Phenotypic characterization of rhizobia isolates and distribution of *Burkholderia* rhizobia in the Core Cape Subregion.

by

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Declaration

I hereby declare that all of the work in the document is my own. The thesis is submitted for the degree of Master of Science in the Department of Botany, University of Cape Town. It has not been for any degree or examination at any other university.

Kolisa Yola Sinyanya

Abstract

The Core Cape Subregion (CCR) is well known for its low nutrient and low pH soils, which harbour a variety of alpha and beta- Proteobacteria associated with a diversity of legume species. Soil bacteria are important for ecological processes and are influenced mostly by edaphic factors such as salinity and pH, and climatic conditions such as temperature. Recent studies have shown that nitrogen fixing *Burkholderia* form associations with legumes members of, among others, tribes Crotalarieae, Podalyrieae and Indigofereae. Selected rhizobia that included *Burkholderia* and *Mesorhizobium*, the two largest rhizobia genera in the isolated in the CCR, and representing beta- and alpha- Proteobacteria were phenotypically characterized to determine the tolerances to abiotic conditions. In a second study, glasshouse trapping experiments were conducted using legume species *Podalyria calyptrata* and *Indigofera filifolia* grown in 13 soils collected from diverse localities of the CCR, to determine the phylogenetic distribution of *Burkholderia* species in diverse soils of the CCR.

To phenotypically characterize rhizobia isolated from a previous study, 29 isolates from representative legume-nodules of 13 different localities were grown under laboratory conditions. Isolates were phenotypically characterized for colony morphology, growth temperature, carbon source, salinity and pH tolerance. Morphological results revealed that majority of the tested isolates were white opaque, rod shaped and fast growing. Exceptions were found in colour where five strains produced a milky pigment, two were watery translucent, observation of bacteriod-shape among six symbionts, and one slow growth isolate that grew after 7 days. The standard Mannitol medium proved to be the best carbon source because all 29 isolates grew successfully, followed by glucose (27 isolates), sucrose medium (25 isolates) and least in starchy medium (19 isolates). Twenty four endosymbionts tolerated temperatures of 30°C, 13 isolates at 35°C and only 12 tolerated 40°C. The standard growth temperature of 25°C was the most favourable temperature for all rhizobia isolates. Majority of the strains responded exceptionally well to NaCl concentrations between 0 and 1% whereas at higher sodium concentrations (>1.5%), the tolerance decreased rapidly. Only 7 isolates responded to

2% NaCl and 3 isolates at NaCl of 3%. Optical density tests showed a higher tolerance for acidic conditions at pH 3 and 4 in *Mesorhizobium* when compared to *Burkholderia*. At pH 5, *Bradyrhizobium* had the highest growth, at pH 6 and pH 8 *Mesorhizobium* had the highest growth. The characterization showed that there is phenotypic diversity among the different strains studied and that they were predominantly white opaque, rod shaped, fast growing, and grow well in media with Mannitol as the carbon source. Symbionts grew best in temperatures of 25°C, salinity concentrations between 0 and 1% and *Mesorhizobium* had the highest tolerance towards low acidic conditions.

The study further investigated the distribution of nodule forming *Burkholderia* species in soils from different sites of the CCR. This was done by collecting soil from 13 localities of the CCR and conducting glasshouse trapping experiments using *Podalyria calyptrata* and *Indigofera filifolia* as host plants. Nodules from the glasshouse grown plants were collected and rhizobia isolated and sequenced for standard markers. Isolates from 6 locations were distributed in 5 different clades of the Bayesian consensus tree based on recombinase A (recA). Both *P. calyptrata* and *I. filifolia* formed symbioses with *Burkholderia* strains regardless of the geographic origin of the soils. The soils used for the trapping were also analysed for macronutrients, available phosphorus and pH. To determine similarities and/or differences in the nutrient concentration of soils from the various sites, univariate and multivariate approaches were employed. The univariate analysis which tested the equality of means of each nutrient element and pH revealed that the 6 sites analysed had significantly different nutrients ($P<0.05$) levels. Principal component analysis (PCA) showed that the localities were separated by soil nutritional status where soil from Jonkershoek, Rhodes Memorial and St Helena Bay soils clearly separated from each other while soils from Bainskloof, Hopsvallei, and Stilbaai showed similarities. The study revealed that *Burkholderia* symbionts cover a wide range of geographic localities and are not restricted by soil type, nutrient content or legume species. Overall, the characterization study showed that there is phenotypic diversity among the different strains studied and *Burkholderia* are common, diverse and *I. filifolia* and *P. calyptrata* have a preference for *Burkholderia* in the Core Cape Subregion.

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Chapter 1

General Introduction

The Core Cape Subregion

The Core Cape Subregion (CCR), previously known as the Cape Floristic Kingdom or the Cape Floristic Region, is the area of about 90 760 km^2 (Manning & Goldblatt, 2012) surrounding the coastline along the south-western tip of the African continent (Figure 1.1). The CCR lies between latitudes 31° and 34°30′S and represents the smallest of the six floral kingdoms of the world with outstanding diversity and endemism that has attracted vast scientific research (Bond & Goldblatt, 1984). This temperate biodiversity hotspot, accommodates a unique assemblage of about 9300 vascular plant species (ferns and fern allies, gymnosperms and flowering plants), about 69% of which are endemic to the Subregion (Forest et al. 2007; Mittermeier et al. 1998; Myers et al., 2000). In addition, five of South Africa's 12 endemic plant families and 109 endemic genera are restricted to the CCR (Goldblatt & Manning, 2000; Manning & Goldblatt, 2012). The high endemism indicates that majority of the local biodiversity originates mainly from the confines of the CCR (Goldblatt & Manning, 2000). Fabaceae in the CCR incorporates approximately 764 species (634 (83%) endemism) in 43 genera (8 of the genera known to be endemic) belonging to 18 tribes (Goldblatt & Manning, 2002).

Geologically, the Cape Region comprises of soils from pre-Carboniferous age (more than 400 mya) rock (Manning & Goldblatt, 2012). The region is primarily made up of a mosaic of sandstone and shale substrates from which varieties of soil types result. The edaphic diversity is a product of soil diversity which includes local areas made up of limestone and granite derived soils. Erosional patterns vary on two rock types, coarse grained sandy soils and richer clay soils, resulting in mountains consisting primarily of sandstone rocks and valleys of shale (Cowling et al. 2009). Granitic schists are found in deep valleys, in mid slope and along the west coast, whereas limestones are exposed near the coast along the southern coast, from the Agulhas Peninsula to Mossel Bay. The physical and chemical differences of the soil type play a vital role as an environmental determinant of plant distribution with primary functions that include nutrient supply, moisture retention and anchorage (Forest et al. 2007; Goldblatt, 1997). Therefore, the CCR landscape is a mosaic of coastal limestones and deep sands with valleys of shale and granite soils, and is one of the five temperate Mediterranean-type systems (Barbero et al. 1992). Climate variability is characterized by intensely seasonal winter rainfalls and hot, arid summers, on the west of the CCR. The eastern part of the biodiversity region is a summer rainfall region with aseasonal rainfall (most of the rain is in the summer but there is some winter rain), with cooler summers and most of the precipitation, while the southern coast is seasonal and receives rainfall throughout the year (Carlson et al.2011; Manning & Goldblatt, 2012).

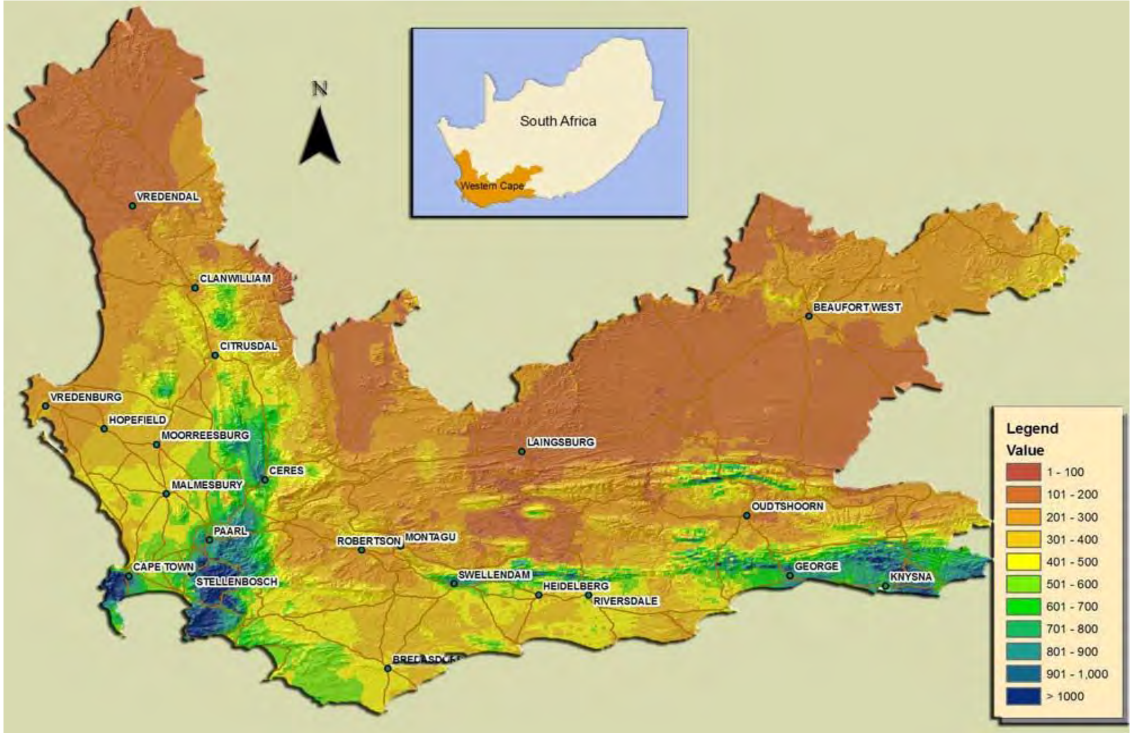

Figure 1.1: The Core Cape Subregions mean annual rainfall (based on Wallace, 2003).

Floristic structure of the CCR consists of approximately 20 families (Manning & Goldblatt, 2012) whereby Asteraceae and Fabaceae (Leguminosae) are the two largest families, making up about 20% of the total plant species found in the area. Fabaceae is the third largest and one of the most widely distributed plant families of the world (Moulin et al, 2001; Sprent, 2012). Leguminous plants are predominantly beneficial for both human consumption and soil fertility. The family has high economic significance due to the ability to fix atmospheric nitrogen which takes place through a symbiotic interaction between host plants and soil bacteria, jointly known as rhizobia. Nevertheless, some genera of legumes are known to have less nodulation successes than others. Most nodulations have been recorded in subfamilies Papilionoideae, Mimosoideae and less in Caesalpinioideae (Sprent, 2007). Cape legumes mostly belong to Papilionoideae which are widely distributed, while other species inhabit specific regions of the floristic region (Elliott et al. 2007; Lemaire et al. 2015).

Rhizobia diversity

Collectively, rhizobia refers to soil bacteria capable of fixing nitrogen by forming symbiotic associations with both leguminous and some non-leguminous plants, parasponia in the family Ulmaceae (Kempel et al. 2009; Udvardi & Poole, 2013; Young & Haukka, 1996). Rhizobia are known to play a substantial role in legume performance and the overall ecosystem productivity. The nitrogen which is provided by these bacteria improves the nutritional quality of plants and can also be utilised in the synthesis of defence compounds which may include alkaloids (nitrogen based) and glucosinolates (nitrogen and sulphur based) (Kempel et al. 2009).

Phylogenetically, rhizobia are paraphyletic, with their distribution intermingled with non-symbiotic, non-rhizobial bacteria. They are dispersed within distantly related lineages of alpha and beta subclasses of phylum *Proteobacteria* (Figure 1.2) (Dresler-Nurmi et al. 2009; Gyaneshwar et al. 2011). Rhizobia can inhabit both the soil and the nodule with an ability to survive in a facultative interface whereby the rhizobia persist in a free-living stage. Outside the plant, soil bacteria can continue growing, proliferating and multiplying in the rhizosphere for a number of years. This is due to their saprophytic nature when the rhizobia are still in the non-symbiotic stage and exchanging genetic information with other microsymbionts in the soil (Perret et al. 2000; Zahran, 2001).

Rhizobia are distinguished from other living non-symbiotic nitrogen-fixing bacteria by their ability to synthesize Nod factor molecules which form a "molecular key" to communicate with their host legume plant (Dakora, 1994; Perret et al. 2000; Franche et al. 2009). All rhizobia were first classified into one genus, *Rhizobium* but the classification criteria was later divided into fast-growing *Rhizobium* and slow growing *Bradyrhizobium* (Willems, 2006). Classification into slow growing and fast growing was deliberated by means of sequence divergences of small 16S rRNA and large 23S rRNA subunits of ribosomal RNA (Figure 1.2) (Dresler-Nurmi et al. 2009; Willems & Collins, 1993). Rhizobia which lead to nodulation in legumes were conventionally known to be limited to a narrow number of genera in the family Rhizobiales (Alpha-proteobacteria) (Sahgal & Johri, 2003). Rhizobia included in the Alpha-proteobacteria are classified according to growth rate and consists of the fast growing genera *Agrobacterium, Allorhizobium, Mesorhizobium, Rhizobium, Sinorhizobium, Devosia, Methylobacterium, Ochrobactrum*, *Phyllobacterium* and the slow growing genera *Azorhizobium* and *Bradyrhizobium* (De Meyer et al. 2011; Sahgal & Johri, 2003; Young & Haukka, 1996)*.* Beta-proteobacteria is made-up of aerobic bacteria which include genera *Burkholderia* and *Cupriavidus* (Makkar & Casida, 1987; Zahran, 2001). Beta-rhizobia were first unpredictably discovered in South African papilinoids (Moulin et al. 2001). The strain was isolated from *Aspalathus carnosa* which was thought to be nodulated by alpha-rhizobia belonging to the genus *Bradyrhizobium*. Phylogenetic analyses of 16S rRNA sequence data led to discovery that the isolate were closely related to already identified strains: *Burkholderia kururiensis* (96.9% identity), *B. brasilense* (96.8% identity) and *B. graminis* (96.8% identity) and belonged to the β-subclass of Proteobacteria.

