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Symbiotic nitrogen fixation efficiency of native rhizobia in selected South African legume crops

BY

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DECLARATION

I Khumbudzo Ndhlovu hereby declare that this MSc thesis entitled “Symbiotic nitrogen fixation efficiency of native alpha and beta rhizobia on selected important legumes in South Africa” is submitted in partial fulfilment of the requirement for the degree MSc Microbiology at the University of Pretoria and is my own work, except where duly acknowledged. I also certify that no plagiarism was committed in writing this thesis.

Signature _____

Date _____

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Abstract

This Master's dissertation reports about the screening and characterization of selected alpha and beta rhizobial isolates from wild legumes in South Africa for their nodulation and nitrogen fixation properties on the cultivated legumes lucern (*Medicago sativa* L), cowpea (*Vigna unguiculata* L) and siratro (*Microptilium atropurpeum D.C*) under glasshouse and field conditions. The rhizobia were initially *in-vitro* characterized for their tolerance to various abiotic stresses and most of the strains were found to be tolerant to extremes of environmental factors such as acidity, aluminium toxicity, salinity and temperature. They were then screened for nodulation and nitrogen fixation efficacy under glasshouse and field conditions. Additional *in-vitro* screening for essential plant growth promoting traits including the production of siderophores, indole acetic acid, ACC-deaminase and phosphate solubilization was conducted. Most of the isolates from the wild legumes, i.e., 7 strains (6 *Bradyrhizobium* and 1 *Paraburkholderia* nodulated cowpea, 1 *Bradyrhizobium* strain nodulated lucerne and 13 strains (3 *Paraburkholderia* and 10 *Bradyrhizobium* strains) nodulated siratro, in the glasshouse experiment with a statistically significant number of nodules ($p > 0.05$). Plant biomass, including fresh weight and dry weight, were significantly improved by *Bradyrhizobium* strains 10BB and Arg68 in cowpea and siratro compared to un-inoculated controls. Five strains for cowpea, six strains for siratro and one strain for lucerne were selected as the best strains for field trial. After harvest, cowpea plant biomass were significantly increased when inoculated with *Bradyrhizobium* sp. Arg68 followed by *Paraburkholderia* sp. KB15 with significant increase in the amount of fixed nitrogen. There was significant difference in the amount of nitrogen fixed when inoculated with different strains of rhizobia. In siratro, plant biomass was increased after inoculation with *Bradyrhizobium* sp. Fp1c strain followed by *Bradyrhizobium* sp. 10BB although the amount of nitrogen fixed had significant different and same applies to lucerne with no nodules formed on control plant. All of the *Bradyrhizobium* strains tested positive for the presence of *nifH* gene while *Bradyrhizobium* strains Arg68 and Arg62 strains contained the *nodC* genes. The study has generated important baseline data, which can be used for further development of the rhizobial strains as legume inoculants for cowpea, siratro and lucerne, but warrants further nodulation screening study in these

and other legumes of similar cross inoculation groups with cowpea, lucerne and siratro.

Keywords: Legume, Nodulation, nitrogen fixation, environmental stress, *Bradyrhizobium*, *Paraburkholderia* and PGPR

Table of content

List of figures

List of tables

List of abbreviations

%NDFA	Nitrogen derived from the atmosphere
µ	Micro
ACC	1-aminocyclopropane-1-carboxylate
ADP	Adenosine di phosphate
Al	Aluminium
ANOVA	Analysis of variance
ARC	Agricultural Research Council
ATP	Adenosine tri phosphate
BNF	Biological Nitrogen Fixation
BTB	Bromothymol Blue
Ca	Calcium
CAS	Chromo Azurol Sulfonate
CEC	Cation Exchange Capacity
$CuCl_2$	Copper chloride
DNA	Deoxyribonucleic Acid
EDTA	Ethelenediamine tetra-acetic acid
Et al	others
Fe^{3+}	Iron
$FeCl_3.6H_2O$	Iron Chloride Hexahydrate
g	gram
G	Guanine
GLM	General Linear Model
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric Acid
HDTMA	Hexadecyltrimethyl Ammonium Bromide
IAA	Indole Acetic Acid

ISCW	Institute of Soil, Climate and Water
K_2SO_4	Potassium Sulphate
KH_2PO_4	Potassium Phosphate Monobasic
L	Litre
LSD	Least Significance Difference
Mg	Magnesium
$MgSO_4$	Magnesium Sulphate
$MnCl_2.4H_2O$	Manganese Chloride
N	Nitrogen
NaCl	Sodium Chloride
$NaMO_4.2H_2O$	Sodium Molybdate
NaoH	Sodium Hydroxide
NB	Nutrient Broth
NH_3Cl	Ammonium Chloride
NN	Nodule Number
NO	Nitric oxide
Nod	N-acetylglucosaminyl transferase
O-F	oxidative- fermentation
P	Phosphorus
PCR	Polymerase Chain Reaction
PGPR	Plant Growth Promoting Rhizobacteria
PH	Potential of Hydrogen
PVK	Pikovskaya
RMM	Rhizobium Minimal Medium
S	Sulphur
SARCC	South African Rhizobium Culture Collection
SAS	Statistical software

SDW	Shoot Dry Weight
SFW	Shoot Fresh Weight
T	Thiamine
TDW	Total Dry Weight
TFW	Total Fresh Weight
TY	Triptone
W/V	Weight Per Volume
WDCM	World Data Centre for Microorganisms
YMA	Yeast Mannitol Agar
YMCR	Yeast Mannitol Congor Red
$ZnSO_4$	Zinc Sulphate
α	Alpha
β	Beta
$\delta^{15}N$	delta N

CHAPTER 1

BACKGROUND AND JUSTIFICATION OF THE STUDY

1.1. Introduction

The ever-increasing world population has brought many challenges in agriculture and food security that prompted researchers to investigate research strategies for increasing food and forage supply. In addition to the current climate change and the challenges in soil micro- and macro- nutrient availability, farmers have to deal with several other limiting factors in the soil that hamper crop production.

Nitrogen (N) is one of the essential elements and an important limiting factor in agricultural production systems globally. Although 78% of atmospheric air is nitrogen (in the form of dinitrogen), it is usually unavailable to plants due to the strong covalent bonds between the two nitrogen atoms (N≡N). Plants therefore require mineral nitrogen. Transformation of atmospheric nitrogen through the process of the nitrogen cycle contributes about 3 x 10^9 million tonnes per year on a global basis. On the other hand, nitrogen can be fixed industrially using the Haber Bosch process, which contributes about 100 million tonnes of fixed nitrogen annually to fulfil the requirement of agricultural plants (Jenkinson 2001). Production of artificial nitrogen fertilizers using this method, and its continuous application, has negative and unpredicted impacts on the environment. This is because the consumption of fossil fuel during production of these fertilizers results in high amounts of carbon dioxide released into the atmosphere. Nitrogen-containing by-products of these fertilizers may also leach into ground water. This ultimately causes the contamination of soil, water and natural areas, thus posing a threat to animal and human health (Vitousek 1997). The use of chemical fertilizers is predicted to increase further in the future due to an increasing demand by farmers for high yield, increased profit and to meet the demand for food around the world (Subba-Rao 1980; Reece et al., 1985).

Legumes, which belong to the family Fabaceae constitute about 19, 000 species including the agriculturally important food and forage plants such as *Glycine max* (soybean), *Vigna unguiculata* L (cowpea), *Phaseolus vulgaris* (beans), *Pisum sativum* (pea), *Cicer arietinum* (chickpeas), *Medicago sativa* (alfalfa), *Arachis hypogaea* (peanut), *Macroptilium atropurpureum* DC (siratro) and white clover (*Trifolium repens L*). The cultivation of these legumes by the application of bio-fertilizers, formulated products of microbial inoculants such as rhizobia reduces the use of chemical fertilizers that not only

pollute the environment (Ghimire 2002) but also affect soil health. These natural bio-fertilizers are inexpensive sources for sustainable growth of legumes. Therefore, inoculation of legumes with rhizobial strains is an important strategy for increasing plant production. During harvesting, the plants release nitrogen back into the soil that may be utilized by other crops and enhances the fertility of the soil. Generally, the rhizobium-legume symbiosis enhances and ensures sustainable agriculture by improving legume productivity and also reducing the use of chemical fertilizers that cause various adverse effect on the soil.

In order to fulfil this requirement and ensure sustainable food security globally, there is an increasing tendency of adopting legume cultivation, as these plants have the unique ability to reduce the use of chemical fertilizers while benefiting the environment. Legumes are widely distributed in nature, particularly in the tropics, thus contributing to the development of food and other agricultural products, as well as preventing soil erosion and minimizing environmental stresses. Wild legumes interact with microorganisms and develop an important function in the ecosystem and derive high percentages of nitrogen that are comparable to cultivated legumes such as forage and grain legumes like *M. sativa* and *T. repens* (Lindstrom et al., 2010). In addition, wild legumes can typically withstand several environmental conditions including drought, salinity, temperatures as well as aluminium toxicity. Also, the rhizobia isolated from wild legumes are not only capable of nodulating cultivated legumes, they also have the ability to survive under different conditions and therefore have huge application in agriculture (Zahran 1999).

The rhizobium-legume symbiosis contributes to the soil quality by increasing nitrogen availability and thus crop productivity (Peoples et al., 1995). However, the symbiosis typically faces environmental stresses, which affects the physiology of both partners. Like the plant partner, rhizobia also differ greatly regarding their tolerance to abiotic stress (Zahran 1999). Therefore, there is a need for screening and identifying wild strains of rhizobia tolerant to these stresses. The use of such strains as inoculants of agriculturally important legumes will likely increase crop yields and while at the same time improving soil health.

1.2 Significance of legume crops in agriculture

Grain legumes are second after cereals in yielding protein-enriched seeds, which are consumed as nutritionally healthy food. In this research, focus was on cowpea, selected

as one of the most economically important grain legumes for small-scale farmers and households in South Africa. Cowpea is commonly used as food and forage legume in semi-arid and tropical regions and is cultivated throughout the world. In addition to being a food and forage legume, it also improves soil fertility. In South Africa, there is no sufficient data indicating the production of cowpea and quantity produced and the area of production (DAFF 2009; 2014), because it is only cultivated for local household. However, the production of cowpea is not high as compared to other staples (e.g. cereals, wheat and sorghum) in South Africa due to lack of economic support from government and the agricultural industry. As a result, cowpea still needs a lot of attention in terms of attaining high yield and reducing constraints in its production (Asiwe 2009). If the yield is to be improved, more research is needed to find the most well adapted varieties and inoculants that would be useful for production.

Apart from other forage legumes, lucerne and siratro are also agriculturally important legumes, and they are primarily used as feed for high producing dairy cattle (Bulang et al., 2006). Lucerne, which is regarded as an invasive species (Cook et al., 2005), was brought to South Africa from France around 1850, and it is referred to as the king of hay (Ehsanpour and Razavizaden 2005). Both lucerne and siratro are easy to grow and because of their long taproots, they are very tolerant to abiotic stress because they can access nutrient and water easily in the soil. Lucerne can remain close to 4-5 years in the soil and can be harvested 3-4 times per year (Rafinska et al., 2016). As food for livestock, lucerne has contributed a lot to the production of high producing dairy cattle since it was planted in South Africa as compared to siratro. Besides its contribution to the cattle production, lucerne has high financial profit and wide adaptability (Cook et al., 2005). Lucerne can be stored for very long periods and used during dry or winter seasons. There is no data recorded in South Africa for the production of siratro. Both the species are known to improve soil quality and reducing soil erosion.

Generally, in South Africa, inoculation with different strains has been conducted in the past, but greater emphasis has been given mostly to certain economically important legumes such as soybean. Some of these inoculations resulted in failure to nodulate the legume species (Bloem 1998), while some have been shown to nodulate non-target species. Furthermore, there is also not enough information to farmers because most are still using chemical fertilizers. The use of inoculant technology needs broad-scale introduction to small-scale farmers, and in doing so increase crop productivity during harsh environmental conditions.

1.3 Problem statement

Most cultivated legumes are sensitive to environmental changes, including various abiotic stresses that negatively affect their rhizobial symbionts, and hence their symbiotic performance. Native rhizobia that have a symbiotic association with different wild legumes, on the other hand, may be able to adapt more readily to such changing environmental conditions and may have the capacity to tolerate various abiotic stresses. One important strategy in the search for highly effective nitrogen fixing rhizobia for use in agriculture is to isolate native rhizobia from wild legumes and screen them for their symbiotic performance in economically important food and/or forage legumes.

1.4. Aim of the study

The major aim of this study was to evaluate the efficacy of native strains of α and β-rhizobia (obtained from the root nodules of wild legumes) on nodulation and nitrogen fixation in selected economically important legumes including lucerne, siratro and cowpea.

1.5. Objectives

1) To characterize native α- and β-rhizobial isolates of wild legumes for their tolerance against various abiotic stresses, including extremes of pH, acidity, salinity, aluminium toxicity and temperature.
2) To characterize these isolates for their plant growth promoting traits.
3) To screen these isolates for their nodulation and nitrogen fixation efficiency in cowpea, lucerne and siratro under glasshouse conditions.
4) To screen these isolates for their nodulation and nitrogen fixation efficiency in cowpea, lucerne and siratro under field conditions.

1.6. Reference

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Nitrogen (N) is one of the essential nutrient elements required by all cellular organisms for growth. Plants and microorganisms require nitrogen in large quantities as it is a major component for proteins, nucleic acids and other cellular constituents (Novoa and Loomis 1981). However, due to the three covalent bonds between two N atoms in dinitrogen (N_2) plants cannot easily utilize it directly from the atmosphere. Dinitrogen can be converted in many ways such as lightning, biological nitrogen fixation (BNF) and the Haber borsch process where the end-product can be useful for plant growth. Shortage of fixed nitrogen in the soil typically manifests in plants where their leaves turn yellow, while branching and growth may also be reduced (Wolfe et al., 1988; Ougham et al., 2005; Boddey et al., 1997).

Legumes constitute the major group of plants that are prominent due to their nitrogen fixation ability in association with soil microbes and are the most diverse flowering plants that produce simple dry fruits from their seedpod (LPWG, 2013). They are adapted to various ecosystems such as terrestrial, dessert and tropical rain forest ecosystems (Doyle and Luckow 2003; Lavin et al., 2005; Lewis et al., 2005). Legumes which generally include grain and forage legumes as well as forest trees are mostly cultivated for human and animal consumption in rural areas as they are rich in protein, carbohydrates and minerals. Legumes are also used as medicines, oil productions, timber species and firebreaks as well as soil enhancing green manure (Lewis et al., 2005). In total, cultivated legumes in arable lands contribute about 25% of crop production in the world (Kinkema et al., 2006).

A group of bacteria capable of fixing atmospheric nitrogen in a symbiotic association with several species of legumes are referred to as rhizobia. Beijerinck (1888) originally isolated the first members of this group. Rhizobia are non-spore forming gram- negative bacteria that are widely distributed across diverse ecosystems (Abd-alla et al., 2016). They are phylogenetically diverse with a range of different physiological properties amongst them (Shin et al., 2016). All produce the enzyme nitrogenase that catalyses in the conversion of atmospheric nitrogen into ammonia (Kaminski et al., 1998).

Plant roots exudate certain flavonoids that attract the rhizobia and result in the production of lipochitooligosaccharides, signalling molecules known as Nod factors by rhizobia (Kim and Rees 1994). These flavonoids and Nod factors provide a means of communication between the legume and the bacterium, which is crucial for the process of nodule formation. However, legumes differ in the flavonoids they secrete and the Nod factor (produced by the products of the common nodulation genes *nodABC*) of rhizobia also differ. Particular legumes typically only interact with particular rhizobia. For the latter, specificity for particular legumes is conferred by the so-called host specificity genes, of which more than 30 are already known to science (Perret et al., 2000). Once the two symbiotic partners have recognized each other, the bacterium will enter into the root cells, which is followed by rapid division of the host cells to form nodules that are highly specialized plant organs (Dudley et al., 1987). Inside the nodules, the rhizobia covert atmospheric nitrogen into ammonium, which is a form that is usable by the plants for the synthesis of amino acids and nucleotides, while the plants, in turn, provide the bacteria with sugars in the form of photosynthate (Glick 2003).

Due to their nitrogen fixing ability, rhizobia play vital roles in agricultural systems where they are important for improving growth and yield of legumes, increasing soil fertility, and nutrient cycling in the ecosystem (Somasogaren and Hoben 1994; Yates et al., 2008). The symbiotic association between legumes and rhizobia has a huge impact on the success of legumes and the natural environment. This association gives them an added advantage over non-leguminous plants (Kabahuma 2013). The legume-rhizobium symbiosis is the major source of fixed nitrogen in the world, and it increases the fertility of soil and quality of agricultural lands, reduce soil erosion, improve crop production and is crucial for agricultural and environmental sustainability (Arrese-igor et al., 2011).

2.2. Biological Nitrogen Fixation

Biological nitrogen fixation (BNF) is a process in which atmospheric nitrogen is converted into ammonia, a usable form that can be absorbed by plants in the form of nitrate (Buyer and Kaufman 1996). The process occurs in various environments such as fresh water, soils and within or outside the root of the plants. It is second after photosynthesis in terms of its importance in plant growth and development (Vance and Graham 1995). In the agricultural setting, about 80% of BNF comes from the symbiosis between legumes and rhizobia. Studies have shown that rhizobia increase plant growth

directly by fulfilling the requirement of the legumes through the process of BNF and indirectly by increasing the nutrient uptake through plant hormone production such as auxin, cytokinin and gibberllins (Kohler et al., 2006).

Nitrogen gas contributes about 78% in the atmosphere and is an essential nutrient that limits agricultural production in the world (Valentine et al., 2011). The legume-rhizobium symbiosis provides biologically fixed nitrogen, which is important for sustainable land management, and crop productivity (Al-Falih 2002). Apart from directly fulfilling the nitrogen requirement of legumes, biological nitrogen fixation also contributes to soil quality by increasing the fixed nitrogen soil for the next crop. Many poor farmers do not have access to expensive artificial nitrogen fertilizers, and the inoculation of legumes with rhizobia is therefore a cheaper alternative. Legume inoculation with rhizobia has become an agriculturally important practice for many years where agricultural lands have continuously improved overtime (Castroux et al., 2001). Many rhizobial strains isolated from wild legumes have proved useful as inoculants of agriculturally important legumes (Yates et al., 2004). In other words, BNF is a crucial component of agricultural production systems, where inoculation with rhizobia not only improves crop yield and quality, but also assist farmers in saving money by reducing the need for artificial fertilizers. Many studies have shown that inoculation with highly effective indigenous nitrogen fixing isolates increases legume quality and yield as well as soil fertility (Pirlak and Kose 2009; Shaharoona et al., 2006).

