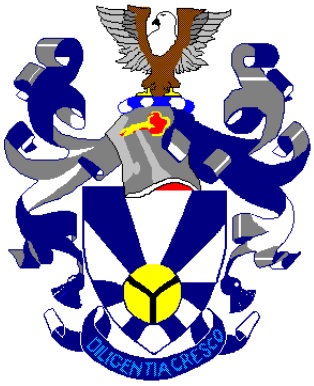

University of Zululand

Phenotypic diversity of indigenous rhizobia nodulating different morphotypes of *Vachellia karroo*

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Phenotypic diversity of indigenous rhizobia nodulating different morphotypes of *Vachellia karroo*

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DECLARATION

I the undersigned do hereby declare that this thesis is my own original work. It has not been submitted for any degree or award in any university and does not contain any work of another person unless otherwise stated. The thesis is being submitted for the degree of Master of Science under the department of Agriculture, University of Zululand.

Signature:

Nkazimulo Ngwenya

Date:

DEDICATION

To the most loving and supporting people in my life

My parents Mr and Mrs David Ngwenya

My husband Silethemba Dube

My grandparents Mr and Mrs Wilson Dube

And

My siblings Andile Prosper Ngwenya, Sisasenkosi Ngwenya and Anele Ngwenya

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ABSTRACT

Vachellia karroo (sweet thorn) is one of the important pasture legumes in agriculture due to its ability to fix nitrogen through its symbiotic relationship with rhizobia. The nitrogen fixed through this biological way is more beneficial than chemical fertilizers as it does not have any harmful effects on the environment, and it is free. There is limited information on the rhizobia nodulating *V. karroo* growing in South African soils. Therefore, the isolation and determination of phenotypic characteristics of indigenous rhizobia nodulating *V. karroo* is of paramount in improving symbiotic effectiveness and thus enhance nitrogen fixation.

Four different morphotypes of *V. karroo* obtained from four areas of South Africa with different agro-ecological conditions namely Kei Mouth, Richards Bay, Leeu Gamka and Tshwane were each grown in four soils, also obtained from these areas. Before planting the morphotypes, the chemical properties of the soil were first assessed and an experiment was conducted to isolate rhizobia in the soil to determine their presence. The morphology of the nodules of *V. karroo* morphotypes was observed after three months of growth. Thirty two rhizobia strains were isolated from the *V. karroo* morphotypes, two from each morphotype and soil combination. Fresh rhizobia cultures of each strain were used for the Gram test, bromomythyl blue test, determination of generation time and for colony morphology. Notable results from the soil chemical analysis was that soils from Richards Bay and Leeu Gamka were alkaline and those from Kei Mouth and Tshwane were acidic. Results of nodule morphology revealed that nodule morphology was influenced by morphotype and not by soil of origin. All the 32 isolates were Gram negative, 87.5% were fast growing rhizobia with below four hours of generation time and they turned bromomythyl blue indicator yellow while 12.5% were slow growing with a generation time of more than six hours and turned bromomythyl blue indicator blue. All the isolates which were slow growing were expectedly from acidic Tshwane soil as slow growing rhizobia prefer acidic conditions. The colonies for all isolates were generally mucoid, shiny and round. The phenotypic characteristics of the 32 rhizobia isolates were then studied. Variation was noted in the isolates' response to temperature, pH, salinity, antibiotic resistance and heavy metal resistance. However, the isolates' response to carbon source utilization was almost the same as they all utilized more than three quarters of the tested carbon sources. When grown under a temperature range of 5 to 45^0C, all the isolates grew in 15 to 30^0C. 18.75%, 87.5%, and 28.13% grew at 5, 40, 45^0C respectively. The temperature tolerance of the isolates was not related to the climatic

conditions of soil of origin nor to those of the host morphotypes. None of the tested isolates grew at pH 3 and all grew at pH 6-7. At pH 4, 5, 8 and 9 the percentages of isolates which grew were 22, 38, 91 and 51%, respectively. Isolates extracted from the alkaline soil from Richards bay were unable to grow at low pH of 3-5. However, for the other soils no such relationship was found and also no relationship was found between morphotypes and growth behaviour with respect to pH. The growth performance of the 32 *Vachellia karroo* rhizobia isolates in relation to salinity varied greatly in all isolates and was not related to the salinity characteristics of the soils from which they were extracted. Fast growing rhizobia were more tolerant to high salt concentrations compared to the slow growing rhizobia. In this research most rhizobia isolates exhibited resistance to antibiotics which was not correlated to conditions of geographic origin and host morphotype and the same pattern was noted in their resistance to heavy metals and utilisation of carbon sources. The dendrogram obtained from the computer numeric analysis of the isolates' phenotypic characteristics produced six clusters at 83% level of relative similarity. This revealed the high variation of indigenous rhizobia. The clustering of isolates showed some degree of relatedness to the soil of origin and host morphotype contrary to their behaviour under each single treatment.

The last study focused on the ability of *V. karroo* rhizobia from each morphotype to cross nodulate with other morphotypes or with other *Vachellia* species (*V. nilotica* and *V. tortilis*). All the rhizobia strains from the four morphotypes of *V. karroo*, two from *V. nilotica* and *V. tortilis* were able to form symbiosis nodules with the other morphotypes and with other *Vachellia* species. These results confirm that *V. karroo* rhizobia are able to form symbiosis with other morphotypes of the host species and also with other species of *Vachellia*. The same was true for the host plants, they were not specific in terms of the rhizobia nodulating them.

From this study, it was discovered that there the four *V. karroo* morphotypes are generally not distinct in terms of rhizobia nodulating them. It was also noted that behavior of rhizobia under certain conditions is not co-related to soil of origin. Furthermore a wide diversity in characteristic was noted in the rhizobia nodulating *V. karroo* which is advantageous when used in the selection of rhizobia which are tolerant to certain adverse condition, symbiotic effectiveness and competitive ability.

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List of Abbreviations and symbols

Al	Aluminium
BNF	Biological nitrogen fixation
BTB	Bromothymol blue dye
CFU	Colony forming units
Co	Cobalt
Cu	Copper
CR	Congo red dye
GT	Generation time
HCl	Hydrochloric acid
K_2HP0_4	Dipotassium phosphate
KM	Kei Mouth
KZN	Kwa-Zulu Natal province
LG	Leeu Gamka
$MgSO_4$.$7H_2O$	Magnesium sulphate
Mn	Manganese
NaOH	Sodium hydroxide
N_2	Nitrogen
NH_3	Ammonia
Ni	Nickel
RB	Richards Bay
Rpm	Revolutions per minute

TS	Tshwane
UPGMA	Unweighted Pair Group Method with Arithmetic mean
YEMA	Yeast extract mannitol agar
YEMB	Yeast extract mannitol broth
Zn	Zinc

CHAPTER 1

General Introduction

1.1 Background

Nitrogen (N) is an essential nutrient that is requires in large quantities, but it is usually deficient in most soils. Despite the high abundance (about 78%) of molecular nitrogen (N_2) in the atmosphere (Ingenbleek, 2006), its deficiency is still one of the major constraints in agriculture (Srivastava *et al*., 2012). This is because plants can only assimilate nitrogen when it is reduced from N_2 to ammonia (NH_3) (Lodwig *et al*., 2003, Lewis, 1992).

Realising this problem, scientist developed the Haber process to reduce atmospheric N_2 industrially (Chatt, 1977) for agricultural crop production. This has been followed by an entrenched practice to apply nitrogen to crop and pasture plants in the form of urea, ammonia or nitrate fertilizers. There are, however, disadvantages associated with the use of these fertilizers. They include high manufacturing costs that are eventually passed on to farmers (Bohlool *et al*., 1992) and the release of hazardous gases during the manufacturing process, which pollute the air and eventually results in the formation of acid rain (Pearce, 2009).

In addition to these problems, excess nitrogen is leached down to the water table where it pollutes ground water and / or is washed by rain water to rivers and other surface water bodies, which leads to eutrophication (Wagner, 2011). These constraints associated with the use of artificial fertilizers call for an alternative affordable and environmentally friendly way of enhancing soil nitrogen content, such as biological nitrogen fixation (BNF).

Biological nitrogen fixation is a process whereby bacterial micro-organisms fix nitrogen in the soil by reducing atmospheric nitrogen (N_2) to NH_3 (Wagner, 2011). These micro-organisms can fix nitrogen either when free-living or when in symbiosis with plants (Shridhar, 2012). The symbiotic fixation is the most relevant contributor to plant biological nitrogen fixation (Cooper and Scherer, 2012). The organisms involved consist of Frankia and Rhizobia, which form symbiosis with non-leguminous and leguminous plants, respectively (Cooper and Scherer, 2012). On the other hand leguminous plants are most beneficial to agriculture because they add nitrogen to the soil which is easily accessed by other plants

(Cooper and Scherer, 2012). This inevitably makes the symbiosis that rhizobia forms with legumes more significant in nitrogen fixation compared to fixation by other microorganisms.

Legumes of importance in agriculture which form symbiosis with rhizobia range from cultivated crops to trees in pasture lands. Of the leguminous trees involved in rhizobia symbiosis, *Vachellia* species are among the preferred legumes to fix nitrogen in pasture and cultivated agricultural lands (Macedo *et al*., 2008). *Vachellia* species were formerly known as *Acacia* species until 2005, when the name was formally changed in an International Botanic congress held in Vienna. At this congress it was proposed and later declared in the year 2011 that the genus name *Acacia* be used only for Australia native plants, and the rest of the *Acacias* in the world be classified under a different genus *Vachellia* (Robin and Carruthers, 2012).

South Africa is endowed with a wide variety of *Vachellia* species which might be beneficial to BNF in both pasture and cultivated lands. *Vachellia karroo* is one of the most common and important *Vachellia* species in Southern Africa (Hayward, 2004) . The species provides valuable browsing in rangelands, and is often used in alley cropping to increase soil nitrogen. It is one of the pioneer tree species on disturbed land, and hence is sometimes used as a pioneer species in re-vegetation programs in Africa because of its beneficial symbiosis with rhizobia (Zahran, 2001).

The preference to use *Vachellia* species for fixing nitrogen is due to their fast growth rate, high nodulation rates and the large amount of nitrogen they fix in comparison to other leguminous trees (Aronson *et al*., 1992). However, there is an information gap concerning the natural nodulation of the *Vachellia* species by rhizobia species found in soils where *Vachellia* grows (Burdon *et al.*, 1999, Alshaharani and Shetta, 2015). Diversity of rhizobia is influenced by host plant provenance (Liu *et al*., 2005). Thus, it is not only important to analyse rhizobia from different geographic areas, but also to determine their interaction with host plants growing in these areas for better characterisation (Bakhoum *et al*., 2014).

Previous research suggests that there is specificity in the symbiosis of *Vachellia* species and rhizobia, which implies that *Vachellia* species can only form this relationship with specific rhizobia and the same is true for rhizobia (Dreyfus and Dommergues, 1981, Turk and Keyser, 1992) Nonetheless, it has also been reported that both *Vachellia* and rhizobia can be promiscuous, for example rhizobia forms symbiosis with various species of *Vachellia* and

vice versa (Pérez-Fernández *et al*., 2008). Rhizobia symbiotic diversity has also been noted among some of the *Vachellia* species (Ba *et al*., 2002), (Bakhoum *et al*., 2015). Consequently, due to the wide variety of *Vachellia* species and rhizobia, there is still an information gap in the nature of their relationship (Burdon *et al*., 1999).

Vachellia karroo has various morpho-types (Taylor and Barker, 2012), which grow in regions of diverse climatic and soil conditions. Due to the diversity noted in this species and in the conditions where it grows, it is assumed that it may have different rhizobial preferences in different areas where it grows.

An important discovery of plant-rhizobia symbiosis is that their specificity rests on the diversity of both the host and the bacteria (Lindström *et al*., 2010). *Vachellia karroo* is a very important tree in South Africa used in the rehabilitation of lands with low nitrogen levels (Boyes, 2004). It does this by fixing its own nitrogen, and thus is able to establish where other trees fail. It is also used in alley cropping in agro-ecosystems (Everson *et al*., 2009). Therefore, the core activities of this research were to explore phenotypic diversity of rhizobia nodulation morphologically diverse populations of *V. karroo* from different habitats that differ widely in climate and soil types.

Many studies have been done on *Vachellia karroo* in relation to its contribution towards agriculture. However, there is limited information on its symbiotic relationship with rhizobia and the nature of rhizobia nodulating it, especially in South African soils. The use of *V. karroo* in relation to nitrogen fixation necessitates detailed studies on the nodulation of this species and the nature of the rhizobia nodulating it.

There is clearly limited information on *V. karroo's* symbiotic relationship with rhizobia and the nature of rhizobia nodulating it, especially in South African soils. Therefore, the core activities of this research were to explore phenotypic diversity of rhizobia nodulating morphologically diverse populations of *V. karroo* from different habitats that differ widely in climate and soil types.

1.2 Objectives

The objectives of the study were:-

a) To examine the nodulation of morphologically diverse species of *V. karroo* by rhizobia found in soils from different agro-ecological regions of South Africa where *V. karroo* grows.

b) To phenotypically characterize indigenous rhizobia that nodulate the *V. karroo* morphotypes growing in different soil types.

c) To determine if the rhizobia from the *V. karroo* morphotypes cross nodulates with other *V. karroo* morphotypes and with other *Vachellia* species.

CHAPTER 2

Literature review

2.1 Importance of Biological Nitrogen Fixation in African soil

Most of the African soils have little organic matter and very low nitrogen content because of high temperatures and increased annual burning which promote rapid decomposition of plant materials (Fynn *et al*., 2003). Hence, acute nitrogen deficiency in the soils has prompted the need to improve biological nitrogen fixation in order to restore and improve nitrogen content of the soil (Vance *et al*., 2002). Research has revealed that biological nitrogen fixation is the largest single enhancer of soil nitrogen (Zahran, 2001). Approximately, 50-70 Teragram (Tg) of nitrogen is fixed through biological nitrogen fixation per year globally.

Earlier research has also revealed that 50% of the nitrogen needed by terrestrial plants per year can be fixed by the *Rhizobium*-legume symbiosis (Vadalkalto and Petterson, 2006). Furthermore, crops grown after legume crops benefit from the nitrogen fixed and give a yield which is equivalent to crops supplied with 300 kg/ha of nitrogen fertilizer (Gilbert, 2012). It is also noteworthy that leguminous trees growing in pasture lands increase the nitrogen content of pasture grasses (Velde, 2009). Pasture and fodder legumes contribute an estimated 12-25 Tg of nitrogen to this amount (Herridge *et al*., 2008). Among the essential pasture legumes that contribute significantly to nitrogen fixation, especially in Africa, are leguminous trees of the genus *Vachellia*.

2.2 The Diversity of *Vachellia*

The genus *Vachellia* is composed of about 1352 species with very diverse morphological and genetic attributes (Leafy *et al*., 2006). The hierarchical classification of *Vachellia* is presented in Figure 2.1. In Southern Africa alone there are about 100 different species of *Vachellia* (Van Wyk and Van Wyk, 1997).

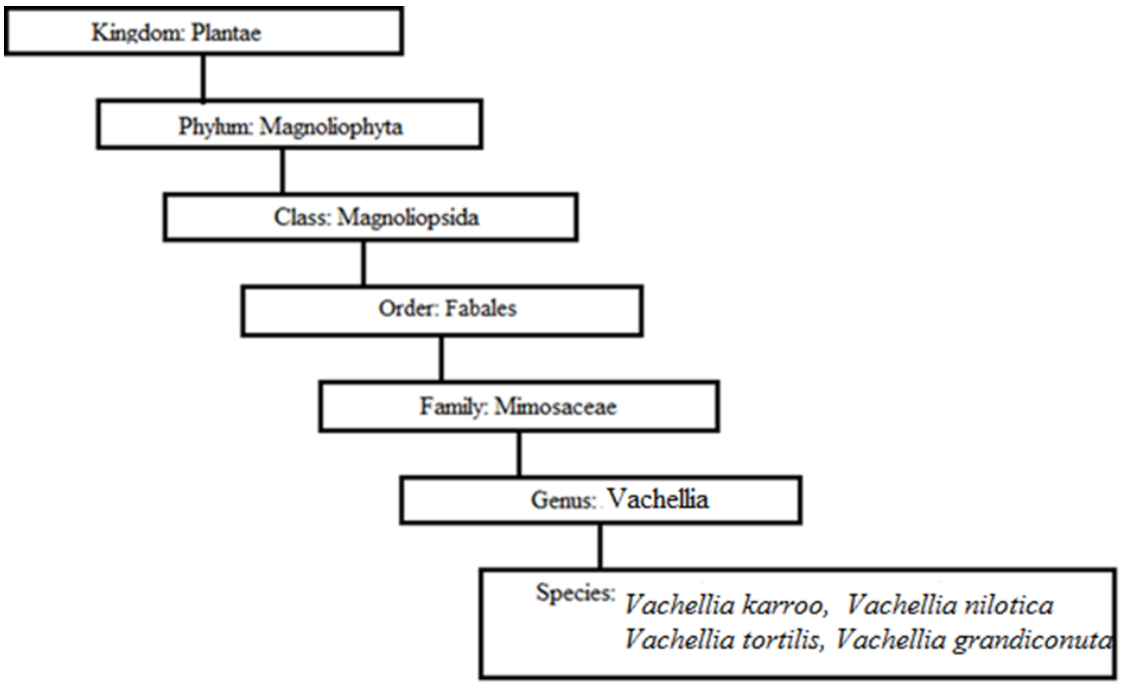

Figure 2.1 Hierarchal classification of Vachellia (Kyalangalilwa *et al*., 2013)

The most common species of *Vachellia* in South Africa are *V. karroo (V. natalitia), V. nilotica, V. tortilis, and V. grandicornuta*. Of these, *V. karroo* has a number of variants or morphotypes (Table 2.1), and is the subject of the present study. The morphotypes presented in the table are from areas of different agro-ecological conditions. Richards Bay and Kei Mouth are in the coastal regions, where rain falls throughout the year (Mboumba, 2006). However, the soil diversity is noted between the two sites. Richards Bay is dominated by alkaline, sandy to loam soils (Scott *et al*., 1993) with minimum and maximum temperatures ranging from 10 to 28ºC and the annual rainfall is at 1200 mm (Archibald and Bond, 2003). By contrast Kei Mouth with a temperature range of 14 to 24ºC is dominated by clay black soils. Of the other two sites, one is a moderate rainfall area (Tshwane) with a temperature range of 5 to 37ºC and mean annual rainfall of 674 mm and the other (Leeu-Gamka) has an average annual rainfall of 150 mm, with temperatures ranging from 5 to 40ºC.

Table 2.1: Description and distribution of *V. karroo* morphotypes in South Africa as summarised by (Barnes *et al*., 1996)

Morphotype description	Region in South Africa where morphotype is found
Greyish-white barked tree or shrub Have long spines. Very fast growing. Long, constricted pods	Richards Bay (KwaZulu-Natal (KZN)
Shrub of about 1 m high. Generally thin	Kei Mouth (Eastern Cape)
Shrub Fire resistant	Leeu Gamka (Western Cape)
Tall trees of 3- 15m high Little branching Brownish to dark bark Constricted pods	Tshwane (Gauteng)

Vachellia karroo is used for wood, timber, livestock feed, and to control soil erosion (Van Wyk and Van Wyk, 1997), and is therefore known as a multipurpose tree (Wolde-meskel *et al*., 2004). It is also used to rehabilitate deforested areas and to restore soil fertility through nitrogen fixation (Chirwa *et al*., 2008, Wolde-meskel *et al*., 2004). *Vachellia karroo* is generally easy to cultivate and is adapted to many soil types and various climatic conditions, inclusive of alkaline and drought prone areas (Degefu *et al*., 2011). Its nodulation occurs in all the soil types where it grows, confirming the presence of compatible nitrogen fixing bacteria in these soils.

2.3 Nitrogen fixing microorganisms

Nitrogen fixing microorganism include aquatic bacteria (e.g *Cyanobacteria spp.*), free-living soil bacteria (e.g. *Azotobacter spp.*), bacteria that form symbiosis with leguminous plants (e.g. Rhizobia) (Wagner, 2011) and Frankia, which form symbiosis with non-leguminous actinorhizal plants such as alders (Huss-Danell, 1997). The symbiotic forming bacteria collectively known as rhizobia surpass the contribution of other nitrogen fixing micro-

organisms (Wagner, 2011). Rhizobia are highly competitive, most efficient and significant nitrogen fixing bacteria (Terpolilli *et al*., 2012).

2.4 Legume- rhizobia symbiotic relationship

Legumes of the Fabaceae family are the only plants that are able to form symbiotic relationships with rhizobia. In this relationship, the rhizobia infest roots of the legumes and become established inside root nodules, in which they fix nitrogen (Suominen *et al*., 2003). Nodule bacteria which are rhizobia that form nodules with legumes are currently classified into 6 genera, namely:, viz; *Rhizobium, Bradyrhizobium, Mesorhizobium, Sinorhizobium, Azorhizobium,* and *Allorhizobium* (Wagner, 2011).

2.4.1 Establishment of Legume-rhizobia symbiosis

The process of nodule formation is known as nodulation. Nodulation is a very co-ordinated effort between the legume and rhizobia bacteria in the soil and requires co-ordinated expression of several bacterial and plant genes (Santos *et al*., 2000). The nodulation process begins with both the rhizobia and legumes releasing and exchanging chemical signals to initiate the relationship (Badri *et al*., 2009).

This molecular interdomain communication between the legume and rhizobia happens around the plant root in the rhizosphere (Cooper, 2007). The molecular cross-talk subsequently leads to the binding of the rhizobia to the host cell, and its uptake into the plant root through the root hair. Upon entrance into the root cell rhizobia survive, replicate and differentiate into a nitrogen fixing form (Jones *et al*., 2007).

2.4.2 Role of Nod-genes in rhizobia-legume recognition

Leguminous plants exude from their roots into the soil a number of flavonoids, which act as signals in the rhizobia-legume cross-talk (Dakora and Phillips, 2002). These flavonoids are diverse and include chalcones, flavonols, isoflavones, flavanones, flavones, and anthocyanidins (Wasson *et al*., 2006). The flavonoids exudes trigger the transcriptional activator NodD in rhizobia which is a type of a Lys R regulator (Wassem *et al*., 2008).

NodD stimulates the expression of nodulation genes (*nod, nol, noe*) by binding upstream of the nod box sequence inducing DNA bending and transcription (Geurts and Bisseling, 2002). The nodulation genes catalyse the synthesis of lipochitin oligosaccharide signal molecules (Nod factors) (Castillo *et al*., 1999). Nod factors promote the deformation of root hairs and

the formation of an infection thread (Cárdenas *et al*., 1998) leading to the establishment of the symbiotic relationship (Santos et al., 2008).

