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Characterization of rhizobia and their symbiotic performance on selected important legumes under abiotic stress conditions

by

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(2050271)

Thesis

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in

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Supervisor: Prof Karl Rumbold

Co-supervisor: Dr Ahmed Hassen

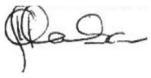
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Declaration

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Characterization of rhizobia and their symbiotic performance on selected important legumes under abiotic stress conditions

Abstract

Introduction: Legumes are a source of proteins in human diet and a staple food for many cultures globally as an inexpensive meat alternative. They form a symbiotic relationship with rhizobia to form root nodules. Inside the nodules, the rhizobia fix atmospheric nitrogen (N_2) by the biological nitrogen fixation (BNF) process. This provides a supply of nitrogen to plants. Lately, farmers are more open to the use of rhizobia inoculants due to the availability of effective and quality products in the market. These inoculants improve yields at a low cost when compared to chemical or artificial fertilizers. Symbiotic genes (*nodA* and *nodC*), the ACC deaminase enzyme (encoded by *acdS* gene) and exopolysaccharides (*exoR* gene) are essential for the rhizobium-legume association, particularly under abiotic stress conditions for effective nodulation.

Aim: The current study aims at isolating stress tolerant SARCC (South African Rhizobium Culture Collection) isolates to be applied as inoculants under abiotic stress conditions.

Materials and methods: Rhizobia spp. tolerance to various levels of temperature, acidity/alkalinity (pH5, pH7 and pH9), heavy metals (50mM, 100mM and 150mM concentrations of $AlCl_3.6H_2O$) and salinity (50mM, 100mM and 150mM concentrations of NaCl) stresses were investigated. Phylogenetic characterization of the isolates was determined using nucleotide sequence analysis of the 16S rRNA, *exoR* gene, *acdS* gene, the house keeping *recA* gene, as well as *nodA* and *nodC* symbiotic genes. A glasshouse nodulation efficacy test under normal and abiotic stress conditions was also conducted.

Results: This study reports tolerance to abiotic stresses and the phylogenetic characterization of 40 rhizobia strains previously isolated from the root nodules of *Medicago sativa, Trifolium repens, Lupinus albus, Vigna unguiculata* and *Phaseolus vulgaris.* The isolates exhibited significant variations in their tolerance to abiotic stresses. Using molecular approaches, some of the rhizobia isolates have been detected to possess certain beneficial traits involved in the reduction of abiotic stresses such as the *acdS* (9 isolates) and *exoR* (5 isolates) genes. The symbiotic genes *nodC* (7 isolates) and *nodA* (16 isolates) were detected as well. Phylogenetic analyses based on 16S rRNA gene, housekeeping genes (recA) revealed that the isolates belongs to five genera: *Sinorhizobium, Bradyrhizobium, Rhizobium, Mesorhizobium* and *Aminobacter*. Under glasshouse nodulation efficacy test, effective isolates provided the highest plant biomass and number of nodules under normal conditions, acidity/alkalinity and salinity abiotic stresses when compared to the un-inoculated controls.

Conclusion: Amid the increasing threats of the global climate change and the rising abiotic stresses, these current results provide baseline information in the selection of rhizobia for use as inoculants under abiotic stressed conditions in South Africa.

Key words: Rhizobia, abiotic stress, biological nitrogen fixation, nodules

Research outputs

Publication forming part of PhD thesis:

A manuscript has been submitted to Letters in Applied Microbiology journal:

L.S. Khambani, A.I. Hassen, and K. Rumbold (2023). Characterization of *Rhizobia* spp. and detection of essential traits that promote nodulation of legumes under abiotically stressed conditions. Letters in Applied Microbiology journal (submitted).

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List of abbreviations

SARCC	South African rhizobium culture collection
PGP	Plant growth promoters
UV	Ultraviolet
ACC	1-Amino cyclopropane 1-carboxylic acid
LPS	Lipopolysaccharides
EPS	Exopolysaccharides
DNA	Deoxyribonucleic acid
BNF	Biological nitrogen fixation
nodA	N-acyltransferase
nodC	N-acetylglucosaminyltransferase
recA	Recombinase A
ARC-PHP	Agricultural research council-plant health protection
RNA	Ribonucleic acid
NCBI	National Center for Biotechnology Information
nodD	Nodulation proten D
LCOs	Lipo-Chitooligosaccharides
YMCR	Yeast mannitol congo red
YMB	Yeast mannitol broth
RMM	Rhizobium minimal medium
CRD	Completely Randomized design
ANOVA	Analysis of variance
LSD	Least significant difference
bp	Base pair
BLAST	Basic local alignment search
ML	Maximum likelihood
NJ	Neibhour Joining

NCD	National communicable diseases
PEG	Polyethylene glycol

CHAPTER 1

INTRODUCTION

1.1 Classification of Rhizobia

Rhizobia are root nodulating bacteria found in the soil and form symbiotic relationships with legumes, this enables them to fix atmospheric nitrogen (Aminu *et al*., 2015). They are classified into two diverse groups which are represented by the α- and β-proteobacteria species in 18 genera (Poole *et al*, 2018; De Lajudie *et al*., 2019). The α-proteobacteria are more diverse and most rhizobia species are *Bradyrhizobium, Allorhizobium, Mesorhizobium, Azorhizobium, and Rhizobium* and *Sinorhizobium (Ensifer)* (Maynaud, *et al*., 2012) and *Microvirga* genera, (Ardley *et al*., 2012). The β-proteobacteria genus was recently discovered and it comprises of *Cupriavidus* and *Burkholderia* species (Gyaneshwar *et al*., 2011) and *Paraburkholderia tuberum* (formerly *Burkholderia tuberum*) which was amongst beta-rhizobia species recognized first (Sawana *et al*., 2014). Thus, α and β-rhizobia distinguishes each class of the symbionts (Compant *et al*., 2008). Moreover, several studies have reported *Pseudomonas* sp.classified as γ-Proteobacteria in black locust (Shiraishi *et al*., 2010).

1.1.1 Alpha-rhizobia (α-rhizobia)

The α-rhizobia are *Hyphomicrobiales* (formerly known as *Rhizobiales*) distributed in 17 genera of 7 famalies and these microsymbionts are common in legumes (Hordt *et al*., 2020). *Mesorhizobium*, *Rhizobium*, *Bradyrhizobium* and *Sinorhizobium* species are the most common

out of the 17 genera and the majority of rhizobia species. The α-rhizobia commonly found in common bean (*Phaseolus vulgaris*), mung bean (*Vigna radiata*), chickpea (*Cicer arietinum*), soybean (*Glycine max*), alfalfa (*Medicago sativa*), peanut (*Arachis hypogaea*), and pea (*Pisum sativum*) legumes (Chen *et al*., 2020). *Ensifer* (non-symbiotic) and *Sinorhizobium* (symbiotic) are currently comined and should be classified into two genera (pre-existing). This is based on their phenotypic and distinct genomic properties and their separation into two phylogenetically distinct clades (Fargozi *et al*., 2020).

1.1.2 Beta-rhizobia (β-rhizobia)

The β-rhizobia were isolated from tropical legumes, e.g *Mimosa* species, and other legumes. The β-rhizobia are less diverse and were discovered much later (Moulin *et al*., 2001; Liu *et al*., 2020). Moreover, they are currently classified into three genera i.e. *Cupriavidus*, *Paraburkholderia* and *Trinickia* belonging to the *Burkholderiaceae* family (Estrada-de los Santos *et al*., 2018). The *Paraburkholderia* and *Trinickia* genera were described based on the whole genome analyses of some former *Burkholderia* species (Dobritsa and Samadpour, 2016; Estrada-de los Santos *et al*., 2018).

1.1.3 Gamma-rhizobia (γ-rhizobia)

The γ-rhizobia remain disputed and must be authenticated further. In *Pseudomonas* species (Shiraishi *et al*., 2010), γ-rhizobia were first described in black locust (*Robinia pseudoacacia*) root nodules isolates and *Mesorhizobium* species commonly nodulates black locust (Kang *et al*., 2012). The *nodC, nodA*, *nifD* and *nifH* (symbiotic genes) sequences of these *Pseudomonas* species were similar to rhizobia species, indicating that these genes were possibily acquired via lateral transfer (Shiraishi *et al*., 2010). Moreover, *Pseudomonas* isolates were possibly

Mesorhizobium and β-rhizobia (mixed cultures). Subsequently, *Pseudomonas* in *Trifolium* legume root microbiome was abundant and bacteria without *nodA* or *nodC* genes are common in legumes root nodules (De Meyer and Willems 2012; (Hartman *et al*., 2017).

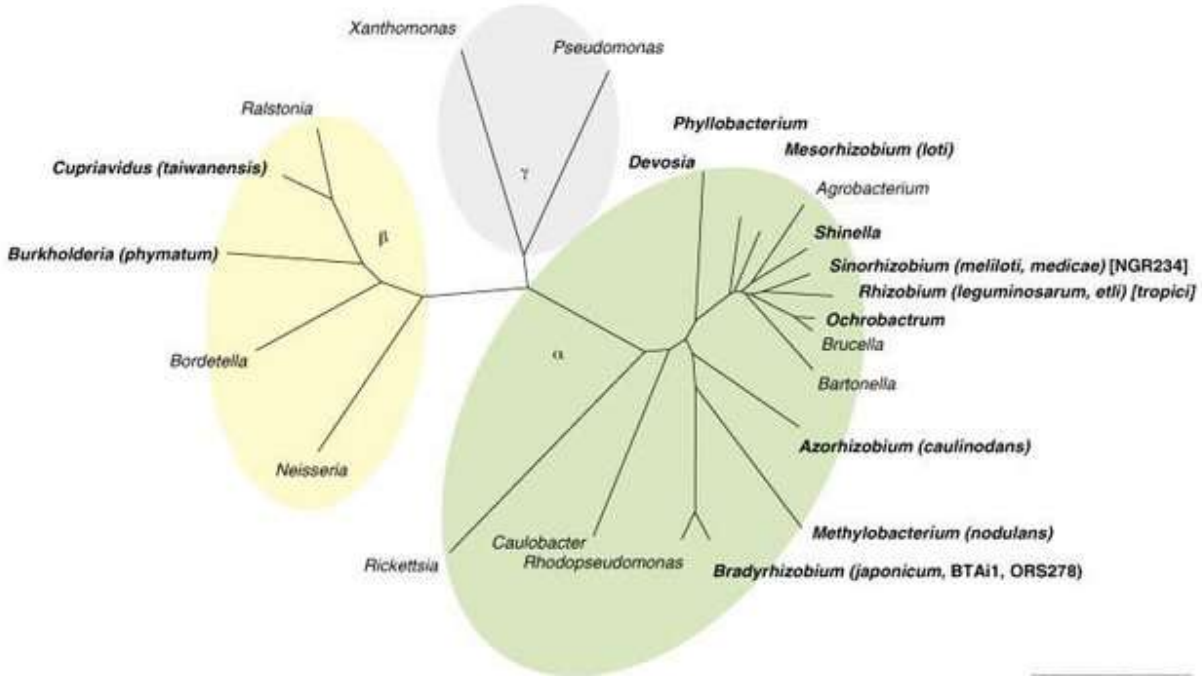


Figure 1.1. The phylogenetic tree constructed from the 16S rRNA gene sequences of Proteobacteria. Rhizobia genera are indicated in bold. Image adapted from (Barreda and Fikri-Benbrahim, 2014).

1.2 Role of Rhizobia

Rhizobia species invade their legume hosts through root hairs, sites where the lateral root emerge or root epidermis and induces legume root nodules development. The rhizobia inside the root nodules of the host then fix atmospheric nitrogen (N_2) in the presence of nitrogenase whereby nitrogen is converted to ammonium (NH_4^+). The nitrogenase activity determines the amount of ammonium supply given to plants by rhizobia. High nitrogenase activity will produce a high amount of nitrogen that is utilized by the legume host plant and ultimately enters the earth's food web to improve plant growth. In exchange, rhizobia acquire carbohydrates, proteins, and oxygen

produced by the legume plants to live and breed (Sprent *et al*., 2013; Aminu *et al*., 2015). The genus *Rhizobium* was the first group to be described in nitrogen fixing bacteria; it is the largest genus of rhizobia accommodating 112 species and has been used frequently as legumes nitrogen-fixing bacteria (Mousavi, 2016; De Lajudie *et al*., 2019).

1.3 Nodulation and nitrogen fixation genes

The symbiotic nitrogen fixation process is dependant on the nitrogen fixing and nodulation genes, mostly the *nod*, *nif* and *fix* genes in the rhizobia genera. The accessory genes of the bacteria encode the Nod and Nif proteins and these genes are found in the transmissible genetic elements. Thus, they can be frequently transferred within the bacterial species genus and not frequently between the genera (Remigi *et al*., 2016). Rhizobia strains have the ability to infect compatible legume host and the growth conditions vary, this is based on the symbiotic development stage. These includes the growth conditions in the curled root hair pocket's which are acidic and oxidative (Hawkins *et al*., 2017). Moreover, free-living and symbiotic growth stages require metabolic capabilities and a set of genes which are unique. Therefore, when developing strains to be used in the production of commercial inoculants, one must account for these requirements and other biological properties, including the industrial scale growth and the ability to survive during desiccation (O'Callaghan, 2016).

1.3.1 Nodulation genes

The *nodA, nodB* and *nodC* genes are essential in rhizobia species to synthesize the core structure of nod factor because they encode enzymes that synthesize the lipo-oligosaccharide core. The inactivation of these genes affects the ability of rhizobia spp. to evoke any kind of the plant's symbiotic reaction. This is despite of the legume host species, their infection mode, location and

the type of the nodules they form (Long, 1989; Martinez *et al*., 1990). Moreover, the Nod genes enzymes have various functions. The *nodC* synthesizes the N-acetyl glucosamine which determines the Nod factor backbone synthesis whilst *nodB* is a chito-oligosaccharide deacetylase responsible for removing an N-acetyl group from the Nod factor and *nodA* is an acyltransferase responsible for the N-acylation of the amino-sugar backbone (Atkinson *et al*., 1994; Perret *et al*., 2000; Lopez-Lara and Geiger, 2000).

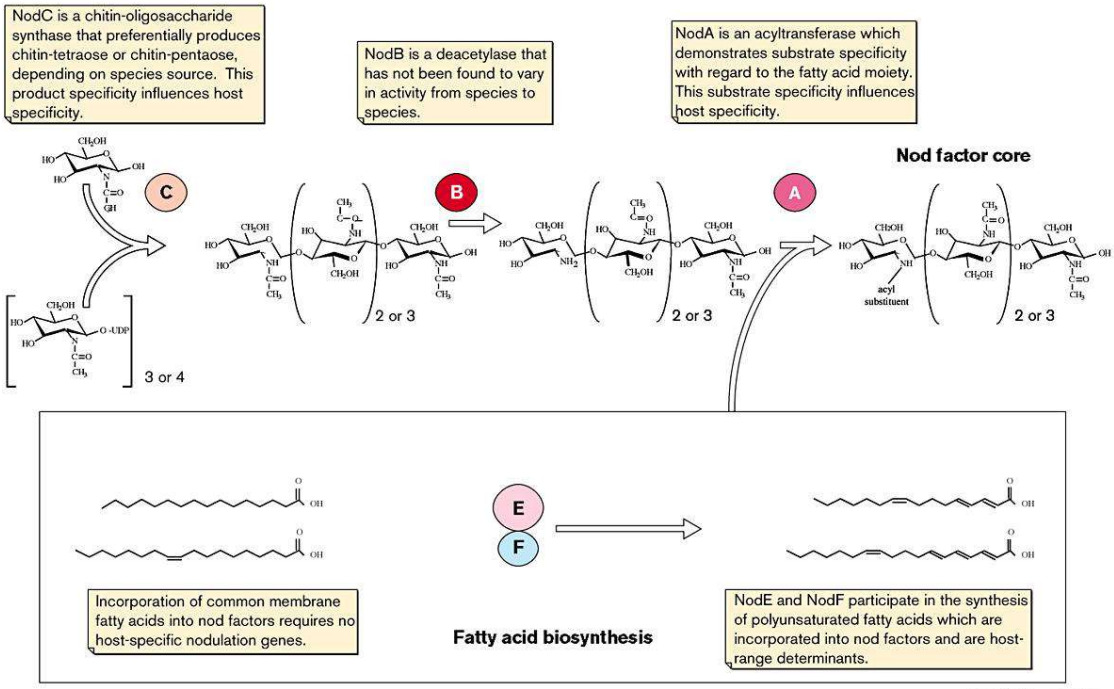


Figure 1.2. The nod factor core sythesis by *nodA*, *nodB* and *nodC* genes, *nodE* and *nodF* host-specific nodulation gene products. Image adapted from (Peters, 1997).

1.3.2 Nitrogen fixation genes

The nitrogen fixation process is driven by a cluster of *nif* gene products (Thiel, 2019). The nitrogen fixing microbes are responsible for carrying out nitrogen fixation in the presence of nitrogenase which reduces nitrogen. Nitrogenase is comprised of two components, i.e. molybdenum iron (MoFe) and iron (Fe) protein and these componets are responsible for various functions. The molybdenum protein reduces nitrogen and the iron protein provides the electron during the nigrogen fixation process (Frank, 2014). The nitrogenase enzyme contains up to 20 genes that encode amino proteins sequences in proteins, but the main structural genes are *nifK* *nifD* and *nifH*. The *nifH* gene is the Fe protein structural gene mostly essential in studies focusing on the ecology and evolution of nitrogen-fixing bacteria. The structural genes encoding α and β subunits of FeMo protein are *nifD* and *nifK* (Raymond *et al*., 2004; Dos Santos *et al*., 2012). The *nif* gene regulation is controlled by *nif* and *nifL* whereby *nif* is the positive activator and *nifL* is a negative regulator. However, the overall expression of the *nif* gene is regulated by present levels oxygen (as indicated in Figure 1.3) and nitrogen (Monson *et al*., 1995).

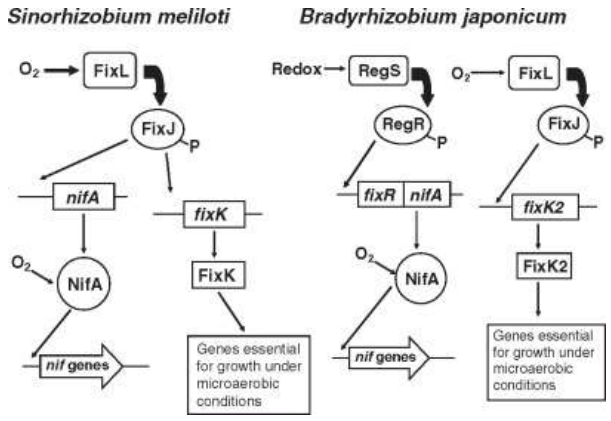


Figure 1.3. Oxygen level controls the expression of *nifA* and *fixK* expression in *Sinorhizobium meliloti* using *fixL* and *fixJ* regulators. Under low O_2 conditions, *fixJ* regulator is phosphorylated and expresses *nifA* and *fixK*. In *Bradyrhizobium japonicum*, *fixLJ* controls the expression of *fixK2* and its downstream targets. The *nifA* senses low O_2 levels and activate *nif*-genes expression. Image adapted from (Stacey, 2007).

1.4 Problem identification

Increasing profit and crop yield for farmers to meet food demand in Africa is challenging in the farming systems, specifically low-input (Tian and Yu, 2019). Moreover, the increasing cost and the environmental impact of inorganic nitrogen inputs have led to the reappraisal of legumes role. Legumes host rhizobia that fix atmospheric nitrogen, reducing the need for inorganic application (Harris and Ratnieks, 2022). Legumes establishes a symbiotic relationship with α and β-rhizobia where abundant atmospheric nitrogen (N_2) is converted into N inside the nodules through the biological nitrogen fixation process (Udvari and Poole, 2013; Basu and Kumar, 2020). Nitrogen fixation is essential in order to sustain agricultural production systems which benefit millions of smallholder farmers (Franke *et al*., 2018; Ulzen *et al*., 2018; Adjei-Nsiah *et al*., 2019).

There is a need for agricultural technologies to focus on increasing production, system durability and improved profits so that household food security is enhanced for smallholder farmers (Helfenstein *et al*., 2020). The life cycles of rhizobia strains fixing nitrogen are complex whereby various abiotic stress factors may affect the biological nitrogen fixation process and the production of legumes (Poole *et al*., 2018; van Loon *et al*., 2018). Generally, rhizobia species are free-living in the rhizospheric soil, which provides unique nutritional and stress conditions where they must compete with other bacteria to form stable microbial population (Hinsinger *et al*., 2009; Li *et al*., 2016). Although rhizobia have numerous traits that promote plant growth, arguably, the bacterial traits that facilitate legume nodulation especially under abiotic stress conditions are the possession of ACC deaminase enzyme and production of exopolysaccharides. These traits are key features in legume nodulation and in plant's response to a vast range of abiotic stresses, thereby optimizing legumes and rhizobia interaction during nodulation. To help

overcome crop production limitations in stress prone areas, the screening and application of rhizobia strains that are stress tolerant could be a viable option. Rhizobia research on legume symbiosis in South Africa in the past focused mainly on the conventional applied aspects of inoculation with no records of the study of ACC deaminase and EPS impacts on nodulation and symbiotic nitrogen fixation. Therefore, the aim of the current study is to characterize selected rhizobia isolates from the SARCC collection for the occurrence of beneficial traits that promote nodulation and growth of legumes under abiotically stressed conditions.

1.5 Aim and objectives

1.5.1 Aim

Characterization of rhizobia and their symbiotic performance on selected important legumes under abiotic stress conditions.

1.5.1. Specific objectives

- To evaluate rhizobia strains from the South African Rhizobium Culture Collection (SARCC) for their ability to produce ACC deaminase.
- To screen the rhizobia isolates for *in-vitro* abiotic stress tolerance.
- To detect the production of exopolysaccharide from the selected rhizobia strains.
- To characterize the rhizobia isolates for genes involved in ACC deaminase (*acdS*) and exopolysaccharide (*exoR*) production as this are traits that aid in nodulation under abiotic stress conditions.
- To conduct random mutagenesis of the *acdS* and *exoR* gene possessing rhizobia isolates.
- To phylogenetically characterize the rhizobia strains possessing either ACC deaminase or EPS using symbiotic and housekeeping genes.

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CHAPTER 2

Nodulation efficacy test of Rhizobia spp. on selected important legumes *in vivo* under normal and abiotic stress conditions

2.1. Legumes

Legumes belong to the Leguminosae family comprising of over 20,000 species of herbs, climbers, trees and shrubs and only a few species are used for human consumption (Yorgancilar and Bilgicli, 2014; Stagnari *et al*., 2017). They are mainly a source of reduced nitrogen in the last decade and are currently cultivated and consumed worldwide (Goncalves *et al*., 2016; Rajan and Ankur, 2017). In human diet, legumes are good sources of proteins as they provide dietary fibre, unsaturated fats, amino acids, essential minerals, carbohydrates, vitamins, and cheaper when compared to animal proteins (Bouchenak and Lamri-Senhadji, 2013; Annor *et al*., 2014; Rebello *et al*., 2014). Moreover, legumes are called 'poor man's meat' and this is based on their consumption in different regions while taking into account the inverse relationship beween their consumption and income observation (Messina *et al*., 2016). Legumes are a staple food worldwide for numerous cultures and are amongst other first crops cultivated by mankind as inexpensive meat alternatives (Kouris-Blazos and Belski, 2016).

Worldwide, health organizations have recommended legume consumption as a healthy diet. This is because of the role they play in controlling and preventing chronic non-communicable diseases (NCDs), e.g diabetes, cancer and cardiovascular diseases (Iqbal *et al*., 2006; Clifton,

2010; United Nations, 2014). They have a number of beneficial health traits such as hypocholesterolaemia, antiatherogenic, anticarcinogenic and hypoglycaemic properties (Ndidi *et al*., 2014; Messina, 2016). Legumes can also aid in controlling body weight because they give a greater satiety, prevents fat accumulation in the abdomen and lastly, they regulate sugar levels in the blood (Williams *et al*., 2008; Clifton, 2010).

Cultivation of legumes by subsistence farmers at household level could be a source of income. However, irrigation systems and fertilizers are expensive so legumes are excellent crops for local farmers who cannot afford. Therefore, planting legumes reduce soil erosion and because of the legume-rhizobia symbiosis, they are considered an excellent rotation crops (Kalidass and Mahapatra, 2014; Nedumaran *et al*., 2015). Commonly cultivated legumes and subsequently used for human consumption are lentils peas (*Pisum sativum*), (*Lens culinaris),* broad beans (*Vicia faba*), soybeans (*Glycine max*), lupins (*Lupinus*), lotus (*Nelumbo nucifera*), mung bean (*Vigna radiata*), green beans (*Phaseolus vulgaris*) and peanuts (*Arachis hypogaea*) (Anonymous, 2013; Yorgancilar and Bilgicli, 2014). However, just like any other crops, the production of legumes is adversely affected by a variety of abiotic stresses.



Figure 2.1. Variety of legume seeds, *Lens culinaris*, *Pisum sativum*, *Vicia faba*, *Cicer arietinum*, *Glycine max* and *Vigna unguiculata* commonly cultivated for human consumption. Image adapted from (FAO, 2016).

