymes. Previously unknown or misunderstood functions may also be better understood using metagenomics. In addition to this, metagenomics may also reveal the potential a set of genes have in a microbial community (Thomas, Gilbert & Meyer, 2012; Turner, James & Poole, 2013).

Two major approaches are used in metagenomics. The first is the structural approach that analyses the structure of microbial communities including any metabolic interactions. The second is the functional approach that analyses the functional properties of microbiome genetic sequences (Alves *et al*., 2018; Stefanini & Cavalieri, 2018). Software such as SqueezeMeta offer a fast method to analyse data sequences without the need for several preparatory steps (Tamames & Puente-Sánchez, 2019).

Metagenomic analysis begins with the separation of microbial samples from plants followed by DNA extraction from the environment typically done using DNA extraction techniques. Polymerase chain reaction (PCR) will be employed in order to increase DNA quantity to enable next-generation sequencing. Assembly of the generated sequences would follow the sequencing step. Categorising the data into specific species referred to as ‘bins’ using binning tools and metagenomic annotation form the last steps before data analysis (Wu & Ye, 2011; Thomas, Gilbert & Meyer, 2012). Metagenomic analysis of the microbial associations in plants and soils also provides ways to better predict and understand the signalling functions of microbes associated with plant growth promotion. This will give way to metaphenomics, the study of products resulting from expressed functions from metagenomes and the environment (Jansson & Hofmockel, 2018).

This molecular technique has been widely used to characterise both culture-dependent and culture-independent microbial communities. Metagenomic studies done on mung bean (*V. radiata*) revealed that Proteobacteria, Actinobacteria and Deltaproteobacteria species are most abundant in root nodules, roots and rhizospheric respectively (Iyer & Rajkumar, 2017). Metagenomics may also be used to assess the progression of microbiomes over long periods. This is driven by the ability of plants to actively select microbial species within the rhizosphere. Soybean, for example, can actively select microbial communities based on functional needs (Mendes *et al*., 2014). Metagenomics, therefore, can assess the differences by revealing the variations in microbiomes over time. Furthermore, metagenomics of the rhizosphere offers further insight into relationships between plants and microbes (Wolińska *et al*., 2017).

2.4.1. Plant-microbe interactions

Despite the economic and environmental challenges affecting agriculture, there is a need to increase crop production. This has led to the exploration of alternative, environmentally friendly fertilisers. Organic-based fertilisers are a suitable alternative as they improve crop yield while limiting the number of pollutants in the environment. In addition, organic fertilisers may also have anti-pathogenic properties on the growing crops preventing the growth of pathogenic microbes (Chang, Chung & Tsai, 2007; Qiu *et al*., 2012). A combination of organic-based fertilisers and biological fertilisers not only increases crop yield but also improves general soil quality. Biological fertilisers typically consist of nitrogen-fixing, potassium and phosphorus solubilizing bacterial or fungal cells (Jilani *et al*., 2007; Amal *et al*., 2011; Khosro & Yousef, 2012).

The ability of legumes to fix nitrogen is not only essential for crop yields but also improves the general quality of soil. Consequently, legumes may be used in crop

rotation systems to reduce nitrogen runoff while reducing the need for chemical fertilisers. The use of legumes in rice fields saw the use of chemical fertilisers reduced by up to 21.4% (Yu, Xue & Yang, 2014). In addition to this, nitrogen-fixing legumes improve microbial soil diversity contributing to plant growth. Though pathogenic microbes threaten the growth and yield of crops, improved microbial diversity reduces the risk of pathogenic infections (Dias, Dukes & Antunes, 2014; Venter, Jacobs & Hawkins, 2016). Horsegram, for example, is often planted as a preparatory crop as it retains soil and fixes nitrogen efficiently. Pigeon pea and lablab beans are also used in coffee fields in Brazil to supplement the use of chemical fertilisers and further improve nitrogen uptake via biological nitrogen fixation (Mendonça *et al*., 2017; Fuller & Murphy, 2018).

The need to understand the plant-microbe interactions, therefore, becomes crucial especially concerning arid agroecology's such as those found in Namibia. The intention to manipulate the plant microbiome is validated by the benefits identified in stress tolerance observed in PGP microbes (Orozco-Mosqueda *et al*., 2018). However, it has been observed that some bacterial species are plant specific with regards to growth promotion therefore, the specificity of identified microorganisms is worth exploring (Batista *et al*., 2018). It is imperative to isolate, identify and classify bacterial species associated with plant rhizospheres using techniques such as shotgun metagenomics (Finkel *et al*., 2017).

2.4.2. Diversity and factor shaping rhizospheric and plant associated bacteria in arid environments

Within plant tissues, microbes exist in symbiosis with the plant without causing damage to the plant. These microbes achieve this through roots, stems and/or seeds

(Reinhold-Hurek & Hurek, 2011; Suman, Yadav & Verma, 2016). Plant microbial associations include PGP microbiome in the rhizosphere, pathogenic microbes and opportunistic human pathogens (Iyer & Rajkumar, 2017). They form relationships that, more often than not, benefit the plant. The microbes may induce abiotic stress tolerance in plants by triggering the plant to activate stress tolerance systems or producing chemicals that may help alleviate the effects of stress (Lata *et al*., 2018). These PGP microbes are known either as rhizobacteria, rhizobia or endophytes depending on whether they colonise the rhizosphere, nodules, or the inner cells of the plant respectively. Therefore, successful occupation and growth of the microbes contributes to the positive growth of the plant (Verma *et al*., 2010).

Molecular techniques are often employed to characterise PGP microbes. This follows the extraction of DNA from the environment (Sharma *et al*., 2005). Alternatively, bacteria may be isolated and cultured to phenotypically classify bacterial species and assess biochemical functions. In order to determine the ability of bacteria to fix atmospheric nitrogen, isolated bacteria may be grown on tryptone soy agar as initial samples followed by growth in a viscous nitrogen-free medium (Batista *et al*., 2018). In a study by Lawless *et al*. (2018), the isolation of bacteria from Kudzu root nodules was followed by molecular analysis using *nif*H gene and 16S RNA primers. This led to the identification of *Bradyrhizobium diazoefficiens*, *R. etli* and close relatives of *Sinorhizobium* species.

2.4.3. Seed endophytic bacteria influence

Diverse endophytic microbes colonise seeds forming some of the first bacterial associations in a plant's life cycle (López *et al*., 2018). These microbes include both bacteria and fungi (Nair & Padmavathy, 2014; Chimwamurombe, Grönemeyer &

Reinhold-Hurek, 2016). Seed endophytes have been observed to contribute to seed germination and cell elongation (Verma *et al*., 2017; Khalaf & Raizada, 2018). In addition, they form the initial microbial association for the promotion of overall health of plants (Khalaf & Raizada, 2016).

Seed endophytes also can remain quiescent in latent seeds. This means they only become active when germination begins (López *et al*., 2018). Furthermore, seed endophytes may be passed through to progeny (Khalaf & Raizada, 2018) with some changes occurring in the microbiome due to pathogenic infections, environmental changes, or other stresses.

Endophytic bacteria contribute positively towards the general health of plants. Several species and genera have been identified as PGP endophytic bacteria. Analysis of rice seedlings analysed for seed endophytes identified *Enterobacter asburiae*, *Pantoea dispersa* and *P. putida*. These were found to produce auxins, solubilize phosphates and inhibit pathogenic fungi (Verma *et al*., 2017). Through nitrogen fixation (Verma *et al*., 2017), hormone production (Chimwamurombe, Grönemeyer & Reinhold-Hurek, 2016; Khalaf & Raizada, 2018) and antimicrobial activity (Nair & Padmavathy, 2014), endophytes improve abiotic stress tolerance and increase germination rates (Suman, Yadav & Verma, 2016). Furthermore, they are also able to regulate hormone content with respect to needs by the plant thereby improving plant adaptation to environmental strains (Asaf *et al*., 2017). With this, endophytes play a positive role in plant growth promotion contributing towards germination rate and increased biomass in environments that are typically unsuitable for successful plant growth.

2.4.4. Root nodules influence

Root nodules are small structures typically found on legume roots. These nodules are small ranging between 2 and 5 mm containing up to 10^9 bacterial cells (Downie, 2014). Root nodule formation is triggered by simultaneous correlations between plants and their soil environment. The release of Nod factors into the soil by rhizobia temporarily activates plant genes that code for specific hormones (Spaink, 2000; Poehlman *et al*., 2019). Peptide hormones, for example, together with signal receptors and low levels of nitrogen in soil induce nodule formation with close association with nitrogen fixing bacteria (Taleski, Imin & Djordjevic, 2018). However, nodule formation may be negatively affected by absence of specific strains, low quorum and failure to colonise the rhizosphere (Prasanna *et al*., 2017). Though root nodules are mostly colonised by nitrogen fixing rhizobia, other microorganisms may also be found present in the nodules (Martínez-Hidalgo & Hirsch, 2017).

The formation of root nodules with the eventual colonisation by bacteria is not fully understood however, it is known that nitrogen fixation is a result of this process. The process of nodulation is triggered by nitrogen levels in the soil with low levels initiating hormone signalling in the form of C-terminally encoded peptides (Verma *et al*., 2010; Taleski, Imin & Djordjevic, 2018). Nod factors are produced by the bacteria as a response to signal molecules from the plant. These chemical signals include flavonoids which trigger the activation of Nod factor regulatory genes in bacteria (Spaink, 2000). This begins the process of infection with the rhizobial bacteria attached to root hairs. Once plant cell membranes detect the Nod factors, root hair deformation follows. A process that results in the structure known as a nodule (Downie, 2014).

Microbial interactions with roots tend to be location specific. Figure 2.3 below illustrates the specificity of different bacteria in relation to the root system.

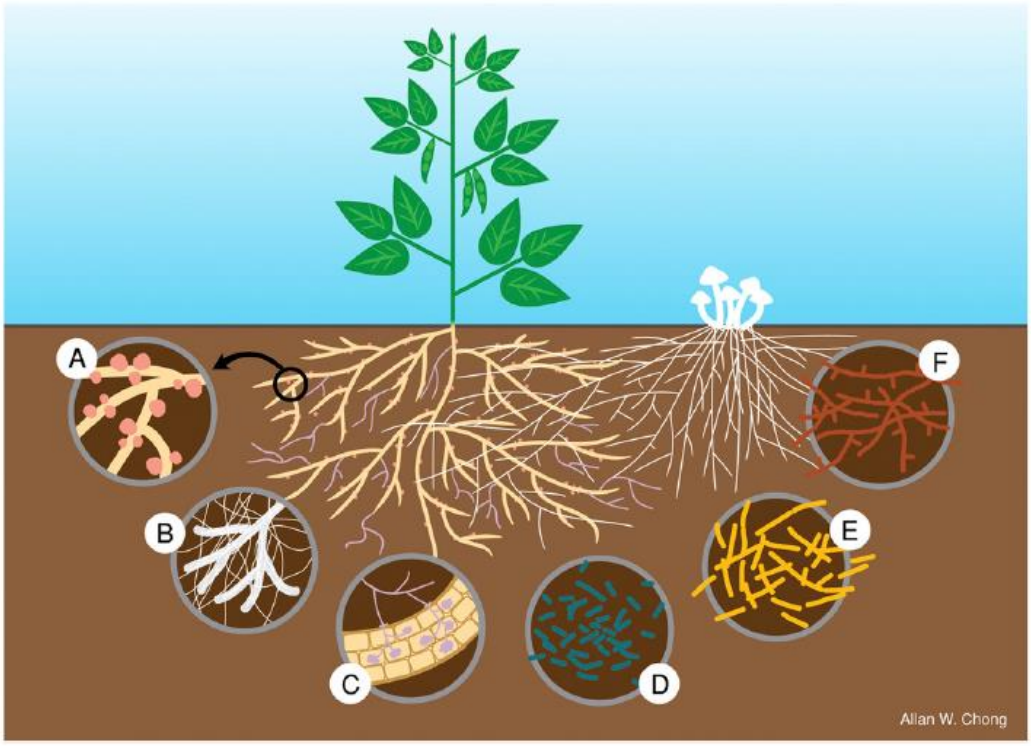

Figure 2.3: Root-nodule interactions with microbes. A- root nodules on plant roots. B- ectomycorrhizal associations with legume tree roots. C- arbuscular mycorrhizal interactions with root cells. D- Gram-negative rhizospheric bacteria which may influence nodule formation. E- Gram-positive bacteria colonise both the rhizosphere and the nodules. F- free living actinomycetes influence plant growth by nitrogen fixation among others. Adapted from (Martínez-Hidalgo & Hirsch, 2017) Illustration by Allan W. Chong.

Bacteria associated with root nodules include *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* (Verma *et al.*, 2010). In addition, species from the *Bacillus*, *Bradyrhizobium* and *Leifsonia* genera have been isolated from legume nodules in semi-arid regions. *Microbacterium* endophytic isolates have also been isolated from root nodules (Nunes *et al.*, 2018; Muresu *et al.*, 2019). The symbioses have the advantage of promoting plant growth by increasing nitrogen uptake and assisting in disease tolerance and resistance. The bacteria may also solubilize phosphate or produce plant hormones which increase plant growth (Busby *et al.*, 2017; Muresu *et*

al., 2019). Plants consequently take advantage of the symbiotic relationship with bacteria present in the soil facilitating formation of root nodules (Lawless *et al*., 2018).

2.4.5. Rhizospheric influence

The rhizosphere is described as the soil region closest to the roots. It acts as a platform for close interaction within the biosphere around the roots of plants (Jha & Saraf, 2015) and is largely influenced by the plant roots themselves (Ai *et al*., 2012; Semenov *et al*., 2020). Therefore, bacteria that colonise the rhizosphere are known as rhizobacteria (Haiyambo, Chimwamurombe & Reinhold-Hurek, 2015).

Through the action of root exudates and essentially chemotaxis (Figure 2.4) the rhizosphere is a microbe rich zone (Orozco-Mosqueda *et al*., 2018; Swarnalakshmi *et al*., 2020). Also referred to as inter-kingdom signalling, chemotaxis forms the basis for initial colonisation of the rhizosphere by microbes (Venturi & Keel, 2016). As a result, it is a site for biological functions including microbial activity (Fernández Lópeza *et al*., 2013) and water regulation (Zhang *et al*., 2020). Both fungal and bacterial organisms form the population of microbes that occupy the rhizosphere (Bui & Franken, 2018; Liu *et al*., 2019; Leontidou *et al*., 2020; Sharma *et al* 2020).

Rhizobacteria possess the unique ability to influence plant systems both directly and indirectly (Enebe & Babalola, 2018). They offer positive support and influence on the crops by performing or facilitating various biological processes. These include solubilisation of inorganic forms of essential compounds (Kaushal & Kaushal, 2015; Puri, Padda & Chanway, 2020), biological nitrogen fixation (Tamagno *et al*., 2018) and antimicrobial activity (Qiu *et al*., 2012; Martínez-Hidalgo & Hirsch, 2017) among others. The microbial community of the rhizosphere, as such, is heavily influenced by microbes present in the general soil mass (Mendes *et al*., 2014).

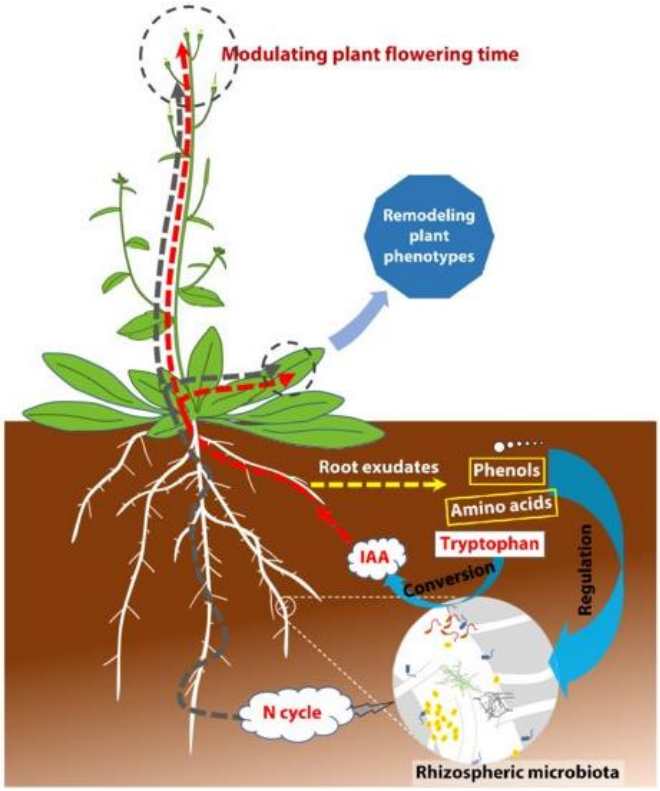

Figure 2.4: Rhizospheric interactions between the environment, microbes, and plant. Adapted from Lu *et al*., (2018).

The rhizosphere forms the primary stage for the exchange of nutrients and compounds between the plants and rhizobacteria. This is made possible by carbon rich root exudates that make the rhizosphere a nutrient rich region. This favours microbial growth (Orozco-Mosqueda *et al*., 2018; Semenov *et al*., 2020). The physical characteristics of the rhizosphere also create a suitable environment to accommodate both aerobic and anaerobic bacteria among others (Jha & Saraf, 2015; Chawngthu, Hnamte & Lalfakzuala, 2020).

One important role played by the rhizosphere is the contribution it makes to water uptake from the bulk soil into plant roots. The uptake of water by plants from the bulk soil is a well understood process, however, the influence of the rhizosphere is often overlooked. Through an intricate interaction between the plant and rhizosphere, water uptake is regulated (Carminati *et al*., 2010). This is initiated by plant roots that have

been observed to produce a gel like substance (mucilage) that is held within the rhizosphere. Mucilage modifies rhizospheric soil properties resulting in improved water storage (Zeppenfeld *et al*., 2017; Zhang *et al*., 2020). Mucilage also has an additional function inducing hydrophobicity in the event of reduced water availability. This allows for a biophysical protection of the plant from drought (Kroener *et al*., 2016).

In addition, research strongly suggests that rhizospheric influence with regards to water uptake may differ depending on age of the roots. This implies, therefore, that distal (younger) roots experience a greater mucilage occurrence to improve water uptake compared to proximal (older) roots (Carminati, 2013). Therefore, the hydraulic properties of the rhizosphere together with root exudates play a crucial regulatory role in water uptake by plants.

Root exudates are nutrient rich carbon sources ideal for microbial communities. They also offer a certain degree of influence on the microbiome (Semenov *et al*., 2020). Due to this influence and its physical properties, the rhizosphere creates an ideal environment for microbes. With this, the rhizosphere is able to house a wide variety of microbes (Fan *et al*., 2018; Town *et al*., 2022), whose compositions are often influenced by plant roots (Essel *et al*., 2019). Distinct differences in microbiomes between the bulk soil and rhizosphere exist, however, the multiplicity decreases around the rhizosphere (Cui *et al*., 2019). In addition, the rhizospheric microbiome is more functionally structured compared to the bulk soil. This strongly points towards ecological stability within the rhizosphere (Zhang *et al*., 2017; Tian *et al*., 2022).

The rhizospheres of all plants are characterised by bacteria from several different genera. These include *Bacillus, Enterobacter* and *Pseudomonas* (Haiyambo,

Chimwamurombe & Reinhold-Hurek, 2015). Some of the most abundant bacterial genera that have been identified within the rhizosphere are *Lactococcus, Nocardioides, Pseudarthrobacter, Rhizobium* and *Streptomyces* (Essel *et al* ., 2019). The rhizosphere of legumes also includes a similar microbial profile. Rhizobacteria isolated from the chickpea rhizosphere include *Azotobacter chroococcum, B. pumilis, B. subtilis* and *P. aeruginosa* (Pandey, Gupta & Ramawat, 2019).

2.4.6. Multi-species microbial interaction

As previously mentioned, bacteria can actively improve the health of plants as PGPB. However, fungi can also promote growth and good health in plants. Arbuscular mycorrhiza, for example, serve as root extensions increasing root functionality. This results in increased surface area for nutrient and water uptake in addition to strengthening of the root-soil structure. *Trichoderma*, for example, improves plant resistance during stress, nitrogen use efficiency and root and root hair development (Lugtenberg *et al*., 2013).

However, mycorrhizal fungi often function in synergy with PGPB improving nutrient availability and nodulation (Swarnalakshmi *et al*., 2020). Interactions between mycorrhizal fungi and bacteria may be function specific. A suitable case in point is the interaction between *Rhizoglomus* irregular and *Pseudomonas* phosphate solubilizing species. Research has found that in synergy, the mycorrhiza and bacteria improve plant growth and phosphorus uptake compared to when bacteria are applied to plants alone (Sharma *et al*., 2020). Moreover, the combination of PGPB and mycorrhiza creates a mutually beneficial symbiotic interaction between them that offers increased action against salt tolerance (Shrivastava & Kumar, 2015; Moreira *et al*., 2020). Research has also found that successful colonisation by PGPB, arbuscular mycorrhiza or

ectomycorrhizal can increase nitrogen fixation by up to 80%. However, this success is highly dependent on the hosts' habitat and environment (Diagne *et al*., 2013). Observations suggest that high phosphorus levels in the soil may inhibit or limit arbuscular mycorrhizal activity resulting in a reduction in root colonisation by the fungi (Smith & Smith, 2011). Similar effects were observed with mycorrhiza associated with *Picconia azorica*, a woody plant of the Azorean Forest. A study by Melo *et al*. (2019), found that different mycorrhiza species require different pH levels to allow for maximum growth and support to *P. azorica*. In addition, they observed a correlation between spore density and altitude with a decrease in spore density being observed with elevation.

Arbuscular mycorrhiza in synergy with beneficial bacteria, therefore, promotes the growth of plants with unmistakable influence from the environment. However, together with PGPB, functionality is improved. This, in no way, takes away the value of PGP by mycorrhiza. Instead, it cements the importance of beneficial interspecies symbiosis.

2.5. Use of bioinoculants in crop improvement

The majority of the farmers in drylands use old farming methods and this has led to failure to cope with increasing populations for food security (O'Callaghan, 2016). The use of modern technology is advantageous and offers farmers opportunities to improve yield. Digitalizing farming systems allows farmers to predict weather and yield, select suitable crops for the area and better manage irrigation systems (Sarker *et al*., 2019). Nuclear technology presents itself as a means to improve yield by use of radioactive isotopes. These are used as tracers and early detectors of the presence of diseases. In addition to this, nuclear technology may be a means to practise sustainable agriculture

by providing green energy (Ebrahimi Sarcheshmeh, Bijani & Sadighi, 2018). The application of technology in agriculture can improve yield by up to 35%, (Kassie, Shiferaw & Muricho, 2011), soil structure (Corbeels *et al*., 2014) and reduce input costs and labour (Rehman *et al*., 2017). However, the greatest challenge is that these modern technologies and farming methods are not accessible to the bulk of the resource-poor farmers in arid lands.

Conservation agriculture may help increase crop production while also improving profitability and reducing soil degradation. This modern system involves minimum tillage, soil cover and crop rotation (Corbeels *et al*., 2014). Some methods are not widely used nor accepted by subsistence farmers largely due to unfamiliarity (Ebrahimi Sarcheshmeh *et al*., 2018). The use of bioinoculants or biofertilisers to improve seeds or varieties is not common in rural Africa (Kassie, Shiferaw & Muricho, 2011; O'Callaghan, 2016). These advancements can improve stress tolerance and yield. However, most rural farmers do not have access to information and services that allow them to successfully assimilate them in their farming practices (Rehman *et al*., 2017).

Bioinoculants or biofertilisers are microorganisms prepared for application to the surface of plants, seeds or mixed with the soil with eventual colonisation of the rhizosphere or endosphere of the plants. They promote plant growth and improve nutrient use and uptake by the plant (Singh, 2013). The identification of PGPB and eventual growth promoting analysis has led to the use of bacteria strains as bioinoculants. These associations may be used in sustainable agriculture to supplement the use of chemical fertilisers.

Inoculation of soil or seeds with bioinoculants improves plant growth of plants. Root length, for example, may be influenced by inoculation of seeds with *A. brasilence* and *P. putida* which are both known to encourage plant growth due to their ability to produce IAA (Shahab, Ahmed & Khan, 2009). Further evidence indicates plant growth improvement by the production of bioactive metabolites of PGPB isolated from the roots of *Salvia miltiorrhiza* (Duan *et al*., 2013). These contribute towards pathogen inhibition and improved disease tolerance and resistance. *B. amyloliquefaciens*, for example, has been found to improve fungal infection tolerance in tomato plants. The inoculation of *B. amyloliquefaciens* in tomato plants has been observed to reduce the effects of *F. oxysporum* sp. *lycopersici* strain (KACC 40032) as seen in Figure 2.5 below. The observations were improved biomass, protein content and root growth compared to samples without *B. amyloliquefaciens* (Shahzad *et al*., 2017).

The use of bioinoculants is further motivated by their environmental benefits. Unlike chemical fertilisers, biofertilisers do not leach into the soil and water nearby, a process known as eutrophication (Wimalawansa & Wimalawansa, 2015; Ouyang *et al*., 2018). However, this may be negatively affected by the chemical composition of the soil. Long term exposure to fertilisers, for example, impacts the rhizospheric microbiome often reducing the diversity of PGP bacteria (Semenov *et al*., 2020).

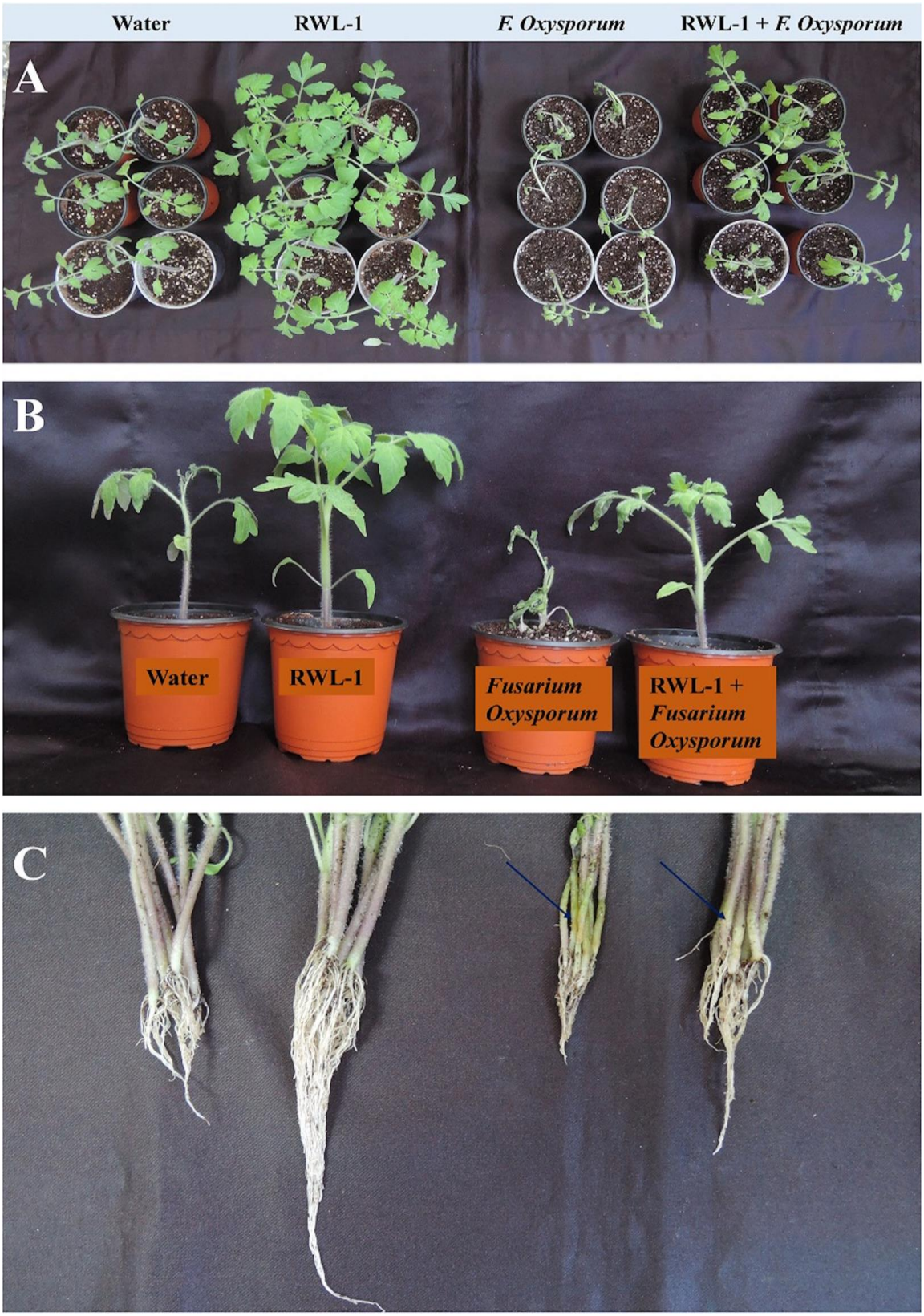

Figure 2.5: The effect of PGPB *B. amyloliquefaciens* (RWL-1) on tomato plants infected with *F. oxysporium*. **A:** Aerial view of tomato plants under different treatment. **B:** Effect of the different treatments on plant structure. **C:** Effect of the different plant treatments on root structure. Adapted from Shahzad *et al*., (2017).

2.6. Microbial plant growth influence

Plant growth promotion by bacteria is characterised by the isolates' ability to perform several biochemical processes that benefit the plant. These characteristics are based on various criteria such as enzyme metabolism, production of organic compounds or how they react to the presence of certain compounds (Haiyambo, Chimwamurombe & Reinhold-Hurek, 2015). There are five main characteristics that are used to determine PGP abilities. These are the presence of 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Puri, Padda & Chanway, 2020), phosphate solubilization (Kaushal & Kaushal, 2015; Gupta & Pandey, 2019a), siderophore production (Bhattacharyya & Jha, 2012; Gamit & Tank, 2014), Indole acetic acid (IAA) production (Goudjal *et al*., 2013; Bhutani, Maheshwari & Suneja, 2018) and antifungal activity (Duan *et al*., 2013; Verma *et al*., 2017). In addition to these, a nitrogen fixing assay, acetylene reduction assay (ARA) (Smercina *et al*., 2019) can also be done. These tests are done based on characteristics that are favourable in promoting plant growth.

2.6.1. Drought stress tolerance

Drought tolerance is an important feature of PGPB as it offers a means to improve crop production during long periods of little to no water. Plant associated microbes help plants tolerate drought by enhancing the plants physiological defenses against drought and producing different types of beneficial biochemicals (Ngumbi & Kloepper, 2016). PGPB have the ability to induce drought tolerance by reducing the accumulation of ethylene, for example, which impedes root elongation and eventual plant growth. This is done by the production of ACC deaminase, an enzyme able to catalyse the ethylene precursor ACC (Vurukonda *et al*., 2016; Delshadi, Ebrahimi & Shirmohammadi, 2017). ACC deaminase also helps plants tolerate drought by

promoting nodule formation (Tsukanova *et al*., 2017). Bacteria found in the *Arthrobacter, Bacillus,* and *Microbacterium* genera actively produce ACC deaminase in plants during water stress (Fadiji, Ayangbenro & Babalola, 2021).

By producing essential amino acids and hormones, PGPB increase the plants defences in cases of drought stress. *Arthrobacter* and *Bacillus* PGPB, for example, contribute to proline production increasing plant growth (Kumari *et al*., 2016). Some *Bacillus* species, like *B. megaterium* and *B. subtilis*, produce cytokinins which are essential in drought stress tolerance (García-fraile, Menéndez & Rivas, 2015). Drought tolerance may also be induced by PGP antioxidant activity. Associated endophytes increase the concentrations of antioxidants such as flavonoids in plant cells. This allows for increased foraging of ROS by the antioxidants to transform them into less harmful forms (Vaishnav *et al*., 2019).

By inducing physical changes to plant systems, PGPB induce physiological defenses against drought. Evidence has shown that PGPR, for example, help improve root systems in the event of drought stress by inducing root elongation and increasing surface area. This improves water uptake (Ngumbi & Kloepper, 2016). *Alcaligenes faecalis, Burkholderia phytofirmans* (Ngumbi & Kloepper, 2016), *Azospirillum brasilense* (Vurukonda *et al*., 2016) strains are known to facilitate root elongation in drought stress conditions. This has been similarly observed in studies of *Paenibacillus polymyxa* SK1 isolated from *Lilium lancifolium* (Khan *et al*., 2020).

2.6.2. ACC deaminase

As a response to various stresses, plants produce hormones that regulate protein production. Ethylene (whose immediate precursor is ACC) is one such hormone and functions as a trigger for seed germination (Penrose & Glick, 2003; Gupta & Pandey,

2019a). An unregulated increase in “stress ethylene” results in the death of shoots and roots leading to the plant to eventually fail to thrive (Singh *et al*., 2015). The presence of ACC deaminase regulates the amount of ethylene in the plant. Furthermore, the presence of ACC deaminase promotes nodule formation supporting plant growth. Some bacterial species produce ACC deaminase that actively breaks down ACC to ammonium and α-ketobutyrate (Belimov *et al*., 2001; Penrose & Glick, 2003; Tsukanova *et al*., 2017).

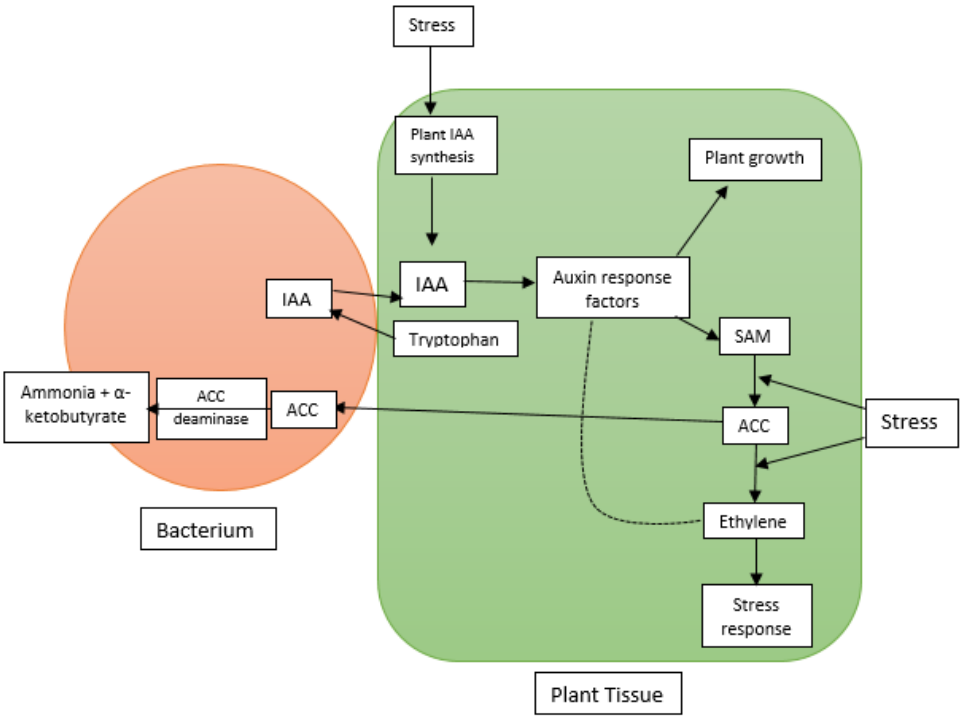

Figure 2.6: The image above as described by Glick (2014) shows bacteria assisted production of ammonia and α-ketobutyrate through the action of ACC deaminase as a response to stress on plants. Abbreviations: ACC - 1-aminocyclopropane-1-carboxylate; IAA - indole acetic acid; SAM - S-adenosylmethionine.

In order to determine the presence of ACC deaminase, bacterial isolates are tested for their ability to utilize ACC as the sole source of nitrogen (in the form of ammonium) (Penrose & Glick, 2003). This is achieved by inoculating the bacterial samples onto augmented Dworkin Foster minimal salt media with added ACC. Growth on these plates would indicate presence of active ACC deaminase. An additional step measures

the activity of the bacteria by determining the amount of α-ketobutyrate and ammonium produced (Ali, Sandhya & Rao, 2014). The process of the production of ammonia and α-ketobutyrate via ACC deaminase activity is shown in Figure 2.6.

Molecular analysis of the isolates via 16S mRNA primers, provides their identities. Some known bacteria species which are capable of hydrolyzing ACC include *P. putida* strain Am2, *P. brassicacearum* strain Am3, *Variovorax paradoxus* strain Bm2, *P. putida* strain Bm3 (Belimov *et al*., 2001), *P. fluorescens* strain FPG3 (Ali, Sandhya & Rao, 2014), *Paenibacillus sp*. strain SG_AIOA2 and *Aneurinibacillus aneurinilyticus* (Gupta & Pandey, 2019a).