The CCR soils harbour a diversity of rhizobia which are within distantly related lineages of alpha (α-rhizobia) and beta (β-rhizobia) subdivisions of Proteobacteria (Dlodlo, 2012; Kanu & Dakora, 2012; Lemaire et al. 2015). All CCR indigenous legumes described to date form a symbiotic association with rhizobia which facilitate nodulation and nitrogen fixation and studies (Ardley et al. 2013; De Meyer et al. 2013; Elliott, et al. 2008; Gerding et al. 2012; Howieson et al. 2013; Lemaire et al. 2015a; Lemaire et al. 2015b; Liu et al. 2014; Moulin et al. 2001) on rhizobia diversity have uncovered these to comprise *Burkholderia, Bradyrhizobium, Mesorhizobium, Rhizobium, Ensifer, Azorhizobia and Mycobacteria*. These rhizobia have been isolated from legumes in tribes Indigofereae, Crotalarieae, Podalyrieae, Phaseoleae, Psoraleeae, Genisteae, Millettieae, Acacieae, Galegeae and Sesbanieae. Cape *Burkholderia* specifically encompasses diverse host associations with lineages from tribes Crotalarieae, Hypocalypteae, Indigoferea, Phaseoleae and Podalyrieae (Lemaire et al. 2015a).

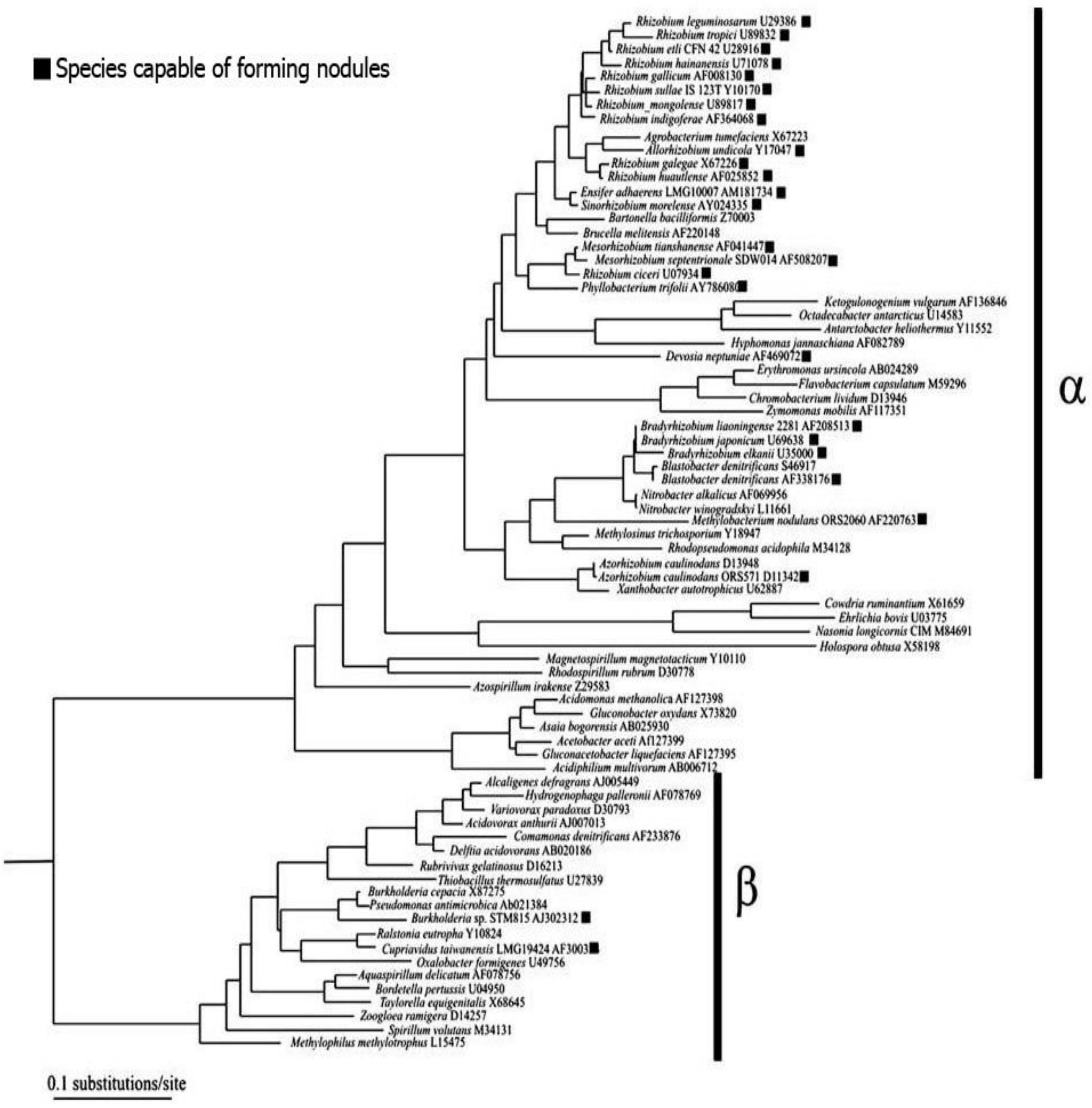

Figure 1.2: Maximum Likelihood tree based on 16S rRNA gene from 75 taxa from α- and β- subdivisions of Proteobacteria (adapted from Dresler-Nurmi et al. 2009).

Biogeography of rhizobia

Microorganisms which include species of rhizobia may be the most widely distributed and diverse organisms globally, but their continental distribution and large-scale ecological ranges are poorly understood. In South African papilinoids, rhizobia are mostly found in the CCR in diverse tribes, including Astragaleae, Crotalarieae, Genisteae, Indigofereae, Millettieae, Phaseoleae, Podalyrieae and Psoraleeae (Beukes et al. 2013; Botha et al. 2002; De Meyer et al. 2013a; Lemaire et al. 2015a; Mavengere et al. 2014; Moulin et al. 2001). Microbial population sizes are enormous and there are low probabilities of small scale extinctions. There are very high dispersal chances and bacteria can be atmospherically dispersed between continents (Fenchel, 2003). For example , a high diversity of rhizobia species have been isolated from diverse niches in mimosoid legumes in Central and South America (Brazil, Mexico,Puerto Rico), Asia (Taiwan) and Australasia (Papua New Guinea) (Geoffrey N. Elliott et al. 2009; Fauvart et al. 2011; Wang et al. 1999). Rhizobial adaptations to the ecosystem can be hindered by soil conditions and by the environmental stresses relating to host specificity and adaptation (Han et al. 2009; Silva et al. 2005). Nemergut et al. (2011) demonstrated that a large majority of rhizobia are confined to limited distributions in specific habitat types. Their investigations also revealed that there is a positive correlation between the abundance of the rhizobia and their distribution within the habitat types. Soil pH plays the most important role in determining the distribution of rhizobial communities (Zhang et al. 2011) whereas abiotic (temperature, soil, pH, and rainfall) and biotic factors (genotype of host plant and distribution of the species) can affect the diversity of the rhizobia species (Lu et al. 2009). Details of effects by abiotic factors on the growth of rhizobia are presented in chapter 2.

Rhizobia growth conditions

In their natural habitats, rhizobia tolerate various environmental stresses including climate change, soil aridity and rigorous agricultural practices (Ruiz-Díez et al. 2012). In the plant-rhizobia symbiosis it is significant to have compatible rhizobia which are tolerant of the environmental stresses including temperature, salinity and pH (Howieson & Ballard, 2004). The compatible rhizobia are essential in effective biological nitrogen fixation which reduces the use of fertilizers thereby improving land remediation and promoting increased plant yields. Effectiveness of the stresses depends on both the rhizobia species and the legume it associates with (Zahran, 1991; Osa-Afiana & Alexander, 1982; Yadav & Vyas, 1973). Studies (Howieson & Ballard, 2004; Slattery et al. 2001) have shown that abiotic factors pose the most stress to the success of rhizobia populations in the soil as proliferations decrease under stressful environment. Several environmental factors shape the structure and activity of rhizobia populations in the rhizosphere with soil pH and temperature often considered to be the major abiotic factors in determining bacterial community diversity.

Rationale

The CCR comprises vast rhizobial diversity of native Fynbos legumes of the papilionoid tribes in both alpha- and beta- lineages and environmental factors, including site elevation, can influence genetic disparity in *Burkholderia* and soil acidity within *Mesorhizobium* (Lemaire *et al.* 2015a). The rhizobia nodulating some legumes in the CCR were isolated and phylogenetically characterized by Dlodlo (2012) and Lemaire, et al (2015a). However, the morphological and phenotypic characteristics of the isolates are not yet known. With the soil and environmental variability of the CCR, it was important, it was important to determine the tolerant levels of the isolates to varying growth conditions. This study therefore phenotypically characterized the isolates morphologically by exposing them to varying growth conditions including different carbon sources, temperature, salinity and pH.Ecological environments of the CCR are vastly diverse as a result of differences in edaphic factors, topography, climatic conditions and the distribution, but their effect on occurrence of *Burkholderia* within the region is not yet well understood. Thus, the study also assessed the occurrence and diversity of *Burkholderia* in the CCR and to determine the specificity of the symbiosis between *P. calyptrata* or *I. filifolia* and *Burkholderia* rhizobia. This was achieved by inoculating the host plants, known to form symbiotic associations with *Burkholderia*, with different soil collected from 13 locations in the CCR.

Aims and objectives

Objectives:

1. To phenotypically characterize selected isolates representing alpha- and beta-proteobacteria from a previous study (Dlodlo, 2012) and examine their ability to induce nodule formation on Siratro.
2. To investigate the distribution and occurrence of *Burkholderia* species isolated from soils collected in diverse sites of the CCR.

Hypotheses:

1. It is expected that beta- rhizobia isolates will be more tolerant of the abiotic factors tested than alpha- rhizobia due to beta-rhizobia association with acidic and infertile soils.
2. It is expected that *Burkholderia* isolates from *Indigofera filifolia* and *Podalyria calyptrata* will be phylogenetically different.

Thesis outline

There are four chapters in this thesis, chapter one being the introduction with objectives and hypotheses. Chapter two presented the results addressing the first objective that is to phenotypically characterize selected isolates representing alpha- and beta- proteobacteria from a previous study (Dlodlo, 2012). Data were collected from experimental set-ups where the isolates were grown in media with varying growth conditions including different carbon sources, temperature, salinity and pH. The results in chapter three were from glasshouse experiment aimed at investigating the occurrence and distribution of *Burkholderia* species in soils from diverse sites of the CCR. Chapters two and three are written as independent chapters, but their introductions supplement the introduction chapter (Chapter 1), with some degree of repetitions. The general discussion and synthesis of the thesis are presented in chapter four.

Chapter 2

Phenotypic characterization of Cape rhizobia

Introduction

Soil bacteria are important for ecological processes and are influenced mostly by edaphic factors such as salinity and pH, and climatic conditions such as temperature. Rhizobia are beneficial bacteria in the soil that form symbiosis with plants predominantly in the family Fabaceae, inducing nodule formation and fixing atmospheric nitrogen. Rhizobia can colonize both the soil and the nodule with an ability to survive in a facultative interface in soil whereby the rhizobia persist in a free-living stage. Outside the plant, soil bacteria can continue growing, proliferating and multiplying in the rhizosphere for a number of years. This is due to their saprophytic nature when the rhizobia are still in the non-symbiotic stage and exchanging genetic information with other microsymbionts in the soil (Perret et al. 2000; Zahran 2001).

In their natural habitats, rhizobia tolerate various environmental stresses which are due to climate variation, soil aridity and rigorous agricultural practices (Ruiz-Díez et al. 2012). Rhizobia populations can be negatively affected by the stressful or competitive environments (Prévost et al. 2012) and the conditions can affect the establishment of an efficient nitrogen-fixing symbiosis (Maâtallah et al. 2002). The acidity of the soil is one of the edaphic factors that affect rhizobia establishment and activities. The pH content of the soil highly depends on the geology and soil type of the area (Howieson & Ballard, 2004) and fast growing rhizobia species are commonly found to proliferate and form wider populations in soils consisting of neutral pH and fine textured soils.

Temperature is significant for the success of plant-rhizobia symbiosis as it impacts almost all the stages of the association (Zahran, 1999). Low temperatures can directly affect rhizobial multiplication, cellular organisation and the uptake of nutrients (Sardesai & Babu, 2001). Organisms therefore use various mechanisms which include altering of membrane fluidity and induction of anti-freeze proteins and cold shock proteins, to adapt to temperature stress (Ermolenko & Makhatadze, 2002; Phadtare et al. 1999; Schumann, Inouye, & Phadtare, 2008). For example, at freezing temperatures (sub-zero) microbes are highly likely to die due to cellular collapse which is associated with DNA and cellular membrane damage (Sreedhar et al. 2006). At high temperatures and depending on the rhizobia species, leghaemoglobin synthesis and nitrogenase activity levels drop (Lira Junior et al. 2005). Some studies (Osa-Afiana & Alexander, 1982; Laranjo & Oliveira, 2011) postulate that rhizobia isolated from tropical legumes tolerate high temperatures with maximum growth temperature ranges of between 30°C and 42°C.