Rhizobia include species of *Bradyrhizobium, Mesorhizobium, Sinorhizobium*, *Rhizobium, Allorhizobium,* and *Azorhizobium*, all of which belong to the alpha sub class of Proteobacteria. Screening for novel strains of rhizobia from wild legumes in the tropical regions have led to new discoveries of rhizobia from the beta sub class of Proteobacteria (Moulin et al., 2001). These include species from the genera *Paraburkholderia*, *Trinickia* and *Cupriavidis* (Bontemps et al., 2010; Chen et al., 2001). In countries such as Brazil, Madagascar, Panama to name a few, *Paraburkholderia* species are the dominant symbionts of the genus *Mimosoea* (Talbi et al., 2010). In South Africa, species of this genus can nodulate papilionoid legumes; a good example is *Paraburkholderia tuberum* which was found nodulating *Cyclopia* species (Elliot et al., 2007). *Paraburkholderia* species are found abundantly in many soils from around the world including South Africa, Australia and Brazil where biological diversity is very broad (Gyaneshwar et al., 2011).

Recently, rhizobia from wild legumes have received more attention in terms of their ability to form nodules (Zahran 2001). The isolation of rhizobia from different legume species has been done in the past, but still need to be described in more detail so as to increase the understanding of this interaction between legumes and bacteria (Beukes et al., 2013; De meyer et al., 2014).

2.3. Plant growth-promoting traits of rhizobia

2.3.1. The production of ACC-deaminase

Higher concentrations of ethylene in leaves and tissues pose a detrimental effect leading to reduced growth depending on the species involved. However, many inhibitory effects of ethylene on plant growth occur because of stressful conditions. Under such conditions, defense related proteins are produced (Glick et al., 2007), some of which can cause plant senescence (Abeles et al., 1992). Several plants produce ethylene as a plant hormone that promotes biological activities for controlling their growth and development (Pierik et al., 2006; Massod et al., 2010; Nazar et al., 2014). In legumes, higher concentrations of ethylene inhibit the formation and normal functioning root nodules (Ferguson and Mathesius 2014). The effect of ethylene on nodule formation by legumes was first discovered by Grobbelaar et al. (1971). Later work showed that ethylene may be involved in several stages of nodule formation, including the first response to bacterial Nod factors and subsequent nodule development (Csukasi et al., 2009; Patrick et al., 2009). Moreover, Oldroyd and Downie 2008 reported that ethylene inhibit the calcium spiking process needed for perception of Nod factors in *Medicago trinculata*.

Several beneficial soil bacteria including the symbiotic rhizobia have mechanisms to modulate ethylene levels in plants root one of which includes an enzyme called 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Ma et al., 2002; Nascimento et al., 2016). ACC-deaminase facilitates growth of the bacteria under abiotically stressed conditions, by regulating the production of ACC, a known precursor in the production of ethylene and thus limits the amount of endogenous ethylene produced. In this way, plants become more resistant to pathogens, salinity, drought and heavy metals thus promoting plant growth (Glick et al., 1994; Goris et al., 2002). Reduced ethylene level (by any biological treatment) increases the distribution of root system that gives access for more water and nutrients (Glick 2014; Lim and Kim 2013). Rhizobia that produce ACC-deaminase are found dispersed widely in range of environments, most commonly

in the soil, thus presenting different levels of ACC deaminase and thereby helping the plant overcome ethylene stress. Many α and β rhizobia encode the *acdS* gene for making ACC-deaminase (Nascimento et al., 2014), and many have been shown to possess ACC-deaminase activity (Onfre-Lemus et al., 2009; Gopalakrishnan et al., 2014). In spite of its importance in plants, it is important to note that not all rhizobia produce this enzyme.

2.3.2. Siderophores production

Iron (Fe^{3+}) is an element found in soil and is required for growth by all cellular organisms. Microorganisms, mainly bacteria, use several mechanisms for obtaining iron from their environment. Several species of bacteria produce different secondary metabolites that are biologically active as iron chelators or siderophores, and as immunosuppressive and antimicrobial compounds (Rondon et al., 2004). For example, under conditions of iron stress, bacteria compete for the available iron in the environment by releasing siderophores that are low molecular proteins whose primary role is to scavenge the iron, thus forming complexes with this essential element (Bellenger et al., 2003). In this way, iron becomes available to iron-dependent enzymes of the bacterium, such as nitrogenase, ferrodoxin, hydrogenase (Wdowia-wrobel et al., 2017).

Siderophore production by plant growth promoting bacteria is a very important strategy to induce plant growth and defence against environmental stresses. As the pH of the soil increases, it negatively affects the availability of iron for plants and microorganisms. Where there is less iron in the soil, microbial siderophores provides the plant with iron to improve plant growth (Crowley 2006). For the past three decades, several strains of soil bacteria belonging to the genera, *Pseudomonas Aeromonas, Azadirachta, Azotobacter, Bacillus, Burkholderia, Pseudomonas, Rhizobium, Serratia and Streptomyces* have been detected to produce siderophores (Kuffner et al., 2008). These bacteria may facilitate plant growth and afford the plant protection from phytopathogens, particularly fungi (Osullivan and Ogara 1992; Solano et al., 2008). In addition to improving plant development and making the plant to be able to survive under stressful conditions, siderophores also help plants absorb radioactive and rhizospheric metal iron at a low concentration, which further enhances their potential roles in agriculture and environmental sustainability (Dimkpa et al., 2009).

2.3.3. Phosphate solubilisation

Phosphorus (P) is an essential element and one of the limiting factors for plant growth (Malhotra et al., 2018). It works hand in hand with nitrogen where both are required by plants as micronutrients for normal growth and functioning, including establishment of the legume-rhizobium symbiosis (Mahantesh and Patil 2011). P is also essential for physiological processes of the plants such as in photosynthesis, cell division, and root system development (Mahantesh and Patil 2011). In addition, it functions as in energy storage and redirecting the Adenosine di and tri phosphate (ADP and ATP) in the plants, which serve as energy currency (Kaviyarasi et al., 2011). It also helps plant tolerate low temperatures. Therefore, P shortage in all agricultural soils generally affects crop yield negatively (Balamurugan et al., 2010; Sagervanshi et al., 2012).

P is available in soil in an organic and inorganic form (Rasool 2011). Its availability in an organic form depends on humus, which is taken up by the plant during nutrient absorption and will be returned back to the soil as organic residues (N, P and S) and these organic matter release inorganic phosphate (Vassileva 1993; Adarsh et al., 2011). Most agricultural soils contain large amounts of P due to irregular application of phosphates fertilizers (Richardson 1994). However, phosphate chemical fertilizers applied in large amounts becomes unavailable to plants due to slow mobilization of this element (Dey 1988; Yadar and Dadarwal 1997). P availability to plants can be improved by applying phosphate solubilizing microorganisms. The introduction of phosphate solubilizing bacteria into the soil is another important and sustainable means to increase the P availability to plant growth and better yield (De-freitas et al., 1997; Rodriquez and Fraga 1999; Richardson 2001; Vessey 2003; Thakuria et al., 2004).

Biofertilizers containing phosphate solubilizing microbes as active ingredients have played important roles in inorganic phosphate solubilization in many agricultural fields. Nevertheless, not all bacteria can be applied for P-solubilisation. Several studies have shown that *Pseudomonas, Rhizobium, Bacillus*, and *Enterobacter* are the most efficient phosphate solubilizers found in the soil (Whitelaw 2000; Rodriguez and Fraga 1999).

2.3.4. IAA production

Indole-3-acetic acid (IAA) is one of the main naturally occurring phytohormones (auxins), which is very essential in plant growth and development especially that of the roots (Yunde 2012). Plants and certain plant growth-promoting bacteria produce this phytohormone. In addition to IAA, other phytohormones including gibberellins, and

cytokinins, are also common traits in microorganisms and they can positively affect cell proliferation in the root and control the metabolic activities of plants (Arora 2013; Ortiz-castro et al., 2003). The production of IAA differs amongst species and is vital for the legume-rhizobial symbiosis (Patten and Glick 1996; Loper and Schroth 1986; Spaepen and Vanderleyden 2011).

Bacteria that produce IAA are known to be involved in improving plant growth by enhancing seed germination and promoting root architecture (Bhardwaj et al., 2014). In addition, bacterial IAA secreted in the root rhizosphere zone induces the production of a secondary messenger, nitric oxide (NO) which in turn triggers the production of other signalling metabolites that lead to improved root growth and development (Molina-Favero et al., 2007).

IAA opens up a greater space of plant roots by increasing their surface area and length to absorb soil nutrients thus improving growth (Vessey 2003). It also loosens up the cell wall to root exudates that provide additional nutrients to support the growth of rhizobacteria (Glick 2012). It increases the number of nodules on roots due to increased root surface area. Several microorganisms isolated from the rhizosphere have shown the ability to produce IAA *in vitro* in the presence of a physiological precursor mainly tryptophan (Caron et al., 1995; Davies 1995). Tryptophan is the main regulator for biosynthesis of IAA found in root exudates (Etesami et al., 2009). It was reported, that the addition of L- tryptophan in the culture medium increases the production of IAA (Akbari et al., 2007).

2.4. Environmental factors that affect BNF

The process of symbiotic nitrogen fixation is affected by various environmental factors. These include soil pH, temperatures, soil salinity, nutrient imbalance as well as aluminium toxicity. Legumes and rhizobia significantly respond differently in their tolerance ability to such stresses (Niste et al., 2013). Several reports have indicated that acidic soil is one of the major environmental factors that affect symbiotic effectiveness in legumes. The process of symbiotic nitrogen fixation is very much sensitive to soil acidity because the interaction of legumes and rhizobia require neutral or slightly acidic soil for effectively fixing nitrogen (Ferreira et al., 2016; Zahran 1999). Both soil acidity and aluminium (Al) reduce development and survival of rhizobia in the soils, as well as establishment of the root nodule, because it disturbs numerous stages in the establishment of symbiosis including molecular signals exchanged between the

symbiotic partners (Hungri and Vargas 2000). Aluminium found in the form of metals in the soil reduce plant growth and changes microbial activities, and because of increased soil acidity in arable lands, aluminium availability is usually one of the most limiting factors (Hede et al., 2001). Increased soil acidity affects the normal functioning of the microsymbionts in the root system resulting in poor growth, which decreased productivity in most crops. Moreover, soil with high acidity reduces the uptake of water by the plants, which in turn reduces growth rates by bringing various metabolic changes similar to those caused by water stress at the cellular, tissue and organ level in plants (Munns 2002; Beck et al., 2007).

With regard to other environmental factors, both rhizobia and legumes are affected by extremes of temperatures in arid and semi- arid regions as well as cold climates (Bansal et al., 2014). Fluctuation in temperature has a negative effect on the establishment of the root nodule. Nodulation and survival of rhizobia in the soil depend on their habitat and their tolerance to temperature changes and extremes (Mohammed et al., 2012). High temperature affects root nodulation in the soil thus inhibiting nitrogen fixation and hence growth. In a study of soybean, for instance, weak growth was observed at 40^0C and at 45^0C and no rhizobia were able to tolerate these temperatures (Chan et al., 2002). Low symbiotic performance has also been reported in *Phaseolus vulgaris* L at high temperatures (Yanyan et al., 2010). The major reason why high temperature affects the nitrogen fixation process is that it directly inhibits nodule establishment and indirectly affects root hair formation leading to decreased nodule sites (Roughly 1970; Piha and Munnus 1987; Frings 1976). In addition, it affects the activity of the nitrogenase enzyme regardless of nutrient and water availability, since it is well known that it functions only at certain optimum temperatures (Graham and Vance 2000).

Drought is another abiotic factor caused by limited water availability in the soil. It decreases the rhizobial population, their metabolism and inhibit nodulation and nitrogen fixation during the dry season (Ledgard and Steele 1992; Zahran 1999; Mohammadi et al., 2012). In the temperate zone, there is high annual temperature range and low moisture availability, rendering the rhizobia that are very sensitive to drought stress and reducing the functioning of the already formed plants nodules (Rao 2014). Rhizobia varies from one species to another in terms of tolerance to drought (Busse and Bottomley 1989). Aerobic bacteria have the ability to use nitrogen oxides as terminal electron acceptor to help them survive under drought conditions. (Abd-alla et al., 2014). However, the occurrence of rhizobial diversity under drought conditions emphasizes the

tolerance of the strains experiencing such conditions depending on the activity, type of the soil as well as moisture content (Jenkins et al., 1989; Waldon et al., 1989; Mabrouk and Belhadj 2010). Nevertheless, drought stress causes morphological changes that lead to reduced infection, initiation of nodule organogenesis and ultimately nodulation, thus reducing the process of nitrogen fixation (Zahran 1999).

Generally, rhizobial sensitivity to drought stress leads to great loss of legume production due to poor nodulation and results in less accumulation of dry matter as affects the various parts of the plant (Long 2001). It decreases the leaf number and cause root shrinking due to water loss. Several studies reported that that some rhizobia have developed strong mechanism to survive under drought stress or low moisture availability by accumulating certain compounds such as potassium ion and glutamine that can be used as a source of growth (Boscari 2002). Therefore, inoculation with some wild rhizobia that are drought tolerant may have some positive impact on legume growth during drought stress.

Another major factor that negatively affects symbiotic nitrogen fixation is poor soil nutrient condition. In agricultural soils, shortage of some elements needed by the plant, affects the nitrogen fixation of the legumes and as a results decrease production. The deficiency of N and P has a well-recognized negative effect on the growth of several legumes with a huge production loss worldwide (Pereira and Bliss 1989). Elements such as calcium and magnesium are limited in acidic soil, where the available potassium is usually also not enough to support growth (Srinivasarao et al., 2003).

2.5. Conclusions

The rhizobium legume symbiosis and the development of inoculants for several economically important legume species have been extensively studied over the past decades worldwide. Most of the rhizobial strains are collected and recovered from the nodules of their legume host that show effective pink nodules with highly developed shoots and roots. These rhizobial strains are thus adapted to the various environmental conditions and limited factors in the wild soils. Nevertheless, in cultivated legume fields, farmers often encounter various problems in the agricultural ecosystem with regard to improving productivity and legume quality. Many of the studies in this respect mainly focused on the development of rhizobial inoculants for food legumes but many recent investigations have also started looking into improving forage legumes using rhizobial inoculants. Thus the use of rhizobial inoculants in the cultivation of both food and forage

legumes is a more sustainable strategy and a perfect alternative to reduce the use of chemical fertilizers that negatively affect the environment.

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CHAPTER 3

MATERIALS AND METHODS

3.1. Legumes and bacterial isolates

The following legumes were used in this study: lucerne (*Medicago sativa* L), cowpea (*Vignia unguiculata* L) and siratro (*Macroptilium artropurpureum* DC). Rhizobial isolates were previously isolated from the vigorous pink nodules of different native legume in South Africa and stored at the collection database at the Department of Biochemistry, Genetics and Microbiology, University of Pretoria. Information regarding the individual isolates and their legume hosts are provided in Table 1.

The bacterial isolates were initially streaked on Yeast Mannitol Congo Red (YMCR) agar that has the following composition (g/L); Mannitol, 10g; KH_2PO_4, 0.5g; MgSO4.7H20, 0.2g; Yeast extract, 0.4g; NaCl, 0.1g; distilled water 1000ml; Congo red, 10ml; pH 7.2±0.2. The plates were incubated at 28^{o}C for 3 to 5 days until white pure colonies were formed. For long term usage, the bacteria were maintained as described below: For each bacterial strain, a pure single colony was streaked onto the same solid medium and incubated as described above, and after sufficient growth, 10ml of 20% glycerol was poured over the pure colonies and mixed very well with sterile swab. The resulting homogenate mixture was transferred into 2 ml Eppendorf tube and stored in an ultra-freezer at -80^{o}C until further used. The isolates were deposited in the South African Rhizobium Culture Collection (SARCC) and were assigned SARCC accession /strain codes (SARCC 722-738) in accordance with principles of the utilization of microbial resources by the World Data Centre for Microorganisms (WDCM).

3.2. Biochemical and Physiological characterization of bacterial isolates

3.2.1. Motility

Motility of the isolates was detected microscopically using a hanging drop preparation. Small thick spots of petroleum jelly were placed on each corner of a cover slip using a wooden toothpick. A drop of log-phase broth culture was mixed and transferred into the centre of the slide and covered with cover slip. The slide were lowered gently to make contact with the petroleum jelly. The slide and cover slip were inverted so that the drop hangs but does not come into the slide and were examined under microscope (Harrigan and Mccance 1966).

3.2.2. Catalase test

The catalase test was done by placing 1 drop of 3% H_2O_2 on a clean slide and suspending a loopful of the bacteria on the drop of the H_2O_2. Formation of gas bubbles in the form of effervescence was used as an indication for a positive test for the enzyme catalase using the protocol described by (Riegel et al., 2006).

3.2.3. Oxidase test

The oxidase test involved the use of an "oxidase reagent" containing 1% (w/v) N,N,N′,N′-Tetramethyl-p-phenylenediamine dihydrochloride. A piece of filter paper, placed in a petri dish, was moistened with distilled water after which 3 drops of the oxidase reagent was added to it. A small part of the pure bacterial colony was scrapped from the YM medium using sterile glass rod and placed onto the filter paper (Shier et al., 2000). Positive test for presence of oxidase was indicated by a colour change of deep blue/ purple and negative result was indicated by no colour change within 10 to 15 seconds.

3.2.4. Oxidative-fermentation test

Hugh & Leifson (1953) were the first to refer to acid production by bacteria from carbohydrates under aerobic conditions only as oxidative and they developed the oxidative fermentative test. The oxidative-fermentation medium had a pH of 7.1 and was composed of 3 g/L peptone, 5 g/L NaCl; 0.3 g/L K_2HPO_4, 3 g/L bacteriological agar, 0.03 g/L bromothymol blue (1 g dissolved in 100 mL distilled water and 3 mL added to 1 L of medium), 10 g/L glucose. Tubes of oxidative-fermentative medium were stab inoculated 1 cm from the bottom with a sterile inoculation loop containing the test organism. Bacteria that can ferment glucose give a fermentative result. The acid produced (pH 6.0) changed the pH indicator bromothymol blue from green to yellow. Yellow on top of the tube indicated oxidation reaction whereas yellow throughout the medium indicated fermentation reaction.

3.2.5. Acid-alkaline production

The production of acid or alkaline substances by the rhizobia was detected by inoculating a pure colony culture of the bacteria on Yeast Mannitol Agar-supplemented with 0.5 % bromothymol blue indicator (YMA-BTB). The plates were incubated at 28^{o}C for 3 days and the production of acid and alkaline was detected by observing colour changes in the YMA-BTB medium (Alberton et al., 2005). Yellow colour is indicative of

acid production whereas green to blue colour change indicates alkaline production. In all the experiment, sterile un-inoculated medium was used as control.