2.4.3 Formation of an infection thread

When recognition has been achieved, rhizobia attaches to the tip of the root hair. In response to a Nod factor released by the rhizobia (Cárdenas *et al*., 1998), an influx of Ca^{2+}, H^{+}, Cl^{-} and K^{+} occurs in conjunction with plasma membrane depolarization at the tip of the root hair (Felle *et al*., 1999). This happens for a period 20-30 seconds, the Ca^{2+} flux is quickly followed by Ca^{2+} spiking in the cytoplasm (Sieberer *et al*., 2009). This process happens within 10 minutes after the exposure of the root tip to the rhizobia nod factor and occurs almost at the same time with the suppression of reactive oxygen (Kanamori *et al*., 2006).

These chemical changes are followed by cortical cell division of the root hair (Terefework, 2002) which leads to swelling, branching and curling of the root hair tip, thus enclosing and wrapping the rhizobia inside the curled root hair (Catoira *et al*., 2001). Development of infection threads subsequently follows, which starts with the rhizobia partially dissolving the cell wall and penetrating the root hair cell (Gage, 2004). The infection thread grows down through the base of the root hair cell. As the rhizobia moves down the infection thread, they release Nod factors which trigger cell division and multiplication of the rhizobia (Gage and Margolin, 2000).

2.4.4 Change from bacteria to bacteriodes

When the infection thread reaches the distal cell wall, the rhizobia become released from the infection thread into the cell cytoplasm of the cells where they cause rapid cell division producing a root nodule (Gage, 2004). With subsequent cell division of the host cell and of the bacteria, the bacteria are transformed into nitrogen fixing forms called bacteriodes (Franche *et al*., 2009). As a result of bacteriod development, the nodule structure becomes fully developed. These bacteriodes are enclosed by a membrane from the plasma membrane (Caiola and Canini, 1993). Nitrogen fixation starts after the formation of bacteriodes in the root nodules. After a series of morphological and physiological differentiation, the bacteriodes are able to reduce atmospheric N_2 by breaking the strong triple bond between the nitrogen atoms (Ghosh *et al*, 2008).

2.5 Nitrogen fixation process

Atmospheric nitrogen diffuses through the infection thread to the bacteroid where it is converted to ammonia (Cheng, 2008). This process involves a series of chemical reactions which use high energy levels and action of an enzyme to break down the massive triple bond between the nitrogen atoms in atmospheric nitrogen.

The reduction of nitrogen is catalysed by the enzyme nitrogenase (Berman-Frank *et al*., 2003). This is an enzyme complex that consists of two proteins namely, dinitrogenase (molybdenum-iron (MoFe) protein) and dinitrogenase reductase (Fe protein) (McLean and Dixon, 1981). The MoFe protein is composed of four subunits (2 alpha and 2 beta) which are joined together by strong non-covalent bond. A series of electron transfers occur within the protein during the reduction of nitrogen. The Fe protein structure complements the previously described MoFe by having two similar subunits, which are bound together by 4Fe-4S bond. These also have an ATP binding site.

Accurately, 16 ATP energy molecules in addition to the enzyme nitrogenase are needed in the successful degradation of 1 molecule of N_2 (Berg *et al*., 2002). As afore mentioned, the ATP used in the process is a result of the degradation of sugars manufactured by the host plant (Mulongoy, 1995).

The chemical reactions involved can be summarised in an equation as follows:-

$$N_2 + 8e^- + 8H^+ + 16ATP \xrightarrow{\text{Nitrogenase}} NH_3 + H_2 + 16ADP + 16P_i$$

Other micro-organism further reduces ammonia as follows:-

$$2\,NH_3 + 3\,O_2 \xrightarrow{\text{Nitrosomonas}} 2NO_2^- + 2\,H^+ + 2\,H_2O$$

$$2\,NO_2^- + O_2 \xrightarrow{\text{Nitrobacter}} 2\,NO_3^-$$

2.6 Abiotic and biotic factors that affect the efficiency of nitrogen fixation

A number of abiotic and biotic factors affect the functioning of rhizobia and legume plant during nitrogen fixation. Under biotic factors the action of the enzyme nitrogenase is the

main limiting factor in nitrogen fixation (Oldroyd and Dixon, 2014). Its action is inhibited by excess oxygen, which is also needed by rhizobia. As a measure to solve this predicament and obtain efficient nitrogen fixation, both the legume and the rhizobium contribute to the production of the protein leghaemoglobin. This protein delivers oxygen in a very controlled manner to rhizobia bacteriods in the root nodules so as to avoid oxygen poisoning of the nitrogenase enzyme (Wagner, 2011). Further, the proper use of energy from photosynthesis to convert N_2 to NH_3 by nitrogen fixing symbionts is essential. This calls for proper combinations of rhizobia and legumes which can be able to fix adequate nitrogen using low energy levels.

Among abiotic factors, any stressful environmental condition such as soil acidity, moisture deficiency, mineral nutrient deficiency and high temperatures, negatively affects nitrogen fixation (Mohammadi *et al*., 2012). Most rhizobia strains isolated from different legumes are sensitive to high temperatures and their survival is reduced as temperatures increase above the optimum (Zahran, 1999). It has been further noted that rhizobia are more sensitive to acidic conditions (Niste *et al*., 2013) in relation to their survival. Presence of heavy metals in the soil in large quantities also negatively affects the growth, abundance, diversity and symbiotic effectiveness of rhizobia (Vasilica *et al*., 2011). This reduces the rhizobia population which leads to the reduction in the nitrogen fixed. Rhizobia utilises a variety of carbon sources in order to improve its growth (Stowers, 1985) and this enhances their survival in the soil which increases the percentage of nitrogen fixed (Bedi and Naglot, 2011).

2.7 Diversity of rhizobia in the soil

Rhizobia population is very high in the soil. Up to 52 isolates have been identified from a single gram of soil sample (Leite *et al*., 2009). High levels of diversity have also been noted among indigenous rhizobia populations (Galli-Terasawa *et al*., 2003). Their diversity can be phenotypic, genotypic (Rai *et al*., 2012) or in terms of their degree of effectiveness (Laranjo *et al*., 2002). The indigenous rhizobia found in the soil can either be effective or non-effective in terms of nitrogen fixation, depending on their compatibility with the plant species growing in that soil (Mhamdi *et al*., 2002).

The physical and chemical properties of the soil also influence the phenotypic and genetic diversity of rhizobia (Garbeva *et al*., 2004). *Vachellia karroo* grows in a wide variety of soil types ranging from loose sandy soils with large particles to clay soils. It also grows under

climates of scarce rainfall (200 mm) to high rainfall (1500 mm), and hence the rhizobial species or strains that naturally nodulate with *V. karroo* may differ in the different agro-climatic regions (Pooley, 1998).

2.8 Diversity of *Vachellia* and rhizobia in relation to their symbiotic relationship

There is wide diversity in rhizobia and *Vachellia* species themselves as well as in their interaction. Species under the genus *Rhizobia* have shown variation in their ability to nodulate with different *Vachellia* species (Wolde-meskel *et al*., 2004) and or nodulating with specific *Vachellia* species (Zerhari *et al*., 2000). Some *Vachellia* species on the other hand have shown some degree of promiscuity in forming effective symbiosis with rhizobia, e.g. *Vachellia seyal* (Diouf *et al*., 2007), whilst some species have shown some degree of specificity (Turk and Keyser, 1992).

Rhizobia diversity is an important aspect in the improvement of nitrogen fixation. In order to improve the efficacy of the symbiotic relationship it is essential to have a wide range of rhizobia strains which can be improved based on their different characteristics (Santos *et al*., 2008). Currently, there is very limited information on the diversity of indigenous rhizobia found in South African soil which nodulate *V. karroo.* It is not known whether the species *V. karroo* is promiscuous or not in its symbiotic relationship with rhizobia. Thus this study seeks to investigate the characteristics of indigenous rhizobia, the isolates with the desired phenotypic characteristics will be recommended as potential inoculants to be used on V. karroo growing in South African soils.

CHAPTER 3

Nodule morphology, growth and morphological characteristics of the isolates

3.1 Introduction

Nodule morphology is one of the important information to obtain in legume-rhizobia symbiosis. The shape and position of the nodules on the root system are mainly determined by the host plant and is not related to the *Rhizobium* strains, (Swelim *et al*., 2010, Zahran, 1998, Burton, 1984). Thus they have been an essential tool in the taxonomy of legumes (Sprent *et al*., 2013). However, the size, colour and the distribution of the nodules on the root system is determined by the *Rhizobium* strains (Burton, 1984).

Nodules formed on the primary root are said to be more efficient in comparison to those formed on the lateral root system (Predeepa and Ravindran, 2010). Difference in nodule number amongst plant species might be due to the adaptability of *Rhizobium* and the host plant to soil conditions (Kficfik and Cevheri, 2013).

There are non-rhizobial bacteria which are able to form nodules with legumes, (Balachandar *et al*., 2007). Thus, in order to distinguish rhizobia from these bacteria and those that might have been caused by contamination, a Gram stain and Congo Red (CR) dye tests are conducted. Therefore the resulting bacteria after these tests which are Gram negative but do not absorb the CR dye are rhizobia.

Culture characteristics are necessary to characterise growth habits of rhizobia (Muthini *et al*., 2013). Fast and slow growing rhizobium bacteria are distinguished on the basis of colour change in yeast mannitol agar (YEMA) containing (bromothymol blue) BTB. Fast growing rhizobia (*Rhizobium*) are acid producers which turn BTB yellow (Kawaka et al., 2014). On the other hand slow growing rhizobia (*Bradyrhizobium*) produce an alkaline reaction which turns BTB indicator blue (Kim *et al*, 1985). The growth rate of the rhizobia is further

assessed by the time taken for the rhizobia population to double, known as generation time (GT). The generation time characterises rhizobia into slow and fast growing groups (Somasegaran and Hoben, 1985).

Colony characteristics (shape, colour and size) and growth rate are also used to classify rhizobia that nodulate leguminous trees into slow and fast growers (Frioni *et al*., 2001). The aim of this study was to assess the relationship between nodule morphology, host plant and soil of origin. It also desired to confirm if the isolates were true rhizobia and to characterise them based on growth rate.

The aim of the study was to find out whether:-

i) *Vachellia karroo* morphotypes have different nodule morphology.
ii) Nodule morphology is related to soil of origin.
iii) *Vachellia karroo* is nodulated by both *Rhizobium* and *Bradyrhizobium*.
iv) *Vachellia karroo* morphotypes vary in the nature of rhizobia nodulating them.
v) Presence of *Rhizobium and Bradyrhizobium* varies with soil type.

3.2 Methodology

3.2.1 Soil and seed collection, and study sites

Soil samples and seeds of *Vachellia karroo* morphotypes were collected from four ecologically different places of South Africa namely: Richards Bay (KwaZulu-Natal 28.46° S, 32.06° E), Kei Mouth (Eastern Cape; 32.4° S, 28.2° E), Leeu Gamka (Western Cape; 32.3° S, 22.3° E) and Tshwane (Gauteng; 25.7° S, 28.2° E) (Figure 3.1). All experiments were conducted at the University of Zululand (28° S, 31° E).

Figure 3.1 Map of South Africa showing regions where the soil samples and seed of the study were obtained. (Source; Wikipedia .org, with some alterations)

Three portions of soil samples were collected per site from a depth of 0-25 cm using an auger. The samples were put into sterile plastic bags, placed in a cooler box and transported to the laboratory for further experiments. The soil samples were ground, sieved and stored in paper bags and sent for soil chemical analysis.

3.2.2 Soil sampling and analysis, and confirmation of the presence of rhizobia in the soil

Samples of the soils were sent to the Soil Fertility and Analytical Services, of the Department of Agriculture, CEDARA, KwaZulu-Natal for chemical analyses. The soil chemical results are indicated in Table 3.2.

3.2.2.1 Preparation of YEMA and YEMB

The yeast mannitol agar (YEMA) in this study was prepared according to Somasegaran and Hoben (1985) (Table 3.2). A 0.025 g quantity of Congo red dye (CR) was added to the YEMA mixture. The YEMA plus CR were altogether mixed in a litre of distilled water and autoclaved at 121°C for 15 minutes before use. The yeast mannitol broth (YEMB) had the same ingredients of the YEMB except the agar.

Table 3.1: The composition of yeast mannitol agar (Somasegaran and Hoben, 1985)

Ingredients	Quantity (gram/L)
Mannitol	10
K_2HP0_4	0.5
$MgS0_4$.$7H_20$	0.2
NaCl	0.1
Yeast Extract	0.5
Agar	15
pH	7.0

3.2.2.2 Extraction and isolation of rhizobia from the soil

Rhizobia were firstly extracted directly from the soil samples to assess their presence in the soil. The soil samples were ground and then sieved. For each sample, 1 g of soil was placed in a conical flask containing 225 ml of YEMB (Table 3.2). The flasks were then covered by aluminium foil and incubated at room temperature for seven days. After incubation, a thin cream film formed at the top of the broth. A loop full of this film was collected and streaked onto plates of YEMA containing Congo red dye under aseptic conditions in a laminar flow hood. The plates were then incubated at 28°C for five days after which bacteria from each of the plates were re-streaked onto three new agar plates to obtain single colonies. The resulting bacterial colonies which did not absorb CR were live rhizobium in the soil samples (Somasegaran and Hoben, 1985).

3.2.3 Plant culture

The plant growth in potted soil was done in a rain protected greenhouse at the Department of Agriculture, University of Zululand. Each of the test soils from the four collection sites was mixed separately with sterilised Hygromix® at a rate of 2:3 by weight of soil and hygromix respectively. The soil mixture was then put into 5 kg plastic pots sterilized with 70% ethanol. The seeds of the four *V. karroo* morphotypes were first put into hot water to surface sterilize them and to break dormancy (Ido, 2011) and then soaked for 24 hours. They were then grown separately in the soil mixtures. Three seeds were planted in each pot and were later thinned into one plant per pot. The potted plants were watered with deionised water and harvested after 90 days of growth for nodule morphology, isolation and characterization of rhizobia.

Figure 3.2 *Vachellia karroo* morphotypes growing in different sample soils

3.2.4 Characterisation of nodule morphology

The morphotypes were carefully uprooted from the soil after 90 days of growth to obtain nodules. The roots were washed thoroughly in running sterile water in order to remove the

soil. Each *V. karroo* morphotype nodulated in all soil types and the nodules were described according to nodule shape, size and attachment to the root system. Nodule shape was categorised into globule, branched and elongated rods. The size (in length) was characterised as small (0-5 mm); medium (5-20 mm); large (above 20 mm). The nodule attachment on the root system was spread over the lateral root system, clustered on the lateral roots or attached on the tap root.

3.2.5 Isolation and culture of rhizobia from nodules

The isolation and culture of rhizobia, and subsequent experiments was done in the laboratory at the University of Zululand.

The roots were washed thoroughly in running sterile water in order to remove soil. Three root nodules were randomly excised from each plant. Each nodule was further surface sterilized to remove other microorganism on its surface (Vincent, 1970). The nodules were washed in 95% ethanol followed by passing it through the flame once and then placed in a petri dish lined with a sterile paper towel. Sterile nodules were held with sterile, blunt forceps and sliced with sterile scalpel. A 1 mm sterile inoculation needle was inserted into the nodule through the cut surface to scoop the rhizobium, which was then streaked onto a YEMA plate containing CR (Table 3.2). This isolation method is known as the needle method (Somasegaran and Hoben, 1985). The agar plates were then incubated at 28°C for five days. Repeated streaking of the rhizobia on new agar plates was done until pure rhizobia colonies were obtained.

The isolates were stored on YEMA slants at 4°C until further use. A total of two rhizobia isolates were randomly selected from each *V. karroo* morphotypes growing in a given soil sample. Given that there were four morphotypes and four soil samples, the total number of selected rhizobial isolates was 32.

3.2.6 Gram test

The Gram stain was done following the procedure of Vincent, 1970**.** A bacterium smear was placed on a slide and heat fixed. It was then be stained with crystal violet indicator, and rinsed after one minute with sterile distilled water. The slide smear was then submerged in iodine solution and allowed to stand for a minute, rinsed with sterile distilled water and then a decolouriser in the form of an acetone alcohol was added onto the slide and allowed to stand for 15 seconds. After this, the slide was again rinsed with sterile distilled water, and further

counter stained with safranin, rinsed again and dried. The slide was then viewed under a high power microscope with a drop of oil added for a clearer view.

3.2.7 Reaction with Congo red dye

Absorption of CR dye was determined using YEMA plates. A mass of 0.25 g of CR dye was first added into 100 ml of distilled water. A 10 ml volume of that stock solution was then added to a litre of YEMA to give a final concentration of $25^{x}10^{-3}$/L. When added to the YEMA, CR dye gives the agar a reddish colour. After being autoclaved at 121°C for 15 minutes, the YEMA +CR were poured into petri dishes. . Rhizobia inoculum from an agar slant was streaked onto the petri dishes with 1 mm inoculation loop under sterile conditions in a laminar flow hood and incubated at 28°C for five days.

3.2.8 Reaction with bromomythyl blue

Bromothymol blue (BTB) was added to a litre of YEMA to make a concentration of $25^{x}10^{-3}$/L. To achieve this concentration, 0.5 g of BTB was added to 100 ml of absolute ethanol. A 5 ml volume of this stock solution was then added to a litre of YMA. Bromothymol blue gives the agar a green colour. The YEMA +BTB were then autoclaved at 121ºC for 15 minutes and poured into petri dishes and let to solidify. An mm loop full of rhizobia which had been preserved in agar slants was then streaked onto the solid media. The plates were incubated at 28ºC for five days. Change of the green media colour was then examined. This procedure was adapted from (Somasegaran and Hoben, 1994). Colony morphology (colour, size, mucosity, appearance and shape) of the isolates was also observed in YEMA after incubation at 28°C for five days.

3.2.9 Generation time of rhizobia isolates

In order to assess the growth rate, 32 rhizobia isolates were inoculated separately into 50 ml YEMB flasks and incubated at 28ºC on a gyratory shaker at 200 rpm. Rhizobial growth was then assessed by measuring optical density at 600 nm after every three hours using a spectrophotometer. The logarithmic phase of growth was then used to calculate the growth rate (Somasegaran and Hoben, 1994).

Formula for generation time:-

$G = t/n$

Where: - G = (generation time), t = (time in hours), n = (number of generations) (Todar, 2011).

3.3 Results

Table 3.2 Chemical analysis of soil samples used in this study

Soil sample	Exch. acidity cmol/L	Total cations cmol/L	Acid/ Salt %	pH (KCl)	Org. C (%)	Nitrogen (%)	Clay (%)
Richards Bay	0.05	5.68	1	7.14	< 0.5	< 0.05	6
Kei Mouth	0.10	6.19	2	4.91	1.8	0.20	17
Tshwane	0.08	7.02	1	5.80	1.5	0.23	53
Leeu-Gamka	0.03	7.91	0	7.70	< 0.5	< 0.05	10

3.3.1 Nodule morphology

Tshwane, Richards Bay and Leeu-Gamka morphotypes had nodules spread on the lateral roots, but additionally Richards Bay and Leeu-Gamka morphotypes had nodules attached on the tap root (Figure 3.3) and the Tshwane morphotypes had nodules which formed clusters in lateral roots (Figure 3.4). Distribution of nodules was spread on the lateral roots (Figure 3.4) for Kei Mouth morphotype in all soil types. Generally, *V. karroo* morphotypes nodulated well in all four test soils (Table 3.3).

Figure 3.3 Nodules of *Vachellia karroo* attached to the tap root

Figure 3.4 Nodules of *Vachellia karroo* spread on the lateral root system

Figure 3.5 Nodules of *Vachellia karroo* forming a cluster in the lateral root system

Branched nodules (Figure 3.6) were observed in *V. karroo* morphotypes alongside with those that were elongate (rod shaped) (Figure 3.7) and round shaped (Figure 3.8). All these shapes of nodules were obtained with all the soil types except the soil from Richards Bay and Tshwane which did not have elongate shaped nodules (Table 3.3). Variation was observed in the shapes of nodules of the different morphotypes. The morphotype from Kei Mouth had all the three shapes of nodule. The morphotypes from Richards Bay and Leeu-Gamka had nodules with all the shapes, but not rod-shaped. While the Tshwane morphotype possessed only the globule shaped nodules. Interesting, both Richards Bay soil and morphotype from Richards bay were not associated with elongated rod-shaped nodules. The frequency ratio of the shapes was 9:5:2 representing globule, branched and elongated rod shaped nodules, respectively. With the exception of Leeu-Gamka morphotypes which had few small nodules in the Leeu-Gamka soil, in the other cases, the morphotypes nodulated well in their soils of origin. All the *V. karroo* morphotypes had both small and medium sized nodules. In addition, Kei Mouth and Richards Bay morphotypes had large branched nodules in Richards Bay and Kei Mouth soils, respectively. When grown in Leeu Gamka and Tshwane soils, all morphotypes did not produce large nodules.

Table 3.3: Nodule shape, size and their attachment on the root system of four *V. karroo* morphotypes growing in four soil samples from different areas

Soil source	V. karroo Morphotype	Dominant nodule shapes	Nodule size	Attachment on the root system
Kei Mouth	Kei Mouth	Elongated rods	Medium	Spread on the lateral roots
Kei Mouth	Richards Bay	Branched	Large	Spread on the lateral roots
Kei Mouth	Leeu-Gamka	Globule	Small	Spread on the lateral roots
Kei Mouth	Tshwane	Globule	Small	Spread on the lateral roots
Richards Bay	Kei Mouth	Branched	Large	Attached on the tap root
Richards Bay	Richards Bay	Globule	Medium	Spread on the lateral roots
Richards Bay	Leeu-Gamka	Branched	Medium	Spread on the lateral roots
Richards Bay	Tshwane	Globule	Small	Spread on the lateral roots
Leeu-Gamka	Kei Mouth	Elongated rods	Medium	Attached on the tap root
Leeu-Gamka	Richards Bay	Branched	Medium	Spread on the lateral roots
Leeu-Gamka	Leeu-Gamka	Globule	Small	Few and spread on the lateral roots
Leeu-Gamka	Tshwane	Globule	Small	Few and spread on the lateral roots
Tshwane	Kei Mouth	Globule	Small	Few and spread on the lateral roots
Tshwane	Richards Bay	Globule	Small	Spread on the lateral roots
Tshwane	Leeu-Gamka	Branched	Medium	Forms a few clusters in the lateral roots
Tshwane	Tshwane	Globule	Medium	Forms a few clusters in the lateral roots

(a)

(b)

Figure 3.6 (a and b). Branched nodules of *Vachellia karroo*

Figure 3.7 *Vachellia karroo* nodules in the shape of elongated rods

(a)

(b)

Figure 3.8 (a and b). Globule shaped *Vachellia karroo* nodules

3.3.2 Confirmation of rhizobia among the isolates extracted

The rhizobia appeared red after staining, meaning that they lost the violet colour of the first stain and retained the red colour of the counter stain, thus confirming them to be Gram negative (Table 3.4). Further, they did not absorb the CR dye and maintained a distinct whitish colour as is expected of rhizobia. Non-rhizobial bacteria were Gram positive. Some which were gram negative absorbed the dye and formed red colonies thus disqualifying them from being rhizobia (Somasegaran and Hoben, 1985). Two rhizobia colonies were then selected from each soil and *V. karroo* morphotype. Thus given that we have four morphotypes and four soil types the selected colonies to be used in the further studies amounted to 32.