2.6.3. Phosphate solubilization

Phosphorus is an essential nutrient required for the growth and development of plants. It is a crucial element in DNA and RNA, adenosine triphosphate (ATP) and phospholipids (Daneshgar *et al*., 2018), thereby positively contributing to photosynthesis, root elongation and nitrogen fixation (Matse *et al*., 2020). Phosphate solubilizing bacteria convert inorganic phosphate (Pi or PO_4^{3-}) into more soluble forms (HPO_4^{2-} or H_2PO_4). Bacteria achieve this by secreting acids that facilitate the solubilization. Succinic acid is one such acid produced by several strains of *B. megaterium* (Suleman *et al*., 2018; Zheng *et al*., 2018).

In order to characterise bacteria for phosphate solubilization, isolates are grown on Pikovaskya's agar plates with 2% inorganic tricalcium phosphate ($Ca_3(PO_4)_2$ (Pandey, Gupta & Ramawat, 2019) or a tris-minimal medium with added zinc phosphate (Shahab, Ahmed & Khan, 2009) and monitored. A positive indication of phosphate solubilization is shown by a clear halo around the colonies depending on media used (Zhao *et al*., 2016). Thereafter, the solubilizing ability is measured using a published

formula (Kumari *et al*., 2016). A molecular technique may also be employed in the identification and characterisation of phosphate solubilising bacteria. This method entails the identification of phosphate solubilising genes in bacterial isolates. Using gene specific primers, genes may be identified (Zheng *et al*., 2018). This, however, is a limited technique as it only indicates the ability of the bacteria to solubilise phosphates but does not reveal the level of expression of the genes.

Bacteria known to solubilize inorganic phosphate include *P. fluorescens*, *P. putida*, *X. maltophilia* (Gupta *et al*., 2014), *E. agglomerans* and *R. leguminosarum* (Bhattacharyya & Jha, 2012). Some studies have identified bacterial strains in co-inoculation studies that improve phosphorus uptake. Improved Phosphorus content was observed when *Rhizobium spp.* strains (CHB1120 and CHB1121) were inoculated with *Azotobacter vinelandii* (strain G31) and *B. aryabhattai* (strain Sb) (Matse *et al*., 2020).

2.6.4. Siderophore production

Siderophores are low molecular weight compounds released by organisms that have a high chelating affinity for ferric iron (iron III). These compounds solubilise ferric iron into more soluble forms (Fe^{3+} complexes) that are more easily taken up by plant cells (Dudeja & Giri, 2014; Gamit & Tank, 2014). As iron is one of the most crucial elements for plant growth and promotion, it is essential for plants to develop ways to acquire usable iron forms. Therefore, siderophore producing rhizobacteria such as *Azadirachta*, *Azotobacter*, *Bacillus*, *Pseudomonas* and *Rhizobium* contribute positively towards plant growth and improvement of chlorophyll content (Gamit & Tank, 2014; Gupta *et al*., 2015). In addition, siderophores play a secondary role in

biocontrol. They achieve this by limiting the amount of iron pathogens can take up (Goswami *et al*., 2014).

Ligands that chelate iron (III) are used to classify and identify siderophores, these include carboxylates, catecholates and hydroxamates (Louden, Haarmann & Lynne, 2011). Chrome azurol S (CAS) agar, with a pH indicator, is often used as a universal identifier for siderophore production tests. Isolates are inoculated onto CAS agar and observed for colour change. The presence of a yellow halo around inoculated isolates indicates siderophore production (Schwyn & Neilands, 1987; Batista *et al*., 2018).

2.6.5. IAA production

The presence of various forms of stresses induces the release of specific PGO hormones. These hormones include cytokinins, gibberellic acid, IAA (Kumar *et al*., 2012). IAA is a PGP auxin that arises as a result of metabolism of tryptophan by bacteria. Previous studies have also identified bacteria that can produce IAA without the use of a tryptophan precursor. It promotes lateral root growth and tissue vascular differentiation (Shahab, Ahmed & Khan, 2009; Goswami *et al*., 2014; Kumari *et al*., 2016). The hormone IAA does this by increasing osmotic activity, increasing water permeability into cells and synthesis of specific proteins promoting cambial activity (Mohite, 2013). Some IAA producing genera include *Azotobacter, Azospirillum, Bacillus, Kocuria, Pseudomonas,* and *Rhizobia* (Bhattacharyya & Jha, 2012; Goswami *et al*., 2014).

IAA production may be assessed from bacterial isolates and quantified using different methods. Microbial analysis of IAA production often follows growth of isolates in Luria-Bertani (LB) broth with tryptophan and incubated while shaking. Samples will

thereafter be centrifuged and supernatant extracted for quantification using spectrophotometer (Rajendran, Patel & Joshi, 2012). Isolates can also be grown in yeast malt dextrose broth and quantification of IAA can then be done using thin layer chromatography (Mohite, 2013). Shahab, Ahmed and Khan (2009) in their published article made use of high performance liquid chromatography (HPLC) instead to quantify IAA. Using HPLC, retention peaks are compared to prepared standards.

2.6.6. Antifungal activity

One of the major threats to crop yield is biotic stress often because of fungal, bacterial, or viral infections. For example, *Colletotrichum lindemuthianum*, a fungus causes anthracnose disease which often results in yield loss. Mung bean is also susceptible to anthracnose infection (Figure 2.7) with losses sometimes reaching up to 60% (Pandey *et al*., 2018). Invasive pests may also contribute to biotic stresses hindering healthy growth and development of plants (Rajesha *et al*., 2010; Singh *et al*., 2012). Antifungal activity of plants by endophytic bacteria, therefore, is beneficial and contributes to PGP activities (Haiyambo, Chimwamurombe & Reinhold-Hurek, 2015).

Antifungal activity of endophytic bacteria may be determined by molecular analysis or microbiological techniques. Molecular analysis of bacterial endophytes with primers allows for the detection of genes that code for the production of antifungal compounds. Previous studies have identified the following genes *phzC-phzD, prnD, pltc, phz, phlD* and *hcnAB* to code for the production of antifungal compounds such as phenazine, phenazine-1-carboxylic acid and pyrrolnitrin (Bahroun *et al*., 2018). Metagenomics may also be used to detect antifungal clones in isolates, however, this method often results in low detection (Burke, 2010).

Antifungal compounds produced by endophytic bacteria actively inhibit growth of pathogenic fungi. Microbial analysis of antifungal activity follows the concept of the inhibitory potential of isolates (Bhattacharyya & Jha, 2012). In order to determine antifungal activity, fungal isolates are grown on potato dextrose agar (PDA) plates co-inoculated with bacterial isolates with antifungal abilities (Rajendran, Patel & Joshi, 2012). Zones of inhibition indicate the degree of efficacy of antifungal compounds produced.

PGPB with antifungal activity can be isolated from different plants. An endophytic bacterium (*P. polymyxa* SK1) isolated from bulbs of the *Lilium lancifolium* was found to possess significant antifungal activity. *P. polymyxa* SK1 was shown to actively inhibit *Botrytis cinerea, Botryosphaeria dothidea, Fusarium fujikuroi* and *F. oxysporum,* all detrimental fungal pathogens (Khan *et al*., 2020). Some *Staphylococcus* strains have been found to reduce drought stress but also inhibit fungal infections in plants (Eid *et al*., 2021). *Streptomyces murinus* is a well-studied endophyte with antifungal activity. The most significant activity has been observed against *Gibberella fujikuroi, Aspergillus niger* and *A. fumigatus* all important plant pathogens (Sun *et al*., 2013).

Figure 2.7: Shows anthracnose on leaves caused by a fungus (Pratap *et al*., 2020).

2.6.7. Acetylene reduction assay

One of the most beneficial characteristics in plant growth is nitrogen fixation. Biological nitrogen fixation (BNF) is the process of supplying available nitrogen to the plant through microbial action. This can be facilitated by bacteria (also referred to as diazotrophs) that fix atmospheric nitrogen (N_2) to more biologically available ammonium form (NH_4^+). This reaction typically occurs in root nodules (Chidebe, Jaiswal & Dakora, 2018). This characteristic is especially crucial for plants growing in nitrogen poor soils. The chemical equation and Figure 2.8 below represent the process of nitrogen fixing. Studies have found that the enzyme nitrogenase catalyses the reaction below (Das & De, 2018; Saiz *et al*., 2019).

$$N_2 + 10H^+ + 8^{e-} \rightarrow 2NH_4^+ + H_2 \text{ (16 ATP)}$$

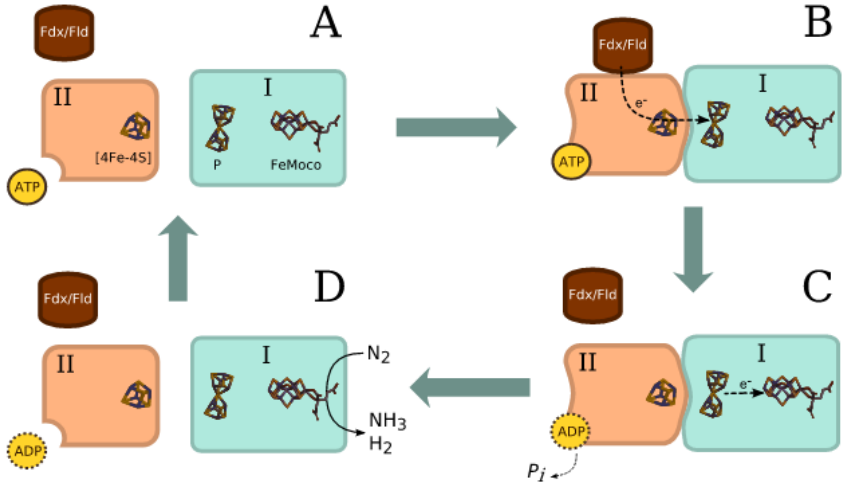

Figure 2.8: Schematic presentation of nitrogen fixation via nitrogenase facilitation. (A) Detached nitrogenase components I (dinitrogenase; MoFe protein) and II (dinitrogen reductase; Fe protein) show II awaiting reduction by adenosine triphosphate (ATP). (B) ATP binds to component II initiating electron transfer from donor [Fdx (ferredoxin) or Fld (flavodoxin)]. ATP binding triggers an allosteric structural change which leads to the components attaching. A flow of electrons occurs from the [4Fe-4S] cluster on II to the P cluster on I. (C) Electrons are further shuttled to the cofactor–iron-molybdenum cofactor (FeMoco) while ATP is hydrolysed to adenosine diphosphate (ADP). D) The two components detach and produce ammonia and H_2 via the reduction catalysed by nitrogenase (Seefeldt, Hoffman & Dean, 2009). Image by R Patrícia.

With respect to nitrogen content, BNF plays a crucial role in improving soil fertility. In addition, it has been documented that close to 80% of all BNF occurrences are through symbiotic bacteria while non-symbiotic activity also contributes significantly (Gothwal *et al*., 2008; Das & De, 2018). Non-symbiotic bacteria also referred to as free living nitrogen fixing (FLNF) bacteria can occur throughout the soil. However, they are often restricted to the rhizosphere due to the availability of carbon from the plant (Smercina *et al*., 2019).

The rate of nitrogen fixation is measured to determine nitrogen fixing abilities of microbes. This is done in one of two ways, acetylene reduction assay (ARA) or $^{15}N_2$ incorporation method (Smercina *et al*., 2019). ARA is based on the reduction activity of nitrogenase enzyme on acetylene to ethylene (Saiz *et al*., 2019). To assess nitrogen fixing activity, isolates are grown on nitrogen free medium with an indicator. Isolates that show growth are thereafter inoculated into nitrogen free broth. This is followed by inoculation and growth in enriched cultures in vials allowing for production of ethylene. The ethylene produced is then measured by gas chromatography (Gothwal *et al*., 2008; Baldani *et al*., 2014).

However, ARA requires the use of a conversion factor to estimate biological nitrogen fixation rate based on the number of moles of ethylene produced. The conversion factor is often approximately 4:1 (Saiz *et al*., 2019). The latter method on the other hand, is more accurate as it measures nitrogen fixation based on the differences in ^{15}N isotope abundance when exposed to $^{15}N_2$ standard samples. However, this method carries a higher risk of contamination (Smercina *et al*., 2019). In addition to these two methods, a microbial bioassay may also be used. In this method, isolates are grown on nitrogen free medium before growth in Jensen's medium. Colony growth is then

monitored and measured using a haemocytometer. A published equation is then used to calculate the rate of BNF (Das & De, 2018).

There exists a catalogue of nitrogen fixing bacteria that play an important role in plant growth promotion. Within that list are *B. pumilis* and *B. subtilis* that have been isolated from the rhizosphere of cauliflower plants. Studies found strains from both species to positively influence plant growth (Kaushal & Kaushal, 2015). *R. larrymoorei, R. oryzae* and *R. undicola* are known to fix nitrogen in association with the legume *Tylosema esculentum* locally known as marama bean (Chimwamurombe, Grönemeyer & Reinhold-Hurek, 2016). Other genera identified include *Bradyrhizobium*, *Mesorhizobium*, *Ensifer*, *Azorhizobium* (Wasai & Minamisawa, 2018) and *Paraburkholderia* (Martínez-Hidalgo & Hirsch, 2017). Table 2.8.1 below summarizes some of the most important species and genera for PGPB.

Table 2.3: Bacterial plant growth-promoting interactions with host plants.

Trait	Effect on plant	Genus/species	Common hosts	References
Phosphate solubilization	Increases phosphate availability	*B. megaterium, E. agglomerans, E. asburiae, Pantoea dispersa, P. putida* and *R. leguminosarum*	*Raphanus raphanistrum, V. radiata, Oryza sativa,* and *Triticum aestivum*	(Bhattacharyya & Jha, 2012; Verma *et al*., 2017; Suleman *et al*., 2018; Zheng *et al*., 2018)
Antifungal activity	Prevents fungal pathogenic infections	*E. asburiae, P. dispersa, B. amyloliquefaciens, P. polymyxa, Streptomyces murinus* and *P. putida.*	*Polygonum cuspidatum,* and *O. sativa, Lilium lancifolium*	(Sun *et al*., 2013; Shahzad *et al*., 2017; Verma *et al*., 2017; Khan *et al*., 2020)
ACC deaminase production	Actively cleaves ACC (precursor to ethylene) to lessen the effects of drought and salt stress	*P. putida, P. brassicacearum, V. paradoxus, P. fluorescens, Paenibacillus sp.* and *Aneurinibacillus aneurinilyticus*	*Pisum sativum, Brassica juncea.* and *T. esculentum*	(Belimov *et al*., 2001; Ali, Sandhya & Rao, 2014; Chimwamurombe, Grönemeyer & Reinhold-Hurek, 2016; Gupta & Pandey, 2019b)

IAA production	Improve cell-water uptake efficiency and protein synthesis during drought and salt stress	*Bradyrhizobium sp., Azospirillum sp., E. cloacae, Bacillus sp., R. leguminosarum* and *Pseudomonas sp.*	*Triticum aestivum, Raphanus raphanistrum, O. sativa,* and *Suaeda fruticose*	(Bhattacharyya & Jha, 2012; Goswami *et al*., 2014)
Siderophore production	Increases availability of iron and reduces available iron to fungal pathogens	*Staphylococcus spp., Microbacterium spp., Pseudomonas spp., Chryseobacterium spp., Burkholderia spp.*, and *Bacillus spp.*	*Paullinia cupana, Salix purpurea, Eleocharis obtuse* and, *V. radiata*	(Olanrewaju, Glick & Babalola, 2017; Batista *et al*., 2018; Oleńska *et al*., 2020)
Biological nitrogen fixation	Increases nitrogen availability especially in nutrient poor soils	*Mesorhizobium spp., Rhizobium spp. and Sinorhizobium spp., B. pumilis, R. larrymoorei, R. oryzae, R. undicola* and *B. subtilis*	*Phaseolus vulgaris, V. angularis, V. subterranea, T. esculentum* and *L. purpureus*	(Verma *et al*., 2010; Kaushal & Kaushal, 2015; Chimwamurombe, Grönemeyer & Reinhold-Hurek, 2016; Andrews & Andrews, 2017)

2.7. Conclusion

Horsegram, mung bean, moth bean, cowpea and dolichos are all valuable legumes not only because of their nutritional compositions but also because of their ability to grow and thrive in arid conditions. The declining state of food security in Africa, therefore, becomes the main driving force behind the need to fully understand these legumes. Increasing the food catalogue of Africa by increasing access to protein rich sustainable food crops as alternatives or suitable options is then of paramount importance. Furthermore, given the dire state of drylands in Africa, it is crucial that subsistence farmers be given crop enhancement options that are affordable and favour the environment in both the short and long run. In conclusion, a study on this scale with these legumes has not been done in Namibia. Given the vulnerability in which Namibia is predisposed to high temperatures and droughts, this research was set to offer potential beneficial alternatives for subsistence and commercial farming. This would also help to alleviate the challenges currently faced due to poor soils and reduced rainfalls.

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CHAPTER 3

Identification and characterisation of rhizobacteria and root endophytes isolated from drought-tolerant legumes in Namibia

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Abstract

Namibia has limited arable land (approximately 1%) which is dominated by nutrient-poor sandy soils. Coupled with the largely arid climate, crop cultivation in Namibia often requires additional nutritive and water support. The present study aimed to determine the plant growth-promoting abilities of bacteria isolated from legume species adapted to arid climates. Root endophytes and rhizobacteria from five legume species (*Vigna radiata*, *Vigna aconitifolia*, *Vigna unguiculata*, *Lablab purpureus*, and

Macrotyloma uniflorum) were identified and assessed for their plant growth promoting properties. These were exopolysaccharide production, antifungal activity, indole acetic acid production, phosphate solubilization, siderophore production, and bacterial nitrogen fixation. Isolates identified were from the Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes phyla and included known plant growth-promoting species such as *Stenotrophomonas pavanii*, *Streptomyces murinus,* and *Enterobacter cloacae*. Rhizobacteria were observed to express more plant growth-promoting traits compared to root endophytes. Siderophore production was observed in most isolates while antifungal activity was largely observed in rhizobacteria. Exopolysaccharide production, however, was observed more in root endophytes than in rhizobacteria.

Keywords: Namibia; Plant growth promotion; Root endophytes; Rhizobacteria, Abiotic stress tolerance Biotic stress tolerance

3.1. Introduction

The effects of climate change in Namibia have been evident for decades. An average increase of 0.2°C per decade has been observed and is projected to continue over the coming years (Reid *et al*., 2008; Mupambwa *et al*., 2021). Furthermore, rainfall and drought events are also projected to become more erratic. These consequences currently influence and will continue to affect all sectors of the Namibian economy (Dube *et al*., 2016). The agricultural sectors in Namibia, both commercial and subsistence, are of great importance for food security and income. More so in the rural areas of Namibia where 57-70% of the population rely on subsistence farming (Angula & Kaundjua, 2015; Braker *et al*., 2015).

The agricultural sector in Namibia has long been vulnerable given that only 1% of the land is arable (Food and Agriculture Organization, 2022). Agricultural activity in the northern regions is limited. The soils are sandy (arenosols) and considered nutrient-poor (organic carbon ~0.4%, nitrogen range ~0.03–0.16% and low phosphorus concentrations that are below detection). These low values may be attributed to the low organic input due to low biodiversity (Liu, Han & Li, 2021). The poor soils dominate the northern parts of Namibia and contribute to the climate change challenges that affect the agricultural sector in the country (Grönemeyer *et al*., 2012). Therefore, there is an urgent need to improve current farming practices to maximise agricultural outputs.

To address these challenges, there is a need to develop economically and environmentally sustainable options that favour both subsistence and commercial farmers. This may be done by the development and use of biofertilisers from plant growth-promoting bacteria (PGPB) (Bakhtiyarifar, Enayatizamir & Mehdi Khanlou, 2021). PGPB are plant-associated bacteria that assist plants with general growth and stress tolerance. This is achieved via the production of compounds that increase nutrient uptake (O'Callaghan, 2016), prevent pathogenic infections (Cherif-Silini *et al*., 2016) and increase abiotic stress tolerance (García-fraile, Menéndez & Rivas, 2015). These PGPB may be used as biofertilisers with lower cost and less environmental impact while attaining a significant effect on higher yield (Khosro & Yousef, 2012).

Several PGPB have been developed as biofertilisers for various crops. Co-inoculations of some *Bacillus sp.* and *Mesorhizobium ciceri* have been used on chickpea crops (Igiehon, Babalola & Aremu, 2019). Improvements in wheat yield by up to 43% have

been noted with the use of *Azotobacter* and *Bacillus* inoculations (Kalayu, 2019). Applications of bioinoculants also include phosphate solubilising bacteria making phosphate available for plants to absorb. It is also important to note that some PGPB work better with certain crops. *Rhizobium* species, for example, are known to improve plant health in legumes and pulses as they fix nitrogen more effectively. On the other hand, *Azospirillum* bioinoculants have been found to be effective biofertilisers on cereals such as sorghum and pearl millet (Nosheen, Ajmal & Song, 2021).

The present study identified and characterised bacterial isolates from five legume species grown in Namibia. The plant growth-promoting (PGP) properties of bacterial symbionts isolated from the root endosphere, and rhizosphere were assessed. The legumes, adapted to arid climates, are mung bean [*Vigna radiata* (L.) R. Wilczek var. radiata], mothbean [*Vigna aconitifolia* (Jacq.) Marechal], cowpea (*Vigna unguiculata* L. Walp), dolichos [*Lablab purpureus* (L.) Sweet var. Lignosus Prain] and horsegram (*Macrotyloma uniflorum* Var. Madhu).

3.2. Materials and methods

3.2.1 Seed material and growth conditions

Analysis was carried out on six (6) accessions from five (5) legume species. The accessions were IC0623025 (*L. purpureus*), Gujarat 5 (*V. unguiculata*), Himala (*M. uniflorum*), IC39399 (*V. radiata*), and two accessions from *V. aconitifolia:* IPCMO-880 and RMB-25. The varieties were planted in pots in a growth chamber. Seeds from each accession were planted into nine (9) 20 cm pots. Each pot with two plants. Samples were watered twice a week receiving approximately 200 mL each. The growth chamber was maintained with 8 daylight hours and 75% humidity. Samples

were monitored for growth and seed production following the methods described by Khandare *et al.* (2020). From each pot, the healthier and stronger plant was selected for isolation of bacterial symbionts.

3.2.1 Isolation of bacteria from the rhizosphere

Rhizosphere sample collection

Plant samples were dug out from pots and roots were aseptically removed from the stem using a sterile scalpel. The roots were weighed and transferred to sterile falcon tubes before adding sterile phosphate buffer saline (PBS) solution (McPherson *et al*., 2018). The tubes were vortexed for 5 minutes to loosen the soil from the roots. Thereafter, the roots were carefully removed with sterile forceps and placed into sterile falcon tubes and stored at 4°C until further processing.

Isolation of rhizobacteria was performed as described by Omar *et al*., (2021) with minor modifications as described below. The soil-PBS mixture was serially diluted up to 10^{-6} using sterile PBS solution. From each dilution, 100 µL was plated onto the following media, yeast extract mannitol (YEM), tryptic soy agar (TSA) and Jensen media. Media compositions, per litre, were: YEM- yeast extract, 1 g; mannitol, 10 g; dipotassium phosphate, 0.5 g; magnesium sulphate, 0.2 g; sodium chloride, 0.1 g; Congo red, 0.025 g; agar, 20 g; 20% TSA- tryptic soy broth, 6 g; agar, 16 g and Jensen -sucrose, 20 g; dipotassium phosphate, 1 g; magnesium sulphate, 0.5 g; sodium chloride, 0.5 g; ferrous sulphate, 0.1 g; sodium molybdate, 0.005 g; calcium carbonate, 2 g; agar, 16 g.

Isolation of bacteria from root endosphere

Root samples previously cut and stored were surface sterilised by washing twice with sterile distilled water followed by incubation for 20 seconds in 70% ethanol. The roots were then incubated for 1 minute in sterile distilled water before being incubated for 30 seconds in 5% sodium hypochlorite. Thereafter, roots were washed six times with sterile distilled water. Surface sterilisation was confirmed by plating 100 µL of the final wash onto TSA plates and incubated at 30°C. Samples showing growth were excluded from further analysis.

Sterilised roots were macerated in 5-10 ml sterile PBS. Thereafter, 100 µL of each macerated sample was inoculated onto 20% TSA, YEM and Jensen media plates. Plates were incubated at room temperature for 3 days before being transferred to a 30°C incubation chamber. Growth was monitored over a period of 5-7 days. Pure cultures were maintained on 20% TSA.

3.2.2 Identification of bacterial isolates

Bacterial isolates were identified following a method similarly described by Pesce, Kleiner and Tisa, (2019). Distinct colonies were inoculated onto 20% TSA and incubated for 24 hours. After which, a single colony was picked from each sample and suspended in 45 µL of sterile distilled water in PCR tubes. These samples underwent a PCR lysis protocol to obtain template DNA as follows: 96°C for 10 minutes. The template DNA was amplified using 16S rRNA universal primers: FDIFuni- 5′AGA GTT TGA TCC TGG CTC3′ and P2Runi- 5′ACG GCT ACC TTG TTA GGA CTT3′. The PCR program used was: 5 minutes of initial denaturation at 98°C, 30 cycles of denaturation at 96°C for 40 seconds, annealing at 54°C for 40 seconds, and extension at 72°C for 90 seconds. A final extension was set at 72°C for 2 minutes.

After PCR, samples were run on gel electrophoresis to confirm amplification and then purified using the EuroClone® spinNaker Gel and PCR DNA purification kit, (Pero, Italy). Amplified samples were sequenced with primers 907r (5′-CCGTCA-ATTCMTTTRAGTTT-3′) (Lane *et al*., 1985) and F785 (5′-GGATTAGATACCC-TGGTA-3′) (Vannini *et al*., 2004). Samples were sequenced by Eurofins Genomics (Ebensburg, Germany). Primary sequence data was run through the National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST) to determine identity. Sequences were submitted to NCBI GenBank, and the accession numbers are listed in Table 3.1.

3.2.3 Characterisation of bacterial isolates

Bacterial phosphate solubilization

Phosphate solubilization ability by bacteria was determined following the method described by Nautiyal (1999) with modifications. Isolates were spot inoculated onto National Botanical Research Institute's phosphate (NBRIP) growth medium (Glucose, 10 g; $Ca_3(PO_4)_2$, 5 g; $MgCl_2 \cdot 6H_2O$, 5 g; $MgSO_4 \cdot 7H_2O$, 0.250 g; KCl, 0.200 g; $(NH_4)_2SO_4$, 0.100 g and agar, 16 g per litre) plates. The inorganic phosphate source was tri-calcium phosphate ($Ca_3(PO_4)_2$). Plates were incubated at 30°C for 7 days.

Bacterial nitrogen fixation

Isolates from the roots and rhizosphere were assessed for their ability to fix nitrogen. This was determined by observing growth on Jensen medium (Das & De, 2018). Isolates were incubated for 72 hours at 30°C.

Bacterial siderophore production

Siderophore production was determined following a modified procedure described by (Schwyn & Neilands, 1987). Chrome azurol S (CAS) agar was prepared with CAS and hexadecyl-trimethylammonium bromide (HDTMA) as indicators. Isolated strains were inoculated onto the medium in duplicates and incubated at 30°C for 5 days in the dark. Siderophore production was scored when a halo of a minimum of 1 mm was observed. Samples showing halos of more than 5 mm were considered superior siderophore producers.

Bacterial antifungal activity

Antifungal activity against *Fusarium graminearum,* a plant pathogen, was tested following the method described by Rajendran *et al.* (2008). Fungal isolates grown on PDA plates were placed onto fresh PDA plates inoculated with the PGPB isolates. The plates and fungal inoculants were monitored for a zone of inhibition over a period of 21 days at 30°C.

Bacteria indole acetic acid production

With modifications, IAA production was determined following the method described by Rajendran, Patel and Joshi, (2012). Pure colonies were grown in 5 mL of 20% Tryptic soy broth over 24 hours at 30°C on a shaker at 220 rpm. Thereafter, 2 mL of each culture were centrifuged. The supernatant (1 mL) was carefully recovered and combined with 2ml of Salkowski's reagent (1 mL of 0.5 M $FeCl_3$ in 50 mL of 35% perchloric acid). To this, 10 µL of orthophosphoric acid was added. Samples were incubated in the dark for 30 minutes. Reading was done at OD_{530}. The standard graph (Figure 3.1 below) was prepared by dissolving 1 mg of IAA in 5 mL of LB broth.

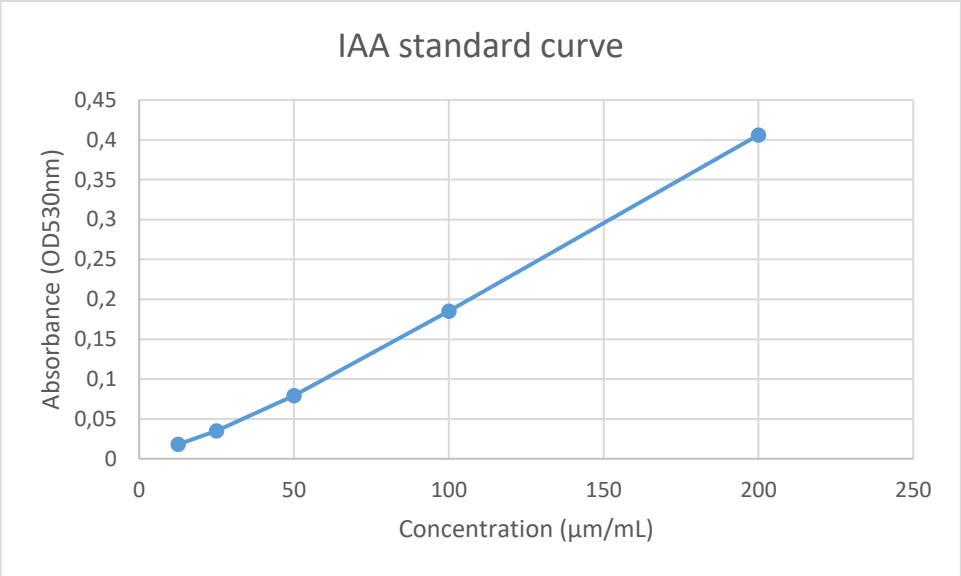

Figure 3.1: IAA standard reference curve based on 1 mg dissolved in 5 mL of LB broth.

Bacterial exopolysaccharide production

EPS production was assessed on YEM (per litre- Yeast extract 0.5 g, Mannitol 4.0 g and agar 16 g) as previously described with modifications. Isolates were inoculated and observed for growth patterns on YEM after 48 hours at 30°C. Positive EPS was scored by the presence of a mucoid textured colonies (Latif *et al*., 2022).

3.2.4 Data analysis

Sequence data were analysed using nucleotide BLAST from NCBI. The searches were made in the 16S ribosomal RNA sequences (Bacteria and Archaea) database and limited to highly similar sequences. Uncultured and environmental sample sequences were excluded from alignments (Zhang *et al*., 2000). Principal component analysis (PCA) was performed using R statistical language version x64 4.1.2 (R Core Team, 2021), factoextra (Lê, Josse & Husson, 2008), and ggplot2 (Wickham, 2009).

3.3. Results

3.3.1 Isolation and identification of bacterial isolates

It was of interest to isolate bacterial strain from five legume species grown in Namibia. As described in the Materials and methods section, a total of 123 isolates based on phenotypic appearance were obtained from the roots and rhizosphere of the five accessions. From this set, 55 isolates were positively identified using 16s rDNA sequencing. Identical strains from the same sample set (root endosphere or rhizosphere for each accession) were excluded from further studies.

A total of 40 identified strains isolated from the roots and rhizosphere of the six accessions were then further studied and analysed for siderophore production, phosphate solubilization, nitrogen fixation, antifungal activity, and IAA production. Identified bacterial isolates were from four phyla, Proteobacteria, Actinomycetota, Bacteroidetes and Firmicutes. Proteobacteria was the most abundant phylum making up 72% of the isolates while 15% were Actinomycetota. The least abundant phyla were Firmicutes and Bacteroidetes which were 8% and 5% respectively.

Genera identified among root endophyte isolates were *Pseudomonas, Enterobacter, Stenotrophomonas, Serratia, Brucella,* and *Herbaspirillum.* Rhizobacteria were more diverse with 10 different genera identified. These were *Serratia, Stenotrophomonas, Streptomyces, Pseudomonas, Staphylococcus, Flavobacterium, Herbaspirillum, Gryllotalpicola, Paenarthrobacter,* and *Brucella* (Table 3.2).

Table 3.1: NCBI BLAST sequence identities.

Species	Common name	Accession	Sample ID	Closest relative	Percent identity (%)	Assigned accession number
Lablab purpureus	Dolichos	IC0623025	DR10	*Pseudomonas aeruginosa*	99,88	ON454260
			DR14	*Lysobacter soli*	99.77	OP564985
			DRhi1	*Serratia nematodiphila*	99,77	ON454259
			DRhi25n	*Stenotrophomonas pavanii*	99,65	ON454254
			DRhi9	*Serratia nematodiphila*	99,43	ON454282
Vigna unguiculata	Cowpea	Gujarat 5	CR10	*Serratia nematodiphila*	99,65	ON454262
			CR22	*Serratia sp.*	96,98	OP503858
			CRhi10	*Streptomyces murinus*	100,00	OP503859
			CRhi15	*Stenotrophomonas maltophilia*	99,19	ON454265
			CRhi18	*Stenotrophomonas maltophilia*	99,30	OP503860
Macrotyloma uniflorum	Horsegram	Himala	HR11new2	*Pseudomonas nitroreducens*	99,65	ON454252
			HR4	*Enterobacter cloacae*	99,06	OP503861

			HR5	*Enterobacter mori*	99,78	OP503856
			HR6	*Enterobacter ludwigii*	99,88	OP503862
			HR7	*Enterobacter cloacae subsp. dissolvens*	99,76	ON454269
			HRhi1	*Streptomyces murinus*	100,00	ON454271
			HRhi12	*Stenotrophomonas sp.*	95,05	OP503863
			HRhi18	*Pseudomonas sp.*	98,59	OP503864
			HRhi4	*Flavobacterium anhuiense*	99,75	ON454274
			HRhi5	*Streptomyces sp.*	97,80	ON454276
Vigna radiata	Mungbean	IC39399	MBR1	*Brucella anthropi*	99,75	OP503865
			MBR14	*Stenotrophomonas pavanii*	99,64	ON454273
			MBR9	*Stenotrophomonas sp.*	94,18	OP503866
			MBRhi10	*Herbaspirillum aquaticum*	99,88	ON454279
			MBRhi14	*Pseudomonas plecoglossicida*	100,00	ON454278

			MBRhi17	*Streptomyces murinus*	100,00	ON454251
			MBRhi20	*Stenotrophomonas pavanii*	99,64	ON454253
			MBRhi3	*Staphylococcus sp.*	88,74	ON454256
Vigna aconitifolia	Moth bean	IPCMO-880	IPCR2	*Brucella sp.*	98,80	OP503857
			IPCR4	*Pseudomonas sp.*	100,00	ON454277
			IPCRhi1	*Staphylococcus sp.*	88,55	ON454270
			IPCRhi18	*Pseudomonas aeruginosa*	100,00	OP503867
			IPCRhi7	*Staphylococcus sp.*	87,54	**
		RMB-25	RMBR1	*Stenotrophomonas pavanii*	99,29	OP503868
			RMBR3	*Brucella sp.*	88,28	**
			RMBR7	*Herbaspirillum frisingense*	99,76	OP503869
			RMBRhi1	*Gryllotalpicola sp.*	92,26	ON454267
			RMBRhi17	*Flavobacterium sp.*	97,81	OP503870

			RMBRhi4	*Paenarthrobacter nicotinovorans*	99,64	ON454266
			RMBRhi6	*Brucella sp.*	98,72	ON454268

**Sequences with low or no similarity to 16S ribosomal RNA and subsequently excluded from NCBI submitted sequences.

3.3.2 Characterisation of the bacterial isolates

The identified isolates were characterised based on six PGP assays. These are siderophore production, phosphate solubilization, nitrogen fixation, antifungal activity, EPS production, and IAA production. The 40 bacterial isolates described above consisted of 17 putative root endophytes and 23 rhizosphere isolates and were all analysed for the PGP traits. The 17 bacterial endophytes exhibited positive activity to at least one trait (Table 3.3). *S. nematodiphila* (CR10) from *V. unguiculata* and *B. anthropi* (MBR1) from *V. radiata* both showed PGP features in four of the assessed traits. However, both exhibited relatively low production of IAA. Of the rhizobacteria isolates, two exhibited no PGP trait. These were *Streptomyces sp.* and *Staphylococcus sp.* unclassified strains. A summary of the results obtained is presented in Table 3.3.