Salinity stress hinders the success of legume yields and consequently, the early stages of plant-rhizobia symbiosis. Constraints of salt stress may include nutrient imbalances which are a result of loss of control on the nutrient uptake. Due to salinity stress, ions speedily expand in the cytoplasm and slow down enzyme activity or they may accumulate in the cell walls and dehydrate the cell. Root hairs may not reach the curling phase or be deformed in some instances, leading to the inhibition of nodule formation (Zahran, 1999). Rhizobia have a higher tolerance for salt compared to leguminous plants, as soil bacteria survive in far more saline environments than their symbiotic partners (Laranjo & Oliveira, 2011). However, the symbiosis can be negatively affected in terms of hampering the growth and proliferation of rhizobia in the rhizosphere. Salinity also reduces root growth of the legume which, further decreases root colonization sites (Kulkarni et al. 2000; Zahran 1991).

The soil environment comprises different types of carbohydrates resulting from plants, and microorganisms. These are available to rhizobia as carbon sources (Reid & Abratt, 2005). For the laboratory culturing of rhizobia, it is important to identify the most appropriate source of carbon for the rhizobia that is inexpensive and easily available. Mannitol is the most widely used source of carbon for culturing rhizobia and is known to induce protection against stressful environments (Wisselink et al. 2002; Vélëz et al. 2007). However, sucrose is the most environmentally abundant disaccharide due to higher plant tissues origin (Kitaoka & Hayashi, 2002).

Lie *et al*., 1987 states that legumes have over the years evolved with their specific rhizobia. This means that legumes are highly likely to form nitrogen fixing association with a wider diversity of rhizobia species when grown in soils from their centres of diversity where a high degree of genetic variation occurs. Lemaire et al., (2015a) studied the rhizobial diversity and host preference of 65 native fynbos legumes of 9 papilionoid legume tribes and revealed that the symbionts belonged to both alpha- (*Azorhizobium*, *Bradyrhizobium*, *Ensifer, Mesorhizobium* and *Rhizobium*) and beta-rhizobial (*Burkholderia*) lineages. The study also reported that rhizobial genera *Burkholderia* and *Mesorhizobium* were the most dominant as they nodulated the widest ranges of native legume hosts. In their study using molecular methods, (Dlodlo, 2012; Lemaire et al. 2015) demonstrated that tribes Crotalarieae and Indigofereae showed the highest promiscuity and formed symbiotic associations with both alpha- and beta-rhizobia. On the other hand, the tribes Psoraleeae and Podalyrieae were exclusively nodulated by *Mesorhizobium* and *Burkholderia* respectively.

This study aimed to phenotypically characterize endosymbionts isolated from a previous study (Dlodlo, 2012) and to investigate their ability to form nodules on *Macroptilium atropurpureum* (Siratro) in order to authenticate them. Selected rhizobia that included *Burkholderia* and *Mesorhizobium*, representing beta- and alpha- Proteobacteria (Figures 2.1, 2.2) were characterized. It was expected that beta rhizobia isolates will be more tolerant to the edaphic and environmental abiotic factors conditions tested than the alpha rhizobia because the beta-rhizobia is associated with acidic and infertile soils (Beukes et al. 2013; Lemaire et al. 2015a; Stopnisek et al. 2013).

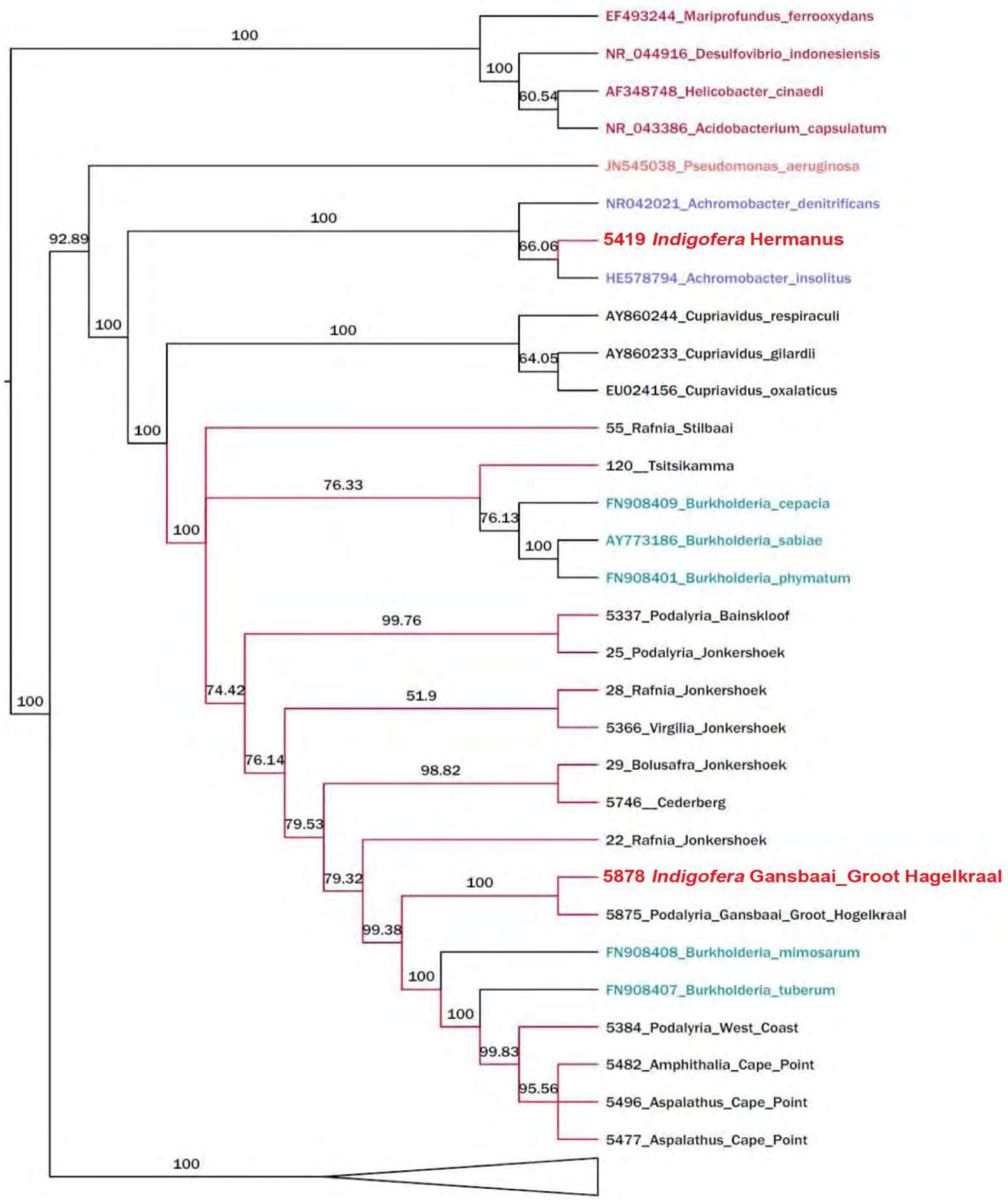

Figure 2.1: Bayesian 50% majority rule consensus tree of 4 main clades (members in beta-Protebacteria) based on CCR symbionts sequences together with reference strains from GenBank (Dlodlo, 2012). Strains used in current study highlighted in red.

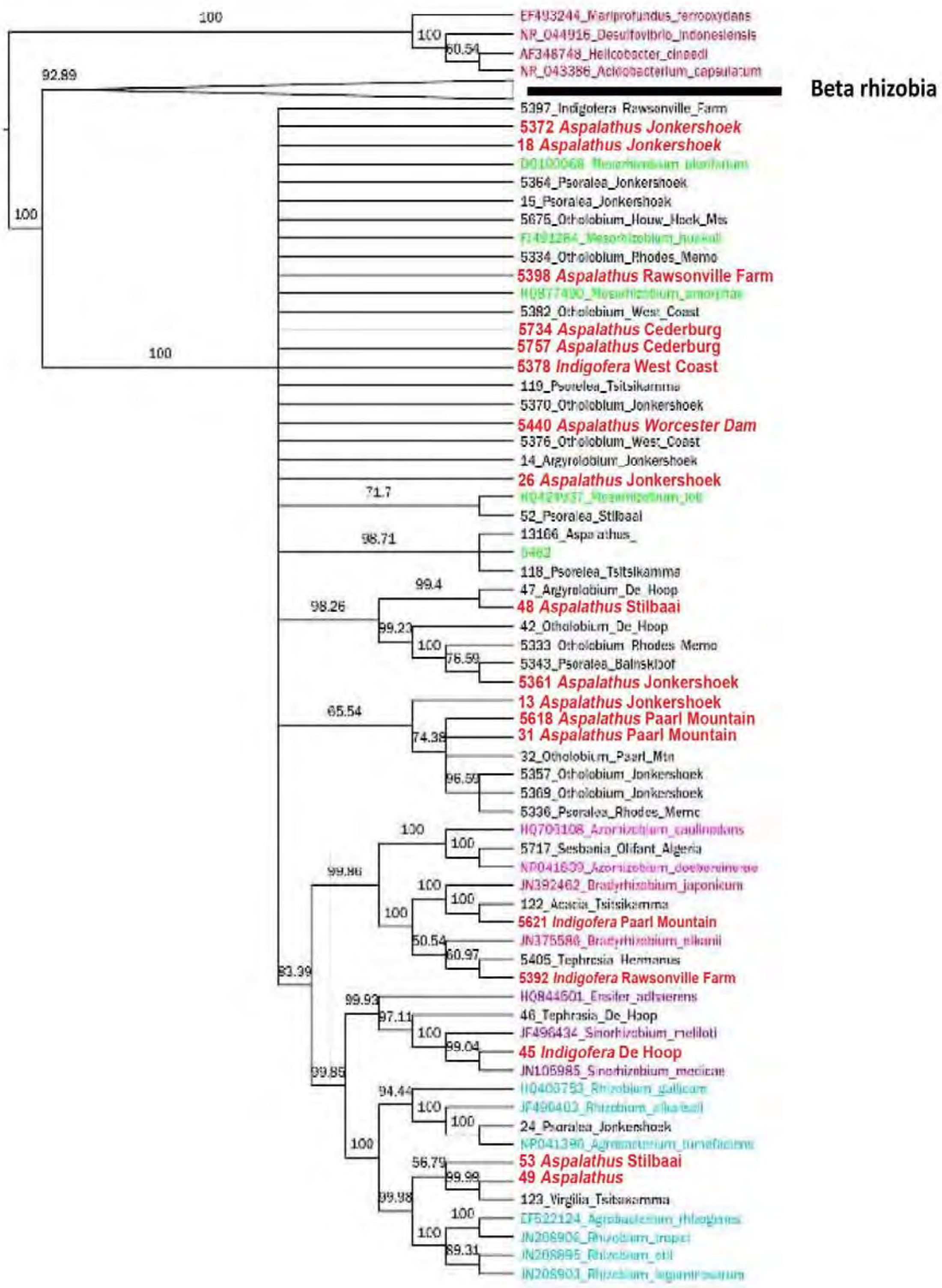

Figure 2.2: Bayesian 50% majority rule consensus tree of 6 main clades (members in alpha-Protebacteria) based on CCR symbionts sequences together with reference strains from GenBank (Dlodlo, 2012). Strains used in current study highlighted in red.

Materials and methods

Rhizobia authentication

Rhizobia authentication was done for selected isolates from *Aspalathus* and *Indigofera* species using the promiscuous crop plant, *Macroptilium atropurpureum* (Siratro), a substitute due to the lack of seeds of original hosts. The Siratro plants were inoculated with 24 strains belonging to both alpha- and beta- Proteobacteria to test nodulation ability (Table 2.1).

The seeds were manually scarified by rubbing seeds between sandpaper and surface sterilized by immersing the seeds in 70% ethanol, and thereafter dipped in 4% hypochlorite solution using a tea strainer (Tiwari et al. 2012). Seeds were thoroughly rinsed in eight changes of sterile distilled water and soaked overnight in boiled water. Seeds were then germinated on water agar plates for about 3 days in a growth chamber regulated to a temperature of 25°C and light intensity of between 120 and 250 $\mu mol.m^{-2}.s^{-1}$. Germinated seeds showing protrusion of a hypocotyl were transplanted into pots filled with sterile acid washed sand. A watering tube was inserted in the midpoint of the pot and a layer of polyvinyl chloride beads were added to cover the sand to minimize airborne contamination. Three replicate pots were used for each of the isolate and each pot planted with 3 seedlings. When the seedlings emerged through the sterile sand and layer of polyvinyl chloride beads, they were inoculated with the rhizobial isolate under a laminar flow hood. Rhizobia inoculum was prepared by growing the isolates in 250 ml sterile conical flasks containing 25 ml of sterile Mannitol growth medium. Each flask was inoculated with each of the 24 isolates and allowed to grow in aerobic conditions on a shaker. Depending on rhizobia strain, isolates were allowed to grow for up to 12 days with daily monitoring to detect any contamination. Plants were watered with sterile distilled water every second day through the watering tubes. The plants were grown (Figure 2.3, 2.4) aseptically in a glasshouse experiment and harvested approximately 8 weeks after planting. At harvest, plants were examined for root nodulation.