3.3. Evaluation of rhizobia for tolerance to various abiotic stresses.

3.3.1. Tolerance to acidity and aluminium

Evaluation of bacterial growth at various pH levels was conducted following the procedures of Keyser and Munns (1979) using a combination of the following media:

A. Micronutrient stock solution in g/L:

$MnCl_{2.}$ $4H_20$ 0.50g; $ZnSO_4$ 0.23g; $CuCl_2$ 0.03g; $NaMO_4.2H_2O$ 0.01g;

B. Phosphate stock solution in 5mL: KH_2PO_4 1, 36g

C. Vitamin stock solution g/100mL: Thiamine HCl 0.4g, Panthothenic acid 0.4g, Biotin 0.0001g.

After preparing all the three solutions, Glycerol 5mL, K_2SO_4 0.131g, Na-glutamate 0.22g, $Mg.SO_4.7H_2O$ 0.07g and EDTA 0.035g, micronutrient solution 5mL, phosphate stock solution 1.0mL, vitamin stock solution 1.0mL and Agar 20,0g were mixed together in 1litre distilled water adjusted to different pH levels ranging from pH=4, pH=7, pH=10 using sterile HCl and NAOH and autoclave for 15 minutes. A loopful of the bacterial colony from a pure culture was scrapped and streaked on the plates and incubated at 28^0C for 3-10 days. The plates were then evaluated for any growth. To evaluate the tolerance of bacterial isolates to aluminium toxicity, the isolates were streaked on Keyser's defined medium containing different concentration of Aluminium (0.02 and 0.04 g) and incubated as above.

3.3.2. Tolerance to temperature

Determination of the temperature sensitivity of the isolates was conducted following the procedure described by Jordan (1984). Pure colonies of each bacterium were streaked on YMA medium containing congo red and incubated at different temperatures of 15^0C, 28^0C, and 37^0C for 3-5 days and observed for optimal growth and temperature tolerance.

3.3.3. Tolerance to salinity

Tests for tolerance to salt were conducted using a simple protocol described in Greenway and Munns (1980). Pure colonies of the bacterial strains were streaked on YMA medium that contains different concentrations of sodium chloride (NaCl): 0.5% (w/v), 1% (w/v) and 1.5% (w/v). The plates were then incubated at 28^{o}C for 3- 5 days and observed for any optimal growth to determine their tolerance to different salt concentration.

3.4. *In vitro* detection of plant growth promoting traits

3.4.1 Detection siderophore production

The universal Chrome Azurol Sulfonate (CAS) assay, initially developed by Schwyn and Neilands (1987), was used to determine the production of siderophores. All the glassware were cleaned using 6M HCl and then rinsed with distilled water to eliminate possible contamination with irons compounds. The assay for siderophore involves three main components: CAS mixture, basal agar medium and glucose stock solution.

For the CAS mixture, three solutions were prepared:

Solution 1: 0.06g of Chrome Azurol Sulfonate (CAS) (Sigma Aldrich, Johannesburg, South Africa) were dissolved in 50mL distilled water

Solution 2: 10.0mL of 1mM $FeCl_3.6H_2O$ (0.0027g of $FeCl_3.6H_2O$ and 83 µL of concentrated HCL in 100mL of distilled water

Solution 3: 0.073g Hexadecyltrimethyl Ammonium Bromide (HDTMA) (Sigma Aldrich) were dissolved in 40mL of distilled water.

All three solutions were mixed and the resulting the dark blue CAS mixture was autoclaved for 15 minutes at 121^{0}C and stored in a plastic container.

For the basal agar medium, 30g of 3-N-morpholino propane Sulphonic acid (sigma Aldrich), 01g of NH_3CL, 0.5g NaCl and 0.3g KH_2PO_4 were dissolved in 830mL of water. The solution was adjusted to 6.8 pH and distilled water was added to 880mL. 20g bacteriological agar was added while stirring the solution and the resulting solution was autoclaved for 15 minutes at 121^{0}C.

For the glucose stock solution, 20g of glucose was added in 100mL of water to make 20% of glucose solution and then autoclaved for 15 minutes.

The autoclaved solutions and media were cooled down to 50^0c in a water bath (Memmert WNB 14). Glucose stock solution was added slowly to the basal media, while stirring together with the CAS solution. After cooling the medium, it was poured in sterile petri dishes. After the media become solid, holes were prepared on the media using the cork borer.

Bacterial isolates were grown in synthetic iron deprived broth (pH 7.2) composed of 10 g/L mannitol; 2 g/L L-glutamic acid sodium monohydrate, 0.5 g/L K_2HPO_4, 0.2 g/L $MgSO_4.7H_2O$ and 0.1 g/L NaCl (Jadhav and Desai 1992). Bacterial cultures were grown by incubating them on a rotary shaker for 24 to 48 hrs. The cultures were centrifuged for 5 minutes at 3 000 rpm, after 60µL of supernatant was added to the holes prepared in the CAS assay plates. The presence of siderophore production was indicated by yellowish colour surrounding the holes on the media within 8- 48 hrs after inoculation. The diameter of the yellow halo zone was measured and recorded.

3.4.2. Detection of IAA production

IAA production was conducted following the protocol of Gordon and Weber 1951. Bacterial isolates were inoculated on yeast mannitol broth (YMB) that consisted of (g/L) 10.0g mannitol; 0.5g K_2HPO_4; 0.2g $MgSO_4$; 0.1g NaCl; 0.5g yeast extract supplemented with 100 µg/mL of L-tryptophan and adjusted to pH of 7.2 before autoclaving. The inoculated medium were incubated at 27^0C ± 1 for 5 days. These cultures were then centrifuged at 3 000 rpm for 30 minutes, after which 2 mL of the supernatant were mixed with 2 drops of Orthophosphoric acid and 4 mL of Salkowski's reagent (50 mL; 35 % perchloric acid and 1 mL of 0.5 M $FeCl_3$) and incubated at room temperature for 25 min. Development of pink colour indicated IAA production and the absorbance of the solutions were read at 530 nm, using spectrophotometer. For the control experiment sterile, yeast mannitol broth were used. The concentration of IAA in the supernatants was determined using a calibration curve and pure IAA (Merck) was used for preparing the standards of 0, 5, 10, 20, 50, and 100 µg/mL. An amount of 10 mg IAA was added to a McCartney bottle of 10 mL acetone in the fume hood and swirled until completely dissolved. This was the 1000 µg/mL stock solution and was used to prepare the different standards concentration mentioned above.

3.4.3 Phosphate solubilisation

The ability of the bacterial isolates to solubilize phosphate was detected by spot plating Pikovskya's (PVK) medium (Pikovskaya 1948) that consisted of (g/L): 0.5 g/L yeast

extract; 10 g/L dextrose; 5 g/L calcium phosphate; 0.5 g/L ammonium sulphate; 0.2 g/L potassium chloride; 0.1 g/L magnesium sulphate; 0.0001 g/L manganese sulphate; 0.0001 g/L ferrous sulphate and 15 g/L agar. Inoculated plates were incubated at 27^0C±for 5 days. The solubilization of tricalcium phosphate was indicated by the formation of a clear halo zone around the bacterial colonies (Ashrafuzzaman et al., 2009). The diameter of zone of clearance surrounding the bacterial colony as well as the diameter of colony were measured after incubation.

3.4.4. ACC deaminase activity

To determine the ACC deaminase activity of the isolates, the protocol described by Glick et al. (1995) was used. Briefly, pure colonies of bacterial cells were transferred to a test tube containing 8mL of Nutrient Broth (NB) with the following composition: D (+) glucose 1g/L; Peptone 15g/L; NaCl 6 g/L; Yeast Extract 3g/L and pH=7.5±0.2. (Merck, South Africa). The tubes were incubated for 3 days on a rotary shaker at 28^0C and 150 rpm. The resulting cultures were centrifuged at 5000 rpm for 5 minutes. The pellets were then washed with 0.85% (w/v) NaCl and transferred to a tube containing 8ml of nutrient broth supplemented with 0.3 M of ACC (1-aminocyclopropane-1-carboxylate). This was then incubated on a rotary shaker (28^0C, 150 rpm) for 3 days. The resulting culture was inoculated on Rhizobium minimal medium (RMM) described by Broughton et al. (1986). On the back of the plates a grid of 2x2 was drawn. Prior to inoculation, 150µl aliquots of 0.3 M of ACC solution was spread on the plates of RMM and incubated at 28^0C for 5 days. Colony formation was observed every 24 hr and the colonies produced during this incubation were then transferred to the same medium under the same experimental conditions. The newly formed colonies on RMM (supplemented with ACC) were considered positive for ACC deaminase activity. Carbonated nitrogen-free RMM without ACC supplement was used as control, where no colonies appeared after 10 days.

3.5. Glasshouse nodulation efficacy test

Seeds of cowpea, lucerne and siratro were surface sterilized using the procedure of Somasegaran and Hoben (1994). Briefly, the seeds were first immersed in 95% ethanol for 30 seconds and 1% (v/v) sodium hypochlorite solution for 1 minute. The excess bleach was drained off and the seed were rinsed with five changes of sterile distilled water. They were then imbibed by soaking them in half-filled sterile water and incubating at 4^0C for 4 hours, and rinse three times with sterile water. The imbibed

seeds were transferred to 0.75% (w/v) water agar plates and incubated in a warm place in the dark for 2-3 days to germinate.

For the nodulation efficacy test, the Leonard jar assembly containing sterile sand and nitrogen free Hoagland solution were used (Somasegaran and Hoben 1994). In each jar, 4 germinated seeds of each legume were planted and each seed was inoculated with 2 mL suspension of the rhizobial culture grown to a concentration level of 1 x 10^8 cfu/mL and the seeds were covered with the sand immediately. Each treatment contained *legume x rhizobium* inoculum and had three replications. The experiment was arranged in a completely randomized design in a controlled glasshouse with day and night temperature of 27^0C and 18^0C respectively and regular watering. Sixty days after planting and inoculation, plants were carefully removed from the Leonard jars and the following data were collected for analysis: shoot fresh weight, shoot dry weight, root fresh weight, root dry weight, nodule dry weight, nodule number and position, nodule colour.

3.6. Field nodulation screening trial

3.6.1. Site selection and soil analysis

Field inoculation trial was conducted around the 2017-2018 growing season at the Agricultural Research Council Zeekoegat experimental farm. The area where the Zeekoegat experimental site is located is characterized by hot summer and cool dry winter with an annual rainfall of 500mm and a slightly alkaline soil.

Prior to planting, soil samples were collected randomly from the different sites at the experimental farm using a soil auger to a depth of 0-15cm to analyse soil chemical properties. These included pH, P, K, Ca and Mg content, as well as Cation exchange capacity (CEC). These analyses were conducted at the Institute for Soil, Climate and Water (ISCW) laboratory of the ARC in Pretoria.

3.6.2. Planting of seed and inoculation with bacteria

In this field inoculation trial, the major aim was to assess the nodulation performance of the rhizobial isolates in the field and to determine the nitrogen derived from the atmosphere by the nitrogen fixation activity of the rhizobia. Before the rhizobial seed treatment and planting, land preparation was done in which furrows of 5m long were

ploughed and prepared 0.5 m apart. Seeds of cowpea, siratro and lucerne were surface sterilized as described before. The seeds were then coated with sterile peat inoculated with the 1 x 10^8 cfu/g of the respective rhizobial strains associated with each legume. The treated seeds were sown in rows at a rate of 2g per row with spacing to provide sufficient planting density. Immediately after planting, each row of plants were inoculated with 75mL of the bacterial suspension as prepared in section 3.5. There were 5 treatments for cowpea, 6 treatments for siratro and 1 treatment for lucerne, all of which were arranged in a randomized block design with three replications. Each legume were grown in three row plots of 7m length with 20cm space between rows and 50 cm between plots.

3.7. Determination of N-fixation using the ^{15}N natural abundance technique

The percentage of plant N derived from the atmosphere (% Ndfa) which is the same as the proportional contribution of biologically fixed nitrogen is calculated from the ^{15}N abundance of the legume and a second non-N_2-fixing reference plant as shown in the following equation (Shearer and Kohl, 1986):

$$\%Ndfa = 100.\ \frac{(\delta 15\text{Nref} - \delta 15\text{Nfixing plant})}{\delta 15Nref - B}$$

δ^{15}N represents the level of δ^{15}N detected in a reference plant growing in the same soil at the same time as the test legume, δ^{15}N fixing plant is the ^{15}N abundance of the legume and B is the ^{15}N abundance (‰) of the legume grown obtaining its entire N from N_2 fixation. The value of B varies for each of the four legumes and is calculated as described below.

$$B = \frac{(\delta 15\text{N whole plant x TN whole plant}) - (\delta 15\text{N seed x TN seed}}{\text{TN plant x TN seed}}$$

δ^{15}N whole plant and δ^{15}N seed are the ^{15}N abundance (‰) of the whole plant and seed and TN is the total N in these compartments.

3.8. Data collection and Statistical Analysis.

Six weeks after planting and inoculation, plants were harvested and the following data were collected to evaluate the nodulation efficacy of the bacterial isolates: total fresh weight, nodule number and total dry weight. Additional data to be collected from the field trial experiment include data on the actual nitrogen fixed from the atmosphere

using the ^{15}N Natural abundance method. All data were analysed using the Analysis of Variance (ANOVA) and the means were compared with the least significance difference $(LSD)_t$ test (at 95% level of significance) of the General Linear Model (GLM) procedure using SAS statistical software (SAS NY, 2003).

3.9. Detection of nodulation (*nodC*) and nitrogen fixation (*nifH*) genes

For extraction of DNA, bacterial strains used in this study were grown on Tryptone broth (TY) on a rotary shaker for 2 to 3 days. DNA was extracted using the wizard® genomic DNA purification kit (Promega Madison, USA) following the manufacturer's instruction. For amplification of the *nodC* genes portion by PCR forward primer (3′ AYGTHGTYGAYGACGGTTC 5′) and reverse primer 5′ CGYGACAGCCANTCKCTATTG 3) were used (Laguerre et al., 2001). For the *nifH* gene portion, the forward primer 5′ GCI WTI TAY GGN AAR GGN GG 3′ and reverse primer 3′GCR TAI ABN GCC ATC ATY TC 5′ (Widmer et al., 1999) were used. Both *nifH* and *nodC* gene amplification were performed in a total reaction volume of 28 µL consisting 23 µL of PCR master mix from Fermentas and 5 µL of extracted genomic DNA. The PCR was performed in an Eppendorf Master Cycler Gradient apparatus (Applied Biosystems, USA) with the following the cycling conditions for *nifH*: initial denaturation of 95^0C for 3 minutes followed by 35 cycles of denaturation 94^0C for 1 min, annealing 55^0C for 1 minute, extension 72^0C for 2 minutes and final extension step at 72^0C for 3 min, and for *nodC*, an initial denaturation of 95^0C for 3 minutes followed by 35 cycles of denaturation at 94^0C for 1 min, annealing 55^0C for 1 minute, extension 72^0C for 2 minutes and final extension step at 72^0C for 3 min. The amplified PCR products were visualised using 0.9% agarose gel electrophoresis stained with ethidium bromide to verify the size with 3µl aliquots of PCR. The PCR products were cleaned-up using QIAquick gel extraction kit (QIAGEN), original primers and the ABI PRISM BigDye Terminator v3.0. Sequencing was performed using Cycle Sequencing Kit on the AB13100 Automated Capillary DNA Sequencer. The resulting sequence were edited using Chromas Lite 2.0. and BioEdit version 5.09 (Hall 1999). Each sequence was compared to those deposited in the Genbank (National Centre for Biotechnology by *blastn* (Altshul et al., 1990) to determine the gene identity. ClustalW version 1.83 (Thompson et al., 1997) was used to generate sequence alignment and those found in the Genbank. MEGA 4 online programme was used in constructing Neighbour-Joining phylogenetic tree after all gaps were removed following the Jukes Cantor and parameter model.

3.10 Reference.

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Chapter 4.

Results and Discussion

4.1. Biochemical and physiological characterization of bacterial isolates

For the motility tests, all the isolates showed different run behaviour on the slides (Table 2) and all bacterial cells grown on the liquid medium observed under the microscope were motile. Of the 16 strains, nine (*Bradyrhizobium* sp. PEAR 76, LEO 170, ARG 62, LEO 121, CHAM 227, and *Paraburkholderia* sp. WK1.1F) were catalase positive. These strains produced the enzyme that allowed conversion of hydrogen peroxide into oxygen and water (Table 2).

Four strains of *Paraburkholderia* (HC1.BA, KB15, *P. tuberum*, *P. aspalathi*) and two strains of *Bradyrhizobium* (FP1C and 32AAV) were oxidase negative (Table 2). No colour change was observed for these bacteria during the oxidase test. Growth of the bacteria on the oxidative-fermentation (O-F) test showed that seven of them were positive (Table 2). After 14 days of growth on this medium, it was found that only two *Bradyrhizobium* strains (32AAV and FP1C) were able to metabolize glucose in the absence of oxygen, while five *Paraburkholderia* strains (N362, *P. aspalathi*, ARG 68, WK1.1F and PEAR 76) could metabolize glucose in the absence of oxygen. Rhizobial strains grown on medium containing bromothymol blue (BTB) as an indicator showed that seven isolates (*Paraburkholderia* sp N362, *P. aspalathi*, WK1.1F, KB15, and *Bradyrhizobium* sp PEAR 76, FP1C, 32AAV) produced an acid reaction (Table 2; Figure 2). The remaining 9 isolates produced an alkaline reaction.

Generally, the rhizobial isolates used in this study demonstrated different biochemical and physiological characteristics when subjected to various tests. This is similar to what has been found previously. For example, Rasool et al. (2015) showed that the rhizobial strains were motile and some of them were oxidative positive, while other studies showed that some strains produce alkali reaction and others produce acid (Hassen et al., 2014; Hernandez cha focht 1984; Moreira et al., 1993; Padmanabhan et al., 1990). Motility of the bacteria is an important aspect as it helps the bacteria to attach themselves to the plant roots and compete for nutrients (Czaban et al., 2007; De weert et al., 2002).

4.2. Evaluation of the bacterial growth at various conditions

4.2.1. Salinity

In this study, *Bradyrhizobium* and *Parabukholderia* differed in their response to salinity (Table 3; Figure 3). Most of the *Paraburkholderia* were tolerant to NaCl concentrations of 0.5%, 1% and 1.5% in the culture medium. Only two *Bradyrhizobium* strains (RP7B and FP1C) originally isolated from *Arachis hypogea* showed tolerance to 0.5%, 1% and 1.5% concentration of salts, while one strain (ARG62) only tolerated 0.5% and 1% of NaCl. The remaining *Bradyrhizobium* strains showed no growth after 7 days an all of the salt concentrations. Similar salinity tolerance results were previously reported for *Rhizobium leguminosarum* in broth culture (Abdel- wahab and Zahran 1979), and for *Ononis arvensis* strains (Wkowiak-wrobel et al., 2017) that were able to grow at 0.5% of NaCl. These results suggest that *Paraburkholderia* isolated from wild legumes might be useful for cultivated legumes because of its tolerance to various concentration to NaCl.