Siderophore production

Assessing the production of siderophores, three strains did not show any growth on the specific media used for siderophore detection after four days of incubation. Most siderophore production was observed in strains isolated from the rhizosphere of *L. purpureus* and isolates from both the roots and rhizosphere of *M. uniflorum*. These were, DRhi9, HR5, HRhi12 and HRhi18 (Table 3.3). Seven strains did not display siderophore production in the assay that was used here. These were, CRhi18, CRhi10, RMBR7, RMBRhi6, RMBRhi17, HRhi5 and IPCRhi7 isolated from *V. unguiculata, V. aconitifolia* (RMB 25 and IPC880), and *M. uniflorum* (Figure 3.2). However, the bacterial strains that did not show siderophore production could have produced them at low levels or the production was possibly stringently regulated.

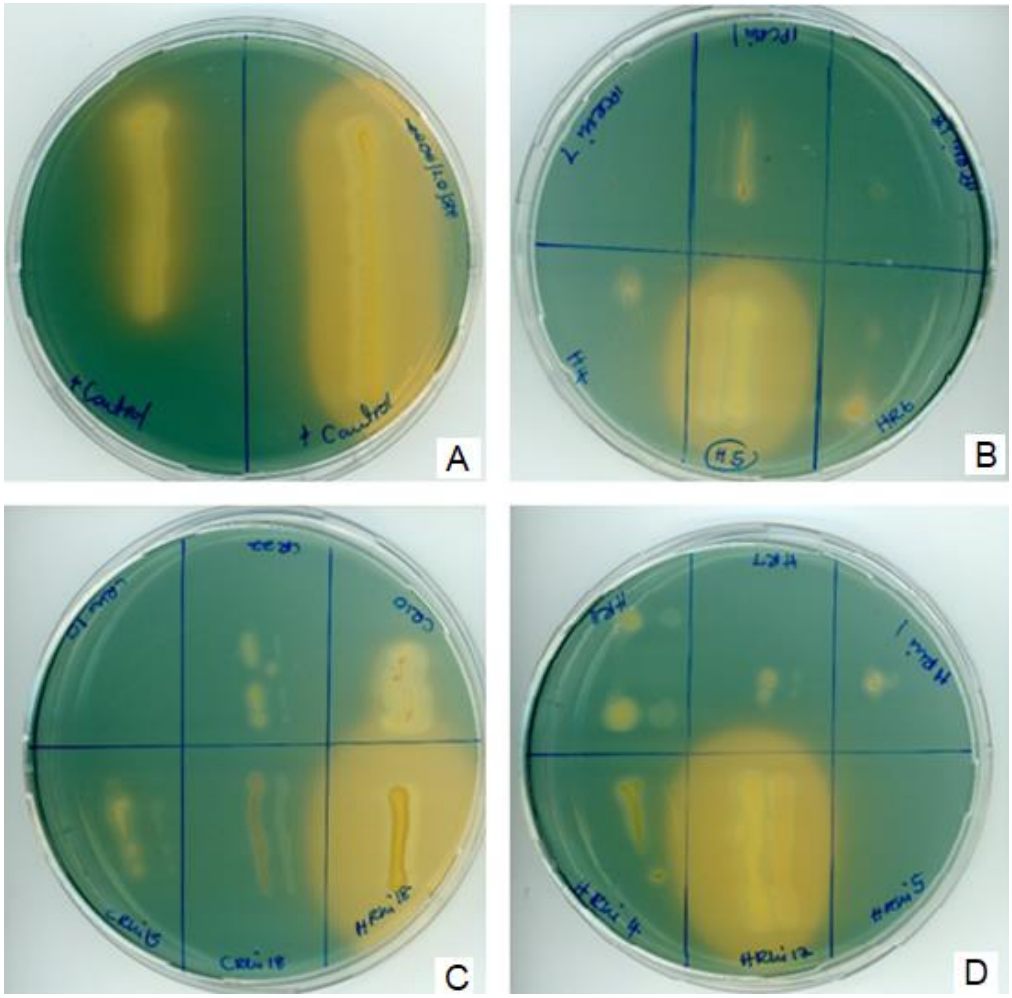

Figure 3.2: Siderophore production by root endophytes and rhizobacteria. **A**: Two siderophore-producing controls (*Escherichia coli* on the left and *Pseudomonas fuscovaginae* on the right) indicate low-producing and high-producing isolates. **B:** High-producing *E. mori* (H5), a root endophyte from *M. uniflorum.* **C:** Siderophore production is observed to be moderate in CR10- *S. nematodiphila* compared to the higher production by a rhizobacterium, *Pseudomonas sp.* (HRhi18). **D:** Different growth patterns of different isolates from roots and rhizosphere of the same legume species i.e., *M. uniflorum.*

Nitrogen fixation

The 40 bacterial strains were assessed for free living nitrogen fixation; 27 isolates indicated low to no growth on Jensen medium indicating that most likely they do not fix atmospheric nitrogen under these conditions (Table 3.3). These were determined to not fix atmospheric nitrogen in free living conditions on Jensen medium. Seven isolates were root endophytes (CR10, RMBR1, HR5, IPCR2, IPCR4, MBR9, MBR1)

while six were rhizobacteria (MBRhi10, DRhi9, CRhi18, RMBRhi1, RMBRhi6, HRhi12) as shown in Table 3.3. *V. aconitifolia* (RMB-25) and *V. radiata* had the most nitrogen-fixing isolates compared to the other accessions.

Phosphate solubilization

It was of interest to determine the phosphate solubilization ability of the bacterial isolates and it was observed in eight of the isolates, three being root endophytes. Three of those isolates were root endophytes. These were *S. nematodiphila* (CR10) from *V. unguiculata* (Shown in Figure 3.3 below), *S. pavanii* (MBR14), and *B. anthropi* (MBR1) from *V. radiata*. Rhizospheric isolates exhibiting phosphate solubilization were *S. nematodiphila* (DRhi9), and *S. pavanii* (DRhi25n) from *L. purpureus*, *Gryllotalpicola sp.* (RMBRhi1) from *V. aconitifolia* (RMB-25), *Stenotrophomonas sp.* (HRhi12), and *Pseudomonas sp.* (HRhi18) from *M. uniflorum* (Table 3.3).

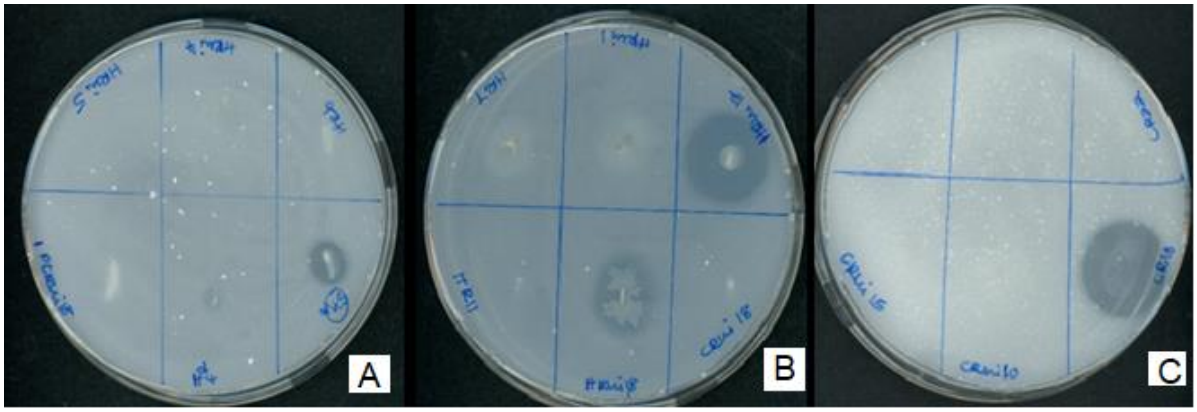

Figure 3.3: Phosphate solubilization by root endophytes and rhizobacteria on NBRIP growth medium. Some solubilizing isolates are H5: *E. mori* (**A**), two unspecified species HRhi18: *Pseudomonas sp.* and HRhi12: *Stenotrophomonas sp.* (**B**) from *M. uniflorum* and CR10: *S. nematodiphila* (**C**) from the root endosphere of *V. unguiculata.*

Bacterial IAA production

This study aimed to identify bacterial isolates that produce the phytohormone IAA. The concentration of IAA produced by the isolates was determined from known concentrations of IAA. Bacterial production of IAA was determined based on known

positive and negative control isolates. Seven isolates were positive for the production of IAA production. These include five isolates from the roots and rhizosphere of *M. uniflorum.* Namely *Streptomyces sp., B. anthropi, Stenotrophomonas sp., P. plecoglossicida,* and *S. pavanii*. IAA production was also observed from rhizobacteria (*S. maltophilia* and *P. nicotinovorans*) from *V. unguiculata* and *V. aconitifolia* (IPCMO-880).

Bacterial antifungal activity

The isolates in this study were also assessed for antimicrobial activity against *F. graminearum.* Most isolates assessed for having anti-fungal activity, did not exhibit an antagonistic effect on a fungal pathogen; an antagonistic effect on *F. graminearum* by the isolates was observed in four isolates (Figure 3.4). Three of the isolates were rhizobacteria isolated from *V. unguiculata, M. uniflorum* and *V. radiata.* These were all provisionally identified as *S. murinus*. The root endophyte was isolated from *M. uniflorum* with its closest identity being *E. cloacae subsp. dissolvens.*

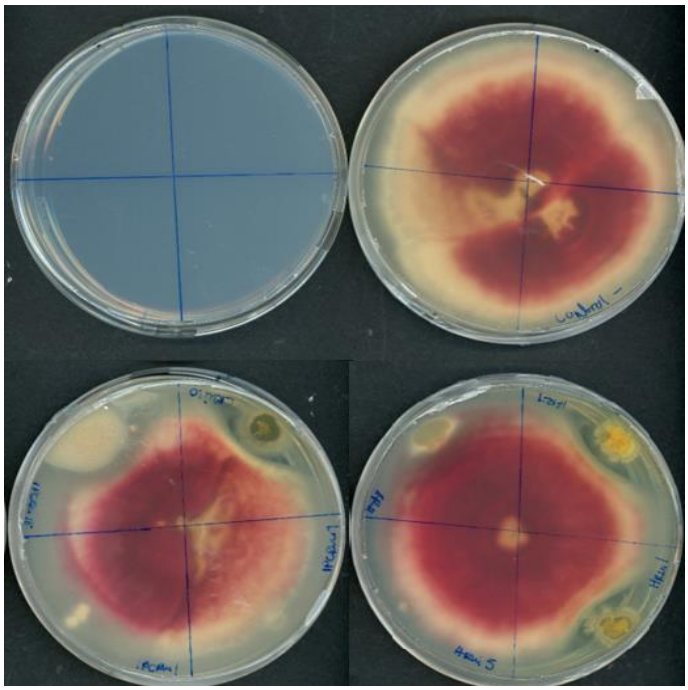

Figure 3.4: *Streptomyces sp.* and *E. cloacae subsp. dissolvens* antifungal activity against *F. graminearum.*

Bacterial exopolysaccharide production

This study also aimed to assess EPS production on YEM agar plates. EPS production was characterised by a mucoid appearance of plated colonies (Figure 3.5). From the 40 isolates assessed, 16 were observed to produce EPS. Interestingly, no isolate from *L. purpureus* was positive for EPS production. One root endophyte and one rhizobacterium from *V. unguiculata* were observed to produce EPSs. This was also observed with *M. uniflorum*. *V. radiata* and *V. aconitifolia* (IPCMO-880) each had three positive isolates (two root endophytes and one rhizobacterium).

Three isolates were root endophytes and three were rhizobacteria (Table 3.3). The EPS-producing isolates from all five accessions were identified as *S. nematodiphila, S. pavanii, Stenotrophomonas sp., B. anthropi, Brucella sp., P. plecoglossicida, Pseudomonas sp., E. mori, H. frisingense, Gryllotalpicola sp., Flavobacterium sp.,* and *Staphylococcus sp.*

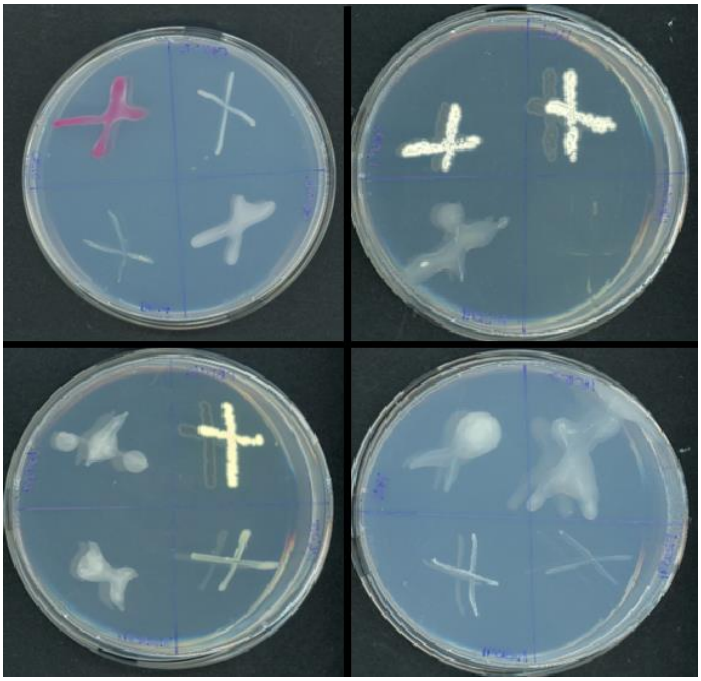

Figure 3.5: Exopolysaccharide production by root endophytes and rhizobacteria. Isolates with distinct 'X' colonies show negative EPS production. Isolates with excessive mucoid colonies show positive EPS production.

Overall plant growth-promoting activity was observed to be higher with root endophytes than with rhizobacteria. To assess correlation, principal component analysis (PCA) was carried out on the number of PGP active strains per sample set. A scree plot (Figure 3.6) was prepared in which the first two components explained 81% of the variation. The subsequent PCA (Figure 3.7) of the variables against the different accessions showed no pattern or correlation. However, PCA of the PGP traits (Figure 3.8) indicate some relatedness. Positive correlation of variables was observed within phosphate solubilization, IAA production, siderophore production and antifungal activity traits in one direction. While EPS production and nitrogen exhibited positive correlation to another direction from the previously mentioned traits.

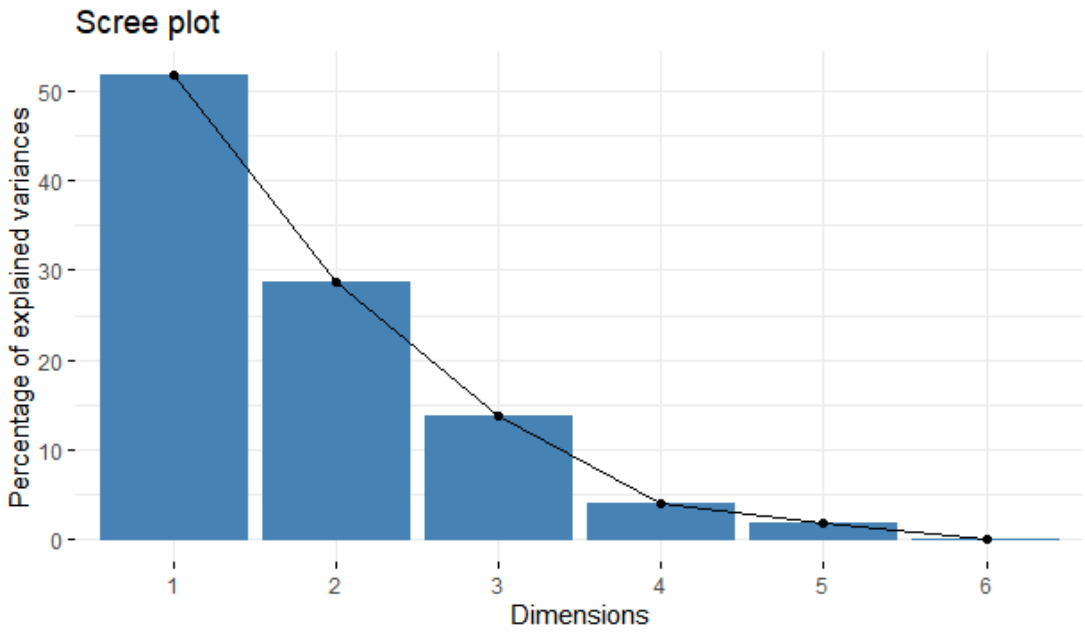

Figure 3.6: Percentage of variances explained by each principal component.

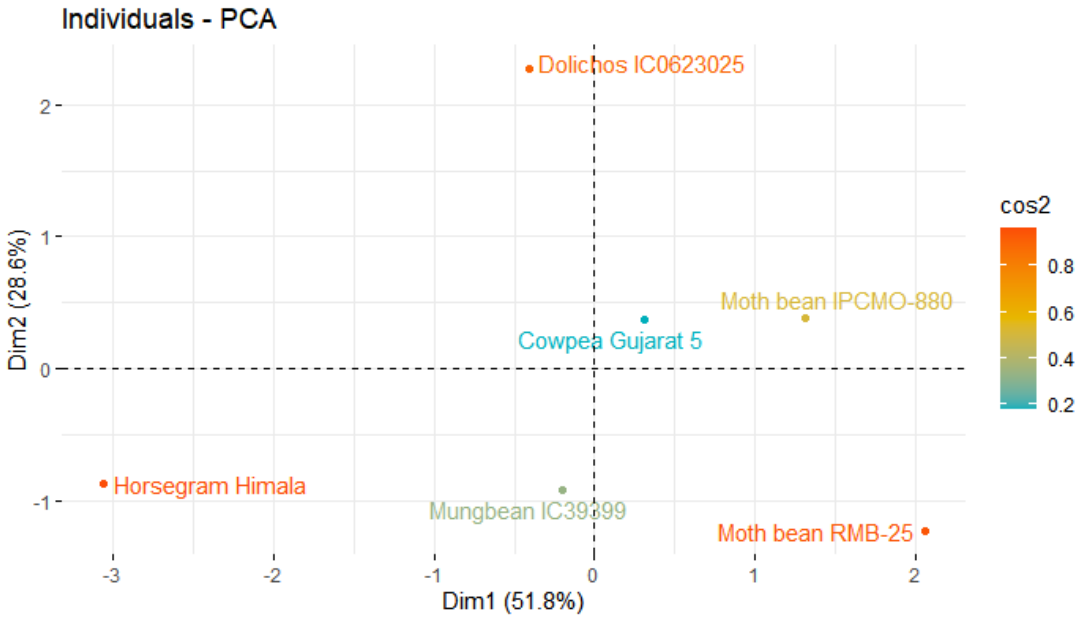

Figure 3.7: PCA presentation of legume accessions showing minimal similarities. Dolichos IC0623025 - *L. purpureus*, Cowpea Gujarat 5 - *V. unguiculata*, Horsegram Himala - *M. uniflorum*, Mungbean IC39399 - *V. radiata*, Moth bean IPCMO-880 - *V. aconitifolia*, and Moth bean RMB-25 - *V. aconitifolia*.

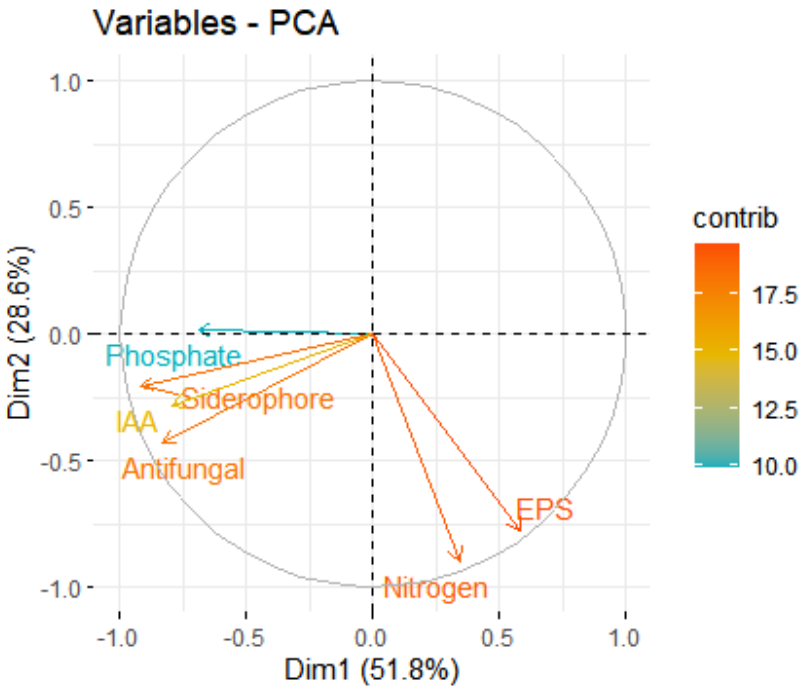

Figure 3.8: PCA graph of PGP traits showing greater positive correlation among phosphate solubilization, IAA production, siderophore production and antifungal activity. These traits appear independent to EPS production and nitrogen fixation which together form a positive correlation.

Table 3.2: PGP traits of isolates from root endosphere and rhizobacteria**.**

Legume Species	Accession	Site	Isolate	Bacterial genus/species	Phos[a]	Sid[b]	Nit[c]	Ant[d]	EPS[e]	IAA[f]
Lablab purpureus	IC0623025	Roots	DR10	*Pseudomonas aeruginosa*	-	+	-	-	-	-
		Roots	DR14	*Lysobacter soli*	-	+	-	-	-	-
		Rhizosphere	DRhi1	*Serratia nematodiphila*	-	+	-	-	-	-
		Rhizosphere	DRhi9	*Stenotrophomonas pavanii*	+	++[1]	+	-	-	-
		Rhizosphere	DRhi25n	*Serratia nematodiphila*	+	+	-	-	-	-
Vigna unguiculata	Gujarat 5	Roots	CR10	*Serratia nematodiphila*	+	+	+	-	+	-
		Roots	CR22	*Serratia sp.*	-	+	-	-	-	-
		Rhizosphere	CRhi18	*Streptomyces murinus*	-	-	+	-	+	-
		Rhizosphere	CRhi15	*Stenotrophomonas maltophilia*	-	+	-	-	-	+
		Rhizosphere	CRhi10	*Stenotrophomonas maltophilia*	-	-	-	+	-	-
Vigna radiata	IC39399	Roots	MBR14	*Pseudomonas nitroreducens*	+	**Un**	-	**Un**	**Un**	-
		Roots	MBR9	*Enterobacter cloacae*	-	+	+	-	+	-

		Roots	MBR1	*Enterobacter mori*	+	+	+	-	+	-
		Rhizosphere	MBRhi20	*Enterobacter ludwigii*	-	+	-	-	-	-
		Rhizosphere	MBRhi17	*Enterobacter cloacae subsp. dissolvens*	-	+	-	+	-	-
		Rhizosphere	MBRhi14	*Streptomyces murinus*	-	+	-	-	+	-
		Rhizosphere	MBRhi10	*Stenotrophomonas sp.*	-	+	+	-	-	-
		Rhizosphere	MBRhi3	*Pseudomonas sp.*	-	-	-	-	-	-
Macrotyloma uniflorum	Himala	Roots	HR4	*Flavobacterium anhuiense*	-	+	-	-	-	-
		Roots	HR5	*Streptomyces sp.*	-	++[1]	+	-	+	+
		Roots	HR6	*Brucella anthropi*	-	+	-	-	-	+
		Roots	HR7	*Stenotrophomonas pavanii*	-	+	-	+	-	-
		Roots	HR11new2	*Stenotrophomonas sp.*	-	+	-	-	-	+
		Rhizosphere	HRhi1	*Herbaspirillum aquaticum*	-	+	-	+	-	-
		Rhizosphere	HRhi4	*Pseudomonas plecoglossicida*	-	+	-	-	-	+

		Rhizosphere	HRhi5	*Streptomyces murinus*	-	-	-	-	-	-
		Rhizosphere	HRhi12	*Stenotrophomonas pavanii*	+	++[1]	+	-	+	+
		Rhizosphere	HRhi18	*Staphylococcus sp.*	+	++[1]	-	-	-	-
Vigna aconitifolia	RMB-25	Roots	RMBR1	*Brucella sp.*	-	+	+	-	+	-
		Roots	RMBR3	*Pseudomonas sp.*	-	+	-	-	+	-
		Roots	RMBR7	*Staphylococcus sp.*	-	-	-	-	+	-
		Rhizosphere	RMBRhi1	*Pseudomonas aeruginosa*	+	-	+	-	+	-
		Rhizosphere	RMBRhi4	*Staphylococcus sp.*	-	+	-	-	-	-
		Rhizosphere	RMBRhi6	*Stenotrophomonas pavanii*	-	-	+	-	+	-
		Rhizosphere	RMBRhi17	*Brucella sp.*	-	-	-	-	+	-
	IPCMO-880	Roots	IPCR2	*Herbaspirillum frisingense*	-	+	+	-	+	-
		Roots	IPCR4	*Gryllotalpicola sp.*	-	+	+	-	+	-
		Rhizosphere	IPCRhi18	*Flavobacterium sp.*	-	+	-	-	+	-
		Rhizosphere	IPCRhi7	*Paenarthrobacter nicotinovorans*	-	-	-	-	-	+

		Rhizosphere	IPCRhi1	*Brucella sp.*	-	+	-	-	-	-

[a] Phosphate solubilization
[b] Siderophore production
[c] Nitrogen fixation
[d] Antifungal activity
[e] Exopolysaccharide production
[f] Indole acetic acid production
[1]Showed greater activity compared to other isolates based on the size of the halo
"–" means showed no production/ "+" means showed production/ "Un" means plant growth-promoting trait could not be determined.

3.4. Discussion

The legumes assessed in this study are typically grown in arid climates and they often form symbioses with microbes that enhance stress tolerance (Sithole, Pérez-Fernández & Magadlela, 2019; Alsharif, Saad & Hirt, 2020). The bacteria isolated and characterised in this study exhibited PGP activities which have the potential to support adaptation to stress conditions. The overall differences observed between legume accessions are attributed to variations in plant metabolism and root exudates that influence plant microbial colonization (Xiao, Chen, *et al*., 2017). Rhizobacteria and endophytes are also capable of improving plant growth and control pathogens have been reported in various studies (Ngumbi & Kloepper, 2016; Orozco-mosqueda, Glick & Santoyo, 2020). In this study, we report that most bacterial isolates from the different legume species were able to show at least one PGP activity *in vitro*.

Siderophore production is a process carried out by many plants. Plants rely in part on rhizobacteria production to increase iron uptake (Francis, Holsters & Vereecke, 2010). Many rhizobacteria and root endophytes produce siderophores in order to obtain iron from the environment (Kaushal & Kaushal, 2015). This was similarly observed in our study on rhizobacteria and root endophytes. However, siderophore production is not exclusive to root endophytes and rhizobacteria; for example, seed endophytes have been identified to produce iron-chelating siderophores. This was observed in seed endophytes from *Tylosema esculentum* grown in Namibia (Chimwamurombe, Grönemeyer & Reinhold-Hurek, 2016). Rhizobacteria, however, exhibited greater siderophore production based on the halo size. This was an expected observation as siderophores produced in the rhizosphere optimise the uptake of soluble iron (Singh, Gera & Kumar, 2018).

PGP traits have been observed to be more frequent in rhizobacteria than in root endophytes (Francis, Holsters & Vereecke, 2010). However, the nitrogen fixation assay in this study identified 53.8% of the potential total nitrogen-fixing isolates to be root endophytes. This may be due to differences in nutrient concentrations between the rhizoplane and root endosphere. Phosphorus content, for example, influences the rate of nitrogen fixation by microbes (Hussain, 2017).

Studies have shown that phosphate solubilizing bacteria are more likely to be isolated from the rhizosphere than any other biosphere associated with plants (Chawngthu, Hnamte & Lalfakzuala, 2020; Sagar *et al*., 2022). This was similarly observed in our results as 62.5% of our isolates exhibited phosphate solubilization activity. Phosphorus is an essential macronutrient required for plant growth and promotion frequently found in insoluble forms in the soil. This may explain the increased activity of phosphate solubilization by rhizobacteria than root endophytes (Batool & Iqbal, 2019).

The production of the phytohormone IAA, varied greatly across all samples. However, positive production was observed in seven samples. This was an expected observation as IAA production is regulated by bacterial metabolism which varies across species and genera (Alkahtani *et al*., 2020; Latif *et al*., 2022). Rhizobacteria were observed to be more likely to produce IAA which is contrary to a previous study. This is because the root endosphere is often found to have high concentrations of tryptophan which promotes the metabolic production of IAA by bacteria (Goudjal *et al*., 2013). However, other studies have observed that higher production of IAA by root endophytes was irrespective of the presence of tryptophan (Alkahtani *et al*., 2020).

Higher concentrations of IAA were observed from *Stenotrophomonas, Enterobacter, Flavobacterium, Staphylococcus* and *Pseudomonas* species. Our results did not concur

with previous studies as *Streptomyces sp*. isolates did not produce any IAA (Goudjal *et al*., 2013). IAA production by *Pseudomonas* species is often antagonised by low phosphorus levels in soils. However, this may be mitigated by the presence of other phosphate solubilizing *Pseudomonas sp.* (Alemneh *et al*., 2020). Based on this observation an assumption can be made about the ability of *Pseudomonas sp.* isolates to produce IAA. The presence of the phosphate solubilizing *P. plecoglossicida* in the soil can induce this positive feedback activity.

Antifungal activity was observed from two species *Streptomyces murinus*, isolated from the rhizosphere and *Enterobacter cloacae subsp. dissolvens* isolated from the root endosphere. Antifungal activity is due to bacterial metabolism and subsequent production of compounds with antagonistic effects on pathogenic fungi (Bahroun *et al*., 2018). Root endophytes are often observed to influence biocontrol compared to rhizobacteria. This is due to the root endosphere being compartmentalised from the environment (Mutungi *et al*., 2022). Our results, however, showed greater antifungal activity by rhizobacteria.

A previous study on the antifungal activity of *E. cloacae* on *Fusarium* pathogens found it to be inactive. Fungal inhibition, however, was observed against *Pythium debaryanum* and *Rhizoctonia solani* (Panigrahi & Rath, 2021). This difference may be attributed to changes in secondary metabolites between species and subspecies due to shifts in microbiomes (Turner, James & Poole, 2013). The antagonistic behaviour of *S. murinus* was an expected feature as organisms from the order *Actinomycetales* are known to have antagonistic effects on different fungal species (Martínez-Hidalgo & Hirsch, 2017). It is important to note that the antifungal activity assay presented in this study was against only one fungal pathogen. Therefore, it can be argued that the

antifungal activity of the remaining isolates might be against other fungal plant pathogens. As such, they should not be excluded as not having any antifungal activity as this may vary with different fungal pathogens (Panigrahi & Rath, 2021).

Exopolysaccharides, as hygroscopic compounds, allow bacteria to retain water in water-stressed conditions. They are commonly produced by rhizobacteria and have been identified in previous work (Latif *et al*., 2022). Therefore, EPS production is largely expected from rhizobacteria as they form a biofilm between the soil and roots. This increases root protection from desiccation (Fadiji *et al*., 2022). It is important to note that EPS production may be increased during stress conditions. This means some isolates not showing EPS production after 48 hours on YEM may require increased stress to show EPS production. This trait has been previously observed in *Pseudomonas sp.* (Ashry *et al*., 2022).

3.5. Conclusion

The present study identified and characterised bacterial isolates from five legume species grown in Namibia. The PGP properties of bacterial symbionts isolated from the root endosphere, and rhizosphere were assessed. The isolates assessed in this study were found to be from four phyla with known PGP bacterial genera and species. These were Actinomycetota, Bacteroidetes, Firmicutes and Proteobacteria. Root endophytes were observed to be less diverse compared to rhizobacteria. *Streptomyces, Staphylococcus*, *Flavobacterium, Gryllotalpicola*, and *Paenarthrobacter* were unique to the rhizosphere while *Enterobacter* species were only isolated from the root endosphere of *M. uniflorum.* PGP activity potential was observed to be higher with root endophytes than with rhizobacteria.

Our results suggest that the isolated bacteria from the drought tolerant legumes are endowed with diverse PGP and drought-tolerant traits for the facilitation of plant growth in arid environments. These PGPB may be harnessed for use to improve soil quality and stress tolerance in crops from both biotic and abiotic factors. Therefore, given these observations, it is recommended that isolates DR14, DRhi9 from *L. purpureus*, CR10, CRhi15, CRhi10 from *V. unguiculata*, MBR9, MBR1, MBRhi14, MBRhi17 from *V. radiata*, HR5, HR7, HR11new2, HRhi1, HRhi14, HRhi12, HRhi18 from *M. uniflorum*, RMBR1, RMBRhi4, RMBRhi1 from *V. aconitifolia* accession RMB-25, and IPCR2, IPCR4, and IPCRhi7 from *V. aconitifolia* accession IPCMO-880 be assessed for their PGP abilities and stress tolerance in field trials with other crop species.

3.6. References

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CHAPTER 4

Illumina sequencing of rhizospheric, seed, and root endophytic associated bacteria of drought tolerant legumes in Namibia

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Abstract

Plant growth-promoting bacteria are of increased interest as they offer sustainable alternatives to the more common chemical fertilisers. Research, however, has increased into the use of plant growth-promoting bacteria as bioinoculants to improve yields. Legumes are known to interact with diazotrophic plant growth-promoting bacteria which increase nutrient uptake by increasing availability, prevent pathogenic

infections, and actively fix nitrogen. In the present study, we used the 16S rRNA sequencing approach to determine the structure of rhizosphere, root, and seed endosphere microbiomes of five drought tolerant legume species: *Macrotyloma uniflorum*, *Vigna radiata*, *Vigna aconitifolia*, *Vigna unguiculata* and *Lablab purpureus*. Several important phyla were identified including Actinobacteriota, Bacteroidota, Firmicutes, Proteobacteria and Verrucomicrobiota. Overall, Proteobacteria was the most abundant phylum followed by Actinomycetota. The most important genera identified were *Bacillus*, *Mesorhizobium*, *Pseudomonas*, *Bradyrhizobium* and the *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* group. The relative abundance of these genera varied across sample types and legume species. This study identified important diazotrophs across all the legume species. *Bacillus*, an important plant growth-promoting bacterium, was found to be the most abundant genus among all the niches and legumes species analysed, while *Rhizobium spp*. was particularly enriched in roots.

Keywords: Plant growth-promoting bacteria; Namibia; Microbiomes; Endophytes, Rhizosphere; Bioinoculants

4.1. Introduction

Legumes are an important class of vegetables across the globe. They are highly nutritious and offer significant levels of proteins, fatty acids and other functional compounds (Miedzianka *et al*., 2017). They are an important food crop and are grown on almost 96 million hectares around the world. They are often rainfed and require minimal fertiliser inputs (Swarnalakshmi *et al*., 2020). In Namibia, legumes are mostly grown in the northern regions by smallholder subsistence farmers. However, due to

poor soils and limited resources, farmers often report low yields (Grönemeyer, Hurek & Reinhold-Hurek, 2015).

Legumes, like other plants, are known to have plant growth promoting (PGP) symbiotic assemblages with both bacteria and fungi (Goudjal *et al*., 2013; Liu, Carvalhais, *et al*., 2017). Research has found an increased interest in plant growth-promoting bacteria (PGPB) in recent years. This is due to their low-cost production, low environmental impact and increased performance in agricultural production (Khandare *et al*., 2020; Adeleke, Babalola & Glick, 2021). Nitrogen fixing rhizobia often found in legume root nodules promote plant growth and improve soil quality in the long run. This is in addition to other PGP properties like 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase production (Pandey, Verma & Chakraborty, 2015), antifungal activity (Verma *et al*., 2017), indole acetic acid (IAA) production (Bhutani, Maheshwari & Suneja, 2018) and siderophore production (Bhattacharyya & Jha, 2012; Gamit & Tank, 2014) that may be key factors that contribute to improved plant growth and yield in an arid environment (Adeleke, Babalola & Glick, 2021).

In addition to being part of the general soil health, these bacteria are found in the roots and seeds as endophytes (Deyett & Rolshausen, 2020) and the rhizosphere as rhizobacteria (Xiao, *et al*., 2017). Seeds, through vertical transmission (Truyens *et*., 2015), also contribute to the PGP microbiome thanks to the seed endophytes. Studies on cowpea have identified several PGPB and have also identified it as a viable soil-improving crop (Chidebe, Jaiswal & Dakora, 2018). As such these legumes can be used in crop rotations to reduce nitrogen runoff (Yu, Xue & Yang, 2014) and essentially the need for environmentally harmful chemical fertilisers (Mayer, de Quadros & Fulthorpe, 2019).