3.3.3 Behavior in BTB, GT and colony morphology of the 32 selected rhizobia isolates

In reaction with bromothymol blue (BTB), 28 of the isolates turned yellow while only four of them produced a blue colour. All of the later four colonies that produced a blue reaction were from Tshwane soil, but two of the colonies were from Tshwane morphotype nodules and the other two from Leeu-Gamka morphotype nodules (Table 3.4).

Of the 32 isolates tested, 87.5% were fast growing with a generation time (30 min < GT < 4 h) and 12.5% were slow growers (4h < GT < 9 h). Tshwane soil had an equal number of fast and slow growing rhizobia. All other soils had only fast growing rhizobia (Table 3.5). Of the four morphotypes, two of them (Kei Mouth and Richards Bay) had only the fast growing rhizobia , whilst both Leeu-Gamka and Tshwane had fast and slow growing rhizobia in the ration of 75%:25% respectively (Table 3.6).

All the colonies also appeared round and raised, mucoid and shiny. However, all the slow growing rhizobia exhibited small colonies with 75% being 1 mm or less in diameter on the YEMA after five days of incubation. In comparison, the fast growing colonies had diameters of more than 1 mm, with some diameters being as large as 8 mm (Table 3.4).

Table 3.4: Colony cultural characteristics of rhizobia extracted from four provenances of *V. karroo* grown in soils from Kei Mouth, Richards Bay, Leeu-Gamka, and Tshwane after 5 days of incubation in yeast mannitol agar

Isolate	Colony Diameter in mm	Bromothymol blue	Mean generation time in hours
KSK 1	5.0	Yellow	1.0
KSK 2	8.0	Yellow	1.1
KSRK 1	7.0	Yellow	1.0
KSRK 2	6.0	Yellow	2.0
KSTK 1	5.0	Yellow	1.0
KSTK 2	3.0	Yellow	2.0
KSLK 1	1.0	Yellow	4.0
KSLK 2	7.0	Yellow	2.0
RSK 1	3.0	Yellow	2.0
RSK 2	8.0	Yellow	1.0
RSKK 1	1.0	Yellow	1.0
RSKK 2	9.0	Yellow	1.0
RSLK 1	0.5	Yellow	4.0
RSLK 2	2.0	Yellow	2.0
RSTK 1	1.0	Yellow	2.0
RSTK 2	1.0	Yellow	1.0
LSK 1	5.0	Yellow	1.0
LSK 2	5.0	Yellow	3.0
LSKK 1	4.0	Yellow	1.0
LSKK 2	4.0	Yellow	2.5
LSRK 1	3.0	Yellow	1.5
LSRK 2	1.0	Yellow	0.5
LSTK 1	2.0	Yellow	0.5
LSTK 2	7.0	Yellow	1.0
TSK 1	0.5	Blue	7.0
TSK 2	0.5	Blue	6.0
TSKK 1	4.0	Yellow	3.0
TSKK 2	1.0	Yellow	2.5
TSRK 1	5.0	Yellow	2.0
TSRK 2	4.0	Yellow	2.5
TSLK 1	1.5	Blue	8.0
TSLK 2	1.0	Blue	8.0

Rhizobia isolates: KSK, Kei Mouth soil and karroo; KSRK, Kei Mouth soil and Richards Bay karroo; KSLK, Kei Mouth soil and Leeu-Gamka karroo; KSTS, Kei Mouth soil and Tshwane karroo; RSK, Richards Bay soil and karroo; RSKK, Richards Bay soil and Kei Mouth karroo; RSLK, Richards Bay and Leeu-Gamka karroo; RSTK, Richards Bay soil and Tshwane karroo; LSK, Leeu-Gamka soil and karroo; LSKK, Leeu-Gamka soil and Kei Mouth karroo; LSRK, Leeu-Gamka soil and Richards Bay karroo; LSTK, Leeu-Gamka soil and Tshwane karroo; TSK, Tshwane soil and karroo; TSKK, Tshwane soil and Kei Mouth karroo; TSRK, Tshwane soil and Richards Bay karroo; TSLK, Tshwane soil and Leeu-Gamka karroo

Table 3.5: Percentage of fast and slow rhizobia isolated from Kei Mouth, Richards Bay, Leeu-Gamka, Tshwane using V. *karroo* trap plants

Soil source	%fast growing	%slow growing
Kei Mouth	100	0
Richards Bay	100	0
Leeu-Gamka	100	0
Tshwane	50	50

Table 3.6: Percentage of fast and slow rhizobia extracted from Kei Mouth, Richards Bay, Leeu-Gamka and Tshwane *V. karroo* morphotypes

V. karroo morphotype	% fast growing	%slow growing
Kei Mouth	100	0
Richards Bay	100	0
Leeu-Gamka	75	25
Tshwane	75	25

3.4 Discussion

The Gram negative bacteria and the inability to absorb CR dye confirmed that the 32 isolates extracted from the trap plants were indeed Rhizobia (Somasegaran and Hoben, 1994). Round nodules were observed in *V. karroo* unlike in other *Acacia* species which do not have round nodules (Somasegaran and Hoben, 1994). Nodulation of some *Acacia* species of the same family as *V. karroo* normally occurs along the lateral root system (Allen and Allen, 1936). In the present study, the *V. karroo* isolates also showed preference to the lateral roots with 87.5% forming nodules around the lateral root system.

The shapes of the isolated root nodules also corresponds with the shapes of nodules isolated from other *Acacia* species (Corby, 1971) giving an impression that they are all nodulated by related rhizobia species. Variation of nodule shape was also noted amongst the morphotypes, implying that the different morphotypes have different preferences of rhizobia. However, the variation may also imply that the same rhizobium may produce nodules of different shapes among the morphotypes. In that case, the nodule shape may depend on the type of morphotype. The variation in nodule morhology which was observed in *V. karroo* morphotypes is a characteristic usually observed in different cultivars of a plant (Leite *et al*., 2009). The morphological diversity noted in *Vachellia* rhizobia isolates may also denote a

diversity of isolates in the soil. This wide diversity in morphological characteristics has also been observed in rhizobia isolated from other soils (Jida and Assefa, 2014).

Acacia trees are known to be nodulated by fast and slow growing rhizobia (Dreyfus and Dommergues, 1981). The present study has also provided evidence that like other *Vachellia* species *V. karroo* is nodulated by both the fast growing rhizobia (*Rhizobium species)* and the slow growing rhizobia (*Bradyrhizobium species*) (Cooper et al., 1985).

The distribution of fast and slow growing rhizobia was not correlated with soil type (Table 3.5) or soil chemical characteristics. Generally slow growing rhizobia are more tolerant of acidic soils than fast growing rhizobia (Rao, 1991), and hence expected to get slow growing isolates from acid soil and vice-versa for alkaline soils. Indeed slow growing rhizobia were only isolated from Tshwane soils, which were acidic. However, despite the acidity of Kei Mouth soil, the rhizobia isolated from this soil was only fast growing. Also, 75% of the isolates from Tshwane soil were fast growing despite the acidic nature of the soil.

Nevertheless, the isolates extracted from plants grown in the alkaline soils from Richards Bay and Leeu-Gamka were all fast growing as expected. Overall, these results suggest that there is no behavioural consistency of fast and slow growing rhizobium in relation to their habitation of acid or alkaline soils (Maâtallah *et al*., 2002). It does, however, seem that fast growing rhizobia are more adaptive to soil pH than slow moving rhizobia, since they were found in both acid and alkaline soils. The *V. karroo* morphotypes showed preference to fast or slow growing rhizobia was similar to that of exhibited by the soils of their origin (Table 3.6). However Leeu-Gamka *V. karroo* morphotype which was nodulated by slow growing rhizobia when grown in Tshwane soil.

3.5 Conclusion

The observations obtained from the study bring to a conclusion that nodule morphology is influenced more by the trap plant than the soil in which the plant is growing. It can also be noted that as much as *V. karroo* is nodulated by both *Rhizobium spp.* and *Bradyrhizobium spp.* it is mostly affiliated with the fast growing species of *Rhizobium*. The fast growing rhizobia also exhibit a high ability of adapting to acidic conditions which are usually considered not ideal for rhizobial growth. Different morphotypes of *V. karroo* appeared to be generally nodulated by morphologically similar rhizobia.

CHAPTER 4

Physiological and biochemical characteristics of rhizobia isolates in relation to soil source, the host morphotype and cross nodulation

4.1 Introduction

Rhizobia isolates are subjected to various *in vitro* stressful conditions under different environmental conditions in various geographical regions and ecosystems (Rodrigues *et al*., 2006). However, some rhizobia isolates show no link between their behaviour and their adaptation to various geographic origins and host plants (Boukhatem *et al*., 2012). This is probably because most rhizobia can easily adapt to a wide variety of soil environmental conditions (Janczarek *et al*., 2010). Nevertheless, the growth of rhizobia isolates in different conditions can be used in the selection of strains best adapted to diverse edapho-climatic conditions (Zerhari *et al*., 2000). Furthermore, the response of rhizobia strains to different carbon sources, salinity and ability to grow in diverse temperature values is useful in taxonomic classification of the strains (Zerhari *et al*., 2000).

Soil persistence is an essential attribute of effective rhizobia. The major factors that affect the growth and survival of rhizobia, hence persistence, in the soil are soil temperature and soil pH. Thus, the tolerance of rhizobia to stressful levels of these factors will give an indication of the persistence of the rhizobia in a soil (Mohammadi *et al*., 2012).

Temperature fluctuations in the soil are common (Boonkerd and Weaver, 1982), and they are mainly caused by climate change (Gopalakrishnan *et al*., 2014). Ability of rhizobia to survive in various temperature conditions is therefore an essential component of effective symbiosis. The optimum temperature range for the growth of most rhizobia strains is usually from 25 to 31°C (Somasegaran and Hoben, 1994), below and above which some fail to survive. High temperatures reduce the survival and persistence of rhizobia population available for nitrogen fixation by causing death of some (Al-Falih, 2002). Low temperatures also reduce rhizobia activity (Maheshwari, 2010). However, a reasonably number of isolates tolerant to cold and high temperatures have also been isolated from various soils (Prévost *et al*., 2003, Bansal *et al*., 2014). Generally, rhizobia isolated from geographical areas of high temperatures are

usually tolerant to high temperatures (Eaglesham and Ayanaba, 1984, Rodrigues *et al*., 2006). Nevertheless, tolerance of rhizobia to extreme temperatures also depends on the host plant (Nandal *et al*., 2005).

Acidic soil conditions are a major constraint to rhizobium-legume symbiosis (Al-Falih, 2002) as they reduce nodulation (Muthini *et al*., 2013). Optimum pH for the growth of rhizobia ranges from 6-8 (Brenner *et al*., 2005). Extreme high and low pH values negatively affect rhizobia survival (Rinaudi *et al*., 2006). The reduction in the rhizobia population eventually leads to reduced nitrogen fixation efficiency (Al-Falih, 2002). Previous studies have found correlation between growth of rhizobia in low pH with the acidic conditions of soils of their origin (Brígido, 2012). Thus, there is need to identify rhizobia tolerant to extreme pH values in order to achieve nitrogen efficiency in legume crops growing in acidic or alkaline soils. This has been achieved in other *Acacia* species (Mohamed *et al*., 2000) and other legume plants (Frioni *et al*., 2001) where rhizobia tolerant of low pH have been isolated. In the present study, isolates of rhizobia which were obtained through trap plants growing in soil collected from sites that differed considerably in temperature regimes and soil pH were tested for their tolerance to stress pH and temperature levels.

Salinity is one of the major stresses in crop production worldwide (Ladeiro, 2012). Saline soils usually have low nutrient levels which makes nitrogen fixation very essential in these areas (Lippi *et al*., 2000). Thus, identification of native rhizobia tolerant of saline condition is important for the improvement of nitrogen fixation. Zahran(1999) have shown that saline conditions inhibit the multiplication and rate of colonisation of Rhizobia. Nevertheless, indigenous *Rhizobia* which are tolerant to high salt conditions have been isolated from *Vachellia* trees (Zahran, 2001). Rhizobia from saline soils are expected to be adapted to their soil conditions and thus should be tolerant of high salt levels. However, some rhizobia isolates from non-saline soils have been found to tolerate saline conditions (Liu, 2008).

Different rhizobia strains vary in their resistance to antibiotics, thus making antibiotic resistance an essential tool in determining rhizobia diversity (Somasegaran and Hoben, 1994; Obaton, 1971). Information on the resistance of rhizobia to antibiotics is also used as a basis to determine rhizobia survival in the soil, invasiveness and its ability to form effective symbiosis with host plant (Milicic *et al*., 2006). The use of antibiotic resistance as a marker technique in rhizobia is inexpensive and uses simple equipment as compared to other marker

techniques (Somasegaran and Hoben, 2012). It has been shown that the ability of rhizobia to be resistant to certain antibiotics is influenced by the climatic and soil conditions of its place of origin (Alexander *et al*., 2006; Xavier *et al*., 1998).

Heavy metals are always present in all soils, but their concentrations differ from soil to soil as influenced by soil pH, iron and aluminium oxide content, clay content, organic matter and cation exchange capacity (Hernandez *et al*., 2003). Some heavy metals like aluminium have been found to inhibit rhizobial growth even at very low concentrations (Paudyal *et al*., 2007). Other metals such as Fe and Mo have also inhibited growth of Rhizobia at medium and high concentrations, respectively (Paudyal *et al*., 2007). The toxicity of heavy metals such as Al, Mn and Fe is further aggravated by soil acidic conditions which increase the solubility of these metals (Al-Faliah, 2002). Thus, the ability of rhizobia to be resistant to heavy metals helps them to survive in heavy metal contaminated soils (Satyanarayana and Johri, 2005). The ability of rhizobia to be resistant to heavy metals improves its population in the soil and symbiotic effectiveness as these are adversely affected by heavy metals (Vasilica *et al*., 2011). Some of the rhizobia tolerant to heavy metals have been isolated from some soils (Abd-Alla *et al*., 2012). Thus screening rhizobia for our legume crops using this criterion will greatly improve the efficiency of nitrogen fixation.

Carbon sources used by rhizobia range from all sugars (e.g. glucose, fructose, sucrose, lactose, maltose), sugar alcohols (e.g. M-inositol, mannitol) and organic acids (e.g. malic acid) (Stowers, 1985). Carbon source utilisation by rhizobia has been known to be influenced by conditions of soil of origin (Andrade *et al*., 2002). The ability to utilise a variety of carbon sources indicates the capacity of rhizobia to compete with other soil microorganisms for nutrients (Germida, 1988) and maintain a high population for effective nodulation. Thus, the ability to utilise a variety of carbon sources gives an insight into rhizobia's competitive ability and this has been an important aspect in the classification of rhizobia (Mirza *et al*., 2007).

The objective of this study was to characterise the physiological and biochemical characteristics of isolates extracted from different soils using different *V. karroo* morphotypes as trap plants. The physiological characteristics tested were effect of temperature, pH, sodium chloride, antibiotics and heavy metals on the growth of the isolates. In addition the ability to assimilate different carbon sources was also tested.

Information on the ability of rhizobia to nodulate different host is very essential in the description of new rhizobia species (Graham *et al*., 1991). The promiscuity and or specificity of rhizobia is believed to be helpful in the revision of legume taxonomy (Gaur and Sen, 1979). It has also been used as a basis for selecting and developing strains for inoculation (Ahmad, 1996). Legume species occupying a vast range of habitats have been found to be promiscuous in their nodulation by rhizobia (Sharma *et al*., 2005). Furthermore, research by other scientists has revealed both promiscuity and specificity relationship of tree legumes and rhizobia nodulating them (Herrera *et al*., 1985).

Little knowledge is known about the promiscuity and or specificity of *V. karroo* and the rhizobia nodulating them. The ability of *V. karroo* morphotypes to form symbiosis with a wide host range of rhizobia and those nodulating other species will increase its ability to colonize new habitats (Rodríguez-Echeverría *et al*., 2003). The aim of this study was to determine the ability of *V. karroo* rhizobia to nodulate with different *Vachellia* species and the ability of *V. karroo* to be nodulated by rhizobia nodulating other *Vachellia* species.

The hypotheses tested were:-

i) Tolerance of rhizobia isolates to temperature and pH is correlated with conditions of their geographic origin and the host morphotype they nodulate.
ii) Rhizobia strains from different soils have a preference to different salt levels.
iii) Rhizobia strains isolated from different morphotypes show preference to different salt levels.
iv) Rhizobia isolated from different soils and morphotypes differ in their resistance to antibiotics.
v) Heavy metal resistance in rhizobia is correlated with soil of origin and varies in different host plants.
vi) Ability of rhizobia to use different carbon sources is correlated with soil of origin and varies with host plant.
vii) Rhizobia from different V. karroo morphotypes cross nodulate among the morphotypes and with other Vachellia species.
viii) Morphotypes of V. karroo nodulate with rhizobia extracted from V. nilotica and V. tortilis.

4.2 Materials and Methods

4.2.1 Effect of incubation temperature

To determine the ability of the isolates to grow in different temperatures, YEMA was prepared (Table 3.2) and poured into sterile petri dishes after autoclaving. Rhizobia inoculum was collected from agar slants using a 1mm inoculation loop. The agar plates were inoculated by the loop using the streak plate method (Perdomo *et al*., 1995) under aseptic conditions. The agar plates were then incubated at different temperatures of 5, 15, 25, 30, 40 and 45°C (Lupwayi and Haque, 1994) for 5 days. Growth was then recorded qualitatively (+) for growth and (-) for no growth.

4.2.2 Assessment of tolerance to low and high pH

To determine the effect of pH on rhizobia, the pH of YEMA was adjusted to 3, 4, 5, 6, 7, 8 and 9 using 0.5 M stock solution of NaOH or HCl. The media was then autoclaved and poured into agar plates for cooling. Rhizobia were grown separately in YEMB for three days, after which a serial dilution of the bacterial population was made to a concentration of 10^3 colony forming units per millilitre (cfu/mL). The agar plates with different pH concentrations were then inoculated with 0.1 ml of the rhizobia concentration using the spread plate method. The agar plates were incubated at 28°C for five days and then assessed for rhizobial growth. The bacteria were also grown in agar plates with pH 7, and these were used as control. The experiment was replicated thrice for each pH value. Growth was recorded qualitatively, (+) for growth and (-) for no growth.

4.2.3 Effect of sodium chloride concentration on Rhizobia growth

Conical flasks with 70 ml YEMB were prepared and inoculated separately with the 32 rhizobia isolates using an inoculation loop. These were incubated at 28°C in a rotary shaker for five days, after which serial dilution of rhizobia growing in the broth was done under sterile conditions.

For the serial dilution 10 ml test tubes were filled with 9 ml of sterile distilled water. One mm quantity of the bacterial suspension was added to the first tube. The tube was vortexed to mix the contents after which 1 ml of the mixture was extracted and added to the second test tube with 9 ml distilled water. The test tube was vortexed and 1 ml of the mixture added to the third test tube. After vortexing the third test tube the final bacterial concentration was 10^3cfu/mL. One ml of the final bacterial concentration was then used to inoculate 70 ml

conical flasks containing YEMB which was altered to different levels of NaCl. The different levels of salt were achieved by adding NaCl to get 0.005; 0.010; 0.050; 0.100 and 0.200 M NaCl. After adjusting to the desired NaCl concentrations, the YEMB was autoclaved before inoculation. The inoculated flasks were incubated in a rotary shaker for three days, after which growth was assessed by measuring absorbance using a spectrophotometer at 600 nm wavelength.

4.2.4 Tolerance to antibiotics

Yeast mannitol agar plates were used in the testing for intrinsic antibiotic resistance of the 32 isolates from the four *V. karroo* morphotypes. The YEMA was prepared to a volume of 600 ml for each of the antibiotics tested. The agar was autoclaved and maintained at 40°C in a hot water bath. The antibiotics are denatured by high heat thus they were separately filter sterilized using Millipore filter membranes with a pore size of 0.22 µm. The pore size used is known to exclude smallest bacteria which might be found in the mixture. Filtering was done under aseptic conditions (Dart, 1996).

Following filter sterilisation the antibiotics were each added separately to 600 ml of YEMA maintained at a temperature of 40°C. The following antibiotics and their concentrations in the YEMA were used: ampillicin (100 µg/mL), neomycin (10 µg/mL), ciprofloxacin (30 µg/mL), chloramphenicol (25 µg/mL) and gentamycin (50 µg/mL). The antibiotics were added into YEMA and mixed together by thorough shaking. The YEMA containing the antibiotic was then poured into petri dishes and allowed to solidify. A sterile inoculation loop was used to collect the rhizobia from the preserving agar slants, and then streaked onto the solidified. Growth was scored qualitatively after five days of incubation of the inoculated plates at 28°C.

4.2.5 Tolerance to heavy metals

Eight heavy metals were each first filter sterilised as they are damaged by high heat. The filter membranes used were those used in the filter sterilisation of antibiotics, Section 4.2.4. The heavy metals were each added to desired concentration to a litre of sterilised YEMA maintained at 40°C in a hot water bath. The heavy metals and the concentration tested were (µg/mL): $ZnCl_2$ (50), $CoCl_2$ (25), $CuCl_2.2H_2O$ (100), $AlCl_3$ (250), $MnCl_2$ (500), $NiSO_4$ (100)). The agar was mixed with the heavy metals by shaking vigorously, and then poured into petri dishes and allowed to solidify. Each of the 32 isolates were then inoculated separately on the solidified agar plates by transferring to the agar 0.5 µL of the rhizobia

isolates which had been growing in YEMB for three days. The plates were incubated at 28°C for five days, after which qualitative growth was assessed and recorded.

4.2.6 Utilisation of carbon source

For testing the ability of the isolates to utilise carbon sources, a carbohydrate free medium similar to YEMA was used with the exception that the amount of yeast extract was modified to 5 mg/L. The medium was first autoclaved and maintained at a temperature of 40°C in a hot water bath. The carbon sources were first mixed with water to a concentration of 10% (w/v). The solutions were then sterilised by filtering using a Millipore filter membrane of 0.22 μm pore size. The carbon sources used in this study were glucose, maltose, fructose, lactose, sucrose, glycerol, M-Inositol, mannitol, malic acid.

Filter sterilisation, the carbohydrates were added to the autoclaved medium to make the desired final concentration of 1 g/L. The mixtures of the agar and solutions of the carbohydrates were mixed thoroughly by shaking vigorously. The agar was then poured into agar plates and allowed to solidify and then inoculated with 0.5μL of the rhizobia isolates which had been growing in YEMB for three days. The plates were incubated at 28°C, and growth was assessed after five days and recorded qualitatively. Procedures of this method were done using the guidelines of Somasegaran and Hoben(1994).