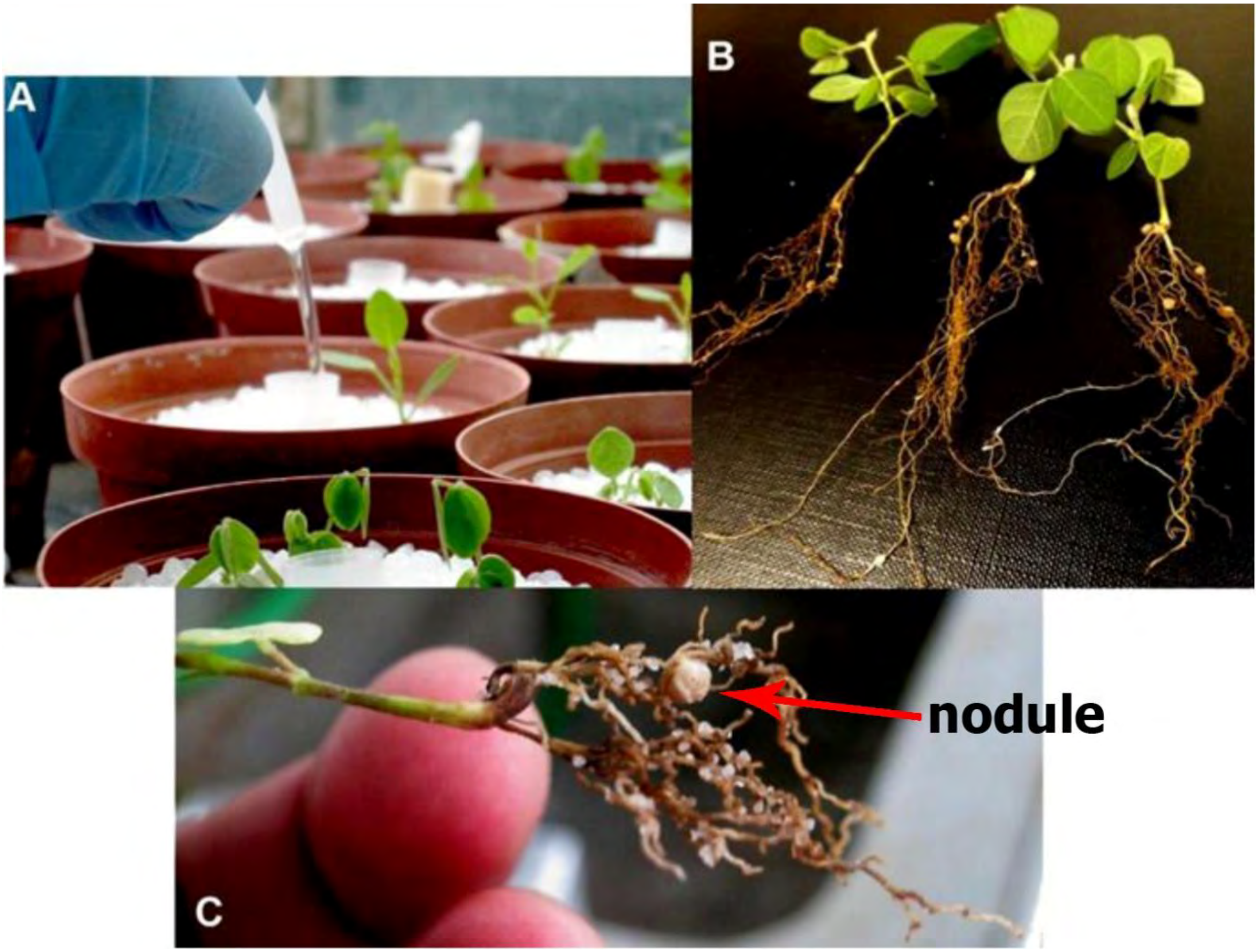

Figure 2.3: A- Inoculated plants in pots covered with beads to prevent contamination and watering tube inserted for irrigation. B- Siratro seedlings after harvest bearing nitrogen fixing nodules, C- Siratro root nodule.

Figure 2.4: Authentication experiment: Watering (through a watering tube) of Siratro plants growing in the glasshouse

Table 2.1: Authentication experiments of CCR rhizobia strains showing original tribe and species name, collector voucher number and nodulation status on Siratro (*Macroptilium atropurpureum*) plants. / = unknown author of species name, + = strain nodulation.

Original tribe and species	Isolate/ collector	Nodulation status	Isolate origin
Crotalarieae			
***Aspalathus astroites* L.**	18- Dlodlo	+	Jonkershoek
***Aspalathus bracteata* Thunb.**	5618- Muasya	+	Paarl Mountain
***Aspalathus callosa* L.**	5477- Muasya	+	Cape Point
***Aspalathus carnosa* L.**	5496- Muasya	+	Cape Point
***Aspalathus ciliaris* L.**	108- Dlodlo	+	Cederberg
***Aspalathus ciliaris* L.**	5361- Muasya	+	Jonkershoek
***Aspalathus cordata* (L.) R.Dahlgren**	13- Bello	+	Jonkershoek
***Aspalathus ericifolia* L.**	5352- Muasya	+	Jonkershoek
***Aspalathus ericifolia* L.**	31- Dlodlo	+	Paarl Mountain
***Aspalathus laricifolia* P.J.Bergius**	5372- Muasya	+	Jonkershoek
Aspalathus sp.	48- Dlodlo	+	De Hoop
Aspalathus sp.	49- Dlodlo	+	De Hoop
Aspalathus sp.	53- Dlodlo	+	Stilbaai
Aspalathus spicata* Thunb*.	5398- Muasya	+	Rawsonville Farm
Aspalathus spicata* Thunb*.	5440- Muasya	+	Worcester Dam
***Aspalathus uniflora* L.**	5734- Muasya	+	Cederberg
***Aspalathus uniflora* L.**	26- Dlodlo	+	Jonkershoek
Asplathus perfoliata (Lam.) R.Dahlgren	5757- Muasya	+	Cederberg
Indigofereae			
***Indigofera angustifolia* L.**	5878- Muasya	+	Groot Hagelkraal
***Indigofera frutescens* L.f.**	5392- Muasya	+	Rawsonville Farm
***Indigofera gracilis* Spreng.**	5621- Muasya	+	Paarl Mountain
Indigofera sp	5378- Muasya	+	St Helena Bay
Indigofera sp.	45- Muasya	+	De Hoop
***Indigofera superba* C.H.Stirt.**	5419- Muasya	+	Hermanus

Phenotypic characterization of rhizobia

In the study by Dlodlo (2012), rhizobia were isolated from roots nodules of about 65 legume species from 15 genera in 20 localities of varying soils types of the Cape Floristic Region. A global positioning system (GPS) was used to record the coordinates of all the localities. Only the viable nodules attached to growing hosts were collected. Soil samples were also collected and allocated voucher numbers and stored in sterile plastic bags for further rhizobia isolation. Host plant samples were collected for the purpose of herbarium specimens and each plant was allocated a voucher number. In the current study, the isolates from representative legume-nodules originated from 13 different localities (Dlodlo, 2012) of the CCR (Figure 2.5) were used. The localities included Rawsonville, Hermanus, Paarl Mountain, Cederberg, De Hoop, St Helena Bay, Groot Hagelkraal, Jonkershoek, Rhodes Memorial, Stilbaai and Bainskloof (Table 2.2, Figure 2.5). Alpha- (*Bradyrhizobium, Ensifer, Mesorhizobium and Rhizobium*) and beta-Proteobacteria (*Burkholderia*) isolates from nodules of *Aspalathus, Indigofera, Podalyria* and *Psoralea* hosts were selected. In GenBank, blast searches were conducted to identify which isolates are closely matched with the sequenced DNA of the studied strains (Dlodlo, 2012; Lemaire et al. 2015a). Selection of isolates was based on plant host diversity to cover four genera (Crotalarieae, Indigofereae, Podalyrieae, and Psoraleeae) and their distribution to cover a wide-area in the CCR.

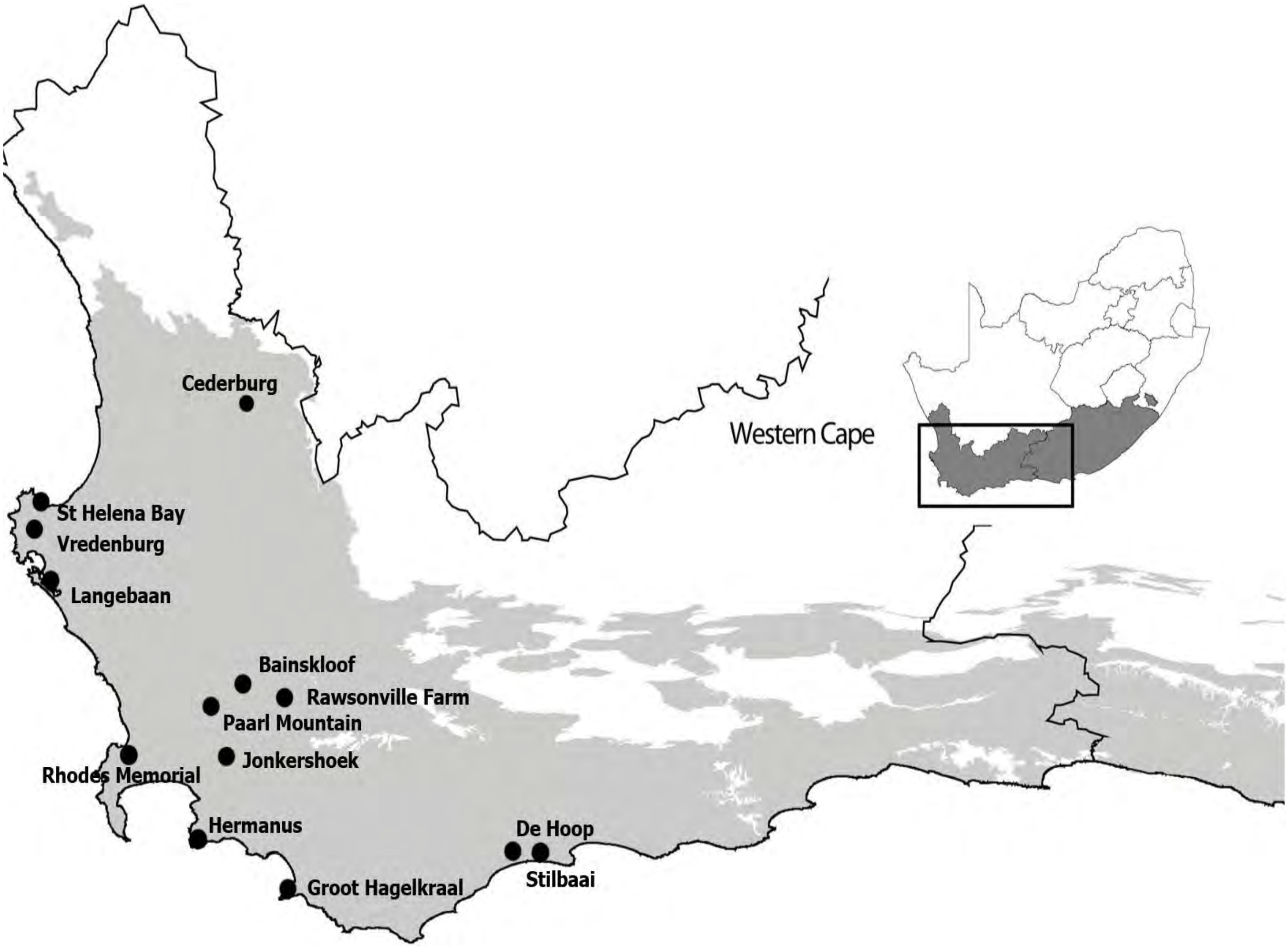

Figure 2.5: Map of South Africa showing the geographic distribution of sampling sites in the Core Cape Subregion. (Adapted from Lemaire et al., 2015a, Photoshoped in CS5).

Morphological characterization

Isolates were phenotypically characterized for colony morphology, growth temperature, carbon source, salinity and pH tolerance following the techniques outlined in Tiwari et al. (2012). The strains were grown in yeast extract Mannitol Agar (YEMA) plates or YEM broth depending on the specific test. Morphological characteristics that were assessed included the rhizobia shape, colour and time to full colony size (days). The YEMA nutrient medium contained (g/L): 10, Mannitol; 0.5 K_2HPO_4; 0.2, $MgSO_4.7H_2O$; 0.1, NaCl; 0.5, Yeast Extract and 15, agar (Tiwari et al. 2012). The medium was autoclaved at 121˚C for 20 minutes for sterilization. After autoclaving, the sterile nutrient agar medium was allowed to cool down for about 30 min and poured into sterile petri dishes (Figure 2.6). The isolates stored in glycerol stock at -80˚C were obtained and cultured on the plate following the protocol outlined by Vincent (1970). A loop-full of rhizobia from glycerol stock was aseptically streaked onto the yeast extract Mannitol agar (YEMA) plates. The dilution streaking pattern to isolate single colonies was used. Plates were incubated at 25˚C (Figure 2.7) for 4-12 days and observed daily to monitor rhizobial growth and possible contamination.