2.2 Climate change and abiotic stress

Agriculrural practices and crops have evolved over the years due to climate change which is constantly altering the environment. This directly affects the agricultural sector as it is mostly vulnerable. Agricultural production such as complex legume crop management, especially nitrogen nutrition is affected by climate change as it causes significant fluctuations in rainfall and temperature (Banerjee and Adenaeuer, 2014; Vadez *et al*., 2012; Hu *et al*., 2017; Peng *et al*., 2020). In order to maintain soil health as agriculture faces climate change, sustainable land management practices have been known for centuries. These practices are potential mitigation strategies for climate change and have garnered new attention (Tilman *et al*., 2002; Norris and Congreves, 2018). Crop rotation restores nutrients in the soil and this is an ancient land management strategy. Legumes (e.g soybean, alfafa and peanut) are essential in crop rotations to increase the nitrogen content in the soil, thus reducing the application of synthesized nitrogen fertilizers (Foyer *et al*., 2019). However, this is not possible using legumes alone; they rely on rhizobia (Gibson *et al*., 2008; Oldroyd *et al*., 2011).

Moreover, human society depends on agriculture for basic means of living, however, agriculture's sustainable development is directly related to the development and survival of the human society (Banerjee and Adenaeuer, 2014; Peng *et al*., 2020). The production and distribution of plants is influenced greatly by various abiotic stresses (salinity, drought, low or high temperature) which are the negative impacts of non-living factors on living organisms in a specific environment, thus reducing crop yield and poor plant growth worlwide (Acquaah, 2012). These abiotic stressors have the ability to restrict the global use of arable lands and negatively impact production in agriculture (He *et al*., 2018). The advesre impacts in relation to abiotic stresses are more severe in Africa and South Asia and already these countries are experiencing

inadequate amount of food (Hasegawa *et al*., 2018). However, rhizobia plays a vital role under abiotic stress to aid in plant growth whereby the root system is modified, the mobilization and essential elements uptake is enhanced and physiological parameters are modulated (Egamberdieva *et al*., 2018). The development of rhizobia inoculants with an increased abiotic stress tolerance is of paramount importance for mitigating challenges related to climate change (Wongdee *et al*., 2021). Therefore, in order to adjust to climate change depicted by rainfall variability, feasible strategies are within reach. These include rhizobia inoculation, planting varieties that are resilient to water stress and timely planting to reduce drought impact (Tumuhairwe *et al*., 2018).

2.3. Nitrogen fixation

Nitrogen is an essential element required for good crop yield in agriculture (Andrews *et al*., 2013; Dall'Agnol *et al*., 2014). All eukaryotic organisms use inorganic nitrogen in an ammonium (NH_4) or nitrate (NO_3) form (Giller, 2001; Loganathan *et al*., 2014), the abundant amount of nitrogen in the atmosphere is in a N_2 form which cannot be readily utilized by plants. Rhizobia and archaea bacteria reduces nitrogen into ammonia through the nitrogen fixation process (Giller, 2001; Lindstrom and Mousavi, 2010). In agriculture, the biological nitrogen fixation technology is currently used to overcome challenges associated with the depleted of soil fertility and to reduce the application of inorganic fertilizers (Tairo and Ndakidemi, 2013; Nyoki and Ndakidemi, 2014).

Nitrogen fixation is an affordable and inexpensive way to enhance crop yield when compared to the nitrogen chemical fertilizers (Jonah *et al*., 2012; Nyoki and Patrick, 2013; Mfilinge *et al*., 2015). Inoculating legumes with rhizobia in agriculture is an essential nitrogen fixing technology

to improve productivity and yield of different legume crops while enhancing the fertility of the soil (Deshwal and Chaubey, 2014). Most legumes can take in and absorb NO_3^- and NH_4^+, however they can also obtain their required N amount from symbiotic microorganisms capable of fixing nitrogen (France *et al*., 2009; Sprent, 2009; Andrews *et al*., 2011; Andrews *et al*., 2013). These microorganisms are termed "rhizobia" and are able to form nodules which are specialized structures on their host's roots where rhizobia fixes atmospheric N_2 into NH_3 in the presence of nitrogenase (Sprent and Sprent, 1990; Andrews *et al*., 2009).

2.4. Nodule formation

Nodules are specialized organs located on legume's species roots and stems and are associated with symbiotic nitrogen fixing bacteria, rhizobia. Rhizobia occupy an exclusive ecological niche for biological nitrogen fixation which reduces atmospheric dinitrogen to ammonia. Legume-rhizobium symbiosis enables rhizobia to convert nitrogen into a readily available state to be used by the host legume for their growth which in turn provides a built in supply of nitrogen fertilizer for almost all legumes worldwide (Martinez-Romero, 2003; Brewin, 2010; Oldroyd *et al*., 2011). Each and every nodule can contain up to 10^9 rhizobia in a niche, this is perfect for reducing N_2 by providing the bacteria with malate (carbon supply) and a low O_2 environment (Downie, 2014). The legume and rhizobia association is mostly studied as one of the beneficial plant microbe interactions and it basically begins with a molecular interaction between the two partners. This interaction is induced by the initial signal exchange whereby when nitrogen is scarce in the soil, legume roots exude flavonoids and isoflavonoids (phenolic compounds) into the rhizospheric soil (Peters and Verma, 1990; Liu and Murray, 2016). Rhizobia sense these molecules and activate the nodulation protein D (nodD) which is a transcriptional regulator; this

causes genes responsible for Nod factors synthesis to be transcribed (D'Haeze and Holsters, 2002).

Nod factors are lipo-chitooligosaccharides (LCOs) that are host determinant and interact with flavonoids (Downie, 1998) and recognised by receptors in the root cells plasma membrane. In plants, Nod factors recognition induces molecular and physiological responses, e.g. the build up of genes induced during the symbiotic nitrogen fixation, curling of the root hair and the infection thread formation (Gage, 2004). The role of the infection thread is to facilitate root hair penetration (Szczyglowski *et al*., 1998). The cortical and pericycle cell division occurs simultaneously, resulting in nodule formation (Figure 2.2). Rhizobia bacteria then travels through the infection facilitated by the division of bacterial cell and ultimately freed into the induced nodule primordium cells (Calvert *et al*., 1984; Mathews *et al*., 1989). Nitrogen (N_2) is fixed into ammonia (NH_3) in the bacteroids, then utilised by the plants for amino acids and proteins synthesis (Poole *et al*., 2018).

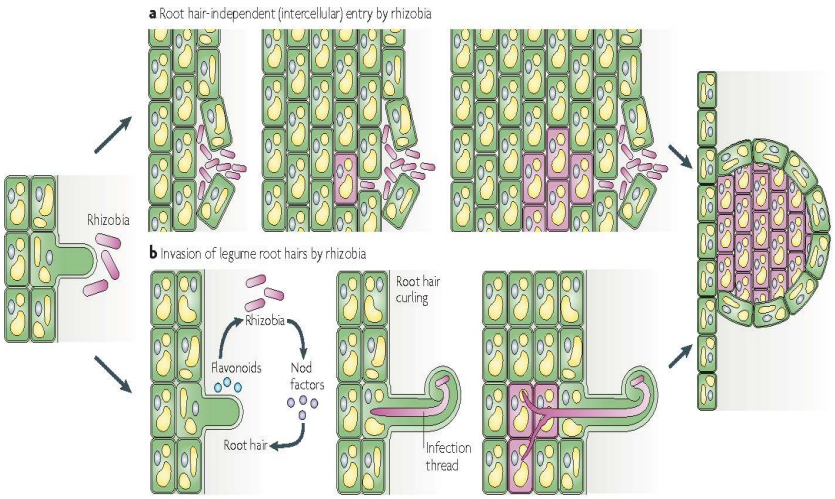


Figure 2.2. Schematic overview of the nodule development processes. The signals released from the host legume triggers a chain of events which allows rhizobia to invade root cells of the plant. Successful signaling leads to cortical cell divisions and nodule development. Image adapted from (Deakin and Broughton, 2009).

Legumes have two types of nodules i.e. determinate and inderterminate. The meristem activity is the main difference between the nodules (Kereszt *et al*., 2011). The determinate nodules meristem is short-lived with a spherical shape (Figure 2.3b), for example they are generally produced by tropical legumes e.g soybean (*Glycine max*) and bird food (*Lotus japonicas)*. The indeterminate nodules meristem is persistent and tip-localised; resulting in a continuous nodule growth and a cylindrical shape with branches (Figure 2.3a). They are mainly produced by temperate legumes, e.g. pea (*Pisum sativa*), white clover (*Trifolium repens*) and barrel clover (*Medicago truncatula*) (Sprent, 2009).

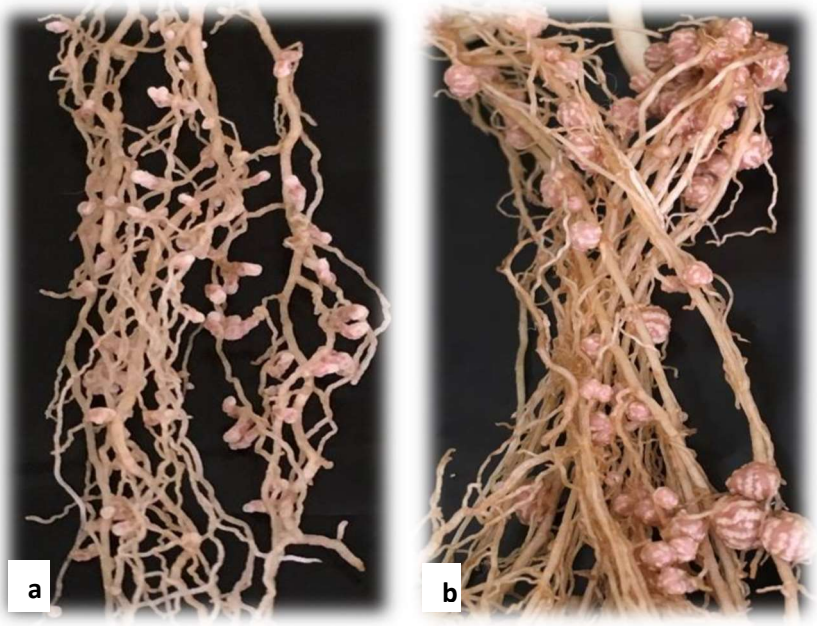


Figure 2. 3. Types of nodules, indeterminate nodules of *Trifolium repens* (a) and derterminate nodules of *Vigna unguiculata* (b). Image adapted from the current research.

When soils are devoid of native rhizobia that fix atmospheric nitrogen, the BNF technology becomes successful provided the isolation, screening and characterization of indigenous soil rhizobia strains is properly done. This may result in the discovery of novel and effective strains

with a potential to be used in the production of inoculants (Lindstrom *et al*., 2010). The selection and isolation of stress tolerant rhizobia strains may enhance growth in legume plants through nodulation and the ability of plants to fix nitrogen under abiotic stresses (Zou *et al*., 1995). Moreover, the selection of rhizobia strains that are effective is a crucial aspect to ensure that biological nitrogen fixation benefits are obtained (Biswas *et al*., 2008).

The selection of host plants in this study was based on the availability of isolates from the legumes of choice in the SARCC, legumes commonly cultivated in South Africa and their importance. The following host plants were selected, i.e. *Vigna unguiculata, Phaseolus vulgaris*, *Medicago sativa, Trifolium repens* and *Lupinus albus. Vigna unguiculata* is a staple crop in most African countries and serves as food for human consumption and fodder for livestock (Singh *et al.,* 2003; Ndema, 2010). *Phaseolus vulgaris* is a protein power house and an affordable grain legume highly consumed globally and their production is central, ensuring food security in Africa and Latin America's poor households (Siddiq and Uebersax, 2012; Wheeler and Von Braun, 2013; Ron *et al*., 2015; Bitocchi *et al*., 2017). *Medicago sativa* is a perennial legume crop grown worldwide with high effects on soil fertility, quality forage, nutritional quality and protein content. It is the most important forage crop, provides protein to dairy and beef, cattle, sheep, horses, birds and other livestock (Huyghe, 2003; Radovic *et al*., 2009; Li and Brummer, 2012). *Trifolium repens* is a forage crop consisting of about 240 species, high protein and minerals when compared to other grasses and species of *Trifolium*. It has the ability to fix atmospheric nitrogen and improves the quality of the feed (Shrestha, 2002; Ellison *et al*., 2006). *Lupinus albus* is a temperate legume with agronomic potential due to high protein content in the seeds and positive effect on soil fertility. It has become popular as protein source in the last decade,

used for animal and human feed and manure in agriculture (Rosolem *et al*., 2002; Jensen *et al*., 2004).

The South African Rhizobium Culture Collection (SARCC) based in Pretoria, South Africa hosts a large collection of rhizobia strains previously collected from various root nodules of legumes. A study done on legumes of South Africa using one of the strains from the collection has resulted in the development of rhizobia inoculants that were commercialized and currently available in the local markets (Strijdom, 1998). Abiotic stresses reduce plant biodiversity and this result in nutrients imbalance. Poor soil fertility limit crop production and is a challenge worldwide regarding food security (Vanlauwe *et al*., 2015; Chasek *et al*., 2019; Zhu *et al*., 2020). However, legume hosts are able to survive and grow under abiotic stresses; this improves when stress-tolerant rhizobia strains inoculate legumes (Wei *et al*., 2008; Kajic *et al*., 2019). Therefore, the present research work aims to perform a nodulation efficacy test on selected rhizobia strains from the collection under normal and abiotic stress conditions.

2.5. Materials and methods

2.5.1 Sampling

Rhizobia isolates (25) previously collected from the root nodules of *Medicago sativa, Trifolium repens Lupinus albus, Phaseolus vulgaris* and *Vigna unguiculata* and deposited at the South African Rhizobium Culture Collection (SARCC) were used in this study. Pure cultures of rhizobia isolates were originally deposited by various reseachers and stored at -80 ℃ (ultra-low freezer) in 20% glycerol stocks. The collection is at the Agricultural Research Council-Plant Health and Protection (ARC-PHP), Biological Nitrogen Fixation (BNF) unit, Pretoria, South Africa.

2.5.2 Rhizobia isolates and growth media

A total number of 25 rhizobia strains isolated from the root nodules of *Medicago sativa, Trifolium repens, Lupinus albus, Vigna unguiculata* and *Phaseolus vulgaris* as the target hosts were used in this study. The isolates were cultured onto yeast mannitol agar plates supplemented with 10% Congo red (YM-CR). The YM-CR media composition (g/L) consisted of: 10g mannitol, 0.5g K_2HPO_4, 0.1g NaCl, 0.2g $MgSO_4.7H_2O$, 0.4g yeast extracts, 15g agar, 10 mL congo red, pH 6.8-7.2 and autoclave at 121 ℃ for 20 min. The isolates were grown on YM-CR agar plates and incubated for 2-3 days at 26 ± 2 ℃ until colony formation.

2.6. Glasshouse nodulation efficacy test

2.6.1. Seeds germination

The seeds of *Medicago sativa, Trifolium repen, Lupinus albus, Vigna unguiculata* and *Phaseolus vulgaris* host plants were surface sterilized as described by (Somasegaran and Hoben, 1994). Briefly, the seeds were firstly immersed in 95% ethanol for 30 seconds and 1% sodium hypochlorite solution for 1 minute. The excess bleach on the seeds was drained off and the seeds were rinsed repeatedly (5x) using sterile distilled water. To imbibe the seeds, they were soaked for 4 hours in sterile water and rinsed twice. The seeds were then placed in a petri dish (10-15 seeds per plate, depending on the size of the seed) containing sterile 0.75% (w/v) water agar (10 mL). Subsequently, the plates were incubated for 2-3 days at 28 ºC to germinate or until the radicle emerge (Figure 2.4).

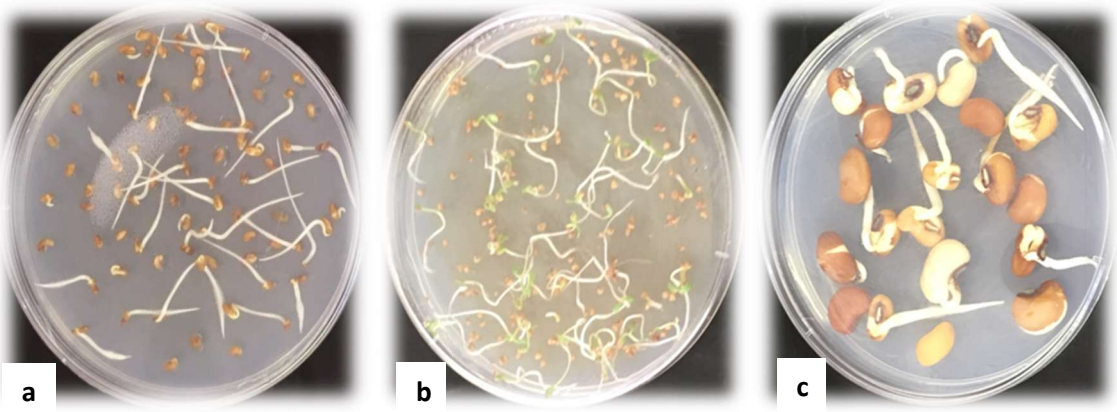


Figure 2.4. Germination and development of *Medicago sativa* (a), *Trifolium repens* (b) and *Vigna anguiculata* (c) seedlings on water agar to be transplanted in Leonard jars. Image adapted from the current research.

2.6.2 Bacterial inoculum preparation

Rhizobial isolates were cultured onto YMCR agar plates and incubated at 28 ± 2 ℃ for 2-3 days. A single colony of the freshly grown culture for each isolate was further inoculated into 50 mL of yeast mannitol broth (YMB). The inoculated flasks were incubated at 28 ± °C for 2-3 days at 120 rpm on a rotary shaker until visible growth; this was indicated by the broth turning milky. The bacterial inoculum was prepared as previously described by Hassen and Labuschagne (2010) with modifications, the final cell concentration of each rhizobia isolate was adjusted with 0.85 % of sterile saline solution to ± 1 x 10^8 cfu/mL using the T60 spectrophotometer (OD_{600nm} of ≥ 0.8-1). The adjusted cell concentration of each isolate was used as an inoculum.

2.6.3 Glasshouse nodulation efficacy test

The Leonard jar assembly containing sterile sand and nitrogen free Hoagland solution (Hoagland and Arnon, 1938) was used. Three seedlings per legume were transplanted per jar (Leonard, 1943). The seedlings (*Medicago sativa, Trifolium repen, Lupinus albus, Vigna unguiculata* and *Phaseolus vulgaris*) in each jar were inoculated with 2 mL suspension of the rhizobia culture

with a concentration level of ±1 x 10^8 cfu/mL and the seedlings were covered with sand immediately. This was done for the nodulation efficacy test under normal conditions. To induce the salinity, acidity/alkalinity abiotic stress conditions, three seedlings per legume were transplanted per jar (Leonard, 1984), containing Hoagland's solution with added different NaCl concentrations (50mM, 100mM and 150mM) to induce salinity stress condition. The acidity/alkalinity condition was achieved by adjusting the Hoagland solution to pH5, pH7 and pH8. The jars were covered with half a petri dish to protect against contamination and the covers were removed when the seedlings reached a ± 2 cm height. Each treatment contained a legume seedlings (*Medicago sativa, Trifolium repen, Lupinus albus, Vigna unguiculata* and *Phaseolus vulgaris)* and rhizobia inoculum with three replications. Un-inoculated Leornard jars were the negative controls and only contained the nitrogen free solution. The inoculated (experiment) and non-inoculated (negative control) were both supplied with Hoagland solution (Broughton and Diworth, 1971). All treatments were replicated 3 times and the experiment was arranged in a completely randomized design (CRD) in a controlled glasshouse with day and night temperature of 27 ºC and 18 ºC respectively (Figure 2.5). The Leonard jars assemblies were maintained for five to eight weeks in the glasshouse during which N-free nutrient solution in the reservoir was monitored. The N-free nutrient solution may be depleted during the glasshouse trial and new solution may be added into reservoir, this depends on the size and growth conditions of the plants. After eight weeks, plants were carefully removed from the Leonard jars and the roots were rinsed thoroughly with water to remove the sand. Nitrogen deficiency in non-inoculated plants and treatments that failed to form nodules upon inoculation was evident by the lack of vibrant green colour in the leaves. The leaves were pale yellow and the overall growth of the

plants was stunted. Furthermore, the following data was evaluated: shoot fresh biomass, shoot dry biomass and number of nodules.

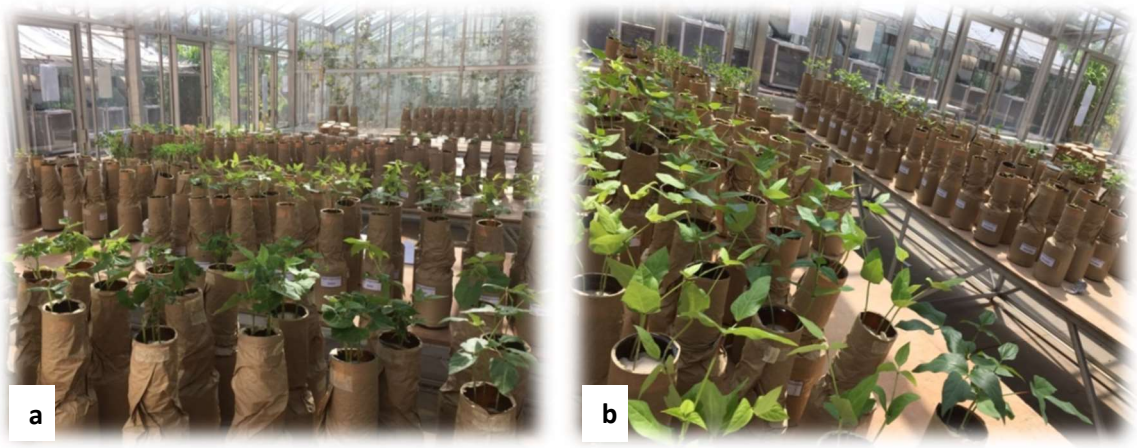


Figure 2.5. Nodulation efficacy test under glasshouse conditions (a &b). Image adapted from the current research.

2.6.4 Statistical analysis

The analysis was conducted with the help of a statistician at ARC. The glasshouse gnotobiotic study data (nodule number, fresh biomass and dry biomass) gathered were subjected to analysis of variance (ANOVA) using the general linear model procedure (PROC GLM) in SAS-9.4 statistical software (SAS, 2016). The mean values were compared by using the least significant difference (LSD) test and Duncan's Multiple Range test at 5 % ($p \leq 0.05$) level of significance.

2.7 Results

The 25 rhizobia isolates tested for authentication trial in this study were confirmed as the same root nodule microsymbionts of legumes from which they were initially isolated as they resulted in nodulation upon inoculation. The rhizobia isolates nodulated their original host legumes i.e. *Medicago sativa* (strain RAK1, RF12, RF14, RF15, RF35 and RF36) , *Trifolium repens* (strain SAC1, SAC2, SAC3 and SR4), *Lupin* (strain VK10) , *Vigna unguiculata* (strain XBD2, XBQ5,

XCR6, XCV14, XFH1, XFH12, XFH13, XFH14, XFH3 and XS21) and *Phaseolus vulgaris* (strain UC10, UC11, UC12 and UD5). Nodulation test on *Medicago sativa, Trifolium repens, Vigna unguiculata* and *Phaseolus vulgaris* resulted in the formation of pink nodules. Legumes without inoculation (negative control) showed nitrogen deficiency symptoms whereby the plant leaves turned pale yellow (chlorosis) with stunted growth and did not form any nodules.

It was evident that the most effective treatments resulted in the formation of leaves dark green in colour and the negative controls formed leaves that were pale green to yellow in colour. However, the symbiotic efficiency of the isolates in their original host plants was reduced in some of the legumes when grown under abiotic stress conditions *in vivo*, especially under 150 mM NaCl and pH9 stresses. The results revealed that most isolates exhibited tolerance to 50 mM NaCl, 100 mM NaCl, pH 5 and pH 7 stresses while few isolates could not tolerate most of the abiotic stresses tested. In addition to the difference in the leaves and nodules color, the inoculated plants also showed a clear difference in terms of plant growth where the un-inoculated plants resulted in stunted growth (Figure 2.7b-j).

Although nodules were formed on most of the inoculated host legumes, the performance of nodution varied based on the response of each rhizobia isolates. The nodules were mainly located on the tap roots, forming clusters of nodules and only a few nodules were located on lateral roots. The nodule size, colour and shape varied amongst the legumes. *Medicago sativa* and *Trifolium repens* resulted in indeterminate nodules whereas *Vigna unguiculata*, *Phaseolus vulgaris* and *Lupinus albus* resulted in determinate nodules. The number of nodules recorded ranged from 3.38 to 59.11 per legume plant under normal conditions. The highest number of nodules was formed by rhizobia isolates RF36 (32.28) and isolate RF12 (29.11) inoculated on

Medicago sativa, Isolate SAC3 (59.11) and isolate SAC1 (38.89) inoculated on *Trifolium repens* and isolate XS21 (35.55) and XBQ5 (31.11) inoculated on *Vigna unguiculata* (Table 2.1).

The number of nodules formed and fresh biomass decreased with an increased salt concentration in the legume hosts. Isolate XCV14 inoculating *Vigna unguiculata*, UC10 inoculating *Phaseolus vulgaris* and VK10 inoculating *Lupinus albus* displayed no nodulation in all salt concentrations tested. Higher salt concentrations (100 and 150 mM) also impaired nodulation in isolate RF12 inoculating *Medicago sativa* and UC10 inoculating *Phaseolus vulgaris*. The isolates (XS21, XFH13, XFH14, XBD2 and XBQ5) inoculating *Vigna unguiculata* and isolates (RF15, RF35 and RF36) inoculating *Medicago sativa* were tolerant to salinity and resulted in nodulation at various NaCl concentrations tested (Table2.1-2.3 and 2.5).