Soil acidity caused by chemical imbalances results in changed growth patterns of plants and sometimes interferes with the functioning of rhizobia. Rhizobia, particularly the *Paraburkholderia* strains examined here, can survive under different concentration of salinity whereas three strains of *Bradyrhizobium* could tolerate such conditions. However, Zeghari et al. (2000) reported that strains for *Acacia* and *Prosopis* can tolerate up to 500 Mm NaCl whereas other strains are inhibited at 100Mm NaCl (Singleton et al., 1982; Yelton et al., 1983). Several rhizobia species that tolerate high acidity conditions are already identified (Graham et al., 1994). Inoculation with such salt tolerant strains isolated from wild legumes may be a good strategy to enhance plant growth in abiotically stressed saline soil.

4.2.2. Acidity and Aluminium toxicity

Out of 16 strains examined, only eleven were able to grow at pH 4 on Keyser defined medium (*Paraburkholderia aspalathi* and *Bradyrhizobium* sp CHAM227 could not) (Table 3; Figure 4). However, they showed some growth on YMA containing congo-red. Most of the strains examined tolerated pH 9, except for *Paraburkholderia aspalathi* and *Paraburkholderia steynii* which did not grow at high pH. Tolerance to pH and acidity may differ amongst strains and most researchers reported that legumes species fail to nodulate at pH less than 5 (Andrew 1978). The production of exopolysaccharides might increase the acid tolerance. Soares et al. (2014) previously reported the adaptation to acidity by tropical *Bradyrhizobium* strains and this adaptation and tolerance to acidic

condition could be due to the production of exopolysaccharides by a number of these *Bradyrhizobium* strains (Cunningham and Munns 1984). Miguel and Moreira (2001) also reported that certain B*radyrhizobium* strain grew better at pH4. In the current study, the *Paraburkholderia* and *Bradyrhizobium* strains grew well at pH 9 but differed amongst strains. Tolerance of rhizobial strains isolated from the wild have a positive impact on the growth of cultivated legumes and in most cases the rhizobia vary greatly with the tolerance to pH as observed previously (Zahran et al., 1999; Appunu et al., 2005). In the current study, fast growing *Paraburkholderia* strains were more tolerant to acid conditions than slow growing *Bradyrhizobium*.

In the tests for Aluminium tolerance by the bacterial isolates, one *Paraburkholderia* strain (HC1.BA) did not grow under Keyser defined medium containing different concentration of aluminium (Table 3; Figure 5). This means that it is very sensitive to high aluminium concentration whereas all the other strains were able to grow on the medium containing aluminium concentration of 0.04g/L to 0.02g/L of $AL_2(SO_4)_3$. On the other hand, most of the *Bradyrhizobium* strains were able to grow under high aluminium concentration but differ in their growth rate. Ferreira et al. 2012 reported that rhizobial strains showed different growth rates when exposed to medium containing aluminium concentration although at higher concentration there was reduced growth. High aluminium concentration can be toxic to the survival of rhizobia, which can cause changes in cellular metabolism. It was also demonstrated in other studies that, fast growing rhizobia could tolerate high concentration of aluminium as compared to B*radyrhizobium (*Arora et al., 2010). We have detected that many of the slow growing *Bradyrhizobium* strains were able to tolerate higher concentrations of aluminium probably due to their adaptation to varying environmental changes in the wild soil habitat.

4.2.3. Temperature

It has been found that rhizobia grow well at temperature of 28±2^{0}C in culture medium, although many are unable to grow below or above their optimum temperatures (Graham 1992). In this study, all the examined bacteria were able to grow at 28^{0}C (Table 3; Figure 6). However, *Paraburkholderia tuberum* and *Paraburkholderia aspalathi* could not grow at a temperature of 15^{0}C, while strains CHAM 227, ARG 68, HC1.BA and ARG 62 grew very slowly under cold conditions. At 37^{0}C, *Paraburkholderia* strain HC1.BA and *Paraburkholderia tuberum* could not tolerate high temperatures.

Except for strain Pear76, all the *Bradyrhizobium* strains tolerated the temperature conditions tested. This is consistent with the fact that *Bradyrhizobium* is present in most South African soils, where temperatures ranges from 0^0C to 40^0C.

Temperature is one of the main limiting factors for rhizobial growth and nodulation effectiveness (Graham 1992). It plays an important role in BNF in the legume-rhizobium symbiosis. We found in this study that most of the bacterial strains were able to grow even at 37^0C. In general, abiotic stresses pose detrimental effect on the plant leading to reduced growth and symbiotic performance in the legume-rhizobia interaction. Tolerance of *Bradyrhizobium* and *Paraburkholderia* strains to the abiotic stresses tested in this study indicated that these strains could have evolved mechanisms to survive under various environmental conditions while growing in the wild.

4.3. Plant growth-promoting traits

4.3.1. Screening for siderophore production

Siderophore production by microorganisms play a very important role for plant growth and development (Haas and Defago 2005). It has been reported in many studies that there is an essential relationship between siderophore production by rhizobia and effectiveness of nitrogen fixation especially in soils where iron (Fe^{+3}) availability to plants is very low. Therefore, the deficiency of iron can limit nodule development, nodule biomass and nitrogenase activity (Duhan et al., 1998). Siderophores produced by bacteria result in the binding of insoluble iron (Fe^{+3}) and make it available to plants for their normal functioning and growth (Mietzner and Morse 1994; Kraemar et al., 2006). However, in this study, none of the bacterial strains screened for siderophore production on CAS agar medium showed the ability to produce siderophores (Table 4). By contrast, several siderophore producing rhizobia and other PGPR strains including *Paraburkholderia* have been reported in other studies (Antoun et al.,1998; De los santos-villalobos et al., 2012).

4.3.2. Screening for phosphate solubilizing bacteria

Phosphorus (P) is the second most nutrient limiting element for growth in terrestrial plants followed by nitrogen. Although there is a large amount of P in the soil, it is usually unavailable to plants as most of it is immobilized in the soil (Stevenson and Cole 1999). In the current study, an *in-vitro* test was used to screen the bacterial isolates for

phosphate solubilisation on Pikovskaya (PVK) media. The ability to solubilize phosphate was detected based on the formation of clear halos of which the diameter were measured (Table 4; Figure 7). These halos are the results of tri-calcium phosphate solubilisation because of the organic acids produced by the bacterial strains. In this study, six strains (*Paraburkholderia aspalathi* 4mm, *Paraburkholderia sophoroidis* WK1.1F with a 32mm halo, *Paraburkholderia kirstenboschensis* KB15 with a 19mm halo, *Paraburkholderia virgiliae* with a 14mm halo, *Bradyrhizobium* CHAM227 with a 19mm halo and *Bradyrhizobium* ARG62 with a 14mm halo) were able to solubilize phosphate. The largest halos were formed by *Parabukholderia* strain WK11.1Ffollowed by strains KB15 and *Bradyrhizobium* CHAM227.

Phosphate solubilizing bacteria are ubiquitous although they occupy less than 5% of the living space. Microorganisms isolated from wild legumes may contribute to enhanced growth in cultivated legumes due to their ability to solubilize phosphorus but the amount of solubilisation differ amongst species. The activity of P solubilisation and phosphorus availability to plants depend on the ability of the microorganisms to multiply in the environment as bacterial growth depends on the soil organic matter content for solubilisation of P and making it available to plants (Kim et al., 1998a). This again affected by many abiotic stresses such as the pH of the soil, soil moisture and other climatic conditions (Tisdale et al., 1993; Barber 1995).

Inoculation with P solubilizing bacteria promote P uptake and crop productivity (Gyaneshwar et al., 2002; Fankem et al., 2008). The use of these bacteria can reduce the use of chemicals by 50% (Yazdan et al., 2009). Furthermore, plant growth promoting rhizobia improves BNF, P uptake and enhance growth (Ponmurugan and Gopi 2006). Linu et al. (2009) reported that certain strain of *Paraburkholderia* were better at promoting the growth of cowpea and it was previously shown by Pandey et al. (2005) to have phosphate solubilisation, ACC deaminase activity and nitrogen fixing ability. As explained below, adequate amounts of P in the soil during planting and organic matter increased the development and enhance the performance of legume symbiosis on lucerne, cowpea and siratro. It is thus likely that P, together with organic matter, increased the functioning of nodules, which also caused the greater the amount of shoot fresh weight accumulated on wild strains (Fatima et al., 2006).

4.3.3. Screening for production of indole acetic acid (IAA)

Most of the bacterial isolates capable of nodulating legumes produce IAA in the presence of L-Triptophan as a precursor as it is involved in multiple processes (Mutluru and Konada 2007). The production of IAA by bacterial strains contribute to plant growth by increasing the number of nodules, accelerate germination, and development of an early process of symbiosis. However, none of the strains tested in this study produced IAA. This is in contrast to many previous studies which, reported that several rhizobial strains *Bradyrhizobium* and *Rhizobium* are able to produce IAA (Mohite 2013; Ghosh et al., 2015; Bal et al., 2013).

4.3.4. Screening for ACC deaminase production

The production of ACC-deaminase was detected by growing the isolates on a media containing ACC as a sole nitrogen source and by checking the growth and colony diameter comparison between the experimental plates. In this study, all of the bacteria tested were able to use ACC as a source of nitrogen as observed on the experimental plates, an indication that the bacteria are able to produce the enzyme ACC deaminase (Table 4). The isolates on the plate with a greater colony diameters were considered as strong producers of ACC-deaminase (Balgiran et al., 2008), although they differed in their growth rate. This is consistent with previous rhizobial studies where such differences among strains were also observed (Balgiran et al., 2008). In fact, ACC-deaminase production is much more common in free-living soil bacteria (Khan et al., 2016). However, several strains of nitrogen fixing rhizobia belonging to different genera (*Rhizobium, Bradyrhizobium, Sinorhizobium and Mesorhizobium*) have also been demonstrated to produce ACC deaminase with superior nodulation of their legume host (Kong et al., 2015). The wild isolates of rhizobia that are tolerant to various abiotic stressed conditions in this study all showed this important PGPR trait. This also concurs with the finding by Brigado et al. (2013) that *Mesorhizobium* spp. improved growth in chickpea plants under salt stressed condition because they possessed the ability to express the ACC deaminase gene.

4.4. Glasshouse trial

Six weeks after inoculation of cowpea, lucerne and siratro seeds with the various rhizobial bacteria included in this study, data were collected. That included total fresh weight, total dry weight, nodule number and colour as well as plant vigour in terms of

leaf colour (Tables 5-6). The following strains were found to be efficient in the glasshouse for cowpea: *Bradyrhizobium* sp. 10BB, ARG68, CHAM227, LEO170, LEO121, ARG62, 32AAV, and *Paraburkholderia* sp. KB15. For siratro the following strains were efficient: *Paraburkholderia* sp KB15, P TUBERUM, HC1.BA, and *Bradyrhizobium* 32AAV, 10BB, PEAR76, ARG62, ARG68, CHAM227, LEO121, LEO170, RP7B, and FP1C. Of the strains tested (Table 1), only *Bradyrhizobium* strain Rp7B, originally isolated from nodules of *Arachis hypogea* were found to be compatible with lucerne (Table 1). Significant differences were observed among the isolates in terms of nodulation and plant growth promotion based on the parameters recorded and many of the isolates resulted in statistically significant ($p<0.05$) growth promotion compared to the uninoculated control.

Both siratro and cowpea were nodulated by almost the same bacterial strains such as *Bradyrhizobium* sp 10BB, CHAM227, 32AAV, ARG62, ARG68, LEO121, LEO170 and one *Paraburkholderia* sp. KB15. The strain which nodulated lucerne was also found to be efficient on siratro showing the promiscuous nature of the strains.

Five isolates were selected, after statistical analysis, as the best performing strains in terms of shoot fresh weight, root fresh weight and number of nodules formed on cowpea (Table 5). Compared to all other strains, inoculation with *Bradyrhizobium* sp.10BB in the glasshouse test resulted in the most statistically significant ($p<0.05$) increase in the number of nodules formed and accumulated biomass followed by *Bradyrhizobium* ARG68 and CHAM227 ($p<0.05$). On the other hand, uninoculated control cowpea plants grew more slowly than the inoculated plants. *Paraburkholderia* strain KB15 was also able to form nodules, although it was not as strong as compared to *Bradyrhizobium* 10BB strain but it could form nodules and the leaves were also green compared to control. Inoculation with strains 10BB and CHAM227 resulted in significantly more root dry weight in comparison with other strains and the uninoculated control (Table 5, Figure 9).

The results obtained in this study confirmed the generally accepted promiscuous nature of cowpea which form nitrogen fixing root nodules with a diverse group of symbiotic bacteria mainly with members of the slow growing *Bradyrhizobium* as well as *Paraburkholderia*. In a previous experiment, glasshouse nodulation screening conducted by Guimaraes at al. (2012) resulted in nodulation of cowpea by 62, out of 119 different strains of rhizobia isolated from agricultural soils in Western Amazon. According to the finding by these researchers, the strains mainly belong to the genus

Bradyrhizobium, but also showed that they are genetically very diverse with high species diversity. The fact that *Paraburkholderia* strain KB15 nodulated cowpea in the current study is supported by a previous finding that reported the nodulation of cowpea by certain *Burkholderia* strains (Guimaraes et al., 2012).

With regard to the glasshouse nodulation experiment in siratro, 13 strains were able to nodulate this forage legume (Table 6). *Bradyrhizobium* strains 10BB and CHAM227 resulted in statistically significant results in terms of nodulation and growth enhancement ($p<0.05$) in the glasshouse inoculation test as compared to other strains. They were followed by *Bradyrhizobium* strains PEAR76 and ARG62. Many of the other tested strains also nodulated this legume efficiently, but differ in the number of nodules formed as observed during harvesting.

Several reports exist that siratro is a promiscuous legume host and forms symbiosis with *Bradyrhizobium*, *Rhizobium* and *Sinorhizobium* species (Lima et al., 2009). In other words, the nodulation of siratro is not only confined to the microsymbionts of *Bradyrhizobium*. Like cowpea, siratro also shows varying degrees of promiscuity but unlike cowpea, it is nodulated by a wide range of rhizobium species including both the alpha and beta proteobacteria. For instance, in a study by Angus et al. (2013), siratro was nodulated by the *Paraburkholderia tuberum* STM678 and other *Paraburkholderia* strains and this important legume could be used as an excellent model in a study of the legume- rhizobium symbiosis under a wide range of environmental conditions. In our study too, we have found that two β-rhizobial strains: *Paraburkholderia steynii* and *Paraburkholderia tuberum* were symbiotically effective and compatible with siratro as indicated by plant vigour with green leaves and more developed shoots.

Unlike the nodulation screening on cowpea and siratro, only one of the tested bacteria nodulated lucerne under glasshouse conditions. Lucerne, which is commonly known for its nodulation specificity and affiliation with *Sinorhizobium* species (*Ensifer*) (Liu 2014), was effectively nodulated by *Bradyrhizobium* strain RP7B. Judging by the very high specificity of lucerne to *Sinorhizobium* (*Ensifer* spp), this nodulation by *Bradyrhizobium* strain RP7B in the current study warrants further detailed glasshouse nodulation authentication study, as well as additional molecular characterization of the bacterial strain to elucidate the symbiotic genes involved in this nodulation.

From all the strains used in this study, two strains, *Paraburkholderia* N362 and *Paraburkholderia aspalathi* failed to nodulate all three legume species. Generally, the

statistically higher plant biomass and effective nodulation (several pink nodules) of the cowpea, siratro and lucerne by many of the wild strains in this study could be an indication that these strains have the potential to improve legume productivity with their efficient nitrogen fixation and growth promotion ability.

4.5. Field screening trial

4.5.1. Soil physicochemical properties

The experiment was conducted at the Zeekoegat experimental station in Roodeplaat East of Pretoria, which contain soil with a texture of clay loamy. The exchangeable cations of the soil were Mn 37. 7 Mg/kg; P 16.824 Mg/kg; N-NO_3 14.022 mg/kg; Na 0.273 mg/kg; K 0.929 mg/kg; Ca 4.505 mg/kg.

The soil collected from the Zeekoegat area has good characteristics for bacterial growth. The soil texture was found to be clay loamy with a pH ranging from 6.8 to 7.1 at randomly selected soil sample sites, and have no negative effect on the root growth as well as the survival and functioning of the rhizobia. The physical and chemical properties of the soil determine the interaction of legume rhizobia symbiosis in the environment (Soares et al., 2014). It was found that Zeekoegat soil has high percentage of organic content. Highest amount of organic carbon and high CEC offers large nutrients reserve for survival of bacteria and their interaction (Hassen et al., 2014). Generally, the soil from Zeekoegat had low amounts of aluminium in which soils from randomly selected sites were found to contain Al concentrations ranging from 0.031 to 0.051cmol/kg. High level of aluminium disturb the growth of the plants, nodulation and root elongation. Level of soil P was found to be adequate for the bacterial survival. The soil appeared to have low level of Mn. The total extractable cations was found be high in soil sample with the average amount 14.022 mg/kg. The amount soil nitrate varies between the samples with the average amount of 14.02 mg/kg.

4.5.2. Rhizobial selection and field inoculation

Based on the results of the glasshouse trials, specific strains were selected for inclusion in the field experiment. The strains used for inoculating cowpea were *Bradyrhizobium* strains CHAM227, ARG68, 32AAV, 10BB and *Paraburkholderia* strain KB15. For siratro, the six strains used were *Bradyrhizobium* strains 10BB, CHAM227, FP1C, ARG62, 32AAV, and PEAR7. For lucerne, *Bradyrhizobium* strain RP7B was used. Six weeks after planting, plants were harvested for nodulation efficacy and plant biomass

evaluation. Data collected include total fresh weight, total dry weight and nodule number (Table 8; Figure 9).

For cowpea, inoculation with *Parabukholderia* strain KB15 and *Bradyrhizobium* strain ARG68 accumulated significantly ($p<0.05$) more dry weight than in the treatments with other strains and their roots have more nodules (Table 8; Figure 10). Besides the differences in dry weight, inoculation of the legumes under field condition resulted in the formation of some nodules, which were pink in colour showing that they have the ability to fix nitrogen. However, it has been detected that there is fierce competition for nodulation between the inoculated strain and indigenous rhizobia, which also formed nodules in the control treatments (see below). However, these nodules seem to be mostly competitive and not effective in fixing nitrogen as most of them look white in colour and the biomass measurement from the control treatments was significantly lower. *Bradyrhizobium* 32AAV appeared to be the poorest performer among the five isolates tested. Poor nodulation in plants inoculated by the wild strains may be due to high temperature experienced by the bacteria during planting which will have caused the denaturation of the bacterial nitrogenase enzyme.