4.2.7 Cross nodulation

This experiment was carried out at the University of Zululand, Department of Agriculture greenhouse. Rhizobia isolates were obtained by growing trap trees in four soils from Kei Mouth, Richards Bay, Leeu-Gamka and Tshwane. The trap plants were four *V. karroo* morphotypes also from Kei Mouth, Richards Bay, Leeu-Gamka and Tshwane. The morphotypes were grown only in the soils of their origin, for example, Richards Bay morphotypes were grown in Richards Bay soil. After 90 days of growth the trap trees were uprooted and rhizobia were extracted as described in Section 3. 2. 5. Only one isolate was obtained from each morphotypes to make a total of four isolates which were used in this study. In addition, seed of *V. nilotica* and *V. tortilis* were collected from Hluhluwe (28° S 32° E) in KwaZulu-Natal (KZN). These were used to check the specificity and or promiscuity of *V. karroo* and its rhizobia. The seeds were grown in soils from the areas they were collected.

The *V. karroo* rhizobia isolates were tested to nodulate other morphotypes from which it was not extracted from by cross inoculation. Furthermore the *V. karroo* rhizobia were tested

through cross inoculation with other *Vachellia* species namely *Vachellia nilotica* and *Vachellia tortilis* for their ability to nodulate other species. In addition isolates extracted from *V. nilotica* and *V. tortilis* were cross nodulated with *V. karroo* morphotypes.

4.2.7.1 Seed inoculation with Rhizobia

The six rhizobia isolates were grown in yeast mannitol broth for five days under aseptic conditions as described in section 3.2.2.2. Seeds from each of the four *V. karroo* morphotypes (Kei Mouth, Richards Bay, Leeu-Gamka and Tshwane) and the other two from *V. nilotica* and *V. tortilis* were pricked with a needle to allow for water absorption and then surface sterilized to eliminate bacteria and break dormancy according to IDO, 2011. The surface sterilized seeds were inoculated with the rhizobia isolates in YEMB culture by coating them with the broth (Deaker et al.,2004; Erker and Brick, 2012). Each test rhizobia isolate was used to inoculate seeds of the different *V. karroo* morphotypes, *V. nilotica* and *V. tortilis.*

4.2.7.2 Plant culture

The Hygromix® was sterilized by autoclaving at 120°C for 15 minutes and then 3 kg sterile plastic pots were filled with sterilized growth medium. Five inoculated seeds of each plant were planted in the pots which were later thinned into one. The experiment was replicated thrice, the replicates being in form of three pots. The experiment was laid out in a Randomised Complete Block design. The plants were watered with deionised water and inspected for effective nodulation after 60 days. A control of all *V. karroo* morphotypes, *V. nilotica* and *V. tortilis* was also planted which had seed which were not inoculated with rhizobia

4.2.7.3 Determining the effectiveness of the rhizobia

Number of nodules was used as a measure of the effectiveness of cross nodulation. This was followed by observing the colour of the nodules by cutting them into two. A pink colour was a sign of the presence of rhizobia and the ability to fix nitrogen (Somasegaran and Hoben, 1994). The shape of the nodules was also recorded to determine if different isolates produced different shaped nodules and also to check if module shape varied with trap plant.

4.2.8 Data analysis

The quantitative data from the effect of salt concentrations on the growth of rhizobia isolates was analysed by two way ANOVA of GENSTAT version 12.0. The phenotypic characteristic were analysed by a computer cluster analysis and a dendrogram was constructed using the unweighed pair group method with the average (UPGMA) clustering method of GENSTAT version 12.

4.3 Results

4.3.1 Effect of temperature on the growth of rhizobia isolates

All *Rhizobia* isolates from the *V. karroo* morphotypes grew in temperatures ranging from 15°C to 30°C (Table 4.1). Almost all isolates (87.5%) grew at 40°C, except LSK2; TSK1; TSK2 and TSLK1. Only six isolates: KSLK2; RSK1; RSLK2; LSTK1; TSK1 and TSK2, were able to grow at the lowest temperature of 5°C. However, only KSK2; KSRK1; KSRK2; RSK1; RSK2; RSKK1; RSTK1; RSTK2 and TSRK2 isolates could grow at 45°C where the majority were extracted from Richards Bay soil. All isolates from Leeu-Gamka soil did not grow at 45°C. Only *Rhizobium* isolate from Richards Bay and Karroo (RSK1) could grow at temperature range of 5-45°C.

It is also interesting to note that rhizobia which formed symbiosis with the *V. karroo* morphotype from Leeu-Gamka in all the soil types also did not grow at the temperature of 45°C. All isolates extracted from the nodules of Kei Mouth *V. karroo* morphotype, irrespective of soil origin, did not grow at 5°C. There was at least one isolate tolerating a given temperature from the isolates extracted from Richards Bay and Tshwane *V. karroo* morphotypes.

4.3.2 Effects of pH on the growth of rhizobia isolates

Almost all rhizobia isolates grew at pH range of 6-8, except for RSKK1; LSTK1 and TSLK2 isolates whose growth was only limited to a pH range of 6-7 (Table 4.1). The KSRK2; LSRK1; LSRK2; TSK1; TSK2 and TSRK1 isolates grew at a pH range of 4-9, while growth of TSLK1 ranged from pH 4 to pH 8. None of the isolates grew at pH 3, and only 22% and 38% grew at pH 4 and 5, respectively. None of the isolates extracted from Richards Bay soil grew at low pH of 3-5. However the Richards Bay morphotype formed symbiosis with rhizobia isolates which tolerated pH 4-5 when grown in Kei Mouth, Leeu Gamka and

Tshwane soils. The remaining soils and morphotypes had at least one isolate tolerating pH 4-9.

Table 4.1: Effects of temperature and pH on the growth of 32 rhizobia isolates extracted from Kei Mouth, Richards Bay, Leeu-Gamka and Tshwane soil using four *V. karroo* morphotypes from the same areas

Isolate	Incubation temperature (°C)						pH						
	5	15	25	30	40	45	3	4	5	6	7	8	9
KSK 1	-	+	+	+	+	-	-	-	+	+	+	+	+
KSK 2	-	+	+	+	+	+	-	-	+	+	+	+	+
KSRK 1	-	+	+	+	+	+	-	-	-	+	+	+	+
KSRK 2	-	+	+	+	+	+	-	+	+	+	+	+	+
KSTK 1	-	+	+	+	+	-	-	-	+	+	+	+	+
KSTK 2	-	+	+	+	+	-	-	-	-	+	+	+	+
KSLK 1	-	+	+	+	+	-	-	-	-	+	+	+	-
KSLK 2	+	+	+	+	+	-	-	-	-	+	+	+	+
RSK 1	+	+	+	+	+	+	-	-	-	+	+	+	+
RSK 2	-	+	+	+	+	+	-	-	-	+	+	+	-
RSKK 1	-	+	+	+	+	+	-	-	-	+	+	-	-
RSKK 2	-	+	+	+	+	-	-	-	-	+	+	+	-
RSLK 1	-	+	+	+	+	-	-	-	-	+	+	+	-
RSLK 2	+	+	+	+	+	-	-	-	-	+	+	+	+
RSTK 1	-	+	+	+	+	+	-	-	-	+	+	+	+
RSTK 2	-	+	+	+	+	+	-	-	-	+	+	+	+
LSK 1	-	+	+	+	+	-	-	-	-	+	+	+	-
LSK 2	-	+	+	+	-	-	-	-	-	+	+	+	-
LSKK 1	-	+	+	+	+	-	-	-	-	+	+	+	-
LSKK 2	-	+	+	+	+	-	-	-	+	+	+	+	-
LSRK 1	-	+	+	+	+	-	-	+	+	+	+	+	+
LSRK 2	-	+	+	+	+	-	-	+	+	+	+	+	+
LSTK 1	+	+	+	+	+	-	-	-	-	+	+	-	-
LSTK 2	-	+	+	+	+	-	-	-	+	+	+	+	+
TSK 1	+	+	+	+	-	-	-	+	+	+	+	+	+
TSK 2	+	+	+	+	-	-	-	+	+	+	+	+	+
TSKK 1	-	+	+	+	+	-	-	-	-	+	+	+	+
TSKK 2	-	+	+	+	+	-	-	-	-	+	+	+	-
TSRK 1	-	+	+	+	+	-	-	+	+	+	+	+	+
TSRK 2	-	+	+	+	+	+	-	-	-	+	+	+	+
TSLK 1	-	+	+	+	-	-	-	+	+	+	+	+	-
TSLK 2	-	+	+	+	+	-	-	-	-	+	+	-	-
% of (+ve) isolates	18.75	100	100	100	87.5	28.13	0	22	38	100	100	91	59

Rhizobia isolates: KSK, Kei Mouth soil and karroo; KSRK, Kei Mouth soil and Richards Bay karroo; KSLK, Kei Mouth soil and Leeu-Gamka karroo; KSTS, Kei Mouth soil and Tshwane karroo; RSK, Richards Bay soil and karroo; RSKK, Richards Bay soil and Kei Mouth karroo; RSLK, Richards Bay and Leeu-Gamka karroo; RSTK, Richards Bay soil and Tshwane karroo; LSK, Leeu-Gamka soil and karroo; LSKK, Leeu-Gamka soil and Kei Mouth karroo; LSRK, Leeu-Gamka soil and Richards Bay karroo; LSTK, Leeu-Gamka soil and Tshwane karroo; TSK, Tshwane soil and karroo; TSKK, Tshwane soil and Kei Mouth karroo; TSRK, Tshwane soil and Richards Bay karroo; TSLK, Tshwane soil and Leeu-Gamka karroo

4.3.3 Effect of salt concentration on the growth of rhizobia isolates

The 32 rhizobia isolates showed significant difference ($p<0.05$) in their growth in different salt concentrations (0.005, 0.01, 0.02, 0.05, 0.1, 0.2 M of NaCl) (Table 4.2). All the isolates grew in all concentrations, but exhibited marked variations in the rate of growth among the concentrations tested. Preference to the different salt concentrations was indicated by the highest growth recorded amongst all the concentrations. None of the isolates showed high growth in the media without NaCl. Both slow and fast growing rhizobia were represented in the preferences for low (0.005 M) and high (0.2 M) concentrations of salt.

A total of 31.25% of the isolates showed preference to low salt between 0.005 and 0.01 M NaCl. A slightly larger proportion of 34.38% had their maximum growth recorded in medium salt concentrations of 0.02 and 0.05 M of NaCl. Also, the same proportion (34.38%) had maximal growth at high salt concentrations of 0.1 and 0.2 M NaCl. The isolates preference to different salt concentrations was not related to conditions of the soil of their origin and host. Acidic soils of Kei Mouth and Tshwane which have low salt content had isolates extracted from them which exhibited high growth in high salt concentration. The reverse was true for Richards bay and Leeu-Gamka soils, which despite having high salt concentration had isolates preferring low salt concentration. Thus, the isolates' growth behaviour did not reflect any adaptation to their soil conditions.

The rhizobial growth showed significant difference ($p<0.05$) in relation to the soil source of the isolates (Table 4.3). Likewise the origin of the *V. karroo* morphotypes used as a trap tree significantly ($p<0.05$) influenced the rhizobial growth (Table 4.4). The isolates preference to different salt concentrations was not related to soils of their origin and host morphotype. However the mean growth for isolates obtained from both Kei Mouth soil and Kei Mouth morphotype was highest under 0.005 M NaCl. Similarly the mean growth of rhizobia isolates from Richards Bay soil and trap morphotype was highest under 0.02 M NaCl (Table 4.3 and 4.4).

Table 4.2: The effect of different salinity levels on the growth of 32 rhizobia isolates extracted from four provenances of V. karroo grown in soil samples from Kei Mouth, Richards Bay, Leeu-Gamka, and Tshwane

Rhizobia Isolate	Salinity treatment							Mean growth
	0M	0.005M	0.01M	0.02M	0.05M	0.1M	0.2M	
KSK 1	1.19	2.14	1.86	1.77	1.71	1.53	1.28	1.64
KSK 2	1.36	1.87	1.44	1.36	1.32	1.27	1.15	1.40
KSRK 1	1.04	1.15	1.17	1.19	1.21	1.23	1.14	1.16
KSRK 2	1.54	1.80	1.48	1.42	1.35	1.28	0.98	1.55
KSLK 1	1.29	1.35	1.59	1.82	1.65	1.51	1.34	1.51
KSLK 2	1.28	1.35	1.36	1.36	1.44	1.41	1.39	1.37
KSTK 1	1.12	1.17	1.20	1.22	1.26	1.24	1.15	1.20
KSTK 2	1.19	1.29	1.31	1.32	1.34	1.75	1.33	1.36
RSK 1	1.19	1.38	1.41	1.73	1.39	1.32	1.32	1.39
RSK 2	1.59	1.74	1.73	1.70	1.63	1.64	1.44	1.64
RSKK 1	1.11	1.25	1.27	1.37	1.14	1.14	1.03	1.19
RSKK 2	1.37	1.72	1.69	1.61	1.47	1.47	1.43	1.54
RSLK 1	1.08	1.15	1.26	1.34	1.42	1.68	1.83	1.40
RSLK 2	0.91	1.02	1.13	1.15	1.26	1.13	0.89	1.07
RSTK 1	1.05	1.15	1.17	1.31	1.40	1.34	1.27	1.24
RSTK 2	1.09	1.20	1.26	1.34	1.41	1.35	1.27	1.27
LSK 1	1.10	1.38	1.40	1.42	1.31	1.20	1.04	1.26
LSK 2	1.07	1.22	1.20	1.24	1.24	1.27	1.21	1.21
LSKK 1	1.50	1.56	1.60	1.61	1.71	1.59	1.47	1.58
LSKK 2	1.60	1.67	1.61	1.63	1.64	1.67	1.58	1.63
LSRK 1	1.46	1.58	1.68	1.53	1.50	1.45	1.22	1.49
LSRK 2	1.01	1.12	1.30	1.22	1.12	1.10	1.09	1.14
LSTK 1	0.83	1.03	1.06	1.10	1.13	1.14	1.16	1.07
LSTK 2	1.12	1.20	1.22	1.31	1.18	1.14	1.07	1.18
TSK 1	0.92	0.94	1.20	1.05	1.20	1.24	1.27	1.12
TSK 2	0.95	1.11	1.09	1.11	1.23	1.26	1.14	1.13
TSKK 1	1.17	1.41	1.86	1.78	1.31	1.21	1.04	1.40
TSKK 2	1.11	1.15	1.27	1.28	1.33	1.41	1.15	1.24
TSRK 1	1.14	1.22	1.25	1.23	1.19	1.10	0.94	1.15
TSRK 2	1.04	1.06	1.07	1.14	1.67	1.14	1.24	1.20
TSLK 1	1.19	1.19	1.27	1.19	1.17	1.12	0.89	1.15
TSLK 2	0.99	1.10	1.11	1.18	1.33	1.45	1.02	1.17

Mean growth	1.18	1.33	1.36	1.38	1.36	1.34	1.24	1.31

Significance($Lsd_{0.05}$) : Salinity treatment : 0.004612
Rhizobia isolate: 0.009861
Salinity treatment ^{x} rhizobia isolate: 0.026091

Rhizobia isolates: KSK, Kei Mouth soil and karroo; KSRK, Kei Mouth soil and Richards Bay karroo; KSLK, Kei Mouth soil and Leeu-Gamka karroo; KSTS, Kei Mouth soil and Tshwane karroo; RSK, Richards Bay soil and karroo; RSKK, Richards Bay soil and Kei Mouth karroo; RSLK, Richards Bay and Leeu-Gamka karroo; RSTK, Richards Bay soil and Tshwane karroo; LSK, Leeu-Gamka soil and karroo; LSKK, Leeu-Gamka soil and Kei Mouth karroo; LSRK, Leeu-Gamka soil and Richards Bay karroo; LSTK, Leeu-Gamka soil and Tshwane karroo; TSK, Tshwane soil and karroo; TSKK, Tshwane soil and Kei Mouth karroo; TSRK, Tshwane soil and Richards Bay karroo; TSLK, Tshwane soil and Leeu-Gamka karroo

Table 4.3: The effect of different salinity levels and soil of origin on the growth of rhizobia isolates

Salinity treatment	Origin of soil				Mean growth
	Kei mouth	Richards bay	Leeu-Gamka	Tshwane	
0	1.25	1.17	1.21	1.06	1.18
0.005	1.52	1.33	1.35	1.15	1.33
0.01	1.43	1.37	1.39	1.26	1.36
0.02	1.43	1.44	1.38	1.25	1.38
0.05	1.41	1.39	1.35	1.30	1.36
0.1	1.40	1.39	1.32	1.24	1.34
0.2	1.35	1.32	1.23	1.09	1.24
Mean growth	1.40	1.34	1.32	1.20	1.31

Significance($Lsd_{0.05}$) : Salinity treatment : 0.05935
Soil source: 0.04487
Salinity treatment ^{x} soil source: 0.11870

Table 4.4: The effect of different salinity levels and origin of *V. karroo* trap morphotype on the growth of rhizobia isolates

Salinity treatment	Origin of *V. karroo* trap morphotype				Mean growth
	Kei mouth	Richards bay	Leeu-Gamka	Tshwane	
0	1.30	1.25	1.11	1.03	1.18
0.005	1.60	1.38	1.22	1.14	1.33
0.01	1.58	1.39	1.30	1.19	1.36
0.02	1.55	1.40	1.34	1.22	1.38
0.05	1.45	1.38	1.35	1.27	1.36
0.1	1.41	1.28	1.35	1.31	1.34
0.2	1.27	1.30	1.20	1.21	1.24
Mean growth	1.45	1.34	1.27	1.20	1.31

Significance($Lsd_{0.05}$) : Salinity treatment : 0.05538
Trap morphotype : 0.04186
Salinity treatment ^{x} trap morphotype : 0.11076

4.3.4 Tolerance to antibiotics

Of the 32 rhizobia isolates that were tested 50% were resistant to ampicillin, whilst 68.75% 84.38% were resistance to chloramphenicol and gentamycin, respectively (Figure 4.1). About 28 and 44% of the isolates were resistant to neomycin and ciprofloxacin respectively. Each soil type had at least one strain of rhizobia resistant to each of the antibiotics. Also, less than 50% of the isolates from each soil were resistant to neomycin. Rhizobia isolates from Leeu-Gamka were all resistant to gentamycin. The *V. karroo* morphotype from Leeu-Gamka was nodulated by rhizobia that were susceptible to neomycin but resistant to gentamycin. All morphotypes had equal numbers of isolates that were resistant to ampicillin (Table 4.5). Isolates LSTK1 and LSTK2 were resistant to all the antibiotics (Table 4.6).

The cluster analysis based on the intrinsic antibiotic resistance revealed five clusters. Cluster one and two comprised of isolates from all soils and morphotypes. Isolates found in cluster three comprised of isolates from Kei Mouth and Tshwane morphotypes grown in Richards Bay and Leeu-Gamka soils. Cluster four had rhizobia isolated from Richards Bay morphotype grown in Tshwane soil. Cluster five on the other hand had rhizobia from all soils and morphotypes except from Leeu-Gamka soil and morphotype (Figure 4.2).

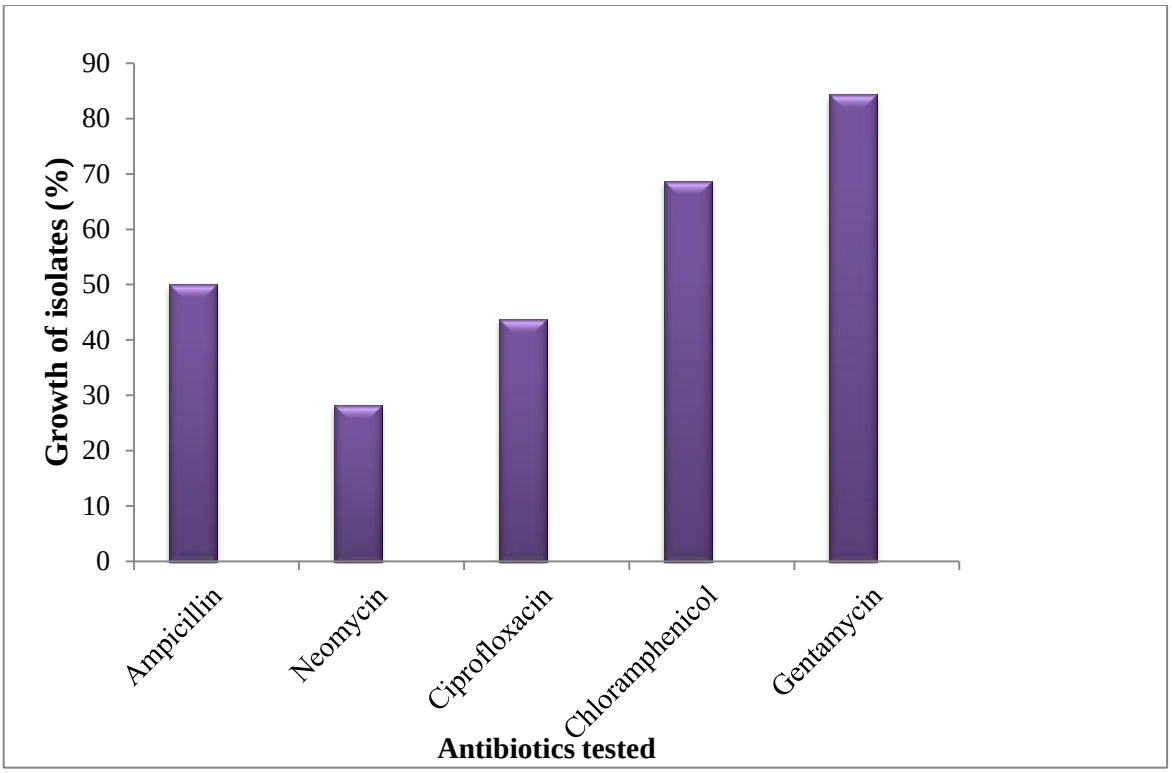

Figure 4.1: Percentage of *V. karroo* rhizobia isolates that were resistant to antibiotics used in this study (ampicillin, neomycin, ciprofloxacin, chloramphenicol and gentamycin)

Table 4.5: The percentage resistance to five antibiotics by *V. karroo* rhizobia isolates which were extracted from four *V. karroo* morphotypes grown in four soils collected from Kei Mouth, Richards Bay, Leeu-Gamka and Tshwane

Antibiotics	% of resistant isolates							
	Soil source of isolates				Host morphotype			
	Kei Mouth	Richards Bay	Leeu Gamka	Tshwane	Kei Mouth	Richards Bay	Leeu Gamka	Tshwane
Ampicillin	12.5	25.0	87.5	75.0	50.0	50.0	50.0	50.0
Neomycin	25.0	25.0	37.5	25.0	37.5	25.0	0.0	50.0
Ciprofloxacin	25.0	37.5	62.5	50.0	37.5	62.5	37.5	37.5
Chloramphenicol	50.0	75.0	87.5	62.5	62.5	75.0	75.0	62.5
Gentamycin	75.0	87.5	100.0	75.0	87.5	75.0	100.0	75.0