The negative effect of salinity on fresh biomass was mostly significant in *Trifolium repens*, *Lupinus albus* and *Phaseolus vulgaris* at 100 and 150 mM NaCl concentrations. *Vigna unguiculata* and *Medicago sativa* showed the most tolerance and the fresh biomass was consistent throughout the NaCl concentrations. Rhizobia inoculation also significantly increased the fresh biomass of the inoculated plants under acidity and alkalinity stress treatment when compared to the non-inoculated. Isolates inoculating *Vigna unguiculata* exhibited more tolerance to acidity or alkalinity conditions as there was a noticeable difference on the fresh biomass under increased or decreased pH stress. The fresh biomass of *Lupin albus*, *Trifolium repens* and *Phaseoulus vulgaris* was decreased at pH 9 and pH 5 stresses, plants exhibited lower fresh biomass (Table 2.6-2.10).

Significant ($p \leq 0.05$) differences amongst the isolates in terms of nodulation, shoot fresh and dry biomass were observed, this was in comparison to the non-inoculated controls under normal and

abiotic stress conditions (Table 2.1-2.10). Under normal conditions, a significant difference between the inoculated and non-inoculated controls in terms of fresh and dry biomass was observed. Isolate XFH12 and XBD2 exhibited the highest fresh biomass (8.730g and 8.729g respectively) on *Vigna unguiculata*, followed by UD5 and UC11 (8.154g and 7.957g respectively) on *Phaseolus vulgaris*. Isolate SAC3 and SAC2 (1.823g and 1.703g respectively) also exhibited the highest fresh biomass on *Trifolium repens***,** isolate RF35 and RF15 also exhibited 1.579g and 1.575g respectively inoculating *Medicago sativa*.



Figure 2.6. Effect of inoculation of *Vigna inguiculata* and *Trifolium repens* with rhizobia strains. *Vigna unguiculata* inoculation with isolate XFH13 and *Trifolium repens* inoculation with isolate SAC2 resulted in healthy looking shoots and roots with many pink nodules (left) as compared to uninoculated plants (right) with no nodules and stunted growth with yellow leaves in each case, a&g) Normal conditions, b&h) 50 mM NaCl, c) 100 mM NaCl, i) 150 mM NaCl, d&j) pH 5, e&k) pH 7 and f&l) pH 9.

Table 2.1. Evaluation of the authentication test of rhizobia strains inoculated on *Vigna inguiculata* for nodulation and plant biomass

Rhizobia strains	*0 stress (normal conditions*			*Salinity stress (50mM NaCl)*			*Salinity stress (100mM NaCl)*			*Salinity stress (150mM NaCl)*		
	Nodule number	*Fresh biomass (g)*	*Dry biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry biomass (g)*
XBD2	27.28^{cd}	8.729^{a}	1.087^{a}	22.11^{e-j}	6.643^{e-i}	0.752^{c-h}	20.00^{i-k}	6.642^{e-i}	0.565^{h-o}	20.00^{d-f}	6.643^{d}	0.583^{h-n}
XBQ5	31.11^{b}	8.392^{ab}	0.998^{ab}	21.44^{g-j}	7.215^{d-f}	0.669^{d-k}	18.67^{j-l}	6.087^{g-j}	0.515^{j-q}	20.67^{h-k}	6.198^{g-j}	0.527^{j-q}
XCR6	16.11^{l}	6.233^{g-j}	0.759^{c-g}	11.89^{mn}	5.955^{ij}	0.473^{l-r}	9.22^{n}	4.980^{kl}	0.517^{j-q}	12.00^{mn}	4.978^{kl}	0.428^{m-r}
XCV14	10.22^{n}	4.987^{k}	0.506^{k-q}	0.00^{o}	4.358^{l-o}	0.401^{n-r}	0.00^{o}	3.989^{no}	0.363^{p-r}	0.00^{o}	3.756^{no}	0.352^{qr}
XFH1	21.17^{h-j}	5.753^{j}	0.706^{d-i}	12.78^{mn}	5.782^{j}	0.519^{j-q}	10.44^{n}	4.731^{k-m}	0.480^{k-r}	11.78^{mn}	4.728^{k-m}	0.421^{m-r}
XFH12	25.33^{c-f}	8.733^{a}	0.995^{ab}	15.22^{lm}	7.331^{c-f}	0.698^{d-j}	23.11^{e-i}	6.626^{f-i}	0.515^{j-q}	18.78^{j-l}	6.695^{e-h}	0.553^{i-p}
XFH13	27.89^{c}	7.371^{d}	0.785^{c-f}	24.44^{d-g}	7.993^{bc}	0.727^{d-i}	21.78^{e-j}	6.008^{h-j}	0.629^{e-l}	21.78^{e-j}	6.008^{j-j}	0.433^{m-r}
XFH14	27.89^{c}	5.768^{j}	0.799^{cd}	20.44^{h-k}	6.738^{e-g}	0.513^{j-q}	20.67^{h-k}	5.938^{ij}	0.430^{m-r}	17.66^{kl}	5.940^{ij}	0.503^{k-r}
XFH3	23.28^{e-h}	8.241^{ab}	0.887^{bc}	0.00^{o}	4.801^{k-m}	0.432^{m-r}	11.33^{n}	4.839^{k-m}	0.378^{o-r}	9.67^{n}	4.454^{k-n}	0.478^{l-r}
XS21	35.55^{a}	8.324^{ab}	1.059^{a}	19.67^{i-k}	8.358^{ab}	0.789^{c-e}	25.33^{c-e}	7.347^{c-e}	0.599^{e-m}	25.33^{c-e}	7.191^{d-f}	0.638^{d-l}
CONTROL	0.00^{o}	3.894^{no}	0.393^{p-r}	0.00^{m}	4.268^{m-o}	0.371^{p-r}	0.00^{o}	3.974^{no}	0.354^{qr}	0.00^{m}	3.662^{o}	0.315^{r}
$LSD_{0.05}$	3.7460	0.7062	0.0190	3.7460	0.7062	0.0190	3.7460	0.7062	0.0190	3.7460	0.7062	0.0190
Pr>F	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table 2.2. Evaluation of the authentication test of rhizobia strains inoculated on of *Medicago sativa* for nodulation and plant biomass

Rhizobia strains	*0 stress (normal conditions)*			*Salinity stress (50mM NaCl)*			*Salinity stress (100mM NaCl)*			*Salinity stress (150mM NaCl)*		
	Nodule number	*Fresh biomass(g)*	*Dry biomass (g)*	*Nodule number*	*Fresh biomass(g)*	*Dry biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry biomass (g)*
RAK1	24.22^{f-h}	0.4095^{k}	0.1367^{cd}	20.33^{k}	0.894^{fg}	0.072^{hi}	21.33^{g-k}	0.922^{e-g}	0.094^{f-h}	13.00^{l}	0.558^{ij}	0.053^{ij}
RF12	29.11^{bc}	0.6903^{hi}	0.0767^{g-i}	11.00^{l}	0.798^{gh}	0.071^{hi}	0.00^{m}	$0.210^{[}$	0.011^{l}	0.00^{m}	0.210^{l}	0.011^{j}
RF14	19.78^{k}	1.4273^{c}	0.1473^{c}	20.11^{k}	1.609^{b}	0.132^{c-e}	25.67^{d-f}	1.426^{c}	0.157^{bc}	0.00^{m}	0.426^{jk}	0.041^{jk}
RF15	23.94^{f-j}	1.6700^{b}	0.1682^{ab}	21.33^{g-k}	1.668^{ab}	0.157^{b}	31.33^{ab}	1.657^{ab}	0.179^{ab}	12.33^{l}	1.212^{d}	0.114^{d-f}
RF35	27.72^{cd}	1.7940^{a}	0.1820^{a}	24.33^{e-g}	1.564^{bc}	0.136^{cd}	27.33^{c-e}	1.537^{bc}	0.156^{bc}	21.00^{g-k}	1.203^{d}	0.118^{d-f}
RF36	32.28^{a}	0.9468^{ef}	0.1112^{ef}	11.33^{l}	1.068^{de}	0.100^{fg}	24.00^{e-i}	1.416^{c}	0.141^{cd}	20.67^{jk}	1.395^{c}	0.136^{cd}
CONTROL	0.00^{m}	0.2035^{l}	0.0193^{kl}	0.00^{m}	0.186^{l}	0.013^{kl}	0.00^{m}	0.153^{l}	0.012^{l}	0.00^{m}	0.120^{l}	0.008^{l}
$LSD_{0.05}$	3.561	0.1703	0.0276	3.561	0.1703	0.0276	3.561	0.1703	0.0276	3.561	0.1703	0.0276
Pr>F	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table 2.3. Evaluation of the authentication test of rhizobia strains inoculated on *Phaseolus vulgaris* for nodulation and plant biomass

Rhizobia strains	*0 Stress (normal conditions)*			*Salinity stress (50mM NaCl)*			*Salinity stress (100mM NaCl)*			*Salinity stress (150mM NaCl)*		
	Nodule number	*Fresh biomass (g)*	*Dry biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry biomass (g)*
UC10	12.389^{b}	8.855^{b}	0.8927^{cd}	13.00^{b}	6.969^{ef}	0.679^{fg}	0.00^{f}	6.727^{f}	0.673^{g}	0.00^{f}	5.634^{gh}	0.511^{hi}
UC11	20.666^{a}	9.595^{a}	0.9672^{bc}	5.78^{de}	7.773^{cd}	0.744^{e-g}	12.44^{b}	7.530^{c-e}	0.784^{ef}	10.00^{bc}	5.295^{hi}	0.495^{hi}
UC12	22.000^{a}	9.877^{a}	1.000^{b}	0.00^{f}	7.060^{e-f}	0.687^{b}	0.00^{f}	7.197^{d-f}	0.738^{b}	0.00^{f}	4.974^{i}	0.468^{hi}
UD5	12.611^{b}	9.588^{a}	1.0812^{a}	4.56^{e}	7.466^{c-e}	0.680^{fg}	8.111^{cd}	8.083^{c}	0.827^{a}	8.67^{cd}	6.045^{g}	0.556^{h}
CONTROL	0.00^{f}	7.320^{de}	0.7392^{e-g}	0.00^{f}	5.073^{hi}	0.437^{i}	0.00^{f}	5.110^{hi}	0.493^{hi}	0.00^{f}	4.770^{i}	0.458^{hi}
$LSD_{0.05}$	3.033	0.640	0.1066	3.033	0.640	0.1066	3.033	0.640	0.1066	3.033	0.640	0.1066
Pr>F	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table 2.4. Evaluation of the authentication test of rhizobia strains inoculated on *Trifolium repens* for nodulation and plant biomass

Rhizobia strains	*0 Stress (normal conditions)*			*Salinity stress (50mM NaCl)*			*Salinity stress (100mM NaCl)*			*Salinity stress (150mM NaCl)*		
	Nodule number	*Fresh biomass (g)*	*Dry biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry biomass (g)*
SAC1	38.89^{b}	1.2878^{e}	0.1207^{d}	15.00^{ef}	1.409^{d}	0.138^{b}	12.22^{fg}	0.375^{i}	0.031^{g-i}	12.89^{e-g}	0.418^{hi}	0.036^{g}
SAC2	32.89^{c}	1.7033^{b}	0.1643^{ab}	10.00^{gh}	0.686^{g}	0.061^{f}	4.78^{i}	0.210^{k}	0.011^{ij}	12.22^{fg}	0.503^{h}	0.058^{f}
SAC3	59.11^{a}	1.8227^{a}	0.1778^{a}	21.00^{d}	1.550^{c}	0.153^{bc}	5.89^{i}	0.346^{ij}	0.031^{g-i}	16.00^{e}	0.368^{i}	0.033^{gh}
SR4	32.56^{c}	1.0657^{f}	0.0972^{e}	7.33^{hi}	0.419^{hi}	0.045^{fg}	0.00^{j}	0.225^{jk}	0.014^{h-j}	16.00^{e}	0.502^{h}	0.046^{fg}
CONTROL	0.00^{j}	0.1408^{kl}	0.0128^{j}	0.00^{j}	0.078^{l}	0.006^{j}	0.00^{j}	0.059^{l}	0.004^{j}	0.00^{j}	0.043^{l}	0.008^{j}
$LSD_{0.05}$	3.140	0.1276	0.0208	3.140	0.1276	0.0208	3.140	0.1276	0.0208	3.140	0.1276	0.0208
Pr>F	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table 2.5. Evaluation of the authentication test of rhizobia strains inoculated on *Lupinus albus* for nodulation and plant biomass

Rhizobia strains	*0 Stress (normal conditions)*			*Salinity stress (50mM NaCl)*			*Salinity stress (100mM NaCl)*			*Salinity stress (150mM NaCl)*		
	Nodule number	*Fresh biomass (g)*	*Dry biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry biomass (g)*
VK10	3.3872^{a}	6.487^{b}	0.7132^{a}	0.00^{b}	7.313^{a}	0.489^{b}	0.00^{a}	4.232^{c}	0.360^{cd}	0.00^{b}	4.212^{c}	0.387^{c}
CONTROL	0.00^{b}	3.984^{c}	0.3700^{c}	0.00^{b}	6.156^{b}	0.696^{a}	0.00^{a}	3.254^{d}	0.298^{cd}	0.00^{b}	3.154^{d}	0.286^{d}
$LSD_{0.05}$	0.889	0.502	0.090	0.889	0.502	0.090	0.889	0.502	0.090	0.889	0.502	0.090
Pr>F	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table2.6. Effect of Acidity/alkalinity on nodulation and plant biomass of *Vigna unguiculata* with or without rhizobia inoculation

Rhizobia Strains	*Acidity/alkalinity (pH 5)*			*Acidity/alkalinity (pH 7)*			*Acidity/alkalinity (pH 9)*		
	Nodule number	*Fresh biomass (g)*	*Dry biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry Biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry biomass (g)*
XBD2	20.89^{h-j}	7.943^{hi}	0.822^{e-g}	26.55^{b-e}	9.392^{b-d}	0.897^{b-f}	24.67^{d-g}	8.323^{f-h}	0.904^{b-f}
XBQ5	25.78^{c-f}	10.179a	1.053^{a}	26.78^{b-d}	9.313^{b-e}	0.891^{b-f}	22.00^{g-i}	7.520^{i-k}	0.810^{fg}
XCR6	4.56^{m}	5.183^{o-q}	0.523^{l-n}	6.55^{m}	5.183^{o-q}	0.528^{l-n}	16.56^{kl}	5.420^{n-p}	0.506^{mn}
XCV14	0.00^{n}	5.025^{pq}	0.496^{mn}	0.00^{n}	5.320^{o-q}	0.516^{l-n}	0.00^{n}	5.982^{mn}	0.613^{j-l}
XFH1	23.89^{e-g}	7.610^{ij}	0.731^{g-i}	18.33^{jk}	5.610^{no}	0.540^{k-m}	15.44^{l}	5.527^{n-p}	0.538^{k-m}
XFH12	23.55^{f-h}	9.835^{a-c}	0.969^{a-c}	21.11^{hi}	9.894^{ab}	0.986^{ab}	19.78^{ij}	7.027^{j-l}	0.634^{i-k}
XFH13	25.33^{c-f}	8.794^{e-g}	0.868^{c-f}	26.67^{b-d}	8.239^{gh}	0.868^{c-f}	32.22^{a}	7.955^{hi}	0.808^{f-h}
XFH14	16.33^{kl}	7.131^{jk}	0.705^{h-j}	28.00^{bc}	6.492^{kl}	0.678^{ij}	32.56^{a}	8.259^{f-h}	0.946^{b-d}
XFH3	16.89^{kl}	6.973^{kl}	0.656^{ij}	20.33^{ij}	6.974^{kl}	0.697^{ij}	26.78^{b-d}	8.023^{hi}	0.849^{d-f}
XS21	27.67^{bc}	8.840^{d-f}	0.887^{b-f}	28.78^{b}	9.801^{a-c}	0.922^{b-e}	29.00^{b}	9.269^{c-e}	0.988^{ab}
CONTROL	0.00^{n}	4.186^{r}	0.431^{n}	0.00^{n}	5.034^{o-q}	0.501^{mn}	0.00^{n}	4.766^{qr}	0.463^{mn}
$LSD_{0.05}$	2.702	0.206	0.103	2.702	0.206	0.103	2.702	0.206	0.103
Pr>F	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table 2.7. Effect of Acidity/alkalinity on nodulation and plant biomass of *Medicago sativa* with or without rhizobia inoculation

Rhizobia Strains	*Acidity/alkalinity (pH 5)*			*Acidity/alkalinity (pH 7)*			*Acidity/alkalinity (pH 9)*		
	Nodule number	*Fresh biomass (g)*	*Dry biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry biomass (g)*
RAK1	14.67^{d}	0.218^{kl}	0.028^{gh}	24.67^{ab}	0.490^{e}	0.050^{d-f}	11.56^{ef}	0.339^{h-j}	0.038^{e-g}
RF12	13.67^{de}	0.383^{f-i}	0.040^{d-g}	22.33^{a-c}	0.497^{e}	0.057^{d}	11.89^{ef}	0.265^{jk}	0.039^{e-g}
RF14	21.56^{c}	0.430^{e-g}	0.049^{d-f}	22.44^{a-c}	1.072^{b}	0.119^{a}	10.00^{f}	0.372^{g-i}	0.054^{de}
RF15	12.78^{de}	0.335^{ij}	0.033^{fg}	24.89^{a}	1.003^{b}	0.112^{a}	12.11^{d-f}	0.452^{ef}	0.074^{c}
RF35	21.33^{c}	0.356^{g-i}	0.035^{fg}	22.44^{a-c}	1.195^{a}	0.120^{a}	24.89^{a}	0.411^{f-i}	0.039e-g
RF36	22.00^{bc}	0.412^{f-h}	0.043^{d-g}	24.89^{a}	0.827^{c}	0.083^{bc}	24.78^{a}	0.605^{d}	0.092^{b}
CONTROL	0.00^{g}	0.133^{m}	0.009^{i}	0.00^{g}	0.121^{m}	0.007^{i}	0.00^{g}	0.172^{lm}	0.014^{hi}
$LSD_{0.05}$	2.742	0.076	0.017	2.742	0.076	0.017	2.742	0.076	0.017
Pr>F	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table 2.8. Effect of Acidity/alkalinity on nodulation and plant biomass of *Phaseolus vulgaris* with or without rhizobia inoculation

Rhizobia Strains	*Acidity/alkalinity (pH 5)*			*Acidity/alkalinity (pH 7)*			*Acidity/alkalinity (pH 9)*		
	Nodule number	*Fresh biomass (g)*	*Dry biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry biomass (g)*
UC10	21.11^{b}	7.088^{b-d}	0.728^{cd}	6.33^{f}	7.590^{bc}	0.838^{bc}	3.45^{fg}	5.124^{h}	0.472^{gh}
UC11	28.33^{a}	6.782^{c-e}	0.667^{de}	0.00^{g}	7.861^{b}	0.720^{cd}	0.00^{g}	5.149^{gh}	0.473^{gh}
UC12	0.00^{g}	9.421^{a}	1.001^{a}	17.00^{cd}	6.726^{c-e}	0.748^{b-d}	0.00^{g}	5.351^{f-h}	0.556^{e-g}
UD5	14.00^{de}	6.206^{d-f}	0.649^{d-f}	17.67^{bc}	7.207^{bc}	0.886^{ab}	11.00^{e}	6.030^{e-g}	0.557^{e-g}
CONTROL	0.00^{g}	4.975^{hi}	0.471^{gh}	0.00^{g}	6.129^{ef}	0.500^{f-h}	0.00^{g}	4.169^{i}	0.369^{h}
$LSD_{0.05}$	3.581	0.884	0.157	3.581	0.884	0.157	3.581	0.884	0.157
Pr>F	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table 2.9. Effect of Acidity/alkalinity on nodulation and plant biomass of *Trifolium repens* with or without rhizobia inoculation

Rhizobia Strains	*Acidity/alkalinity (pH 5)*			*Acidity/alkalinity (pH 7)*			*Acidity/alkalinity (pH 9)*		
	Nodule number	*Fresh biomass (g)*	*Dry biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry Biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry biomass (g)*
SAC1	29.56^{ab}	0.478^{hi}	0.043^{ef}	30.89^{ab}	0.777^{d}	0.079^{c}	9.33^{e}	0.593^{fg}	0.053^{de}
SAC2	25.22^{cd}	0.439^{i}	0.041e-g	33.33^{a}	1.650^{a}	0.138^{a}	11.11^{e}	0.591^{fg}	0.049^{e}
SAC3	28.11^{b-d}	0.660^{ef}	0.070^{cd}	29.11^{bc}	1.560^{b}	0.144^{a}	8.33^{e}	0.477^{hi}	0.079^{c}
SR4	33.56^{a}	0.540^{gh}	0.054^{de}	25.00^{d}	1.124^{c}	0.116^{b}	9.33^{e}	0.679^{e}	0.070^{cd}
CONTROL	0.00^{f}	0.0342^{j}	0.0267^{fg}	0.00^{f}	0.3367^{j}	0.0380^{e-g}	0.00^{f}	0.3180^{j}	0.0233^{g}
$LSD_{0.05}$	4.023	0.076	0.018	4.023	0.076	0.018	4.023	0.076	0.018
Pr>F	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table 2.10. Effect of Acidity/alkalinity on nodulation and plant biomass of *Lupinus albus* with or without rhizobia inoculation

Rhizobia Strains	*Acidity/alkalinity (pH 5)*			*Acidity/alkalinity (pH 7)*			*Acidity/alkalinity (pH 9)*		
	Nodule number	*Fresh Biomass (g)*	*Dry biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry Biomass (g)*	*Nodule number*	*Fresh biomass (g)*	*Dry biomass (g)*
VK10	4.00^{a}	6.649^{a}	0.635^{a}	0.00^{b}	6.740^{a}	0.650^{a}	0.00^{b}	4.022^{c}	0.494^{b}
CONTROL	0.00^{b}	5.652^{b}	0.5513^{b}	0.00^{b}	5.102^{b}	0.492^{b}	0.00^{b}	3.412^{c}	0.366^{c}
$LSD_{0.05}$	1.453	0.678	0.082	1.453	0.678	0.082	1.453	0.678	0.082
Pr>F	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

2.8 Discussion

This study evaluated the nodulation efficacy of 25 rhizobia isolates from SARCC under normal and abiotic stress conditions. Although a total number of 40 isolates were selected from the SARCC, only 25 isolates were evaluated for the nodulation efficacy based on the availability of legume seeds of their original hosts and their previous performance under normal conditions in a glasshouse nodulation efficacy test. The authentication of rhizobia is required to screen effective native rhizobia isolates to determine their symbiotic efficiency. The glasshouse nodulation efficacy test in this study revealed that inoculating *Medicago sativa, Trifolium repens, Vigna unguiculata* and *Phaseolus vulgaris* with rhizobia isolates previously isolated from these legumes induced nodulation on their original host. The current results concur with previous studies reporting that inoculation with efficient rhizobia strains can significantly increase nodulation (Hungria and Mendes, 2015; Tounsi-Hammami *et al*., 2016). Osei *et al*. (2018) reported that rhizobia can induce nodule formation subsequently capable to fix atmospheric nitrogen is commonly used to evaluate the inherent relation between rhizobia and their legume hosts. Control legumes or legumes without inoculation did not form any nodules revealing that there was no external contamination during the glasshouse experiment. The absence of contamination is crucial for the bacteria nodulating legume authentication experiment (Ogutcu *et al*., 2008; Hassen *et al*., 2014).

Among the SARCC isolates tested, most isolates inoculating *Vigna inguiculata* were highly effective and resistant to the abiotic stresses tested, followed by isolates inoculating *Medicago sativa* and *Trifolium repens.* Isolates inoculating *Lupinus albus* and *Phaseolus vulgaris* were the least effective. Effective isolates in most of the abiotic stresses tests provided the highest plant biomass and number of nodules when tested under acidity or alkalinity and salinity conditions.

This finding concurs with results by Beshah and Assefa (2019), they reported that stress tolerant isolates have the ability to thrive under various environmental stresses as they can occupy root nodules, fix atmospheric nitrogen to improve crop production.

Nodulation varied based on the legume species. Amongst the legumes, *Vigna unguiculata*, *Trifoloium repens*, *Phaseolus vulgaris* and *Medicago sativa* plants were the most nodulated when compared to *Lupinus albus* which was the least nodulated. This could be as a result of genetic differences among the species, it has been demonstrated that legume species and microsymbionts interactions are highly specific (Qiang *et al*., 2013). Moreover, although nodulation was observed in most of the inoculated legumes and the nodules were pink, however, some nodules were not pink in color and the un-inoculated treatments did not form any nodules. A number of authors have demonstrated that for a bacterial isolate to be classified as a legume nodulating bacteria, its identity must first be confirmed through plant nodulation efficacy test on relevant legume host. Therefore, nodulation is used as a confirmatory test to determine whether the bacteria have the ability to nodulate legumes or not (Lafay and Burdon, 2007; Arora *et al*., 2017).

In the current study, aunthetication test revealed that most of the rhizobia isolates inoculating *Medicago sativa, Trifolium repens, Vigna unguiculata* and *Phaseolus vulgaris* resulted in pink nodules formation. Nodules that are actively fixing nitrogen contain leghaemoglobin and its presence is indicated by the red color on the interior of the nodules, which shows whether the bacteria is alive and active (Virtanen *et al*., 1947). Leghaemoglobin is a red haemeprotein specifically accumulated in nodule; it plays a vital role in nitrogen fixation and this is achieved by protecting nitrogenase from inactivation by molecular oxygen (Appleby, 1984). Moreover, pink nodules contain *nifH* genes and expreses them actively, these genes codes for the synthesis

of nitrogenase enzymes used to reduce N to NH_3 (Rondon *et al*., 2007). A previous study by Farid and Navabi (2015) also revealed that most of the root nodules were pink in colour; indicating the presence of a protein containing iron (leghaemoglobin) which plays an important role during effective nitrogen fixation process. The current results also revealed that the color of *Medicago sativa, Trifolium repens* and *Vigna unguiculata* leaves inoculated with rhizobia were dark green. Morever, *Phaseolus vulgaris* showed nodulation but the leaves were not dark green; they were pale green to yellow. A study by Degefu *et al*. (2018) revealed similar results to the current findings; their study was based on the authentication and efficacy test of *Vigna unguiculata*. The study's findings reported that, isolates effective in fixing atmospheric nitrogen results in deep green leaves and deep red or pink nodules. Dungu (2017) also reported that cow pea formed dark green leaves upon inoculation with *Bradyrhizobium strains* when compared to the non-inoculated plants which formed yellowish leaves.