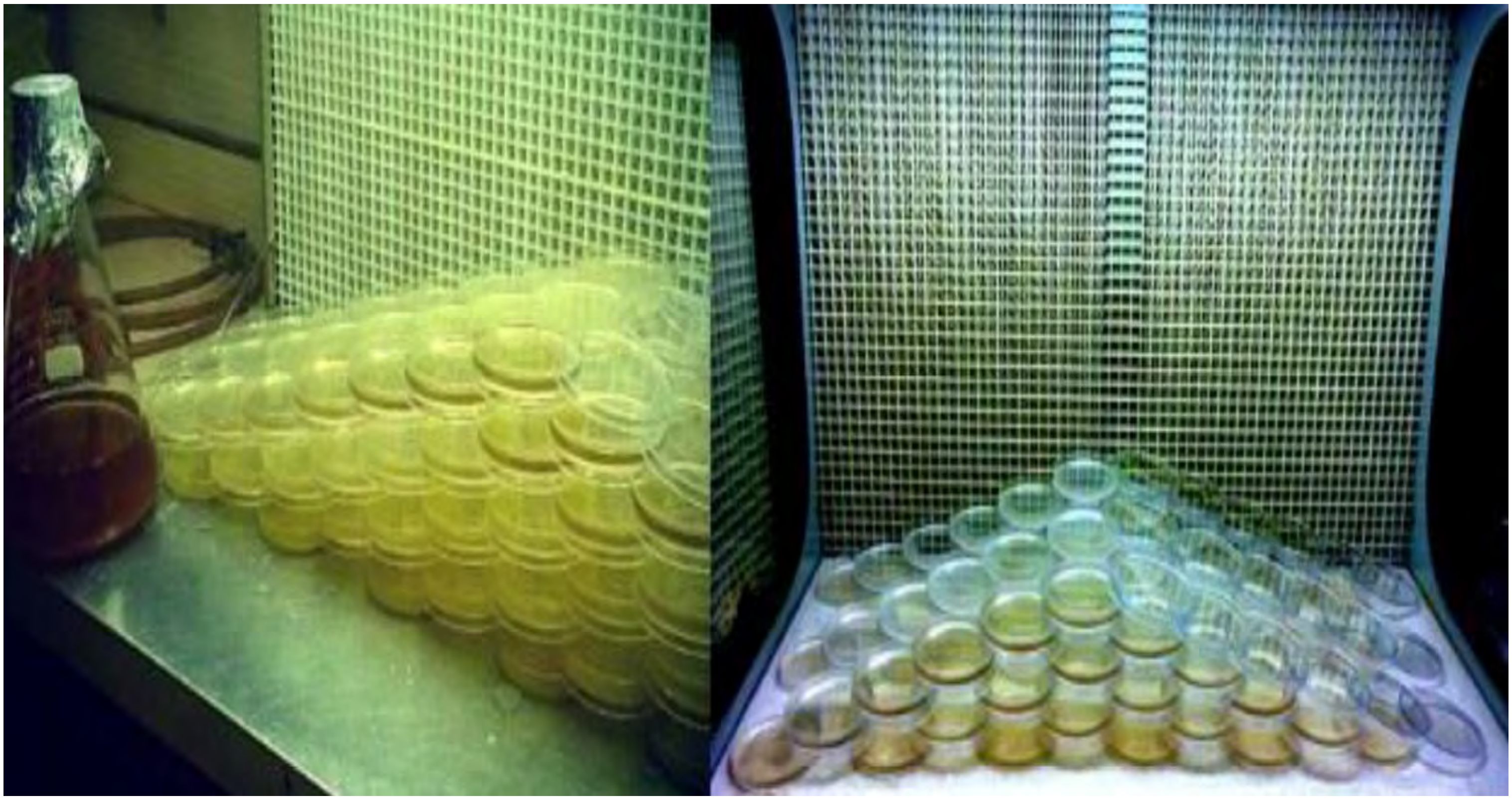

Figure 2.6: YEMA plate after pouring

Table 2.2: Selected rhizobial strains, host plant, location substrate and voucher number, precipitation and temperature data (Dlodlo 2012, Lemaire et. al 2015a). The temperature and rainfall precipitation data for the locations were extracted from WorldClim database (Hijmans et al. 2005) using global positioning system (GPS) readings.-=no data available

Rhizobia genus	**Host plant**	**Location**	**Substrate**	**Rhizobia voucher #**	**Annual precipitation (mm)**	**Precipitation of the warmest quarter (mm)**	**Max temperature of the warmest period (˚C)**
Bradyrhizobium	*Indigofera frutescens* L.f.	Rawsonville Farm	Alluvium	5392	726	68	28
Bradyrhizobium	*Indigofera ericifolia* L. subsp. Ericifolia	Paarl Mountain	Granite	5621	1017	87	28
Burkholderia	*Podalyria calyptrata* (Retz) Willd.	Stilbaai	Limestone	1a	-	-	-
Burkholderia	*Podalyria calyptrata* (Retz) Willd.	Rhodes Memorial	Shale	1b	1128	112	26
Burkholderia	*Podalyria calyptrata* (Retz) Willd.	Jonkershoek	Alluvium	1c	994	90	29
Burkholderia	*Podalyria sericea* (Andrews) R. Br.	Langebaan	Granite	2	289	24	25
Burkholderia	*Podalyria spicata*	Groot Hagelkraal	Acid sand	3	521	65	23
Burkholderia	*Indigofera filifolia* Thunb.	Jonkershoek	Alluvium	44	994	90	29
Burkholderia	*Indigofera superba* C.H. Stirt.	Hermanus	Sandstone	5419	741	88	23
Burkholderia	*Indigofera sp.*	Cederberg	Sandstone	5746	-	-	-
Burkholderia	*Indigofera sp.*	St Helena Bay	Granite	5378	289	24	25
Burkholderia	*Indigofera angustifolia* Thunb.	Groot Hagelkraal	Acid sand	5878	521	65	23
Ensifer	*Indigofera sp.*	De Hoop	Limestone	45	-	-	-
Mesorhizobium	*Aspalathus cordata* (L.) Dahlg.	Jonkershoek	Alluvium	13	994	90	29
Mesorhizobium	*Aspalathus astroites* L.	Jonkershoek	Alluvium	18	994	90	29
Mesorhizobium	*Aspalathus uniflora* L. subsp. Uniflora	Jonkershoek	Alluvium	26	994	90	29
Mesorhizobium	*Aspalathus ericifolia* L.subsp. ericifolia	Paarl Mountain	Granite	31	-	-	-
Mesorhizobium	*Aspalathus sp.*	De Hoop	Limestone	48	-	-	-
Mesorhizobium	*Otholobium hirtum* (L.) C.H. Stirt.	Paarl Mountain	Granite	32	-	-	-
Mesorhizobium	*Otholobium hirtum* (L.) C.H. Stirt.	Rhodes Memorial	Shale	5334	1128	112	26
Mesorhizobium	*Otholobium hirtum* (L.) C.H. Stirt.	Vredenburg	Granite	5382	283	21	25
Mesorhizobium	*Otholobium virgatum* (Burm.f.) C.H. Stirt.	Rhodes Memorial	Shale	5333	1128	112	26
Mesorhizobium	*Otholobium virgatum* (Burm.f.) C.H. Stirt.	Jonkershoek	Alluvium	5370	994	90	29
Mesorhizobium	*Otholobium bracteolatum* (Eckl. & Zeyh.) C.H.Stirt. subsp. limnophilum	De Hoop	Granite	42	-	-	-

Mesorhizobium	*Otholobium asarina* (Berg.) Salter	Jonkershoek	Alluvium	15	994	90	29
Mesorhizobium	*Otholobium sp.*	Stilbaai	Limestone	52	-	-	-
Mesorhizobium	*Psoralea pinnata* L.	Rhodes Memorial	Shale	5336	1128	112	26
Mesorhizobium	*Psoralea rigidula* C.H.Stirt.	Bainskloof	Sandstone	5343	905	77	26
Rhizobium	*Otholobium gigantea* M.N. Dludlu, A.M. Muasya & C.H. Stirt.	Jonkershoek	Alluvium	24	994	90	29

Results were recorded by microscopically observing colonies for shapes, and visual observation of the colonies for colour. To test for temperature tolerance of the rhizobial isolates, YEMA plates were inoculated by aseptically streaking isolates from glycerol stock onto the medium using a sterile loop full of rhizobia. The streaked plates were incubated in temperature conditions of 4, 15, 20, 25, 30, 35, 40 and 53°C for up to 12 days (Figure 2.7). Positive results were recorded by checking visually for rhizobial growth on the agar plate.

Figure 2.7: Rhizobia incubation chambers set at particular temperatures.

Salt tolerance was characterised by culturing strains in YEMA (g/L) medium containing 0 (0%), 0.1 (0.01%), 0.5 (0.05%), 1 (0.10%), 10 (1%), 20 (2%), and 30 (3%) NaCl concentrations. A loop full of sterile rhizobia from glycerol stock was aseptically streaked onto yeast extract Mannitol agar plates. The dilution streaking pattern to isolate single colonies was used and bacteria were allowed to grow at 25°C for up to 12 days depending on the strain. Positive results were recorded based on the number of plates with rhizobial growth.

The carbon test entailed growth of isolates on agar with different sources of carbon including glucose, sucrose, starch and Mannitol. Each carbon source was prepared separately in 1L distilled water (see Table 2.3). Media were autoclaved at 121°C for 15 minutes. Each of the carbon sources were cultured with the rhizobial isolates by aseptically streaking with a loop full of rhizobia from the glycerol stock. The streaked plates were incubated at 25°C for up to 12 days as the sampled population contained slow and fast growing rhizobia isolates. Results were recorded based on the number of plates with rhizobia growth in the four different carbon sources.

Table 2.3: Amount of each carbon source for the media for the carbon sources experiment. Other components of the growth medium include (i.e. g/L) 0.5, K_2HPO_4 0.2, $MgSO_4.7H_2O$; 0.1, NaCl; 0.5, Yeast Extract; 15, Agar

Glucose (g/L)	**Sucrose (g/L)**	**Starch (g/L)**	**Standard medium/ Mannitol (g/L)**
40, dextrose	20, sucrose	3, starch	5, Mannitol
10, peptone	5, peptone		

To test for pH tolerance, rhizobia were grown in yeast extract Mannitol broth (YEM) adjusted with buffers to pH 3, 4, 5, 6, 7, 8 and the control was set at 6.8 (i.e. pH for the media commonly used for rhizobia cultures). For each pH, a distinct chemical combination was added to the culture medium (per 100 ml) to obtain a specific pH as follows: pH 3: 55.5 mL 0.2M acetic acid and 1 mL 0.2M sodium acetate; pH 4: 41 mL 0.2M acetic acid and 9 mL 0.2M sodium acetate; pH 5: 14.8 mL 0.2M acetic acid and

35.2 ml 0.2M sodium acetate; pH 6: 17.9 mL 0.1M citric acid and 32.1 ml 0.2M Na2HPO$_4$, pH 7: 6.5 mL 0.1M citric acid and 43.5 ml 0.2M Na2HPO$_4$ and pH 8: 2.65 mL 0.2M NaH_2PO_4 and 47.3 ml 0.2M Na2HPO$_4$ (Gerhardt, 1994). The YEM contained (g/L): 10, Mannitol; 0.5 K_2HPO_4; 0.2, $MgSO_4.7H_2O$; 0.1, NaCl and 0.5, Yeast Extract. The pH in each treatment was confirmed using a pH meter (Figure 2.8B). The broths contained in McCartney vials (Figure 2.8A) were autoclaved at 121°C for 20 minutes to sterilize the growth medium. A loop full of sterile rhizobia from the glycerol stock was aseptically inoculated into yeast extract Mannitol (YEM) broth in the McCartney vials. In 25°C room, the broths cultured in the vials were shaken at 120 rpm to maintain aerobic conditions and to mix the nutrients with the inoculants. The rhizobia cultures were allowed to grow for up to 12 days depending on the growth period of the strain. Culture growth was monitored by visual observation. At the end of the growth period of each strain, rhizobia growth was determined by measuring the degree of turbidity formed by the growing strains in the McCartney bottle. This was done by measuring the optical density of the broth at 600 nm using a spectrophotometer (Sohbatzadeh et al. 2010;Youvan et al. 1997). Strains which took approximately 12 days to develop, for example only started showing turbidity on day 10 and the intensity of the cloudiness was measured on day 12.

Figure 2.8: McCartney bottle (A) used or rhizobia growth during the characterization for pH, and (B) is the pH meter.

Results

Rhizobia authentication

All 24 strains tested on Siratro, a host plant known to be symbiotically promiscuous, nodulated.

Morphology

Rhizobial growth period and shape: Twenty five symbionts which make up roughly 86% of the rhizobia strains grew within 3-5 days (fast growing). Only one isolate (3%) of the rhizobia grew after 7 days (7-12; slow growing rhizobia) and was isolated from an *Otholobium virgatum* from Rhodes Memorial site (Table 2.4). Majority of the isolates

(23 of the 29; 79%) were rod-shaped. Only six (21%) were bacteriod-shaped isolated from Indigofereae (*Indigofera frutescens*) and Psoraleeae (*Otholobium bracteolatum* and *Psoralea spp.*) collected from 5 CCR sites (Table 2.4).

Colour: Majority of isolates (22 of the 29, 76%) produced a white opaque colour from 4 different legume tribes and 11 diverse geographic sites. Five strains, approximately 17% produced a milky pigment from predominantly Podalyrieae (*Podalyria calyptrata* and *Podalyria spicata*) and an *Indigofera* species*.* These strains originated from Cederberg, Groot Hagel kraal, Rhodes Memorial and Stilbaai. Only two (7%) of the rhizobia had a watery translucent pigment. The Beta-rhizobia generally showed more phenotypic colour plasticity as the various strains resulted in three (white opaque, milky and watery translucent) colour forms (Table 2.4). Alpha-rhizobia had less diverse colour response variety as the isolates observed exhibited only two colour variations which are milky and watery translucent.

Carbon Source: The bacteria that were isolated from nodules of Indigofereae from Cederburg and of Psoraleeae from Stilbaai did not grow well on glucose compared to Mannitol medium; 93% versus 100% of the isolates.

In sucrose medium, the response rate slightly decreased with 25 isolates (86%) responding to the carbon source. The few isolates that did not respond to sucrose were from Indigofereae in Cederberg and St Helena Bay and Psoraleeae in Stilbaai*.* There was a considerable decline of response to starch (Table 2.4, Figure 2.9 B) as only 19 isolates (66%) grew in the starchy medium.