In siratro, it was found that *Bradyrhizobium* strain FP1C has achieved high total dry weight whereas in the glasshouse strain 10BB was the one that achieved high total dry weight followed by *Bradyrhizobium* CHAM227 (Table 8; Figure 11). In the field trial, there were no statistical differences between 10BB and 32AAV in terms of nodule number. Highest nodule number is mostly linked to the shoot biomass and the chlorophyll content of the plant. *Bradyrhizobium* strains 10BB and FP1C also had similar nodule numbers. However, siratro generally had slow rate of germination which might have been due to soil type and other soil chemical properties.

Lucerne inoculated with *Bradyrhizobium* strain RP7B had statistically significant difference in the number of nodules compared to the uninoculated control ($p<0.05$) (Table 8; Figure 12). However, shoot dry weight and fresh weight were not statistically different from that of the control. Reports from other studies also indicated that lucerne is nodulated by *Ensifer melliloti* (Liu 2014). and also by other species of rhizobia (Wigley et al., 2015). Nevertheless, there is no data indicating the nodulation of lucerne by *Paraburkholderia* species so far.

Untreated soil contains high number of indigenous rhizobia, however the inoculated plants showed significant difference in terms of most of the parameters measured

compared to uninoculated control. Similar results were reported by Kyei-boahen et al. (2017) in which rhizobial strains inoculated in the field resulted in significant improvement in the symbiotic properties and growth in cowpea compared to uninoculated treatments. Nodulation efficacy and shoot dry weight increase in legumes indicate the efficiency of the inoculated strains in fixing nitrogen. Nevertheless, indigenous soil rhizobia often affect the effectiveness of the introduced strain (Thies et al., 1991). In this study, some of the strains, such as *Bradyrhizobium* ARG68, were particularly highly competitive and such competiveness depends on the ability to tolerate various conditions such as pH, temperature, acidity and salinity and specificity of the strain.

Recently, the competitiveness for nodule formation and symbiotic performance of different *Burkholderi*a species (*now named Paraburkholderia*) including (*B. diazotrophica*, *B. mimosarum*, *B. phymatum*, *B. sabiae*, *B. symbiotica* and *B. tuberum)* has been conducted in the legumes cowpea, siratro and beans (Lardi et al., 2017). The result of the study indicated that one of the strains, except for *Burkholderia phymatum* was able to form nodules competitively in all three legumes. However, this experiment was conducted only in a defined controlled condition, thus no field inoculation data is available to date using *Paraburkholderia* strains to inoculate cowpea, siratro or lucerne. Therefore, the observation in our study that certain *Paraburkholderi*a strains were able to nodulate cowpea under field condition is the first report, which warrants further investigation on the nodulation genes and additional field work to prove and elucidate the symbiotic properties in detail.

4.6. Estimation of nitrogen fixation using the natural abundance technique

As part of the determination of the nitrogen fixation efficiency of the inoculated rhizobia in lucerne, cowpea and siratro under field condition, estimation of the nitrogen fixed from the atmosphere was conducted using the natural abundance technique. Tables 9 and 10 shows the amount of nitrogen assimilated from the nitrogen-fixing legume relative to the non-fixing reference plant, which is maize. The amount of nitrogen fixed by the legumes differed from one species to another.

In this studies, no significant differences were observed for cowpea in terms of the amount of nitrogen derived from the atmosphere ($p<0.05$) when inoculated with *Bradyrhizobium* strains a well as the control ($p<0.05$). *Paraburkholderia* KB15 fixed the highest amount of nitrogen when compared to other strains, and there was a positive

correlation between the nodule dry weight and nodule number (r=0.91). This strain was followed by *Bradyrhizobium* ARG68. There were no significant differences in the delta N between the inoculated plants, which indicates that the selected strains have positive impacts on BNF when use in the field (Guimaraes et al., 2008). However, there were significant differences in the amount of nitrogen fixed, indicating that the strains likely compete with those already established in the soil and that can be plant growth promoter to increase yield. To increase the efficacy of the tested strains, it will take time based on the amount of nitrogen fixed per plant, or alternatively, unless if the inoculation can be applied every time during planting. This was due to the high amount of high ^{15}N enrichment of the maize reference plant resulting in lower estimates of %Ndfa to the inoculated cowpea plant.

For siratro, significant differences were observed between plants inoculated with various *Bradyrhizobium* strains (Table 10). Those inoculated with strain 10BB had the highest amount of nitrogen derived from the atmosphere. Although the results were observed in the field and other strains having accumulated the same amount nitrogen derived from the atmosphere *Bradyrhizobium* strains (32AAV, CHAM227, FP1C and PEAR76). There was no significant differences in the N content accumulated by the plant inoculated with the selected strains. Similar results were also observed on soybean inoculated with *Bradyrhizobium* strains where there was no different in N accumulation between the strains tested (Guimaraes et al., 2008), although there was only one strains which accumulated high amount of N from the atmosphere. *Bradyrhizobium* 10BB strain was found to be the one fixing more nitrogen compared to other strains. Compatibility of the strain on the legume play a vital role in the process of biological nitrogen fixation and other climatic factors.

Lucerne plants inoculated with *Bradyrhizobium* were not significantly different from the control and reference in terms of the amount of nitrogen derived from the atmosphere and the plant N content. There were also no differences in the accumulation of ^{15}N enrichment between the non-fixing reference plant and the inoculated plants. These data indicate that the plants had access to similar N pools, or there was spatial variation in the ^{15}N abundance (Guimaraes et al., 2008). The reference plant shoot tissue showed positive values in ^{15}N abundance. The %Ndfa is mostly affected by competition of other fixing microorganisms in the environment (Sanford et al., 1999).

In most previous cases, natural abundance was used to estimate N_2 fixation by legumes (Sanford et al., 1994, 1995; Bolger et al., 1995; Peoples et al., 1995, and Riffkin et al.,

1999) even though the legumes differ in their ability to access nitrogen. The intake of N from the soil by these three legumes examined in the current study did not appear to increase nitrogen fixation because %Ndfa was not significantly different. Because the plants were showing growth and nodules, this can be due to the already existing nitrogen in soil. The distribution of the root, plant demand varies continuously and impacts root functioning (Unkovich 2000). In this study, habitat conditions played a vital role in the ability in adaptation rhizobacteria to increase N up take and nitrogen fixation with rhizobia isolated from wild legumes. BNF depend on the symbiotic relationship between the legume, rhizobia and environment (Moreira and Siqueira 2006). This is consistent with the results of the current study, where the amount of nitrogen fixed by the plant in the field on each legume were not significantly different. As legumes are the nitrogen fixers, they do not compete for mineral N with non-fixing plants (Sanford 1995), thus making it difficult to compare the sparing of N from the soil between the two plants as the reference plant also accumulated high amounts of nitrogen.

4.7. Detection of the *nodC* and *nifH* genes

To verify that the rhizobial strains contained at least one of the common nodulation (*nodC*) and nitrogen fixation genes (*nifH*), primers unique to these sequences were used for PCR-amplification. The presence of the *nodA* gene has already been detected for some of the *Bradyrhizobium* strains used in this study (Beukes et al., 2016). A 250 base pair (bp) fragment of the *nifH* gene was detected for *Bradyrhizobium* strains. A 300 bp fragment of the *nodC* genes was detected in *Bradyrhizobium* ARG62 and ARG68 (Figure 13). The *nifH* and *nodC* genes could not be amplified for the *Paraburkholderia* strains in this study because of the primers used. However, the genes encoding nodulation and nitrogen fixation have been identified in the genome sequences of these *Paraburkholderia* strains (as part of an ongoing PhD study at the university of Pretoria).

Amplified PCR product of *nifH* genes consensus sequences were used to draw a neighbour joining phylogenetic trees (Figure 14). All the clusters represent a monophyletic group of *nifH* genes of *Bradyrhizobium* divided into five major branches. Clade I was represented by only *Bradyrhizobium sp* 32AAV with 100% bootstrap support. The second clade represent three of the *Bradyrhizobium* strains (LEO121, 10BB, CHAM227) that form a monophyletic group with *B. viridifuturi* strain and SEMIA6146. Strains Arg62 and Arg68 were closely related in their *nifH* sequences.

In this study, the phylogenetic tree constructed from the *nifH* sequences represent a well-supported group of *Bradyrhizobium* species that were isolated from different legume species. This is in accordance with the report by Moulin et al. (2004) and Stepkowski et al. (2007) who showed that all diazotrophic bacteria contain *nifH* genes. It was also indicated in other studies that there is a high diversity in *Bradyrhizobium* species based on the *nod* and *nif* genes (Bala et al., 2003; Menna et al., 2009a and b; Menna and Hungria 2010).

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CHAPTER 5

GENERAL DISCUSSION

This study presents the characterization of a group of alpha and beta rhizobia isolated from different wild legumes of the Papilionoideae and Mimosoideae family and their nodulation ability on cultivated legumes. The characterization involves study of their symbiotic property (nodulation and nitrogen fixation) on selected cultivated legumes including cowpea, lucerne and siratro as well as how they respond to various environmental stresses. The bacterial strains were assessed *in-vitro* for their tolerance to various abiotic stresses such as soil acidity, aluminium toxicity and different temperature conditions and responded differently to the different environmental stresses. They have been found tolerant to the various abiotic stresses and responded differently in their symbiotic efficiency.

Symbiotic nitrogen fixation depends on the interaction between the legume and its rhizobial symbionts for the formation of nodules. In poorly acidic soils, many *Paraburkholderia* strains were reported to nodulate legumes and were found tolerant to abiotic stresses with the ability to fix nitrogen in some wild legumes (Garau et al., 2009; Bontemps et al., 2010; Gyaneshwar et al., 2011). Tolerance to acidity by various *Bradyrhizobium* strains has been previously reported but most of the strains were adapted to a pH level of 7. Each isolate responded differently depending on the type of medium used. In Keyser defined medium, three isolates were not able to grow under pH of 4 and five strains were not able to grow under pH of 7 and 9. In this study, *Parabukholderia* strain KB15 was able to tolerate higher aluminium concentrations and was efficient in nodulating cowpea.

In addition to pH, temperature has an important effect on the growth of rhizobia related to the root hair infection and nodulation (Graham 1992). All the rhizobia in this study grew well at an optimum temperature range of 28-30^{0}C, suggesting that they are adapted to harsher environmental conditions. Moreover, most of the bacteria were able to grow at a temperature as low as 15^{0}C and as high as 37^{0}C, as these rhizobia were isolated from the root nodules of wild legumes, some of the isolates have shown different useful characteristics that enable them to grow and survive on harsh environmental conditions. In this study *Paraburkholeria* strain KB15 was capable of

surviving under various conditions namely salinity, acidity and temperatures tested in the laboratory and it was able to nodulate cowpea in the glasshouse.

The legume crops cowpea, lucerne and siratro that provide food and forage for animal and human consumption face many challenges that result in huge crop losses. Among these challenges are various abiotic stresses such as salinity, drought, and temperature. The nodulation and nitrogen fixation response of the legumes after inoculation with different strains of *Paraburkholderia* and *Bradyrhizobium* was slightly different between the glasshouse and field trials. This is mostly because of the soil physicochemical characteristics at the Zeekeogat experimental station where the field inoculation trial was conducted. Therefore, there were a number of limiting factors that created an unfavourable condition for the survival and functioning of most of the rhizobial strains tested. However, some of the effective isolates from the glasshouse screening experiments (e.g., *Bradyrhizobium* strains ARG68 and 10BB) have shown effective nodulation and improved growth in the field inoculation trial on cowpea and in siratro. The *Paraburkholderia* strain KB15 and *Bradyrhizobium* strains 10BB, CHAM227, 32AAV have beneficial plant growth promoting traits to withstand abiotic stress and production of ACC. Soares et al. (2014) reported that *Bradyrhizobium* strains UFLA3-164, UFLA3-153 and a strain of *Paraburkholderia* could perform best in tolerance to abiotic stress and were good competitors to indigenous strains. Santos et al. (2013) also reported that there was nodulation on siratro under high temperatures and water deficits.

Cowpea is not only used as food, but also a forage and is mostly nodulated by *Bradyrhizobium* to increase growth (Yoseph et al., 2017). However, should some of the strains from this study be applied as inoculants, they will have to be applied more than once to ensure that resident native rhizobia in the soil do not outcompete them for nodule occupancy.

Apart from fixing atmospheric nitrogen, rhizobia also have also such important PGPR properties such as phosphate solubilisation and production of the enzyme ACC, siderophore and IAA production (Bashan and Holguin 1997). Therefore, production of ACC deaminase by the isolates in the current study might have a positive effect on the nodulation and nitrogen fixation resulted in increased shoot and root growth on cowpea, siratro and lucerne, thus releasing plant stress and improving growth. Not all isolates have the ability to solubilize phosphorus, their other plant growth promoting traits may increase nutrient availability to the plants in the rhizosphere (Vessey 2003). This

particularly include phosphate solubilization abilities of the *Paraburkholderia* (WK1.1f, KB15, N362) and *Bradyrhizobium* (32AAV and CHAM227) strains examined and their corresponding positive impact on the growth of cowpea and siratro.

Soil is an important component for plant growth. However, infertile soil negatively affects such growth as the legume-rhizobia symbiotic association largely depends on the fertility and soil type. The results of the field trial in this study showed that there were native rhizobial populations present in the soils even though there was no history of cowpea and siratro cultivation on these sites. Nevertheless, the high amount of nitrogen fixed by the *Bradyrhizobium* strains ARG68, 10BB and RP7B (because of the 15N abundance assimilated in all three legumes) means their application to these legumes can improve plant growth. As mentioned above, the bacterial strains can enhance plant growth in number of ways: tolerance to abiotic stress such as salinity, temperature and aluminium toxicity, improving iron availability, reducing the amount of ethylene level as well as phosphorus solubilisation.

The *Bradyrhizobium* strains in the current study (RP7B, 10BB, CHAM227, 32AAV, ARG68 and ARG62) were able to nodulate more than one legume, which shows the promiscuous nature of the strains as observed under both glasshouse and field condition. Strain 10BB was originally isolated from *Acacia dealbata* grown in South Africa, produced ACC-deaminase, and was able to nodulate cowpea and siratro effectively, followed by strain CHAM 227 that was originally isolated from a species of *Chamacrista* in South Africa. Neither of these strains were able to nodulate lucerne, which was only nodulated by *Bradyrhizobium* strain RP7B (originating from *Arachis hypogea*). *Bradyrhizobium* strains 10BB and CHAM227 are not phylogenetically related but they belong to the *Bradyrhizobium japonicum* lineage (Beukes et al., 2016), which is also the case for strain RP7B, although it is closely related to *B. japocum* strain CCBAU 45002 in its *nifH* gene. It was reported by Angus et al. (2013) that the ability to nodulate more than one species may be due to flavonoids produced by the plant. *Bradyrhizobium* strain RP7B performed better in the field, and this might be based on its tolerance to various abiotic stress and its ability to compete with other native rhizobia in the soil. There are several legume species which are promiscuously nodulated by different groups of rhizobia, while a number of others are very specific in their rhizobial requirement for nodulation (Ferro et al., 2000; Laranjo et al., 2014)

In this study, the characterised *nifH* gene sequences were very similar among the *Bradyrhizobium* strains analysed. This is consistent with the conserved function of the

protein encoded by this gene, where it is essential for the conversion of nitrogen to ammonia (Fischer 1994; Smith and Gallon 1993). The same is also true for the *nodC* gene, which encodes an N-acetylglucosaminyltransferase involved in the production of the Nod factor recognized by the host legume (Schlaman et al., 1998). Because of the highly essential function of the protein encoded, these genes were very similar among the strains examined. Their presence is also consistent with the fact that they were all able to nodulate the legumes tested and were able to establish efficient nitrogen fixing symbiosis with them.

Many studies have investigated inoculants for tolerating harsh conditions on different legumes around the world (Dita et al., 2006), but their exploitation in agriculture for replacing chemical fertilisers is very slow (Abd-Alla et al., 2017). This may be due to a lack of information to farmers, especially in the rural areas and developing countries. Still, more development of isolation of strains is needed because some legumes prefer a specific symbiont. Inoculation of crop legumes with wild strains may be useful because they have the ability to fix nitrogen and survive to various conditions where the crop is planted. This is a cheaper method, which can be used and more sustainable for agriculture to improve soil fertility and restore the soil damaged by agricultural chemical fertilizers applied to improve growth.

Plant biotechnology has become an important aspect in agriculture by bringing ideas for improving agriculture. Biotechnology approaches have provided information and has worked on varieties of food and forage legumes in terms of tolerance to abiotic stress, plant growth, nodulation and nitrogen fixation.

As hypothesized in section 1.4 above, it was found out that one *Bradyrhizobium* strain isolated from *Arachis hypogea* was able to nodulate both lucerne and siratro. Almost 81% of wild strains used in this study were able to nodulate siratro and 54% of the strains were able to nodulate cowpea in the glasshouse. Interestingly, none of the wild rhizobia strains were able to form nodules on soybean during the initial screening (results not shown). This is due to the fact that South Africa soil probably lack rhizobial strains which are compatible in nodulating soybean plant (Bloem 1998). Based on the information collected in this study it can be concluded that bacterial strains tolerating stress, producing plant growth promoting traits and that is able to form nodules on crop legumes could pave a way in developing inoculants that are able to work under different conditions, which will be an alternative method of chemical fertilizers.

In a concluding remark, this MSc study focused on the screening of rhizobia initially isolated from wild legumes for their nodulation and nitrogen fixation efficacy on both food and forage legumes. It is the first study in South Africa that has generated important baseline data that can be used for further development of the rhizobial strains as legume inoculants for cowpea, siratro and lucerne. According to the data so far gathered, *Bradyrhizobium* strain RP7B has a huge potential for future inoculant development for lucerne cultivation while strains KB15,10BB and ARG68 have promising potential for siratro and cowpea respectively under abiotically stressed soils in South Africa. Judging by the fact that these effective isolates were isolated from the rhizosphere of wild growing legumes, it is anticipated that they could possibly nodulate and fix atmospheric nitrogen on other cultivated legumes that grow under harsh environmental conditions. Hence, the results obtained in this study warrant further nodulation screening study in these and other legumes of similar cross inoculation groups with cowpea, lucerne and siratro.

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TABLES

Table 1. List of rhizobia isolated form wild legumes used in this study.