The current findings demonstrated that inoculating *Medicago sativa, Trifolium repens, Vigna unguiculata, Lupinus albus* and *Phaseolus vulgaris* with rhizobial isolates enhanced nodulation and nitrogen fixation. This in turn improves nitrogen concentration on the plant's shoot and biomass. The results of the glasshouse nodulation efficacy test demonstrated significant increases in nodulation, fresh and dry biomass. Previous reports indicated that efficient rhizobia strains stimulate the formation of more nodules which provide legumes with more fixed nitrogen resulting in greater biomass yield, increased nodulation, nitrogen fixation and plant growth (Thalikarathna *et al.*, 2019).

Nodulation and plant growth were highly affected by different concentrations of NaCl in this current study. Plants growth was retarded with increased NaCl concentration compared to plant growth and nodulation under normal conditions. The relationship between legumes and rhizobia

under saline conditions to be successful requires isolates that can show tolerance to higher NaCl concentration. The impact of salt depends on plant species, salinity levels and ionic composition (Yadav *et al.,* 2010). *Vigna unguiculata* exhibit tolerance to high temperature, drought, high salinity, ability to reduce soil erosion and fix atmospheric nitrogen, therefore it is used in sustainable farming (Timko *et al.,* 2007; Agbicodo *et al.,* 2009; Merwad *et al.,* 2018). Munns and Tester (2008) also revealed that compared to other crops, alfalfa is relatively tolerant to salt stress. These current results also revealed that tolerance to higher NaCl concentrations was observed in isolates XFH13, XFH14, XS21, XBD2 and XBQ5 inoculating *Vigna unguiculata*, isolates RF15, RF35 and RF36 inoculating *Medicago sativa.*

In comparison with the glasshouse authentication test results under normal conditions, plants showed a decrease in a number of nodules, fresh and dry biomass plant biomass under abiotic stress conditions. However, inoculation of the legume plants with rhizobial isolates increased plant tolerance to saline conditions when compared to the negative controls (non-inoculated) with the same Hoaglands solution. The current results obtained from this study revealed that isolate XFH13 (*Vigna unguiculata*), RF35 (*Medicago sativa*), UC11 (*Phaseolus vulgaris*), and SAC1 (*Trifolium repens*) resulted in maximum nodulation under 50, 100 and 150 mM NaCl concentrations. Swaraj and Bishnoi (1999) reported that rhizobia thrive under saline conditions because of the ionic adjustment they make. Furthermore, rhizobia cope more efficiently with salinity in comparison with their host legumes. However, their rate of growth and survival of rhizobia varies under saline conditions as this is based on the type of strain used. Khaitov *et al*. (2020) have also recently reported that legume inoculation with rhizobia decreases ethylene levels under saline and water deficit conditions. However, this increases plants tolerance to stress, increases nodulation and photosynthesis. In this sudy, tolerance to salinity also varied per

strain used to inoculate their original host under saline conditions and inoculation enhanced plant tolerance to salinity and nodulation when compared to the non-inoculated.

Enhanced nodulation resulting from rhizobia inoculation was positively associated with fresh biomass. Some rhizobia isolates were more efficient based on the fresh and dry biomass and the number of nodules formed. The maximum growth-promoting activity based on the plant's fresh biomass was recorded in isolate XFH12 (8.730g), XBD2 (8.729g) inoculating *Vigna unguiculata,* SAC3 (1.823g), SAC2 (1.703g) inoculating *Trifolium repens*, RF35 (1.579g), RF15 (1.575g) inoculating *Medicago sativa* and UC10 (8.154g), UC11 (7.957g) inoculating *Vigna unguiculata*. These rhizobia isolates improved significantly both nodulation and fresh biomass. The glasshouse test results further indicated that efficient rhizobia strains stimulate more nodules formation and consequently improved plant growth. There were no nodules formed on the roots of legumes that were not inoculated (negative control) with rhizobia isolates revealing that there was no contamination.

Previous reports also revealed that due to the absence of rhizobia in the non-inoculated treatments (inorganic nitarate/ammonium limiting conditions), plants resulted in no nodule formation which decreased the biomass of the plants (Fatima *et al*., 2007; van Noorden *et al*., 2016). Increased salinity in the substrate has been previously reported to decrease the number of nodules and size in *Vigna inguiculata* (Al-Saedi *et al*., 2016). This decreases in the presence of rhizobia in the rhizosphere because the synthesis exopolysccharides, glucans and lipopolysaccharides (LPS) is inhibited and these are essential superficial molecules produced by the bacteria required for their interaction with plants (Tewari and Sharma, 2020). Therefore, the nodule number and mass, leghaemoglobin synthesis and nitrogenase activity decreases as the synthesis of exopolysaccharides, glucans and lipopolysaccharides is inhibited (Sunita *et al*.,

2019). Although *Vigna inguiculata* was more tolerant to salinity, isolate XCR6, XFH3 and XFH1 resulted in a decreased number of nodules and isolate XCR6 did not nodulate at all. Our current result also revealed similar results for some isolates inoculating *Medicago sativa*, *Phaseolus vulgaris*, *Trifolium repens* and *Lupin albus*. The decrease in the number of nodules with the increase in NaCl concentration (salinity) was not only observed in *Vigna inguiculata*. Some of the nodules were not pink, indicating a decrease in leghaemoglobin and nitrogenase activity.

Salinity affects plant growth, fresh and dry plant biomass (Raptan *et al*., 2001; Yupsanis *et al*., 2001; Goulam *et al*., 2002; Lobato *et al*., 2009). However, this can be overcomed by the treatment of plants with rhizobacteria, this will increase the germination, root and shoot growth, overall plant biomass, seed weight, and the yield under saline conditions (Joseph *et al*., 2007; Yasmin *et al*., 2007). Ertan *et al*. (2008) reported that radish plants treated with rhizobacteria increased growth parameters significantly under saline and normal conditions. Current results in this study are similar to these finding, most of the isolates were able to significantly increase plant growth and nodulation under salinity and normal conditions, results similar to this were reported in beans (Yildrim and Taylor, 2005). Moreover, Keneni *et al*. (2010) reported that isolates tolerant to higher NaCl concentrations are extremely competitive when they colonize the rhizosphere and nodulate their host plants in adverse environmental conditions, e.g. soils affected by salt. Therefore, the tolerance to NaCl concentration by these isolates mentioned above in this current study can potentially enhance the growth and yield of legumes under saline prone conditions.

Previously, it has been reported that soil acidity affects the persistence of rhizobia in the soil, plant's rhizosphere and nodulation efficiency in tropical areas (Graham *et al*., 1994). However,

in the current study, most of the isolates were tolerant to acidic pH as they were still able to form nodulation at pH 5. This could be due to the fact that adaptive strategies designed to minimize damage induced by acid have been evolved by rhizobia because acid tolerance mechanisms that are inducible are important to symbionts growing in acidic soils (Foster, 2000). In most instances, the transcriptional activation of genes is due to the bacterial response and products that helps cope with physicochemical stress. At least 15-20 genes have been reported to contribute to rhizobia acid tolerance (Foster, 2000; Vinuesa *et al*., 2003). To be specific, *actA, exoR, actP, actR, lpiA,* and *phrR* genes are important for the growth of rhizobia at low pH (de Lucena *et al*., 2010). In this study, the *exoR* gene was detected in isolate UC11, SAC1, SAC2 and SR4 (Chapter 3) and these isolates had the ability to form nodulation at a low pH of 5.

Soil reaction, pH in particular influences numerous soil properties and processes that affect plant growth and this can be considered as a variable key. The activity of microorganisms, nutrients solubility and availability are vital processes that are pH dependent. For an example, in acidic soils, most micro-nutrients are more available than in neutral-alkaline soil conditions and this generally favors plant growth (Loncaric *et al*., 2008). In this study, isolates were more sensitive to alkaline pH when compared to the acidic pH. However, some isolates showed tolerance under acid and alkaline stresses as they resulted in plant roots nodulation and an increased plant growth when compared to non-inoculated plants. This was evident in isolates XS21, XFH12, XFH13, XS21, XBQ5, XFH3 and XFH12 inoculated on *Vigna unguiculata* and isolates RF12, RF14, RF15, RF36 and RAK1 inoculated on *Medicago sativa* (Table 6 and 7). Farissi *et al*. (2014) revealed that environmental conditions which are highly acidic and alkaline limit rhizobia growth and the successful nitrogen fixation with the legume host. Higher soil pH negatively affects the growth and survival of rhizobia because the essential mineral nutrients required by

rhizobia are no longer available. Moreover, some of the micro-nutrients and elements that are not essential can be toxic when available at a higher concentration. In contrast, although the availability of macro-nutrients is increased, the availability of phosphorus and micro-nutrients is reduced, resulting in lower levels which can in turn negatively affect plant growth (Jiang *et al*., 2017). Therefore, in agricultural production, it is best to select rhizobia isolates capable of tolerating acid and alkaline stresses.

Legume inoculation with rhizobia isolates varied per legume at different pH stresses. Isolate XCV14 did not show any response to inoculation. In a previous study, Giller (2001) reported the absence of some legumes responses to rhizobia inoculation under various environments. This can be due to the host plant's inherent characteristics and the type of rhizobia species used, including the nitogen fixation sensitivity to the acidity, dryness, salinity, low fertility and high temperatures of the soil (Graham *et al*., 1994; Wolde-meskel *et al*., 2018). In this study, the isolates showed more sensitivity to alkaline conditions as they resulted in reduced nodulation or no nodulation upon inoculating their original legume hosts. The survival or multiplication of rhizobia is greatly influenced by alkaline or acidic agricultural soil. Agricultural fields generally have an average pH of 7.0-8.5. Alkaline stress can hinder rhizobia growth and subsequently the establishment of a symbiotic relationship with legumes. Therefore, it makes good agricultural practice to select rhizobia isolates that are tolerant to alkaline conditions and capable of nodulating legumes (Farissi *et al*., 2014). Isolates XS21, XFH12, XFH13, XBQ5, XFH3 and XFH12 all inoculated on *Vigna unguiculata* and isolates RF36, RF12, RF14, RF15, RF36 and RAK1 on *Medicago sativa* in the current study were the most tolerant to acid and alkaline conditions.

2.9 Conclusion

The current study confirmed that the selected rhizobia strains preserved at the SARCC formerly isolated from the root nodules of *Trifolium repens, Lupinus albus, Vigna unguiculata, Medicago sativa* and *Phaseolus vulgaris* have the ability to nodulate their original host under normal and abiotic stress conditions. The results of the glasshouse nodulation efficacy test conducted following the Koch's postulate experiment demonstrated significant ($p \leq 0.05$) increases in nodule number, fresh and dry biomass under normal and abiotic stress conditions in comparison with the non-inoculated control. The nodulation efficacy and the biomass of the plants declined under abiotic stress conditions. However, some of the isolates (RF36, RF12, RF14, RF15 and RAK1) inoculating *Medicago sativa*, isolates (XS21, XFH12, XFH13, XBQ5 and XFH3) inoculating *Vigna inguiculata* and isolates (SAC1, SAC2 and SAC3) inoculating *Trifolium repens* showed more tolerance. Therefore, these results provide baseline information in the use of rhizobia as inoculant and that these isolates might have a practical advantage when selecting rhizobia isolates to promote sustainable agricultural production systems under abiotic stress conditions.

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CHAPTER 3

Characterization of *Rhizobia* spp. and Detection of Essential Traits that Promote Nodulation of Legumes under Abiotically Stressed Conditions

This chapter has been submitted as a manuscript for publication in *Letters in Applied Microbiology* journal

Characterization of Rhizobia spp. and Detection of Essential Traits that Promote Nodulation of Legumes under Abiotically Stressed Conditions

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3.1 Significance and impact of the study

Legumes are important sources of protein in human diet and are mostly grown by smallholder farmers in South Africa. However, their growth and yield is severely affected by the amount of nitrogen available in the soil and abiotic stresses. Rhizobia promote legume plant growth, can fix atmospheric nitrogen and mitigate multiple abiotic stresses, these roles have been documented. Moreover, rhizobia have proven to be environmentally friendly with minimal production costs and environmental hazards concerned with the use of inorganic nitrogen fertilizers. Therefore, the development of rhizobia inoculants with increased resistance to abiotic stress is critical to alleviate climate change challenges.

3.2 Abstract

The growing interest in using rhizobia as inoculants in sustainable agricultural systems has prompted the screening of rhizobia species for beneficial traits that enhance nodulation and nitrogen fixation under abiotic stressed conditions. This study reports the tolerance to abiotic stresses and the phylogenetic characterization of 40 rhizobia strains previously isolated from the root nodules of *Medicago sativa, Trifolium repens, Lupinus albus, Vigna unguiculata* and *Phaseolus vulgaris*. Rhizobia spp. tolerance to various levels of temperature, acidity/alkalinity, heavy metals ($AlCl_3.6H_2O$) and salinity (NaCl) stresses were investigated. The isolates that showed significant tolerance to these stresses were characterized for the possession of *acdS* and *exoR* genes, essential in triggering stress tolerance and enhancing nodule formation in legumes. Phylogenetic characterization of the isolates was determined using nucleotide sequence analysis of the 16S rRNA, the house keeping *recA* gene, as well as *nodA* and *nodC* symbiotic genes. The isolates belong to *Sinorhizobium, Bradyrhizobium, Rhizobium Mesorhizobium* and *Aminobacter* genera, which have exhibited significant variations in their tolerance to abiotic stresses. Amid the increasing threats of the global climate change and the rising abiotic stresses, these current results provide baseline information in the selection of rhizobia for use as inoculants under abiotic stressed conditions in South Africa.

Keywords: *abiotic stress, legume, Rhizobia, phylogeny, ACC-deaminase, nodulation*

3.3 Introduction

Abiotic stresses are extremes of temperature, drought, salinity, metal toxicity and acidic soils negatively affecting several agricultural crops by decreasing crop productivity and yield globally. Plant growth is severely affected by these stresses, starting from germination to the maturing stage, resulting in severe yield loss ranging between 50 and 82% depending on the crop (Kang *et al*., 2014, Xu *et al*., 2014; Chodak *et al*., 2015; Negrao *et al*., 2017; Nadeem *et al*., 2018; Nadeem *et al*., 2019). Although plants are sessile in nature and cannot escape from these stresses, they have evolved stress resistance and tolerance adaptations that aid them to cope under abiotic stress conditions (Pfalz *et al*., 2012; Upadhyaya and Panda, 2013). Plants are able to sense stresses and react in ways, which are more favorable to their survival. Moreover, plant stress adaptive responses are triggered by exposure from the past environmental conditions. However, various mechanisms help plants overcome abiotic stresses in such a manner that can modify response to stresses they have encountered before (Crane *et al*., 2011; Ahmad *et al*., 2015; Hilker *et al*., 2015; Jiang *et al*., 2016).

Rhizobia are Gram-negative soil bacteria that belong to alpha and beta sub class of Proteobacteria which are able to fix atmospheric N_2 by forming symbiotic relationships with legumes. Thus, rhizobia are essential in the agricultural ecosystem since they result in improved legume production and reduced application of inorganic nitrogen fertilizers (MacLean *et al*., 2007; Bomfeti *et al*., 2011; Orrell and Bennett 2013; Berrada and Fikri-Benbrahim, 2014). Many of the legume-rhizobium interaction are governed by the legume host specificity to a unique species or strain of rhizobia, whereby the specificity is facilitated by recognition between plant receptor and rhizobia elicitor interactions which trigger signal transduction pathways in the plant host. This ensures that the same rhizobia strains infect and nodulate a specific species of legume

host plant (Yang *et al.,* 2010; Wang *et al.,* 2012; Batista *et al.,* 2015; Tang *et al,.* 2016; Fan *et al.,* 2017). However there is reports now that indicate the existence of various legume species which also nodulate promiscously by more than one rhizobia species and vice versa (Hassen *et al*., 2020).

There is an increasing global interest to use rhizobia as inoculants in sustainable production of legumes (Santos *et al*., 2019). This has prompted screening studies of rhizobia strains elsewhere for their ability to form legume-rhizobia symbiosis to improve growth and crop yield. In doing so, it is vital that the effective rhizobia strains selected to be developed into legume inoculants can be characterized and identified to at least the genus level of the taxonomic rank. In the past decades, rhizobia have been characterized based on conventional morphological, physiological, and biochemical methods. Serological and molecular methods have been developed also due to the conventional methods failing to identify strains within a species (Berrada *et al*., 2012). Accurate, faster and efficient molecular techniques have been developed to distinguish different microbial genera, species, and strains (Wolde-Meskel *et al*., 2004; Ismail *et al*., 2013). During the last two decades, rapid developments in the characterization of microorganisms using molecular techniques have led to radical revision and re-organization of previous bacterial phylogenies (Macrae, 2000; Lindstrom *et al*., 2010).

Characterization of rhizobia is a complex process whereby the identity of strains must be revised frequently as new rhizobia species and genera are constantly being discovered. However, the *ad hoc* committee (International Committee on Systematic of Prokaryotes) recommends the use of symbiotic genes, 16S rRNA, DNA-DNA hybridization, housekeeping and symbiotic genes as the molecular criteria used to delineate species. This committee only focuses on species definition re-evaluation (Stackebrandt *et al*., 2002; Pongsilp, 2012). In bacterial taxonomy, the 16S rRNA

gene sequence analysis is commonly used to measure the relation of organisms above species level (Martens *et al*., 2008); however, it can lack the resolving power at species level and below (Zinga *et al*., 2017). Hence to overcome such limitations, it is better to use protein-encoding genes with higher sequence divergence levels when compared to the rRNA genes (Martens *et al*., 2008; Aserse *et al*., 2012; Peix *et al*., 2015; Rouhrazi *et al*., 2016). Other studies have used housekeeping genes, nodulation and symbiotic genes to assess the diversity of rhizobia and phylogeny in different geographic locations, this is based on their high-resolution characteristic (Ampomah and Huss-Danell, 2016; Wang *et al*., 2016).

Lately, agricultural systems improve crop productivity and yield using rhizobia. The application of rhizobia has increased whereby worldwide, several farmers have developed awareness about the use of microbial inoculants due to the availability of effective and quality products in the market. These inoculants improve yield at an affordable cost when compared to chemical or artificial fertilizers (Santos *et al*., 2019). This study has made use of several strains of rhizobia spp. currently deposited at the South African Rhizobium Culture Collection (SARCC). These deposited strains were originally collected and previously isolated from nitrogen fixing effective pink root nodules of wild and cultivated legumes. Some of the isolates from the SARCC have already been commercialized and developed into inoculants. This is evident, for instance, in the previous development of *Bradyrhizobium japonicum* strain WB74 (for soybean), *Sinorhizobium meliloti* strain RF14 (for lucern), *Bradyrhizobium japonicum* strain XS21 (for groundnut) and *Rhizobium tropici* strain UD5 (for beans). The effective formulation of soybean inoculant developed in South Africa by Stimuplant Pty Ltd. from strain WB74 in 1998 is still being used throughout the country. The products registration numbers are L5799 Act 36 of 1947 and L9012 Act 36 of 1947 and are available as powdered and liquid formulations, respectively. Moreover,

although some strains have already been developed into inoculants, it is important to continuously screen for possible isolates with key mechanisms to mitigate the increasing abiotic stresses. Therefore, this study is important for effective screening of SARCC strains which can lead to the selection of elite isolates that could be potentially developed into commercial inoculants to enhance the sustainable production of legumes even under abiotic stress conditions.

The selection of stress tolerant rhizobia strains has a good potential in the development of bio-inoculants. Therefore, this study evaluated the tolerance of the rhizobia strains from the SARCC to abiotic stresses such as salinity, temperature, acidity/alkalinity and heavy metal as these stresses affect the nodulation and nitrogen fixation process. Isolates, which showed these tolerances to various stresses, were characterized by the presence of the *acdS* and *exoR* genes essential for abiotic stress tolerance and nodule formation. The phylogenetic and taxonomic positions of the rhizobia strains was determined using sequence analysis of 16S rRNA, housekeeping gene (*recA*) and symbiotic genes (*nodC* and *nodA*).

3.4 Materials and Methods

3.4.1 Sample collection and growth conditions

A total number of 40 rhizobia isolates previously collected from the root nodule of the legumes dry bean (*Phaseolus vulgaris*), lucerne (*Medicago sativa),* lupin *(Lupinus albus),* clover *(Trifolium repens)* and cowpea *(Vigna unguiculata)* and deposited at the South African Rhizobium Culture Collection (SARCC) were used. The isolates were grown on Yeast Mannitol Congo Red (YM-CR) agar plates (Vincent, 1970) and incubated for 2-5 days at 26 ± 2 ºC until colony formation.

3.4.2 In vitro assays for various abiotic stress tolerances

A total number of 40 isolates were evaluated for growth under various abiotic stress conditions: temperature, salinity, heavy metals, and pH tolerance. For tolerance to temperature evaluation the isolates were streaked on YMCR agar plates and incubated at 16, 28, and 36 °C (Lupwayi and Haque, 1994; Jordan, 1984). Fresh cultures of rhizobial isolates were streaked on YMCR agar plates and incubated at different temperatures of 16, 28, and 36 °C. For each stress evaluated, the experiment was conducted in three replicates. In order to accomplish the heavy metal tolerance of the isolates, the isolates were streaked on YMCR agar plates supplemented with various concentrations (50, 100 and 150 mM) of aluminium chloride. The ability of the isolates to exhibit tolerance to acidity or alkalinity was determined by growing each isolate on YMCR agar adjusted to the pH of 5, 7 and 9 respectively using 1N NaOH and 0.1N HCl as described by Bernal and Graham, (2001). Salinity tolerance was determined by streaking the isolates on YMCR supplemented with different concentrations (50, 100 and 150 mM) of sodium chloride (NaCl). Tolerance to abiotic stress was determined by the presence of bacterial growth after incubating the plates at incubated at 26 ±2 ºC for 3-7 days except of temperature tolerance where the inoculated plates were incubated at different temperatures of 16, 28, and 36 °C. After 3-7 days of incubation, rhizobia growth was observed. The results were recorded as + for poor growth, ++ for moderate growth, +++ for good growth and – for no growth.

3.4.3 Phylogenetic characterization of bacterial isolates

The total genomic DNA was extracted from 1 mL Yeast Mannitol Broth (YMB) culture suspension of the selected rhizobia isolates using the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA) following the manufacturer's instruction. The DNA was used as a

template in the amplification of 16S rRNA*, nodC, nodA, recA, acdS* and *exoR* genes. The PCR amplification of 16S rRNA*, nodC, nodA, recA, acdS* and *exoR* genes were performed in a total reaction mixture of 25 µL. The reaction mixture consisted of 0.5 µL of forward and reverse primers (10 µM) of the gene of interest, 2.5 µL green go Taq flexi buffer (5X), 1.5 µL $MgCl_2$ (25 mM), 2.5 µL dNTP's (10 mM), 2.5 µL of extracted genomic DNA , 0.125 µL Taq G2 flexi DNA polymerase (5u/µL) and 16.875 µL of nuclease free water. The PCR was performed in an Eppendorf Master Cycler Gradient apparatus (Applied Biosystems, USA).

Primer pair, 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1485R (5′-TAC GGY TAC CTT GTT ACG ACTT-3′) were used to amplify the 16S ribosomal RNA (rRNA) gene (Lane, 1991). Primer pair, recA-63F (5′-ATC GAG CGG TCG TTC GGC AAG GG-3′) recA 504R (5′-TTG CGC AGC GCC TGG CTC AT-3′) were used to amplify the *recA* (DNA recombinase A) gene (Gaunt *et al*., 2001).

Primer pair, nodA-1 (5′-TGC RGT GGA ARN TRN NCT GGG AAA-3′) and nodA-2 (5′-GGN CCG TCR TCR AAW GTC ARG TA-3′) were used to amplify the nodA gene (Palaniappan *et al*., 2010). Primer pair, nodCf (5′-GCT GCC TAT GCA GAC GATG-3′) and nodCr (5′-GGT TAC TGG CTT TCA TTT GGC-3′) were used to amplify the nodC gene (Laguerre *et al*., 2001). Primer pair, F1936f (5'-GHG AMG ACT GCA AYW SYG GC-3') and F1939r (5' - GARGCRTCGAYVCCRATCAC-3') were used to amplify the *acdS* gene (Blaha *et al*., 2006). Primer pair exoRFw (5′-GTT GCC GCC TGC CTG AGA TGA AC-3′) and exoRRW (5′-GAG AGC AGC GCG TTG ACG AAG AAG-3′) were used to amplify *exoR* gene (Mazur *et al*., 2011). The amplified PCR products were visualized using 1 % agarose gel electrophoresis stained with ethidium bromide to verify the size. The PCR samples were sent to Inqaba Bioech Laboratories, South Africa, Pretoria for sequencing.