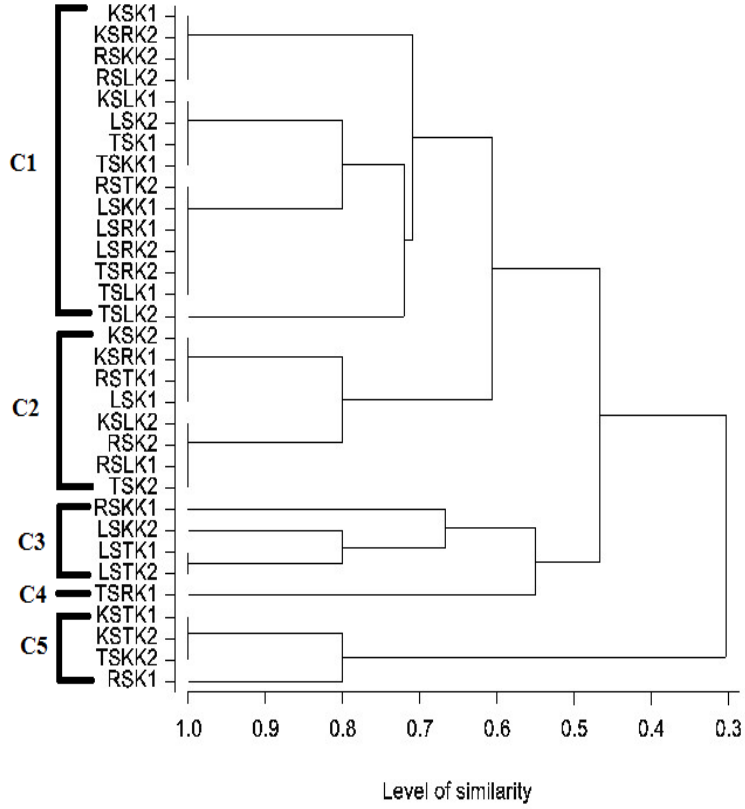

Figure 4.2: Dendrogram highlighting the relationship of rhizobia nodulating different morphotypes of *V. karroo* isolated from 4 different agro-ecological regions of South Africa based on intrinsic antibiotic resistance

Rhizobia isolates: KSK, Kei Mouth soil and karroo; KSRK, Kei Mouth soil and Richards Bay karroo; KSLK, Kei Mouth soil and Leeu-Gamka karroo; KSTS, Kei Mouth soil and Tshwane karroo; RSK, Richards Bay soil and karroo; RSKK, Richards Bay soil and Kei Mouth karroo; RSLK, Richards Bay and Leeu-Gamka karroo; RSTK, Richards Bay soil and Tshwane karroo; LSK, Leeu-Gamka soil and karroo; LSKK, Leeu-Gamka soil and Kei Mouth karroo; LSRK, Leeu-Gamka soil and Richards Bay karroo; LSTK, Leeu-Gamka soil and Tshwane karroo; TSK, Tshwane soil and karroo; TSKK, Tshwane soil and Kei Mouth karroo; TSRK, Tshwane soil and Richards Bay karroo; TSLK, Tshwane soil and Leeu-Gamka karroo

Table 4.6: Intrinsic antibiotic resistance, heavy metal resistance and Carbon source utilisation of 32 rhizobia isolates extracted from Kei Mouth, Richards Bay, Leeu-Gamka and Tshwane soils using 4 *V. karroo* morphotypes from the same areas

	Intrinsic antibiotic resistance					Heavy metal resistance						Carbon source utilisation									
Isolates	Ampicillin	Neomycin	Ciprofloxacin	Chloramphenicol	Gentamycin	Zinc	Cobalt	Copper	Aluminium	Manganese	Nickel	Glucose	Maltose	Fructose	Lactose	Sucrose	Mannitol	Glycerol	M-Inositole	Malic acid	Negative control
KSK 1	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	-
KSK 2	-	-	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	-
KSRK 1	-	-	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+
KSRK 2	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
KSTK 1	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
KSTK 2	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
KSLK 1	+	-	-	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	-	+
KSLK 2	-	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	-
RSK 1	-	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+
RSK 2	-	-	-	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+
RSKK 1	+	+	-	-	+	+	+	-	+	+	-	+	+	+	+	+	+	-	+	-	+
RSKK 2	-	-	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	-	+	-	+
RSLK 1	-	-	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+
RSLK 2	-	-	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	-	+
RSTK 1	-	-	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+
RSTK 2	+	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+
LSK 1	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
LSK 2	+	-	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+
LSKK 1	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
LSKK 2	+	+	-	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	-	+
LSRK 1	+	-	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+
LSRK 2	+	-	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	-	+
LSTK 1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
LSTK 2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
TSK 1	+	-	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	+
TSK 2	-	-	-	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	-	-	-
TSKK 1	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
TSKK 2	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
TSRK 1	+	+	+	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+
TSRK 2	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
TSLK 1	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
TSLK 2	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+

Rhizobia isolates: KSK, Kei Mouth soil and karroo; KSRK, Kei Mouth soil and Richards Bay karroo; KSLK, Kei Mouth soil and Leeu-Gamka karroo; KSTS, Kei Mouth soil and Tshwane karroo; RSK, Richards Bay soil and karroo; RSKK, Richards Bay soil and Kei Mouth karroo; RSLK, Richards Bay and Leeu-Gamka karroo; RSTK, Richards Bay soil and Tshwane karroo; LSK, Leeu-Gamka soil and karroo; LSKK, Leeu-Gamka soil and Kei Mouth karroo; LSRK, Leeu-Gamka soil and Richards Bay karroo; LSTK, Leeu-Gamka soil and Tshwane karroo; TSK, Tshwane soil and karroo; TSKK, Tshwane soil and Kei Mouth karroo; TSRK, Tshwane soil and Richards Bay karroo; TSLK, Tshwane soil and Leeu-Gamka karroo

4.3.5 Tolerance to heavy metals

About 37.5% of the 32 *V. karroo* rhizobia isolates were resistant to toxicity of all heavy metals tested (Figure 4.3). Of the 32 isolates 60, 63 and 85% were resistant to toxicity of copper, nickel and cobalt respectively. Moreover 97% of the isolates were tolerant to zinc, aluminium or manganese. Each soil and morphotype had at least one rhizobium isolate which tolerated all the heavy metals. In all soils, there were more than 50% of isolates that were resistant to toxicities of all heavy metals with the exception of the Richards Bay soil in which

25% of isolates were resistant to copper toxicity. A similar pattern was noted for the morphotypes. In almost the entire morphotypes ≥50% of the isolates extracted from them were resistant to toxicity of all heavy metals except those for Kei Mouth and Richards Bay morphotypes in which lesser proportions were resistance to copper and nickel, respectively (Table 4.11). Isolates KSK 2, KSRK 2, KSTK 1, KSTK 2, KSLK 2, LSK 1, LSTK 1, LSTK 2, TSK 1, TSKK 1, TSKK 2 and TSLK 1 exhibited resistance to toxicity of all the heavy metals within the concentration ranges tested. Other isolates were susceptible to toxicity of at least three heavy metals, Table 4.5.

A dendrogram with four clusters of rhizobia isolates was produced when the 32 isolates were clustered based on their ability to tolerate heavy metal toxicity. Cluster one comprised of isolates from all soils and morphotypes and comprised almost all. Cluster two had the same composition apart from not have isolates nodulating Tshwane morphotype. Cluster three had only isolates from Richards Bay and Tshwane soils and their morphotypes, with cluster four having an isolate from Leeu-Gamka soil and Kei Mouth morphotype (Figure 5.4).

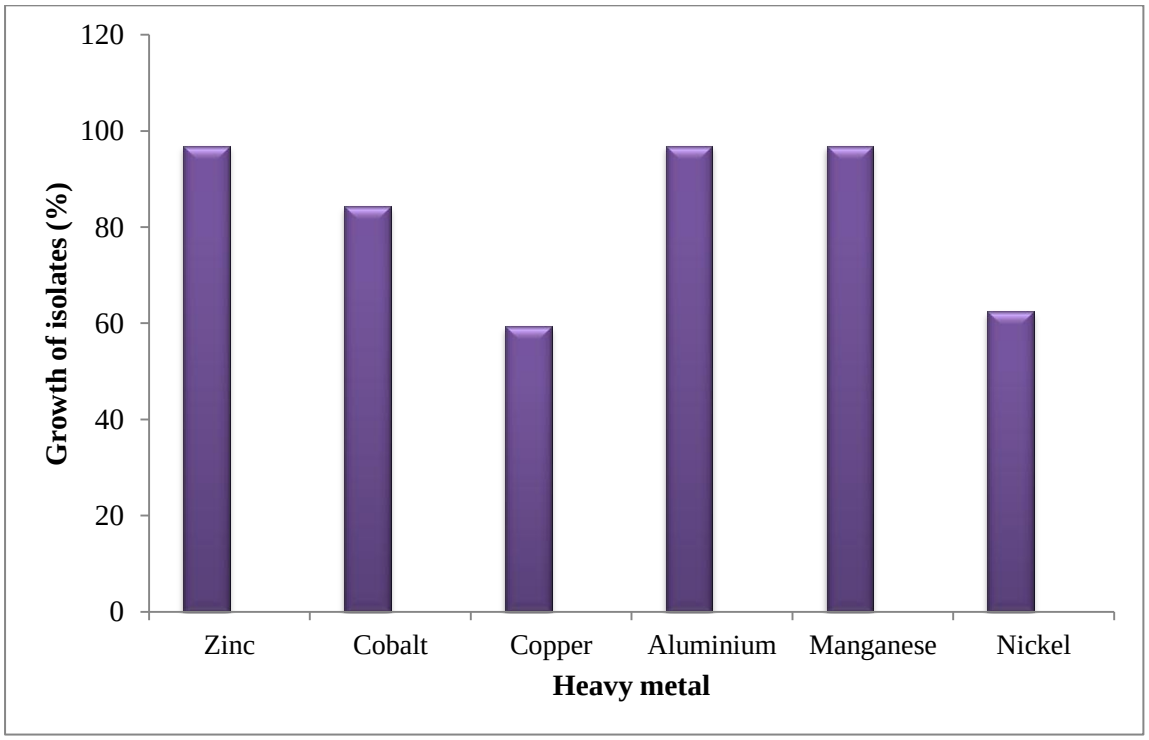

Figure 4.3: Proportions of the 32 V. karroo rhizobium isolates that were tolerant of toxicity of heavy metals Zinc, Cobalt, Copper, Aluminium, Manganese and Nickel

Table 4.7: The percentage resistance to six heavy metals by *V. karroo* rhizobia isolates which were extracted from four *V. karroo* morphotypes grown in four soils collected from Kei Mouth, Richards Bay, Leeu-Gamka and Tshwane

Heavy metals	% of resistant isolates							
	Soil source of isolates				Host morphotype			
	Kei Mouth	Richards Bay	Leeu Gamka	Tshwane	Kei Mouth	Richards Bay	Leeu Gamka	Tshwane
Zinc	100.0	87.5	100.0	100.0	100.0	87.5	100.0	100.0
Cobalt	100.0	62.5	100.0	75.0	100.0	75.0	87.5	75.0
Copper	75.0	25.0	50.0	87.5	37.5	62.5	75.0	62.5
Aluminium	100.0	100.0	87.5	100.0	87.5	100.0	100.0	100.0
Manganese	100.0	100.0	87.5	100.0	87.5	100.0	100.0	100.0
Nickel	75.0	50.0	62.5	62.5	75.0	37.5	50.0	87.5

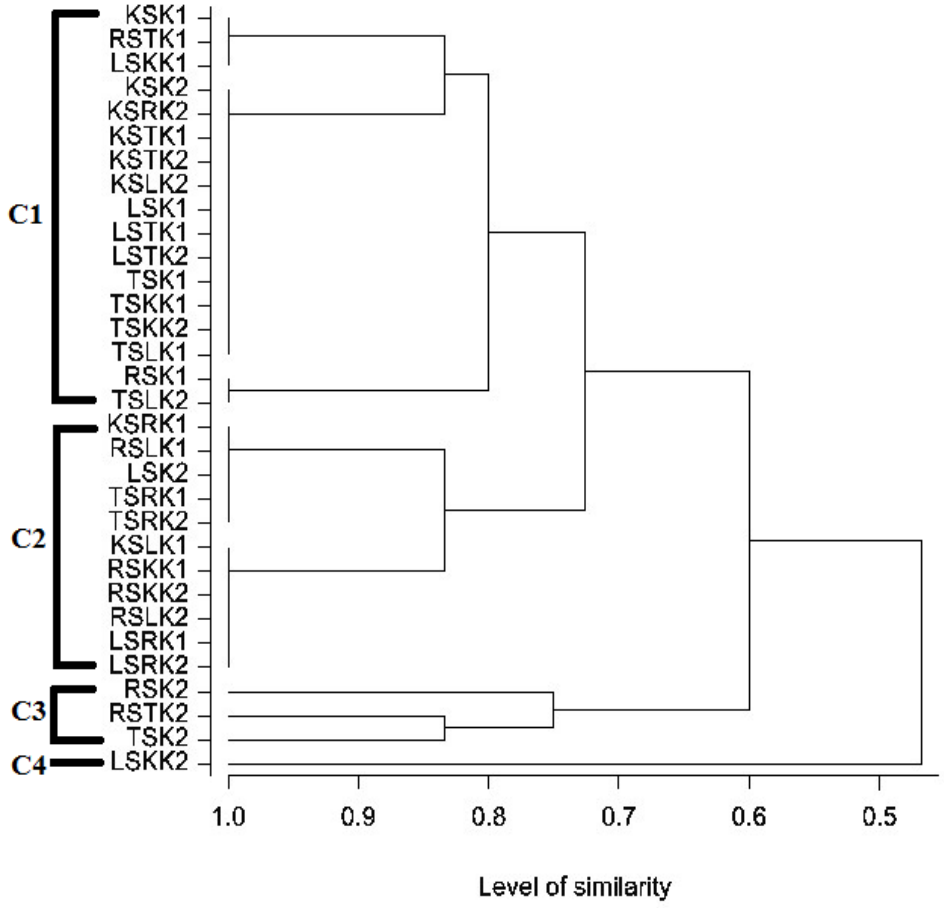

Figure 4.4: Dendrogram highlighting the similarity of rhizobia nodulating different morphotypes of *V. karroo* isolated from 4 different agro-ecological regions of South Africa based on their resistance to heavy metals.

Rhizobia isolates: KSK, Kei Mouth soil and karroo; KSRK, Kei Mouth soil and Richards Bay karroo; KSLK, Kei Mouth soil and Leeu-Gamka karroo; KSTS, Kei Mouth soil and Tshwane karroo; RSK, Richards Bay soil and karroo; RSKK, Richards Bay soil and Kei Mouth karroo; RSLK, Richards Bay and Leeu-Gamka karroo; RSTK, Richards Bay soil and Tshwane karroo; LSK, Leeu-Gamka soil and karroo; LSKK, Leeu-Gamka soil and Kei Mouth karroo; LSRK, Leeu-Gamka soil and Richards Bay karroo; LSTK, Leeu-Gamka soil and Tshwane karroo; TSK, Tshwane soil and karroo; TSKK, Tshwane soil and Kei Mouth karroo; TSRK, Tshwane soil and Richards Bay karroo; TSLK, Tshwane soil and Leeu-Gamka karroo

4.3.6 Utilisation of Carbon source

All the isolates utilised glucose, lactose, sucrose and mannitol, 94% of the isolates utilised maltose, glycerol and m-Inositol, 97% utilised fructose (Figure 4.12). Only 9% of the isolates were able to utilise Malic acid. Rhizobia extracted from Kei Mouth and Richards Bay was all unable to use malic acid but isolates nodulating morphotypes were able to utilise malic acid. On the other hand isolates nodulating morphotypes from Leeu-Gamka and Tshwane were unable to utilise malic acid contrary to the ability of isolates from these soils to utilise the acid, Table 4.8. Rhizobia strains isolated from Kei Mouth and Richards Bay soil did not grow in malic acid, yet interesting enough, their morphotypes when grown in Leeu-Gamka and Tshwane soil were nodulated by isolates which were grew in malic acid. Isolates LSKK1, LSRK1 and TSRK2 were the only isolates which were able to utilise all carbon sources, Table 4.5.

In the dendrogram showing cluster analysis only two clusters were formed, Figure 4.6. All isolates clustered under cluster one with the exception of KSLK2 which clustered alone under cluster two.

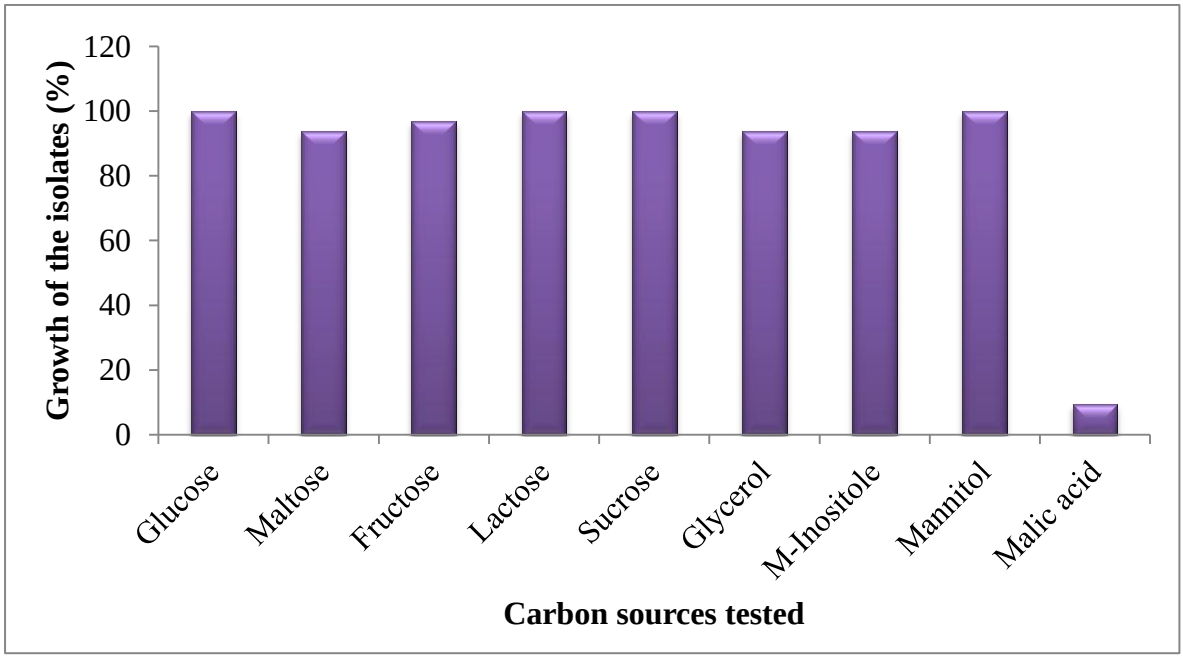

Figure 4.5:Percentage growth of rhizobia isolates showing their utilisation of different carbon sources

Table 4.8: The percentage utilisation of carbon sources by *V. karroo* rhizobia isolates which were extracted from four *V. karroo* morphotypes grown in four soils collected from Kei Mouth, Richards Bay, Leeu-Gamka and Tshwane

Carbon source	% of isolates that utilised carbon sources							
	Soil source of the isolates				Host morphotype			
	Kei Mouth	Richards Bay	Leeu Gamka	Tshwane	Kei Mouth	Richards bay	Leeu Gamka	Tshwane
Glucose	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Maltose	87.5	100.0	100.0	87.5	87.5	100.0	100.0	87.5
Fructose	87.5	100.0	100.0	100.0	100.0	100.0	87.5	100.0
Lactose	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Sucrose	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Glycerol	100.0	75.0	100.0	100.0	75.0	100.0	100.0	100.0
M-Inositol	87.5	100.0	100.0	87.5	100.0	100.0	87.5	87.5
Mannitol	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Malic acid	0.0	0.0	25.0	12.5	12.5	25.0	0.0	0.0

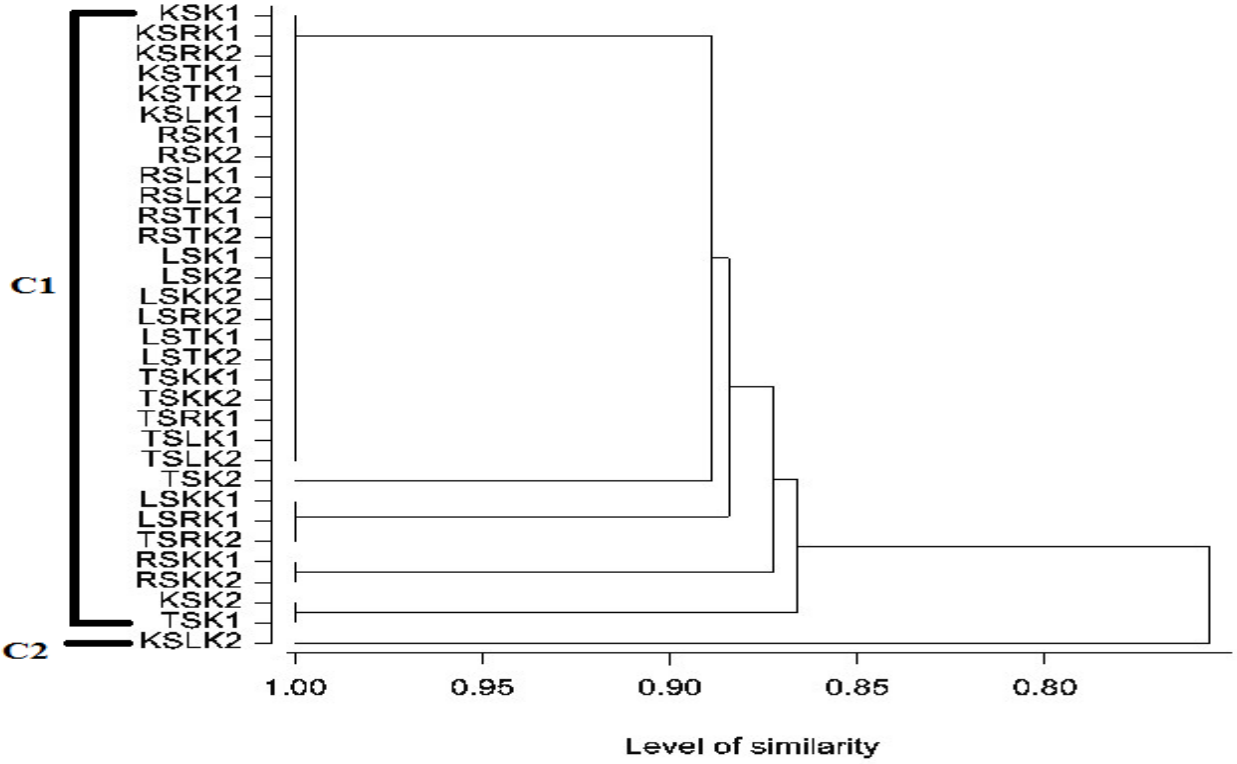

Figure 4.6: Dendrogram highlighting the similarity of rhizobia nodulating different morphotypes of *V. karroo* isolated from 4 different agro-ecological regions of South Africa based on their utilisation of carbon sources.