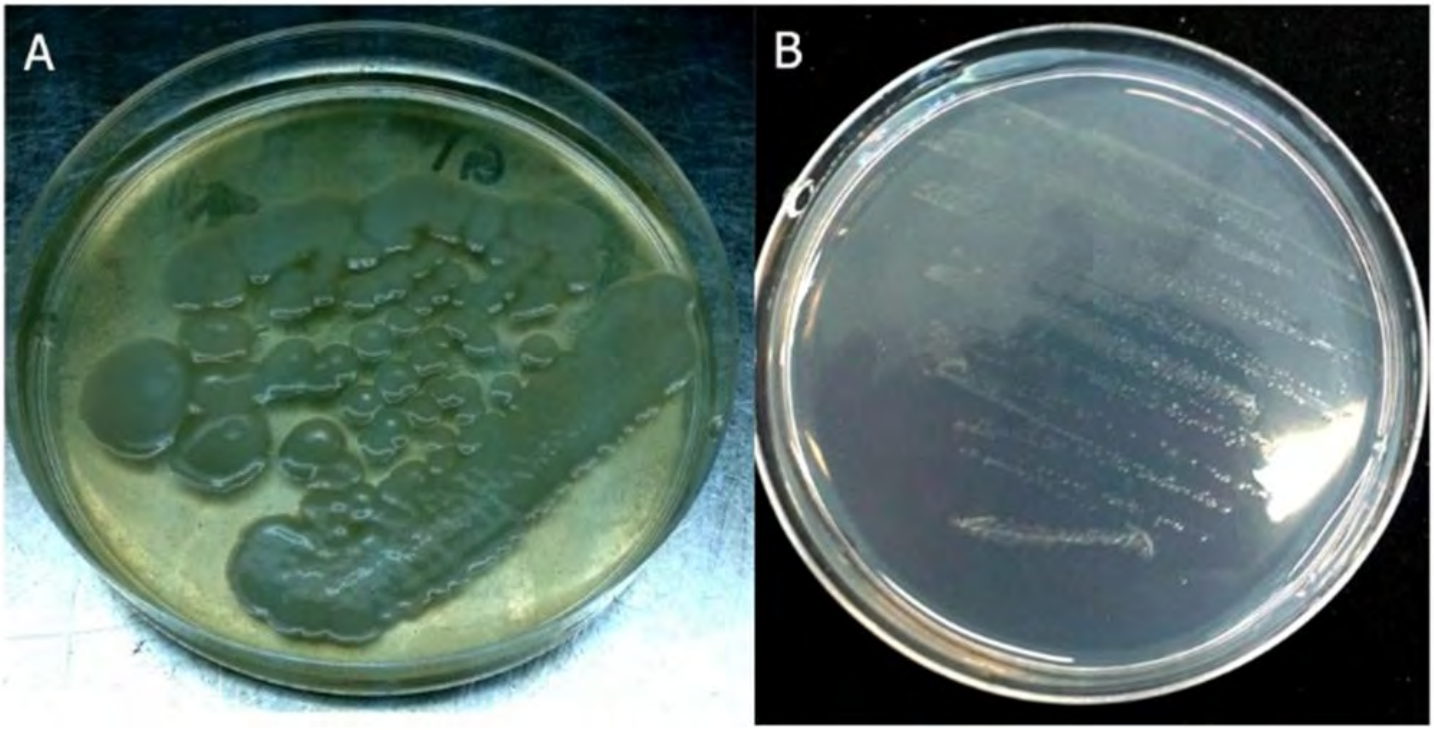

Figure 2.9: Rhizobial isolates of *Burkholderia* isolated from *A. uniflora* in Jonkershoek cultured on glucose medium (A) and starch medium (B).

Temperature tolerance: At the standard growth temperature of 25°C, all 29 (100%) of the rhizobia isolates grew successfully (Table 2.5). Of the 29 isolates, 24 (83%) endosymbionts tolerated temperatures of 30°C. These isolates were originated from all the 4 legume genera used and were from all 13 CCR localities. At 35°C, 13 (45%) tolerated the temperature. At 40˚C, 12 (41%) isolates belonging to Indigofereae, Podalyrieae, Crotalarieae and Psoraleeae from 9 different localities tolerated the conditions (Table 2.5). Neither beta- nor alpha- rhizobia tolerated temperature extremes of 53˚C.

Salt tolerance: Majority of the strains (27 out of 29, 93%) responded exceptionally well to NaCl concentrations between 0 and 1% whereas at higher sodium concentrations, the tolerance decreased rapidly (Table 2.5). Twenty seven strains (93%) from the diverse hosts and localities tolerated medium with no NaCl treatment with the exception of strains from *Indigofera sp.* (Cederberg) and *Psoralea asarina* (Jonkershoek) which failed to grow in these conditions. At the standard sodium chloride concentration of 0.01%, 28 (97%) of the isolates grew successfully in the treated growth medium with the exception of one isolate from *Psoralea asarina* in Jonkershoek. Twenty six (90%) of the tested rhizobia isolates tolerated salt treatment at 0.05%. At 0.10% 28 isolates, 97% of the rhizobia responded to the treatment with the exclusion of a strain from Rhodes Memorial. At 1%, 27 strains showed tolerance to the saline medium but 2 isolates from Jonkershoek and Stilbaai did not respond to the treatment. At 2% salt concentration, only 7 (24%) of the isolates tolerated the high salt content.

Table 2.4: Morphology of the studied isolates grown in Mannitol medium at 25°C and in different carbon sources. Glu= Glucose, St= Starch, Su= Sucrose, Man= Mannitol.

Rhizobia	**Host**	**Site**	**Strain Voucher #**	**Rhizobia Morphology**			**Carbon Source**			
				Shape	**Growth Rate (days)**	**Colour**	**Glu**	**St**	**Su**	**Man**
Bradyrhizobium	*I. frutescens*	Rawsonville Farm	5392	Bacteriod	3-5	White opaque	+	+	+	+
Bradyrhizobium	*I. gracilis*	Paarl Mountain	5621	Rod	5-7	White opaque	+	-	+	+
Burkholderia	*Po. calyptrata*	Stilbaai	1	Rod	3-5	Milky	+	-	+	+
Burkholderia	*Po. calyptrata*	Rhodes Memorial	1	Rod	3-5	Milky	+	-	+	+
Burkholderia	*Po. calyptrata*	Jonkershoek	1	Rod	3-5	Milky	+	-	+	+
Burkholderia	*Po. sirecea*	Langebaan	2	Rod	3-5	White opaque	+	+	+	+
Burkholderia	*Po. spicata*	Groot Hagelkraal	3	Rod	3-5	Milky	+	+	+	+
Burkholderia	*I. filifolia*	Jonkershoek	44	Rod	3-5	White opaque	+	+	+	+
Burkholderia	*I. superba*	Hermanus	5419	Rod	3-5	White opaque	+	+	+	+
Burkholderia	*Indigofera sp.*	Cederberg	5746	Rod	3-5	Milky	-	-	-	+
Burkholderia	*Indigofera sp.*	St Helena Bay	5378	Rod	3-5	White opaque	+	+	-	+
Burkholderia	*I. angustifolia*	Groot Hagelkraal	5878	Rod	3-5	White opaque	+	-	+	+
Burkholderia	*A. uniflora*	Jonkershoek	26	Rod	3-5	White opaque	+	+	+	+
Burkholderia	*A. astroites*	Jonkershoek	18	Rod	5-7	Watery translucent	+	+	+	+
Ensifer	*Aspalathus sp.*	De Hoop	45	Rod	3-5	White opaque	+	+	+	+
Mesorhizobium	*A. ericifolia*	Paarl Mountain	31	Rod	3-5	White opaque	+	+	+	+
Mesorhizobium	*Aspalathus sp.*	De Hoop	48	Rod	3-5	White opaque	+	+	+	+
Mesorhizobium	*O. hirtum*	Paarl Mountain	32	Rod	3-5	White opaque	+	+	+	+
Mesorhizobium	*O. hirtum*	Rhodes Memorial	5334	Rod	3-5	White opaque	+	+	+	+
Mesorhizobium	*O. hirtum*	Vredenburg	5382	Rod	3-5	White opaque	+	+	+	+
Mesorhizobium	*O. virgatum*	Rhodes Memorial	5333	Rod	7-12	Watery translucent	+	+	+	+
Mesorhizobium	*O. virgatum*	Jonkershoek	5370	Rod	3-5	White opaque	+	-	+	+
Mesorhizobium	*O. bracteolatum*	De Hoop	42	Bacteriod	3-5	White opaque	+	+	+	+
Mesorhizobium	*Ps. asarina*	Jonkershoek	15	Bacteriod	3-5	White opaque	+	+	+	+
Mesorhizobium	*Psoralea sp.*	Stilbaai	52	Bacteriod	3-5	White opaque	-	-	-	+
Mesorhizobium	*Ps. pinnata*	Rhodes Memorial	5336	Bacteriod	3-5	White opaque	+	+	+	+
Mesorhizobium	*Ps. rigidula*	Bainskloof	5343	Bacteriod	3-5	White opaque	+	+	+	+
Mesorhizobium	*A. cordata*	Jonkershoek	13	Rod	5-7	White opaque	+	+	+	+
Rhizobium	*Ps. gigantea*	Jonkershoek	24	Rod	3-5	White opaque	+	-	+	+

Table 2.5: Growth of rhizobia isolates in various temperatures and in media with different concentration of salt. - = No rhizobial, + = positive rhizobial growth

Rhizobia	**Host**	**Site**	**Strain voucher #**	**Temperature (˚C)**								**Salt tolerance (%)**						
				4	**15**	**20**	**25**	**30**	**35**	**40**	**53**	**0**	**0.01**	**0.05**	**0.10**	**1**	**2**	**3**
Bradyrhizobium	*I. frutescens*	Rawsonville Farm	5392	-	-	-	+	+	+	+	-	+	+	+	+	+	+	+
Bradyrhizobium	*I. gracilis*	Paarl Mountain	5621	-	-	-	+	+	+	+	-	+	+	+	+	+	-	-
Burkholderia	*Po. calyptrata*	Stilbaai	1	-	-	+	+	+	+	+	-	+	+	+	+	+	-	-
Burkholderia	*Po. calyptrata*	Rhodes Memorial	1	-	-	-	+	+	+	+	-	+	+	+	+	+	-	-
Burkholderia	*Po. calyptrata*	Jonkershoek	1	-	-	-	+	+	+	+	-	+	+	+	+	+	-	-
Burkholderia	*Po. sirecea*	Langebaan	2	-	-	-	+	+	+	+	-	+	+	+	+	+	-	-
Burkholderia	*Po. spicata*	Groot Hagelkraal	3	-	-	-	+	+	+	+	-	+	+	+	+	+	-	-
Burkholderia	*I. filifolia*	Jonkershoek	44	-	-	-	+	+	+	+	-	+	+	+	+	+	+	+
Burkholderia	*I. superba*	Hermanus	5419	-	-	-	+	+	+	+	-	+	+	+	+	+	+	+
Burkholderia	*Indigofera sp.*	Cederberg	5746	-	-	-	+	+	-	-	-	-	+	+	+	+	-	-
Burkholderia	*Indigofera sp.*	St Helena Bay	5378	-	-	-	+	+	-	-	-	+	+	+	+	+	-	-
Burkholderia	*I. angustifolia*	Groot Hagelkraal	5878	-	-	-	+	+	-	-	-	+	+	+	+	+	-	-
Burkholderia	*A. uniflora*	Jonkershoek	26	-	-	-	+	-	-	-	-	+	+	+	+	+	-	-
Burkholderia	*A. astroites*	Jonkershoek	18	-	-	-	+	-	-	-	-	+	+	+	+	+	-	-
Ensifer	*Aspalathus sp.*	De Hoop	45	-	-	-	+	+	+	-	-	+	+	+	+	+	-	-
Mesorhizobium	*A. ericifolia*	Paarl Mountain	31	-	-	-	+	+	-	-	-	+	+	+	+	+	-	-
Mesorhizobium	*Aspalathus sp.*	De Hoop	48	-	-	-	+	-	-	-	-	+	+	+	+	+	-	-
Mesorhizobium	*O. hirtum*	Paarl Mountain	32	-	-	-	+	+	-	+	-	+	+	+	+	+	-	-
Mesorhizobium	*O. hirtum*	Rhodes Memorial	5334	-	-	-	+	+	-	-	-	+	+	+	+	+	+	-
Mesorhizobium	*O. hirtum*	Vredenburg	5382	-	-	-	+	+	+	-	-	+	+	+	+	+	-	-
Mesorhizobium	*O. virgatum*	Rhodes Memorial	5333	-	-	-	+	+	-	-	-	+	+	+	+	+	-	-
Mesorhizobium	*O. virgatum*	Jonkershoek	5370	-	-	-	+	+	-	-	-	+	+	+	+	+	+	-
Mesorhizobium	*O. bracteolatum*	De Hoop	42	-	-	+	+	+	-	+	-	+	+	+	+	+	-	-
Mesorhizobium	*Ps. asarina*	Jonkershoek	15	-	-	-	+	+	+	-	-	-	-	+	+	-	-	-
Mesorhizobium	*Psoralea sp.*	Stilbaai	52	-	-	+	+	-	-	-	-	+	+	-	+	+	-	-
Mesorhizobium	*Ps. pinnata*	Rhodes Memorial	5336	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-
Mesorhizobium	*Ps. rigidula*	Bainskloof	5343	-	-	-	+	+	-	+	-	+	+	-	+	+	+	-
Mesorhizobium	*A. cordata*	Jonkershoek	13	-	-	-	+	+	-	-	-	+	+	-	+	+	+	-
Rhizobium	*Ps. gigantea*	Jonkershoek	24	-	-	+	+	+	+	-	-	+	+	+	+	+	-	-

pH tolerance: Figures 2.10 A shows the response of isolates to acidic condition set at pH 3. Under these pH levels, a *Mesorhizobium* (5370) isolated from *Otholobium virgatum* in Jonkershoek had the highest optical density (OD) of 1.1 at 600 nm. The second highest OD at 1.0 was also from a *Mesorhizobium* (26), isolated from *Aspalathus uniflora* also growing in Jonkershoek. A third highest OD value of 0.7 was for *Mesorhizobium* (5336) strain from a *Psoralea pinnata* in Rhodes Memorial. For the growth medium set at pH 4, a *Mesorhizobium* (48) isolated from an *Aspalathus sp.* in De Hoop had the highest OD of 0.5. A *Burkholderia* (26) isolated from *Aspalathus uniflora* and a *Mesorhizobium* (44) isolated from *I. filifolia*, both in Jonkershoek had an OD of 0.2. The third highest OD of 0.1 was observed from a *Burkholderia* (5746) isolated from an *Indigofera sp*. in Cederberg.