The 16S rRNA, *acdS*, *nodC*, *nodA, recA* and *exoR* gene sequence were edited using Bioedit v.7.2.5 (Hall, 1999). The BLASTn program (Altschul *et al*., 1990) was used in nucleotide similarity search by comparing the sequences of the gene of interest to those on NCBI GeneBank (Benson *et al*., 2017). The edited sequences were aligned using ClustalW (Thompson *et al*., 1994) in which portions of sequences that could not be aligned properly were removed from the data matrix used for the phylogenetic analysis. All phylogenic trees were constructed using MEGA 7 software based on the bootstrap analysis of 1000 replicates to calculate the statistical significance of the phylogenetic tree branches (Kumar *et al*., 2018) and the evolutionary history was inferred using maximum likelihood (ML) and neighbor- joining (NJ). The phylogenetic trees were generated by the Kimura 2-parameter model to calculate evolutionary distances (Kimura, 1980). The nucleotide sequence were deposited in the GenBank at the National Center for Biotechnology Information (NCBI) and their accession numbers are listed as follows: (16S rRNA OM835959-OM835997), (*acdS* ON597815-ON597822*)*, (*nodC* ON721594-ON721599*)*, (*nodA* ON721600-ON721614*), (recA* ON673110-ON673140*)* and (*exoR* ON533660-ON533664*)*.

3.5 Results and discussion

This current study reports the characterization of 40 rhizobia spp. obtained from the South African Rhizobium Collection (SARCC) and their potential in mitigating various abiotic stresses that can affect legume nodulation and growth. Using phenotypic and molecular approaches, some of the rhizobia isolates have been detected to possess key beneficial traits such as ACC deaminase activity and exopolysaccharides production. These traits are involved in the mitigation of abiotic stresses (Hussain *et al*., 2014; Nascimento *et al*., 2016). The 16S rRNA gene was amplified using the polymerase chain reaction (PCR) which yielded a single fragment

of approximately 1500 bp length product. Moreover, phylogenetic analysis of the 16S rRNA gene indicated that the rhizobia isolate formed four distinct major clades belonging to *Sinorhizobium, Bradyrhizobium, Rhizobium, Mesorhizobiu,* and *Aminobacter* genera (Figure 3.1).

In clade I, isolates SAC2, SR4, SAC3, SAC1, UC11, UC12, UC10, XFH1, XFH3, XS34, UD5, XBD2 and XHH1 formed a clustered with *Rhizobium* spp. In clade II, isolates XCR6, RAK1, RF36, RF35, RF12 and RF14 formed a cluster with *Sinorhizobium* spp. In clade III, isolates XAP2, XHK1, XAP5, XEL1, XCV14, XEL2, XHJ18 and XFL2 formed a cluster with *Mesorhizobium* spp. In clade IV, isolates XHT1, XFH3, VK10, XFH14, XCG2, XFH12, XS21, WB74, USDA 122, WB1, XBQ5 and XDF5 formed a cluster with *Bradyrhizobium spp*. Isolate XFL2 was closely related to *Aminobacter aminovorans* hence the 16S rRNA sequence reference strain of *A. aminovorans* NR_025301 was also included in the 16S rRNA sequence alignment used to construct the phylogenetic tree. However, the phylogenetic tree revealed that the isolate was grouped with the *Mesorhizobium* spp. Estrella *et al.* (2009) also found that although 16S rRNA analyses revealed that isolate BA135 was closely related to *M. chacoense* LMG PR5 as they were grouped together, 16S rRNA sequencing revealed that BA135 had greater similarity (99.7%) to the *Aminobacter aminovorans* strain DSM6450 than *M. chacoense* LMG PR5 with 97.6% similarity. Moreover, according to McDonald *et al*. (2005), the genus *Aminobacter* has nerver been reported to nodulate legumes but it is phylogenetically related to *Mesorhizobium*, however, Estrella *et al*. (2009) were the first to report a member of the *Aminobacter* genus with the ability to form a symbiotic relationship.

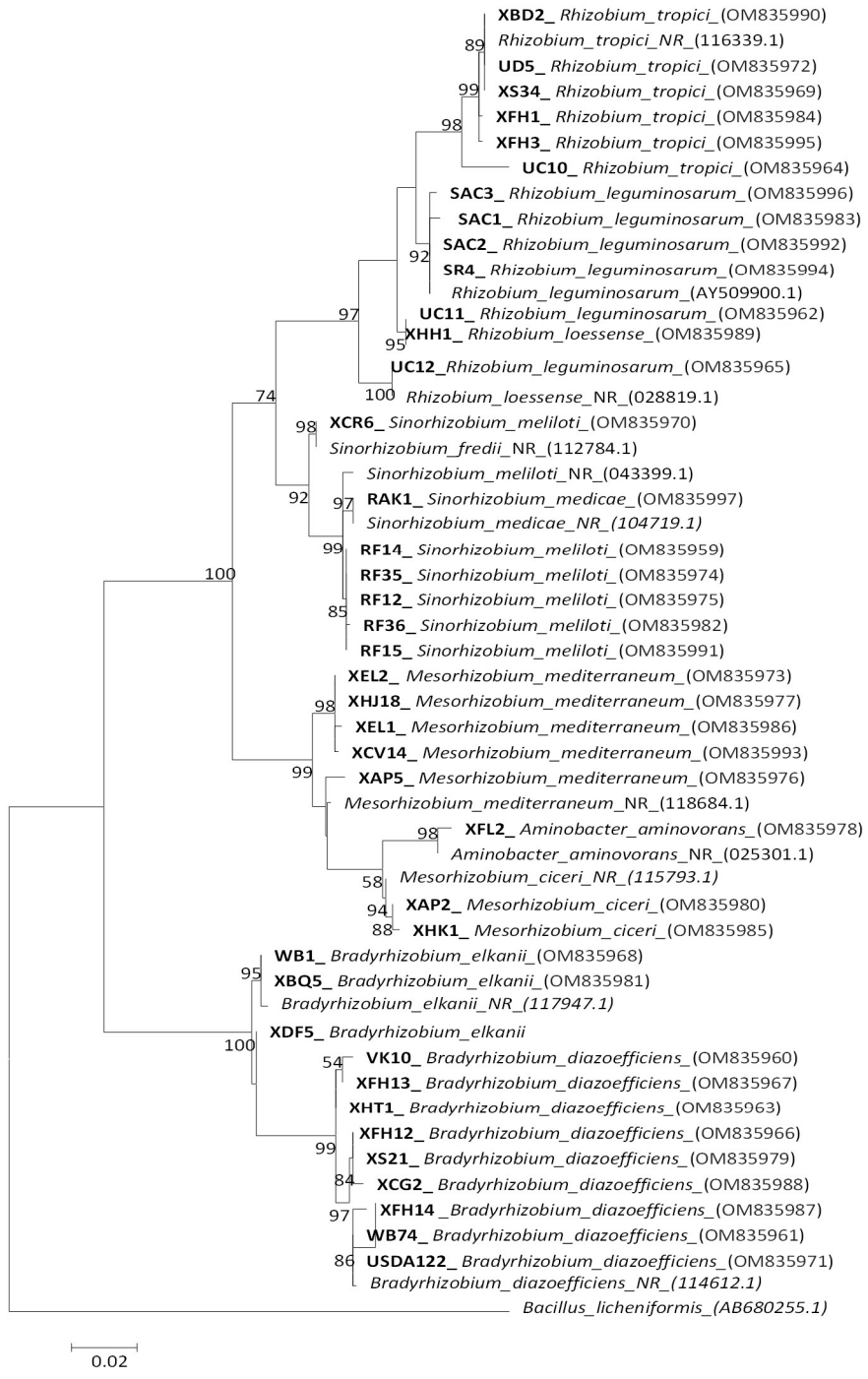


Figure 3.1. Maximum likelihood phylogenetic tree of 16S rRNA sequences of rhizobia isolates with 12 reference strains obtained from the GenBank. The isolates used in this study aligned themselves with *Mesorhizobium*, *Rhizobium*, *Sinorhizobium, Aminobacter* and *Bradyrhizobium* genera.

Analyzing the 16S rRNA gene sequences also revealed that *Bradyrhizobium* spp. and *Rhizobium* spp. were the most dominant whereby out of the 40 isolates characterized, 12 isolates were identified as *Bradyrhizobium* and 13 isolates as *Rhizobium*. In this study, the rhizobia strains identified as *Bradyrhizobium* spp. were originally isolated from root nodules of lupin *(Lupinus albus)* and cowpea *(Vigna unguiculata).* Previous reports have indicated that *Bradyrhizobium* species form efficient symbiotic relationships with most of the legumes and they are important for ecology and economy (Van Insberghe *et al*., 2015; Sprent *et al*., 2017). *Bradyrhizobium* is the most diverse rhizobia genus and an ancestral symbiont highly distributed as a free-living bacterium in soil and even in places where legumes are absent (Delmont *et al*., 2012; Okubo *et al*., 2012; Parker *et al*., 2015; Guha *et al*., 2016). This explains why *Bradyrhizobium* species were the most dominant genera amongst the isolates which were characterized in the current study.

Analysis of 16S rRNA gene sequences is an excellent tool used in molecular characterization of bacteria and is widely used to determine their phylogenetic relationship and taxonomic position for closely related groups. However, 16S rRNA sequence analysis is unable to determine neighbours because various species can share or have 16S rRNA sequences that are almost identical (Silva *et al*., 2012; Ismail *et al*., 2013; Yan *et al*., 2017; Klepa *et al*., 2019; Helene *et al*., 2020). Due to these limitations, protein-encoding genes have been proposed as alternative phylogenetic markers and *recA* is one of the housekeeping gene used frequently to trace the evolutionary history of bacteria (Gaunt *et al*., 2001; Rivas *et al*., 2009; Kalita and Małek, 2017; Huang *et al*., 2018). Thus, to confirm the phylogeny of the isolates analyzed using the 16S rRNA gene sequences, additional phylogenetic characterization using analysis of the *recA* (DNA recombinase A) gene sequences was performed.

In this study, a fragment of approximately 500bp *recA* gene was detected in only 33 isolates out of the 40 tested. The *recA* genes for the remaining isolates could not be sequenced effectively with all optimization procedures. Like in the 16S rRNA gene, phylogenetic construction using the *recA* grouped the isolates into four clades belonging to *Sinorhizobium, Bradyrhizobium, Rhizobium* and *Mesorhizobium* genera (Figure 3.2). Isolates for which the BLAST alignment search resulted in 97% -100% similarities to *Rhizobium* spp (UC11, UC1O, UC12, XS34, UD5, SAC1, XFH1, XBD2, SR4, SAC2, SAC1 and XFH3) were grouped in Clade I. Clade II, isolate XEL2, XAP5, XHJ18, XAP2, XHK1, XEL1 and XCV14 showed 99-100% similarity to *Mesorhizobium* spp. Clade III, isolate VK10, WB74, XHT1, XFH12, XFH13, USDA 122, XDF5, XFH14 and XCG2 showed 99-100% similarity to *Bradyrhizobium* spp. Clade IV, isolate XCR6, RF35, RF12, RF36 and RF15 showed 99-100% similarity to *Sinorhizobium* spp. Previously, *recA* was used to produce phylogenetic trees that complement the phylogenies of 16S rRNA. The gene was used individually or together with other housekeeping genes (Gaunt *et al*., 2001; Martens, 2007; Aserse *et al*., 2012; Dall Agnol *et al*., 2014). The sequence analysis of *recA* gene compared to the 16S rRNA gene sequence analysis revealed that the isolates belong to the same genera.

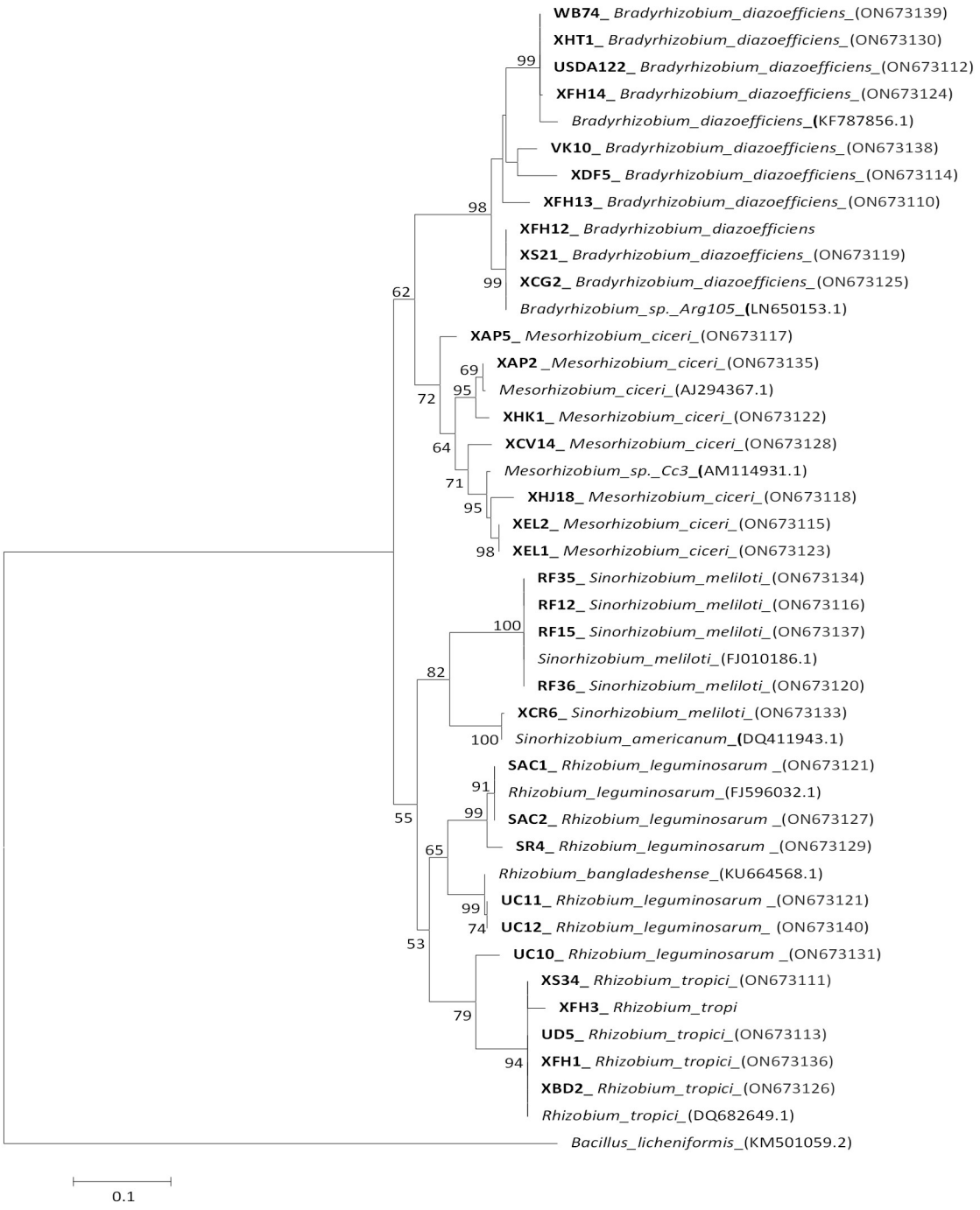


Figure 3.2. Maximum likelihood phylogenetic tree of *recA* sequences of rhizobia isolates with 10 Reference sequences were obtained from the GenBank. The isolates used in this study aligned themselves with *Mesorhizobium, Rhizobium, Sinorhizobium* and *Bradyrhizobium* genera.

To get insightful information concerning the genetic diversity of the symbiotic nitrogen fixing genes found in the SARCC, we analyzed the *nodA* and *nodC* sequences. In this current study 40 isolates were tested, 660bp fragments of the *nodA* gene were successfully amplified in 16 isolates whereas 480bp fragments *nodC* were only successfully amplified in seven isolates. Moreover, 21 isolates tested for nodulation authentication under glasshouse conditions resulted in nodulation even though *nodA* gene was not amplified in all isolates. The sequence similarity of *nodA* gene with reference type sequence varied from 71- 99%. Isolate XFH1, XBD2, SAC2, SR4, XFH3 and SAC3 aligned with *Rhizobium* spp, isolate RF35, RF15, RF14 and RAK1 aligned with *Sinorhizobium* spp and isolate XAP5 aligned with *Mesorhizobium* spp (Figure 3.3).

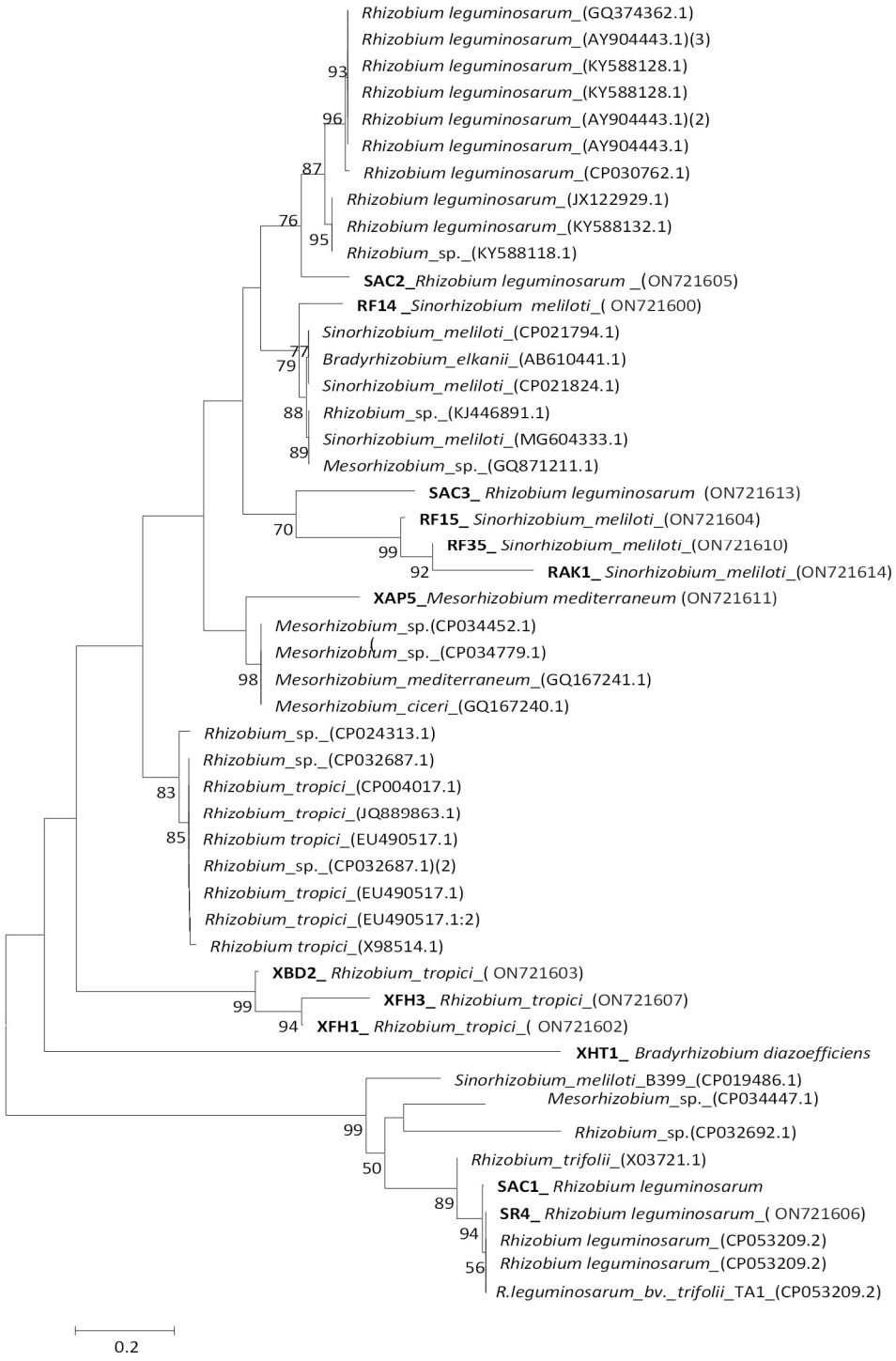


Figure 3.3. The Neighbor-Joining phylogenetic tree of *nodA* sequences of rhizobia isolates with 36 reference sequences obtained from the GeneBank. The isolates used in this study align themselves with *Rhizobium*, *Mesorhizobium* and *Sinorhizobium* genera.

In this study, *nodA* gene was not amplified in most of the rhizobia strains even after using different set of primers (TsnodA2 and TsnodA3) but could nodulate their host legume during the nodulation authentication test. According to Howieson *et al*. (2013) rhizobia isolates with the inability to amplify *nodA* gene does not imply that they are ineffective endophytes. However, this might be due to degenerated primers causing primer mismatches, the Nod factor-independent symbiotic signaling pathway and the release of a plasmid carrying symbiotic determinants upon cultivation. Previous studies have also indicated failure to amplify *nodA*, however the rhizobia strains had the ability to nodulate their host legumes during the authentication experiments. This clearly indicates that these strains harbor nodulation-related genes or they use an alternative nodulation signaling pathway (Giraud *et al*., 2007). Recently, Bopape *et al*. (2020) reported the nodulation of the legume *Cajanus cajan* by *Rhizobium tropici* SARCC-755 isolated from native soil. According to the study, this rhizobia strain does not contain any of the common nodulation genes, but whole genome sequence analysis showed the rhizobium probably uses purine biosynthetic pathways for inducing nodules on its legume host.

The amplification of the *nodC* gene produced an expected fragment of 480 bp in six isolates. The sequence analysis of isolate UD5, XS34, XFH1, XBD2 and XFH3 showed 100% similarity to *Rhizobium tropici*. Isolate WB1 showed 90 % similarity to the genus *Bradyrhizobium*. The isolates clustered into two distinct clades in which clade I represents the *nodC* belonging to the *Rhizobium* spp. (WB1, UD5, XS34, XFH1, XBD2 and XFH3) with a 100% similarity whereas clade II represents the *Bradyrhizobium* (WB1) *nodC* types at 90% similarity with reference strains (Phylogenetic tree not shown). The *nodC* gene in this study was only successfully amplified in six isolates even after using different set of primers (nodcF and nodc1, nodcF and nodc1). Similar reports also exist where *nodC* PCR amplification of *Mimosa* nodulating rhizobia

was possible only for a few of the strains used in that study (Bontemps *et al*., 2010). Failure to amplify *nodC* gene could be because the genes are located on the plasmid, and after nodulation the plasmid could be lost as it becomes a genetic burden for the bacteria (Lenart-Boron *et al*. 2019). Wdowiak-Wrobel *et al*. (2017) observed that *Bradyrhizobium* strains (ORS278 and BTAi1) which had the ability to form a symbiotic relationship with *Aeschynomene species* did not harbour any *nodABC* genes but was still able to develop a symbiotic relationship with legume plants.

The 558 bp fragments of *acdS* gene of were only amplified in nine isolates even after trying various primers (acdSR1 and acdSF1, Deg ACCr and Deg ACCf, acdSR2 and acdSF2). According to the maximum likelihood (ML) phylogenetic tree of the *acdS* gene, the isolates formed two clades (Figure3.4). Isolate VK10, WB74, XHT1, XFH12, XFH13, USDA 122, XS21, and XFH14 had 94-100 % nucleotide similarity with *Bradyrhizobium* spp. Isolate RF36 had 98 % nucleotide similarity with *Sinorhizobium* sp. The *acdS* gene in this study was amplified in nine isolates and eight of the isolates were identified as *Bradyrhizobium* spp. This concurs with a previous report by Nascimento *et al*. (2014), revealing that the ACC deaminase genes are highly widespread and transmitted between *Bradyrhizobium* spp. and *Paraburkholderia* spp. According to Nascimento *et al*. (2018) the presence of the *acdS* gene in *Bradyrhizobium* spp. and *Paraburkholderia* spp. results from previous ancestral selection relating to the ability of these species to internally colonize plants and modulate the levels of plant ethylene for regulating plant's basal defense response. Moreover, the ACC deaminase enzyme is essential for the rhizobia-legume association, and this has been indicated in previous studies, particularly under abiotic stress conditions (Ma *et al*., 2004; Brigido *et al*., 2013; Nascimento *et al*., 2016; Belimov *et al*., 2019).