The legumes in this study (horsegram (*Macrotyloma uniflorum* Var. Madhu), mung bean [*Vigna radiata* (L.) R. Wilczek var. radiata], moth bean [*Vigna aconitifolia* (Jacq.) Marechal], cowpea (*Vigna unguiculata* L. Walp) and dolichos [*Lablab purpureus* (L.) Sweet var. Lignosus Prain]) are of great importance to Namibia. They exhibit some level of drought tolerance, growing in arid regions (Tiwari *et al*., 2018; Lestari *et al*., 2019; Parkash Kaundal *et al*., 2019; Sadeghipour, 2019). This characteristic is crucial for Namibia as it is an arid country with poor sandy soils (Strohbach, 2013; Strohbach & Kutuahuripa, 2014). Moreover, these legumes offer superior nutritional support given the high protein content and other nutritional benefits (Miedzianka *et al*., 2017).

The protein content of dolichos ranges between 18.8 and 24.5% (Pranesh & Ramesh, 2019; Purwanti, Prihanta & Fauzi, 2019). Horsegram on the other hand contains lower concentrations of protein with an average amount of 20.8% with comparable mineral concentrations that range between 210.3-290.0 mg/100 g for calcium and 8.1-10.0 mg/100 g for iron (Bhartiya *et al*., 2017; Patil & Kasturiba, 2019). Species within the *Vigna* genus also offer competitive nutritional values. The protein content of mung bean ranges between 21.0-23.3% (Yi-Shen, Shuai & Fitzgerald, 2018) while moth bean (*V. aconitifolia*) typically ranges between 18.9-26.1% (Bhardwaj & Hamama, 2016; 2017; Badami, Kasturiba & Ag, 2019). Compared to the other two species in the *Vigna* genus, cowpea (*V. unguiculata*) seeds record the highest protein content with ranges from 20.3% to 39.4% and with an approximate average content of 24.3% (Chikwendu, 2015; Gonçalves *et al*., 2016).

Despite this, the cultivation of these legumes in Namibia is limited. Cowpea, for example, has historically performed poorly in Namibia despite its nutrient-rich seeds

and soil nitrifying properties (Ajayi, Gbadamosi & Olumekun, 2018). This is due to several reasons including recurring droughts (Horn, Ghebrehiwot & Shimelis, 2016). A recent study, however, found that the use of *Bradyrhizobium*-based bioinoculants in cowpea fields in northern Namibia improves yield and biomass (Luchen *et al*., 2018). Therefore, analysis of the microbial communities associated with these legumes could provide more information useful for the development of bioinoculants.

Metagenomic analysis represents a valid technique to unveil and characterise legume microbiomes (Bragg & Tyson, 2014). It allows for the characterisation of microbes with respect to their structure and functionality (Tamames & Puente-Sánchez, 2019), offering a way to identify and analyse functional genes in microbiomes. In addition to this, metagenomics also reveals the potential genes harboured by a microbial community (Thomas, Gilbert & Meyer, 2012; Turner, James & Poole, 2013).

In this study, microbiomes associated with six accessions of five drought tolerant legumes; horsegram (*M. uniflorum*), mung bean (*V. radiata*), moth bean (*V. aconitifolia*), cowpea (*V. unguiculata*) and dolichos (*L. purpureus*) were metagenomically analysed for their microbiomes. This study aimed to identify and classify plant growth-promoting bacteria found in the roots, seeds, and the rhizosphere of drought-tolerant legumes.

4.2. Materials and methods

4.2.1. Study design

Seeds obtained from the National Bureau of Plant Genetic Resources' (NBPGR), India in July 2019 were used in this study. Seeds of six accessions from the five species were used. The accessions were Himala (*M. uniflorum*), IC39399 (*V. radiata*), Gujarat (*V. unguiculata*), IC0623025 (*L. purpureus*) and two accessions from *V. aconitifolia*

which were IPCMO-880 and RMB-25. The seeds were surface sterilised as previously described by Chimwamurombe, Grönemeyer, and Reinhold-Hurek, (2016) with modifications. Seeds were washed twice with sterile distilled water before being incubated in 70% ethanol for 20 seconds. The seeds were washed again with sterile distilled water. Seeds were incubated in 5% NaOCl for 30 seconds before being washed with excess sterile distilled water. Surface sterilisation was verified by inoculating 100 µL of the final wash onto sterile LB agar plates for both roots and seeds. Samples with growth were excluded from further analysis.

To determine seed endophytes, seeds were placed in falcon tubes with sterile saw dust (Figure 4.1) and germinated in sterile conditions at 30°C. After 7 days, germinated roots and shoots were surface sterilised as above. The assessment of rhizospheric and root endophytic microbiomes was done from potted plants with soil obtained from Bagani, Kavango East. Samples were grown in a growth chamber maintained at 25% humidity, 30°C with 12-hour light cycles at the University of Namibia. Seeds (two) from each accession were planted into eight pots to have two plants growing in each pot. Individual pots were kept 10 cm apart while pots of different accessions were kept 50 cm apart (Figure 4.1). Pots were watered twice a week receiving a uniform amount of water (200 mL). After six weeks, four plants that showed the least necrosis and greatest plant growth were selected for metagenomic analysis. Bulk soil was metagenomically analysed to present a reference (control) of the overall microbial profile of the Bagani soil.

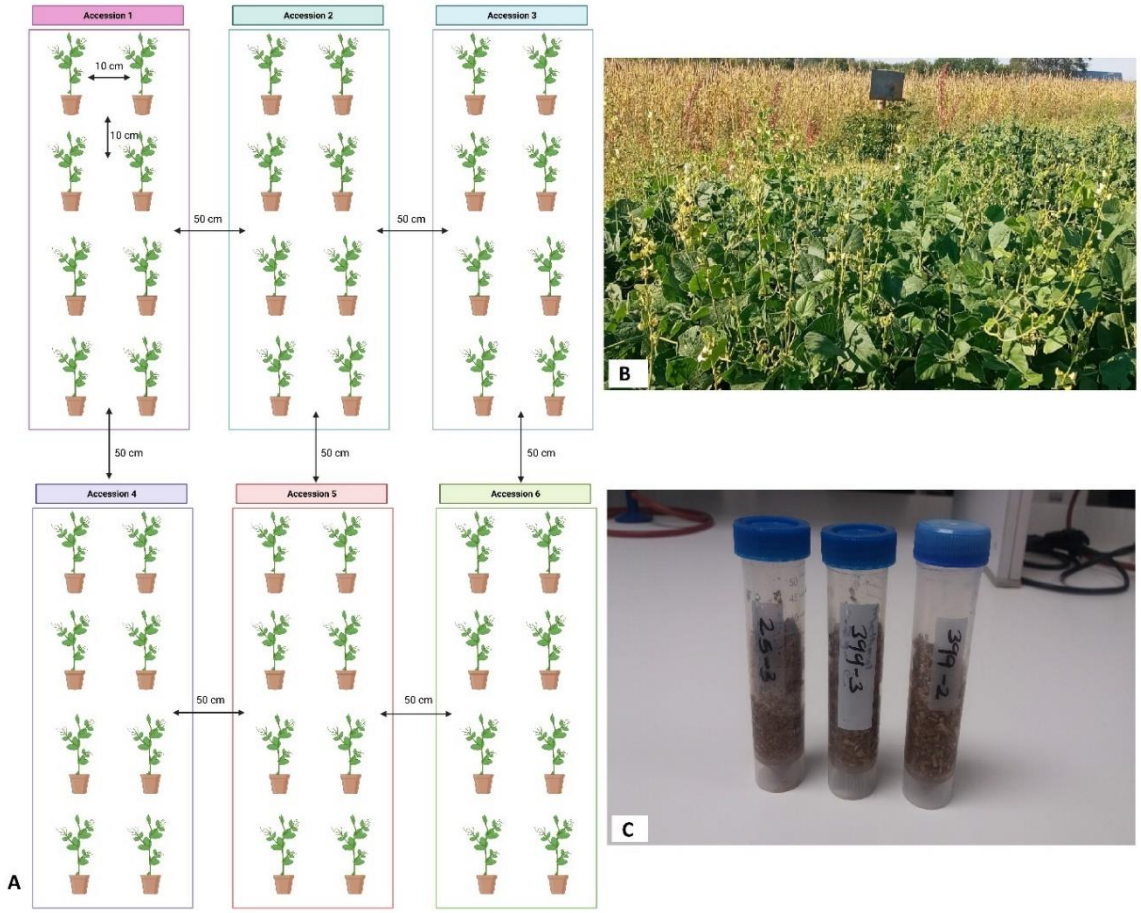

Figure 4.1: A) Potting strategy used for the 6 accessions. **B**) Bagani Research Station study field in Bagani in the Kavango East region in the north-eastern parts of Namibia. **C**) Falcon tubes in which surface sterilised seeds were germinated in sterile sawdust.

DNA extraction from seeds, roots and rhizospheric soil samples

Roots were carefully uprooted and prepared for DNA extraction following the method described by Grönemeyer, Burbano, Hurek, & Reinhold-Hurek (2012). Roots were aseptically cut off from the rest of the plant and placed in 14 mL sterile falcon tubes. To these tubes, 10 mL of sterile phosphate buffer (per litre - KH_2PO_4 6.75 g; K_2HPO_4 8.75 g) was added. Samples were vortexed for 5 minutes to remove the rhizospheric soil around the root before being centrifuged for 10 minutes at 10,000 x g. Root samples were carefully removed, and surface sterilised as above. (The remaining soil in the transport buffer was reserved for DNA isolation from the rhizosphere).

Thereafter, DNA was extracted using QIAGEN® DNeasy® Plant Mini Kit (Qiagen, USA, Valencia, CA) following the manufacturer's instructions.

Tubes containing rhizospheric soil and transport buffer from the previous step were centrifuged for 5 minutes at 10,000 x g. The supernatant was carefully removed, avoiding the pellet. DNA was extracted from rhizospheric soils using Zymo Research™ Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, CA) following the manufacturer's instructions. DNA from all samples was quantified using the ThermoScientific NanoDrop (NanoDrop One UV-Vis Spectrophotometer, Thermo Scientific, USA). Samples were sequenced individually and grouped according to sample type, accession, and legume species.

4.2.2. Library preparation

16S rRNA metabarcoding and Illumina sequencing

Microbiome sequencing library preparation was done following the Illumina MiSeq System manual (Illumina, 2013). DNA samples were amplified using 16S amplicon PCR primers: forward = 5'TCGTCGGCAGCGTCAGATGTGTATAA-GAGACAGCCTACGGGNGGCWGCAG3' and 16S Reverse Primer = 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTT-CTAATCC3' (Klindworth *et al.*, 2013). The following PCR parameters were used; 95°C for 5 minutes, 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds before a final extension of 72°C for 5 minutes and held at 4°C.

PCR products underwent clean-up to remove free primers and primer dimers. This was followed by attachments of dual indices and Illumina sequencing adapters using the Nextera XT Index Kit according to the manufacturer's protocol. The PCR products underwent a second clean-up procedure as above. The prepared libraries were

quantified using the Qubit® dsDNA High Sensitivity Assay Kits as per the manufacturer's manual (Freed & Silander, 2020). All PCR products were diluted to 4 nM and aliquots of 5 μL of diluted DNA from each library were pooled together and sent to sequence by Illumina Miseq sequencing.

4.2.3. Data analysis

Amplicon data processing

The sequenced amplicon profiling data were processed with workflow based on DADA2 (v1.12.1, https://github.com/Guan06/DADA2_pipeline) (Callahan *et al*., 2016). Forward and reverse reads were demultiplexed. Raw sequencing reads were subsequently truncated to 260 bp (forward) or 240 bp (reverse) and filtered with the command maxN = 0, maxEE = c (2,2), truncQ = 2, rm.phix = TRUE. After learning the error rates, amplicon sequence variants (ASVs) were generated by merging the corrected forward and reverse reads, and chimeras were removed.

Community diversity analysis at the phylum level

Merged reads were aligned to the SILVA database implemented in the QIIME2 package as described by Bolyen *et al*. (2019). Taxonomic annotation at different taxonomic levels ranging from phylum to genus was performed based on ASV composition and relative abundance. Community richness, diversity indices and rarefaction curves were determined using the QIIME diversity core-metrics-phylogenetic command for alpha and beta diversity analysis in the QIIME2 package. We estimated the Shannon diversity (H´) operational taxonomic units' (OUT) richness indices using the package Phyloseq in R (McMurdie & Holmes, 2013). Statistical

analysis for alpha diversity was done with the function Kruskal.test or pairwise.Wilcox.test in the R base. For beta-diversity analyses, OTU tables were normalized by the variance stabilizing transformation (VST) method using the package DESeq2 in R. Bray-Curtis distance was calculated from the normalized OTU tables using the function ordinate of the R package Vegan (Oksanen *et al*., 2019). Principal coordinate analysis (PCoA) and canonical analysis of principal coordinates, (CAP) analysis using the unweighted Unifrac distance was calculated using the plot_ordination function from the R package Phyloseq and Vegan. Permutational multivariate analysis of variance (PERMANOVA) was determined with the function adonis in the R package Vegan and a maximum of 999 permutations. Sequences were submitted to NCBI and were assigned the reference accession PRJNA834937.

4.3. Results

Sequencing data

A total of 4670 taxa were identified from the soil, rhizosphere, root, and seed samples sequenced. Low abundance taxa with less than 50 reads among all the samples (3387) were removed from further analysis. These taxa represented 220 genera. Reads annotated as chloroplast made up 70% of the sequences and were excluded from the data set. The total number of reads was 246230, ranging from 2 to 16517. No operational taxonomic units (OTUs) were identified as Archaea.

The highest number of reads, as shown in Table 4.1, were from bulk soil samples. Average reads were found to be lowest in seed samples as shown in Table 4.1 below. The lowest number of reads were from *V. aconitifolia* (IPCMO-880) seeds with 2 reads. The highest average number of reads were found in *M. uniflorum* seeds approximating 5981.

Table 4.1: Average number of sequences counts subset by species.

Species	Common name	Accession	Sample	Average sequence counts	Average number of reads
Vigna unguiculata	Cowpea	Gujarat 5	Roots	60554	3151
			Rhizosphere	62236	12381
			Seeds	67704	18
Vigna radiata	Mungbean	IC39399	Roots	58101	7954
			Rhizosphere	57657	13165
			Seeds	61987	8
Macrotyloma uniflorum	Horsegram	Himala	Roots	64509	11628
			Rhizosphere	70659	13569
			Seeds	59136	5981
Lablab purpureus	Dolichos	IC0623025	Roots	56299	6902
			Rhizosphere	39893	7417
			Seeds	61631	2512

Vigna aconitifolia	Mothbean	IPCMO-880	Roots	54052	4659
			Rhizosphere	70433	12497
			Seeds	57302	2
		RMB 25	Roots	32144	2469
			Rhizosphere	39101	7313
			Seeds	54861	5
			Bulk soil	60788	13625

The diversity of microbial communities within samples was compared and shown by the alpha diversity plot. Species richness was highest in bulk soil and rhizosphere samples. A trend was observed as a decrease in diversity with bulk soil being the most diverse. This was followed by the rhizosphere, roots, and finally seeds with the least diversity. The lowest diversity was found in seed samples. The diversity within these seeds was particularly low for *V. aconitifolia*, *V. radiata* and *V. unguiculata* in which the diversity was approximately zero as shown by the Alpha-Diversity (Shannon) plot Figure 4.2.

The diversity between samples by PCA (shown in Figure 4.3) was found to be influenced largely by seed endophytes with the least number of reads. Distance measurements showed an absence of significant differences among bulk soil, rhizosphere, and root samples. However, a significant difference was observed between seeds and the rest of the sample types. The seeds, mostly showing values greater than 0.25 on axis 1, had the least influence on variation. The PCoA based on the unweighted UniFrac distance measure showed that seed samples formed a distinct cluster to bulk soil, rhizosphere, and root samples. To infer significant differences among seeds and the rest of the root samples, we performed a PERMANOVA test on the unweighted UniFrac distances comparing different groups (with 999 permutations in all tests). Significant differences were detected for seeds samples compared to rhizosphere ones (pseudo-$F = 7.9$, $p = 0.001$), seeds samples compared to bulk soil ones (pseudo-$F = 6.9$, $p = 0.003$) and seeds samples compared to root samples (pseudo-$F = 5.7$, $p = 0.002$) shown in supplementary Figure S1 (Appendix B).

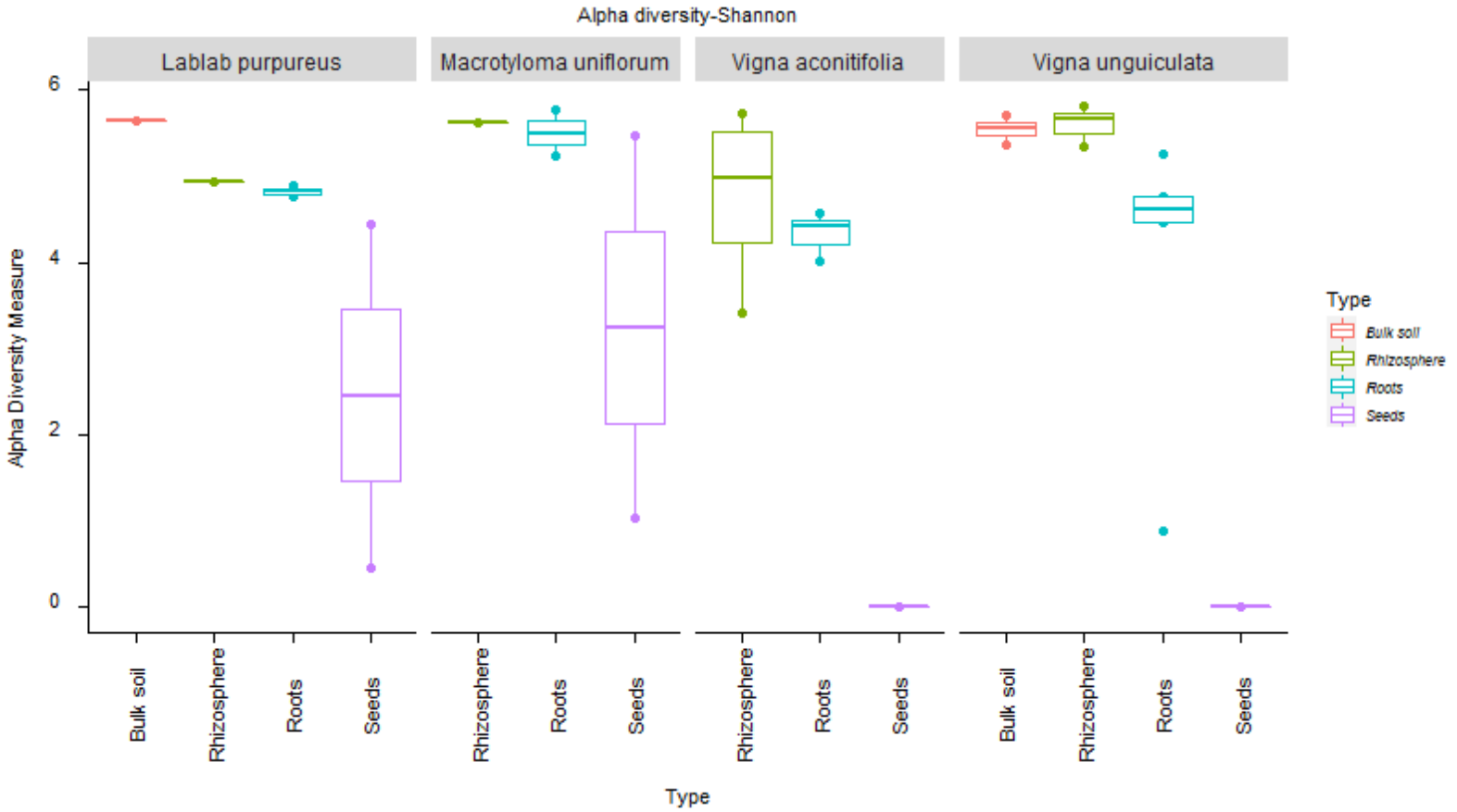

Figure 4.2: Shannon index of the microbiome in the rhizosphere soil, seed, bulk soil, and roots of legume. The larger the Shannon index, the better the sample uniformity. In the case of the same species richness, the greater the uniformity of each species in the community, the greater the diversity of the community.

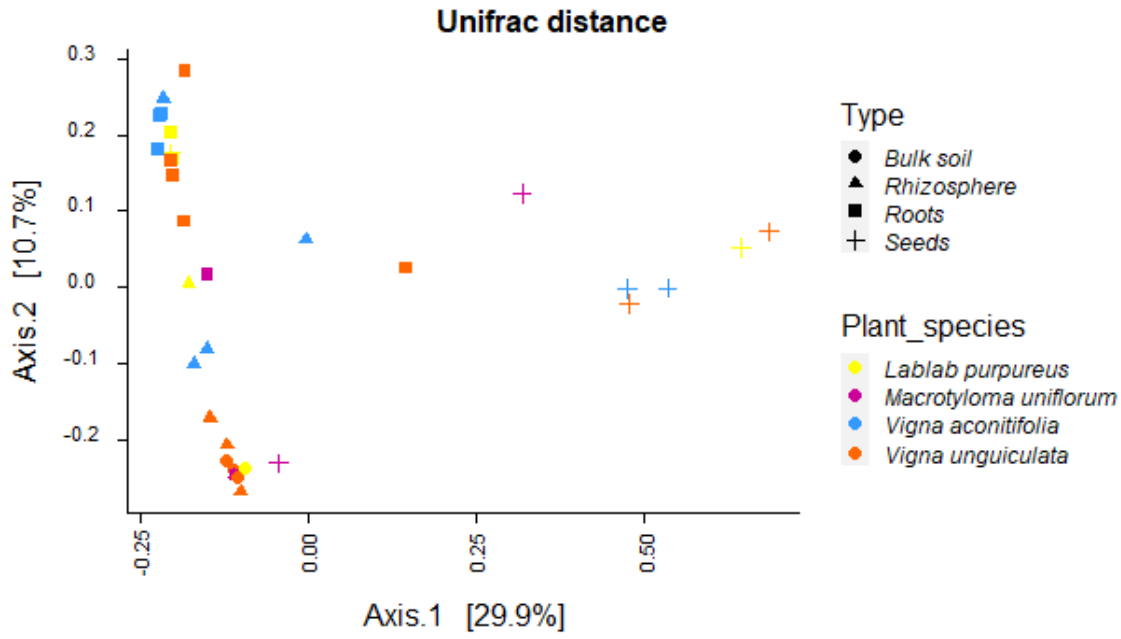

Figure 4.3: PCoA based on unweighted unifrac distance calculated on rhizosphere, roots, seeds, and bulk soil samples. Statistical significance has been inferred using PERMANOVA (see Supplementary Figure S1).

Structure of bacterial communities

Several different phyla were identified from the samples analysed. The most abundant phyla as seen in Figure 4.4 were Proteobacteria (14-52%), Firmicutes (5-24%), and Actinobacteriota (5-24%). On the other hand, Elusimicrobiota, RCP2-54, FCPU426 and WPS-2 were the least abundant taxa. Proteobacteria was the most abundant phylum across all samples. It was identified in all samples but the seeds of *V. aconitifolia*, *V. radiata* and *V. unguiculata*. The phylum Proteobacteria was found most abundant in the rhizosphere and roots of *V. aconitifolia* accessions. Figure 4.5 shows the abundance of the top 13 phyla in the different samples and species. The phyla observed to make up less than 1% were glommed together. Seed samples of *V. aconitifolia*, *V. radiata* and *V. unguiculata* had the lowest relative abundance and diversity of the different phyla.

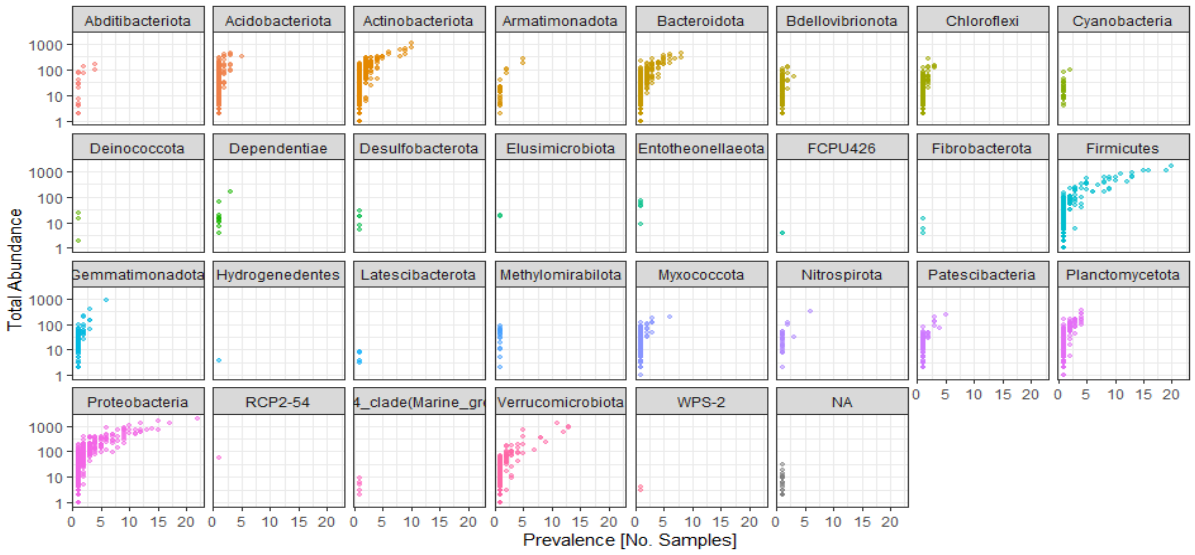

Figure 4.4: Overall abundance of phyla identified.

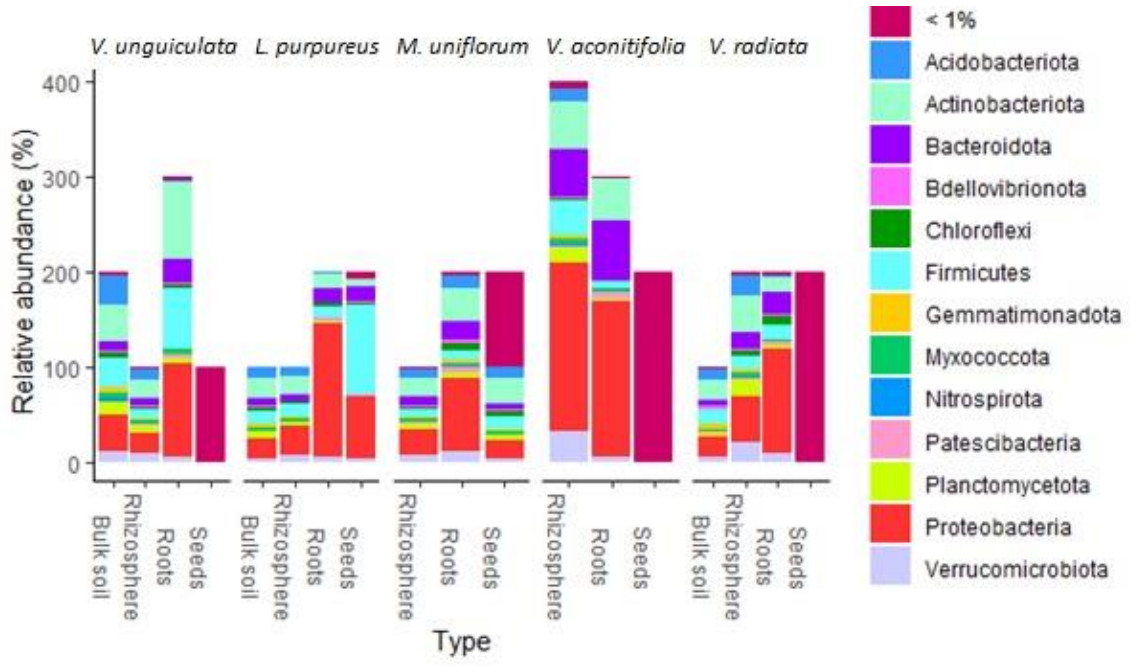

Figure 4.5: Phylum abundance according to sample type of the top 13 phyla.

The microbiomes at the genus level were dominated by *Acidibacter*, *Ammoniphius*, *Bradyrhizobium*, *Bacillus*, *Flavobacterium*, *Mesorhizobium*, *Pseudomonas* and *Streptomyces* (Figures 4.6 and 4.7). Uncultured groups, WD2101 soil group, 67-14 and RB41 are non-specific isolates that were also identified within the sequences. The most abundant genera identified in the samples include *Bacillus, Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* genus group, uncultured group and

Niastella. The heatmap (Figure 4.7) further details the differences in the abundance of the 50 most abundant taxa.

In *L. purpureus* samples, the genus *Bacillus* was the most predominant particularly in seeds. In *M. uniflorum* samples, the most abundant genera were *Bacillus*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* genus group and an uncultured group. These observations were in line with those in *V. aconitifolia* samples in addition to *Luteolibacter* which was abundant in rhizosphere samples. In *V. radiata*, however, the *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* genus group was most abundant in root samples while the rhizosphere had more of the uncultured genus group and *Candidatus Udaeobacter*. *V. unguiculata* roots had the highest abundance of *Bacillus* and *Streptomyces* compared to other species. Like *V. radiata* roots, *V. unguiculata* roots had a high amount of the uncultured genus group and *Candidatus Udaeobacter*.

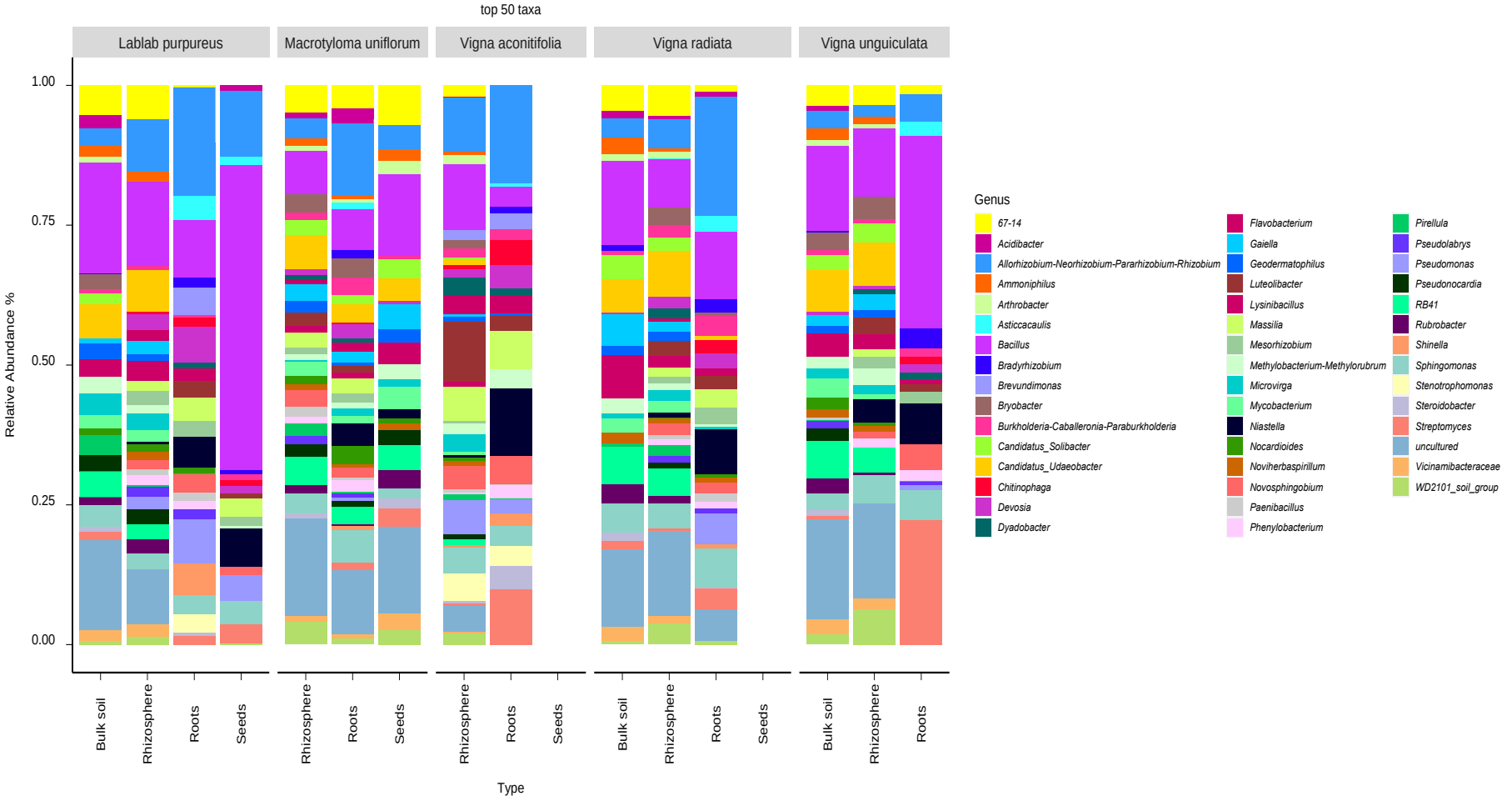

Figure 4.6: Genera abundance according to sample type of the top 50 genera.

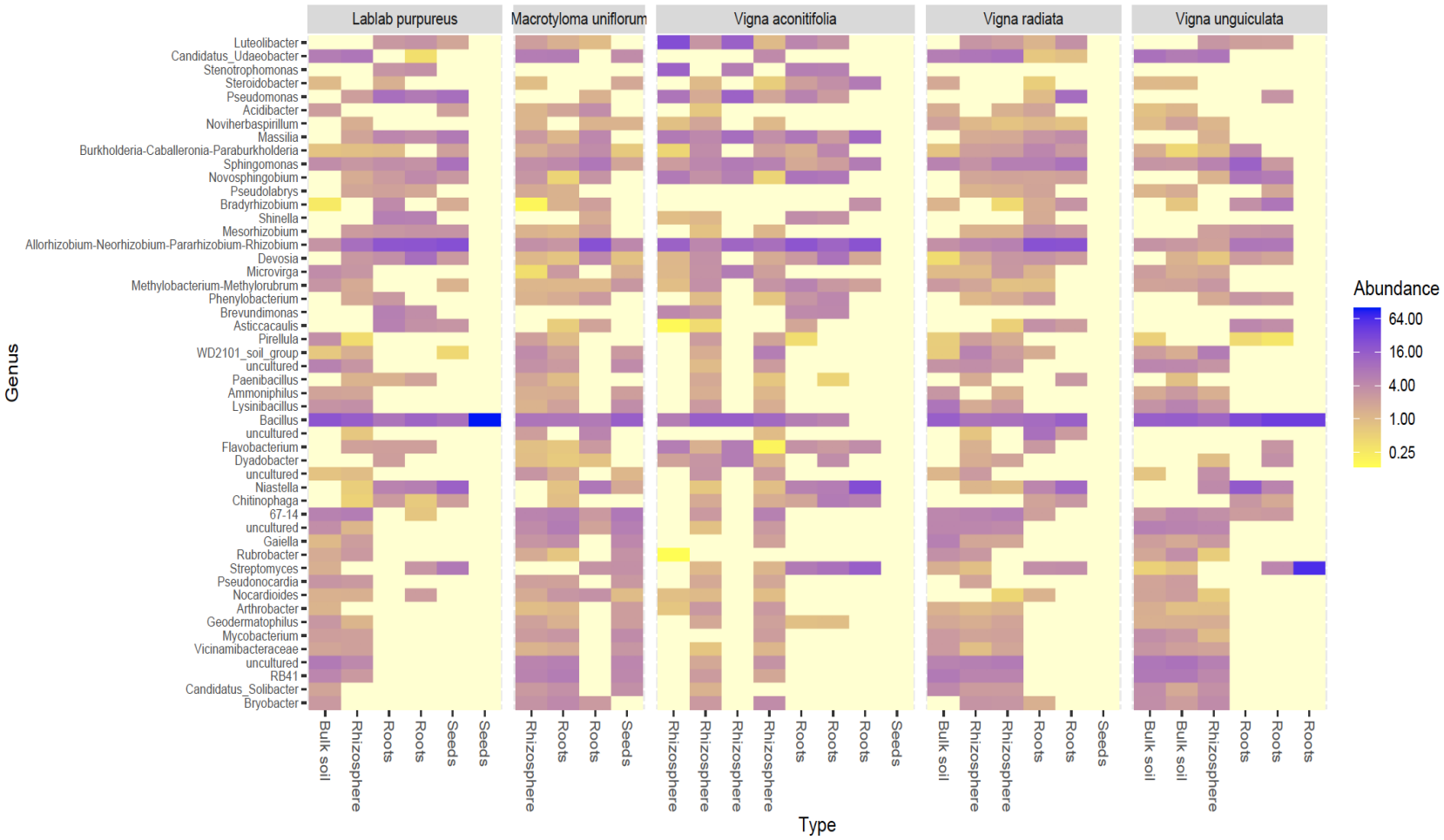

Figure 4.7: Heat map with the relative abundances of the bacterial genera in the five different legume crops and rhizosphere soil, and the endophytes of the roots and seeds.