In acidic conditions of between pH 5 (Figure 2.10 B), strain 5621, a *Bradyrhizobium* isolated from *I. gracilis* in Paarl Mountain, had a highest OD of 0.4. The second highest ODs of 0.1 at pH 5 were observed from a *Mesorhizobium* (52) isolated from *Psoralea sp*. in Stillbaai and a *Burkholderia* (5878) isolated from *I. angustifolia* in GrootHagelkraal. In medium of pH 6, OD 0.3 was the highest (5370) for a *Mesorhizobium* isolated from *Otholobium virgatum* in Jonkershoek. The second highest OD readings of 0.2 were from two *Mesorhizobium*, 42 isolated from *O. bracteolatum* in Jonkershoek and 5343 isolated from *Ps. rigidula* in Bainskloof.

The highest tolerance strain in neutral medium at pH 7 recorded an OD of 0.4 from a *Burkholderia* (5378) from *Indigofera sp.* in St Helena Bay. Second highest optical densities were 0.2 for 5621 a *Bradyrhizobium* isolated from *I. gracilis* in Paarl Mountain and a *Mesorhizobium* (15) from *Ps. asarina* in Jonkershoek.

In alkaline conditions of pH 8 (Figure 2.11 A), the highest optical density readings were obtained for a Mesorhizobium (52) strain with an OD of 0.7, isolated from *Otholobium sp.* in Stilbaai. The second highest OD of 0.4 for pH 8 were observed from a *Bradyrhizobium* (5392) isolated from *Indigofera frutescens* in Rawsonville. A

Burkholderia (5878) isolated from *I. angustifolia* in GrootHagelkraal had a reading of 0.3.

The pH control which was set at 6.8 (Figure 2.11 b) showed that two *Mesorhizobium* (13- *A. cordata* and 18- *A. astroites*) isolated from soils collected in Jonkershoek, had the two highest ODs of 0.7 and 1.3, respectively. The third highest OD was from 5621 a *Bradyrhizobium* isolated from *I. gracilis* in Paarl Mountain.

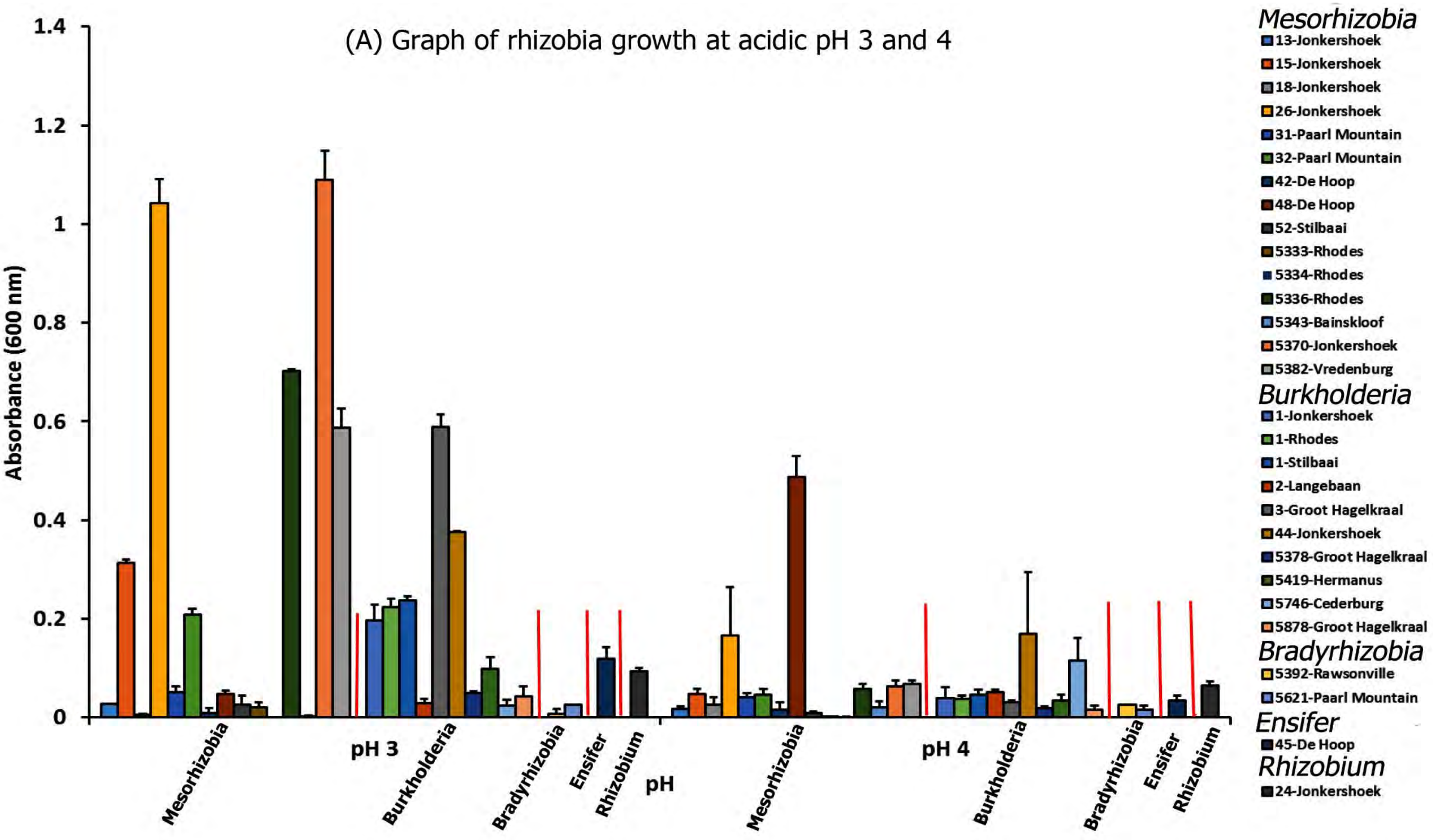

Figure 2.10 (A): Graphs of rhizobia growth in acidic conditions of pH 3 and 4. Vertical lines separate the different rhizobia genera.

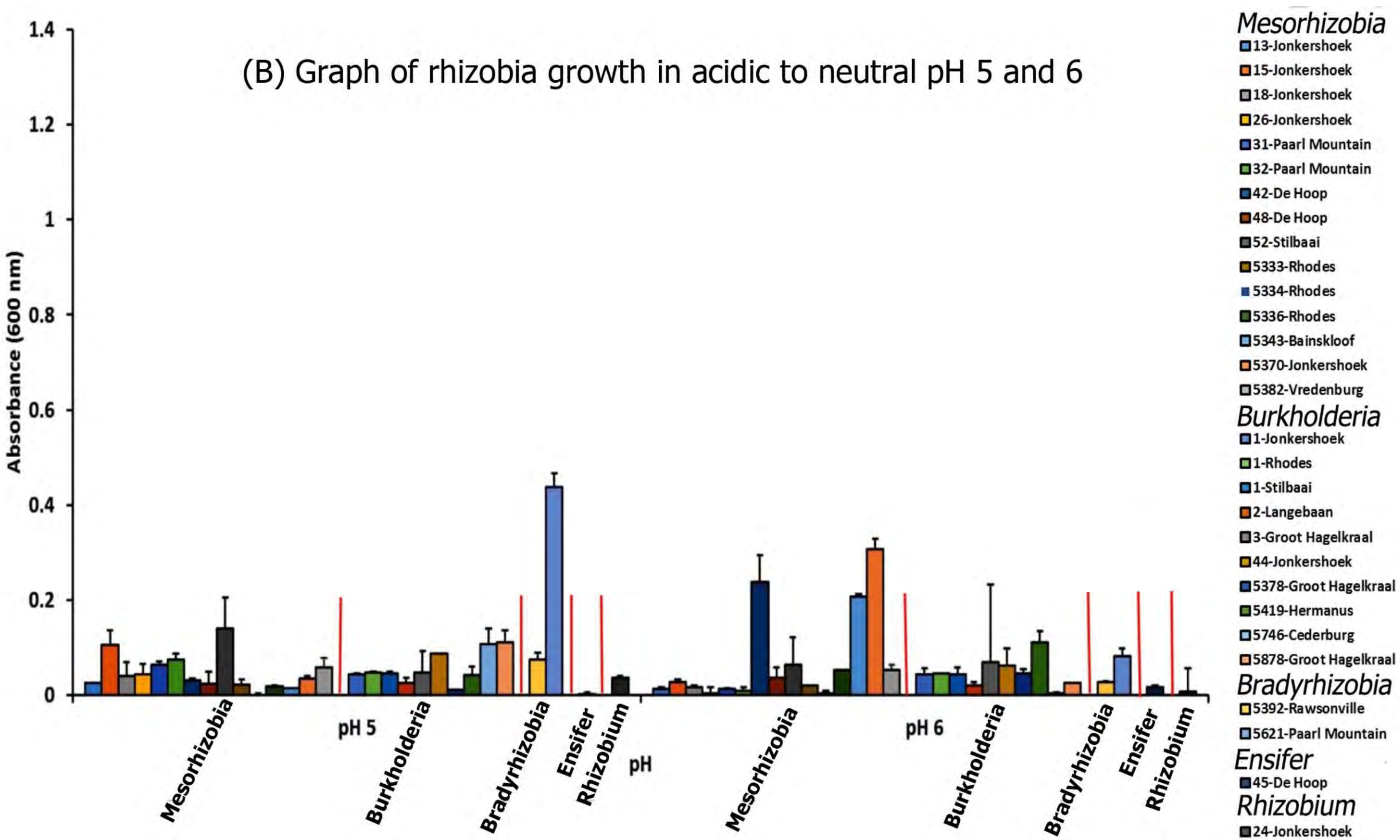

Figure 2.10 (B): Rhizobial growth in acidic to neutral pH of 5 and 6. Vertical lines separate the different rhizobia genera.

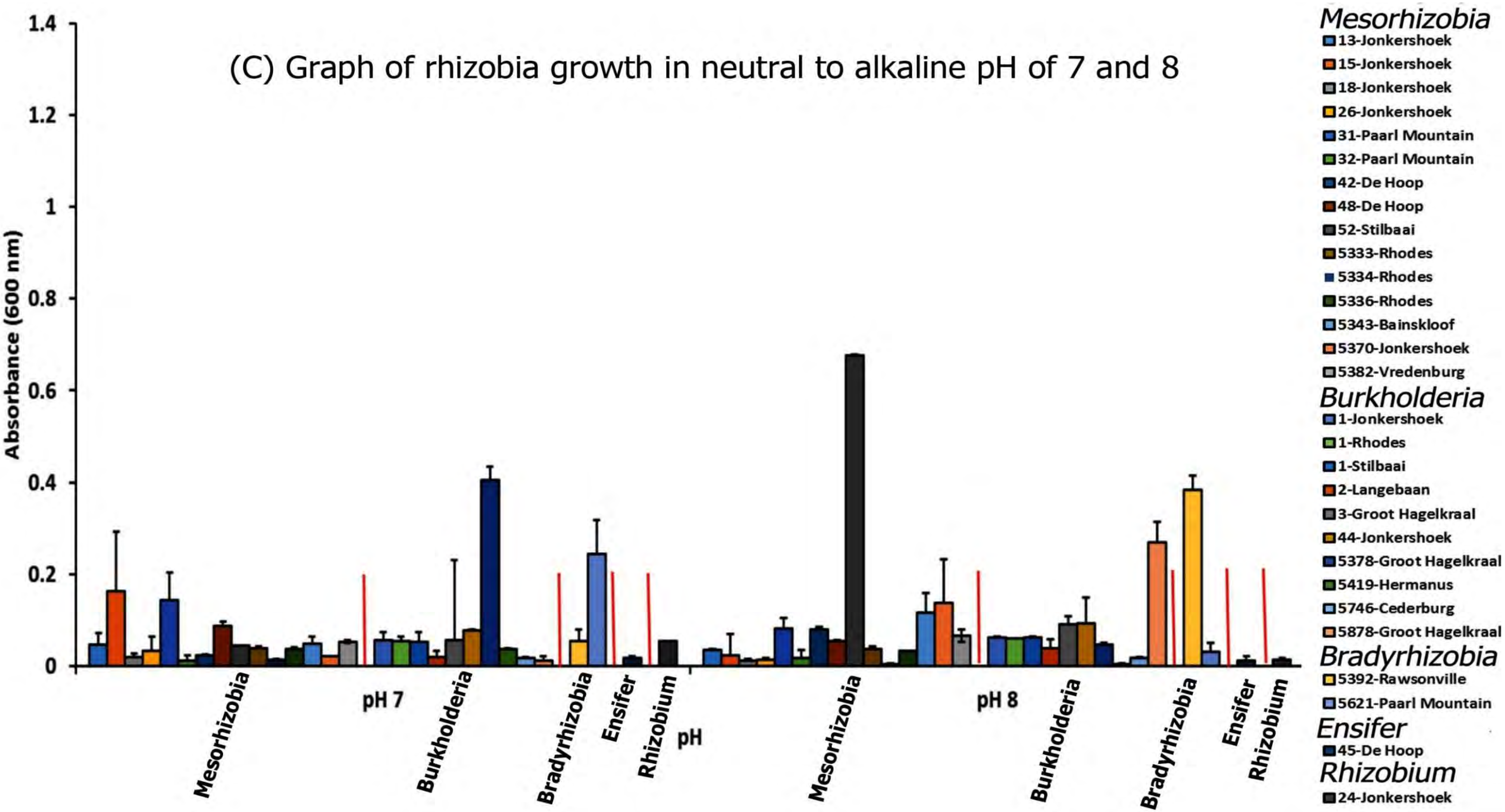

Figure 2.11 (C): Rhizobial growth in neutral and alkaline pH of 7 and 8 respectively. Vertical lines separate the different rhizobia genera