Isolate	Original legume host	Subfamily
Paraburkholderia virgiliae N362^{T}	*Virgillia aroboides*	Papilionoideae
Paraburkholderia aspalathi VG1CT/LGM2731^{T}	*Aspalaths abietina*	Papilionoideae
Paraburkhoderia kirstenboschensis KB15^{T}	*Hypocalyptus sophoroides, H. oxalidifolius, H. coluteoidis, virgillia oroboides*	Papilionoideae
Paraburkholderia tuberum STM678/LMG21444^{T}	*Cyclopia falcate, C.galiodes, C.genistoides, C intermedia, C pubescens*	Papilionoideae
Paraburkholderia vachelliae 44.1^{T}/LMG28730^{T}	*Vachellia karroo*	Mimosoideae
Paraburkholderia steynii HC1.ba^{T}/LMG28730^{T}	*Hypocalyptus sophoroides*	Papilionodeae
Paraburkholderia sophoroidis WK1.1f^{T}/LMG28731^{T}	*Hypoatalyptus sophoroides*	Papilionoideae
Bradyrhizobium sp. 32AAV	*Acacia mearnsii*	Mimosoideae
Bradyrhizobium sp. 10BB	*Acacia dealbata*	Mimosoideae
Bradyrhizobium sp. Pear76	*Pearson obovata*	Papilionoideae
Bradyrhizobium sp. Arg62	*Argyrobium sericeum*	Papilionoideae
Bradyrhizobium sp. Arg68	*Argyrolobium sericeum*	Papilionoideae
Bradyrhizobium sp. Cham227	*Chamaecrista sp.*	Papilionoideae
Bradyrhizobium sp. Leo121	*Leobordea Divaricate*	Papilionoideae
Bradyrhizobium sp. Leo170	*Leobordea lanceolata*	Papilionoideae
Bradyrhizobium sp. Rp7b	*Arachis hypogaea*	Papilionoideae
Bradyrhizobium sp. Fp1c	*Arachis hypogeae*	Papilionoideae

Table 2. Physiological characterization of bacterial isolates.

Isolate	Catalase test	Oxidase test	O-F test	Motility	Acidity / alkalinity
P. virgiliae N362^T	+	+	+	+	Acid
P. aspalathi VG1CT/LGM2731^T	-	-	+	+	Acid
P. kirstenboschensis KB15^T	-	-	-	+	Acid
P. tuberum STM678/LMG21444^T	-	-	-	+	Alkaline
P. steynii HC1.ba^T/LMG28730^T	-	-	-	+	Alkaline
P. sophoroidis WK1.1f^T/LMG28731^T	+	+	+	+	Acid
Bradyrhizobium sp. 32AAV	+	-	+	+	Acid
Bradyrhizobium sp. 10BB	-	+	-	+	Alkaline
Bradyrhizobium sp. Pear76	+	+	+	+	Acid
Bradyrhizobium sp. Arg62	+	+	-	+	Alkaline
Bradyrhizobium sp. Arg68	-	+	+	+	Alkaline
Bradyrhizobium sp. Cham227	+	+	-	+	Alkaline
Bradyrhizobium sp. Leo121	+	+	-	+	Alkaline
Bradyrhizobium sp. Leo170	+	+	-	+	Alkaline
Bradyrhizobium sp. Rp7b	-	+	-	+	Alkaline
Bradyrhizobium sp. Fp1c	+	-	+	+	Acid

(+) catalase, oxidase, O-F test, motility, and (-) no reaction. The isolates produce acid or alkaline reaction.

Table 3. Evaluation of the bacterial growth at various conditions of acidity, temperature, salinity, and aluminium toxicity.

Isolate	Ph			Temperature (^{0}c)			Salinity (NaCl)			Aluminium $Al_2(SO4)$ concentration	
	4	7	9	15^{0}c	28^{0}c	37^{0}c	0.5%	1%	1.5%	0.04g $Al_2(SO_4)$	0.02g $Al_2(SO_4)$
P. virgiliae N362	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++
P. aspalathi VG/LGM2731^T	-	-	-	-	+	-	++	+	+	-	-
P. kirstenboschensisKB15	+++	+++	+++	++	+++	+++	++	++	+	+++	+++
P. tuberum STM678/LGM2144	-	+	+	-	++	-	+	+	+	+	-
P. steynii HC1.BA	-	-	-	-	++	-	+	+	+	-	-
P. sophoroidisWK1.1F	-	++	++	-	++	+++	+	++	+	+++	+++
Bradyrhizobium sp. 32AAV	+	++	+	++	++	++	-	-	-	++	++
Bradyrhizobium sp. 10BB	+	+	+	++	++	++	-	-	-	++	++
Bradyrhizobium sp. Pear76	++	+	++	+	++	-	-	-	-	++	++
Bradyrhizobium sp. Arg62	++	++	++	+	++	++	+	+	-	++	++
Bradyrhizobium sp. Arg68	++	++	++	+	++	++	-	-	-	+	+
Bradyrhizobium sp. Cham22	-	++	++	+	++	++	-	-	-	+	+
Bradyrhizobium sp. Leo121	+	++	++	++	++	++	-	-	-	+	+
Bradyrhizobium sp. Leo170	+	++	++	++	++	++	-	-	-	+	+
Bradyrhizobium sp. Rp7b	+	+	+	+	+	+	+	+	+	++	++
Bradyrhizobium sp. Fp1c	+	+	+	+	+	+	+	+	+	+	+
Control	-	-	-	-	-	-	-	-	-	-	-

Slight growth (+), moderate growth (++), effective growth (+++), no growth (-)

Table 4. Results recorded selected major in-vitro for plant growth promoting traits.

Isolate	Siderophore production	Phosphate solubilisation	ACC deaminase	Indole-3-acetic acid
P. virgiliae N362^T	-	++	++	-
P. aspalathi VG1CT/LGM2731^T	-	+	++	-
P. kistenboschensis KB15^T	-	+	++	-
P. tuberum STM678/LMG21444^T	-	_	++	-
P. steynii HC1.ba^T/LMG28730^T	-	_	++	-
P. sophoroidis WK1.1f^T/LMG28731^T	-	+++	++	-
Bradyrhizobium sp. 32AAV	-	_	++	-
Bradyrhizobium sp. 10BB	-	_	++	-
Bradyrhizobium sp. Pear76	-	_	++	-
Bradyrhizobium sp. Arg62	-	_	++	-
Bradyrhizobium sp. Arg68	-	_	++	-
Bradyrhizobium sp. Cham22	-	++	++	-
Bradyrhizobium sp. Leo121	-	_	++	-
Bradyrhizobium sp. Leo170	-	_	++	-
Bradyrhizobium sp. Rp7b	-	_	++	-
Bradyrhizobium sp. Fp1c	-	_	++	-
Control	-	-	-	-

*Slight growth (+), moderate growth (++), effective growth (+++), no growth (-).

Table 5. Nodulation screening and measurements of selected growth promotion parameters in cowpea (*Vigna unguiculata*) after inoculation with rhizobial isolates from wild legumes in a glasshouse experiment.

Isolate	SFW(g)	SDW(g)	RFW(g)	RDW(g)	NDW(g)	NN
P. virgiliae N362^{T}/LMG	3.709^{bcd}	0.524^{efg}	1.225^{abc}	0.349^{efg}	0.037^{cd}	21.667^{bc}
P. aspalathi VG1CT/LGM2731^{T}	2.894^{cdef}	0.357^{efg}	1.215^{abc}	0.231^{fgh}	0.000^{e}	0.000^{d}
P. kirstenboschensis KB15^{T}	2.366^{defg}	0.676^{cde}	2.119^{ab}	0.603^{cd}	0.050^{bc}	19.333^{dc}
P. tuberum STM678/LMG21444^{T}	2.843^{cdef}	0.268^{fg}	1.534^{ab}	0.526^{cde}	0.019^{a}	8.333^{cd}
P. steynii HC1.ba^{T}/LMG28730^{T}	3.413^{bce}	1.029^{bc}	1.888^{ab}	0.587^{cde}	0.028^{cde}	12.000^{dc}
P. sophoroidis WK1.1f^{T}/LMG28731^{T}	3.181^{bcd}	0.864^{cd}	1.836^{ab}	0.379^{defg}	0.030^{cde}	12.667^{bc}
Bradyrhizobium sp. 32AAV	5.214^{ab}	0.425^{efg}	2.028^{ab}	0.608^{bc}	0.050^{bc}	31.000^{ab}
Bradyrhizobium sp. 10BB	6.322^{a}	1.545^{a}	1.496^{ab}	0.588^{cde}	0.016^{a}	45.000^{a}
Bradyrhizobium sp. Pear76	2.547^{defg}	0.252^{fg}	1.855^{ab}	0.432^{def}	0.019^{a}	7.000^{cd}
Bradyrhizobium sp. Arg62	3.212^{bcde}	0.369^{efg}	2.492^{a}	0.939^{a}	0.048^{bcd}	31.333^{a}
Bradyrhizobium sp. Arg68	5.020^{abc}	1.373^{ab}	1.496^{abc}	0.265^{fgh}	0.0426^{bcd}	42.333^{a}
Bradyrhizobium sp. Cham227	3.051^{bcdef}	0.311^{efg}	1.788^{ab}	0.354^{efg}	0.073^{b}	31.667^{ab}
Bradyrhizobium sp. Leo121	1.132^{fg}	0.483^{def}	1.526^{ab}	0.395^{def}	0.000^{e}	0.000^{d}
Bradyrhizobium sp. Leo170	3.049^{bcdef}	0.030^{g}	2.453^{a}	0.861^{ab}	0.017^{a}	22.000^{be}
Bradyrhizobium sp. Rp7b	1.420^{efg}	0.322^{efg}	1.541^{ab}	0.368^{defg}	0.000^{e}	0.000^{d}
Bradyrhizobium sp. Fp1c	0.302^{g}	0.035^{g}	0.186^{c}	0.059^{h}	0.000^{e}	0.000^{d}
Control	0.994^{fg}	0.166^{fg}	1.118^{abc}	0.150^{gh}	0.000^{e}	0.000^{d}
$LSD_{0.05}$	2.251	0.398	1.309	0.244	0.032	18.443
Pr>F	0.360	0.550	0.525	0.808	0.583	0.74

*SFW= Shoot Fresh Weight; SDW= Shoot Dry Weight; RFW= Root Fresh Weight; RDW= Root Dry Weight; NDW= nodule dry weight; NN= nodule number. Mean values are significantly different indicated by different letters within the column at $p<0.05$.

Table 6. Nodulation screening and measurements of selected growth promotion parameters in siratro (*Microptilium atropurpureum*) after inoculation with rhizobial isolates from wild legumes in a glasshouse experiment.

Rhizobia	SFW(g)	SDW(g)	RFW(g)	RDW(g)	NDW	NN
P. virgiliae N362^{T}/LMG	1.347^{efgh}	0.348^{ef}	3.014^{ab}	0.250^{cd}	0.057^{e}	42.667^{b}
P. aspalathi VG1CT/LGM2731^{T}	0.173^{h}	0.033^{f}	0.070^{e}	0.023^{d}	0.000	0.000^{e}
P. kirstenboschensis KB15^{T}	0.662^{fgh}	0.097^{ef}	0.208^{e}	0.041^{d}	0.016^{fg}	10.000^{de}
P. tuberum STM678/LMG21444^{T}	0.258^{h}	0.042^{f}	0.192^{e}	0.056^{d}	0.022^{fg}	12.333^{cd}
P. steynii HC1.ba^{T}/LMG28730^{T}	0.302^{h}	0.073^{ef}	0.147^{e}	0.035^{d}	0.033^{ef}	16.000^{cd}
P. sophoroidis WK1.1f^{T}/LMG28731^{T}	0.517^{gh}	0.058^{f}	0.492^{e}	0.071^{d}	0.034^{ef}	40.667^{b}
Bradyrhizobium sp. 32AAV	4.998^{bc}	0.475^{ef}	3.617^{a}	1.218^{a}	0.095^{cd}	53.333^{b}
Bradyrhizobium sp. 10BB	6.523^{a}	1.609^{ab}	2.322^{c}	0.611^{b}	0.106^{bc}	71.667^{a}
Bradyrhizobium sp. Pear76	4.827^{bc}	1.278^{bc}	1.497^{cd}	0.601^{b}	0.087^{cd}	51.000^{b}
Bradyrhizobium sp. Arg62	5.277^{ab}	0.983^{cd}	1.629^{c}	0.485^{bc}	0.103^{bc}	51.000^{b}
Bradyrhizobium sp. Arg68	4.939^{bc}	1.216^{bc}	1.746^{c}	0.464^{cd}	0.127^{ab}	34.532^{c}
Bradyrhizobium sp. Cham227	6.317^{ab}	1.038^{cd}	1.992^{c}	0.465^{bc}	0.142^{a}	69.667^{a}
Bradyrhizobium sp. Leo121	2.986^{de}	1.056^{c}	1.966^{c}	0.700^{b}	0.059^{de}	22.333^{cd}
Bradyrhizobium sp. Leo170	3.208^{cd}	0.977^{cd}	1.636^{c}	0.619^{b}	0.041^{ef}	20.667^{cd}
Bradyrhizobium sp. Rp7b	6.361^{ab}	1.983^{a}	1.725^{c}	0.507^{bc}	0.041^{c}	40.342^{c}
Bradyrhizobium sp. Fp1c	2.200^{def}	0.553^{de}	0.658^{de}	0.223^{cd}	0.083^{cd}	50.000^{b}
Control	0.366^{gh}	0.072^{ef}	0.269^{e}	0.040^{d}	0.000	0.000^{e}
$LSD_{0.05}$	1.648	0.488	0.855	0.3181	0.029	13.641
Pr>F	0.339	0.750	0.343	0.323	0.057	0.626

*SFW= Shoot Fresh Weight; SDW= Shoot Dry Weight; RFW= Root Fresh Weight; RDW= Root Dry Weight; NDW= nodule dry weight; NN= nodule number. Mean values are significantly different indicated by different letters within the column at $p<0.05$.

Table 7. Nodulation screening and measurements of selected growth promotion parameters in lucerne (*Medicago sativa*) after inoculation with rhizobial isolates from wild legumes in a glasshouse experiment.

Isolate	SFW(g)	SDW(g)	RFW(g)	RDW(g)	NDW(g)	NN
P. virgiliae N362^{T}/LMG	0.253^{b}	0.031^{cb}	0.210^{bc}	0.038^{bcde}	0. 000^{b}	0.000^{b}
P. aspalathi VG1CT/LGM2731^{T}	0.166^{b}	0.025^{cb}	0.243^{b}	0.0253^{cde}	0.000^{b}	0.000^{b}
P. kirstenboschensis KB15^{T}	0.367^{b}	0.050^{bc}	0.023^{b}	0.066^{ab}	0.000^{b}	0.000^{b}
P. tuberum STM678/LMG21444^{T}	0.169^{b}	0.021^{c}	0.022^{b}	0.051^{abcd}	0.000^{b}	0.000^{b}
P. steynii HC1.ba^{T}/LMG28730^{T}	0.027^{b}	0.028^{bc}	0.228^{b}	0.029^{abcd}	0.000^{b}	0.000^{b}
P sophoroidis WK1.1f^{T}/LMG28731^{T}	0.387^{b}	0.060^{b}	0.017^{bc}	0.020^{cde}	0.000^{b}	0.000^{b}
Bradyrhizobium sp. 32AAV	0.950^{b}	0.016^{c}	0.065^{c}	0.015^{cde}	0.000^{b}	0.000^{b}
Bradyrhizobium sp. 10BB	0.197^{b}	0.030^{bc}	0.258^{b}	0.034^{bcde}	0.000^{b}	0.000^{b}
Bradyrhizobium sp. Pear76	0.393^{b}	0.045^{bc}	0.263^{b}	0.034^{bcde}	0.000^{b}	0.000^{b}
Bradyrhizobium sp. Arg62	0.019^{b}	0.030^{cb}	0.235^{b}	0.052^{abcd}	0.000^{b}	0.000^{b}
Bradyrhizobium sp. Arg68	0.160^{b}	0.030^{cb}	0.167^{bc}	0.029^{abcd}	0.000^{b}	0.000^{b}
Bradyrhizobium sp. Cham227	0.193^{b}	0.029^{bc}	0.250^{b}	0.044^{bcde}	0.000^{b}	0.000^{b}
Bradyrhizobium sp. Leo121	0.130^{b}	0.017^{c}	0.172^{bc}	0.013^{de}	0.000^{b}	0.000^{b}
Bradyrhizobium sp. Leo170	0.140^{b}	0.017^{c}	0.216^{bc}	0.032^{bcde}	0.000^{b}	0.000^{b}
Bradyrhizobium sp. Rp7b	1.458^{a}	0.210^{a}	0.421^{a}	0.087^{a}	0.0163^{a}	15.666^{a}
Bradyrhizobium sp. Fp1c	0.142^{b}	0.020^{c}	0.198^{bc}	0.010^{c}	0.000^{b}	0.000^{b}
Control	0.158^{b}	0.023^{c}	0.153^{bc}	0.021^{cde}	0.000^{b}	0.000^{b}
$LSD_{0.05}$	2.036	0.036	0.153	0.039	0.426	1.232
Pr>F	0.191	0.052	0.045	0.095	0.426	0.975

*SFW = Shoot Fresh Weight; SDW = Shoot Dry Weight; RFW = Root Fresh Weight; RDW = Root Dry Weight; NDW = nodule dry weight; NN = nodule number. Mean values are significantly different indicated by different letters within the column at $p<0.05$.

Table 8. Total fresh and total dry weight, as well as the number of nodules found on roots of cowpea (*Vigna unguiculata*) and siratro (*Macroptilium artropurpureum*) and lucerne (*Medicago sativa*) after inoculation with rhizobia in the field.

Isolate	**Siratro**			**Cowpea**			**Lucerne**		
	Fresh (g)	*Dry (g)*	*Nodule number*	*Fresh (g)*	*Dry (g)*	*Nodule number*	*Fresh (g)*	*Dry (g)*	*Nodule number*
Bradyrhizobium sp 10BB	4.920^{abcd}	0.813^{bc}	5.000^{abcd}	47.35^{bc}	5.871^{cde}	14.000^{abcde}	-	-	
Bradyrhizobium sp FP1C	6.083^{abc}	1.106^{ab}	5.333^{abcd}	-	-	-	-	-	
Bradyrhizobium sp PEAR76	3.717^{cd}	0.395^{bc}	3.500^{bcdef}	-	-	-	-	-	
Bradyrhizobium sp 32AAV	4.370^{bcd}	0.869^{abc}	5.000^{abcd}	36.33^{c}	4.390^{de}	10.000^{cdef}	-	-	
Bradyrhizobiumsp Cham227	2.923^{bcd}	0.490^{bc}	5.300^{abcd}	50.84^{abc}	6.027^{bcde}	25.33^{ab}	-	-	
Bradyrhizobium sp Arg62	3.155^{bcd}	0.54^{bc}	2.667^{defg}	-	-	-	-	-	
Bradyrhizobium sp Arg68	-	-	-	50.62^{abc}	6.188^{abcd}	19.000^{abcde}	-	-	
Paraburkholderia sp KB15	-	-	-	51.46^{abc}	6.520^{abcd}	13.000^{abcde}	-	-	
Bradyrhizobium sp Rp7B							7.590^{a}	1.286^{ab}	6.000^{a}
Control	2.433^{cd}	0.431^{bc}	0.667^{fg}	34.28^{c}	4.220^{de}	11.333^{cdef}	5.677^{abc}	1.306^{ab}	0.000^{b}
$LSD_{0.05}$	3.423	0.565	2.045	*22.874*	*2.946*	*12.943*	5.56	1.304	3.737
Pr >F	0.938	0.437	0.962	*0.177*	*0.033*	*0.030*	5.566	0.685	0.766

Values are significantly different indicated by different letters within the column are significantly different at p<0.05.