Rhizobia isolates: KSK, Kei Mouth soil and karroo; KSRK, Kei Mouth soil and Richards Bay karroo; KSLK, Kei Mouth soil and Leeu-Gamka karroo; KSTS, Kei Mouth soil and Tshwane karroo; RSK, Richards Bay soil and karroo; RSKK, Richards Bay soil and Kei Mouth karroo; RSLK, Richards Bay and Leeu-Gamka karroo; RSTK, Richards Bay soil and Tshwane karroo; LSK, Leeu-Gamka soil and karroo; LSKK, Leeu-Gamka soil and Kei Mouth karroo; LSRK, Leeu-Gamka soil and Richards Bay karroo; LSTK, Leeu-Gamka soil and Tshwane karroo; TSK, Tshwane soil and karroo; TSKK, Tshwane soil and Kei Mouth karroo; TSRK, Tshwane soil and Richards Bay karroo; TSLK, Tshwane soil and Leeu-Gamka karroo

4.3.7 Numerical cluster analysis

A numerical cluster analysis of the phenotypic characteristics was carried out and a dendrogram was constructed using the Unweighted pair group method (UPGMA) analysis of the GENSTAT statistical version 12.

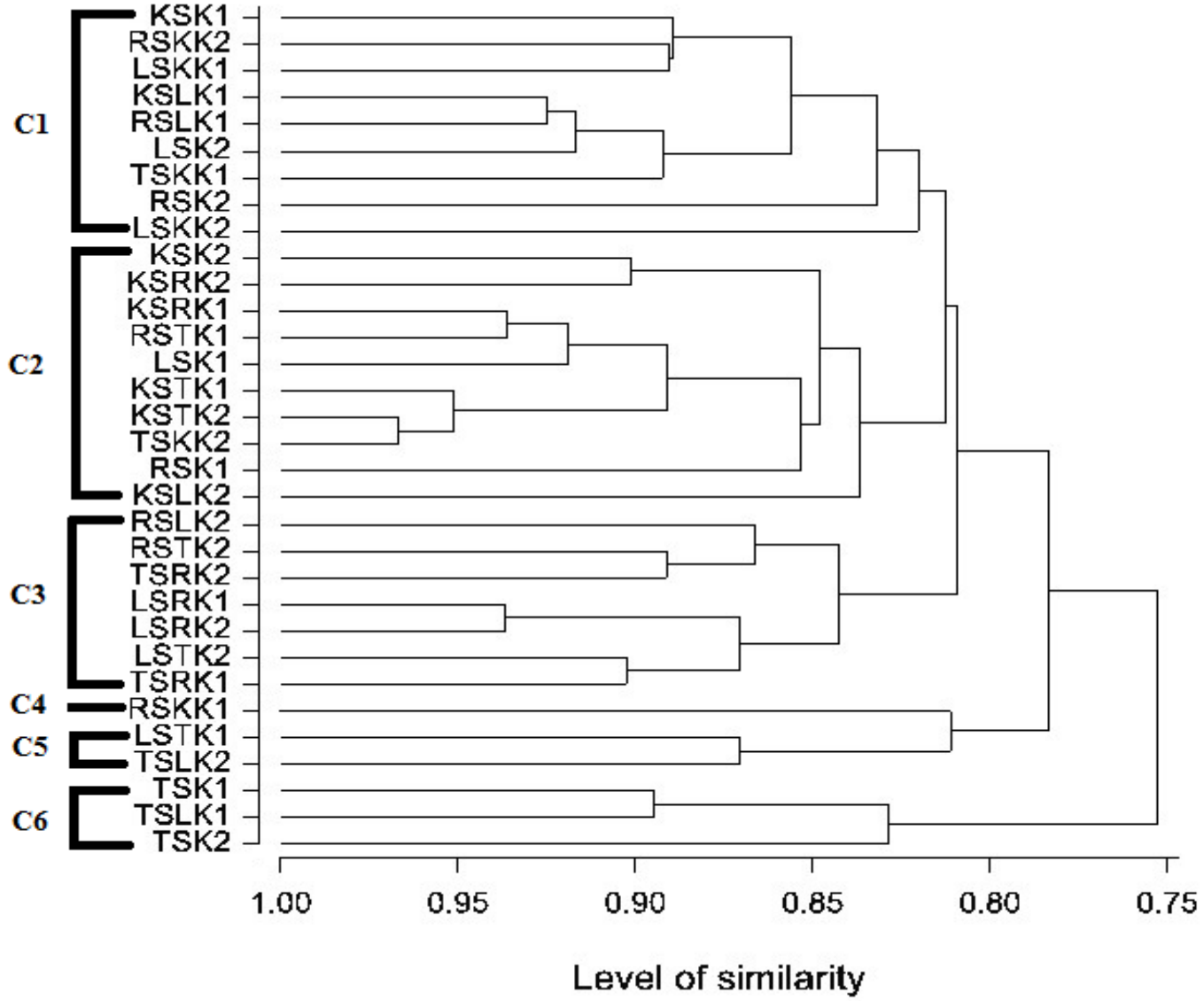

Figure 4.7: Dendrogram highlighting the phenotypic similarity of 32 *V. karroo* rhizobia isolated from four *V. karroo* morphotypes growing in Kei Mouth, Richards Bay, Leeu-Gamka and Tshwane soils

Rhizobia isolates: KSK, Kei Mouth soil and karroo; KSRK, Kei Mouth soil and Richards Bay karroo; KSLK, Kei Mouth soil and Leeu-Gamka karroo; KSTS, Kei Mouth soil and Tshwane karroo; RSK, Richards Bay soil and karroo; RSKK, Richards Bay soil and Kei Mouth karroo; RSLK, Richards Bay and Leeu-Gamka karroo; RSTK, Richards Bay soil and Tshwane karroo; LSK, Leeu-Gamka soil and karroo; LSKK, Leeu-Gamka soil and Kei Mouth karroo; LSRK, Leeu-Gamka soil and Richards Bay karroo; LSTK, Leeu-Gamka soil and Tshwane karroo; TSK, Tshwane soil and karroo; TSKK, Tshwane soil and Kei Mouth karroo; TSRK, Tshwane soil and Richards Bay karroo; TSLK, Tshwane soil and Leeu-Gamka karroo

Table 4.9: Grouping of rhizobia isolates according to their phenotypic characteristics

Phenotypic characteristics	**Cluster 1** (9 strains)	**Cluster 2** (10 strains)	**Cluster 3** (7 strains)	**Cluster 4** (1 strain)	**Cluster 5** (2 strains)	**Cluster 6** (3 strains)
Generation time						
(30 min < GT < 4 h)	7	10	7	1	1	0
(4h < GT < 9 h)	0	0	0	0	1	3
Temperature (^{0}C)						
5	0	2	1	0	1	2
15	9	10	7	1	2	3
25	9	10	7	1	2	3
30	9	10	7	1	2	3
40	8	10	7	1	2	0
45	1	5	2	1	0	0
pH						
3	0	0	0	0	0	0
4	0	1	3	0	0	3
5	2	3	4	0	0	3
6	9	10	7	1	2	3
7	9	10	7	1	2	3
8	9	10	7	0	0	3
9	2	8	7	0	0	2
Highest growth in NaCl (M)						
0.00	0	0	0	0	0	0
0.005	3	2	0	0	0	0
0.01	1	0	3	0	0	1
0.02	1	2	1	1	0	0
0.05	1	3	3	0	0	0
0.1	2	3	0	0	1	1
0.2	1	0	0	0	1	1
Intrinsic antibiotic resistance						
Ampicillin	5	0	6	1	2	2
Neomycin	1	4	2	1	1	0
Ciprofloxacin	3	1	7	0	2	1
Chloramphenicol	9	3	6	0	1	3
Gentamycin	9	6	6	1	1	3
Heavy metal resistance						
Zinc	8	10	7	1	2	3
Cobalt	8	9	6	1	1	2
Copper	3	9	6	0	2	2
Aluminium	8	10	7	1	2	3
Manganese	8	10	7	1	2	3
Nickel	5	9	2	0	2	2
Carbon source						
Glucose	9	10	7	1	2	3
Maltose	9	9	7	1	2	2
Fructose	9	9	7	1	2	3
Lactose	9	10	7	1	2	3
Sucrose	9	10	7	1	2	3

Mannitol	9	10	7	1	2	3
Glycerol	8	10	7	0	2	3
M-Inositol	9	9	7	1	2	2
Malic acid	1	0	2	0	0	0

The results from the numeric analysis, exhibit that below 0.83 level of similarity, the *V. karroo* rhizobia isolates were grouped into six distinct clusters. Cluster one (KSK1, TSKK1, RSKK2, LSKK1, KSLK1, LSK2, RSLK1, RSK2, LSKK2) (Figure 4.7) comprised of only fast growing rhizobia, which were isolated from all soil types. The cluster also consisted of rhizobia extracted from all morphotypes except Tshwane morphotype. Isolates in this cluster did not grow in low temperature of 5°C. All but one of the isolates grew in extremely high temperature of 40°C, however only one isolate could survive in 45°C. Seven out of nine of the isolates in this cluster did not grow in very acidic conditions (less than or equal to pH 5) and below and all grew in pH 8 but failed to grow in pH 9. Furthermore the isolates showed tolerance to the antibiotics gentamycin and chloramphenicol under the tested concentrations but did not grow in ciprofloxacin and neomycin. Majority of the isolates (six out of nine) failed to grow in the presence of copper.

Cluster two (KSK2, KSRK1, KSRK2, RSTK1, LSK1, KSTK1, KSTK2, TSKK2, RSK1, KSLK2) (Figure 5.7) comprised of only fast growing rhizobia, which were isolated from all soil types and morphotypes. Two of the isolates grew at 5°C, all grew in 40°C and 50% of them grew at 45°C. Almost all the isolates grew in high pH of 9 and failed to grow in acidic conditions. Highest growth of these isolates was in the medium to high salt concentration (0.05-0.1 M NaCl). All of the isolates were susceptible to growth inhibition by ampicillin and ciprofloxacin and all but three were susceptible to chloramphenicol. Almost all the isolates were resistant to all heavy metals tested, and none of isolates utilised malic acid (Table 4.9).

Cluster three (RSLK2, RSTK2, TSRK2, LSRK1, LSRK2, LSTK2, TSRK1) (Figure 4.7) consisted of isolates from three soil types and morphotype. Isolates extracted from Kei Mouth soil and morphotype were not part of this cluster. The isolates in this cluster were similar to those of Cluster two and three in that they were all fast growing. Most isolates in this cluster grew in pH 4 to 9, the highest growth of isolates in this cluster was found in the low to medium salt concentration (0.005-0.02 M NaCl). Most of the isolates were resistant to four antibiotics and all but two were sensitive to neomycin. All but two of the isolates in this

cluster had their growth inhibited by nickel. Only three out of 32 isolates assimilated malic acid and two of the three isolates are found in Cluster three.

Cluster four contained only one isolate (RSKK1) extracted from Richards bay soil using Kei Mouth trap morphotype. This isolate was also fast growing, grew in extremely high temperature of 45°C. This isolate grew in pH 6 to 7 and could not grow in pH below and above this range. In addition, the isolate did not grow in two of the tested antibiotics, namely chloramphenicol and ciprofloxacin. It could not also grow in the presence of copper and nickel.

Cluster five had both slow and fast growing rhizobia and consisted of only two isolates (LSTK1, TSLK2) (Figure 4.7). Like the isolate in cluster four, the isolates only grew in pH 6 to 7. They had highest growth in the highest salt concentration of 0.1 M and 0.2 M NaCl and generally grew in all antibiotics and heavy metals and could assimilate all the tested carbon sources with the exception of malic acid.

The slow growing rhizobia isolates extracted from Tshwane soil growing in Tshwane morphotype (TSK1, TSK2) and Leeu Gamka morphotypes (TSLK1) were the components of Cluster 6 (Figure 4.7). Two thirds of these isolates grew in very low temperature of 5°C and all the isolates did not grow in extreme high temperatures of 40 to 45°C. All the isolates grew in a wide pH range of extremely acidic to alkaline conditions of 4-9. Most of the isolates grew in ampicillin and all grew in the presence of gentamycin and chloramphenicol.

All the rhizobia strains from *V. nilotica* and *V. tortilis* were able to form symbiosis with the four *V. karroo* morphotypes. Also,. Likewise the *V. karroo* morphotypes did not show any specificity in their symbiosis with rhizobia as they cross-nodulated among themselves and with with *V. nilotica* and *V. tortilis* (Table 4.10). Nonetheless some strains were some few exceptions in which cross nodulation did not occur.

The strain from Richards Bay soil and Richards Bay *V. karroo* morphotypes was the only strain which nodulated with all morphotypes *V. nilotica* and *V. tortilis*. The nodules formed comprised of two shapes, namely globule and branched. Nodule shape was also not confined to any strain, with the exception of the strain from *V. tortilis* which only produced globule nodules in all the morphotypes. Other strains produced both globule and branched nodules. Likewise the nodule shape was not related to the trap plant for most morphotypes. An exception was however noted on Leeu-Gamka morphotype and *V. nilotica* which only produced globule shaped nodules regardless of the strain nodulating them. No nodules were observed from the control plants.

Table 4.10: Cross nodulation assessment within *Vachellia karroo* morphotypes as well as with *V. nilotica* and *V. tortilis*

Source plant	Test plant	Average number of nodules	Shape of nodules
KM *V. karroo*	KM *V. karroo*	7	Branched
KM *V. karroo*	RB *V. karroo*	26	Globule
KM *V. karroo*	LG *V. karroo*	0	
KM *V. karroo*	TS *V. karroo*	2	Globule
KM *V. karroo*	*V. nilotica*	2	Globule
KM *V. karroo*	*V. tortilis*	2	Branched
RB *V. karroo*	RB *V. karroo*	16	Branched
RB *V. karroo*	KM *V. karroo*	15	Globule, Branched
RB *V. karroo*	LG *V. karroo*	4	Branched
RB *V. karroo*	TS *V. karroo*	11	Globule, Branched
RB *V. karroo*	*V. nilotica*	3	Globule
RB *V. karroo*	*V. tortilis*	8	Globule, Branched
LG *V. karroo*	LG *V. karroo*	2	Globule
LG *V. karroo*	KM *V. karroo*	2	Globule
LG *V. karroo*	RB *V. karroo*	0	
LG *V. karroo*	TS *V. karroo*	2	Globule
LG *V. karroo*	*V. nilotica*	0	
LG *V. karroo*	*V. tortilis*	2	Globule
TS *V. karroo*	TS *V. karroo*	3	Globule
TS *V. karroo*	KM *V. karroo*	2	Branched
TS *V. karroo*	RB *V. karroo*	0	
TS *V. karroo*	LG *V. karroo*	5	Globule
TS *V. karroo*	*V. nilotica*	0	
TS *V. karroo*	*V. tortilis*	26	Globule, Branched
V. nilotica	*V. nilotica*	36	Globule, Branched
V. nilotica	KM *V. karroo*	0	
V. nilotica	RB *V. karroo*	0	
V. nilotica	LG *V. karroo*	12	Branched
V. nilotica	TS *V. karroo*	2	Globule
V. nilotica	*V. tortilis*	13	Branched
V. tortilis	*V. tortilis*	2	Globule

V. tortilis	KM *V. karroo*	2	Globule
V. tortilis	RB *V. karroo*	0	
V. tortilis	LG *V. karroo*	0	
V. tortilis	TS *V. karroo*	2	Globule
V. tortilis	*V. nilotica*	2	Globule

KM (Kei Mouth), RB (Richards Bay), LG (Leeu Gamka), TS (Tshwane)

4.4 Discussion

4.4.1 Effect of temperature on the growth of rhizobia isolates

Some of the rhizobia isolates nodulating *V. karroo* morphotypes tolerated extremely high temperatures of 45°C. Such rare species of rhizobia isolates tolerating extremely high temperatures have also been found to nodulate other legume plants (Bansal *et al*., 2014). No association was found between the temperature preferences of rhizobia isolates and the temperatures prevailing in areas of origin. On the contrary, areas of moderate temperatures (Richards Bay and Kei Mouth) had a higher number of rhizobia which tolerated high temperatures. In certain previous studies, isolates nodulating other *Vachellia* species have also been shown to have no correlation between their tolerance to temperature and the climatic conditions of their geographic origin (Boukhatem et al., 2012, Zerhari et al., 2000).

Also among the 32 tested isolates no link was found in their preference to different temperatures and the host *V. karroo* morphotypes. In each soil sample, rhizobia were extracted which grew in different temperature ranges indicating rhizobia diversity in relation to temperature tolerance (Brockwell *et al*., 2005) regardless of location. This diversity can be taken advantage of in selecting rhizobia strains for specific areas with characteristic temperatures. One isolate extracted from Richards Bay *V. karroo* morphotype growing in Richards bay soil (RSK1) was outstanding in that it tolerated very low and extremely high temperatures of 5 to 45°C.

4.4.2 Response of rhizobia isolates to different pH levels

A relationship between the pH of soil of origin and the growth behaviour of isolates with respect to pH was noted in Richards Bay soil. Richards Bay soils are alkaline and isolates extracted from this soil were unable to grow at low pH of 3-5. This seemed to concur with other findings which have shown a relationship between tolerance of rhizobia to pH in culture media with pH of origin soils of the isolates (Brígido and Oliveira, 2013, Jida and Assefa,

2014). However, rhizobia isolated from other soils tolerated both acidic and alkaline conditions regardless of the pH conditions of the soil from which they originated.

There was diversity of the isolates extracted from the same morphotype in their tolerance to acidic and alkaline conditions. The behaviour of isolates in the given pH values was not inclined to pH of their host morphotype place of origin. This diversity in tolerance of rhizobia to pH has been noted for rhizobia nodulating other legume species (Appunu and Dhar, 2006; Zahran, 1999). Among the tested isolates there were slow and fast growing rhizobia that tolerated acidic pH of 4-5 as reported by Mpepereki *et al*., (1997). A high proportion (75%) of the slow growing rhizobia was tolerant of low pH whilst only 32% of fast growers were low pH tolerant. This suggested that most of the slow growing rhizobia are more tolerant to acidic pH as compared to fast growing, a trend that has also been noted by other researchers (Cooper *et al*., 1985; Graham *et al*., 1994).

4.4.3 Growth of rhizobia isolates in different salinity levels

The growth performance of 32 *Vachellia karroo* rhizobia isolates in relation to salinity was not related to the salinity characteristics of the soils from which they were extracted. This phenomenon has also been noticed in other rhizobia species (Bhardwaj, 1975; Jida and Assefa, 2014; L'taief et al., 2007). In general fast growing rhizobia were more tolerant to high salt concentrations as compared to slow growing rhizobia, which was the phenomenon that was also identified in rhizobia nodulating other *Vachellia* species (Zerhari *et al*., 2000). However, this characteristics of *Vachellia* rhizobia is contrary to what has been understood in other rhizobia that fast growing rhizobia are more tolerant to high salt concentration compared to slow growing rhizobia (Berrada *et al*., 2012).

Isolates extracted from Kei Mouth soil had the highest mean growth in comparison to isolates from other soils. The 0.02 M NaCl concentration also had the highest mean growth amongst other concentrations, indicating that most isolates preferred this concentration. In addition to revealing the preference of both low and high salt concentrations exhibited by rhizobia extracted from the same soil and morphotype the mean growth based on the soil source and trap morphotype revealed the most preferred concentration. Isolates from Kei Mouth soil and morphotype generally prefer low salt concentration, when the salt concentration was increased the mean growth decreased.

A medium salt concentration preference was revealed in isolates from Richards Bay soil and morphotype. The salt preference of Lee Gamka soil isolates was different from that of Lee Gamka trap morphotypes with the former having the highest mean growth under low concentration and the later under medium concentration. Same applies to isolates from Tshwane soil which had the highest mean growth under medium concentration whilst isolates extracted from Tshwane morphotype had highest mean growth on high concentration. Isolates RSLK1, RSLK2, TSK1 and TSKK2 are recommended for further tests as they showed the ability to grow the best in the high salt concentration (0.2 M NaCl).

4.4.4 Rhizobia resistance to toxicity of different antibiotics

In relation to their resistance to antibiotics, the rhizobia isolate clusters were generally not according to the soils from which they originated. This is contrary to reports by other researchers that climatic and soil conditions of an area influence the resistance to antibiotics by rhizobia. In this research most rhizobia isolates exhibited resistance to antibiotics which was not correlated to conditions of geographic origin and host morphotype as reported on chickpea (Alexandre *et al*., 2006) and cowpea (Moreira *et al*., 2006) rhizobia.

However isolates from Lee Gamka soil displayed a degree of relationship with soil of origin in that all isolates from this soil were resistant to gentamycin. This trend of correlation of isolates with soil of origin has also been stated by Zilli *et al*., (2004) when looking at the intrinsic antibiotic resistance of rhizobia. The pattern of rhizobia intrinsic antibiotic resistance relationship with soil of origin might suggest the presence of these antibiotics in their native environments thus they are adapted to them, or their sensitivity might be a signal of the absence of the antibiotics in the soil (Bedi and Naglot, 2011).

4.4.5 Effect of toxicity of heavy metals to rhizobia growth

Approximately 37.5% of the strains grew in all the heavy metals at the concentrations used in this study, which is in harmony with behaviour of other *Vachellia* rhizobia (Bakhoum *et al*., 2014). Rhizobia from the same soils varied in their growth in diverse heavy metals and when clustered according to their ability to grow in these heavy metals, there was no clear pattern in the correlation of their behaviour to soil of origin was observed. The same pattern was observed in the link between the growth of the isolates in heavy metals and the place of origin of the trap morphotypes.

The soil from which the rhizobia isolates were extracted did not influence the clustering of the isolates in relation to their ability to tolerate heavy metals. This might be due to the fact that when grown in culture rhizobia are understood to be able to grow in higher concentrations of heavy metals than those normally found in the soil of origin (Saikia, 2008, Chaudri *et al.*, 1993). Capacity of rhizobia and their host plants to resist toxicity of heavy metals marks them as important tools in the rehabilitation of heavy metal contaminated soils (Satyanarayana and Johri, 2005).