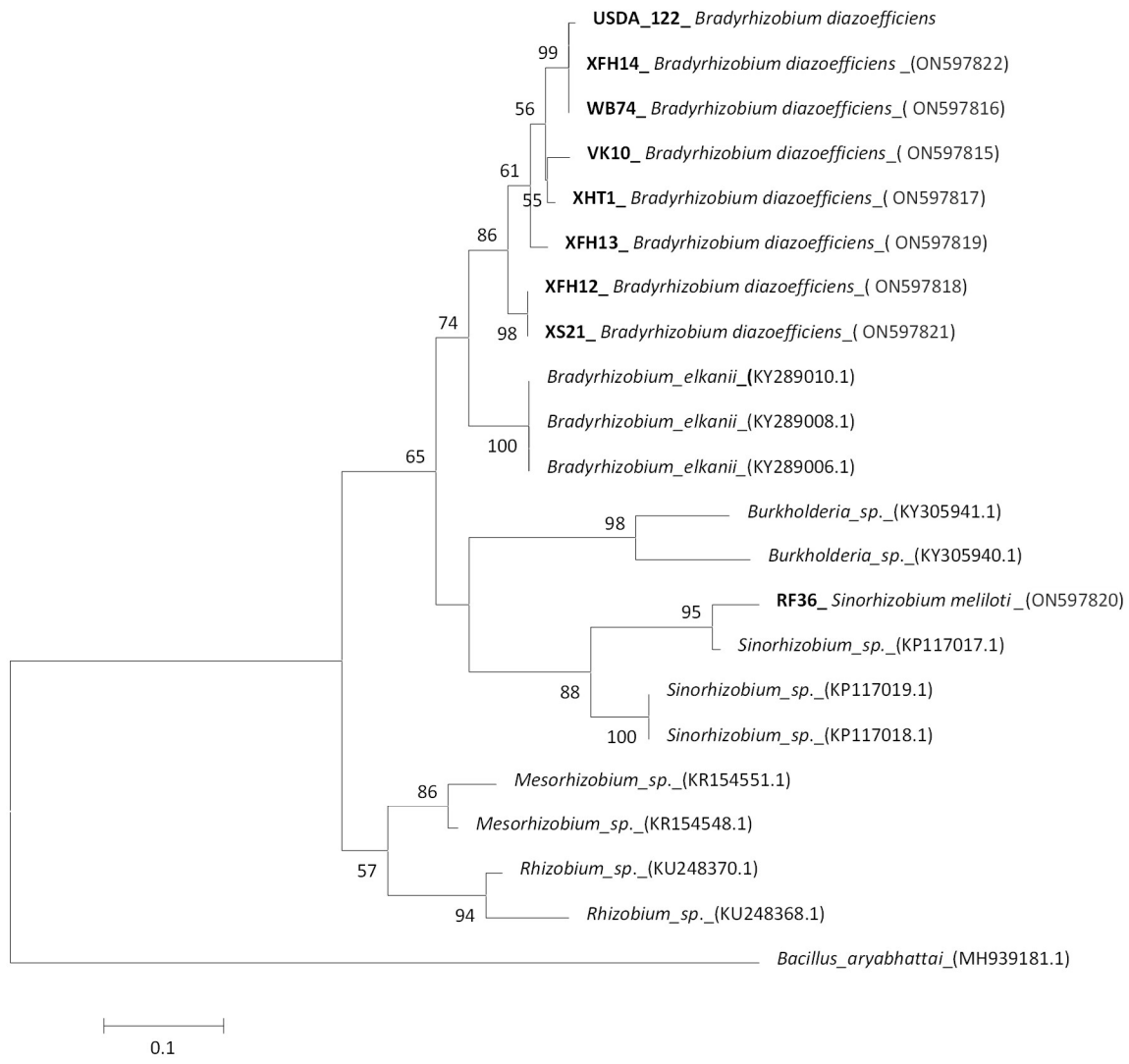


Figure 3.4. Maximun likelihood phylogenetic tree of a*cdS* sequences of rhizoba isolates 13 Reference sequences were obtained from the GenBank. The isolates used in this study aligned themselves with *Bradyrhizobium* and *Sinorhibium* genera.

The effort to amplify the *exoR* gene using a wide range of primers (*exorFW* and *exorRW, Pexor3* and *Pexor1, mucRR* and *mucRF, mucR antisense* and *mucR sense, Pexoy1 1610R* and *Pexoxy 588*) was only successful for five isolates out of the 40 tested. For the five isolates all belonging to *Rhizobium* spp. (SAC1, SAC2, SR4, UC11, UC12), a 416 bp fragment of the *exoR* gene was

amplified. The *exoR* gene sequences showed a 97-99% similarity to the *exoR* gene of *Rhizobium* spp. (Figure 3.5).

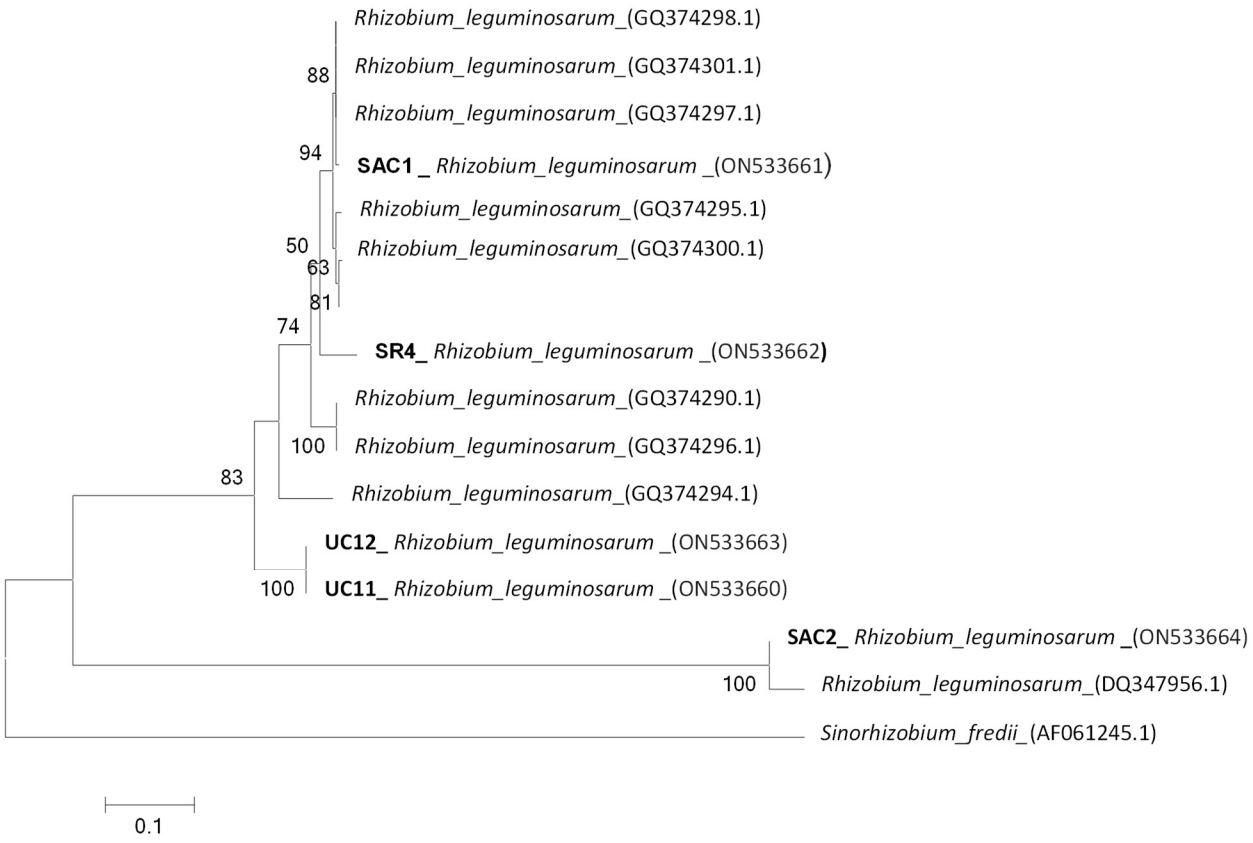


Figure 3.5. Maximum likelihood phylogenetic tree of *exoR* sequences of rhizobia isolates with 11 Reference sequences were obtained from the GenBank. The isolates used in this study aligned themselves with *Rhizobium* genera.

In our current study, *exoR* gene was amplified in rhizobia isolates previously collected from the root nodule of the legumes *Trifolium repens* and *Vigna unguiculata*. Previous studies have revealed that the production of exopolysaccharides is required by legumes (e.g *Medicago*, *Pisum*, *Trifolium*, *Leucaena*, and *Vicia* spp) to form effective nodulation; this is in addition to Nod factors and flavonoids (Janczarek and Urbanik-Sypniewska, 2013). The *exoR* gene has been

previously detected on the chromosome of *Rhizobium leguminosarum* bv. *Viciae* (Tomlinson *et al*., 2010). Previous findings have revealed that the symbiotic function of EPS in rhizobia with the ability to induce determinate nodules have also shown that EPS plays a signalling role at the late stages of infection thread and bacterial release in symbiosis with *Lotus japonicus* (Kelly *et al*., 2013). Moreover, the exposure of *S. meliloti* to acidity up regulate genes involved in the biosynthesis of exopolysaccharide and down regulate genes related to motility and chemotaxis (Hellweg *et al*., 2009).

Forty rhizobia isolates were characterized and screened for their tolerance to high and low temperatures, salinity, heavy metals, and acidity or alkalinity. A screening test for temperature tolerance revealed that 50% of the tested isolates could effectively grow at varying temperatures of 16 ℃, 28 ℃, and 36 ℃ (Table 1). Moreover, only 5 isolates could not grow at 36 ºC; however, all isolates resulted in slight to effective growth at 16 ºC. In our study, all isolates tested also exhibited sufficient growth at 28 ℃ as it has been described by Somasegaran and Hoben (2012) that most rhizobia species can optimally grow at a temperature of 28 ℃. Temperature is a major determining factor for bacterial community diversity and influences the survival and persistence of rhizobia strains in soil (Abd-Alla *et al.,* 2014). High temperature reduces nitrogen fixation; this is mainly because the nitrogenase activity is reduced (Ormeno-Orrillo *et al*., 2016; Liu *et al*., 2018). Therefore, selecting rhizobial strains that are temperature tolerant may be an efficient way to mitigate temperature-induced stress in host plants (Yadav and Nehra, 2013).

The response of most rhizobia strains to saline stress varies and bacterial growth can be inhibited at 100 mM NaCl (Zahran, 2001), hence the NaCl concentration ranging between 50-150 mM

NaCl was chosen for this study. The screening assay for salinity tolerance revealed that 13 isolates exhibited good growth in a culture medium supplemented with 50, 100 and 150 mM NaCl concentrations. As expected, the isolates were more tolerant to 50 mM NaCl, a study by Niste *et al*. (2015) reported that a small amount of salt does not affect the growth of rhizobia that much as it was observed in their data and the data obtained in this study. Table 1 shows 18 isolates with no growth in 150 mM NaCl supplemented medium. Although rhizobia strains are salt-tolerant in general, their growth is affected by NaCl treatment and strains exhibited a significant variations in salt tolerance (Zahran *et al*., 2003; Laranjo and Oliveira, 2011; Moussaid *et al*., 2015; Niste *et al*., 2015).

This study also revealed that the ability to resist salinity decreased with an increase in concentration where the isolates showed tolerance to 50 mM NaCl but their growth was reduced or completely inhibited at 150 mM NaCl. Kucuk and Kivanc (2008) have indicated that rhizobia from chickpea nodules vary in their tolerance to NaCl, some strains may grow at a high NaCl concentarion such as 500 mM, whereas others growth may even be inhibited at 100 mM NaCl. Our results indicated that six *Sinorhizobium* strains (XCR6, RF14, RF35, RF12, RF15 and RAK1) and five *Rhizobium* strains (XS34, XBD2, UC10, UD5 and XFH1) were tolerant to 150 mM NaCl. A study by Niste *et al*. (2015) revealed that *Sinorhizobium meliloti* (SmM2) was highly salt tolerant and can withstand up to 300 mM NaCl. Moreover, in *Sinorhizobium meliloti* (SmM1) the differences between, 50 mM, 100 mM and 150 mM NaCl were not considerable but a significant decrease in bacterial growth was evident at 300 mM NaCl after 72 hours incubation.

Rhizobia strains WB74, UC11, UC10, UC12, XFH12, WB1, XS34, UD5, XHH1, SAC2, XFH3 and XFH1 showed tolerance to acid/alkaline conditions as they grew efficiently at pH 5, 7 and 9. Rhizobia strains RF14, RF12, XAP5, RF15, RF36 and RAK1 could not grow at pH 5 whereas

only rhizobia strain XEL1 could not grow at pH 9. Previous reports by other researchers indicated that there is no correlation between the soil pH from which the organism was isolated and its tolerance to high or low levels of pH (Ruiz-Diez *et al*., 2009). In the current study, we evaluated the ability of the rhizobia strains for their tolerance to acidity was detected, in which isolates RF14, RF12, RF36, RF15 and RAK1, which were all identified as *Sinorhizobium* spp. were inhibited at pH 5. *Sinorhizobium meliloti* has been reported as the most sensitive to low level of pH or high acidity amongst the nodule-inducing bacteria (Correa *et al*., 2001; Ramirez-Bahena *et al*., 2015). However, isolates UC10, UC11, XS34, UD5, XFH1, XHH1, XBD2, SR4 and XFH3 which were all identified as *Rhizobia* spp. demonstrated good growth at pH 5, 7 and 9. The overall results indicated that the optimum growth for all rhizobia isolates was at pH 7 and the minimal growth was exhibited at pH 9. Previous similar findings by Wadhwa *et al*. (2017) also revealed that optimum pH for rhizobia growth in their study was between 6 and 7 and the minimal growth was exhibited at pH 8 and 9. A slight adjustment or variation in the medium pH level may affect rhizobia growth Deora and Singhal (2010) and this was observed in the current study as rhizobia growth was affected based on the pH of the media.

The other important abiotic stress tolerance for which the *Rhizobia* spp. were detected in the current study was aluminium concentration. Aluminium toxicity has been found to reduce the growth of several soil microbes including rhizobia, which are very sensitive to this metal compared to their host legumes. Generally, aluminium toxicity poses serious challenges to legumes as it interferes with the symbiotic processes, reduces the ability of the rhizobia to nodulate and fix atmospheric nitrogen (O'Hara and Glenn, 1994; Ferreira *et al*., 2012; Mendoza-Soto *et al*., 2015). Although many of the isolates in this study were found to be less tolerant to $AlCl_3.6H_2O$, other isolates exhibited significant tolerance and good growth at 50 mM

$AlCl_3.6H_2O$ and still showed moderate growth at 100 mM $AlCl_3.6H_2O$. The rhizobia strains that showed effective tolerance to 50 mM $AlCl_3.6H_2O$ were *Bradyrhizobium* sp. XS34, *Rhizobium* sp. UD5, *Rhizobium* sp. XFH1, *Bradyrhizobium* sp. XBD2 and *Rhizobium* sp. XFH3, while no isolate was found to be tolerant to 150 mM $AlCl_3.6H_2O$. It was observed in several studies that high concentration of Al^{3+} can be detrimental to the bacteria fixing nitrogen in the soil or culture medium (Kinraide and Sweeney, 2003; Rohyadi, 2006; Ferreira *et al*., 2012). Similarly, the increase in $AlCl_3.6H_2O$ concentration in our *in-vitro* growth study resulted in the suppression of several rhizobia isolates with minimum or no growth at all.

Bradyrhizobium sp. XS34, *Rhizobium* sp. XFH1, *Rhizobium* sp. XBD2 and *Rhizobium* sp. XFH3 were also acid tolerant at pH 5 and previous research by (Graham *et al*., 1994; Ferreira *et al*., 2012) indicated that rhizobia strains from the same origin were highly tolerant to acidity and up to a concentration of 1 mmol Al^{3+} L^{-1}. Based on the current results, the isolates were both fast and slow growers and their growth was significantly affected by the different concentration of $AlCl_3.6H_2O$ indicating that Al toxicity is extremely toxic to the growth of most rhizobia. Arora *et al*. (2010) also reported that aluminium and copper metals are very toxic to the enzyme activity and rhizobia species. All abiotic stress data tolerance is presented in table 1.

Table 1 SARCC isolates exhibiting tolerance to various abiotic stress *in-vitro*

	Salinity			*Heavy meatal ($AlCl_3.6H_2O$)*			*Acidity/Alkalinity*			*Temperature*		
Isolates	50 mM	100 mM	150 Mm	50 mM	100 mM	150 mM	pH 5	pH 7	pH 9	16 °C	28 °C	36 °C
RF14	+++	+++	+++	+	-	-	-	+++	+++	+++	+++	+++
VK10	++	-	-	-	-	-	+	+++	+	+++	+++	++
WB74	++	+	-	-	-	-	+++	+++	+++	+++	+++	+++
UC11	+++	+	-	-	-	-	+++	+++	+++	+++	+++	+++
XHT1	+	-	-	-	-	-	++	+++	++	++	+++	+++
UC10	+++	+++	+++	+	-	-	+++	+++	+++	+++	+++	+++
UC12	+++	+	-	-	-	-	+++	+++	+++	+++	+++	+++
XFH12	+++	-	-	+	+	-	+++	+++	+++	+	+++	+++
XFH13	++	-	-	+	+	-	++	+++	++	++	+++	+++
WB1	++	+	-	+	-	-	+++	+++	+++	++	+++	+++
XS34	+++	+++	+++	+++	++	-	+++	+++	+++	+++	+++	+++
XCR6	+++	+++	+++	+	-	-	+	+++	+++	++	+++	+++
USDA 122	+++	+	-	-	-	-	++	+++	++	+	+++	+++
UD5	+++	+++	+++	+++	-	-	+++	+++	+++	+++	+++	+++
XDF5	++	++	-	+	-	-	++	+++	++	+	+++	+++
XEL2	+++	++	++	+	-	-	+	+++	++	++	+++	-
RF35	+++	+++	+++	++	+	-	+	+++	+++	+++	+++	+++
RF12	+++	+++	+++	+	-	-	-	+++	+++	+++	+++	+++
XAP5	-	-	-	-	-	-	-	+++	++	++	+++	-
XHJ18	++	++	+	+	-	-	++	+++	++	++	+++	-
XFL2	+++	+++	+++	++	+	-	+	+++	+++	+++	+++	+++
XS21	+++	-	-	+	-	-	+++	+++	++	+	+++	+++

Isolate	*Salinity*			*Heavy meatal ($AlCl_3.6H_2O$)*			*Acidity/Alkalinity*			*Temperature*		
	50 mM	100 Mm	150 Mm	50 mM	100 mM	150 mM	pH 5	pH 7	pH 9	16 °C	28 °C	36 °C
XFH14	+++	-	-	+	-	-	+++	+++	++	++	+++	+++
XCG2	++	-	-	+	+	-	++	+++	++	+	+++	++
XHH1	+++	+++	+	++	-	-	+++	+++	+++	+++	+++	+++
XBD2	+++	+++	+++	+++	++	-	+++	+++	+++	+++	+++	+++
RF15	+++	+++	+++	+	-	-	-	+++	+++	+++	+++	+++
SAC2	+++	-	-	-	-	-	+++	+++	+++	+++	+++	+++
XCV14	+++	+++	+	++	-	-	++	+++	+++	+++	+++	++
SR4	++	-	-	+	-	-	++	+++	+++	+++	+++	+
XFH3	+++	+++	+++	+++	++	-	+++	+++	+++	+++	+++	+++
XAP2	+++	+++	++	++	-	-	++	+++	++	+++	+++	-
XBQ5	++	++	-	-	-	-	++	+++	++	+	+++	+++
RF36	+++	+++	+++	+	-	-	-	+++	+++	+++	+++	+++
SAC1	+++	+	-	-	-	-	+++	+++	++	+++	+++	+++
XFH1	+++	+++	+++	+++	++	-	+++	+++	+++	+++	+++	+++
XHK1	+++	+++	++	+	-	-	++	+++	+++	+++	+++	+++
XEL1	+++	++	++	+	-	-	+	+++	-	++	+++	-
SAC3	+	-	-	-	-	-	+	+++	++	++	+++	+++
RAK1	+++	+++	+++	-	-	-	-	+++	+++	+	+++	+

+++ = Good growth, ++ = Moderate growth, + = Poor growth, - = No growth

This study revealed that several strains of the root nodulating rhizobia spp. deposited at the SARCC have an immense potential for use as bio-inoculants in the production of legumes under various abiotic stressed conditions, particularly the isolates inoculating *Vigna ungucuilata* as they showed most tolerance to the abiotic stresses induced under glasshouse conditions (glasshouse nodulation test results not shown). The isolates, with huge genetic diversity and possession of essential abiotic stress tolerance genes such as the a*cdS* and *ExoR* have exhibited medium to maximum tolerance to various abiotic stresses. The data gathered have therefore generated valuable baseline information about the rhizobia strains at the SARCC, which warrant further screening of the isolates under actual field conditions before being commercialized as inoculants in sustainable legume production.

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3.7 Conflicts of interest

The authors declare that there is no competing of interest.

3.8 Funding information

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3.9 Author contributions

LSK designed, conducted the laboratory experiments, analyzed sequences and wrote the manuscript. AIH and KR read and edited the draft manuscript.

3.10 References

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CHAPTER 4

SCREENING RHIZOBIA FOR ACC-DEAMINASE AND EXOPOLYSACCHARIDES PRODUCTION, SELECTION OF *acdS* AND *exoR* GENES SYMBIONTS USING MUTAGENESIS

4. Tolerance of Rhizobia spp. to abiotic stress conditions

Rhizobia spp. in general comes across adverse environmental conditions particularly those related to abiotic stresses within the soil rhizosphere that negatively affect the symbiotic process. However, rhizobia have some key tolerance mechanisms against abiotic stresses hence their application is of paramount importance because these abiotic stresses are the major constraints in sustainable agriculture. The major mechanisms developed by the symbiotic rhizobia and free-living rhizobacteria to tolerate abiotic stresses and thrive includes bacterial exopolysaccharides and 1-aminocyclopropane-1-carboxylate deaminase production (Timmusk and Nevo, 2011; Kim *et al.,* 2013; Timmusk *et al.,* 2014). These mechanisms assist rhizobia and other beneficial bacteria to execute their plant growth promoting traits under stress conditions (Gopalakrishnan *et al.,* 2015).

4.1 1-Aminocyclopropane-1-carboxylate (ACC) deaminase

Ethylene is an important phytohormone and a central regulator during nodulation but when over produced under stressful conditions it can inhibit plant growth or death especially for seedlings.

In plants, ethylene induces multiple physiological changes at a molecular level, takes part in the defense and symbiotic activities induced mainly by the bacteria (Belimov *et al.,* 2001; Saleem *et al*. 2007; Sharma *et al.,* 2013; Guinel, 2015; Guinel, 2015, Larrainzar *et al*., 2015; Van de Poel *et al*., 2015; Berrabah *et al*., 2018). The *acdS* gene encodes the enzyme ACC deaminase which regulates the production of plant ethylene by metabolising 1-aminocyclopropane-1-carboxylic acid (immediate precursor) into ammonia and α-ketobutyric acid, which in turn facilitates plant root or shoot growth (Figure 4.1) (Argueso *et al*., 2007; Glick, 2014).Therefore, the production of ACC deaminase is beneficial to plants as they become resistant or tolerant to abiotic stresses (Glick, 2005).

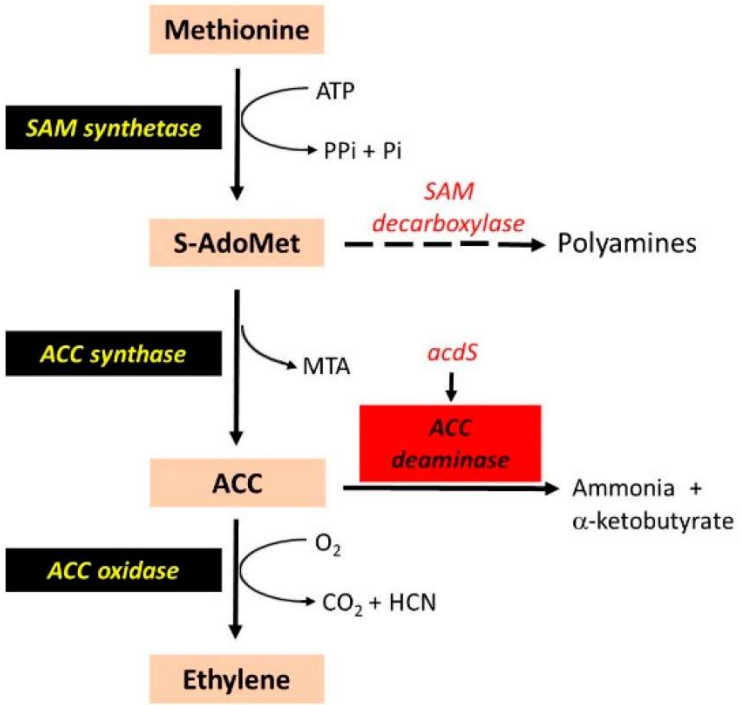


Figure 4. 1. Ethylene synthesis pathway and ethylene level reduction through substrate mobilization (Goyal *et al*., 2021).

Generally, ethylene and ACC inhibit the nodulation process, which is initiated by the rhizobia symbionts (Guinel, 2015). Together with other hormones, ethylene modulates disease, abiotic

stress response and negatively regulates the nodulation process. The control of ethylene synthesis and perception presents opportunities to enhance the ability of *Rhizobium* to nodulate. In addition to nodulation, the manipulation of ethylene response can also have a positive effect on abiotic stress tolerance. Moreover, previous studies have reported that ACC and ethylene takes part in the symbiosis process, e.g initial response to bacterial nod factors, nod factors signal transduction, formation of the infection thread, nodule development and and plays a major role in senescence (Argueso *et al*., 2007, Guinel, 2015; Larrainzar *et al*., 2015). For example, the application of ethylene or ACC reduces the formation of nodule in various legumes (Okazaki *et al*., 2004). Rhizobia producing ACC-deaminase breaks down the ACC, this releases and supply energy and nitrogen. In turn, this alleviates ethylene's effects which minimizes plant stress and enhances plant growth (Glick, 2005).

Numerous genetic elements take part in ethylene production and sensitivity in some legumes when compared to other plants (Penmetsa *et al*., 2008; Desbrosses and Stougaard, 2011; Miyata *et al*., 2013). Amongst endophytic and rhizosperic bacteria, the *acdS g*ene is one of the mostly diffused genes and it encodes the ACC deaminase enzyme which cleaves the plant-produced ACC. This ACC is the ethylene precusor and it reduces plant's response to infection (Nascimento et al., 2014; Singh et al., 2015; Checcucci *et al.,* 2017). The *acdS* structural gene has been found in various organisms, including the symbiotic rhizobia species (Singh *et al.,* 2015). According to Ma *et al*. (2003) the *acdS* gene positively affects the nodulation process and thus *R. leguminosarum* bv. *Viciae* 128C53K *acdS* mutant strain inoculating *P. sativum cv*. Sparkle resulted in a decreased (reduced by 25%) nodulation.

4.2 Exopolysaccharides (EPS)

Exopolysaccharides are complex high molecular weight biopolymers made up of sugars and lipids, and extracellular DNA proteins (Rana and Upadhyay, 2020). Generally, exopolysacharides are found on the cell surface or culture medium (Barbosa *et al*., 2004) and are the most significant group of biopolymers present in a capsular form or slimy layer depending on their location (Freitas *et al.,* 2014; Ates, 2015). The production and composition of exopolysccharides enhances the bacterial resistance to abiotic stress (Sandhya and Ali, 2015). Previous reports revealed that the production of exopolysaccharides is a common trait in Rhizobia spp. and they are produced in large quantities. There is a wide variety of EPS composition, these are produced by different species of bacteria (Skorupska *et al.,* 2006; Monteiro *et al.,* 2012). The EPS secreted by many Rhizobia spp. are associated with the root hair infection, response to environmental stresses and nodule formation.