Table 9. The mean values of nitrogen fixed derived from the atmosphere, nitrogen fixed and the delta N for lucerne and cowpea.

	Cowpea							Lucerne						
Isolates	**No of plant**	**Dry Mass**	**DM/5M^2**	**δ15N (‰)**	**Plant N content**	**%Ndfa**	**N fixed/ Kg/ha**	**No of Plant**	**Dry Mass**	**DM/5M^2**	**δ15N (‰)**	**Plant N content**	**%Ndfa**	**N Fixed/ Kg/ha**
32aav	21.333	7.540^{a}	142.54^{a}	6.363^{b}	29.237^{ab}	17.177^{a}	285.3^{a}	-	-	-	-	-	-	-
Cham227	22.333	8.830^{a}	146.56^{a}	6.833^{b}	26.697^{ab}	19.527^{a}	306.0^{a}	-	-	-	-	-	-	-
10bb	21.000	6.637^{ab}	85.90^{abc}	6.563^{b}	24.470^{ab}	15.017^{a}	229.9^{ab}	-	-	-	-	-	-	-
Kb15	20.33	7.027^{ab}	111.20^{ab}	5.860^{b}	25.437^{ab}	22.303^{a}	354.1^{a}	-	-	-	-	-	-	-
Arg68	21.333	9.153^{a}	155.73^{a}	6.316^{b}	33.420^{a}	17.593^{a}	334.9^{a}	-	-	-	-	-	-	-
RP7b	-	-	-	-	-	-	-	16.667^{a}	2.486^{ab}	40.223^{a}	8.020^{A}	9.873^{a}	16.417^{a}	46.81^{a}
Control	10.333	3.581^{b}	50.43^{bc}	6.083^{b}	15.893^{ab}	24.230^{a}	94.2^{ab}	13.333^{a}	1.506^{b}	20.483^{Bb}	6.77^{B}	9.850^{a}	14.337^{a}	21.77^{ab}
Ref plant	5.000	3.736^{b}	18.68^{c}	8.020^{a}	9.370^{b}	-	-	5.000^{b}	3.736^{a}	18.682	6.726	5.385	-	-
$LSD_{0.05}$	3.593	3.541	71.62	1.248	19.949	13.558	260.89	3.336	1.806	17.448	0.674	7.933	18.268	28.703
Pr>F	0.954	0.692	0.097.	0.638	0.0012	0.896	0.821	0.025	0.046	0.046	0.0097	0.032	0.026	0.672

Mean values are significantly different indicated by different letters within the column at p<0.05.

Table 10. The mean values of nitrogen fixed derived from the atmosphere, nitrogen fixed and the delta N for siratro.

Isolate	No of plant	DM	DM/5M^2	δ 15N (‰)	Plant N content	%Ndfa	N fixed
32aav	15.663^{bc}	0.990^{bc}	11.48^{b}	7.016^{bc}	3.693^{b}	13.057^{ab}	14.620^{ab}
Arg62	12.667^{cd}	1.523^{bc}	15.23^{ab}	7.476^{ab}	1.386^{c}	5.403^{bc}	1.232^{d}
Pear76	14.333^{bcd}	2.093^{abc}	20.69^{ab}	7.026^{bc}	3.993^{bc}	9.933^{ab}	12.421^{abc}
Fpic	17.333^{ab}	1.733^{bc}	18.45^{ab}	7.016^{bc}	2.660^{bc}	10.007^{ab}	9.037^{bcd}
10bb	20.000^{a}	2.156^{abc}	24.30^{ab}	6.516^{c}	3.840^{bc}	14.513^{a}	22.127^{a}
Cham227	16.667^{abc}	3.116^{ab}	26.45^{a}	6.926^{bc}	2.290^{bc}	13.570^{ab}	12.383^{abc}
Control	10.667^{d}	1.48^{bc}	14.83^{ab}	6.330^{c}	1.384^{c}	13.990^{ab}	4.237^{cd}
Ref1	5.000^{e}	3.736^{a}	18.45^{ab}	8.020	9.85^{a}	-	-
$LSD_{0.05}$	4.017	1.836	22.04	0.750	2.102	9.632	9.754
Pr>F	0.117	0.092	0.416	0.067	0.198	0.173	0.03

Mean values are significantly different indicated by different letters within the column at p<0.05.

FIGURES

Figure 1. Maize (*Zea mays L*) plant affected by abiotic stress in the field, especially drought and high temperatures (Gakpo 2018).

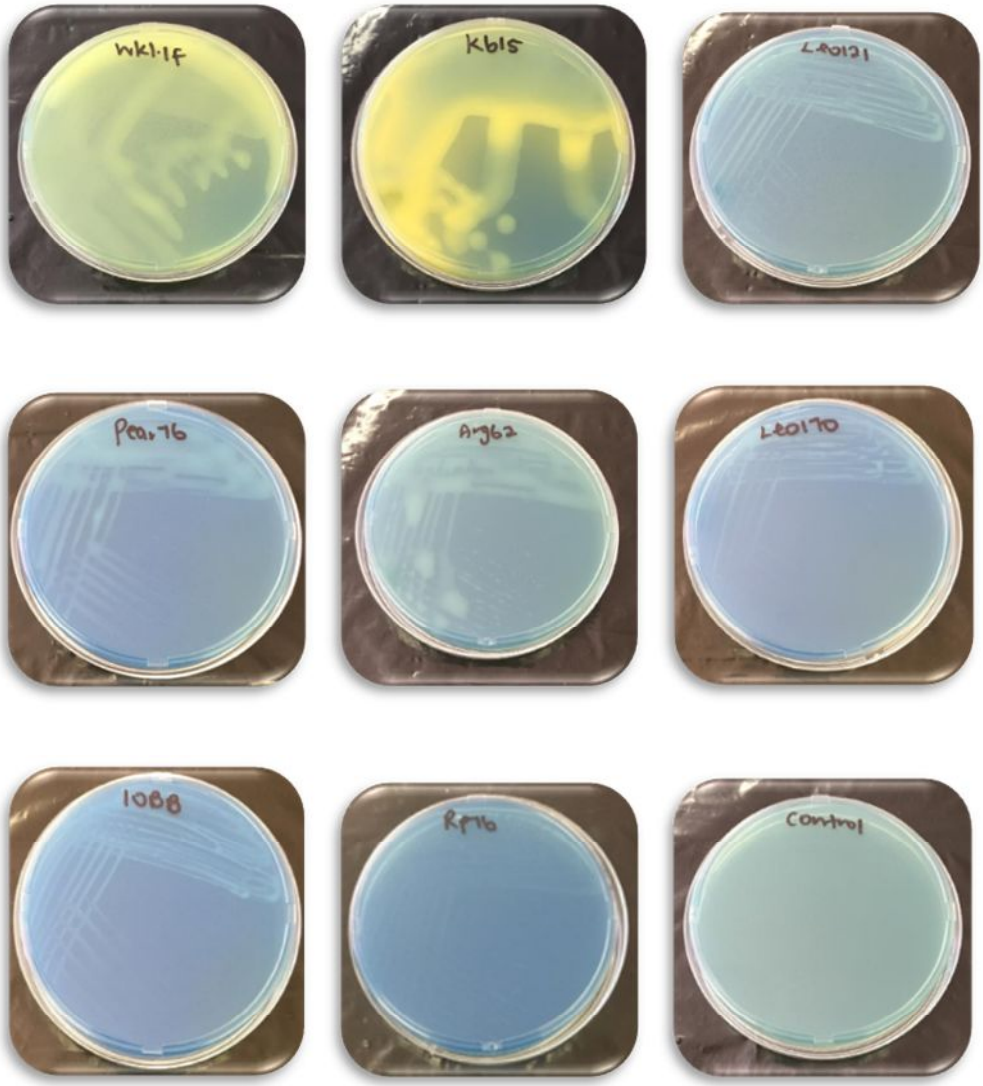

Figure 2. Growth of rhizobia producing different reaction on YMA-BTB medium for observing acid-alkaline reaction.

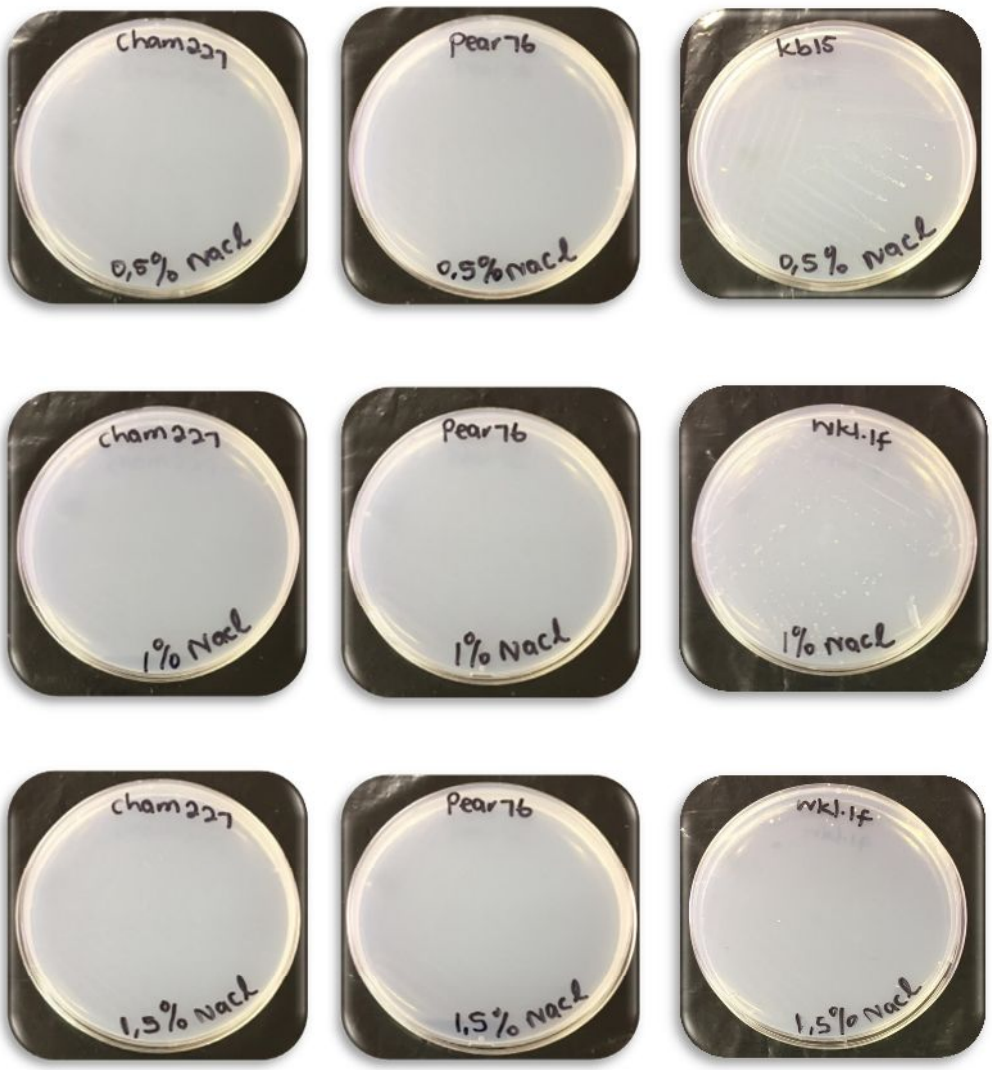

Figure 3. Rhizobial tolerance under different concentration of salts.

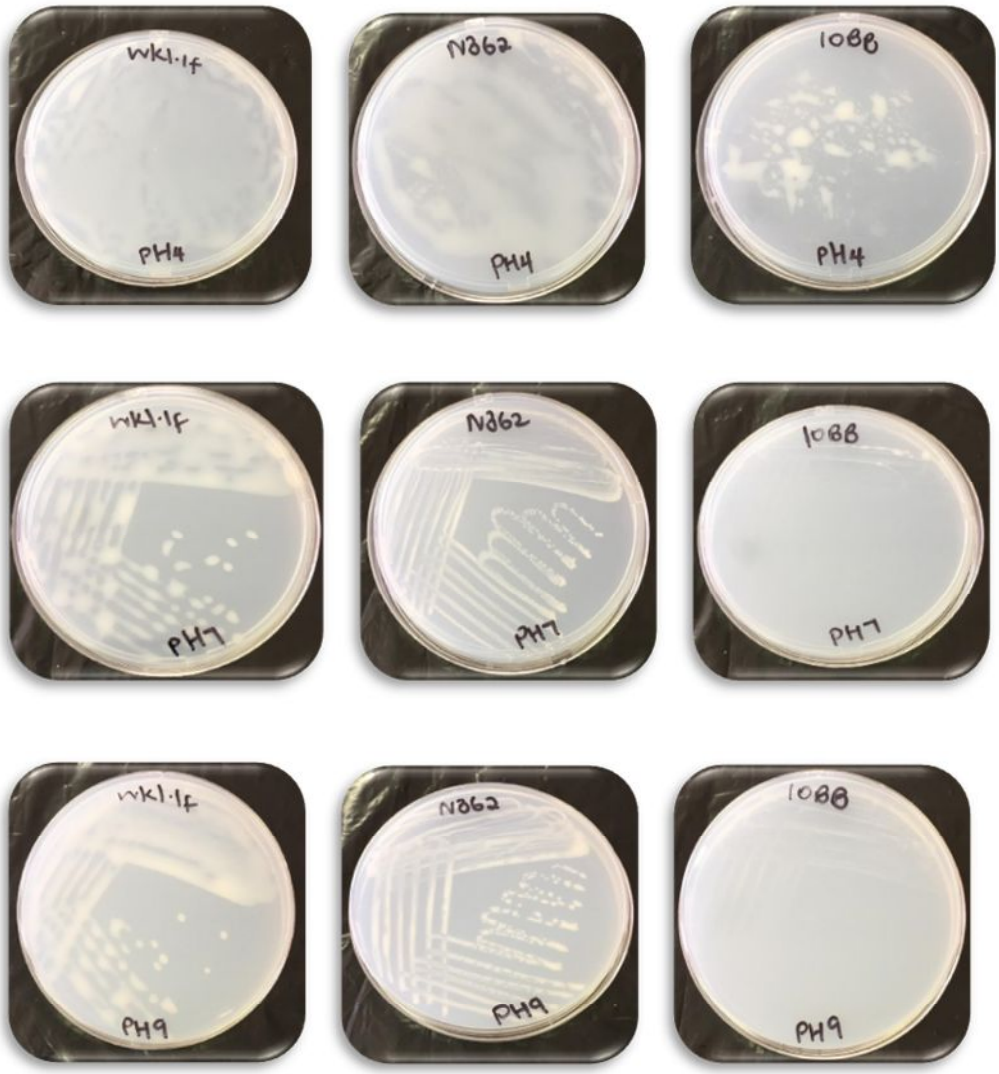

Figure 4. Growth of rhizobia strains on Keyser defined media adjusted to pH 9, 7 and 4 showing optimal growth.

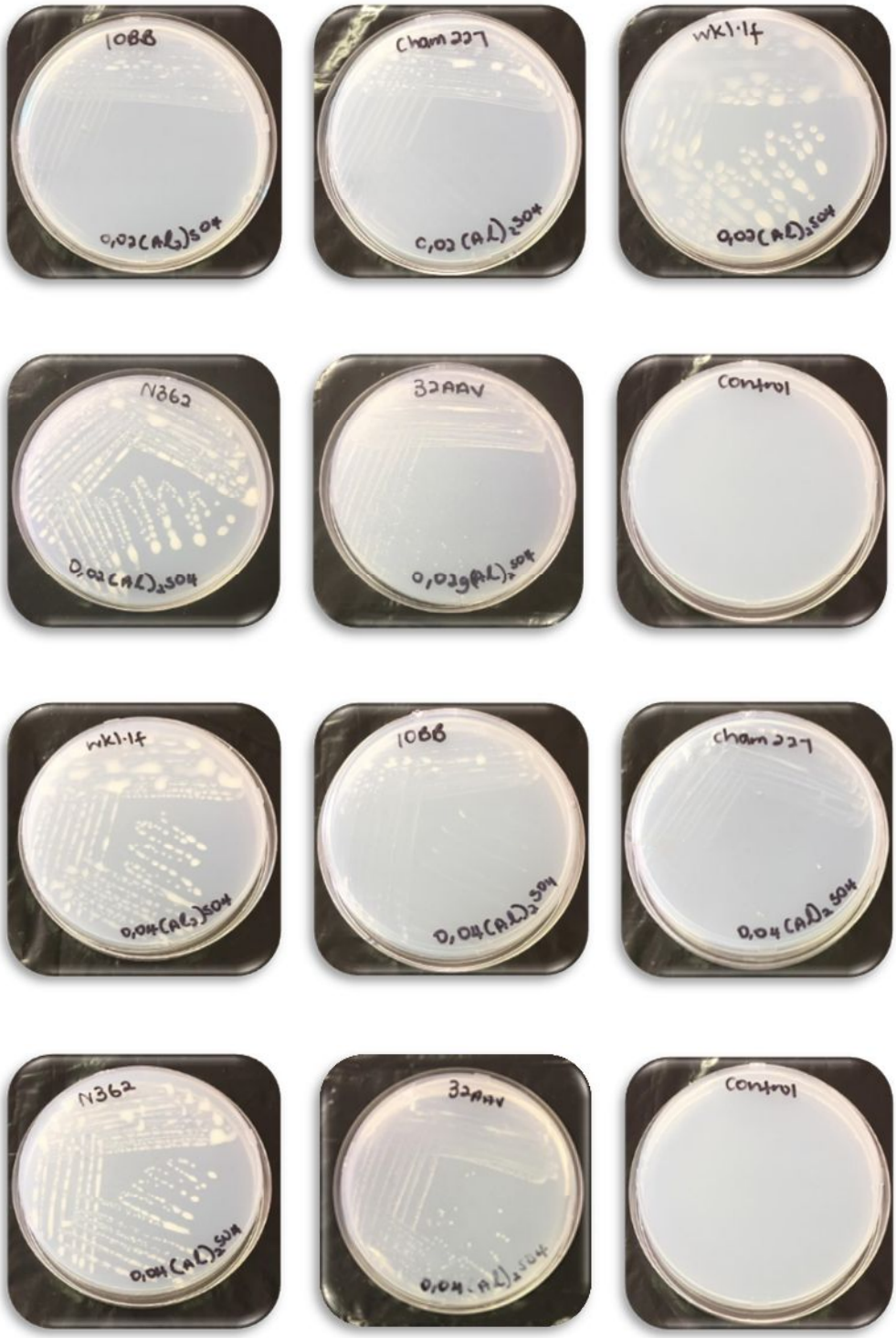

Figure 5. Detection of tolerance to aluminium toxicity of the bacterial isolates when grown under different concentration of aluminium on Keyser's defined medium**.**