The abundance of the *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* genus group was found to range from 0.2 to 1.8% (Figure 4.8). The *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* genus group was found to be least abundant in *V. unguiculata* and *M. uniflorum* while the highest amounts were observed in *V. radiata* and *L. purpureus*. Though present in both bulk soil and the rhizosphere, *Bradyrhizobium,* was found in relatively low amounts ranging from 0.25 to 1 relative to the heatmap (Figure 4.7).

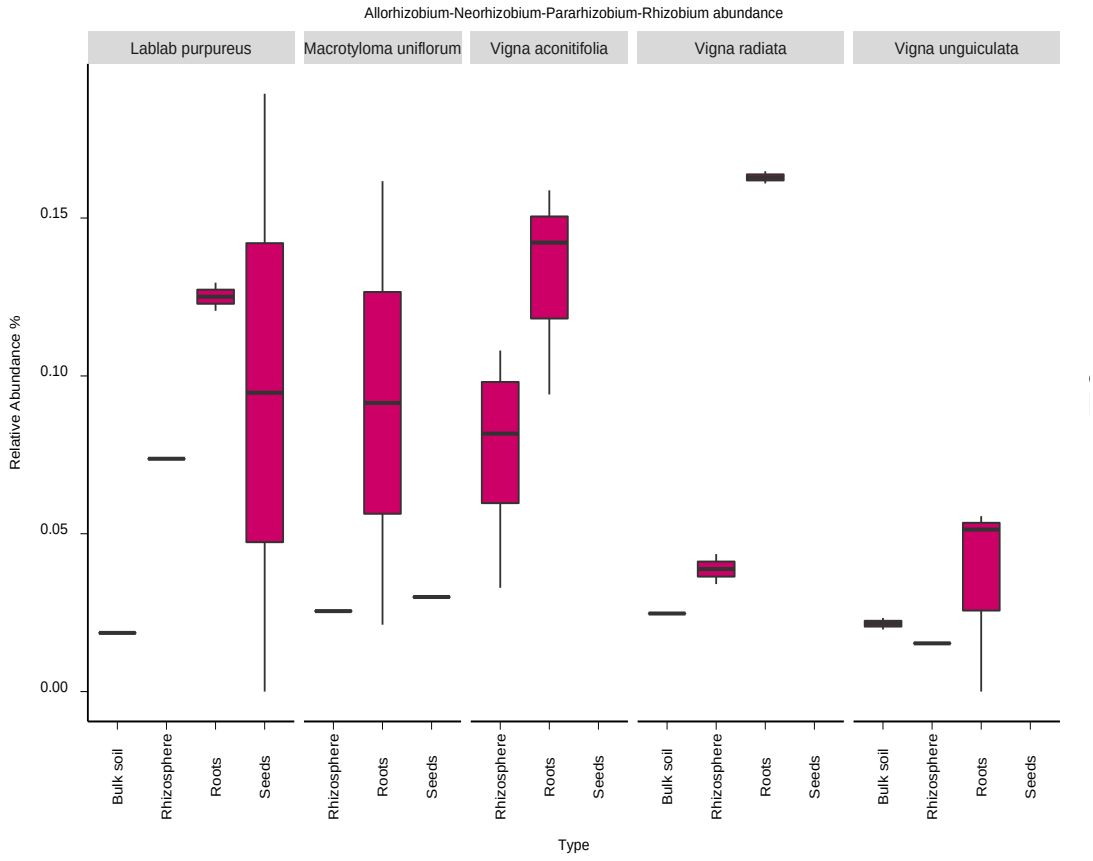

Figure 4.8: Relative abundance of *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* group.

Core microbiome

To examine the existence of an identifiable common core microbiome (Lozupone *et al*., 2007), a core as the group of members shared among the microbial community was defined. It represented the core by overlapping areas in the circles in a Venn

diagram at 97% identity (Figure 4.9). Consequently, 1034, 1633, 1144, and 310 OTUs were identified in the bulk soil, rhizosphere, roots, and seeds respectively. As shown in Figure 4.9, 548 OTUs were shared among the four groups, occupying 13.6% of all OTUs. These shared taxonomic members can be regarded as the core microbiome of roots, seeds, and soil as well as the rhizosphere.

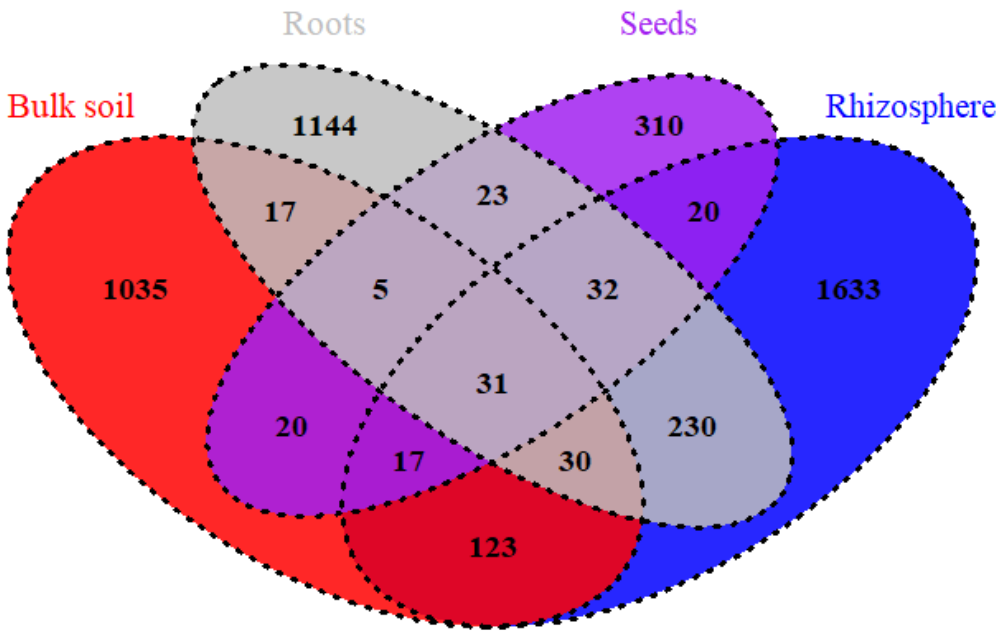

Figure 4.9: Venn diagram of shared and unique genera between all the microbiomes observed in this study.

4.4. Discussion

Microbiome studies offer a path to analyse a complete microbiome via culture-independent methods, providing a full picture of the total number of members of a microbial community. This analysis method allows for the exploration of microbial communities in plants and their functions in comparison with other similar plant-microbiomes (Gururani *et al.*, 2021). In this study, the microbiomes of 6 accessions from 5 legume species were analysed to extrapolate the plant-microbial interactions of both culture-dependent and independent bacteria. However, one major limitation was

identified. This study used 16S sequencing which limited the identification of *nifH* genes specific for nitrogen fixation Therefore, strains with specific genes associated with PGP traits could not be defined (Turner, James & Poole, 2013).

Significant differences in diversity were observed between sample types. The greatest biodiversity was observed in bulk soil samples as expected (Essel *et al*., 2019). The degree of diversity decreased significantly between the bulk soil and the rhizosphere reflecting the specific selectivity of roots and root exudates (Deyett & Rolshausen, 2020). PGPB, therefore, may differ across different plant species, varieties, and different plant niches within the same host. The diversity further decreases from the rhizosphere to root endophytes. This is largely due to the selective ability of very specialized bacteria to colonize the root systems (Vacheron *et al*., 2013).

PGPB are found in several different phyla with different characteristics. Actinobacteria, Bacteroidota and Verrucomicrobiota are phyla that make up the most common soil bacteria. These are often found in great abundance in the soil and rhizosphere of legumes (Saleem *et al*., 2018). Studies have also identified Proteobacteria and Actinobacteria constituting up to 54.90% and 32.00% respectively (Mitter, Freitas & Germida, 2017; Pang *et al*., 2022). These observations were similar to the results obtained in this study. Proteobacteria, as shown in Figure 4.4, was the most abundant phylum in all samples.

Actinobacteria (also referred to Actinomycetota) and Firmicutes are both Gram-positive phyla with a high G-C and low G-C content respectively. PGPB found under Actinobacteria include strains from *Streptomyces*, *Arthrobacter* and *Nocardia* genera. *Bacillus* and *Paenibacillus*, on the other hand, are important Firmicutes diazotrophs (Francis, Holsters & Vereecke, 2010). The genus *Bacillus* is of particular importance

as it was strongly represented in *L. purpureus* seeds and *V. unguiculata* roots with a high abundance reflected on the heatmap. This study observed *Bacillus* being the most abundant genus across all *V. radiata* samples. Previous studies have also found *Bacillus spp*. along with *Arthrobacter* to be dominant in the rhizosphere of *V. radiata* (de los Reyes *et al*., 2020). *Streptomyces* strains were poorly represented in most samples except for in *V. unguiculata* roots. This genus was least represented in *V. aconitifolia* samples.

The analysis in this study found Proteobacteria to be the most abundant phylum dominating the root endosphere. By comparison, the abundance of Proteobacteria was relatively less in the bulk soil and rhizosphere in most samples. Root exudates are known to influence both the rhizosphere and root endosphere (Fernández-González *et al*., 2019). As a result, the soil microbiome composition often differs from the rhizosphere and root endosphere. This supports a study that found this phylum most abundant in the root endosphere (Mitter, Freitas & Germida, 2017).

This phylum contains several diazotrophic genera identified by the presence of *nifH* genes (Hurek, Egener & Reinhold-Hurek, 1997). These include *Rhizobium, Sphingomonas* (Fernández-González *et al*., 2019), *Bradyrhizobium*, *Burkholderia* (Xiao, Fan, *et al*., 2017) and *Pseudomonas* (Beckers *et al*., 2017). In bulk soil, *Bradyrhizobium* was observed to be in low abundance compared to other genera. However, higher levels were observed in roots and rhizosphere samples. *Pseudomonas* species were found in greater abundance in the rhizospheres compared to other sample types. These genera are often found in root nodules pointing to their nitrogen-fixing properties. *Rhizobium* and *Bradyrhizobium* are symbionts of *V. radiata* with increased abundance in root nodules (Hakim, Imran & Mirza, 2021).

The genus *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* was identified in bulk soil, rhizospheres, and root endospheres with a lesser presence in seeds. This genus is of particular importance as it is a diazotrophic genus known to have non-cyanobacteria species. It is often found within soils but associated species are often found in roots contributing to nitrogen fixation (You *et al*., 2021). As PGPB, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* species have been observed to improve sugarcane weight and sucrose content in the plants (Pang *et al*., 2022). In addition to the PGP properties of *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, species within this genus have been found to positively contribute to soil bioremediation. A study found some species actively reducing the amount of di(2-ethylhexyl) phthalate, an environmental contaminant used in plastic manufacture (Bai *et al*., 2020).

4.5. Conclusion

Several important PGPB phyla were identified from all the samples. These include Actinobacteria, Bacteroidota, Firmicutes and Proteobacteria. Within these groups, diazotrophic genera were identified. These legumes, grown in poor sandy soils of Bagani, were found to actively recruit PGPB. Recruitment was found to be selective for bacteria known to promote plant growth. These include *Rhizobium*, *Bradyrhizobium*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Pseudomonas* and *Bacillus*. Significant differences were not observed between the rhizosphere and roots. The low reads in seeds resulted in a significant difference in biodiversity.

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CHAPTER 5

Exploring culture-dependent and culture-independent root nodule endophytes of drought-tolerant legumes grown in Namibia

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Abstract

The nodule microbiome plays a key role in the fitness and behaviour of its host legume plants. Legume root nodules contain beneficial bacteria that can fix atmospheric nitrogen among other plant-beneficial activities. Studies solely based on culture-dependent techniques have overlooked most microbial diversity. The present study described the concomitant use of culture-dependent and -independent techniques to

characterise nodule endophytic microbiomes from six legume accessions grown in soil obtained from Bagani, Kavango East. Nodule endophytes were grown on modified soil extract agar and yeast extract mannitol agar before being characterised for plant growth-promoting properties. A total of 41 isolates were assessed for their ability to fix atmospheric nitrogen, and produce indole acetic acid, siderophore and exopolysaccharides. They were also assessed on their ability to solubilize phosphates and antagonise fungal growth. From the 41 isolates, 34 isolates tested positive for at least one plant growth-promoting trait. Isolates, MB3.1, H14, M25-11, M8-16.1 and M8-16.2 showed the most potential as plant-growth promoters. Using 16S rRNA metagenomic Illumina MiSeq sequencing, the relative abundance of the nitrogen-fixing *Bradyrhizobium* genus was determined in four drought-tolerant legume species. Metagenomic analysis of the nodule microbiomes produced a total of 17364 reads. The genus *Bradyrhizobium* was found to be the most abundant in the nodule microbiomes. It was most abundant in *L. purpureus* and *M. uniflorum* making up 99.2% and 98% of the total bacterial microbiomes respectively. The characterisation of nodule microbiomes using one method cannot be considered conclusive. The methods used in this study allow for the analysis of both culture-dependent and -independent bacteria.

Keywords: Root nodule endophytes; Nodule microbiome; Stress tolerance; Drought-tolerant legumes; Namibia

5.1. Introduction

Food insecurity is of increasing concern given the socioeconomic challenges, which include increased land degradation and social unrest, affecting agricultural production (Li, 2018; Prăvălie *et al*., 2019). In addition, the world population is estimated to

increase to 9.7 billion by 2050 (United Nations Department of Economic and Social Affairs Population Division, 2022). This means the food demand is also projected to double (Dias, Dukes & Antunes, 2014). However, global food security is also currently threatened by climate change (Tahat *et al*., 2020; Hubert *et al*., 2022), worsening land degradation (Pacheco *et al*., 2018), and an increase in agricultural input costs including rising fertiliser costs (Schnitkey *et al*., 2021). Therefore, there is a need to utilise more climate-adapted crops to relieve the food security demand.

Arid climate-tolerant legumes are nutritious alternatives that can be used to help address food insecurity. In addition, they also tend to improve soils over time. Therefore, they offer an additional solution to land degradation. Essentially, incorporating these legumes in crop rotation practices would result in improved soil health (Gan *et al*., 2015; Reckling *et al*., 2016). Legumes consequently make a compelling argument for the elimination of environmentally harmful chemical fertilisers (Gururani *et al*., 2021; Town *et*., 2022). There is a clear indication that a replacement of chemical fertilisers with more environmentally sustainable practices also reduces input costs without negatively affecting yields. This is evident even in the case of partial substitutions with organic fertilisers (Tang *et al*., 2022). As such, the maintenance of good soil health is a critical requirement for optimum soil fertility essential for crop production (Tahat *et al*., 2020). This can be achieved by exploiting beneficial plant-microbe interactions.

Bacteria that can fix atmospheric nitrogen are classified as diazotrophs. They perform an important role and sustainably contribute close to 65% of biologically fixed nitrogen in the agricultural sector (Liu *et al*., 2018). Diazotrophs are known to form symbiotic relationships with arid climate tolerant legumes in the root endosphere and

nodules (Mirza & Rodrigues, 2012). Some common legumes grown in Africa associated with diazotrophs include *Vigna unguiculata* L. Walp, *V. subterranean* L. Verdc, *Macrotyloma geocarpum* Harns, *Phaseolus vulgaris* L., *Glycine max* L. Merr (Jaiswal & Dakora, 2019), and *Lablab purpureus* (L.) Sweet (Grönemeyer, Bünger & Reinhold-Hurek, 2017). Several bacterial genera associated with these arid-tolerant legumes are classified as diazotrophs. These include *Rhizobium* (Kifle & Laing, 2016), *Burkholderia*, *Polaromonas* (Fan, Weisenhorn, Gilbert, Shi, *et al*., 2018), *Mesorhizobium, Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (You *et al*., 2021) and the well-known nodule symbiont *Bradyrhizobium* (Zhao, Xu & Lai, 2018).

The diversity and composition of root nodules is highly dependent on the soil microbial and chemical profile in addition to the highly plant specific root exudates (Han *et al*., 2020; Rocha *et al*., 2020). Despite this, *Bradyrhizobium* is often the most abundant genus in root nodules (Ormeño-Orrillo & Martínez-Romero, 2019). Microbiome analysis of root nodules reveals the rich diversity of the nodule microbiome. Some common nodule endophytes include *Nitrobacter* (Mayhood & Mirza, 2021), *Sinorhizobium* (Zheng *et al*., 2020), and *Bacillus* (Rocha *et al*., 2020).

This study, with the above-mentioned knowledge in mind, sought to isolate, identify and characterise nodule endophytes from six accessions of five arid tolerant legumes grown in Namibia. It also aimed to present the first report on the composition of the *Bradyrhizobium* genus within microbiomes of drought-tolerant legume root nodules in Namibia using 16S Illumina sequencing technology. The legumes are dolichos [*L. purpureus* (L.) Sweet var. Lignosus Prain], mung bean [*V. radiata* (L.) R. Wilczek var. radiata], cowpea (*V. unguiculata* L. Walp), horsegram (*M. uniflorum* Var. Madhu), and mothbean [*V. aconitifolia* (Jacq.) Marechal]. The factors considered for plant

growth-promotion (PGP) in this study were nitrogen fixation, siderophore production, indole acetic acid (IAA) production, exopolysaccharide (EPS) production, phosphate solubilization, and antifungal activity against *Fusarium graminearum.*

5.2. Materials and methods

5.2.1. Seed material and growth conditions

Analysis was carried out on six accessions from five legume species shown in Figure 5.1 [IC0623025 (*L. purpureus*), Gujarat 5 (*V. unguiculata*), Himala (*M. uniflorum*), IC39399 (*V. radiata*), and two accessions from *V. aconitifolia*: IPCMO-880 and RMB-25]. Microbiome analyses were carried out on four legume accessions belonging to four legume species from a sample cache of six accessions belonging to five legume species. The accessions selected were IC0623025 (*L. purpureus*), Gujarat 5 (*V. unguiculata*), Himala (*M. uniflorum*), and IPCMO-880 (*V. aconitifolia*). Samples from *V. aconitifolia* (RMB-25) and *V. radiata* (IC0623025) were excluded as nodule mass could not be attained for DNA extraction. Legumes were planted in eight pots with soil obtained from Bagani, Kavango East. Samples were grown in pots in a greenhouse at the University of Namibia, Windhoek main campus. Humidity was maintained at 25% with natural light hours during the months of February and March. The plants were watered twice a week each receiving 200 ml of water. Plants were grown for at least 5 weeks before harvesting root nodules.

Nodules were harvested and prepared for isolation following a similar method previously described (Dhali *et al*., 2021). Plants were carefully uprooted from pots and washed with tap water to remove excess soil. Nodules were removed using a sterile scalpel. Harvested nodules were washed twice with sterile distilled water followed by incubation for 20 seconds in 70% ethanol. The nodules were incubated for 1 minute in

sterile distilled water before being incubated for 30 seconds in 5% sodium hypochlorite. Thereafter, nodules were washed with excess sterile distilled water. Surface sterilisation was confirmed by plating 100 µL of the final wash onto Luria-Bertani (LB) plates and incubated at 30°C. Samples showing growth were excluded from further analysis.

5.2.2. Isolation and characterisation of endophytes

Sterilised nodules were macerated in 5-10 ml sterile phosphate buffer saline (PBS). Thereafter, 100 µL of each macerated sample was inoculated onto yeast extract mannitol (YEM) and modified soil extract agar (SEA) plates. Media compositions, per litre, were: YEM- yeast extract, 1 g; mannitol, 10 g; dipotassium phosphate, 0.5 g; magnesium sulphate, 0.2 g; sodium chloride, 0.1 g; Congo red, 0.025 g; agar, 20 g; modified SEA- yeast extract, 1 g; mannitol, 10 g; agar, 16 g; soil extract, 200 ml; pH 7. Soil extract - 160 g air-dried soil (collected from Bagani) and 0.4 g sodium carbonate suspended in 1 L distilled water and filtered to remove soil debris. Plates were incubated at room temperature for 3 days before being transferred to a 30°C incubation chamber. Growth was monitored over a period of 5-7 days. Pure cultures were maintained on YEM and modified SEA plates.

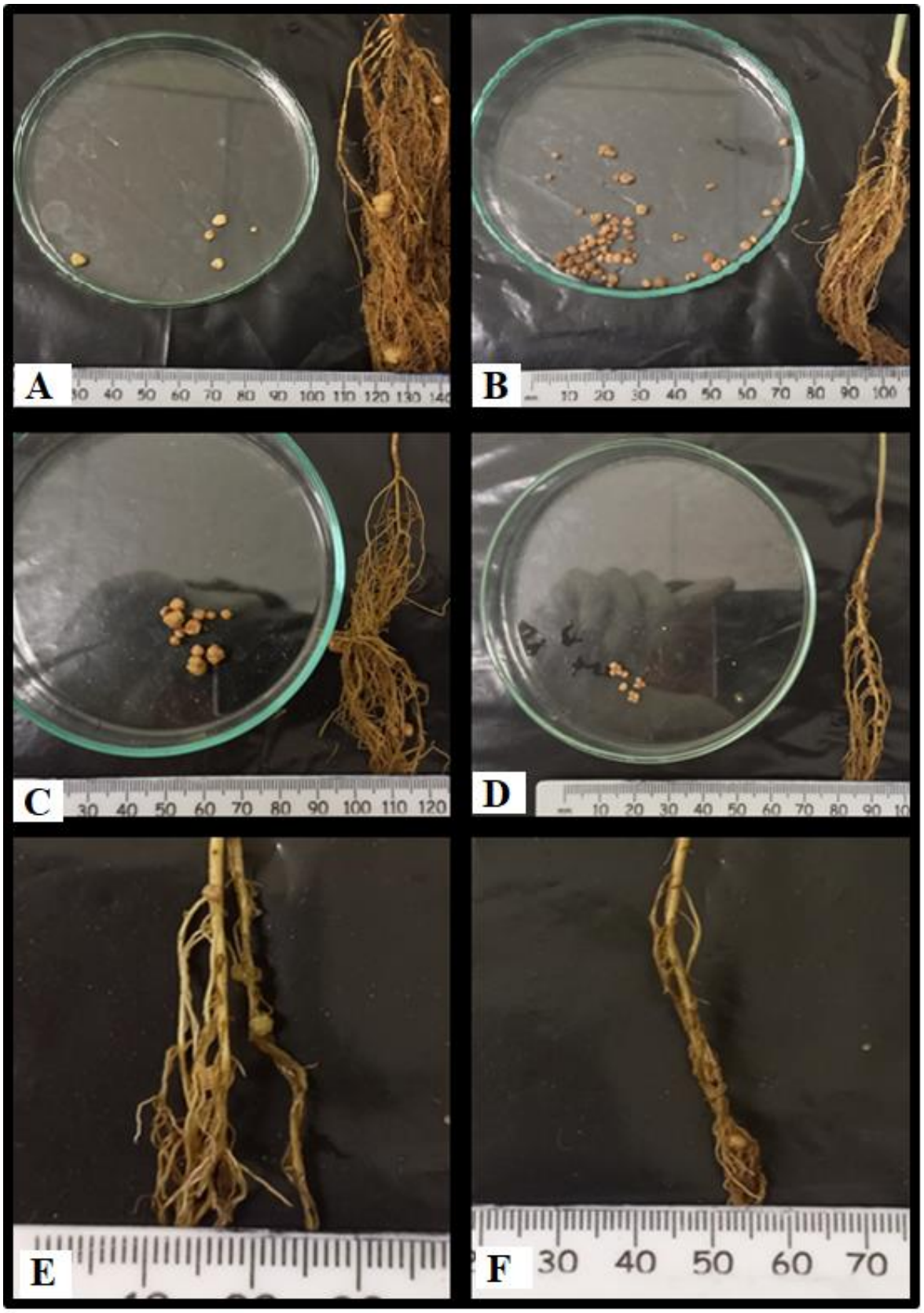

Figure 5.1: Nodules from the legume species **A**: *L. purpureus (*IC0623025*)* **B**: *V. unguiculata* (Gujarat 5) **C**: *M. uniflorum* (Himala) **D**: *V. aconitifolia* (IPCMO-880) **E**: *V. aconitifolia* (RMB-25) **F**: *V. radiata* (IC39399).

Identification of isolates

Isolates were identified following a method similarly described by Pesce, Kleiner and Tisa, (2019). Samples were lysed (96°C for 10 minutes) and the template DNA was amplified using 16S rRNA universal primers: FDIFuni- 5′-AGA GTT TGA TCC TGG CTC-3′ and P2Runi- 5′-ACG GCT ACC TTG TTA GGA CTT-3′. The PCR program used was as follows: 5 minutes of initial denaturation at 98°C, 30 cycles of denaturation at 96°C for 40 seconds, annealing at 54°C for 40 seconds, and extension at 72°C for 90 seconds. A final extension was set at 72°C for 2 minutes.

After PCR, samples were purified using the EuroClone® spinNaker Gel and PCR DNA purification kit (Pero, Italy). Amplified samples were sequenced with primers 907r (5′-CCGTCAATTCMTTTRAGTTT-3′) (Lane *et al*., 1985) and F785 (5′-GGATTAGATACCCTGGTA-3′) (Vannini *et al*., 2004). Samples were sequenced by Eurofins Genomics, Germany. Primary sequence data was run through the National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST) to determine identity. Accession numbers obtained after submission onto GenBank are shown in Table 5.1.

Phosphate solubilization

Phosphate solubilization was determined following the method described by Nautiyal, (1999) with modifications. Isolates were spot inoculated onto National Botanical Research Institute's phosphate (NBRIP) growth medium (Glucose, 10 g; $Ca_3(PO_4)_2$, 5 g; $MgCl_2 \cdot 6H_2O$, 5 g; $MgSO_4 \cdot 7H_2O$, 0.250 g; KCl, 0.200 g; $(NH_4)_2SO_4$, 0.100 g and agar, 16 g per litre) plates. The inorganic phosphate source was tri-calcium phosphate ($Ca_3(PO_4)_2$). Plates were incubated at 30°C for 7 days.

Nitrogen fixation

Nodules endophytes were assessed for their ability to fix nitrogen. This was determined by observing growth on Jensen medium (Das & De, 2018). Isolates were incubated for 72 hours at 30°C.

Siderophore production

Siderophore production was determined following a modified procedure described by (Schwyn & Neilands, 1987). Chrome azurol S (CAS) agar was prepared with CAS and hexadecyl-trimethylammonium bromide (HDTMA) as indicators. Isolated strains were inoculated onto the medium in duplicates and incubated at 30°C for 5 days in the dark. Siderophore production was scored when a halo of a minimum of 1 mm was observed. Samples showing halos of more than 5 mm were considered superior siderophore producers.

Bacterial antifungal activity

Antifungal activity against *F. graminearum,* a plant pathogen, was tested following the method described by Rajendran *et al.* (2008). Fungal isolates grown on potato dextrose aga (PDA) plates were placed onto fresh PDA plates inoculated with the isolates. The plates and fungal inoculants were monitored for zones of inhibition over a period of 21 days at 30°C.

Indole acetic acid production

With modifications, IAA production was determined following the method described by Rajendran, Patel and Joshi, (2012). Pure colonies were grown in 5 mL of 20% tryptic soy broth over 24 hours at 30°C on a shaker at 220 rpm. Thereafter, 2 mL of each culture was centrifuged. The supernatant (1 mL) was carefully recovered and

combined with 2 ml of Salkowski's reagent (1 mL of 0.5 M $FeCl_3$ in 50 mL of 35% perchloric acid). To this, 10 µL of orthophosphoric acid was added. Samples were incubated in the dark for 30 minutes. Reading was done at OD_{530}. The standard graph (Figure 2) was prepared by dissolving 1 mg of IAA in 5 mL of LB broth.

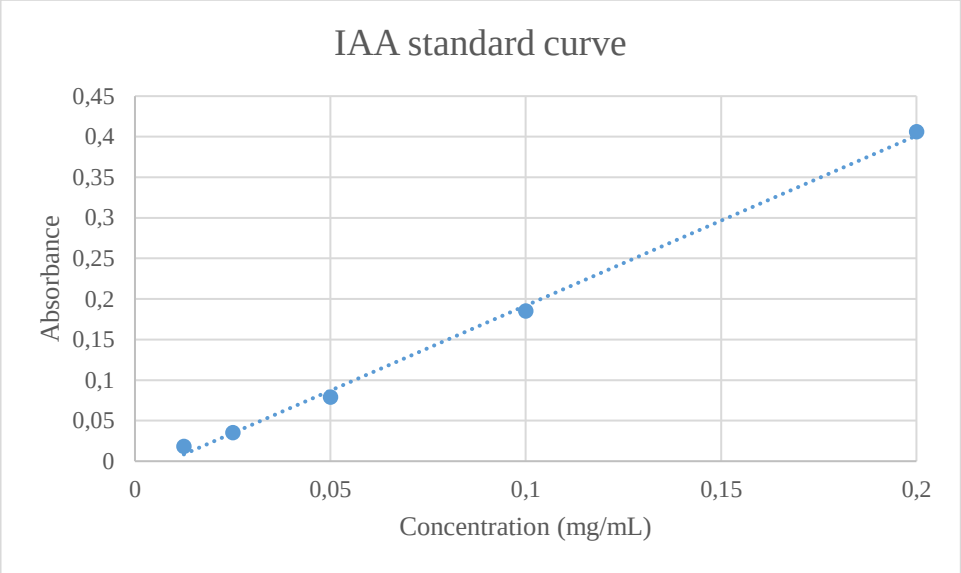

Figure 5.2: Standard curve using IAA used to infer concentrations from spectrophotometer readings.

Exopolysaccharide production

EPS production was assessed on YEM (per litre- yeast extract 0.5 g, mannitol 4.0 g and agar 16 g) as previously described with modifications. Isolates were inoculated and observed for growth patterns on YEM after 48 hours at 30°C. Positive EPS was scored by the presence of a mucoid textured colonies (Latif *et al.*, 2022).

5.2.3. 16S Microbiome sequencing library preparation

16S rRNA metabarcoding and Illumina MiSeq sequencing

A total of 11 samples from four legume species were analysed. DNA was extracted from surface sterilised nodules using QIAGEN® DNeasy® Plant Mini Kit (Qiagen, USA, Valencia, CA) following the manufacturer's instructions. Microbiome

sequencing library preparation was done following the Illumina MiSeq System manual (Illumina, 2013). Libraries were quantified using the Qubit® dsDNA High Sensitivity Assay Kits following the manufacturer's manual (Freed & Silander, 2020). Libraries were pulled together into a 1.5mL Eppendorf tube and sequenced using the Illumina MiSeq system.

5.2.4. Data analysis

Sequence data were analysed using nucleotide BLAST from NCBI. The searches were made in the 16S ribosomal RNA sequences (Bacteria and Archaea) database and limited to highly similar sequences. Uncultured and environmental sample sequences were excluded from alignments (Zhang *et al*., 2000). Principal component analysis (PCA) was performed using R statistical language version x64 4.1.2 (R Core Team, 2021), factoextra (Lê, Josse & Husson, 2008), and ggplot2 (Wickham, 2009).

Amplicon data processing

Data clean-up was done by initially excluding all low-quality reads, adapter sequences and low-abundant taxa (<50). Reads identified from chloroplasts and mitochondria were also removed from the data before analysis. The sequenced amplicon profiling data were processed with workflow based on DADA2 (v1.12.1, https://github.com/Guan06/DADA2_pipeline) (Callahan *et al*., 2016). Forward and reverse reads were demultiplexed. Raw sequencing reads were subsequently truncated to 260 bp (forward) or 240 bp (reverse) and filtered with the command maxN = 0, maxEE = c (2,2), truncQ = 2, rm.phix = TRUE. After learning the error rates, amplicon sequence variants (ASVs) were generated by merging the corrected forward and reverse reads, and chimeras were removed.

Community diversity analysis at the phylum level

Merged reads were aligned to the SILVA database implemented in the QIIME2 package as described by Bolyen *et al*., (2019). Taxonomic annotation was performed based on ASV composition and relative abundance. Community richness and diversity indices and rarefaction curves were determined using the QIIME diversity core-metrics-phylogenetic command for alpha and beta diversity analysis in the QIIME2 package. The Chao1 and Shannon diversity (H´) operational taxonomic units' (OTU) richness indices were estimated using the Vegan (Oksanen *et al*., 2019) and Phyloseq packages (McMurdie & Holmes, 2013) in R. Bray-Curtis distance was calculated from the normalized OTU tables using the function ordinate of the R package Vegan (Oksanen *et al*., 2019). The R base version used for analysis was x64 4.1.2 (R Core Team, 2021). The heat map was constructed using the ggplot package in R software (Kassambara, 2016).

5.3. Results

5.3.1 Culture-dependent nodule bacteria identification

Bacterial isolates

A total of 41 isolates were identified in this study. Isolates were identified from three phyla: Firmicutes (also Bacillota), Actinomycetota (also Actinobacteria) and Proteobacteria. Two isolates were grouped as unclassified *Streptomyces* species from the phylum Actinomycetota from *V. unguiculata*. Three isolates were identified as unclassified *Xanthomonas* (phylum Proteobacteria) species from *V. radiata* and *V. aconitifolia* (RMB-25) as shown in Table 5.1. Actinomycetota and Proteobacteria

species each made up 5% and 7% respectively of the total isolates identified (Table 5.1).

Most of the isolates, as shown in Figure 5.3, were from the phylum Firmicutes (88%). The identified isolates all were close relatives to the genus *Bacillus.* In addition to some *Bacillus* species identified, other genera were *Priestia, Paenibacillus, Gottfriedia, Neobacillus, Lysinibacillus, Fictibacillus,* and *Brevibacillus.* A total of 10 different genera were identified. Figure 5.3 below shows the distribution of these genera. Table 5.1 lists all isolates as identified using NCBI.

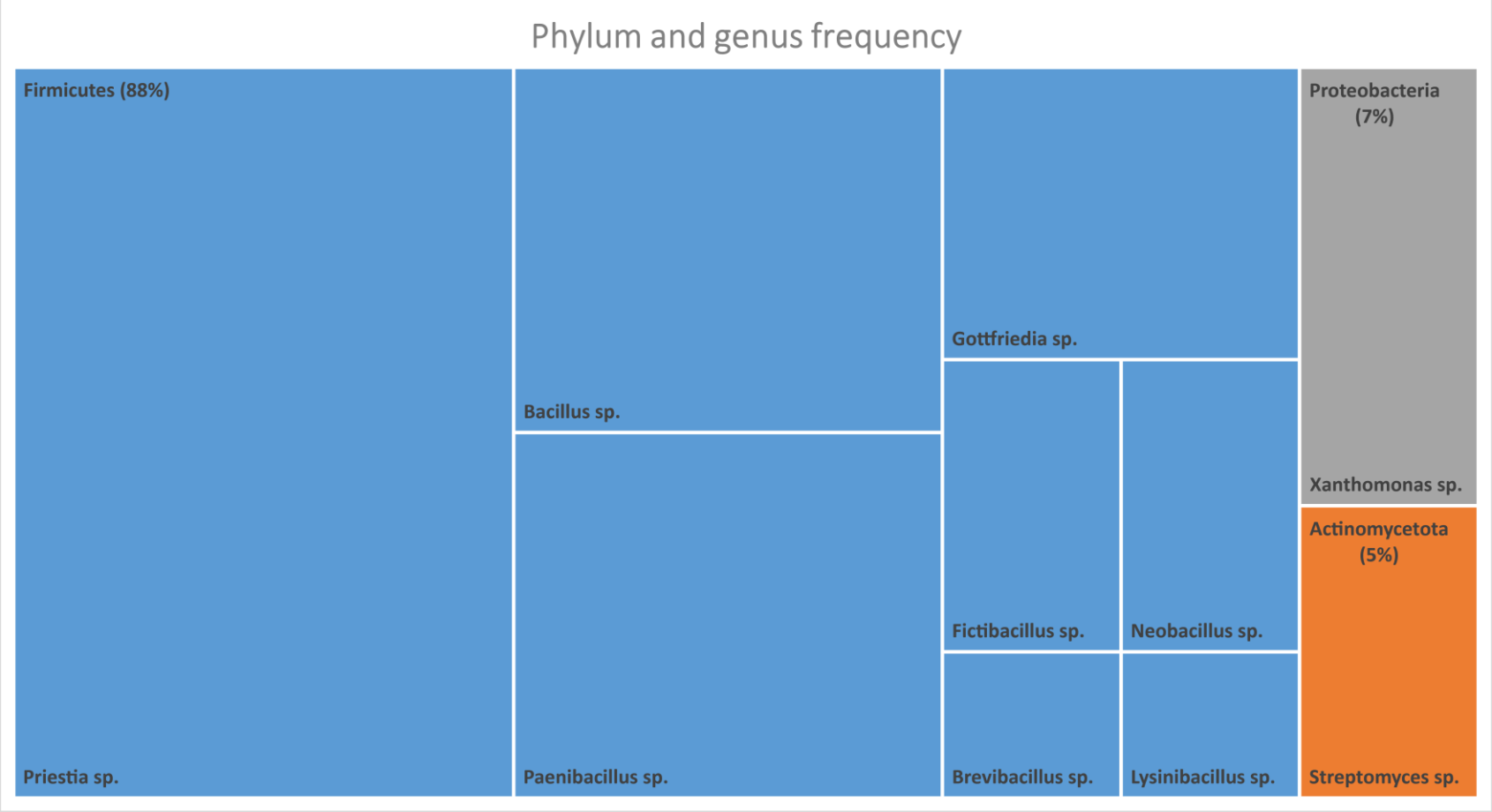

Figure 5.3: Distribution of the different genera identified and the phyla under which they are classified.