Recently, the application and production of different EPS produced by rhizobia have increased. Exopolysaccharides are potentially produced by Rhizobia spp. and they belong to *Sinorhizobium, Mesorhizobium, Bradyrhizobium and Rhizobium* genera (Staudt *et al*., 2012; Monteiro *et al*., 2012; Bomfeti *et al*., 2011). Downie, (2010) has reported large numbers of exopolysaccharides chemical structures within the rhizobia strains. There are two types of exopolysaccarides produced by rhizobia, i.e. EPS I (succinoglycan) and EPS II (galactoglucan) (Reinhold *et al.,* 1994). The difference between these two types is their chemical composition. Galactoglucan is the residue of acetylated glucose and pyruvylated galactose composed of a disaccharide repeating unit (Her *et al.,* 1990). Succinoglycan is composed of one galactose and seven glucose residue molecules and it is modified by substituents, i.e. succinyl, acetyl, and pyruvyl (Reuber and Walker, 1993).

The wild-type EPS in full length is a positive signal that modulates the responses of plant defense while allowing the infection thread to develop (Kelly *et al*., (2013). However, the host plant perceives the short length of EPS as a negative signal that induces defense reactions and blocks the infection thread development. Exopolysacharides are involved in the infection process during nodule formation. This was demonstrated in the symbiosis between alfafa and *Sinorhizobium meliloti*, pea and *Rhizobium leguminosarum*-pea. Exopolysaccharides defective bacterial mutants result in impaired infection thread formation and elongation; this forms uninfected pseudonodules (Stuurman *et al*., 2000; Laus *et al*., 2004, Laus *et al*., 2005). The synthesis of rhizobial exopolysaccharides consists of multiple steps and this process is regulated by *exo/exs, exp,* or *pss* genes at transcriptional and post-transcriptional levels .The *exo* genes encodes proteins that facilitates production, polymerization, and exports of the EPS. These genes are clustered in plasmids in most Rhizobium spp. except in some rhizobia strains such as the *Lotus* symbionts where they are found in the chromosome (Kaneko *et al*., 2000; Janczarek and Skorupska, 2011).

Previous studies have revealed that *Rhizobium leguminosarum* bvs. *trifolii* and viciae, and *Sinorhizobium meliloti* EPS-deficient mutant strains still induces the formation of root nodules upon inoculation of their compatible legume host. However the nodules formed are empty or partially infected root nodule-like structures. Moreover, this nodule cannot fix atmospheric nitrogen effectively (Janczarek and Rachwał 2013). *Rhizobium leguminosarum* RBL5523 *exo*-mutant inoculation of *V. sativa* subsp. *nigra* roots blocked the formation of the infection thread (Laus *et al*., 2004, Laus *et al*., 2005). However, *Rhizobium leguminosarum* and *Sinorhizobium meliloti* strains that are over producing EPS are characterized by significantly occupying root nodules of their host plants and symbiotic effectiveness is enhanced (Janczarek *et al*., 2009;

Lehman and Long 2013). Exopolysaccarides in rhizobia species are responsible for gathering nutrients, formation of biofilm, protection against desiccation and environmental stresses. These functions ensure that the bacteria adapt to the constant changes in soil conditions (Broos *et al*., 2005; Janczarek, 2011).

4.3 Ultraviolet (UV) mutagenesis

Mutagenesis studies are of great importance in plant breeding to enhance physiological properties of any species and genetic engineering random mutagenesis, adaptive laboratory evolution are highly explored for strain improvement (Doan and Obbard, 2012). Random mutagenesis is straight forward and inexpensive. The technique requires little knowledge about the genetic information of the organism, biochemical pathways, genetic regulation and moreover, a few technical manipulations are required and the generated strains does not have any main issues of strict concerns (Bellou *et al*., 2014; Nawrath and Wu, 2018; Ottenheim *et al*., 2018). Random mutagenesis is achieved by exposing organisms to chemical or physical mutagens which alters its DNA. Physical mutagenis occurs when organisms are exposed to X-rays, gamma and ultraviolet (UV) rays (Zayadan *et al*., 2014). The ultraviolet radiation comprises of three categories according to their respective wavelength, i.e. UV-A (320 to 400 nm), UV-B (290 to 320 nm), and UV-C (<290 nm), with the energy-rich UV-C, covering the absorption maximum of DNA, being the most potent mutagen (Suthaparan *et al*., 2018). The 250-290 nm wavelengths induces the deletetion of A-T the base pairs in the DNA or promotes the thymine dymers (Muthuraj *et al*., 2019).

Ultraviolet light form pyrimidine dimers in DNA and RNA which inactivates microorganisms, this can intefere with the transcription and replication process (Cutler and Zimmerman, 2011).

Moreover, numerous mutations are induced by UV radiation cause, .e.g deletions, frameshifts, base substitutions mutations just to name a fewt (Schaaper *et al*., 1987). However, mutagenesis is dependant on period of exposure, the UV exposure intesity and the bacterial cells's physiology (Kostyleva *et al*., 2016). Therefore wild type strains are exposed to UV irradiation for a longest time (Sher *et al*., 2012). There are several reports of *Bradyrhizobium japonicum* mutants which were isolated by chemical or by UV mutagenesis (Dale Noel *et al*., 1982; Stacey *et al*., 1982). However, any genetic changes in Rhizobia spp. may either decrease or increase their ability to fix atmospheric nitrogen. Thus, physical and chemical mutagens can cause mutations at the DNA level (Bhagwan and Akkiraju, 2015).

4.4. Materials and methods

4.4.1. Screening for exopolysaccharide production

Single colonies from the agar plates prepared as discussed in section 2.5.2 (chapter 2) were inoculated into 100 mL Yeast Mannitol Broth (YMB). A total number of 40 isolates were used in this chapter because 40 isolates were originally selected for the study but only few isolates (25) were used in the glasshouse nodulation efficacy test (Chapter 2) based on the availability of legume seeds and their importance. The flasks were incubated at 26 ± 2 ºC for 5-7 days at 120 rpm depending on the growth of the isolate. The bacterial cultures were collected 5-7 days after incubation and centrifuged for 40 minutes at 10 000 rpm. The supernatant was collected from each isolate, mixed with 35% (v/v) of cold ethanol and stored at 4 ºC for 24 h. The mixture was centrifuged for 40 minutes at 10 000 rpm. The presence of EPS was indicated by the presence of a slimy precipitates. These slimy precipitates were collected and dried at 30 ºC for 24 hours. The dried precipitates were weighed and measured mg/ 100 mL culture (Rathaur *et al*., 2012).

4.4.2. The ACC deaminase activity

4.4.2.1 Growth of rhizobia isolates

The isolates were streaked on YM-CR agar plates and incubated for 2-3 days at 30 ºC until colony formation. Test tubes containing 8 mL Tryptone Yeast Extract (TY) broth were inoculated with an individual colony of each strain. The medium composition (g/L) of TY broth consisted of: 5g Tryptone, 3g Yeast extracts, 6g $CaCl_2.2H_2O$ pH 7-7.2 and autoclaved at 121 ºC for 20 minutes. The inoculated test tubes were incubated at 120 rpm in a shaker for 2-3 days at 30 °C.

4.4.2.2 Preparation of ACC solution and Rhizobium Minimal Medium (RMM)

A 0.3 M ACC solution was prepared and filter sterilized using a 0.2 µm membrane. The ACC solution was aliquoted 150 µL per cryovial and frozen at -20°C. The rhizobium minimal medium was prepared as described by Broughton *et al*. (1986).

4.4.2.3 The ACC deaminase activity on RMM agar plates

To measure the activity of ACC deaminase, the bacterial culture of rhizobia isolates (grown in TY medium section 5.4.5.1) were centrifuged for 10 minutes at 11 000 x g. The pellets were washed with 0.85% NaCl solution twice and resuspended in a 5 mL volume of modified M9 minimal medium (Ma *et al*., 2003). The test tubes containing 5 mL M9 medium were incubated at 30 ºC for 36-40h at 120 rpm to induce the ACC deaminase production. The bacterial cells prepared as described above were re-suspended in 50 µL 0.85% NaCl solution. This was used to spot inoculate two different set of RMM plates. A 4 x 4 grid was drawn at the back of RMM agar plates; this was done to indicate the right place of inoculation. Two series of RMM plates were prepared. The first series of plates were not supplemented with anything. The second series of

plates were spread with 150 µL aliquot of 0.3 M ACC solution (as prepared in section 4.4.5.2) as a sole source of nitrogen. The plates were dried prior to inoculation. The two series of plates were inoculated with a 2 µL aliquot of the prepared bacterial suspension and this made a small circle on the RMM plate. Each plate was inoculated with different strains in two replications and the assays were performed in duplicate. The plates were incubated at 30 ℃. The ACC deaminase activity will be determined by the growth on the RMM plates supplemented with 0.3M ACC.

4.4.3 Mutagenesis

4.4.3.1 Ultraviolet (UVC) interval based mutagenesis

The isolates subjected to UVC mutagenesis were selected based on the detection of the *acdS* and *exoR* gene on the isolates (chapter 3). Individual colonies from the overnight grown culture of the rhizobia isolates were selected and transferred to 50 mL yeast mannitol broth (YMB) flasks. The final cell concentration of each rhizobia isolate was adjusted post incubation to ± 1 x 10^8 cfu/mL using the spectrophotometer (OD_{600nm} of ≥0.6). The UV lamp was set at 254 nm and allowed to warm up for 30 minutes before irradiation of the bacterial cultures. The culures were irradiated with UV light for 0, 20, 40, and 60 minutes (Rathaur *et al*., 2012) in the dark with modifications. A sample from each isolate was withdrawn at 20 minutes interval; samples from respective isolates were removed from the light and wrapped with foil. Upon completion of exposure to the UV light, a series of 10 fold dilutions were prepared using 0.9% saline. The sample that was not exposed (0-minute) to the UV light was used as a control. The 100 µL of the prepared dilutions were spread on YMCR agar plates using the spread plating technique and this made final plates for the UV irradiation. The plates were incubated until colony formation at 30 ℃. Post colony formation, the colonies were compared with colonies from the control plates.

The colonies with variant morphology from the control plates were considered as potential mutants and were selected for further screening.

4.4.3.2 Selection of potential mutants from UVC mutagenesis

The selection of potential mutants was based on the ACC deaminase activity and exopolysaccharides production post exposure to the UV light. Presumptive *exoR* and *acdS* gene mutants were analyzed further to confirm possible mutation.

4.4.3.3 Molecular characterization to confirm acdS and exoR genes mutant

The *exoR* and *acdS* genes nucleotide sequence were used to characterize the potential mutants. The total genomic DNA was extracted from 1 mL YMB culture suspension of the selected rhizobia isolates using the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA) following the manufacturer's instruction. The extracted DNA of each Rhizobia isolate was amplified using specific primers for *exoR* and *acdS* genes. The *exoR* and *acdS* gene amplification using PCR were performed in total reaction mixtures of 25 µL as described in section 3.4.3.2. The PCR cycle for the gene was as follow: 95 ℃ for 3 min, 95 ℃ for 1 min, 60 ℃ for 1 min, 72 ℃ for 1 min, 72 ℃ for 5 min, (35 cycles). The PCR cycle for the *exoR* gene was as follow: 95 ℃ for 3 min, 95 ℃ for 1 min, 59 ℃ for 1 min, 72 ℃ for 1 min, 72 ℃ for 5 min, (35 cycles). The amplified PCR products were visualised using 1% agarose gel electrophoresis, stained with ethidium bromide to verify the size.

4.5 Results

The production of EPS formed translucent, cream white and mucoid colonies on YMCR agar plates and an increased viscosity when grown in YMB. The mucoid colonies formed long

filamentous lines when picked from the plate with an inoculation loop (figure 4.1). The EPS production ranged from 0-71.10 mg/100 mL (Table 2.1). The maximum amount of EPS was produced by isolate XAP2 (71.10 mg/100 mL), SAC1 (62.23 mg/100 mL) and SAC2 (50.50 mg/100 mL). Isolates XFH14, XFH12, XFH13, XCG2, RF36, RF35, XBQ5, VK10, XS21, WB74, XFL2 and XFD5 formed no slimy precipitate after the supernatant was mixed with ethanol, meaning that these isolates does not produce exopolysaccharides.

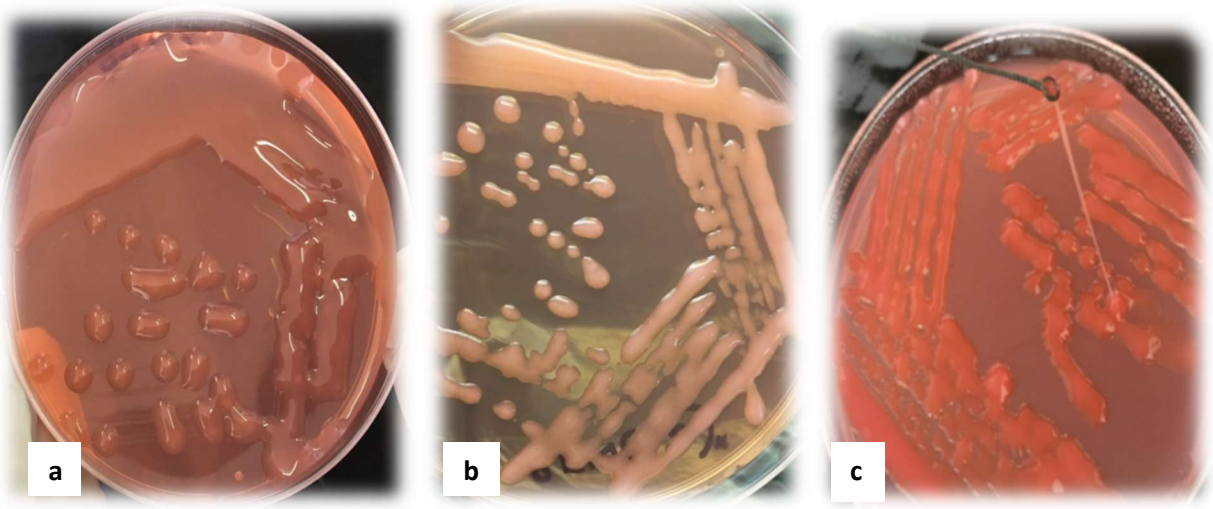


Figure 4.2. Rhizobia isolates UC12 (a), RF15 (b) and SAC2 (c) producing EPS forming mucoid colonies on YMCR agar.

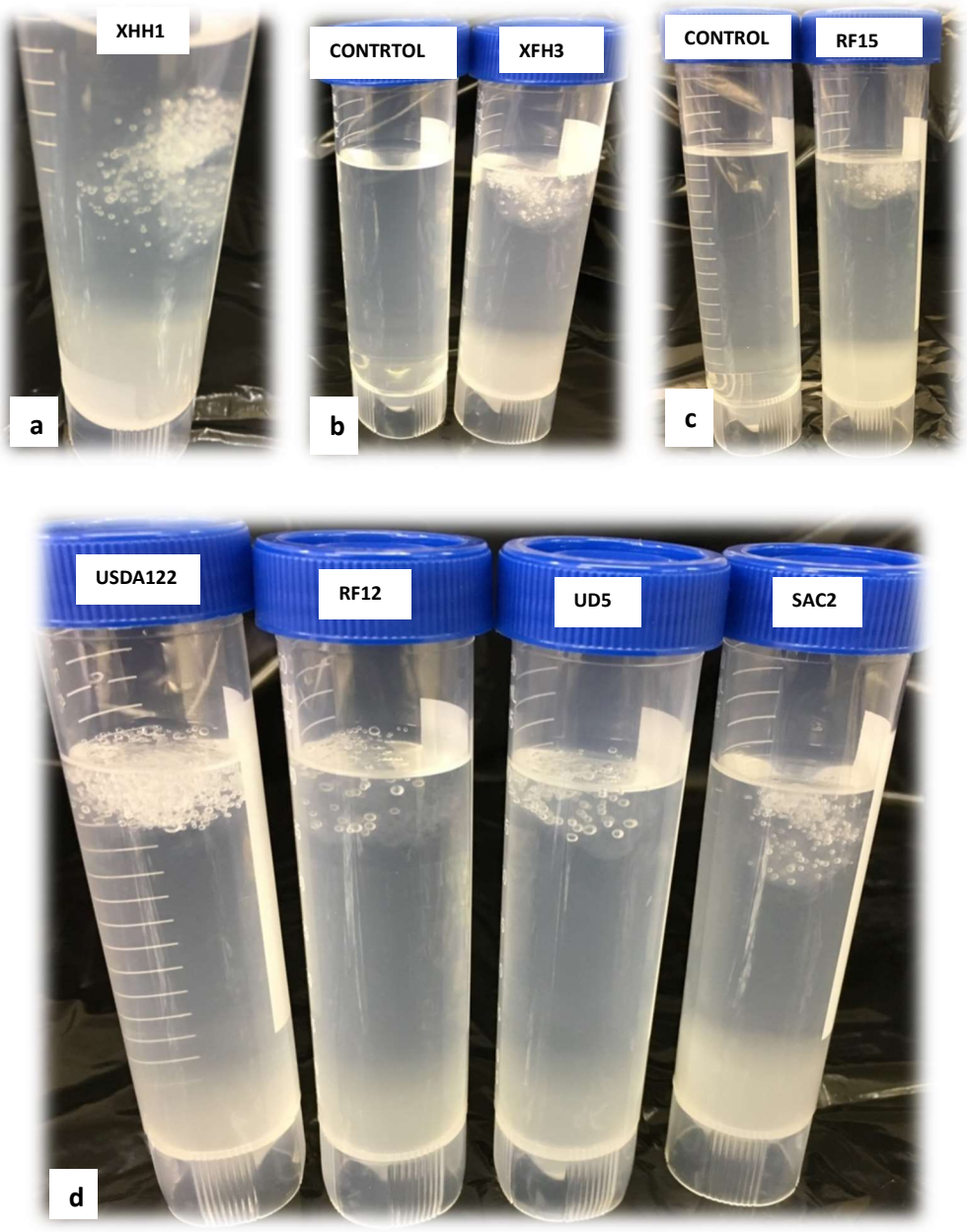


Figure 4. 3. EPS production by the rhizobia isolates XHH1 (a), XFH3 (b), RF15 (c) and USDA 122, RF12, UD5, SAC2 (d) indicated by the presence of a slimy precipitate. The tube without a precipitate indicates a negative control

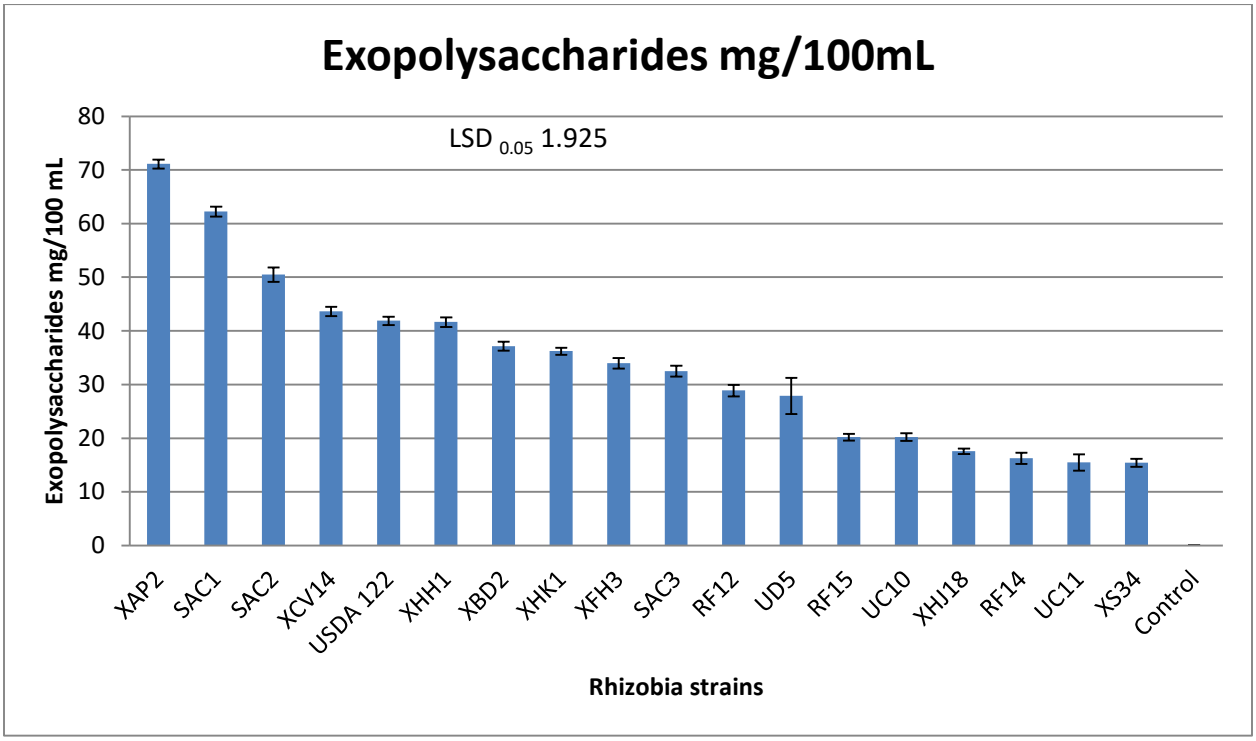


Figure 4.4. The production of exopolyscharrides by rhizobia isolate. Mean data for experiment performed in triplicate and the least signicance difference $LSD_{0.05}$=1.925.

Rhizobia isolates from the South African Rhizobium Collection were also screened for the ACC deaminase activity using the nutrient enrichment method where 1-aminocyclopropane-1-carboxylate (ACC) was used as the sole source of nitrogen. The ability of any isolate to grow on RMM + ACC plates after incubation was related to the ability of the isolates to utilize ACC as the source of nitrogen, implying ACC deaminase activity. A total of 40 isolates were screened, the results indicated that 13 isolates could grow on RMM + ACC plates and the control plate with RMM without any source of nitrogen. However, 27 isolates could only grow on RMM plates without any source of nitrogen and they were considered negative because they couldn't grow in the RMM +ACC meaning that they don't have the ability to produce ACC deaminase in

the presence of ACC. The growth and colony diameter of the isolates grown on two different RMM plates is clearly illustrated in figure 4.5.

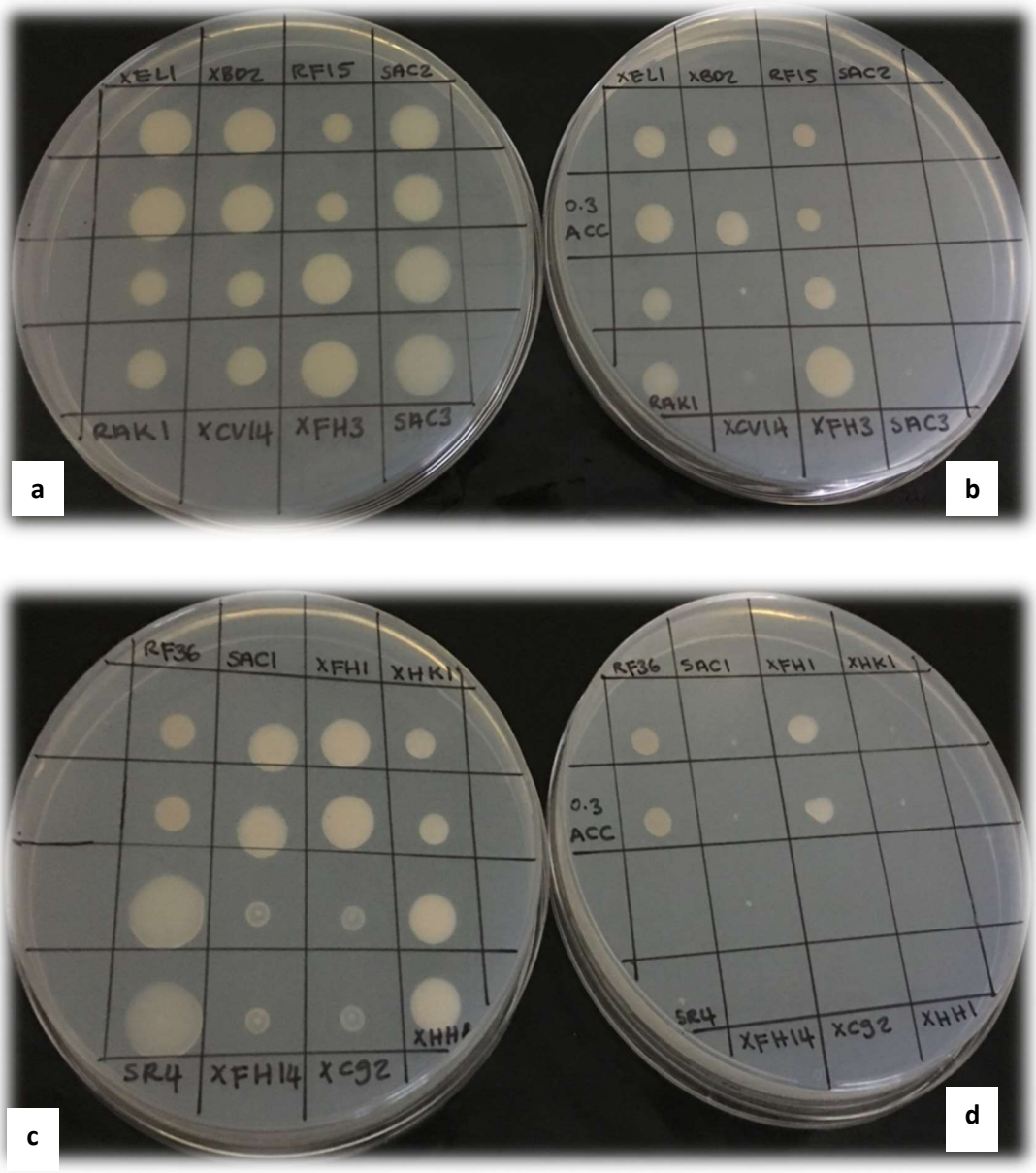


Figure 4.5. Screening of rhizobia isolates for ACC deaminase activity. RMM plate without any source of nitrogen (control) resulted in growth for all the isolates (a&c), RMM+ACC plate (experiment) resulted in growth for isolate RF36, XFH1, XEL1, RAK1,XBD2,RF15 and XFH3, indicating ACC deaminase activity (b&d).