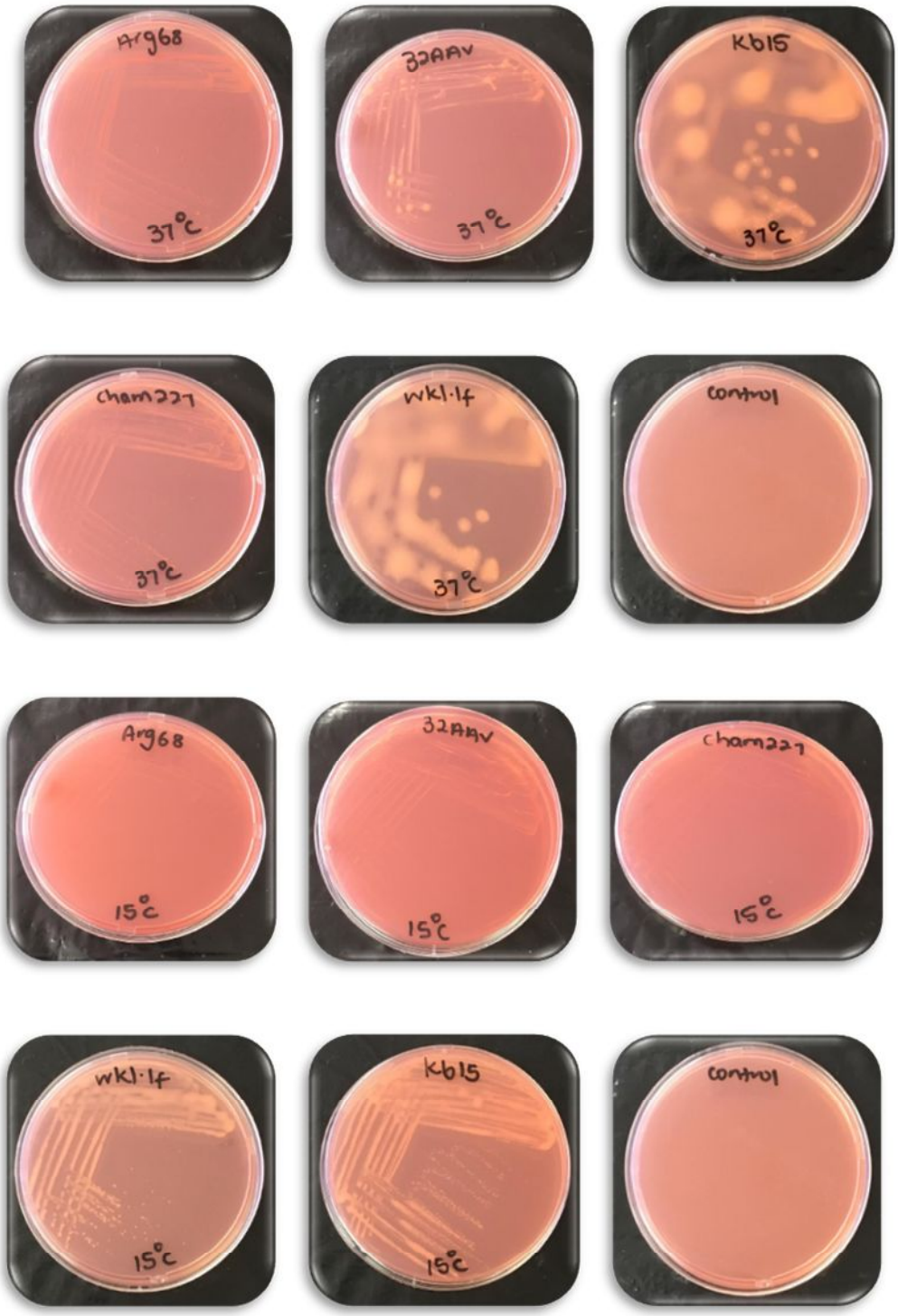

Figure 6. Detection of tolerance of the bacterial isolated to different temperatures (15^0C, 28^0C, 37^0C).

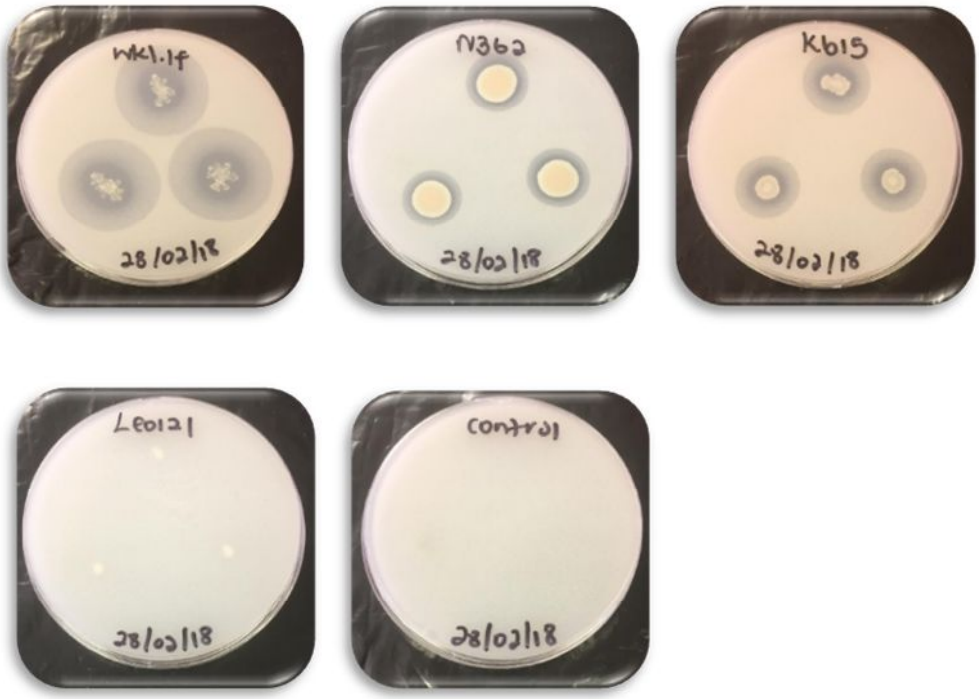

Figure 7. Clear halo zone by phosphate solubilizing *Paraburkholderia* and *Bradyrhizobium* strains grown on Pikovskaya media.

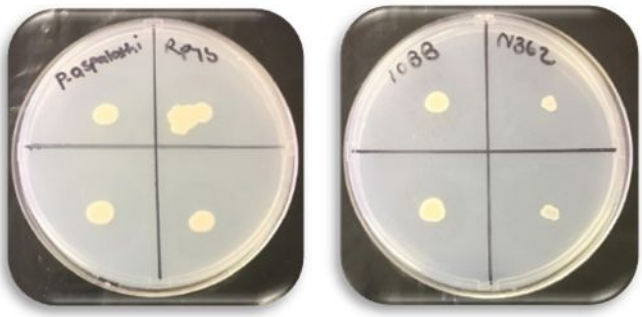

Figure 8. Bacterial screening ACC deaminase production.

Figure 9. Glasshouse test to evaluate the nodulation and nitrogen fixation efficiency of representative bacteria isolates on lucerne, siratro and cowpea. Lucerne inoculated with *Bradyrhizobium* strain RP7B (A and D). Siratro inoculated with *Bradyrhizobium* strain 10BB (B and E). Cowpea inoculated with 10BB strain (C and F).

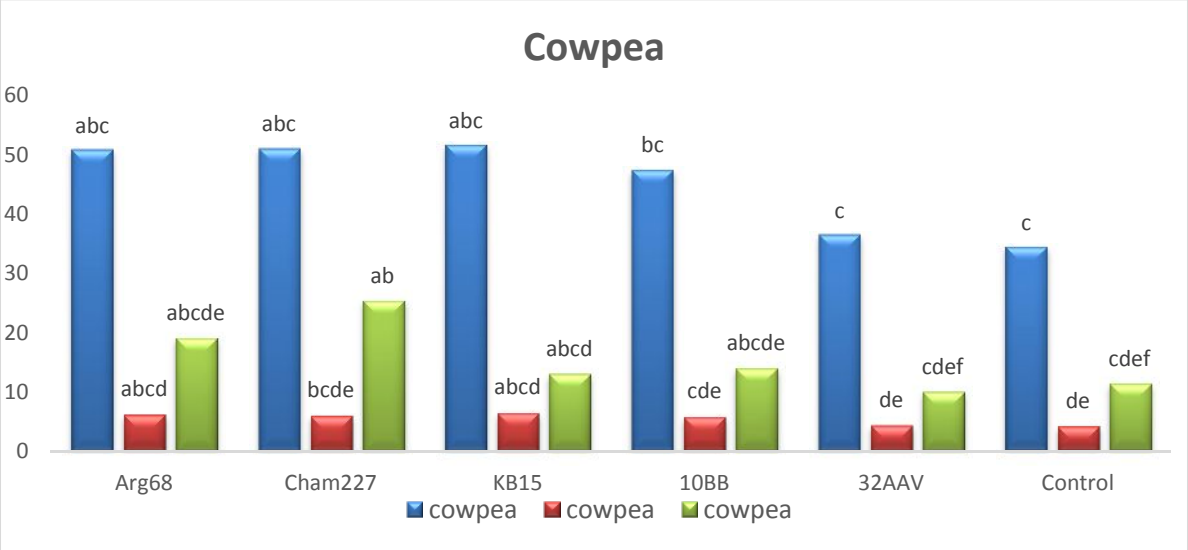

Figure 10. Effect of inoculation of cowpea with wild rhizobia on fresh weight (TFW) in gram, total dry weight (TDW) in gram and nodule number (NN) of five strains of rhizobia tested in the field. Different by letters on bars show significant difference when inoculated with different strains.

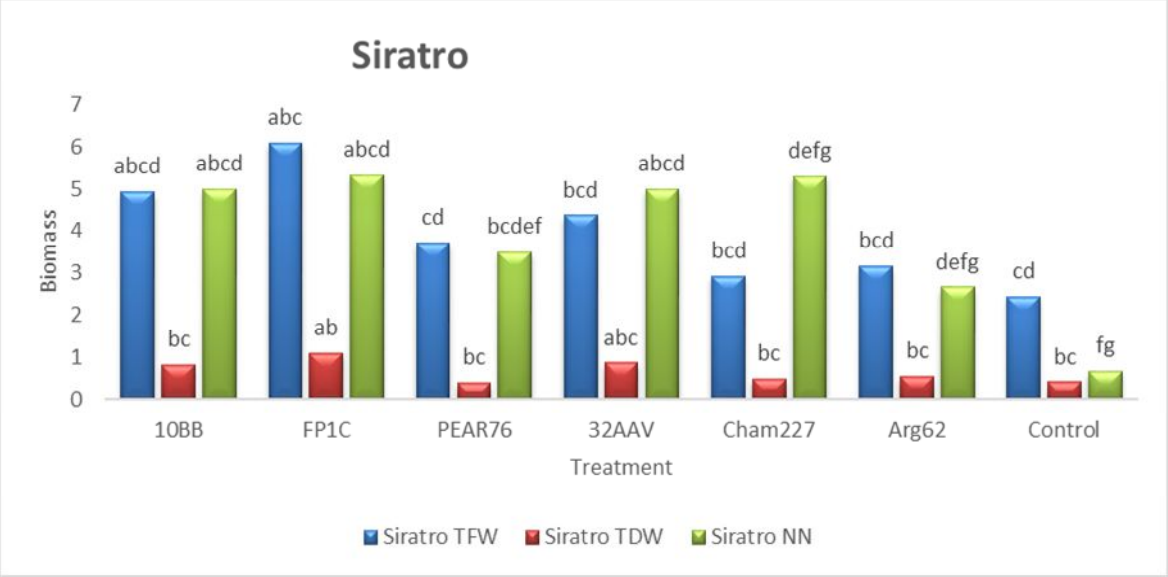

Figure 11. Effect of inoculation of siratro with wild rhizobia on total fresh weight (TFW) in gram, total dry weight (TDW) in gram and nodule number (NN) of six strains of rhizobia tested in the field. Different letters on bars show significant difference when inoculated with different strains.

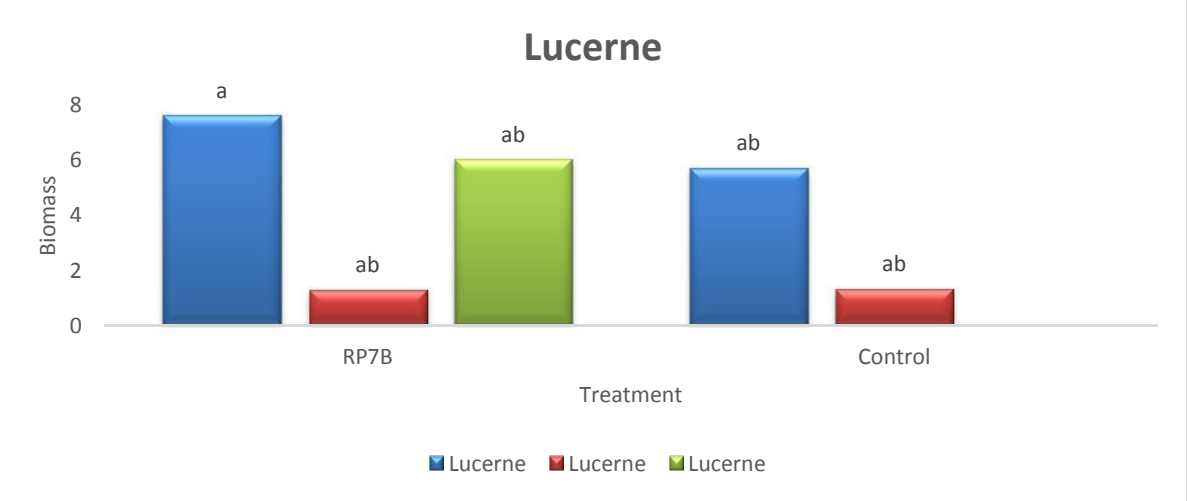

Figure 12. Effect of inoculation of lucerne with wild rhizobia on total fresh weight (TFW) in gram, total dry weight (TDW) in gram and nodule number (NN) of rhizobia tested in the field. Different letters on bars show significant difference when inoculated with strain.

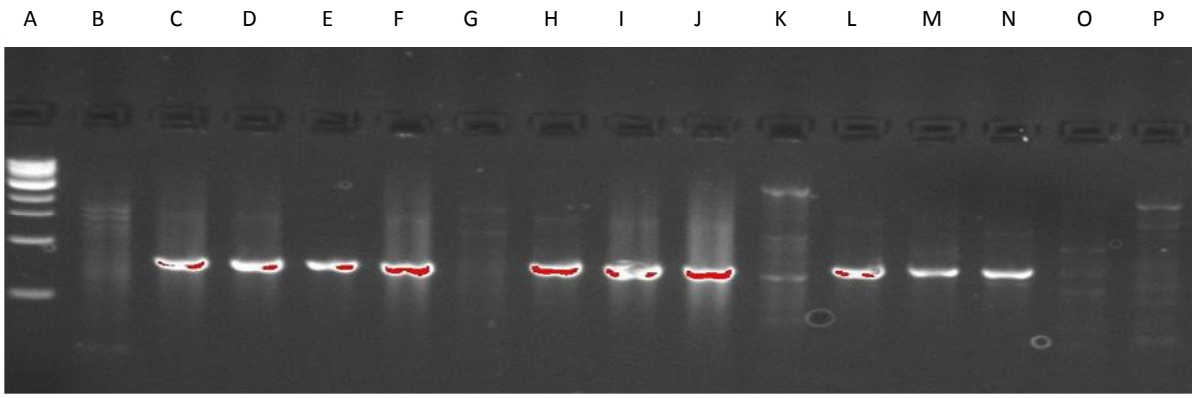

Figure 13. Agarose gel electrophoresis of the amplified *nifH* amplicons. A-1kb ladder; B-WK1.1f; C-32AAV; D-RP7B; E-PEAR76; F-10BB; G-KB15; H-ARG62; I-CHAM227; J-LEO121; K-N362; L-LEO170; M-ARG68; N-FP1C; O- *Paraburkholderia tuberum.*

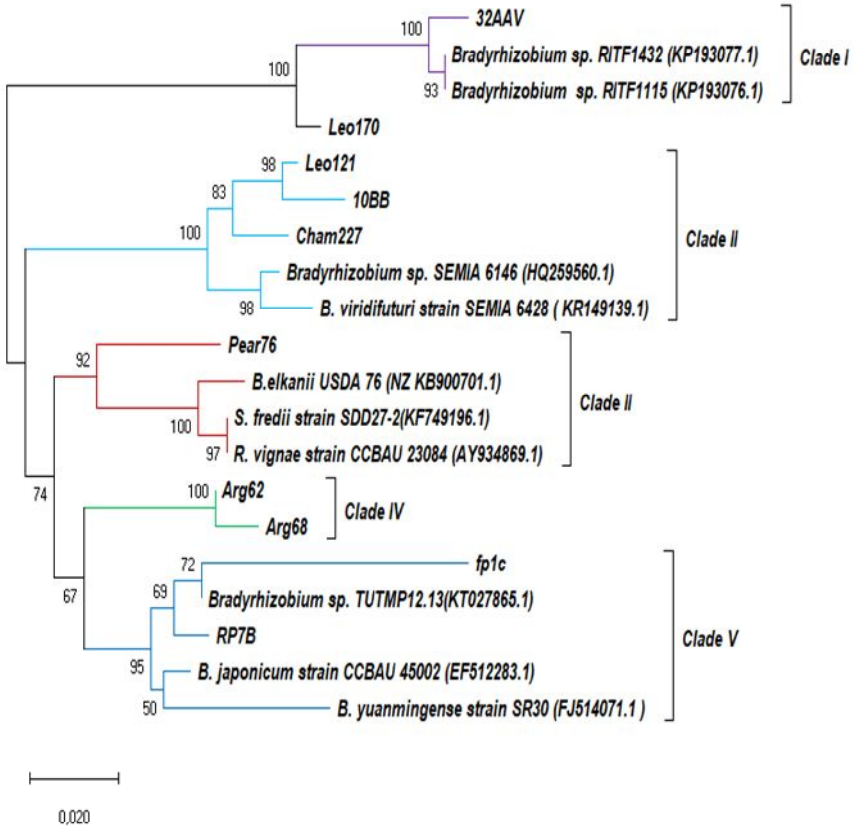

Figure 14. Midpoint rooted Neighbour-joining phylogenetic tree of the *nifH* gene sequences of *Bradyrhizobium*. Bootstrap values are shown at the nodes and the scale bar is 0.020 nucleotide substitutions per site.