4.4.6 Utilisation of diverse carbon sources

The utilisation of carbon sources by the 32 rhizobia strains was irrespective of the soil of origin and the morphotype (Figure 4.6). Moreover, fast and slow growing rhizobia showed no variation between each other in their utilisation of carbon sources. This is contrary to other findings which state that fast growing rhizobia utilise a wide variety of carbohydrates in comparison to slow growing (Sadowsky *et al*., 1983). The utilisation of all carbon sources except for malic acid by a percentage of the tested isolates is a phenomenon widely observed in many rhizobia, their utilisation of different carbohydrates as sole carbon sources is a desired growth characteristic (Laurette *et al*., 2015).

All but one of the isolates clustered together when classified according to their ability to utilise carbon sources. All the rhizobia utilised a wide variety of carbon sources regardless of the soil where they were extracted and the morphotypes they nodulate. This implies that rhizobia naturally tend to utilise a wide variety of carbon sources irrespective of the soil they were isolated from and the plant they nodulate (Laurette *et al*., 2015).

4.4.7 Numeric analysis of all phenotypic and biochemical characteristics

Phenotypic and biochemical variation was noted in the indigenous rhizobia nodulating *V. karroo* as revealed by the formation of six clusters when a numerical analysis of the characteristics was performed. The first two clusters with the largest number of isolates had rhizobia isolates from different soils and nodulating different morphotypes, this variation has previously been noticed in *Vachellia* (Bakhoum *et al*., 2014; Zerhari *et al*., 2000) and Chickpea (Rai *et al*., 2012) rhizobia isolates.

Formation of more than three cluster groups based on numerical analysis of phenotypic characteristics is a very common characteristic of indigenous rhizobia (Chagas Júnior *et al*., 2013; Ramírez-Bahena *et al*., 2009; Sharma *et al*., 2010). The non-correlation of isolates and

their host morphotype and the geographic characteristics of their soil of origin observed in clusters one to three might be due to a wide genetic diversity of rhizobia in each soil sample (Rai *et al*., 2012., Rejili *et al*., 2009)

The isolate from Richards bay soil and Kei Mouth morphotype which clustered alone is a fast growing isolate which had characteristics similar to those exhibited by the slow growing rhizobia isolates. When all the physiological and biochemical characteristics were combined isolates from Leeu Gamka and Tshwane soils and morphotypes were observed to cluster alone in clusters five and six. The notable feature in the isolates from these clusters is that 80% were slow growing rhizobia extracted from Tshwane soil. Amongst all the tested condition, the generation time, temperature, pH, antibiotic resistance and heavy metal resistance were the most effective characteristics in classifying the 32 *V. karroo* rhizobia into different groups. These results are consisted with other findings which have shown tree legume nodulating rhizobia clustering according to their soil origin (Wolde-meskel *et al*., 2004) and that slow growing rhizobia have characteristics distinct from those of fast growing rhizobia (Sadowsky *et al*, 1983).

4.4.8 Cross nodulation

Cross nodulation was observed between *V. karroo* morphotypes as hypothesized. This ability of the morphotypes to share rhizobia is expected since it has been genetically proven by other researchers that the morphotypes are one species (Taylor and Baker, 2012). The similarity in nodule shape further confirms the relationship of the isolates. This ability of the rhizobia strains to form different nodule shapes maybe due to the fact that one strain of rhizobia forms different forms of bacteriods which assume different shapes (Paracer and Ahmadjian, 2000). However the inability of some morphotypes to nodulate with other rhizobia strains from different morphotypes might be a result of incompatibility between the host and the rhizobia.

Moreover the ability of *V. karroo* rhizobia extracted from different morphotype to from effective nodules with *V. nilotica* and *V. tortilis* showed a lack of host specificity in the rhizobia stains (Granada *et al*., 2014), a trait desired in the use of the rhizobia strain as an inoculant and in the successive integration of non-native tree species. Nodule shape has been linked to the host plant and rhizobia (Fred *et al*., 1932). With different species forming different shapes. The similarity in nodule shape of *Vachellia* species gives a suggestion of a strong relationship between different rhizobia species and likewise a similarity between the three *Vachellia* species.

4.5 Conclusion

Vachellia karroo morphotypes nodulated in all the four test soils. This implies that the soils carried rhizobia that could nodulate with any of the four *V. karroo* morphotypes. The soils were dominated by fast growing rhizobia which constituted 87.5% of the total number of extracted rhizobia isolates.

Most of the isolates could not be distinguished based on soil, morphotype or chemical properties of the soils from which they were extracted in terms of their tolerance to extreme pH and temperature values, resistance to antibiotics, tolerance to heavy metal toxicity and salinity or utilization of carbon sources. However relativeness to soil of origin and host morphotype was noted in the four slow growing rhizobia extracted from Tshwane soil using Tshwane morphotype and Leeu Gamka morphotype. The isolates were diverse from others in their tolerance to temperature, pH, resistance to antibiotics and resistance to heavy metals. Yet they were distinct from the other isolates on their growth in different salt concentration and utilisation of carbon sources.

All soils carried rhizobia for *V. karroo* that were diverse in terms of their growth behaviour in response to pH, temperature, antibiotics, heavy metal toxicity, salinity and utilization of carbon sources. The optimum pH range was 6-7, below and above which other isolates failed to grow. The optimum temperature ranged from 15 to 30°C. Isolates which were able to grow at the extreme temperature of 45°C were merely nine. The most preferred salt concentrations were 0.05 and 0.1 M of NaCl. Tolerance to extremely high temperatures and high salt tolerance are important characteristics which are recommended to be used in the selection of inoculants for *V. karroo* in hot and dry regions.

Rhizobia resistance to the given concentration of more than three antibiotics increases their capacity to compete with other bacteria and thus increasing their survival chance in the soil. Tolerance to the given concentrations of heavy metals might be an indication of the presence of these metals in the soils of origin. A test of these heavy metals in the soils is recommended as a further study. The current information of heavy metal resistant characters is to be used in further genetic studies as positive markers. Utilisation of a wide variety of carbon sources by all the tested rhizobia is an indication that the isolates are efficient nutrient competitors.

The ability of *V. karroo* to be nodulated by various rhizobia as observed and the ability of rhizobia strains to tolerate different phenotypic environments makes this study a scientific foundation for the use of *V. karroo* in rehabilitation of soils in diverse agro-ecological regions. Inoculation of *V. karroo* with the right strain of rhizobia improves seedling growth and the establishment of the tree (Galiana et al., 1994). The rhizobia isolates are recommended for further improvement and for use as inoculates in diverse ecological regions.

Non-correlation of *V. karroo* rhizobia to soil of origin and origin of trap tree reveals the wide diversity of these rhizobia. These indigenous rhizobia are to be further improved for use in various soils as they exhibited an ability to form effective nodules in various soil conditions and with the different morphotypes and species. The ability of rhizobia to form effective nodules with different species of *Vachellia* is to be used in species differentiation.

Chapter 5

General Conclusion and Recommendations

Vachellia karroo is one of the plants which have exhibited high levels of phenotypic plasticity by changing its form when grown in different climatic conditions and thereby giving rise to different morphotypes. These morphotypes are physiologically diverse and this study sought to find out if there was any diversity in the rhizobia that nodulate them. As much as these morphotypes are highly diverse morphologically they are genetically the same and results from the study further revealed that they are generally not distinct in terms of the rhizobia nodulating them.

These morphotypes are recommended to be used for fixing nitrogen, land rehabilitation or any other agricultural use in Kei Mouth, Richards Bay, Leeu Gamka and Tshwane areas as they were able to grow and nodulate with rhizobia found in these soils. Furthermore they can also be nodulated with rhizobia extracted from any of the morphotypes and or from other *Vachellia* species as the study revealed that they can cross nodulate with each other and other *Vachellia* species.

The behaviour of rhizobia isolates nodulating *V. karroo* in South Africa had never before been investigated. Thus the results from the study provide useful information regarding the potential of using *V. karroo* for fix nitrogen. .. Thus results from the study are a step in identifying the direction for the selection and improvement of *V. karoo* rhizobia, which can be used as inoculants in areas where they perform best.

Further studies on phenotypic characteristics are recommended to expose more distinct features of the isolates. The formation of six clusters from the rhizobia isolates suggests that many genomic groups of rhizobia nodulating *V. karroo* might be available in South African soils, furthering this study using molecular techniques such as PCR-RFLP performed on 16S-23S rDNA is recommended to authenticate this. Also, further studies are recommended to identify whether the *V. karroo* rhizobia with desirable characteristics can nodulate different species of crop legumes.

Chapter 6

References

Abd-Alla, M. H., Morsy, F. M., El- Enany, A. W. E. & Ohyama, T. 2012. Isolation and characterization of a heavy-metal-resistant isolate of Rhizobium leguminosarum bv. viciae potentially applicable for biosorption of Cd 2^{+} and Co 2^{+}. International Biodeterioration & Biodegradation, 67, 48-55.

Ahmad, N. 1996. Nitrogen economy in tropical soils, Springer.

Al-Falih, A. M. 2002. Factors affecting the efficiency of symbiotic nitrogen fixation by Rhizobium. Pakistan Journal of Biological Sciences, 5, 1277-1293.

Alexandre, A., Laranjo, M. & Oliveira, S. 2006. Natural populations of chickpea rhizobia evaluated by antibiotic resistance profiles and molecular methods. Microbial ecology, 51, 128-136.

Allen, O. & Allen, E. K. 1936. Root nodule bacteria of some tropical leguminous plants: I. Cross-inoculation studies with *Vigna sinensis* L. 1. Soil Science, 42, 61-77.

Alshaharani, T. S & Shetta, N.D. Phenotypic and biochemical characterization of root nodule bacteria naturally associated with woody tree legumes in Saudi Arabia. Journal of Environmental Biology, 36**,** 363.

Andrade, D., Murphy, P. & Giller, K. 2002. The diversity of Phaseolus-nodulating rhizobial populations is altered by liming of acid soils planted with *Phaseolus vulgaris* L. in Brazil. Applied and Environmental microbiology, 68, 4025-4034.

Appunu, C. & Dhar, B. 2006. Symbiotic effectiveness of acid-tolerant *Bradyrhizobium* strains with soybean in low pH soil. African Journal of Biotechnology, 5.

Archibald, S. & Bond, W. J. 2003. Growing tall vs growing wide: tree architecture and allometry of *Acacia karroo* in forest, savanna, and arid environments. Oikos, 102, 3-14.

Aronson, J., Ovalle, C. & Avendano, J. 1992. Early growth rate and nitrogen fixation potential in forty-four legume species grown in an acid and a neutral soil from central Chile. Forest Ecology and Management, 47, 225-243.

Ba, S., Willems, A., De Lajudie, P., Roche, P., Jeder, H., Quatrini, P., Neyra, M., Ferro, M., Prome, J. C. & Gillis, M. 2002. Symbiotic and taxonomic diversity of rhizobia isolated from *Acacia tortilis* subsp. raddiana in Africa. Systematic and Applied Microbiology, 25, 130-145.

Badri, D. V., Weir, T. L., Van Der Lelie, D. & Vivanco, J. M. 2009. Rhizosphere chemical dialogues: plant–microbe interactions. Current Opinion in Biotechnology, 20, 642-650.

Bakhoum, N., Galiana, A., Le Roux, C., Kane, A., Duponnois, R., Ndoye, F., Fall, D., Noba, K., Sylla, S. N. & Diouf, D. 2015. Phylogeny of Nodulation Genes and Symbiotic Diversity of *Acacia senegal* (L.) Willd. And *A. seyal* (Del.) Mesorhizobium Strains from Different Regions of Senegal. Microbial Ecology, 69, 641-651.

Bakhoum, N., Le Roux, C., Diouf, D., Kane, A., Ndoye, F., Fall, D., Duponnois, R., Noba, K., Sylla, S. N. & Galiana, A. 2014. Distribution and Diversity of Rhizobial Populations Associated with Acacia senegal (L.) Willd. Provenances in Senegalese Arid and Semiarid Regions. Open Journal of Forestry, 4, 136.

Bala, A., Murphy, P. & Giller, K. 2004. Classification of tropical tree rhizobia based on phenotypic characters forms nested clusters of phylogenetic groups. West African Journal of Applied Ecology, 6.

Balachandar, D., Raja, P., Kumar, K. & Sundaram, S. 2007. Non-rhizobial nodulation in legumes. Biotechnology and Molecular Biology Review, 2, 49-57.

Bansal, M., Kukreja, K., Sunrja, S. & Dudeja, S. 2014. Symbiotic effectivity of high temperature tolerant mungbean (*Vigna radiata*) rhizobia under different temperature conditions. International Journal of Current Microbioly and Applied Sciences, 3, 807-821.

Barnes, R., Filer, D. & Milton, S. 1996. *Acacia karroo*: monograph and annotated bibliography, Oxford Forestry Institute, Department of Plant Sciences, University of Oxford.

Bayonne Mboumba, G. 2006. Assessment of quantitative and genetic molecular variation of *Acacia karroo* in two extreme populations. Stellenbosch: University of Stellenbosch.

Bedi, M. K. & Naglot, A. 2011. Characterization of Rhizobium isolated from root nodules of *Trifolium alexandrinum*. Journal of Agricultural Technology, 7, 1705-1723.

Berg, J. M., Tymoczko, J. L. & Stryer, L. 2002. Nitrogen Fixation: Microorganisms Use ATP and a Powerful Reductant to Reduce Atmospheric Nitrogen to Ammonia. Biochemistry. 5th Edition. New York: W. H Freeman. Section 24.1

Berman-Frank, I., Lundgren, P. & Falkowski, P. 2003. Nitrogen fixation and photosynthetic oxygen evolution in cyanobacteria. Research in Microbiology, 154, 157-164.

Berrada, H., Nouioui, I., Houssaini, M. I., El Ghachtouli, N., Gtari, M. & Benbrahim, K. F. 2012. Phenotypic and genotypic characterizations of rhizobia isolated from root nodules of multiple legume species native of Fez, Morocco. African Journal of Microbiology Research, 6, 5314-5324.

Bhardwaj, K. 1975. Survival and symbiotic characteristics of Rhizobium in saline-alkali soils. Plant and soil, 43, 377-385.

Bohlool, B., Ladha, J., Garrity, D. & George, T. 1992. Biological nitrogen fixation for sustainable agriculture: A perspective. Plant and soil 141:1-11

Boonkerd, N. & Weaver, R. 1982. Survival of cowpea rhizobia in soil as affected by soil temperature and moisture. Applied and Environmental Microbiology, 43, 585-589.

Boukhatem, Z. F., Domergue, O., Bekki, A., Merabet, C., Sekkour S., Bouzza, F., Duponnois, R., De Lajudie, P. & Galiana, A. 2012. Symbiotic characterization and diversity of rhizobia associated with native and introduced acacias in arid and semi-arid regions in Algeria. FEMS Microbiology Ecology, 80, 534-547.

Boyes, L. 2004. Regeneration failure and the *Acacia karroo* successional pathway in coastal dune forests in KwaZulu-Natal, South Africa. Honours Thesis, University of KwaZulu-Natal, Pietermaritzburg, SA.

Brenner, D. J., Krieg, N. R., Staley, J. T. & Garrity, G. M. 2005. Bergey's manual of systematic bacteriology, vol. 2. The Proteobacteria. East Lansing, USA, 183.

Brigido, C. & Oliveira, S. 2013. Most acid-tolerant chickpea mesorhizobia show induction of major chaperone genes upon acid shock. Microbial Ecology, 65, 145-153.

Brigido, C. C. 2012. Tolerance of Chickpea Mesorhizobia to Acid and Salt Stress. Univ. Évora Portugal, 2012. 1, 2012.

Brockwell, J., Searle, S. D., Jeavons, A. C. & Waayers, M. 2005. Nitrogen fixation in acacias: an untapped resource for sustainable plantations, farm forestry and land reclamation, Australian Centre for International Agricultural Research (ACIAR).pp 132

Burdon, J., Gibson, A., Searle, S. D., Woods, M. & Brockwell, J. 1999. Variation in the effectiveness of symbiotic associations between native rhizobia and temperate Australian Acacia: within-species interactions. Journal of Applied Ecology, 36, 398-408.

Burton, J. 1984. Legume inoculants and their use–a pocket manual, Rome: Food and Agriculture Organization of the United Nations. FAO, Rome (Italy). Land and Water Development Div. FAO, Rome (Italy). Plant Production and Protection Division.

Caiola, M. G. & Canini, A. 1993. Structure and physiology of Cycad coralloid roots. Plant Biosystem, 127, 428-445.

Cardenas, L., Vidali, L., Domínguez, J., Perez, H., Sanchez, F., Hepler, P. K. & Quinto, C. 1998. Rearrangement of actin microfilaments in plant root hairs responding to Rhizobium etli nodulation signals. Plant Physiology, 116, 871-877.

Castillo, M., Flores, M., Mavingui, P., Martinez-Romero, E., Palacios, R. & Hernandez, G. 1999. Increase in alfalfa nodulation, nitrogen fixation, and plant growth by specific DNA amplification in *Sinorhizobium meliloti*. Applied and Environmental Microbiology, 65, 2716-2722.

Catoira, R., Timmers, A., Maillet, F., Galera, C., Penmetsa, R. V., Cook, D., Denarie, J. & Gough, C. 2001. The HCL gene of Medicago truncatula controls Rhizobium-induced root hair curling. Development, 128, 1507-1518.

Chagas Junior, A. F., De Oliveira, L. A., De Castro, H. G., Cornelio, G. D. L., Dos Santos, G. R., Chagas, L. F. B. & Costa, J. D. L. 2013. Isolation and phenotypic characterization of rhizobia that nodulate cowpea in the Cerrado in Tocantins State, Brazil. Journal of Biotechnology and Biodiversity, 4.

Chatt, J. 1977. Prospects for New Nitrogen Fixation Processes. Philosophical Transactions of the Royal Society of London. B, Biological Sciences, 281, 243-248.

Chaudri, A. M., Mcgrath, S. P., Giller, K. E., Rietz, E. & Sauerbeck, D. R. 1993. Enumeration of indigenous Rhizobium leguminosarum biovar trifolii in soils previously treated with metal-contaminated sewage sludge. Soil Biology and Biochemistry, 25, 301-309.

Cheng, Q. 2008. Perspectives in biological nitrogen fixation research. Journal of Integrative Plant Biology, 50, 786-798.

Chirwa, P. W., Akinnifesi, F. K., Sileshi, G., Syampungani, S., Kalaba, F. K. & Ajayi, O. C. 2008. Opportunity for conserving and utilizing agrobiodiversity through agroforestry in Southern Africa. Biodiversity, 9, 45-48.

Cooper, J. 2007. Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue. Journal of Applied Microbiology, 103, 1355-1365.

Cooper, J., Wood, M. & Bjourson, A. 1985. Nodulation of Lotus pedunculatus in acid rooting solution by fast-and slow-growing rhizobia. Soil Biology and Biochemistry, 17, 487-492.

Cooper, J. E. & Scherer, H. W. 2012. Chapter 16 - Nitrogen Fixation. In: PETRA, M. (ed.) Marschner's Mineral Nutrition of Higher Plants (Third Edition). San Diego: Academic Press.

Corby, H. 1971. The shape of leguminous nodules and the colour of leguminous roots. Plant and Soil, 35, 305-314.

Dakora, F. D. & Phillips, D. A. 2002. Root exudates as mediators of mineral acquisition in low-nutrient environments. Plant and Soil, 245, 35-47.

Dart, R. K. 1996. *Microbiology for the analytical chemist*, Royal Society of Chemistry.

Deaker, R., Roughley, R. J. & Kennedy, I. R. 2004. Legume seed inoculation technology—a review. Soil Biology and Biochemistry, 36, 1275-1288.

Degefu, T., Wolde-Meskel, E. & Frostegård, Å. 2011. Multilocus sequence analyses reveal several unnamed Mesorhizobium genospecies nodulating Acacia species and Sesbania sesban trees in Southern regions of Ethiopia. Systematic and Applied microbiology, 34, 216-226.

Diouf, D., Samba-Mbaye, R., Lesueur, D., BA, A. T., Dreyfus, B., De Lajudie, P. & Neyra, M. 2007. Genetic diversity of *Acacia seyal* Del. rhizobial populations indigenous to Senegalese soils in relation to salinity and pH of the sampling sites. Microbial Ecology, 54, 553-566.

Dreyfus, B. & Dommergues, Y. 1981. Nodulation of *Acacia* species by fast and slow growing tropical strains of Rhizobium. Applied and Environmental Microbiology, 41, 97-99.

Eaglesham, A. & Ayanaba, A. 1984. Tropical stress ecology of rhizobia, root nodulation and legume fixation. Current Developments in Biological Nitrogen Fixation, 1-35.

Erker, B. & Brick, M. 2012. Legume Seed Inoculants. Sciences, 5, 06.

Everson, C., Everson, T. & Van Niekerk, W. 2009. Soil water competition in a temperate hedgerow agroforestry system in South Africa. Agroforestry Systems, 75, 211-221.

Felle, H. H., Kondorosi, É., Kondorosi, Á. & Schultze, M. 1999. Elevation of the cytosolic free [Ca^{2+}] is indispensable for the transduction of the Nod factor signal in alfalfa. Plant Physiology, 121, 273-280.

Franche, C., Lindström, K. & Elmerich, C. 2009. Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. Plant and Soil, 321, 35-59.

Fred, E. B., Baldwin, I. L. & Mccoy, E. 1932. Root nodule bacteria and leguminous plants, UW-Madison Libraries Parallel Press.

Frioni, L., Rodrıguez, A. & Meerhoff, M. 2001. Differentiation of rhizobia isolated from native legume trees in Uruguay. Applied Soil Ecology, 16, 275-282.

Fynn, R. W. S., Haynes, R. J. & O'connor, T. G. 2003. Burning causes long-term changes in soil organic matter content of a South African grassland. Soil Biology and Biochemistry, 35, 677-687.

Gage, D. J. 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. Microbiology and Molecular Biology Reviews, 68, 280-300.

Gage, D. J. & Margolin, W. 2000. Hanging by a thread: invasion of legume plants by rhizobia. Current Opinion in Microbiology, 3, 613-617.

Galiana, A., Prin, Y., Mallet, B., Gnahoua, G.-M., Poitel, M. & Diem, H. G. 1994. Inoculation of *Acacia mangium* with alginate beads containing selected *Bradyrhizobium*

strains under field conditions: long-term effect on plant growth and persistence of the introduced strains in soil. Applied and Environmental Microbiology, 60, 3974-3980.

Galli-Terasawa, L., Glienke-Blanco, C. & Hungria, M. 2003. Diversity of a soybean rhizobial population adapted to a Cerrados soil. World Journal of Microbiology and Biotechnology, 19, 933-939.

Garbeva, P., Van Veen, J. & Van Elsas, J. 2004. Microbial diversity in soil: selection of microbial populations by plant and soil type and implications for disease suppressiveness. Annual Review of Phytopathology., 42, 243-270.

Gaur, Y. & Sen, A. 1979. Cross inoculation group specificity in cicer-*Rhizobium* symbiosis. New Phytologist, 83, 745-754.

Germida, J. J. 1988. Growth of indigenous Rhizobium leguminosarum and Rhizobium meliloti in soils amended with organic nutrients. Applied and Environmental Microbiology, 54, 257-263.