Table 5.1: Nodule endophyte identities based on NCBI BLAST.

Species	Common name	Accession	Sample	NCBI BLAST identity	Percent identity (%)	Assigned sequence numbers
Vigna unguiculata	Cowpea	Gujarat 5	C11	*Priestia aryabhattai*	99,89	OP623457
			C3	*Priestia sp.*	97,16	OP623475
			C1	*Paenibacillus sp.*	98,92	OP623474
			C5	*Gottfriedia luciferensis*	99,54	OP623456
			C6	*Priestia sp.*	98,41	OP623476
			CA4	*Streptomyces sp.*	97,51	**
			CA9	*Streptomyces sp.*	98,14	OP623477
Lablab purpureus	Dolichos	IC0623025	D10	*Priestia sp.*	98,63	OP623458
			D14	*Bacillus sp.*	98,06	OP623479
			D7	*Paenibacillus sp.*	92,13	OP623488
			D5	*Priestia sp.*	98,35	OP623478
			D17.1	*Bacillus subtilis subsp. subtilis*	100,00	OP623480

Vigna radiata	Mungbean	IC39399	MB17	*Priestia aryabhattai*	99,89	OP623491
			MB16	*Priestia filamentosa*	99,62	OP623473
			MB1	*Xanthomonas sp.*	93,06	OP623469
			MB14	*Paenibacillus sp.*	94,29	OP623472
			MB11	*Bacillus sp.*	95,45	OP623471
			MB3.1	*Priestia sp.*	95,11	**
			MB8	*Gottfriedia sp.*	93,88	OP623470
Macrotyloma uniflorum	Horsegram	Himala	H14	*Priestia aryabhattai*	99,68	OP623481
			H13	*Neobacillus sp.*	98,70	OP623460
			H4	*Priestia sp.*	93,71	OP623459
Vigna aconitifolia	Mothbean	RMB-25	M25-17	*Gottfriedia luciferensis*	99,76	OP623490
			M25-11	*Lysinibacillus boronitolerans*	99,12	OP623492
			M25-13	*Paenibacillus sp.*	98,28	OP623467
			M25-12	*Xanthomonas sp.*	97,16	OP623466

			M25-10	*Priestia aryabhattai*	99,78	OP623493
			M25-16	*Gottfriedia luciferensis*	99,70	OP623468
			M25-6	*Xanthomonas campestris*	99,56	OP623489
			M25-5	*Fictibacillus aquaticus*	96,37	OP623465
			M25-7	*Fictibacillus gelatini*	96,53	OP623494
			M25-3	*Priestia aryabhattai*	99,09	OP623464
		IPCMO-880	M8-2	*Priestia sp.*	96,99	OP623461
			M8-8	*Neobacillus niacini*	98,94	OP623482
			M8-6	*Paenibacillus sp.*	85,33	OP623462
			M8-21	*Bacillus sp.*	96,75	OP623463
			M8-14	*Paenibacillus polymyxa*	99,78	OP623485
			M8-9	*Priestia aryabhattai*	99,89	OP623483
			M8-13	*Brevibacillus sp.*	97,92	OP623484
			M8-16.1	*Bacillus sp.*	98,00	OP623486

			M8-16.2	*Bacillus proteolyticus*	99,38	OP623487

**Sequences with low or no similarity to 16S ribosomal RNA

5.3.2 Culture-independent microbiome analysis

The sequence data obtained after sequencing the nodules was cleaned to exclude non-bacterial reads and low sequence reads. No OTUs were identified as Archaea. Furthermore, reads annotated as chloroplast and mitochondria were excluded from the data set. Low abundance taxa with less than 50 reads were also removed from the analysis. After these exclusions, a total of 17364 reads were further analysed. Average reads per legume species ranged from 3171 to 5677 with *L. purpureus* having the highest number of reads (Table 5.2). Sequence reads were submitted to NCBI under the BioProject number PRJNA896769.

Table 5.2: Average number of reads for nodule microbiomes.

Legume species	Common name	Accession	Average reads
Lablab purpureus	Dolichos	IC0623025	1892
Vigna unguiculata	Cowpea	Gujarat 5	1071
Macrotyloma uniflorum	Horsegram	Himala	1767
Vigna aconitifolia	Mothbean	IPCMO-880	1586

The alpha diversity plots (Shannon and Chao1) were prepared to visually present the diversity within the different microbiomes. Total richness was estimated using the alpha diversity Chao1 plot (Figure 5.4). The four legume species showed great variation among each other concerning the number of observed species. This variation was also observed within *V. unguiculata* samples. *L. purpureus* and *M. uniflorum* exhibited minimal variation, however, *M. uniflorum* and *V. aconitifolia* (IPCMO-880) were observed to be the most similar in the number of observed species. This was apart from a single *M. uniflorum* sample.

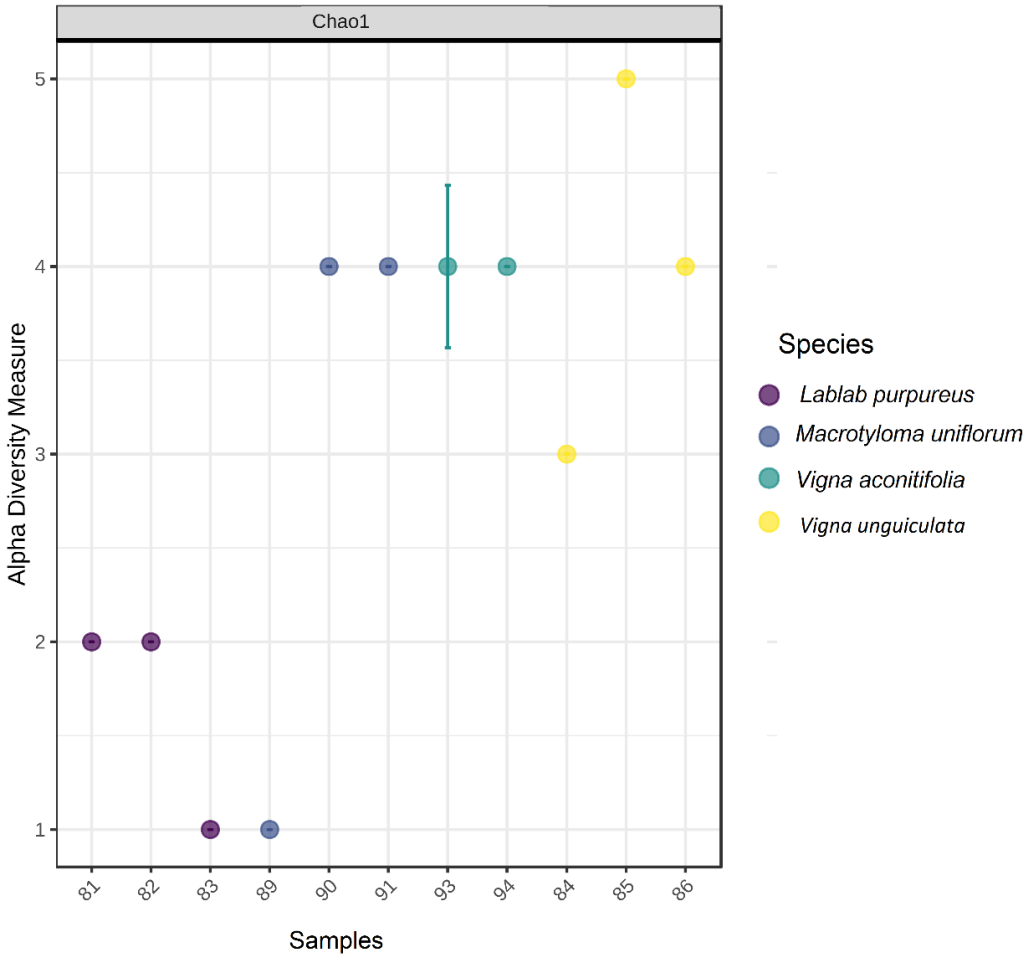

Figure 5.4: Alpha diversity (Chao1) indicating a measure of similarity in observed species within legumes species [*L. purpureus*, *V. unguiculata*, *V. aconitifolia* (accession IPCMO-880) and *M. uniflorum*] and across the complete data set.

The Shannon diversity plot (Figure 5.5) was used to estimate both richness and evenness within the samples. The greatest diversity was in *V. unguiculata* nodules and *V. aconitifolia.* Nodule samples from *M. uniflorum* exhibited both low and high diversity. As shown in the plot the diversity measure varied from 0.0 to approximately 1.0. This implies greater diversity between *M. uniflorum* nodules. A comparison of the four legume species indicated an uneven spread of abundance across the legume species.

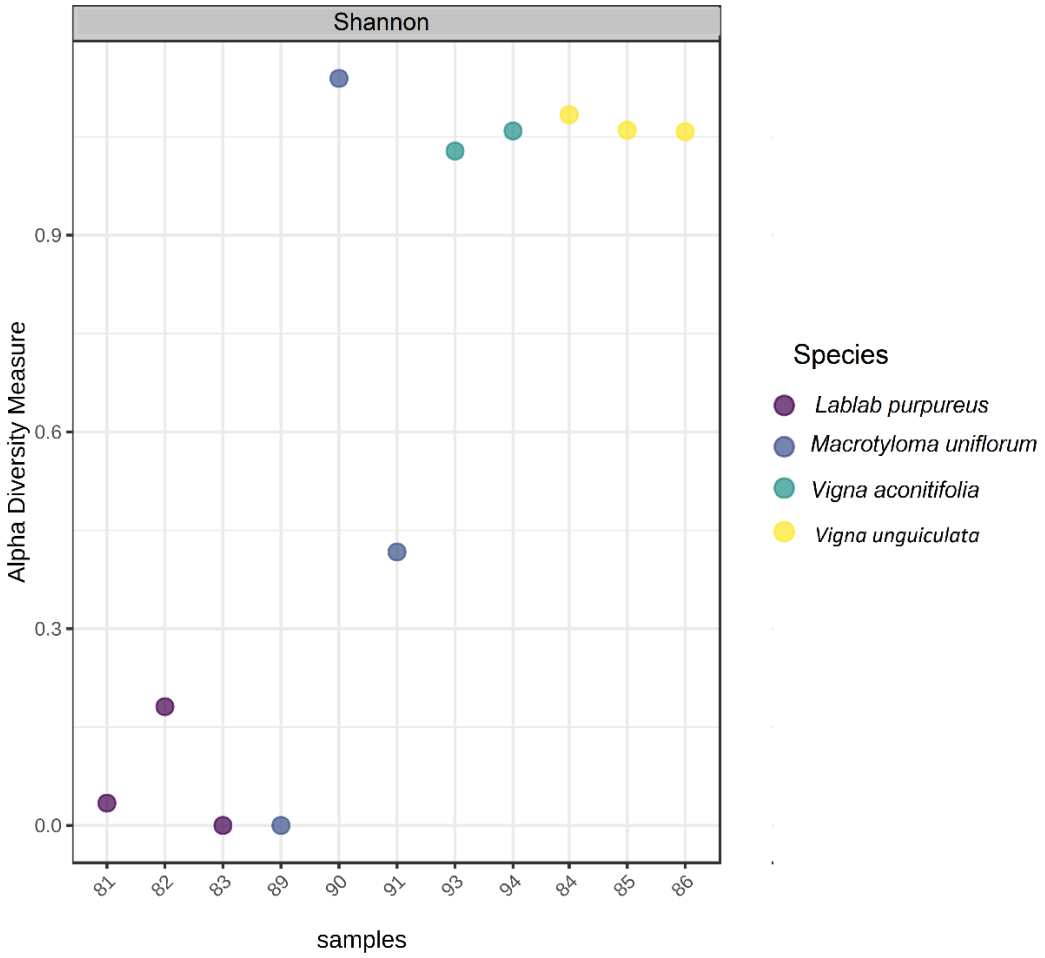

Figure 5.5: Shannon index of the microbiome in the root nodules of *L. purpureus*, *V. unguiculata*, *V. aconitifolia* (accession IPCMO-880) and *M. uniflorum*. The larger the Shannon index, the better the sample uniformity. In the case of the same species richness, the greater the uniformity of each species in the community, the greater the diversity of the community.

The sequence data were analysed to determine differences between and within samples. Differences in taxonomic composition were assessed and graphically shown using the Bray Curtis beta diversity plot (Figure 5.6). As shown in the figure, *V. aconitifolia* and *V. unguiculata* were clustered most closely. This implies strong similarity in taxonomic profiles. The remaining legume species exhibited clear separation from each other.

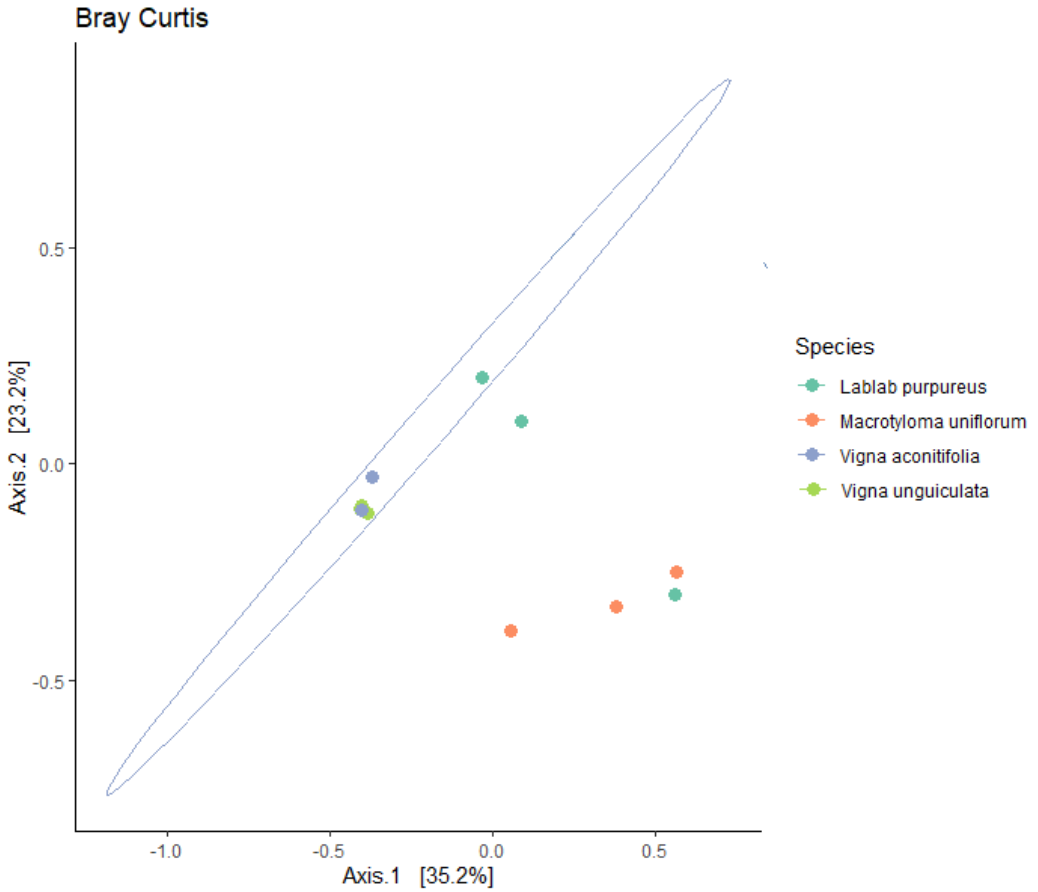

Figure 5.6: Bray Curtis plot of similarities between the nodule microbiomes of legume species.

The top six most abundant bacterial families identified in the root nodules were *Bacillaceae, Chitinophagaceae, Burkholderiaceae, Xanthobacteraceae, Enterobacteriaceae,* and *Xanthobacteraceae_Bradyrhizobium* (which is now officially referred to as *Nitrobacteraceae*). However, as seen in Figure 7, greater abundance was observed with the closely related *Xanthobacteraceae* families. The family *Enterobacteriaceae*, however, was significantly more abundant only in *L. purpureus* nodules and *V. unguiculata* (Figure 5.7). Taxonomic relatedness was observed to be closest between *Xanthobacteraceae_Bradyrhizobium* (*Nitrobacteraceae*) and *Enterobacteriaceae*. Based on the hierarchical clustering shown in Figure 5, these two families also showed close relation with the *Xanthobacteraceae* family.

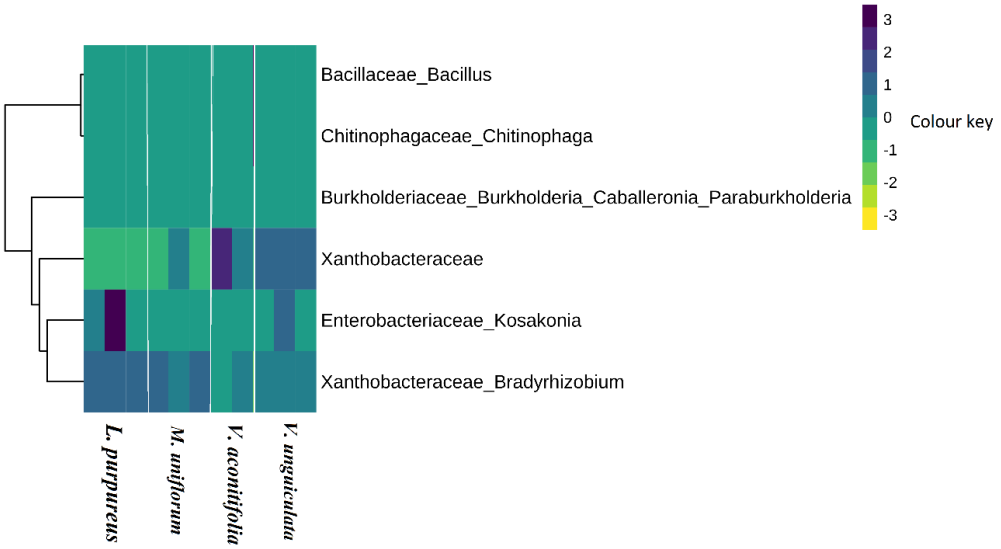

Figure 5.7: Heat map showing hierarchical clustering of the top six most abundant families and related genera from the nodule microbiomes. The map also shows the evolutionary links among the families.

The results observed in the heat map above correlate with observations in the abundance data at the genus level. The genus *Bradyrhizobium* was the most abundant in all samples (Figure 5.8). On average, this genus made up 99.2% and 98% of all bacteria in *L. purpureus* and *M. uniflorum* nodules respectively. In *V. unguiculata*, 72.9% were *Bradyrhizobium* while *V. aconitifolia* (IPCMO-880) had the least amount of *Bradyrhizobium* detected with an average of 69.9%. Other bacteria genera were also detected; however, they could not be classified due to the low number of reads.

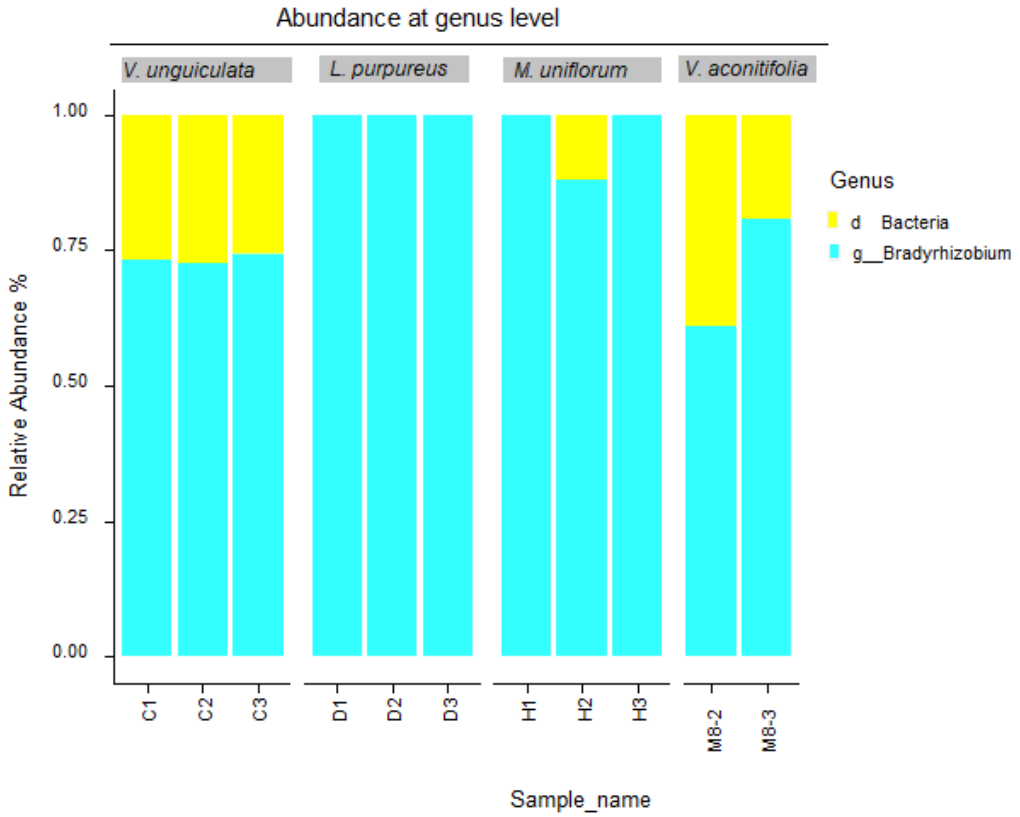

Figure 5.8: *Bradyrhizobium* abundance in the root nodule microbiomes. Unclassified bacteria with low reads are shown in yellow.

5.3.3 Culture-dependent characterisation

Nitrogen fixation

Nitrogen fixation, based on phenotypic growth patterns on Jensen medium, was distinctly observed in eight root nodule endophytes (Table 5.3). These isolates included unclassified *Priestia* species from *L. purpureus* and *V. radiata*. Other isolates identified from *V. radiata* are two unclassified species from the genera *Xanthomonas* and *Paenibacillus*. Two species isolated from *V. aconitifolia* (RMB-25) were identified to fix nitrogen. These were *Priestia aryabhattai* and *Xanthomonas campestris*. *Paenibacillus polymyxa*, a *V. aconitifolia* (IPCMO-880) isolate was observed to also fix nitrogen. No isolate from *V. unguiculata* was observed to fix nitrogen.

Phosphate solubilization

Root nodule endophytes of the six accessions were assessed for their ability to solubilize inorganic phosphate. None of the 41 isolates showed the ability to solubilize inorganic phosphates after 7 days. Figure 5.9 below shows the activity of both positive and negative controls compared to some of the isolates assessed in this study. These results are summarised in Table 5.3.

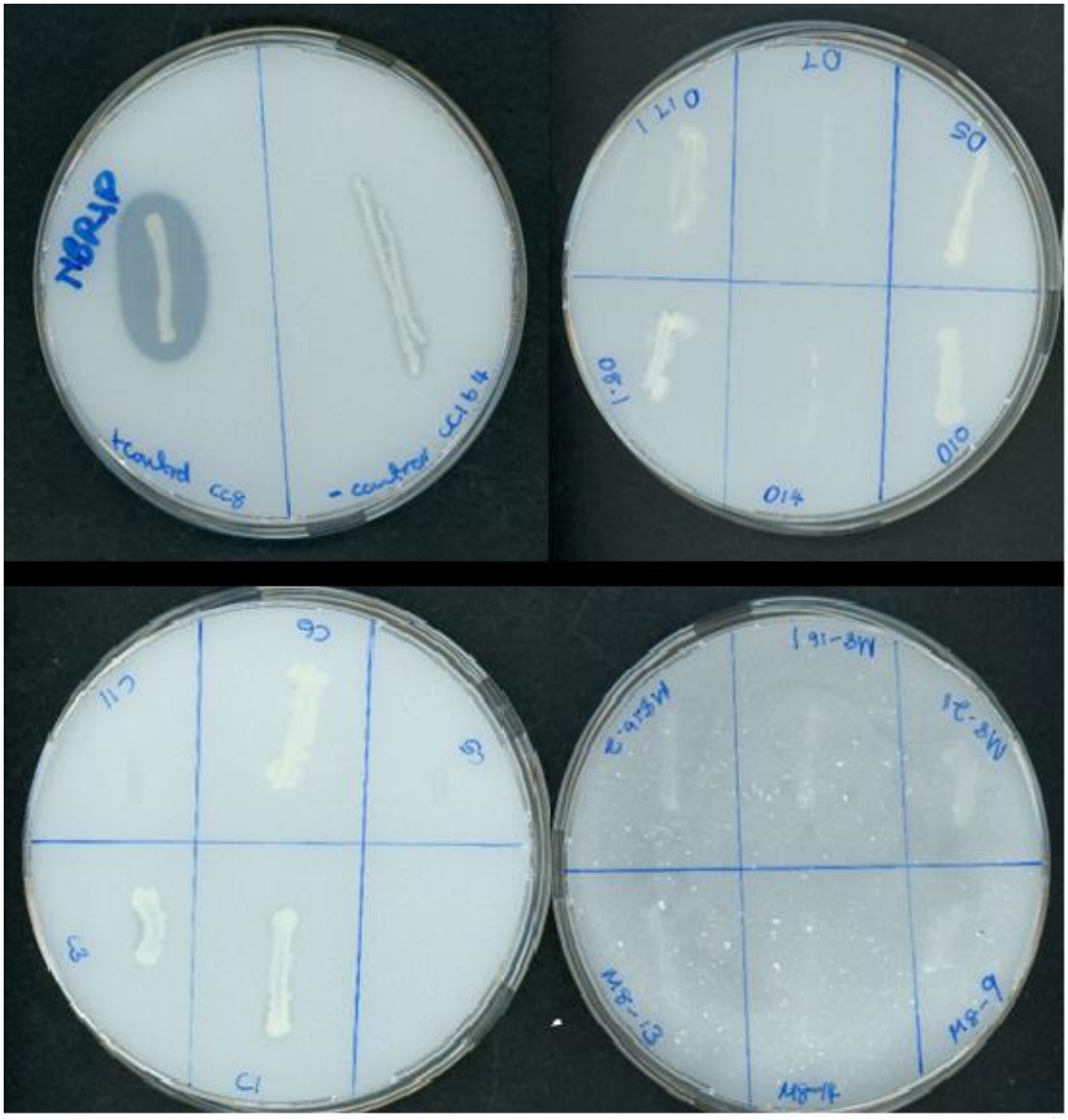

Figure 5.9: Phosphate solubilization assay of isolates grown on NBRIP growth medium.

Siderophore production

Siderophore production assessed on CAS agar was observed in 23 isolates as shown in Table 5.3. Each legume accession had at least one positive isolate. Siderophore-producing isolates were identified from all three phyla in which samples were classified in this study. This includes *Bacillus, Priestia, Xanthomonas, Paenibacillus*, and *Streptomyces* genera. The isolates and closest identities are listed in Table 5.3. As shown in Figure 5.10 below, the orange halo around colonies indicates positive siderophore production.

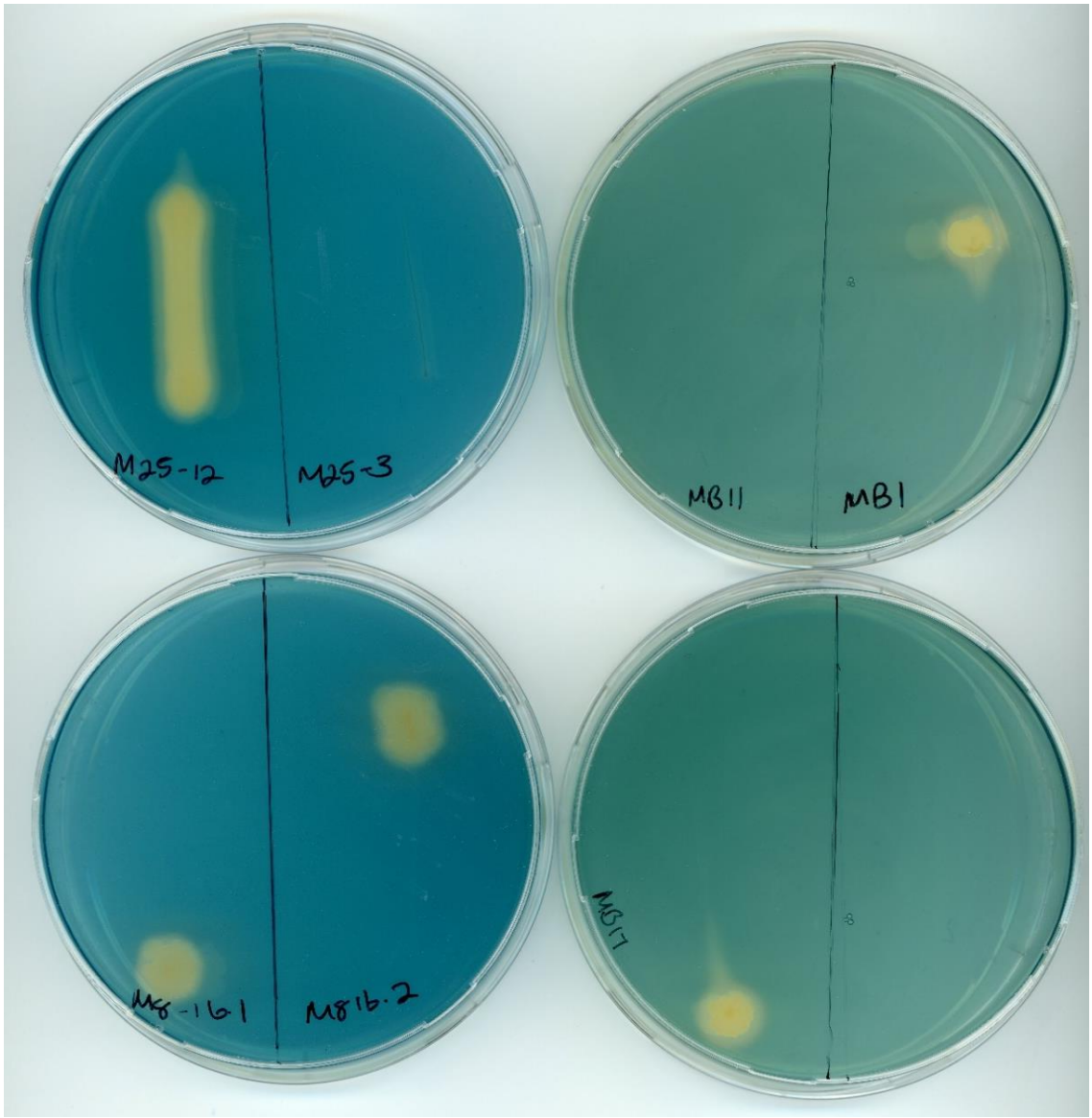

Figure 5.10: Siderophore production by nodule endophytes.

Bacterial antifungal activity

Root nodule endophytes, when assessed against *F. graminearum* showed minimal activity. From 41 isolates, 3 showed antagonistic action against *F. graminearum.* Two *Paenibacillus* species (including *P. polymyxa*) and *B. subtilis subsp. subtilis* were observed to have distinct antifungal antagonistic action. Other isolates were observed to inhibit the growth of *F. graminearum* due to an increased growth rate. However, no distinct antagonistic effect was observed. Figure 5.11 below shows the antagonistic effect of *B. subtilis subsp. subtilis* while *P. aryabhattai* (M8-9) and an unclassified *Paenibacillus sp*. strain showed no antagonistic effect at all.

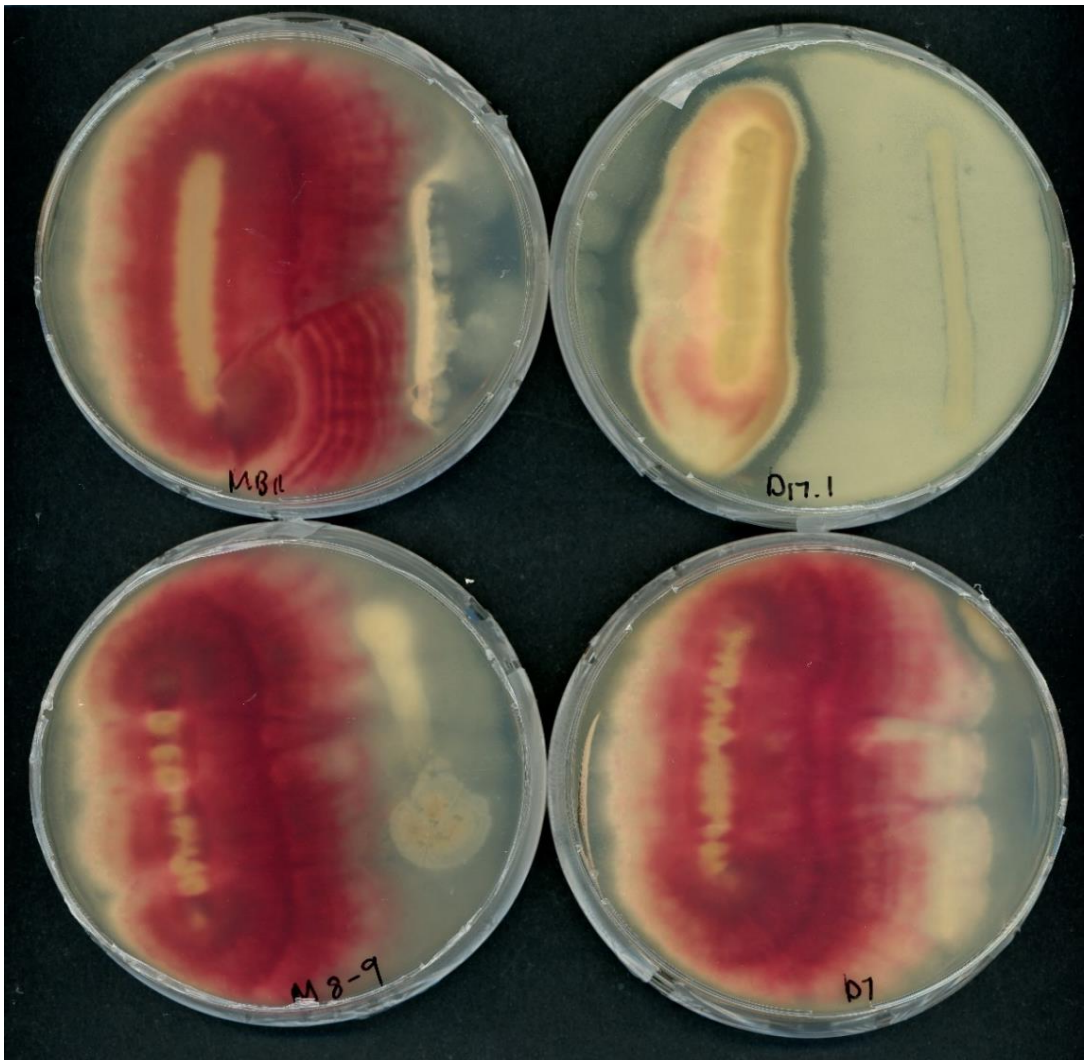

Figure 5.11: Antifungal activity of nodule endophytes against *F. graminearum.*

Bacterial indole acetic acid production

This study aimed to assess IAA production of the nodule endophytes at optical density 530 nm. Concentrations were determined based on a standard concentration of IAA (standard curve graph shown in Figure 5.2). Positive bacterial production of IAA was determined based on known positive and negative control isolates. Based on the positive control (43 µg/mL after 24 hours growth) 14 isolates were observed to produce IAA (Table 5.3). The highest concentration was observed from a *Brevibacillus* unclassified species isolated from *V. aconitifolia* IPCMO-880. The lowest concentration of IAA being produced was observed from an unclassified *Paenibacillus* species isolated from *L. purpureus.*

Exopolysaccharide production

EPS production was determined by the mucoid appearance of colonies on YEM medium (Figure 5.12). Ten isolates showed positive exopolysaccharide production. Two isolates from *M. uniflorum* were observed to produce EPS in some cases. Therefore, their ability to produce EPS could not be determined. These isolates were both *Priestia* species. The most EPS producing isolates were from *V. unguiculata* and *V. radiata* with four samples each. The EPS producing isolates were identified as *Priestia* species - *P. aryabhattai, P. filamentosa* and two unclassified species. Two *Gottfriedia* species including *G. luciferensis* were also identified. The remaining isolates were from the *Brevibacillus,* and *Xanthomonas* genera.