Table 4.1: Screening of rhizobia isolates for the utilization of ACC deaminase

Rhizobia Strains	RMM	RMM + ACC deaminase
RF14	+	+
VK10	+	-
WB74	+	-
UC11	+	-
XHT1	+	-
UC10	+	-
UC12	+	-
XFH12	+	-
XFH13	+	-
WB1	+	-
XS34	+	+
XCR6	+	-
USDA 122	+	-
UD5	+	+
XDF5	+	-
XEL2	+	+
RF35	+	+
RF12	+	+
XAP5	+	-
XHJ18	+	-

XFL2	+	-
XS21	+	-
XAP2	+	-
XBQ5	+	-
RF36	+	+
SAC1	+	-
XFH1	+	+
XHK1	+	-
XEL1	+	+
XFH14	+	-

XCG2	+	-
XHH1	+	-
XBD2	+	+
RF15	+	+
SAC2	+	-
XCV14	+	-
SR4	+	-
XFH3	+	+
RAK1	+	+

+ indicates a positive reaction, - indicates a negative reaction

The effect of UV exposure on rhizobia isolates with the aim of inducing mutations for future functional gene profiling was explored on isolates of interest based on the presence of the *acdS* and *exoR* genes. The selected four rhizobia isolates (SAC1, SAC2, XS21 and RF36) were subjected to UV irradiation treatment at different intervals to explore the effect of random mutagenesis on microbial cells. The results revealed that UV exposure decreased the number of colonies formed depending on the period of exposure in comparison with the 0 minute exposure (control not subjected to UV treatment). Fewer colonies were formed or no colony formation in isolates exposed to UV for a longer period. Generally, rhizobia colonies vary from opaque and cream white colonies with a firm gummy or smooth mucoid texture but there wasn't any major drastic change on the appearance of bacterial colonies on YMCR after UV exposure. Colonies that exhibited slightly less mucoid and big colonies compared to the wild type were screened further to determine possible *exoR* and *acdS* gene mutants.

The *acdS* and *exoR* genes presence was assessed pre- and post-UVC exposure of the isolates. The effect of UV light exposure on the *acdS* gene was confirmed by the ACC deaminase activity of the isolates exposed to UV and the presence or absence of the *acdS* gene was confirmed by PCR to confirm possible *acdS* gene mutants. Isolates RF36 and XS21were selected as possible mutants but they still displayed ACC deaminase activity as they were able to grow on RMM+ACC agar plates like the wild type isoates (Figure 4.6). The polymerase chain reaction also revealed that there was no possible *acdS* mutants as the *acdS* gene was still detected this was evident by the formation of 558 bp formation (Figure 4.7). The *exoR* gene was also still detected on isolate SAC1 and SAC2 the possible selected mutants post UVC exposure, meaning that no possible *exoR* mutants were formed and as a 416 bp band was formed on both wild type and possible mutants (Figure 4.8).

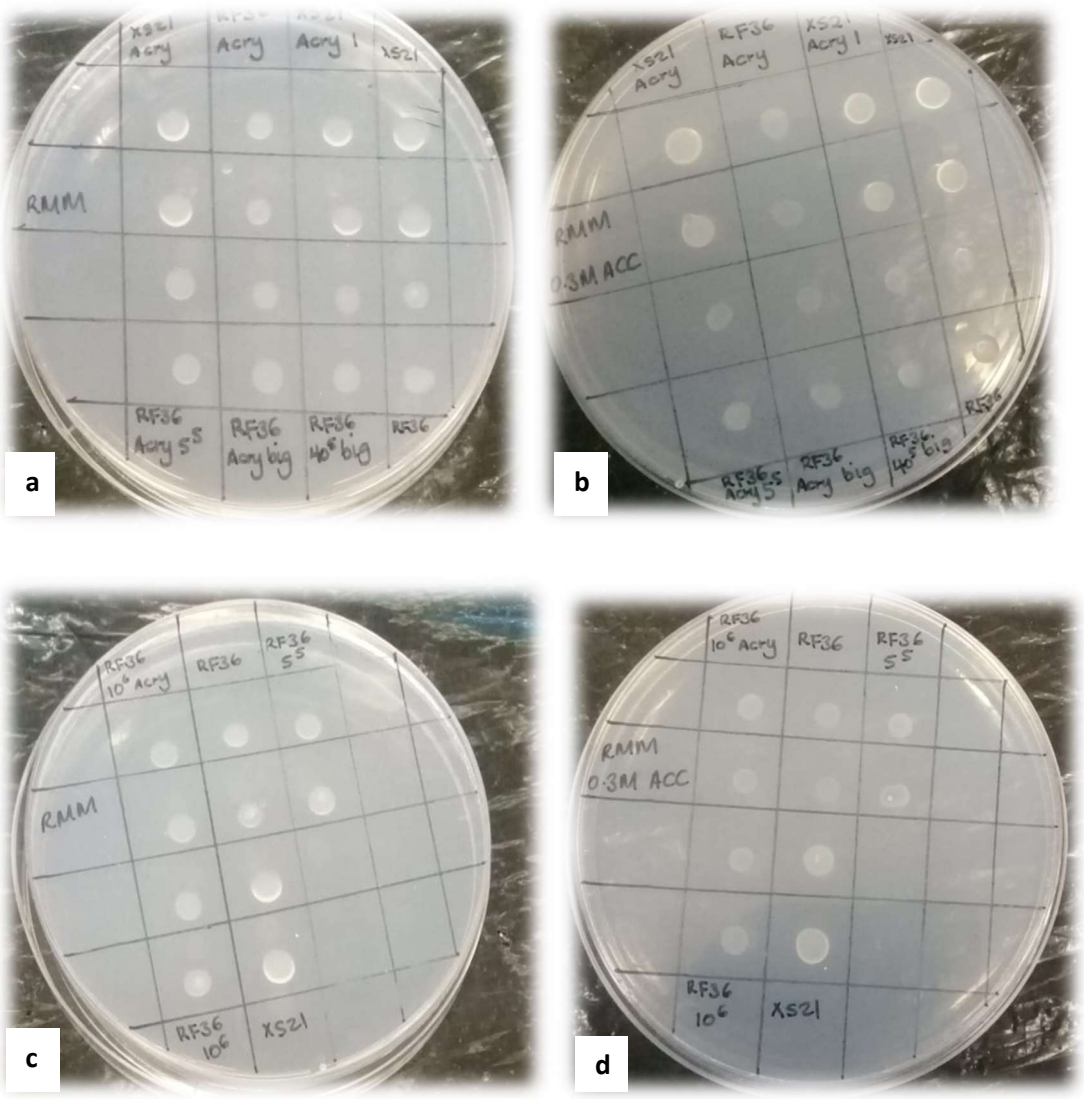


Figure 4.6: Screening of rhizobia isolates for ACC deaminase activity. RMM plate without any source of nitrogen (control) resulted in growth for all the isolates (a&c), RMM+ACC plate (experiment) resulted in growth for isolate RF36 and XS21 treated with UV and wild type strains, indicating ACC deaminase activity (b&d).

Table 4.2: Screening of rhizobia isolates for the utilization of ACC deaminase post UV exposure

Rhizobia strains	**RMM**	**RMM + ACC deaminase**
RF36 wild type	+	+
RF36 acry	+	+
RF36 10^{-6}	+	+
RF36 5^{-5}	+	+
RF36 40^{-5}	+	+
XS21 wild type	+	+
XS21 acry	+	+
XS21 20^{-2}	+	+

+ indicates a positive reaction, - indicates a negative reaction

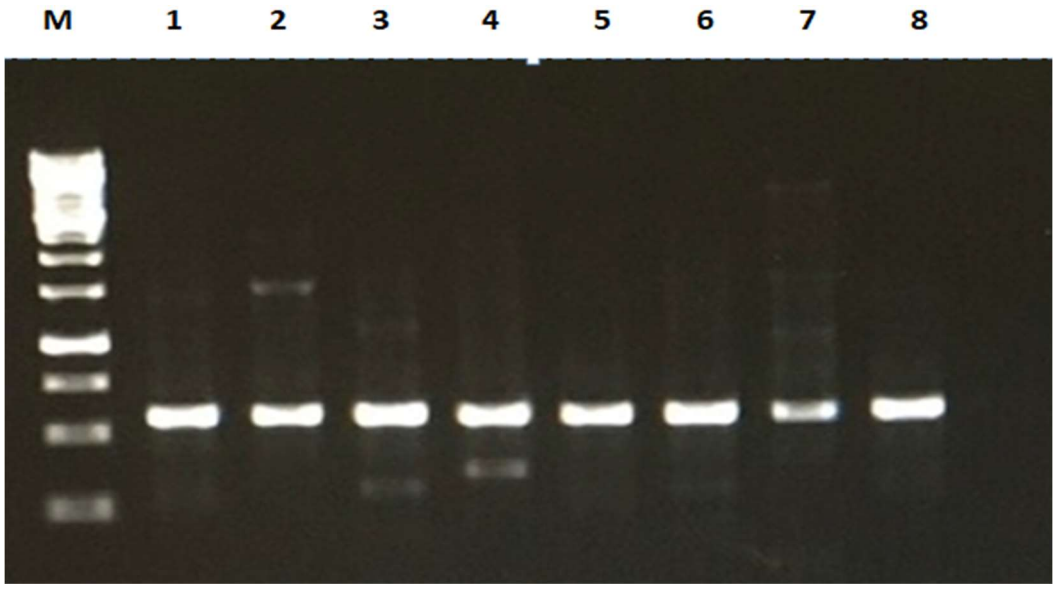


Figure 4.7. 1% agarose gel electrophoresis of *acdS* gene (558 bp). Lane M indicates the DNA ladder (1 kbp), lane 1-2 indicates the positive control (wild type strain XS21 and RF36), lane 3(RF36 acry), 4 (RF36 10^{-6}), 5 (RF36 5^{-5}), 6 (RF36 40^{-5}), 7 (XS21 acry) and 8 (XS21 20^{-2}) indicates the respective isolate XS21 and RF36 selected rhizobium colonies post UV exposure.

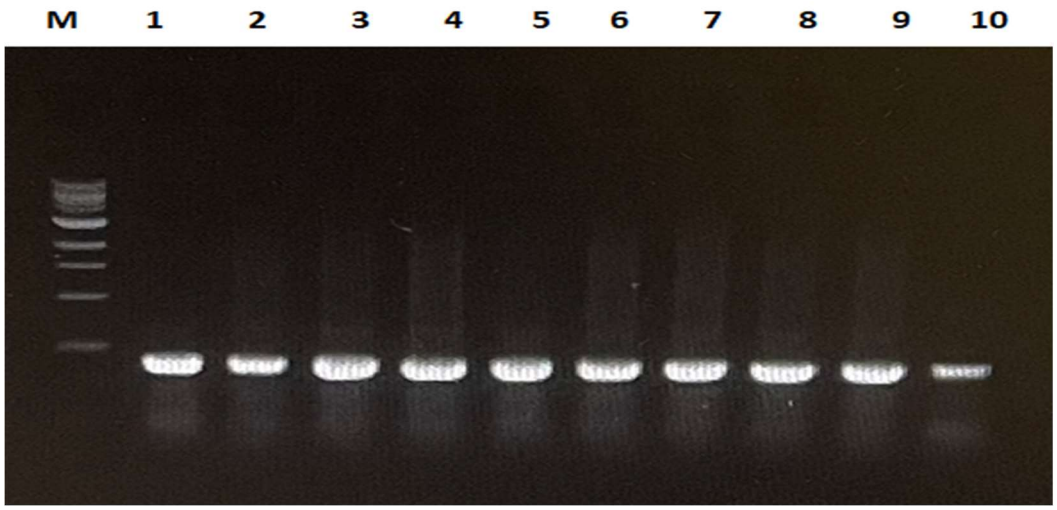


Figure 4.8. 1% agarose gel electrophoresis of *exoR* gene (416 bp). Lane M indicates the DNA ladder (1 kbp), lane 1-2 indicates the positive control (wild type strain SAC1 and SAC2), lane 3 (SAC1 20^{-4}), 3 (SAC1 5^{-6}), 4 (10 SAC1 40^{-5}), 5 (SAC1 acry), 6 (SAC2 20^{-3}), 7 (SAC2 5^{-6}) and 8 (SAC2 acry) indicates the respective isolate SAC1 and SAC2 selected rhizobium colonies post UV exposure.

4.6 Discussion

Rhizobia possess certain beneficial traits associated with the reduction of abiotic stresses in the soil. Examples include exopolysaccharides production and ACC deaminase activity. In our current study 40 isolates were screened for EPS production, 27 isolates showed EPS production when grown in yeast mannitol broth and 13 isolates didn't show any EPS production. The ACC deaminase activity screening revealed that 13 isolates were able to grow on RMM supplemented with ACC as a sole source of nitrogen and 27 isolates couldn't grow. The EPS production was evident by the increased viscosity of the isolates growing on YMB and confirmed by the presence of slimy precipitate. Similar finding was previously reported by Noel (2009), who revealed that rhizobia are well known to produce EPS. Large amounts of polysaccharides are excreteed into the rhizophere and produce vast amounts of EPS when grown in pure cultures, resulting in an increased viscosity. The EPS production varied amongst the isolates and this

concurs with Kumari *et al*. (2009) who reported that the EPS production in rhizobia varies between the bacterial strains, even among the same genus cultivated under the same conditions.

Rhizobia produce a large number of EPS in culture medium when compared to EPS produced under symbiotic conditions (Ghosh and Maiti, 2016). Several studies have revealed that EPS yields vary, depending on the growth curve of the bacteria but the composition does not change throughout the production cycle (Ghosh *et al*., 2005). The process of producing EPS is influenced by the microbial strain and operational conditions e.g. agitation, aeration rate, incubation period, temperature, pH, medium composition, volume and the inoculum age and the composition of cultivation medium. These factors affect the synthesis, yield and the composition of exoplolysaccahrides (Barreto *et al*., 2011; Staudt *et al*., 2012). The isolates cultivated under the same conditions in current research but the EPS production varied per isolate. Isolates producing the maximum amount of exopolysachharides were identified as *Mesorhizobium* (XAP2), *Rhizobium* (SAC1) and *Rhizobium* (SAC2). Several rhizobia strains have been reported as EPS producers and they belong to *Ensifer* or *Sinorhizobium, Rhizobium, Bradyrhizobium,* and *Mesorhizobium* genera (Berrada and Frikri-Benbrahim, 2014; Carareto *et al*., 2014; Marcondes *et al*., 2014).

The ACC producing bacteria were identified by the presence of growth on ACC enriched on medium. In the current study, out of 40 isolates screened, 13 isolates showed ACC deaminase activity by growing on RMM+ACC agar plates. Duan et al. (2009) screened 233 rhizobia strains which were isolated from 30 various soils for their ability to utilize ACC and 27 strains showed the activity of ACC deaminase. A study by Etesami et al. (2014) also reported that a total number of 80 endophytic bacteria isolated from rice plants roots revealed that 28 of these strains exhibited ACC deaminase activity. The isolates with ACC deaminase activity in our study were

identified as *Sinorhizobium* and *Bradyrhizobium.* This concurs with previous studies where it was reported that *Sinorhizobium meliloti* SM11 with the *acdS* is able to produce ACC deaminase (Stiens *et al*., 2006) and *Bradyrhizobium japonicum* strain that can produce ACC deaminase is able to degrade ACC (Murset *et al*., 2012).

Rhizobia strains with the ability to express ACC deaminase can efficiently form nodules capable of nitrogen-fixation up to 40 % more when compared to other strains lacking the ACC deaminase activity (Ma *et al*., 2003; Ma *et al*., 2004). Recent reports revealed that *Rhizobium leguminosarum* bv. viciae 1066S with the ACC deaminase activity enhanced the plant shoot biomass, nodule formation, nitrogen fixation, efficient use of water and the uptake of nutrients in pea plants under water deficient conditions (Belimov *et al*., 2019). Furthermore, rhizobia strains exhibiting ACC deaminase activity enhanced germination in *Glycine max* L. (soybean), this was under drought stress induced by 4% of polyethylene glycol (PEG) (Igiehon *et al.,* 2019). Previous studies have suggested that plants inoculated with rhizobacteria exhibiting ACC deaminase activity aid plants to develop tolerance and can be resistant to drought, salinity, flood, and different pathogens stresses (Ravanbakhsh *et al*.,2017; Ghosh *et al*., 2018; Saikia *et al*., 2018). Thus, ACCdeaminase activity is an essential trait required to promote plant growth under stress conditions (Nadeem *et al*., 2011). Therefore, in our current study, the isolates with ACC deaminase activity have the potential to promote the growth of numerous legume plants, particulalry under abiotic stress conditions.

Amongst other aims, this study also explored the effect of random UV mutagenesis with the possibility to produce *exoR* and *acdS* gene mutants. Currently, rhizobium research has mainly focused on understanding bacterial gene expression and their role in symbiosis. The use of proteomics will likely reveal new techiniques that will help understand the complex interactions

between rhizobia and legumes, and mechanisms used by rhizobia to survive under stressed conditions (Musarrat *et al*., 2010). Ultraviolet (UV) radiation inhibits cell growth and induces gene damage (EPA, 2014). Bose and Venkataraman (1972), Macgrego and Alexander (1971) isolated and characterized UV induced mutant of rhizobia. The development of an effective symbiotic relationship does not dependent only on the directly required genes for symbiosis but also on the type of genes responsible for the production of exopolysaccharides (Downie, 2010; Gibson *et al*., 2008).

Rhizobia mutants that were defecient in distinct polysaccharides production were deficient in symbiosis establisment (Fraysse *et al*., 2003; *et al*., 2004). The *acdS* gene plays an ecological role in plant growth via the reduction ACC levels (Ma, *et al*., 2004; Glick*et al*., 2007; Conforte*et al*., 2010; Nascimento*et al*., 2012a, Nascimento *et al*., 2012c). Previous studies have reported that *Mesorhizobium* sp. MAFF303099 *acdS* mutant decreases the strain's symbiotic performance. The *acdS* mutant strain inoculating *L. japonicus* formed few nodules when compared to *L. japonicus* wild type strain with the *acdS* gene present. Furthermore, the *acdS* gene knockout resulted in a decreased ability of the bacteria strain to nodulate its legume host plant; this was in comparison to the respective wild type strain of the bacteria. Therefore this clearly indicates that the presence of *acdS* gene plays a vital role in the symbiosis efficiency and increases the nodulation of legumes (Uchiumi *et al.,* 2004). The *exoR* and *acdS* genes were still detected on the possible mutants, meaning that no *exoR* or *acdS* gene mutants were formed or the random mutagenesis didn't target our genes of interest.

A study by Gao and Garcia-Pichel, (2011) reported that the organism's resistance to UV radiation could be due to different attributes. These attributes includes the protection or repairing of the damaged DNA, pigments cellular protection and the reduction of damage caused by

photo-oxidative products. The EPS genes empower microorganisms to be tolerant to abiotic stresses (Roberson and Firestone, 1992). Moreover, cell composition and membrane structure are linked to the bacteria's ability to tolerate stress, e.g exopolysaccharide production of bean rhizobia and *Bradyrhizobium* sp. strains is pH tolerance related, this result in an increased legumes symbiotic nitrogen fixation (Donot *et al*., 2012; Razika *et al*., 2012).

Rhizobia strains SAC1 and SAC2 subjected to UV exposure showed the ability to produce exopolysaccharides when grown in YMB and no *exoR* mutant strains were formed post UV exposure. Previous studies have demonstrated that an increase in microbial resistance against UV radiation is due to biofilms (De Carvalho, 2017). This is probably because UV radiation penetration in the biofilm matrix is generally in a sense that only the first few top layers of the microbial cells are exposed to the UV's deleterious effects. Moreover, cells exposed to the UV produces specialized compounds such as mycosporine-like amino acids and carotenoid pigments, providing further protection against UV radiation. A study revealed that compared to the planktonic bacteria, the EPS matrix shield microorganisms physically against radiation (UV-C, UV-B, and UV-A). They allow about 13%, 31%, and 33% UV light to be transmitted, respectively to the microorganisms. Thus, biofilms can effectively protect the microbial cells against UV radiation and exposure (Elasir and Miller, 1998) and this might be the reason why no *exoR* mutants were formed in our study.

4.7 Conclusion

The exposure of bacterial cells to UV radiation for a longer period results in a reduced cell concentration or even cell death. Isolates with *exoR* and *acdS* genes were exposed to UV radiation with the aim to isolate *exoR* and *acdS* mutants strains post UV exposure. However, no

exoR and *acdS* gene mutant strains were detected. This was further confirmed by ACC deaminase activity and polymerase chain reaction meaning that these isolates were tolerant to UV radiation to a certain extent. The role of isolates with ACC deaminase activity (*acdS* gene) and exopolysaccharides (*exoR* gene) production post UV exposure is significant in the agricultural system prone to climate changes. Further studies such as field trial are recommended for isolates to be selected for potential use as inoculants under abiotic stressed conditions.

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CHAPTER 5

GENERAL CONCLUSION

There's a global increase in human population at an alarming rate, resulting in a substantial increase of food production (Koskey *et al*., 2021). However, there's a decline in soil fertility resulting in an inherent low soil fertility status and it is a major challenge in crop production, affecting African smallholder farming areas (Smaling *et al*., 1993, Ncube *et al*., 2009; Fanadzo *et al*., 2010; Njoloma *et al*., 2016). To address this challenge, chemical fertilizers can be used to improve crop yield but they are expensive. Small scale farming communities lack knowledge to apply inorganic fertilizers and they have negative impacts on the quality of water, soil fauna and health (Njira *et al*., 2012; Schroder, 2014; Koskey *et al*., 2021; Yadav *et al*., 2021). Water, soil and air pollutions have been caused by extensive use of inorganic fertilizers. This is as a result of the

destruction of soil physical characteristics, nutrient leaching and accumulation of toxic chemicals in water (Agbede, 2010). Thus, agrochemicals are part of dominant factors causing environmental pollutions and hazardous to human and livestock health (Sharma and Singhvi, 2017).

The increasing cost of inorganic fertilizers and the negative effects they have in the environment has prompted the research of alternative sources of nitrogen. Legumes form symbiotic relationships with rhizobia genera such as *Mesorhizobium, Rhizobium*, *Bradyrhizobium*, *Sinorhizobium* and *Azorhizobium,* this enables them to fix atmospheric nitrogen. This symbiotic relationship has the ability to enhance production in agriculture (Kebede *et al*., 2021). The introduction or use of inoculants is an alternative source of nitrogen, permits effective legume nodulation due to inoculation, atmospheric nitrogen fixation and subsequently increases production in agriculture (Wolde-meskel et al., 2018; Matse et al., 2020; Yadav et al., 2021). The application of microbial inoculants is a promising component as it provides fundumental solutions to agro-environmental challenges. Microbial inoculants enhances nutrients availability and uptake, this promotes plant growth while maintaining the plant's health (Korir *et al*., 2017). However, this requires screening of rhizobia isolates with compatible hosts. This is conducted with the possibility of bacteria showing the potential to be transformed in future into microbial inoculants (Yanni et al., 2016; Koskey et al., 2017; Manasa et al., 2017; Muleta et al., 2017).

There's an increasing interest in smallholder's agricultural farming systems to use rhizobia as inoculants. This in turn has encouraged the identification of rhizobia strains in large numbers. In this current study, a total number of 40 rhizobia strains which were previously collected from legume root nodules of *Trifolium repens, Lupinus albus, Vigna unguiculata, Medicago sativa, Phaseolus vulgaris* were used. These isolates were deposited at the South African Rhizobium

Culture Collection (SARCC) for storage. Phylogenetic analyses which were based on the 16S rRNA and *recA* gene sequences revealed that the isolates belong to four genera: *Sinorhizobium, Bradyrhizobium, Rhizobium* and *Mesorhizobium*. The authentication study of the SARCC isolates confirmed that the isolates were rhizobia and they infect and form nodules on their original hosts and improved plant growth. Glasshouse nodulation efficacy test revealed that the application of rhizobia strains significantly ($P \leq 0.05$) increased fresh biomass, dry biomass, and root nodule number when compared to non-treated legume plants under normal and abiotic stress. Bacterial beneficial genes such as *acdS* and *exoR* were detected in these isolates and are very essential to alleviate abiotic stresses in plants. Symbiotic genes such as *nodA* and *nodC* were also detected in some of the isolates. To date, inoculants containing diverse rhizobia strains have been developed. These inoculants are currently used as biofertilizers which promote the fertility of the soil and legumes symbiotic nitrogen fixation. Based on the results obtained, the isolates can also be potential candidates in the development of inoculants for legumes and possibly other crops. Future studies should focus on further screening of these rhizobia strains on selected legumes under soil conditions where such abiotic stresses as drought, salinity or acidity prevail in South Africa.

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