Geurts, R. & Bisseling, T. 2002. Rhizobium Nod factor perception and signalling. The Plant Cell Online, 14, S239-S249.

Gilbert, N. 2012. African agriculture: Dirt poor. Nature, 483, 525-527.

Gopalakrishnan, S., Sathya, A., Vijayabharathi, R., Varshney, R. K., Gowda, C. L. & Krishnamurthy, L. Plant growth promoting rhizobia: challenges and opportunities. 3 Biotech, 5, 355-357.

Graham, P., Sadowsky, M., Keyser, H., Barnet, Y., Bradley, R., Cooper, J., De Ley, D., Jarvis, B., Roslycky, E. & Strijdom, B. 1991. Proposed minimal standards for the description of new genera and species of root-and stem-nodulating bacteria. International Journal of Systematic Bacteriology, 41, 582-587.

Graham, P. H., Draeger, K. J., Ferrey, M. L., Conroy, M. J., Hammer, B. E., Martinez, E., Aarons, S. R. & Quinto, C. 1994. Acid pH tolerance in strains of *Rhizobium* and *Bradyrhizobium*, and initial studies on the basis for acid tolerance of *Rhizobium tropici* UMR1899. Canadian Journal of Microbiology, 40, 198-207.

Granada, C. E., Strochein, M., Vargas, L. K., Bruxel, M., Sá, E. L. S. D. & Passaglia, L. M. 2014. Genetic diversity and symbiotic compatibility among rhizobial strains and *Desmodium incanum* and *Lotus* spp. plants. Genetics and Molecular Biology, 37, 396-405.

Hayward, R. (2004). The acacia tree: a sustainable resource for Africa. *An overview of research conducted by Oxford Forestry Institute, the Natural Environmental Research Council (NERC) Centre for Ecology and Hydrology and the University of Duncadee 1986 and 2004.* Rowes Printers , P.366.

Hernandez, L., Probst, A., Probst, J. L. & Ulrich, E. 2003. Heavy metal distribution in some French forest soils: evidence for atmospheric contamination. Science of the Total Environment, 312, 195-219.

Herrera, M. A., Bedmar, E. J. & Olivares, J. 1985. Host specificity of *Rhizobium* strains isolated from nitrogen-fixing trees and nitrogenase activities of strain GRH2 in symbiosis with *Prosopis chilensis*. Plant Science, 42, 177-182.

Herridge, D. F., Peoples, M. B. & Boddey, R. M. 2008. Global inputs of biological nitrogen fixation in agricultural systems. Plant and Soil, 311, 1-18.

Huss-Danell, K. 1997. Tansley Review No. 93. Actinorhizal symbioses and their N 2 fixation. New Phytologist, 375-405.

Ido, S. 2011. Evaluation of growth, nodulation and nitrogen fixation of two *Acacia* species under salt stress. World Applied Sciences Journal, 13, 256-265.

Ingenbleek, Y. 2006. The nutritional relationship linking sulfur to nitrogen in living organisms. The Journal of Nutrition, 136, 1641S-1651S.

Janczarek, M., Kutkowska, J., Piersiak, T. & Skorupska, A. 2010. *Rhizobium leguminosarum* bv. trifolii rosR is required for interaction with clover, biofilm formation and adaptation to the environment. BMC Microbiology, 10, 284.

Jida, M. & Assefa, F. 2014. Phenotypic diversity and plant growth promoting characteristics of *Mesorhizobium* species isolated from chickpea (*Cicer arietinum* L.) growing areas of Ethiopia. African Journal of Biotechnology, 11.

Jones, K. M., Kobayashi, H., Davies, B. W., Taga, M. E. & Walker, G. C. 2007. How rhizobial symbionts invade plants: the Sinorhizobium–Medicago model. Nature Reviews Microbiology, 5, 619-633.

Kanamori, N., Madsen, L. H., Radutoiu, S., Frantescu, M., Quistgaard, E. M., Miwa, H., Downie, J. A., James, E. K., Felle, H. H. & Haaning, L. L. 2006. From The Cover: A nucleoporin is required for induction of Ca^{2+} spiking in legume nodule development and essential for rhizobial and fungal symbiosis. Science Signaling, 103, 359.

Kawaka, F., Dida, M. M., Opala, P. A., Ombori, O., Maingi, J., Osoro, N., Muthini, M., Amoding, A., Mukaminega, D. & Muoma, J. 2014. Symbiotic Efficiency of Native Rhizobia Nodulating Common Bean (Phaseolus vulgaris L.) in Soils of Western Kenya. *International Scholarly Research Notices*, 2014.

KfiCfiK, Q. and Cevheri, C. 2013. Nodulation Study of Natural Forage Legume in Semiarid Region, Turkey. Pakistan Journal of Biological Sciences, 17, 535

Kyalangalilwa, B., Boatwright, J. S., Daru, B. H., Maurin, O. & Bank, M. 2013. Phylogenetic position and revised classification of *Acacia sl* (Fabaceae: Mimosoideae) in Africa, including new combinations in *Vachellia* and *Senegalia*. Botanical Journal of the Linnean Society, 172, 500-523.

L'taief, B., Sifi, B., Gtari, M., Zaman-Allah, M. & Lachaâl, M. 2007. Phenotypic and molecular characterization of chickpea rhizobia isolated from different areas of Tunisia. Canadian Journal of Microbiology, 53, 427-434.

Ladeiro, B. 2012. Saline agriculture in the 21st century: using salt contaminated resources to cope food requirements. Journal of Botany, 2012.

Laranjo, M., Branco, C., Soares, R., Alho, L., Carvalho, M. & Oliveira, S. 2002. Comparison of chickpea rhizobia isolates from diverse Portuguese natural populations based on symbiotic effectiveness and DNA fingerprint. Journal of Applied Microbiology, 92, 1043-1050.

Laurette, N. N., Maxémilienne, N. B., Henri, F., Souleymanou, A., Kamdem, K., Albert, N., Dieudonné, N. & François-Xavier, E. 2015. Isolation and Screening of Indigenous Bambara Groundnut (Vigna Subterranea) Nodulating Bacteria for their Tolerance to Some Environmental Stresses. American Journal of Microbiological Research, 3, 65-75.

Leafy, J. K., Singleton, P. W., Scowcroft, P. G. & Borthakur, D. 2006. Symbiotic diversity in the cosmopolitan genus Acacia. Symbiosis, 41, 107-117.

Leite, J., Seido, S. L., Passos, S. R., Xavier, G. R., Rumjanek, N. G. & Martins, L. M. V. 2009. Biodiversity of rhizobia associated with cowpea cultivars in soils of the lower half of the São Francisco River Valley. Revista Brasileira de Ciência do Solo, 33, 1215-1226.

Lewis, O. A. 1992. Plants & Nitrogen, Cambridge University Press.

Lindström, K., Murwira, M., Willems, A. & Altier, N. 2010. The biodiversity of beneficial microbe-host mutualism: the case of rhizobia. Research in Microbiology, 161, 453-463.

Lippi, D., DE Paolis, M., DI Mattia, E., Grego, S., Pietrosanti, T. & Cacciari, I. 2000. Effect of salinity on growth and starvation-survival of a tropical *Rhizobium* strain. Biology and Fertility of Soils, 30, 276-283.

Liu, J., Wang, E. T. & Chen, W. X. 2005. Diverse rhizobia associated with woody legumes *Wisteria sinensis*, *Cercis racemosa* and *Amorpha fruticosa* grown in the temperate zone of China. Systematic and Applied Microbiology, 28, 465-477.

Liu, T.-X. 2008. Soil ecology research developments, Nova Science Publishers. 19-29

Lodwig, E., Hosie, A., Bourdes, A., Findlay, K., Allaway, D., Karunakaran, R., Downie, J. & Poole, P. 2003. Amino-acid cycling drives nitrogen fixation in the legume–Rhizobium symbiosis. Nature, 422, 722-726.

Lupwayi, N. & Haque, I. 1994. Legume-Rhizobium technology manual, environmental science division, International Livestock Centre for Africa, Addis Ababa, Ethopia.

Maâtallah, J., Berraho, E., Munoz, S., Sanjuan, J. & Lluch, C. 2002. Phenotypic and molecular characterization of chickpea rhizobia isolated from different areas of Morocco. Journal of Applied Microbiology, 93, 531-540.

Macedo, M., Resende, A., Garcia, P., Boddey, R., Jantalia, C., Urquiaga, S., Campello, E. & Franco, A. 2008. Changes in soil C and N stocks and nutrient dynamics 13 years after recovery of degraded land using leguminous nitrogen-fixing trees. Forest Ecology and Management, 255, 1516-1524.

Maheshwari, D. K. 2010. Plant growth and health promoting bacteria, Springer Science & Business Media.

Mclean, P. & Dixon, R. 1981. Requirement of nifV gene for production of wild-type nitrogenase enzyme in Klebsiella pneumoniae.

Mhamdi, R., Laguerre, G., Aouani, M. E., Mars, M. & Amarger, N. 2002. Different species and symbiotic genotypes of field rhizobia can nodulate *Phaseolus vulgaris* in Tunisian soils. FEMS Microbiology Ecology, 41, 77-84.

Miličić, B., Delić, D., Kuzmanović, Đ., Stajković, O. & Jošić, D. 2006. Intrinsic antibiotic resistance of different *Bradyrhizobium japonicum* and *Rhizobium galegae* strains. Roumanian Biotechnological Letters,11, 2723-2731

Mirza, B. S., Mirza, M. S., Bano, A. & MALIK, K. A. 2007. Coinoculation of chickpea with Rhizobium isolates from roots and nodules and phytohormone-producing Enterobacter strains. Animal Production Science, 47, 1008-1015.

Mohamed, S., Smouni, A., Neyra, M., Kharchaf, D. & Filali-Maltouf, A. 2000. Phenotypic characteristics of root-nodulating bacteria isolated from Acacia spp. grown in Libya. Plant and soil, 224, 171-183.

Mohammadi, K., Sohrabi, Y., Heidari, G., Khalesro, S. & Majidi, M. 2012. Effective factors on biological nitrogen fixation. African Journal of Agricultural Research, 7, 1782-1788.

Moreira, F. M., Siqueira, J. O. & Brussaard, L. 2006. Soil biodiversity in Amazonian and other Brazilian Ecosystems, CABI.

Mpepereki, S., Makonese, F. & Wollum, A. 1997. Physiological characterization of indigenous rhizobia nodulating *Vigna unguiculata* in Zimbabwean soils. Symbiosis (Philadelphia, Pa.)(USA).

Mulongoy, K. 1995. Technical paper 2: Biological nitrogen fixation. Food and Agriculture Organization of the United Nations (FAO) Corporate Document Repository, ILRI Training Manual, 2.

Muthini, M., Maingi, J. M., Muoma, J. O., Amoding, A., Mukaminega, D., Osoro, N., Mgutu, A. & Ombori, O. 2013. Morphological assessment and effectiveness of indigenous rhizobia

isolates that nodulate *P. vulgaris* in water hyacinth compost testing field in Lake Victoria basin. Methodology.

Nandal, K., Sehrawat, A. R., Yadav, A. S., Vashishat, R. K. & Boora, K. S. 2005. High temperature-induced changes in exopolysaccharides, lipopolysaccharides and protein profile of heat-resistant mutants of *Rhizobium sp*. (Cajanus). Microbiological Research, 160, 367-373.

Niste, M., Vidican, R., Rotar, I. & Rodica, P. 2013. The Effect of pH Stress on the Survival of *Rhizobium Trifolii* and *Sinorhizobium Meliloti* in Vitro. Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Agriculture, 70, 449-450.

Obaton, M. 1971. Utilisation de mutants spontanes resistants aux antibiotiques pout l'etude ecologique des *Rhizobium*. Acad Sci Paris CR Ser D.

Oldroyd, G. E. & Dixon, R. 2014. Biotechnological solutions to the nitrogen problem. *Current opinion in biotechnology,* 26**,** 19-24.

Paracer, S. & Ahmadjian, V. 2000. Symbiosis: an introduction to biological associations, Oxford University Press Oxford.

Paudyal, S., Aryal, R. R., Chauhan, S. & Maheshwari, D. 2007. Effect of heavy metals on growth of *Rhizobium* strains and symbiotic efficiency of two species of tropical legumes. Scientific World, 5, 27-32.

Pearce, F. 2009. The nitrogen fix: breaking a costly addiction. Yale Environment, 360.

Perdomo, F., Echávez-Badel, R., Alameda, M. & Schröder, E. 1995. In vitro evaluation of bacteria for the biological control of *Macrophomina phaseolina*. World Journal of Microbiology and Biotechnology, 11, 183-185.

Pérez-Fernández, M., Valentine, A. & García-Mauriño, S. M. 2008. Rhizobial Diversity Associated with South African Legumes. Biological Nitrogen Fixation: Towards Poverty Alleviation through Sustainable Agriculture. Springer.

Pooley, E. 1998. Trees: Southern African green guide. Southern Book Publishers (Struik Publishing Group), Cape Town.

Predeepa, R. & Ravindran, D. 2010. Nodule formation, distribution and symbiotic efficacy of *Vigna unguiculata L.* under different soil salinity regimes. Emirates Journal of Food and Agriculture, 22.

Prévost, D., Drouin, P., Laberge, S., Bertrand, A., Cloutier, J. & Lévesque, G. 2003. Cold-adapted rhizobia for nitrogen fixation in temperate regions. Canadian Journal of Botany, 81, 1153-1161.

Qureshi, S. T. Phenotypic and molecular characterization of rhizobia nodulating *Cicer arietinum* L. and *Pisum sativum* L. isolated from different areas of Pakistan. Quaid-i-Azam University Islamabad, Pakistan 2010.

Rai, R., Dash, P. K., Mohapatra, T. & Singh, A. 2012a. Phenotypic and molecular characterization of indigenous rhizobia nodulating chickpea in India. Indian Journal of Experimental Biology, 50, 340.

Ramírez-Bahena, M.-H., Velázquez, E., Fernández-Santos, F., Peix, A., Martínez-Molina, E. & Mateos, P. F. 2009. Phenotypic, genotypic, and symbiotic diversities in strains nodulating clover in different soils in Spain. Canadian Journal of Microbiology, 55, 1207-1216.

Rao, N. S. 1991. Current developments in biological nitrogen fixation, Cambridge University Press.

Rejili, M., Lorite, M. J., Mahdhi, M., Pinilla, J. S., Ferchichi, A. & MARS, M. 2009. Genetic diversity of rhizobial populations recovered from three *Lotus* species cultivated in the infra-arid Tunisian soils. Progress in Natural Science, 19, 1079-1087.

Rinaudi, L., Fujishige, N. A., Hirsch, A. M., Banchio, E., Zorreguieta, A. & Giordano, W. 2006. Effects of nutritional and environmental conditions on Sinorhizobium meliloti biofilm formation. Research in microbiology, 157, 867-875.

Robin, L. & Carruthers, J. 2012. National Identity and International Science: The Case of Acacia. Historical Records of Australian Science, 23, 34-54.

Rodrigues, C. S., Laranjo, M. & Oliveira, S. 2006. Effect of heat and pH stress in the growth of chickpea mesorhizobia. Current Microbiology, 53, 1-7.

Rodríguez-Echeverría, S., Pérez-Fernández, M., Vlaar, S. & Finnan, T. 2003. Analysis of the legume–rhizobia symbiosis in shrubs from central western Spain. Journal of Applied Microbiology, 95, 1367-1374.

Sadowsky, M. J., Keyser, H. H. & Bohlool, B. B. 1983. Biochemical characterization of fast- and slow-growing rhizobia that nodulate soybeans. International Journal of Systematic Bacteriology, 33, 716-722.

Saikia, R. 2008. Microbial Biotechnology, New India Publishing.

Santos, J. O., Araújo, A. S., Gomes, R. L., Lopes, A. & Figueiredo, M. V. 2008. Rhizobia-*Phaseolus lunatus* symbiosis: Importance and diversity in tropical soils-A review. Dynamic Soil, Dynamic Plant, 2**,** 56-60.

Santos, P., Fortunato, A., Ribeiro, A. & Pawlowski, K. 2008. Chitinases in root nodules. Plant Biotechnology, 25, 299-307.

Santos, R., Herouart, D., Puppo, A. & Touati, D. 2000. Critical protective role of bacterial superoxide dismutase in Rhizobium–legume symbiosis. Molecular Microbiology, 38, 750-759.

Satyanarayana, T. & Johri, B. 2005. Microbial diversity: current perspectives and potential applications, IK International Pvt Ltd.

Scott, D., Burns, M., Raal, P. & Scholes, R. 1993. Dune sand fertility in relation to vegetation cover, as influenced by dredge mining, at Richards Bay, South Africa. Report to the Division of Forest Science and Technology, Council for Scientific and Industrial Research, Stellenbosch, South Africa.

Sharma, M. P., Srivastava, K. & Sharma, S. K. 2010. Biochemical characterization and metabolic diversity of soybean rhizobia isolated from Malwa region of central India. Plant Soil Environment, 56, 375-383.

Sharma, R. S., Mohmmed, A., Mishra, V. & Babu, C. R. 2005. Diversity in a promiscuous group of rhizobia from three *Sesbania* spp. colonizing ecologically distinct habitats of the semi-arid Delhi region. Research in Microbiology, 156, 57-67.

Shridhar, B. S. 2012. Review: Nitrogen Fixing Microorganisms. International Journal of.

Sieberer, B. J., Chabaud, M., Timmers, A. C., Monin, A., Fournier, J. & Barker, D. G. 2009. A nuclear-targeted cameleon demonstrates intranuclear Ca^{2+} spiking in *Medicago truncatula* root hairs in response to rhizobial nodulation factors. Plant Physiology, 151, 1197-1206.

Somasegaran, P. & Hoben, H. J. 1985. Methods in legume-Rhizobium technology, University of Hawaii NifTAL Project and MIRCEN, Department of Agronomy and Soil Science, Hawaii Institute of Tropical Agriculture and Human Resources, College of Tropical Agriculture and Human Resources.

Somasegaran, P. & Hoben, H. J. 1994. Collecting nodules and isolating rhizobia. Handbook for Rhizobia. Springer.

Sprent, J., Ardley, J. & James, E. 2013. From North to South: A latitudinal look at legume nodulation processes. South African Journal of Botany, 89, 31-41.

Srivastava, A. K., Gaiser, T., Paeth, H. & Ewert, F. 2012. The impact of climate change on Yam (Dioscorea alata) yield in the savanna zone of West Africa. Agriculture, Ecosystems & Environment, 153, 57-64.

Stowers, M. D. 1985. Carbon metabolism in Rhizobium species. Annual Reviews in Microbiology, 39, 89-108.

Suominen, L., Luukkainen, R., Roos, C. & Lindström, K. 2003. Activation of the nodA promoter by the nodD genes of Rhizobium galegae induced by synthetic flavonoids or Galega orientalis root exudate. FEMS Microbiology Letters, 219, 225-232.

Swelim, D., Ali, M. & Elham, I. E.-K. 2010. Some tree-legume-rhizobia are meagerly arising in Egyptian soil. Aust J Basic Appl Sci, 4, 1297-1304.

Taylor, C. & Barker, N. 2012. Species limits in *Vachellia (Acacia) karroo* (Mimosoideae: Leguminoseae): Evidence from automated ISSR DNA "fingerprinting". South African Journal of Botany, 83, 36-43.

Terefework, Z. 2002. Diversity and Phylogeny of *Rhizobium galegae*, and reflections on molecular evolution of rhizobium-legume symbiosis, University of Helsinki.

Terpolilli, J. J., Hood, G. A. & Poole, P. S. 2012. Chapter 5 - What Determines the Efficiency of N2-Fixing *Rhizobium*-Legume Symbioses? In: Robert, K. P. (ed.) Advances in Microbial Physiology. Academic Press.

Todar, K. 2011. Bacterial resistance to antibiotics (page 3). Todar's online textbook of bacteriology**,** 4.

Turk, D. & Keyser, H. H. 1992. Rhizobia that nodulate tree legumes: specificity of the host for nodulation and effectiveness. Canadian Journal of Microbiology, 38, 451-460.

Van Wyk, B. & Van Wyk, P. 1997. Field guide to trees of Southern Africa, Struik Pub.

Vance, C. P., Graham, P. H. & Allan, D. L. 2002. Biological Nitrogen Fixation: Phosphorus-A Critical Future Need? Nitrogen fixation: From molecules to crop productivity. Springer.

Vasilica, S., Gament, E., Cornea, C. P., Voaideş, C., Mirela, D. & Plopeanu, G. 2011. Effects of heavy metal from polluted soils on the *Rhizobium* diversity. Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 39, 88-95.

Vincent, J. M. 1970. A manual for the practical study of the root-nodule bacteria. A manual for the practical study of the root-nodule bacteria.

Wagner, S. 2011. Biological Nitrogen Fixation. Nature Education Knowledge, 2, 14.

Wasson, A. P., Pellerone, F. I. & Mathesius, U. 2006. Silencing the flavonoid pathway in Medicago truncatula inhibits root nodule formation and prevents auxin transport regulation by rhizobia. The Plant Cell Online, 18, 1617-1629.

Wassem, R., Kobayashi, H., Kambara, K., Le Quéré, A., Walker, G. C., Broughton, W. J. & Deakin, W. J. 2008. TtsI regulates symbiotic genes in *Rhizobium* species NGR234 by binding to tts boxes. Molecular Microbiology, 68**,** 736-748.

Wolde-Meskel, E., Terefework, Z., Lindström, K. & Frostegård, Å. 2004. Rhizobia nodulating African *Acacia* spp. and *Sesbania sesban* trees in southern Ethiopian soils are metabolically and genomically diverse. Soil Biology and Biochemistry, 36, 2013-2025.

Xavier, G., Martins, L., Neves, M. & Rumjanek, N. 1998. Edaphic factors as determinants for the distribution of intrinsic antibiotic resistance in a cowpea rhizobia population. Biology and Fertility of Soils, 27, 386-392.

Zahran, H. 1998. Structure of root nodules and nitrogen fixation in Egyptian wild herb legumes. Biologia plantarum, 41, 575-585.

Zahran, H. H. 1999. Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. Microbiology and Molecular Biology Reviews, 63, 968-989.

Zahran, H. H. 2001. Rhizobia from wild legumes: diversity, taxonomy, ecology, nitrogen fixation and biotechnology. Journal of Biotechnology, 91, 143-153.

Zerhari, K., Aurag, J., Khbaya, B., Kharchaf, D. & Filali-Maltouf, A. 2000. Phenotypic characteristics of rhizobia isolates nodulating *Acacia* species in the arid and Saharan regions of Morocco. Letters in applied microbiology, 30, 351-357.

Zilli, J. É., Valisheski, R. R., Freire Filho, F. R., Neves, M. C. P. & Rumjanek, N. G. 2004. Assessment of cowpea rhizobium diversity in Cerrado areas of north eastern Brazil. Brazilian Journal of Microbiology, 35, 281-287.