Figure 5.12: EPS production on YEM medium after 48 hours.

PCA of the number of positive isolates per trait and accession was performed. Phosphate solubilization was excluded from the analysis as no isolate showed positive activity against siderophore production. The percentage of variances was determined to be approximately 70% based on the Scree plot below (Figure 5.13). PCA of correlation between accessions (Figure 5.14) showed no significant correlation between variances across groups. PCA of the PGP traits, however, (Figure 5.15) showed the most positive correlation between nitrogen fixation and exopolysaccharide production. Antifungal activity, siderophore production and IAA also showed similar correlation.

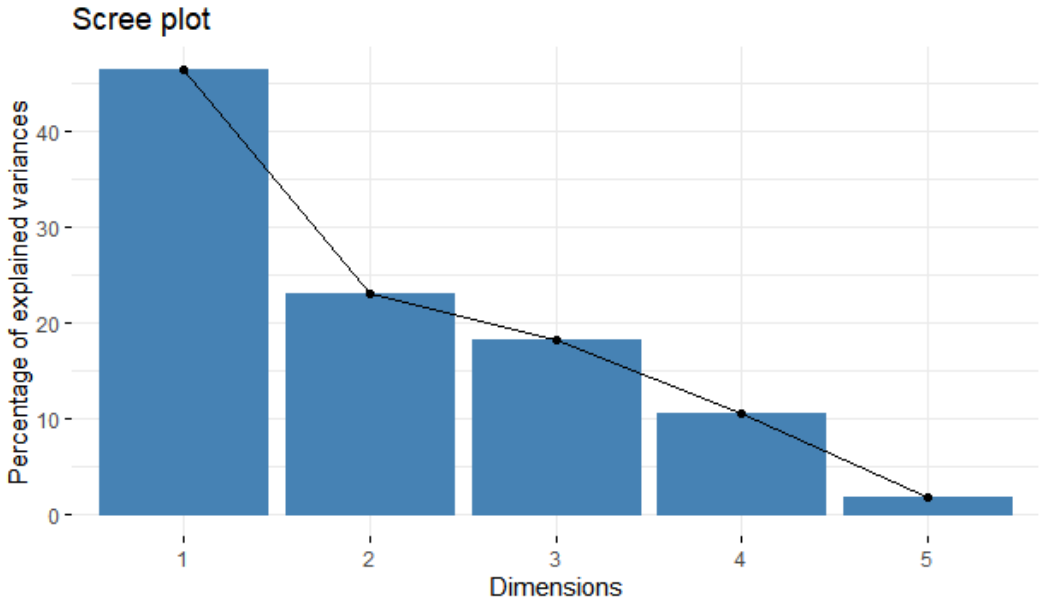

Figure 5.13: S*cree plot* showing the percentage of variances explained by each principal component.

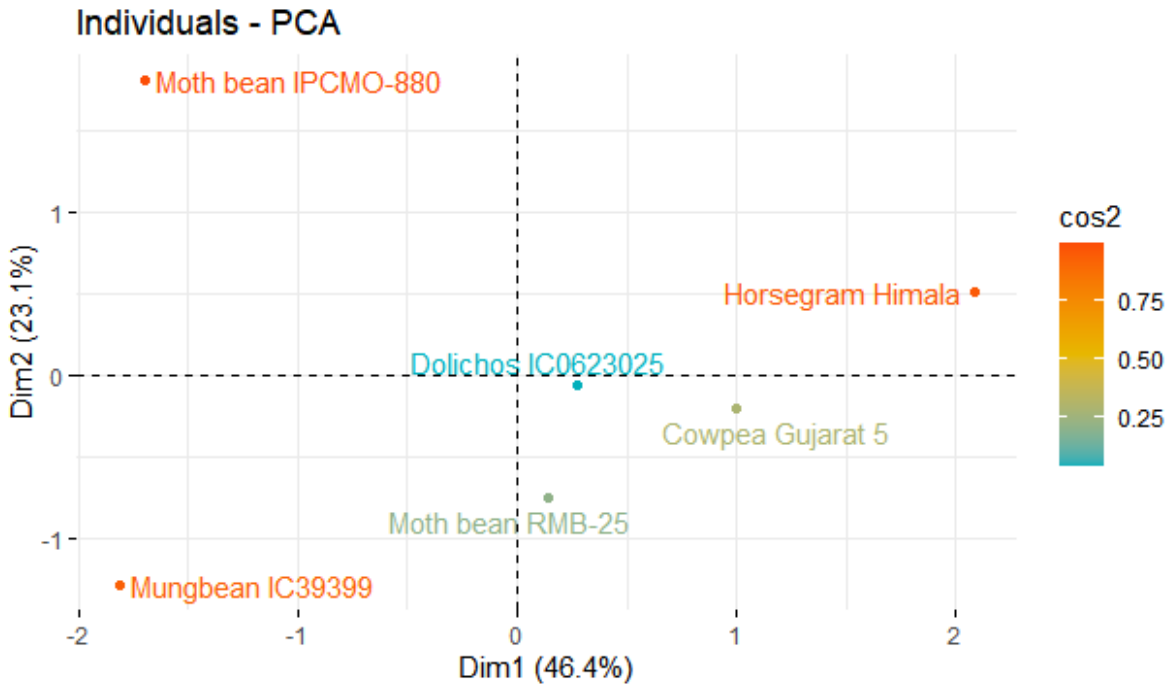

Figure 5.14: PCA presentation of legume accessions showing correlations between legume accessions. Dolichos IC0623025 - *L. purpureus*, Cowpea Gujarat 5 - *V. unguiculata*, Horsegram Himala - *M. uniflorum*, Mungbean IC39399 - *V. radiata*, Moth bean IPCMO-880 - *V. aconitifolia*, and Moth bean RMB-25 - *V. aconitifolia*.

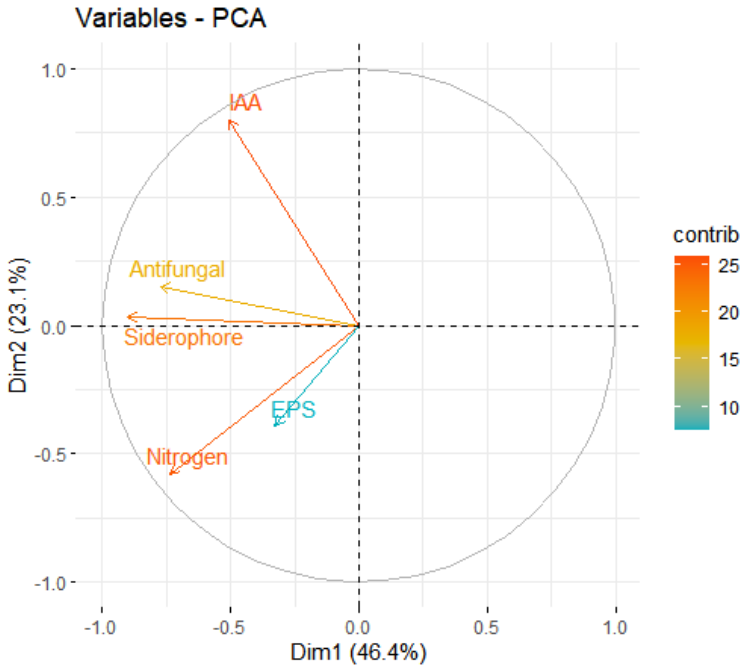

Figure 5.15: PCA graph of PGP traits showing positive correlation between EPS production and nitrogen fixation. Siderophore production, antifungal activity and IAA production were more similarly correlated to each other than the EPS production and nitrogen fixation.

Table 5.3: Plant growth-promoting traits of root nodule isolates.

Legume	**Common name**	**Accession**	**Sample**	**Bacterial genus/species**	**Phos[a]**	**Sid[b]**	**Nit[c]**	**Ant[d]**	**EPS[e]**	**IAA[f]**
Lablab purpureus	Dolichos	IC0623025	D10	*Priestia sp.*	-	-	-	-	-	-
			D14	*Bacillus sp.*	-	+	-	-	-	-
			D7	*Paenibacillus sp.*	-	-	-	-	-	-
			D5	*Priestia sp.*	-	+	+	-	-	-
			D17.1	*Bacillus subtilis subsp. subtilis*	**Un**	+	**Un**	+	-	+
Vigna unguiculata	Cowpea	Gujarat 5	C1	*Paenibacillus sp.*	-	+	-	-	+	-
			C3	*Priestia sp.*	-	-	-	-	+	+
			C11	*Priestia aryabhattai*	-	-	-	-	+	-
			C5	*Gottfriedia luciferensis*	-	+	-	-	+	+
			C6	*Priestia sp.*	-	-	-	-	-	-
			CA9	*Streptomyces sp.*	-	+	-	-	-	-
			CA4	*Streptomyces sp.*	-		-	-	-	-

Macrotyloma uniflorum	Horsegram	Himala	H14	*Priestia aryabhattai*	-	-	-	-	**Un**	+
			H13	*Neobacillus sp.*	-	+	-	-	-	-
			H4	*Priestia sp.*	-	-	-	-	**Un**	+
Vigna radiata	Mungbean	IC39399	MB17	*Priestia aryabhattai*	-	-	-	-	-	-
			MB16	*Priestia filamentosa*	-	+	-	-	+	-
			MB1	*Xanthomonas sp.*	-	+	+	-	+	+
			MB14	*Paenibacillus sp.*	-	-	+	+	-	-
			MB11	*Bacillus sp.*	-	+	-	-	-	-
			MB3.1	*Priestia sp.*	-	+	+	-	+	+
			MB8	*Gottfriedia sp.*	-	+	-	-	+	-
Vigna aconitifolia	Moth bean	RMB-25	M25-17	*Gottfriedia luciferensis*	-	-	-	-	-	-
			M25-11	*Lysinibacillus boronitolerans*	-	-	-	-	-	+
			M25-13	*Paenibacillus sp.*	-	+	-	-	-	-
			M25-12	*Xanthomonas sp.*	-	+	-	-	-	-

			M25-10	*Priestia aryabhattai*	-	-	+	-	-	-
			M25-16	*Gottfriedia luciferensis*	-	-	-	-	-	-
			M25-6	*Xanthomonas campestris*	-	+	+	-	-	-
			M25-5	*Fictibacillus sp.*	-	+	-	-	-	-
			M25-7	*Fictibacillus sp.*	-	+	-	-	-	-
			M25-3	*Priestia aryabhattai*	-	-	-	-	-	-
	Moth bean	IPCMO-880	M8-2	*Priestia sp.*	-	+	-	-	-	-
			M8-8	*Neobacillus sp.*	-	+	-	-	-	+
			M8-6	*Neobacillus sp.*	-	+	-	-	-	-
			M8-21	*Bacillus sp.*	-	+	-	-	-	+
			M8-14	*Paenibacillus polymyxa*	-	-	+	+	-	-
			M8-9	*Priestia aryabhattai*	-	-	-	-	-	+
			M8-13	*Brevibacillus sp.*	-	-	-	-	+	+
			M8-16.1	*Bacillus sp.*	-	+	-	-	-	+

			M8-16.2	*Bacillus proteolyticus*	-	+	-	-	-	+

[a] Phosphate solubilization
[b] Siderophore production
[c] Nitrogen fixation
[d] Antifungal activity
[e] Exopolysaccharide production
[f] Indole acetic acid production
"–" means showed no production/ "+" means showed production/ "Un" means plant growth-promoting trait could not be determined.

5.4. Discussion

This study aimed to identify and classify culturable bacteria from the root nodules of six accessions. In addition, this study assessed the PGP traits possessed by the isolated bacteria. The identification of the isolates was performed with based on the 16S rRNA gene, which in many cases and depending on the genus analysed lacks resolution for the identification at the species level, therefore, other techniques such as MLSA (multi-locus sequence analysis) or whole genome sequencing may be used to identify species or to describe novel species. The isolates identified from the nodules were largely in the phylum Firmicutes now also referred to as Bacillota (Oren & Garrity, 2021). They were mostly in the families *Bacillaceae* and *Paenibacillaceae*. This was an expected observation as it has been documented that *Bacillus* species are the most common non-rhizobial occupants of root nodules of a variety of legumes (Martínez-Hidalgo & Hirsch, 2017).

Streptomyces species were only isolated from *V. unguiculata* while *Xanthomonas* isolates were obtained from *V. radiata* and *V. aconitifolia* (RMB-25) only. This informs towards the specificity of host plants influencing nodule endophytes (Rocha *et al*., 2020). Nevertheless, legume root nodules are typically colonised by rhizobia. This includes *Rhizobium*, *Bradyrhizobium* (Martínez-Hidalgo & Hirsch, 2017), *Mesorhizobium* (Xiao, Chen, *et al*., 2017), *Sinorhizobium*, and *Azorhizobium* (Nosheen, Ajmal & Song, 2021). However, as observed in this study, non-rhizobial species such as *Bacillus* and *Streptomyces* (Bakhtiyarifar, Enayatizamir & Mehdi Khanlou, 2021) may also colonize nodules. In addition, research has also identified the non-rhizobial genera *Acinetobacter* and *Pseudomonas* in root nodules (Hakim *et al*., 2020).

By using Microbiome studies to assess the nodule communities, this study also assessed the differences in culture-dependent and culture-independent bacteria. The present study assessed the relative abundance of the *Bradyrhizobium* genus in microbiomes of *V. unguiculata, V. aconitifolia, M. uniflorum* and *L. purpureus* grown in Namibia. Microbiome studies allow for the analysis of both culture-dependent and culture-independent bacterial groups (Turner, James & Poole, 2013). This is because it accounts for the DNA of all microbes present in a sample. As a result, the data presented in this study provide a microbiome overview inclusive of bacteria which cannot be cultured with current culture methods (Gururani *et al*., 2021).

The methodology followed in this study, may have been limited in selectivity of some rhizobia. This is due to rhizobia typically being slow growing, and, therefore, they may be inhibited by fast growing species (Missbah El Idrissi et al., 2021). Furthermore, evidence has shown that bacteria may have inhibitory effects against each other within the same ecosystem. *Bacillus* species, for example, have been observed inhibiting the growth of *Bradyrhizobium* species (Han *et al*., 2020). This may account for the high percentage of *Bacillus* obtained on SEA and YEM.

Microbiome analysis of the abundance of *Bradyrhizobium* in this study was largely limited by using 16S primer sequencing for universal rRNA genes. As a result, specific PGP genes could not be defined to identify related PGP taxa (Turner, James & Poole, 2013). In addition, the data obtained in study were for nodules at five weeks after planting. Differences, which were not considered in this study, may exist during different growth stages. This is attributed to the different needs a plant may have during early, active, and senescence stages (Hansen *et al*., 2020).

The observation of high abundance of *Bradyrhizobium* genus in the samples was as expected in legume nodules. Previous studies have defined *Bradyrhizobium* sp. in *V. subterraenea* (Puozaa, Jaiswal & Dakora, 2017), *V. unguiculata* (Bünger *et al*., 2018), *Glycine clandestine, G. max* (Klepa *et al*., 2021) and *L. purpureus* (Grönemeyer, Bünger & Reinhold-Hurek, 2017). In addition, the low similarity across the different legume species shown by the Bray Curtis plot (Figure 5.4), was also expected. This observation was further supported by the relative abundance of *Bradyrhizobium*. The abundance across the different legume species showed a clear difference. This is because nodule infection by rhizobia has been shown to be highly specific to host plants (Vacheron *et al*., 2013; Han *et al*., 2020). Furthermore, the different microbial compositions may also be linked to the presence of both biotic and abiotic stress factors. These stress factors may affect nodulation, microbial colonisation, and composition (Gossmann *et al*., 2012). This was not factored in our study, as no plants showed physiological evidence of stress.

The *Xanthobacteraceae_Bradyrhizobium* (*Nitrobacteraceae*) and *Xanthobacteraceae* families were the most abundant. They form the families in which rhizobia genera are found. Literature, however, supposes that *Bradyrhizobium* as a genus is better classified under the family nomenclature *Bradyrhizobiaceae* given the various species and genera that have been proposed under it. Recently, *Bradyrhizobiaceae* has been renamed as *Nitrobacteraceae* citing the more fitting nitrogen fixing ability of the species in this family (Ormeño-Orrillo & Martínez-Romero, 2019). The results obtained in this study were similar to a previous analysis of the nodule microbiome of soybean (*G. max*). *Bradyrhizobium* species contributed more than 99% of the Proteobacteria sequences (Sharaf *et al*., 2019). The taxonomic variations in relative

abundance of *Bradyrhizobium* were expected. They are known to vary across other legume species (Han *et al*., 2020).

Other taxonomically important families were also defined in the nodule microbiomes. The *Bacillaceae*, for example, includes the *Bacillus* genus that is a commonly observed non-rhizobial nodule occupant (Martínez-Hidalgo & Hirsch, 2017). In addition, evidence has also shown that *Bacillus* species can promote plant health and regulate nodule formation (Han *et al*., 2020). Previous work has identified it exhibiting antagonistic activity against plant pathogens (Pang *et al*., 2021). *Burkholderiaceae*, as observed in our study, represented some of the rhizobial species from other bacterial families which may occupy nodules (Rocha *et al*., 2020).

Microbial biochemical assays were used to assess the bacterial PGP traits of the isolates. Bacterial siderophore production was assessed in the isolates from root nodules. Five of the seven isolates from the *V. radiata* nodules were observed to be positive producers of siderophores. However, this is a sharp contrast to a previous study in which no nodule isolates from *V. radiata* were positive for siderophore production (Bhutani, Maheshwari & Suneja, 2018). Siderophore production is widely known to be characteristic of *B. megaterium* and *B. simplex* (Francis, Holsters & Vereecke, 2010). This coincides with our observations from samples D14, M8-16.1 and MB11 (*Bacillus sp.*), D17.1 (*B. subtilis subsp. subtilis*), and M8-16.2 (*B. proteolyticus*). However, in this study siderophore production was also observed in other isolates. This included *Xanthomonas sp*., some *Priestia sp.* such as *P. filamentosa,* and *Paenibacillus sp.*

The isolates were assessed for their antagonistic effect against *F. graminearum*. Bacterial antifungal activity was observed in three isolates, M8-14 (*P. polymyxa*),

MB14 (*Paenibacillus*), and D17.1 (*B. subtilis subsp. subtilis*). These observations were expected as antifungal activity is known to vary within genera (Bakhtiyarifar, Enayatizamir & Mehdi Khanlou, 2021). In addition, *Bacillus* sp. are known to actively inhibit fungal pathogens (Zhao, Xu & Lai, 2018). Phosphate solubilization, in this study, was negative for all isolates. Bhutani, Maheshwari and Suneja (2018), similarly observed minimal phosphate solubilization from a collection of 13 nodule endophytes with only two showing positive activity.

This study also aimed to assess IAA production by the nodule isolates. IAA production was observed to differ largely across the nodule isolates as no distinct trend was noted. Genetic variations may account for this (Bhutani, Maheshwari & Suneja, 2018). Furthermore, the different growth rates of the bacteria may have influenced the production rate as previously observed (Nunes *et al*., 2018). EPS production, on the other hand, is influenced by environmental and nutritional conditions. Therefore, the different requirements of the bacterial isolates, which include temperature and pH, impact the production of EPS (Abd-Alla *et al*., 2018). This alludes to the different observations. Quantifying EPS production under different parameters would have provided more information given the different bacterial requirements (Latif *et al*., 2022)

Atmospheric nitrogen fixation was also assessed based on colony morphology on Jensen medium. It was notably low across all nodule endophytes from the six legume accessions. This may be attributed to the low phosphorus content in the Bagani soils in which samples were grown. Low soil phosphorus content is a well-known contributing factor that often results in reduced nitrogen fixation (Hussain, 2017). However, it is also known that nitrogen fixation occurs optimally in anaerobic

conditions. Hence, rhizobia only fixing nitrogen while in root nodules (Martínez-Hidalgo & Hirsch, 2017). Improved bioinoculant performance for nitrogen fixation may be improved by co-inoculation with phosphorus-solubilising bacteria (Matse *et al*., 2020). Nitrogen fixation was also observed across different genera which include *Priestia, Xanthomonas, and Paenibacillus.* Nitrogen fixation was not uniform in these genera. This assay was limited as it observed biological nitrogen-fixing on solid medium over a period of 72 hours. However, it did not account for differing rates of nitrogen fixation. The use of acetylene reduction assay would better inform towards this (Saiz *et al*., 2019). Furthermore, the identification of nitrogen-fixing genes (*nif* genes) would also highlight isolates with the ability to fix nitrogen (Gaby & Buckley, 2012; Liu, Zhang, *et al*., 2017).

5.5. Conclusion

The present study characterised bacterial isolates and the nodule microbiomes of drought-tolerant legumes grown in Namibia. In addition, microbiome analyses were carried out on the root nodules. Microbiome analysis of the nodules revealed that the top six most abundant taxonomic families were *Bacillaceae, Chitinophagaceae, Burkholderiaceae, Xanthobacteraceae, Enterobacteriaceae,* and *Nitrobacteraceae*. However, the most abundant was *Nitrobacteraceae* (*Xanthobacteraceae_Bradyrhizobium)* which was represented by the genus *Bradyrhizobium*. This was a sharp contrast to the isolated strains which were mostly *Bacillus*, or a close genetic relative (*Priestia, Paenibacillus, Gottfriedia, Neobacillus, Lysinibacillus, Fictibacillus*, and *Brevibacillus*). Characterisation of the nodule isolates found that the root nodules were endowed with bacteria with PGP traits. This informed towards promotion of general plant health and growth and potential

bioinoculant use. Among the PGP traits assayed, only phosphate solubilization was not observed in any of the isolates. Therefore, this study concludes that the root nodules of the legumes assessed harbour a diverse group of endophytes with biofertiliser potential to promote growth and may be employed as biofertilisers in Namibia. Isolates MB3.1 (*Priestia sp.*), H14 (*P. aryabhattai*), M25-11 (*L. boronitolerans*), M8-16.1 (*Bacillus sp.*) and M8-16.2 (*B. proteolyticus*) showed the most potential as plant-growth promoters. They are therefore, strongly recommended to be assessed as bioinoculants in field trials.

5.6. References

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CHAPTER 6

Assessment of early growth response of *Vigna unguiculata* to bioinoculants under drought stress conditions

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Abstract

One of the most important plant growth-promoting traits from bacteria is drought-tolerance. Induced drought-tolerance by plant growth-promoting bacteria (PGPB) is a favourable trait which has been shown to be effective. The present study investigated plant growth promoting abilities of previously isolated bacterial strains (*Bacillus sp., Stenotrophomonas maltophilia, Priestia aryabhattai, Lysinibacillus boronitolerans,* and *Enterobacter mori*). This was achieved by assessing early growth response under drought stress conditions in cowpea (*Vigna unguiculata*) seeds. Surface sterilised *V.*

unguiculata seeds were inoculated with the selected isolates. Growth parameters assessed were germination rate of samples planted and response indices- root length stress index (RLSI) and shoot length stress index (SLSI). Increased root and shoot length were observed in drought-stressed samples of all inoculants except *L. boronitolerans.* The highest average root lengths (51.8 cm and 37.5 cm) were observed in *Bacillus sp.* and *Stenotrophomonas maltophilia* respectively. Average shoot length was highest (80.6 cm and 75.3 cm) in *Bacillus sp.* and *Enterobacter mori* respectively. Samples inoculated with *Bacillus sp.* had the highest RLSIs and SLSIs at 158,4% and 120.3% respectively. *Bacillus sp., S. maltophilia,* and *E. mori* were observed to promote growth under drought stress when applied individually in comparison to control plants that were not treated with the bioinoculants. Therefore, *Bacillus sp., S. maltophilia,* and *E. mori* strains may be assessed in field trials as biofertilisers.

Keywords- Bioinoculants; Drought stress; Sustainable agriculture; Plant growth promotion; Namibia

6.1. Introduction

Dryland agriculture has increased across the globe. Studies indicate that climate change coupled with anthropogenic activities negatively impact agricultural production (Hubert *et al*., 2022). These challenges include but are not limited to water shortage (Zia *et al*., 2021), loss of biodiversity (Pacheco *et al*., 2018), and increased frequency of drought occurrences (Ahmadalipour *et al*., 2019). Namibia is particularly vulnerable to these challenges as it is an arid country, the most arid in the Southern African region (Nijbroek *et al*., 2018).

There is a need, therefore, to utilise environmentally and financially sustainable practices to improve crop stress tolerance and yields. This is crucial for Namibia as it

has limited arable land (approximately 1%) and close to 70% of the entire human population is reliant on subsistence agriculture (Braker *et al*., 2015; Food and Agriculture Organization, 2022). Evidence from research has shown the increasingly harmful effects of pollutant chemical fertilisers on the environment (Batool & Iqbal, 2019). Their increasing cost also makes them economically unsustainable options for rural smallholder and subsistence farmers (Maheshwari, Aeron & Saraf, 2013; Schnitkey *et al*., 2021).

Desert adapted plants are bestowed with microorganisms that play substantial roles in nutrient availability and consequent uptake. Plant-associated bacteria can be harnessed as biofertilizers or bioinoculants to improve plant stress tolerance and improve soil health (Igiehon, Babalola & Aremu, 2019). These plant growth-promoting bacteria (PGPB) are economic and environmentally friendly alternatives to chemical fertilisers (Abd-Alla *et al*., 2018; Eid *et al*., 2021). Furthermore, research has proven their ability to improve crop yields in different crops such as cowpea (*Vigna unguiculata*) and wheat (*Triticum aestivum*) (Luchen *et al*., 2018; Alsharif, Saad & Hirt, 2020).

One of the most important PGP traits is drought tolerance. Bacteria use several multiple pathways to improve drought stress tolerance. This may be achieved by the production of phytohormones like indole acetic acid (IAA) (Khan *et al*., 2020) or the production of exopolysaccharides (Bonatelli *et al*., 2021). Therefore, induced drought tolerance by PGPB is a favourable trait which is effective (Zhang *et al*., 2019). The present study aimed to investigate enhanced plant growth abilities of previously isolated bacterial strains. This was achieved by assessing early vegetative growth response under drought stress conditions in cowpea (*V. unguiculata*) seedlings in a greenhouse setting.

6.2. Materials and methods

6.2.1. Bioinoculant preparation

Preliminary studies on early germination and drought tolerance were done to assess the potential of the bacterial isolates. Previously isolated bioinoculants were chosen based on *in vitro* drought tolerant traits (IAA production). Bioinoculants were prepared following the method described by Azeem *et al*. (2022). Selected bacterial inoculants (listed in Table 6.1) were grown overnight in tryptic soy (TS) broth on a shaker at 175 rpm. After which, 50 mL of each culture was centrifuged at 2000 rpm for 10 minutes. Cultures were centrifuged and cell pellets were washed twice with phosphate buffer saline (PBS). After which, they were resuspended in 40 mL PBS with an OD_{600} between 0.8 and 1.1. A consortium of the 5 bacterial isolates was prepared by mixing 10 mL of each of the resuspended isolates. Table 6.1 shows the isolates used and their plant source.

Table 6.1: Isolates assessed for *in vivo* drought tolerance.

Isolate ID	**Closest relative***	**Plant source**	**Accession**
CRhi15	*Stenotrophomonas maltophilia*	Cowpea (*Vigna unguiculata*)	Gujarat5
HR5	*Enterobacter mori*	Horsegram (*Macrotyloma uniflorum*)	Himala
H14	*Priestia aryabhattai*	Horsegram (*Macrotyloma uniflorum*)	Himala
M25-11	*Lysinibacillus boronitolerans*	Mothbean (*Vigna aconitifolia*)	RMB-25

M816.1	*Bacillus sp.*	Mothbean (*Vigna aconitifolia*)	IPCMO-880

*Identity based on 16S RNA gene sequencing.

6.2.2. Seed preparation

Selected strains were assessed for *in vivo* growth promotion in cowpea (*V. unguiculata*) seeds. Seeds were surface sterilised following a modified method described by Chimwamurombe, Grönemeyer and Reinhold-Hurek, (2016). Seeds were washed twice with sterile distilled water. Thereafter, seeds were incubated in 5% sodium hypochlorite (NaOCl) for 5 minutes before being washed with excess sterile distilled water. Surface sterilised seeds were incubated for 40 minutes in resuspended bacterial cells.

6.2.3. Drought tolerance

Assessment of growth promotion in cowpea seeds was done following modified methods described by Uzma, Iqbal and Hasnain (2022) and Zia *et al*. (2021). Experiments were set up with two control sets: control under normal water without inoculants, and control under stress conditions without inoculants.

Water content was determined following the method described by (Azeem *et al*., 2022). Each pot was filled with approximately 2.9 kg of sandy soil. The weight of watered and dry soil was measured. The control water amount was maintained at 200 mL (100%) and drought stress was induced with 100 mL (50%) water. Pots were watered twice a week for 2 weeks before watering was suspended for 7 days. Trials were performed in triplicates.

Biofertiliser-induced drought stress tolerance in cowpea (*V. unguiculata*) was assessed following the method described by Priya Dharshini *et al*. (2021). Stress tolerance was assessed based on the root and shoot lengths. The germination rate was also assessed from the samples planted. Response indices: root length stress index (RLSI) and shoot length stress index (SLSI), were calculated from the data collected. Response indices of plants under normal conditions were also calculated and compared against stressed plants. These were labelled as root length index (RLI) and shoot length index (SLI).

$$\text{Total germination percentage} = \frac{\text{total germinated seeds}}{\text{total planted seeds}} \times 100$$

$$\text{Root length stress index} = \frac{\text{root length of inoculated plants}}{\text{root length of control plants}} \times 100$$

$$\text{Shoot length stress index} = \frac{\text{shoot length of inoculated plants}}{\text{shoot length of control plants}} \times 100$$

6.2.4. Data analysis

The data obtained were presented as means of the replicates. To determine significance, drought tolerance data sets were analysed using one way ANOVA. This was done using R statistical language version x64 4.1.2 (R Core Team, 2021), tidyverse (Wickham *et al*., 2019), and rstatix (Kassambara, 2022).

6.3. Results

Early growth under drought stress was assessed on *V. unguiculata* grown in pots in a controlled growth chamber. *V. unguiculata* seeds were planted and allowed to grow for 21 days. Thereafter, the number of germinated plants, and root and shoot lengths were documented. Response indices, root length stress index (RLSI) and shoot length stress index (SLSI), were calculated from the data collected. Response indices of plants

under normal conditions were also calculated and compared against stressed plants. These were labelled as root length index (RLI) and shoot length index (SLI).

6.3.1. Germination rate

The average germination rate for samples under drought treatment was 63.1% while samples with normal water conditions were 66.7%. The highest rate was observed in drought-stressed samples inoculated with *S. maltophilia* at 91.7%. The lowest was 41.7% which was observed with samples inoculated with *L. boronitolerans.* The highest germination rate under normal conditions was observed in samples inoculated with *S. maltophilia* and the consortium of isolates. Both had a rate of 83.3%. Table 6.2 presents the germination rates as percentages of total seeds planted.

Table 6.2: Germination rates for the different inoculations under drought stress.

Treatment	**Isolate identity**	**Drought stress (%)**	**No stress* (%)**
Control	No Bioinoculant	75.0	58.3
CRhi15	*Stenotrophomonas maltophilia*	91.7	83.3
HR5	*Enterobacter mori*	50.0	50.0
H14	*Priestia aryabhattai*	75.0	67.0
M25-11	*Lysinibacillus boronitolerans*	41.7	50.0
M8-16.1	*Bacillus sp.*	50.0	75.0
Consortium	All Isolates	58.3	83.3

*Control samples

6.3.2. Drought stress influence on root length

Root length was measured against control plants that had not been inoculated with the selected bacteria. Root length under drought conditions ranged from 5.8 cm to 92.0

cm influenced by *L. boronitolerans* and *Bacillus sp.* respectively. Uninoculated plants had root lengths ranging from 17.0 cm to 46.7 cm. Bioinoculant-treated samples were observed to have thicker roots compared to untreated samples. Samples with average longest roots (*Bacillus sp.* and *S. maltophilia*) are shown in Figure 6.1. Under normal conditions, however, the shortest roots were observed in plants inoculated with a consortium of bacterial isolates (6.3 cm). *Bacillus sp.* inoculated samples had the longest roots in normal conditions (61.7 cm) which, on average, were 23.4% longer than the control. Table 6.3 shows the average root lengths observed in samples.

Figure 6.1: Root length under drought stress of uninoculated samples (control), *Bacillus sp*. (M8-16.1) and *S. maltophilia* (CRhi15).

6.3.3. Drought stress influence on shoot length

Shoot length was observed to range from 12.3 cm (*L. boronitolerans*) to 121.0 cm (*E. mori*) with an average of 64.4 cm under drought stress. Early shoot growth of *V.*

unguiculata under drought stress is shown in Figure 6.2 against untreated samples. Control samples were observed to range from 24.4 cm to 124.1 cm. Under normal conditions, the shortest shoot length was observed on samples inoculated with *S. maltophilia* which was 15.0 cm. This was more than half that of the control shortest shoot length (38.1 cm). However, the longest shoot length was observed in samples inoculated with *L. boronitolerans*. The average dimensions observed are presented in Table 6.3 while Figure 6.3 graphically presents the average root and shoot lengths under both water treatments. The full data set is provided in the supplementary material (Appendix D).

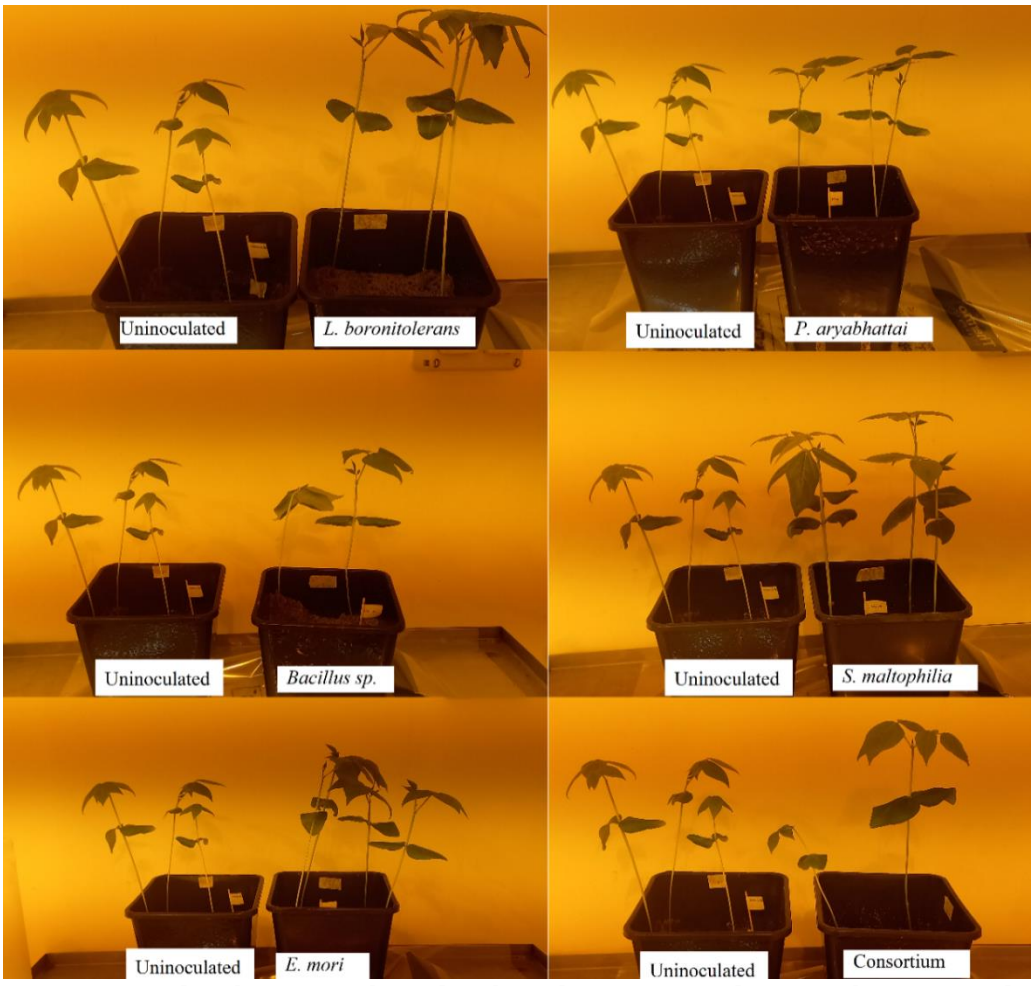

Figure 6.2: Early plant growth under drought stress conditions. Shown are plants inoculated with the bioinoculants with the corresponding uninoculated drought-stressed plants.

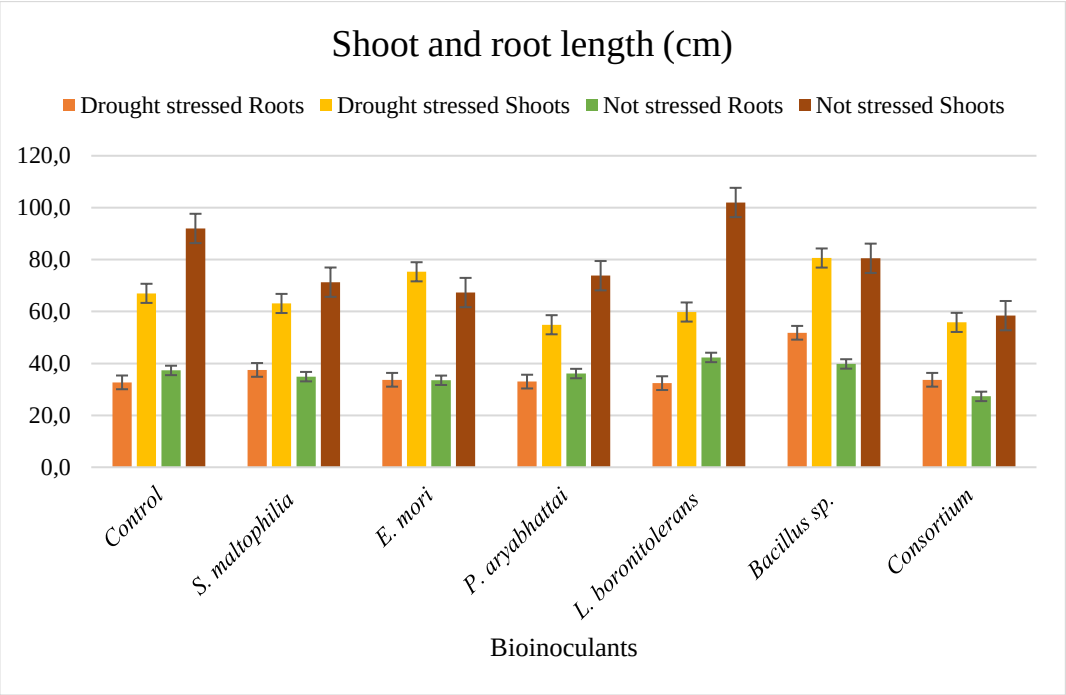

Figure 6.3: Root and shoot lengths of *V. unguiculata* plants under drought stress and normal conditions.

6.3.4. Root and shoot length stress indices

Stress indices, calculated using the previously mentioned equations, are used as measurements for drought tolerance. This was calculated based on root and shoot length against uninoculated samples. The root length stress indices were all above 100% except those inoculated with *L. boronitolerans.* The highest RLSI were observed on drought-stressed samples inoculated with *Bacillus sp.* (158.4%) compared to the uninoculated control samples (Figure 6.4). *Bacillus sp*. also induced the highest SLSI (120.3%). It was observed, however, that drought-stressed plants had higher stress indices compared to the response indices of plants grown under normal conditions (Figure 6.4). This was seen with all bioinoculant treatments except *L. boronitolerans*. The lowest response index was observed on the shoot length of samples inoculated with the consortium of bioinoculants.

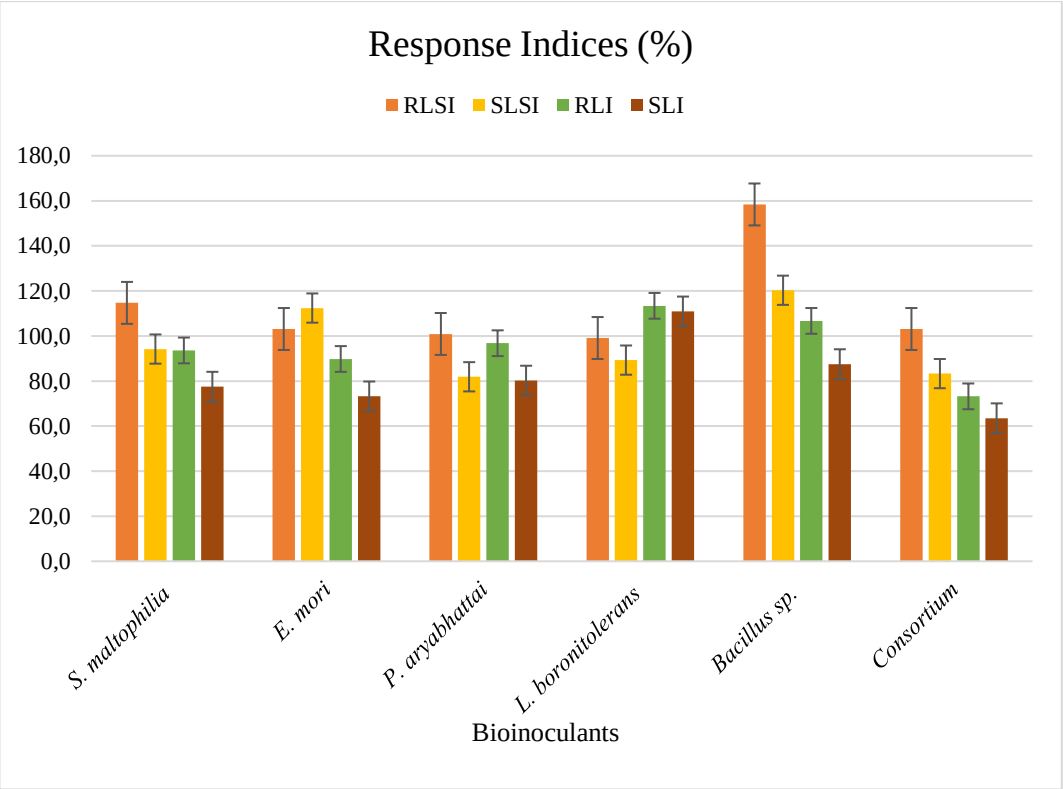

Figure 6.4: Response indices under drought stress and normal water provision. RSLI-Root Length Stress Index; SLSI- Shoot Length Stress Index; RLI-Root Length Index (under normal conditions); SLI- Shoot Length Index (under normal conditions).

The ANOVA result of root length data (under stress conditions) showed that there were no significant differences in the observed responses from all the bioinoculants at 0.05 level. The least significant difference (LSD) test when done also showed no significant differences across the groups except with *Bacillus sp.* Analysis of shoot length (under drought stress) data also revealed no significant difference across groups even after the LSD test. The full one-way ANOVA test results are presented as supplementary material (Appendix D).

Table 6.3: Summary of bioinoculant influence on growth parameters.

		Drought-stressed				No stress			
Isolate ID	**Closest relative**	**Root length (cm)[a]**	**RLSI[1] (%)**	**Shoot length (cm)[b]**	**SLSI[2] (%)**	**Root length (cm)[a]**	**RLSI[1] (%)**	**Shoot length (cm)[b]**	**SLSI[2] (%)**
Control	Uninoculated	32,7	-	67,0	-	37,3	-	92,0	-
CRhi15	*Stenotrophomonas maltophilia*	37,5	114,7%	63,1	94,2%	34,9	93,6%	71,3	77,5%
HR5	*Enterobacter mori*	33,7	103,1%	75,3	112,4%	33,5	89,8%	67,3	73,2%
H14	*Priestia aryabhattai*	33,0	100,9%	54,9	81,9%	36,1	96,8%	73,8	80,2%
M25-11	*Lysinibacillus boronitolerans*	32,4	99,1%	59,8	89,3%	42,3	113,4%	102,0	110,9%
M8-16.1	*Bacillus sp.*	51,8	158,4%	80,6	120,3%	39,8	106,7%	80,5	87,5%
Consortium	All bacterial isolates	33,7	103,1%	55,8	83,3%	27,3	73,2%	58,4	63,5%

[ab] Values show average lengths determined
[1] RLSI- Root Length Stress Index
[2] SLSI- Shoot Length Stress Index

6.4. Discussion

This study, using previously isolated bacteria, intended to assess the ability of five bioinoculants to influence drought stress tolerance and promote early growth. In addition, a co-inoculation assessment was carried out by combining the inoculants to assess them in a consortium. Drought stress was assessed on *V. unguiculata* (cowpea) plants. Growth parameters factored were plant growth across the length of the plant and biomass weight. The main limitation of this study was the inability to fully mimic the natural environment of plants. This is significant because bacteria are known to work in synergy with other microorganisms. As this study was carried out in potted trials, the full interaction of the bioinoculants with environmental microbiomes could not be assessed.

The genus *Stenotrophomonas* is a common plant-associated genus. It has been isolated in root nodules of soybean (*Glycine max*), chickpea (*Cicer arietinum*) and cowpea (*V. unguiculata*) (Abd-Alla *et al*., 2018; Zheng *et al*., 2020). The strain used in this study, however, was obtained from the rhizosphere of *V. unguiculata.* Analysis carried out in this study revealed that under drought stress, plants inoculated with *S. maltophilia* will most likely have deeper roots. Furthermore, though drought stress tolerance was assessed in this study, *S. maltophilia* also improves nutrient availability. It has been suggested that *S. maltophilia* can improve nitrogen availability in plants grown in nitrogen-deficient soils (Alexander *et al*., 2019).

Enterobacter species have been previously identified for their PGP characteristics. *E. mori,* for example, has been observed to positively promote wheat (*Triticum aestivum* L.) growth by lowering ethylene levels (Ludueña *et al*., 2019). The root and shoot length stress indices, however, were both higher under drought dress compared to

under normal conditions. This was an expected result as another species in the *Enterobacter* genus (*E. cloacae*) was observed to induce similar results. *In vitro* experiments found that *E. cloacae* grew well under 40% PEG 6000 and also produced exopolysaccharides essential for drought tolerance (Eke *et al*., 2019). However, PGP activity is well noted to vary across species of the same genus (Ludueña *et al*., 2019). This may explain why samples inoculated with *E. mori* had the second lowest germination rate (50%) despite other species inducing high germination rates (Verma *et al* ., 2017).

Nodule endophytes used in this study include *P. aryabhattai, L. boronitolerans* and *Bacillus sp. P. aryabhattai* is known to possess antimicrobial abilities. Antimicrobial activity has been observed against *Xanthomonas oryzae* and *Rhizoctonia solani* (Nunna & Balachandar, 2022). In addition, it has been observed to promote growth under high salinity (Shahid *et al*., 2022). In our study, however, drought stress was assessed, and samples inoculated with *P. aryabhattai* were observed to increase root length.

L. boronitolerans was noticeably the least active plant growth promoter of the isolates assessed. It had the lowest overall germination rate under both water treatments. This is despite exhibiting *in vitro* plant growth promotion. Furthermore, it was noted that treatments under normal conditions exhibited greater overall growth. As there is limited literature on the assessment of *L. boronitolerans* as a bioinoculant, it may be believed that *in vivo* PGP under drought stress is not often observed. However, as a close *Bacillus* relative, the genus *Lysinibacillus* has been noted to improve plant growth in heavy metal polluted soils (Martínez & Dussán, 2018). In addition, species in this genus have been noted to contain antifungal compounds (Naureen *et al*., 2017).

The *Bacillus* genus is well known to have many PGP species. These include *B. cereus, B. albus* (Ashry *et al*., 2022), *B. tropicus, B. thuringiensis* (Azeem *et al*., 2022) and *B. megaterium* (Khan *et al*., 2020) among many other species. This study observed the isolate identified as *Bacillus sp.* (M8-16.1) from the nodules of *V. aconitifolia* (IPCMO-880) to have the greatest influence on growth under drought conditions. Unclassified *Bacillus spp.* have also been previously observed to improve plant growth by up to 50% (Di Benedetto *et al*., 2019). Other studies have documented chlorophyll content to increase by over 73% and biomass by over 116% (Kifle & Laing, 2016) in *Bacillus sp.* inoculated samples. Furthermore, *Bacillus* species, as rhizobacteria also influence nodulation. This trait is essential for nitrogen fixation (Azizoglu, 2019).

A combination of different bacteria to develop biofertilizers made up of a consortium of different species is known to improve growth. This is because the different isolates with different PGP traits collaborate to improve plant health (Di Benedetto *et al*., 2019). However, biofertilisers developed from multiple species require standardisation as some strains may inhibit the growth of others impeding plant growth (Han *et al*., 2020).

Despite the evidence in this study motivating for these isolates to be used as inoculants, some limitations still exist in the use of biofertilisers in more practical settings. Firstly, their sensitivity and specificity to different temperatures and humidity make storage challenging. As a result, retailers in rural areas are often limited (Yadav & Sarkar, 2018). Furthermore, there is limited data on field trials performed in Namibia with different biofertilisers developed from the host legumes used in this study.

6.5. Conclusion

Given the data presented in this study, *Bacillus sp.*, *S. maltophilia*, and *E. mori* were observed to promote growth under drought stress when applied individually. Therefore, there is compelling evidence to use the present *Bacillus sp.*, *S. maltophilia*, and *E. mori* strains in field trials as biofertilisers. Therefore, it is recommended that these bioinoculants be assessed in the fields in Bagani, Kavango East, Namibia and other arid regions in southern Africa. Furthermore, it is recommended the *Bacillus sp.* isolate be further analysed in genome studies to identify it and further characterise it together with other known isolates.

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CHAPTER 7

7.1. Conclusion

This study presents a comprehensive investigation of the plant growth-promoting (PGP) microbiomes associated with five legume species grown in Namibia. These legumes, *Vigna unguiculata* (cowpea), *V. radiata* (mungbean), *V. aconitifolia* (mothbean), *Macrotyloma uniflorum* (horsegram), and *Lablab purpureus* (dolichos) are arid climate tolerant and have been noted to thrive in such regions. Bacteria from the root nodules, roots, and rhizosphere were isolated, identified, and characterised for their plant growth-promoting traits.

The identified root endophytes and rhizobacteria were found to be from four phyla with known plant growth-promoting bacterial (PGPB) genera. These were Actinomycetota, Bacteroidetes, Firmicutes and Proteobacteria. Root endophytes were observed to be less diverse compared to rhizobacteria. *Streptomyces, Staphylococcus, Flavobacterium, Gryllotalpicola,* and *Paenarthrobacter* were unique to the rhizosphere while *Enterobacter* species were only isolated from the root endosphere of *M. uniflorum.* Nodule endophytes identified were from the Bacillota, Actinomycetota and Proteobacteria phyla. Isolates from the Bacillota phylum were all from the Bacillales order.

This study presented the first account of characterisation of beneficial plant-associated bacteria isolated from these arid climate tolerant legumes grown in Namibia. Potential PGP activity was observed to be higher with root endophytes than with rhizobacteria. Nevertheless, our results suggest that the isolated bacteria from the roots and

rhizosphere of drought tolerant legumes are endowed with diverse PGP and drought-tolerant traits for the facilitation of plant growth in arid environments. This was similarly observed in root nodule isolates, however, none of the isolates were positive for phosphate solubilization. Despite this, characterisation indicated the positive potential of their ability to promote growth in plants particularly with siderophore production and exopolysaccharide production.

The most active PGPB were selected for *in vivo* trials to assess drought tolerance in *V. unguiculata* seeds grown in pots in a growth chamber. *Bacillus sp., Stenotrophomonas maltophilia,* and *Enterobacter mori* were observed to promote growth under drought stress when applied individually. Therefore, there is compelling evidence to proceed with the *Bacillus sp., S. maltophilia,* and *E. mori* strains in field trials as biofertilisers.

This study also presented an overview of the bacterial microbiomes of the root, seed, and nodule endospheres, and the rhizosphere. Analysis of the roots, seeds and rhizosphere identified several important potential PGPB phyla from all the samples. These include Actinobacteria (Actinomycetota), Bacteroidota, Firmicutes and Proteobacteria. Within these groups diazotrophic genera were also identified. Recruitment was found to be selective for bacteria known to promote plant growth. These include *Rhizobium, Bradyrhizobium, Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium, Pseudomonas* and *Bacillus*. Nodule microbiome analysis revealed the genus *Bradyrhizobium* as the most abundant genus in *V. aconitifolia, V. unguiculata, L. purpureus* and *M. uniflorum.* Metagenomic analyses of plant-associated microbiomes of these drought tolerant legumes growing in Namibia had not been done previously.

These legumes, grown in poor sandy soils of Bagani, Kavango East were found to actively recruit PGPB which may be useful for plant stress-tolerance. Both culture-dependent and independent methods revealed that bacteria associated with these legumes are from known PGP genera like *Stenotrophomonas, Bradyrhizobium* and *Bacillus*. Culture dependent methods revealed positive activity of the bacteria *in vitro* that promote nutrient availability (siderophore production, phosphate solubilisation, and nitrogen fixation), drought stress tolerance (IAA production and exopolysaccharide production), and disease tolerance (antifungal activity against *F. graminearum*).

7.2. Recommendations

Given these observations, it is recommended that isolates CRhi15 (*Stenotrophomonas maltophilia*) from *V. unguiculata*, HR5 (*Enterobacter mori*) from *M. uniflorum,* and M8-16.1 (*Bacillus sp.*,) from *V. aconitifolia* (accession IPCMO-880) be assessed for their PGP abilities in field trials in Bagani, Kavango East, Namibia with eventual development into commercial biofertilisers. Further recommendations are made for the isolates DR14, DRhi9 from *L. purpureus*, CR10, CRhi10, CA9 from *V. unguiculata,* MBR9, MBRhi17, MB1, MB3.1 from *V. radiata,* HR5, HRhi12, HRhi18, H14 from *M. uniflorum,* RMBR1, RMBRhi1, M25-11 from *V. aconitifolia* accession RMB-25, and IPCR2, IPCR4, M8-16.1 and M8-16.2 from *V. aconitifolia* accession IPCMO-880 to be assessed for PGP under different biotic and abiotic stresses in other arid regions in southern Africa. It is also recommended that *Bradyrhizobium* species be isolated from nodules using selective media for biofertiliser use. Furthermore, it is recommended that the *Bacillus sp.* (M8-16.1) isolate be further analysed in genome studies to identify it and further characterise it together with other known isolates.

APPENDICES

Appendix A

Supplementary material A.1: Ethical clearance

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UNIVERSITY OF NAMIBIA

ETHICAL CLEARANCE CERTIFICATE

Ethical Clearance Reference Number: SOS-0044 Date: 04 March 2022

This Ethical Clearance Certificate is issued by the University of Namibia Ethics Committee (REC) in accordance with the University of Namibia's Research Ethics Policy and Guidelines. Ethical approval is given in respect of undertakings contained in the Research Project outlined below. This Certificate is issued on the recommendations of the ethical evaluation done by the ethics committee.

Title of Project: CHARACTERISATION AND ISOLATION OF BENEFICIAL BACTERIA IN SELECTED ARID CLIMATE TOLERANT LEGUMES FOR USE IN COMMON LEGUMES IN NAMIBIA

Student: PAIDAMOYO MATARANYIKA

Student Number: 201306809

Supervisor(s): DR. JEAN UZABAKIRIHO; PROF. PERCY CHIMWAMUROMBE

Centre for Research Services

Take note of the following:

1. Any significant changes in the conditions or undertakings outlined in the approved Proposal must be communicated to the ethics committee. An application to make amendments may be necessary.
2. Any breaches of ethical undertakings or practices that have an impact on ethical conduct of the research must be reported to the ethics committee
3. The Principal Researcher must report issues of ethical compliance to the ethics committee (through the Chairperson) at the end of the Project or as may be requested by the ethics committee
4. The ethics committee retains the right to:

i) Withdraw or amend this Ethical Clearance if any unethical practices (as outlined in the Research Ethics Policy) have been detected or suspected,

ii) Request for an ethical compliance report at any point during the course of the research.

The ethics committee wishes you the best in your research.

Dr. Zivayi Chiguvare (Chairperson Ethics Committee)

Prof. Davis Mumbengegwi (Head, Multidisciplinary Research)

Supplementary material A.2: Declaration of Originality

DECLARATION OF ORIGINALITY

This form shall be completed by students for every registered course accompanying every assignment or research project submitted for grading.

Student Name	Paidamoyo Natasha Mataranyika	**Student Number**	201306809
Title of Module/ Course	DOCTOR OF PHILOSOPHY DISSERTATION (MICROBIOLOGY)	**Module Code**	BIO6100
Academic Year	2022	**Semester**	Semester 2
UNAM Campus	Main Campus	**School**	School of Science
Topic of Work	CHARACTERISATION OF BACTERIAL SYMBIONTS IN SELECTED DROUGHT-TOLERANT LEGUMES FOR BIOFERTILISERS DEVELOPMENT FOR USE IN NAMIBIA		
Type of Work	Dissertation		

I declare herewith that this above-mentioned work is my own original work.

Furthermore, I confirm that:

1. this work has been composed by me without assistance
2. I have clearly referenced in accordance with departmental requirements, in both the text and the bibliography or references, all sources (either from a printed source, internet or any other source) used in the work
3. all data and findings in the work have not been falsified or embellished
4. this work has not been previously, or concurrently, used either for other courses or within other exam processes as an exam work
5. this work has not been published.

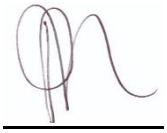
I acknowledge that any false claim in respect of this work will result in disciplinary action in

accordance with the University regulations.

I confirm that I understand that my work may be electronically checked for plagiarism by the

use of plagiarism detection software and stored on a third party's server for eventual future.

comparison.

Student signature:  Date: 29 November 2022

Place: Windhoek, Namibia

Appendix B

Supplementary material B1

Table S1: Sequence accession numbers obtained from NCBI submission for root and rhizosphere isolates.

Sample ID	Submitted sequence ID	Accession
MBRhi17	FIS761_36067610_36067610/1-875	ON454251
HR11new2	FIS771_36067719_36067719/1-901	ON454252
MBRhi20	FIS760_36067603_36067603/1-901	ON454253
DRhi25n	FIS770_36067702_36067702/1-901	ON454254
DR14	GEK974_41709741_41709741	OP564985
MBRhi3	FIS764_36067641_36067641/1-958	ON454256
DRhi1	EYC154_33301540_33301540/1-903	ON454259
DR10	EYC152_33301526_33301526/1-898	ON454260
CR10	EYC159_33301595_33301595/1-901	ON454262
CRhi15	EYC164_33301649_33301649/1-1511	ON454265
RMBRhi4	EYC170_33301700_33301700/1-876	ON454266

RMBRhi1	EYC169_33301694_33301694/1-945	ON454267
RMBRhi6	EYC171_33301717_33301717/1-842	ON454268
HR7	EYC180_33301809_33301809/1-901	ON454269
IPCRhi1	EYC190_33301908_33301908/1-949	ON454270
HRhi1	EYC181_33301816_33301816/1-877	ON454271
MBR14	EYC196_33301960_33301960/1-907	ON454273
HRhi4	EYC182_33301823_33301823/1-882	ON454274
HRhi5	EYC183_33301830_33301830/1-878	ON454276
IPCR4	EYC193_33301939_33301939/1-896	ON454277
MBRhi14	FIS762_36067627_36067627/1-893	ON454278
MBRhi10	FIS763_36067634_36067634/1-894	ON454279
DRhi9	FIS767_36067672_36067672/1-902	ON454282
CR22	EYC161_33301618_33301618/1-1498	OP503858
CRhi10	EYC165_33301656_33301656/1-873	OP503859
CRhi18	EYC163_33301632_33301632/1-1233	OP503860
HR4	EYC178_33301786_33301786/1-898	OP503861
HR5	FXR167_39951671	OP503856
HR6	EYC179_33301793_33301793/1-1521	OP503862
HRhi12	EYC184_33301847_33301847/1-1597	OP503863
HRhi18	EYC185_33301854_33301854/1-1510	OP503864
MBR1	EYC200_33302004_33302004/1-1086	OP503865
MBR9	EYC197_33301977_33301977/1-1238	OP503866
IPCR2	FXR166_39951664	OP503857
IPCRhi18	EYC186_33301861_33301861/1-896	OP503867

RMBR1	EYC175_33301755_33301755/1-907	OP503868
RMBR7	EYC177_33301779_33301779/1-1468	OP503869
RMBRhi17	EYC174_33301748_33301748/1-899	OP503870

Appendix C

Supplementary material C1

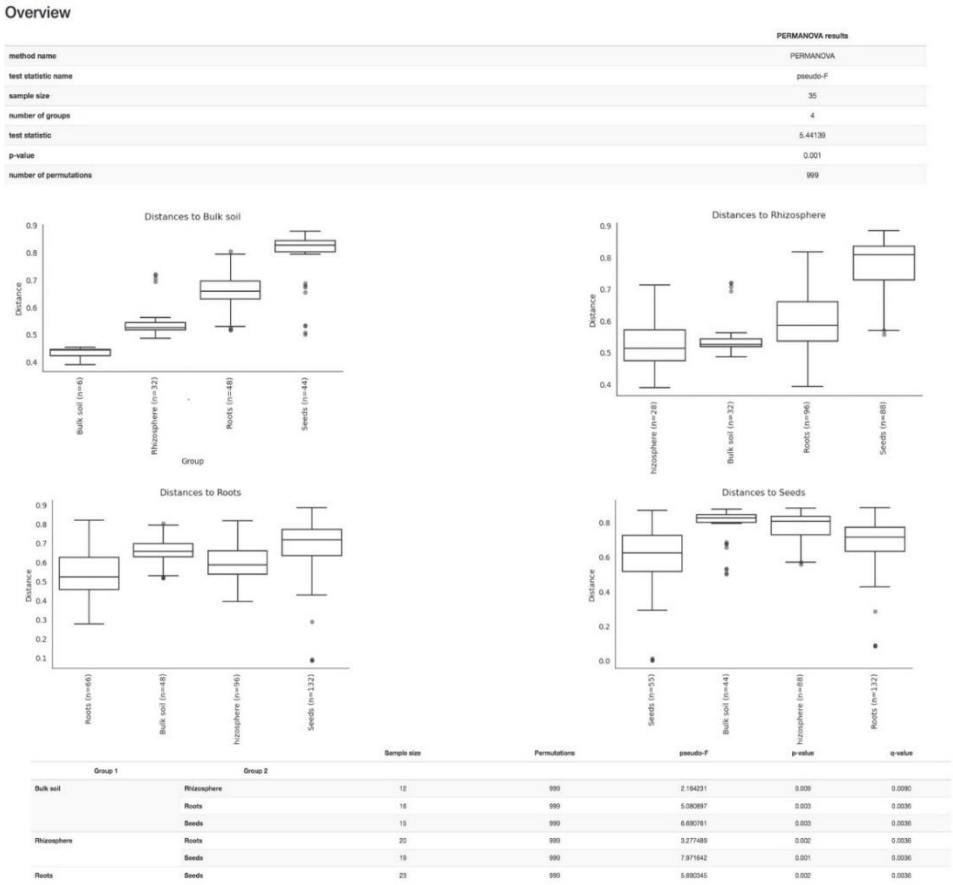


Figure S1: Unweighted UniFrac distances comparing data obtained from bulk soil, rhizosphere, roots, and seeds.

Supplementary material C2

Table S2: Sequence accession numbers from NCBI submission for each sample set.

Accession	Legume species	Site	SPUID
SAMN28085006	*V. unguiculata*	Seed	CS1

SAMN28085007	*V. unguiculata*	Rhizosphere	Crhi
SAMN28085008	*V. unguiculata*	Root	CowRT1
SAMN28085009	*V. unguiculata*	Root	CRo2
SAMN28085010	*V. unguiculata*	Root	CRTS3
SAMN28085011	-	Bulk soil	CBu1
SAMN28085012	-	Bulk soil	CB So2
SAMN28085013	*V. radiata*	Seed	MuBS1
SAMN28085014	*V. radiata*	Seed	MBSS2
SAMN28085015	*V. radiata*	Rhizosphere	MBRhi1
SAMN28085016	*V. radiata*	Rhizosphere	MnBRh
SAMN28085017	*V. radiata*	Root	MBRoot1
SAMN28085018	*V. radiata*	Root	MBRs2
SAMN28085019	-	Bulk soil	MBBulksoil1
SAMN28085020	*M. uniflorum*	Seed	HGS1
SAMN28085021	*M. uniflorum*	Seed	HG2
SAMN28085022	*M. uniflorum*	Rhizosphere	HRhi1
SAMN28085023	*M. uniflorum*	Root	HRoot1
SAMN28085024	*M. uniflorum*	Root	HRs2
SAMN28085025	*L. purpureus*	Seed	DS1
SAMN28085026	*L. purpureus*	Seed	DBSeed2
SAMN28085027	*L. purpureus*	Rhizosphere	DolRhi1
SAMN28085028	*L. purpureus*	Root	DolichosR1
SAMN28085029	*L. purpureus*	Root	DRs2
SAMN28085030	-	Bulk soil	DBeanBS1

SAMN28085031	*V. aconitifolia*	Seed	IPC880S1
SAMN28085032	*V. aconitifolia*	Seed	IPCSS2
SAMN28085033	*V. aconitifolia*	Rhizosphere	IPCRhi1
SAMN28085034	*V. aconitifolia*	Rhizosphere	IP8C8R0h2
SAMN28085035	*V. aconitifolia*	Root	IPCR1
SAMN28085036	*V. aconitifolia*	Root	880IPCRt2
SAMN28085037	*V. aconitifolia*	Seed	RMothBS1
SAMN28085038	*V. aconitifolia*	Seed	RMBean 25SS2
SAMN28085039	*V. aconitifolia*	Rhizosphere	RMBRhizosphere1
SAMN28085040	*V. aconitifolia*	Rhizosphere	RMBRh2
SAMN28085041	*V. aconitifolia*	Root	RMBRoots1

Appendix D

Supplementary material D1

Table S3: Sequence accession numbers obtained from NCBI submission for root nodule isolates.

Sample ID	Submitted Sequence ID	Accession
C1	FXR170_39951701_39951701	OP623474
C11	FXR258_39952586_39952586	OP623457
C3	FXR171_39951718_39951718	OP623475
C5	FXR022_39950223	OP623456
C6	FXR169_39951695_39951695	OP623476
CA9	FXR191_39951916_39951916	OP623477
D10	FXR024_39950247	OP623458
D14	FXR173_39951732_39951732	OP623479

D17 1	FXR177_39951770_39951770	OP623480
D5	FXR172_39951725_39951725	OP623478
D7	GEK972_41709727_41709727	OP623488
H13	FXR049_39950490	OP623460
H14	FXR182_39951824_39951824	OP623481
H4	FXR051_39950513	OP623459
M25 10	FXR179_39951794_39951794	OP623493
M25 11	FXR180_39951800_39951800	OP623492
M25 12	FXR041_39950414	OP623466
M25 13	FXR040_39950407	OP623467
M25 16	FXR043_39950438	OP623468
M25 17	FXR175_39951756_39951756	OP623490
M25 3	FXR047_39950476	OP623464
M25 5	FXR045_39950452	OP623465
M25 6	GEK976_41709765_41709765	OP623489
M25 7	FXR183_39951831_39951831	OP623494
M8 13	FXR189_39951893_39951893	OP623484
M8 14	FXR185_39951855_39951855	OP623485
M8 16 1	FXR188_39951886_39951886	OP623486
M8 16 2	FXR187_39951879_39951879	OP623487
M8 2	FXR052_39950520	OP623461
M8 21	FXR056_39950568	OP623463
M8 6	FXR054_39950544	OP623462
M8 8	FXR186_39951862_39951862	OP623482

M8 9	FXR184_39951848_39951848	OP623483
MB1	FXR034_39950346	OP623469
MB11	FXR036_39950360	OP623471
MB14	FXR035_39950353	OP623472
MB16	FXR033_39950339	OP623473
MB17	FXR176_39951763_39951763	OP623491
MB8	FXR064_39950643	OP623470

Supplementary material D2

Table S4: Assigned accession numbers for sequence reads from NCBI submission (BioProject-PRJNA896769).

Accession	**SPUID**	**Organism**	**Tax ID**
SAMN31566282	D1	*Lablab purpureus*	1297885
SAMN31566283	D2	*Lablab purpureus*	1297885
SAMN31566284	D3	*Lablab purpureus*	1297885
SAMN31566285	C1	*Vigna unguiculata*	1297885
SAMN31566286	C2	*Vigna unguiculata*	1297885
SAMN31566287	C3	*Vigna unguiculata*	1297885
SAMN31566288	H1	*Macrotyloma uniflorum*	1297885
SAMN31566289	H2	*Macrotyloma uniflorum*	1297885
SAMN31566290	H3	*Macrotyloma uniflorum*	1297885
SAMN31566291	M8-2	*Vigna aconitifolia*	1297885
SAMN31566292	M8-3	*Vigna aconitifolia*	1297885

Appendix E

Supplementary material E1

Analysis of variance results: Root length

Table S5: Analysis of variance table - response: Root length.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
BC	6	1838.3	306.38	1.8236	0.1153
Residuals	46	7728.6	168.01		

Table S6: LSD t-test for root length.

	RL	std	R	LCL	UCL	Min	Max
Bacillus sp.	51.81667	29.355300	6	41.16502	62.46831	22.9	92.0
Consortium	33.70000	8.156592	7	23.83849	43.56151	19.5	42.7
Control	32.68889	8.439704	9	23.99186	41.38592	17.0	46.7
Enterobacter mori	33.68333	8.906271	6	23.03169	44.33498	25.5	47.0
Lysinibacillus boronitolerans	32.38000	15.342001	5	20.71171	44.04829	5.8	44.5
Priestia aryabhattai	33.04444	7.909347	9	24.34741	41.74148	23.9	42.8
Stenotrophomonas maltophilia	37.54545	7.825134	11	29.67869	45.41222	21.1	52.0

LSD- least significant difference
Mean square error: 168.0127, BC, means and individual (95%) CI
Alpha: 0.05; DF error: 46
Critical value of t: 2.012896

Table S7: Groups according to probability of means differences and alpha level (0.05).

	RL	**Groups***
Bacillus sp.	51.81667	A
Stenotrophomonas maltophilia	37.54545	B
Consortium	33.70000	B
Enterobacter mori	33.68333	B
Priestia aryabhattai	33.04444	B
Control	32.68889	b
Lysinibacillus boronitolerans	32.38000	b

*Treatments with the same letter are not significantly different.

Supplementary material E2

Analysis of variance results: Shoot length

Table S8: Analysis of variance table- response: Shoot length.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
BC	6	3799	633.16	1.017	0.4262
Residuals	46	28638	622.57		

Table S9: LSD t-test for shoot length.

	SL	**std**	**r**	**LCL**	**UCL**	**Min**	**Max**
Bacillus sp.	80.61667	25.74113	6	60.11258	101.12075	50.9	109.5
Consortium	55.78571	20.58943	7	36.80262	74.76881	33.2	91.0
Control	66.95556	31.20012	9	50.21404	83.69707	24.4	124.1
Enterobacter mori	75.25000	25.15693	6	54.74591	95.75409	45.4	121.0
Lysinibacillus boronitolerans	59.80000	27.24986	5	37.33890	82.26110	12.3	80.8

Priestia aryabhattai	54.90000	20.52791	9	38.15848	71.64152	27.3	100.9
Stenotrophomonas maltophilia	63.10000	23.42738	11	47.95673	78.24327	26.3	110.4

LSD- Least significant difference
Mean square error: 622.572/BC, means and individual (95%) CI
Alpha: 0.05; DF error: 46
Critical value of t: 2.012896

Table S10: Groups according to probability of means differences and alpha level (0.05).

	SL	**Groups***
Bacillus sp.	80.61667	a
Enterobacter mori	75.25000	a
Control	66.95556	a
Stenotrophomonas maltophilia	63.10000	a
Lysinibacillus boronitolerans	59.80000	a
Consortium	55.78571	a
Priestia aryabhattai	54.90000	a

*Treatments with the same letter are not significantly different.