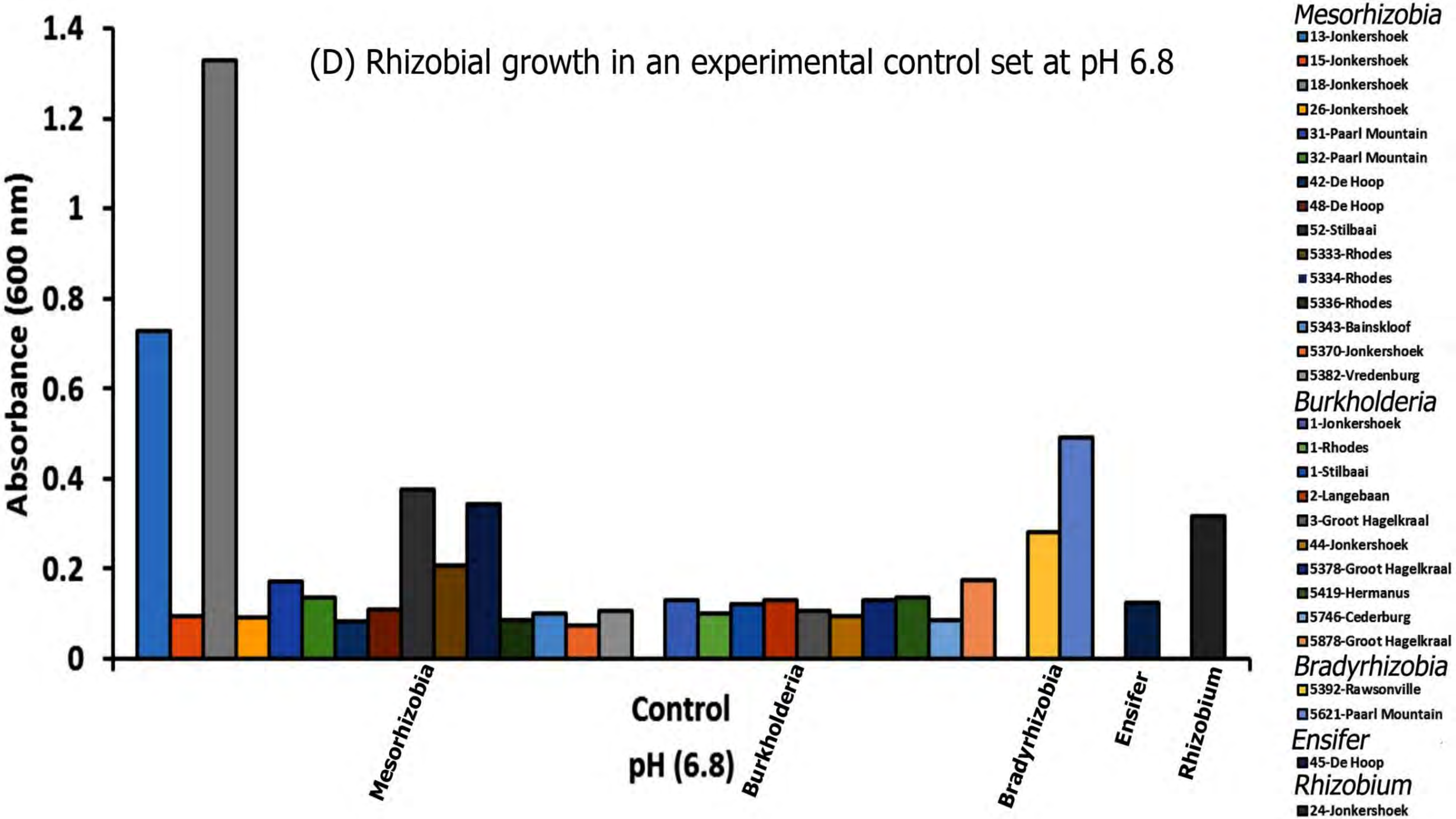

Figure 2.11 (D): Rhizobial isolate growth in control media set at pH 6.8. acidic to neutral pH of 5 and 6. Vertical lines separate the different rhizobia genera

Discussion

The fast growing rhizobia dominated the region and were widespread across the localities. Fast growing rhizobia have been isolated from various geographic locations (Argentina, China, New Zealand, Senegal, Spain, Pakistan, USA, Vietnam, South-Western Australia) in diverse soils and ecological niches (Hafeez et al. 1995; Marsudi, 1999; Saldaña et al. 2003). The high frequency of fast growing rhizobia in Cape soils may suggest that they are better survivors in the acidic and infertile soils of the CCR than slow-growing strains (Lemaire et al. 2015b; Garau et al. 2009). Sprent (1994) observed that selection pressure on rhizobia leads to the evolution of stress tolerant forms, which could mean that fast growth is possibly an evolutionary trait for tolerance to acidity.

Approximately 40% of soil carbon is derived from plant root exudates, where diverse molecules are released into rhizosphere soils including acids, sugars, polysaccharides and ectoenzymes (Marschner, 2012). This suggests that the rhizobia have high tolerance for the media and may have adapted to the provided carbon sources. Rhizobia strains could also be highly compatible with compounds found in the carbon sources which resemble or are the same as the primary and secondary metabolites that make up root exudates (Bais et al. 2006). Rhizobia isolates which had high tolerance for all carbon sources may indicate that they have high activity of both amylase (hydrolyse starch into sugars) and cellulase (hydrolyse cellulose) (Rasul et al. 2012). This means that the strains in these genera could be tested for amylase and cellulase activity and could be utilised in the biocatalyst industry for the industrial production of enzymes. Enzymes are important components in food manufacturing, DNA technology, chemical industry, and in the production of pure amino acids and rare sugars, in the production of fructose and penicillin derivatives. In this study, Mannitol was the predominantly preferred carbon source which may explain why this carbon source is universally preferred. Temperature tolerance was evaluated at temperatures between 4 and 53°C. The majority of the strains (20, making up 69%) are capable of growth at temperatures between 20 and 40°C. This is supported by other studies (Zahran, 2001) which have found that rhizobia are mesophilic and can tolerate temperatures between 10 and 37°C, but high soil or root temperature negatively affects nitrogen fixation (Michiels et al. 1994). At 25°C there was a 100% growth observed, indicating optimum growth for all the strains at this temperature. This is consistent with previous studies (Guar, 1993; Harwani, 2006; Ali et al. 2009) which reported that optimum temperature growth of root nodulating soil bacteria ranged from 25- 35°C. Twelve strains (41%) from Rawsonville, Paarl Mountain, Stilbaai, Rhodes Memorial, Jonkershoek, Langebaan, Groot Hagel kraal, Hermanus and De Hoop did manage to grow at extreme temperatures of 40°C. The isolates from these localities may have adjusted the designs of their bacterial lipopolysaccharides (LPS), allowing them to withstand the temperature stress. Moreover, the strains might have overproduced a protein, namely 65 kDa which can be produced under extreme temperature stresses (Zahran et al. 1994; Zahran, 1999; Zeidler et al. 2010). The results indicate that these rhizobia strains may be used as potential high-temperature tolerant inoculum to rehabilitate degraded ecosystems or in agricultural ecosystems.

Salinity may have an impact on the growth and metabolism of rhizobia, affecting their symbiotic efficiency (Rasul et al. 2012). The data obtained in this study for salt tolerance shows that a majority (93%) of the isolates tested had a tolerance for sodium chloride (up to 1%). However, three (10%) *Indigofera* species were tolerant of 2- 3% sodium chloride content. It may be possible that *Indigofera* species are among the species with the greatest tolerance to saline soils as they were able to withstand salinity levels above 1.5% .Therefore house rhizobia that have salt tolerance traits. Similar results were reported by Rasul et al. (2012) where rhizobial isolates from *Milletia pinnata* in India tolerated salinity concentrations of 3% NaCl. The symbionts which tolerated salinity of greater than 2% NaCl may be suitable for use in areas with high (above 1.5%) sodium chloride contents in the soil.

Cape soils are particularly high in acidity, with pH ranging from pH 2.9 to above 8 (Kanu & Dakora, 2009; Lemaire et al. 2015a)The studied symbionts were isolated from a diversity of legume hosts in various geographic sites of the Cape with pH ranging between 3.3 and 6.5 (Dlodlo, 2012). Some genera of rhizobia such as *Burkholderia* are known to be more tolerant of acidic conditions than other genera as they are fast growing and can increase several yield components (Tran et al, 2006). Novel strains of *Burkholderia* species were found in root nodules of Papilionoideae which habituate acidic soils of the Western Cape- South Africa (De Meyer et al., 2013, 2014, 2011; Garau et al., 2009; J. G. Howieson et al., 2013; Mavengere et al., 2014; Steenkamp et al., 2015). In another study, 16S rRNA gene sequencing revealed that strains isolated from root nodules of *Mimosa* spp. native to acidic soils of north-east Brazil were closely related to *Burkholderia* spp. already identified. However, *Mesorhizobium* dominated growth in the more acidic media of pH 3 and 4 than *Burkholderia* in this study. This shows that *Mesorhizobium* is also acid tolerant and this probably explains its dominance in nodulating more legume species that the *Burkholderia* in the CCR (Lemaire et al. 2015a). In other reports, *Mesorhizobium loti* have also been found to have high acidity tolerance and were capable of withstanding pH levels as low as 4.0 (Rickert et al. 2000) by physiologically adjusting their cells upon exposure to lethal acid stress. Strains from Jonkershoek with voucher numbers 26 and 5370 were more tolerant of acidic stress in pH 3. This may be caused by the presence of "low pH detecting" genes in the isolate which physiologically adjust the strains ability to withstand acid stress. These rhizobia strains are suitable for use in inoculation studies in low pH areas. The control which was set at pH 6.8 showed that *Mesorhizobium* isolates 13 and 18; *Bradyrhizobium* 5621 and *Rhizobium* 24 have a relatively high growth response. It is expected that the same isolates would positively respond with high optical density measurements between pH 6 and 7, however that was not the case. This could be a result of the addition of chemical buffers, which conceivably altered the state of the medium and caused the unexpected result of specific isolates responding differently to the control set at 6.8 and pH treatments (chemically regulated) of between 6 and 7.

Burkholderia isolates were all observed to be rod shaped while 62% of the *Mesorhizobium* symbionts were rod shaped and only a low 38% were bacteriod shaped. This is an implication that *Mesorhizobium* were more morphologically diverse than the *Burkholderia*. *Burkholderia* strains were largely (62%) white opaque and only 38% are characterized by a milky culture pigment. Out of the 13 *Mesorhizobium* tested 12 (92%) were white opaque with only one isolate forming a watery translucent colour. Growth rate characterization of the symbionts showed that both genera were dominantly (92%) made up of fast growing rhizobia. *Burkholderia* are known to be acidophilic, thrive in low nutrient acidic environments and are fast growing (Stopnisek et al. 2013; Aizawa et al. 2010; Marsudi 1999; Saldaña et al. 2003). Furthermore, *Mesorhizobium also* withstand acidic environments (Brígido et al. 2007; Clarisse et al. 2013) and have been found to have intermediate to fast growing properties (Odee et al. 2002; Garc et al. 2007).

Conclusion

Both genera were highly compatible at 100% growth in the universal medium, Mannitol, an indication that Mannitol is the most preferred carbon source many genera. *Burkholderia* also had 100% growth in media with glucose as carbon source, while *Mesorhizobium* had 92% growth success, suggesting that CCR symbionts belonging to both genera may also do well in glucose-rich environments. *Burkholderia* growths were lower in starch (62%) and sucrose (83%) media when compared to *Mesorhizobium* with 85% in *starch* and 92% in sucrose media*.* This shows that *Mesorhizobium* is adapted both starch and sucrose sources of carbon associated with high hydrolysis of cellulose and starch into sugars.

Burkholderia isolates were generally more tolerant of extreme temperature stress as 58% of the isolates could withstand excessive heat of 40°C, while only 23% of *Mesorhizobium* survived under the conditions. This may be due to heat inducible protein regions (Münchbach et al.1999) in the *Burkholderia* symbionts*,* allowing the strains to regulate physiological response to heat stress (Parsell & Lindquist, 1993; Riezman, 2004). Two *Burkholderia* symbionts were highly tolerant of NaCl concentration of between 2 and 3% and *Mesorhizobium* isolates failed to grow at salinity stress of 3% while four strains grew in salinity stress of 2%. Natural selection of NaCl stress tolerant (Rai et al. 2012)*Burkholderia* symbionts in Cape soils may be a driver of the high NaCl tolerance by beta- rhizobia (Park et al. 2010).

The characterization experiments showed that there is phenotypic diversity among the different strains studied and that they were predominantly fast growing. All of the strains preferred Mannitol, supporting its common use in rhizobial laboratories. The isolates were persistent between temperatures 25 and 40°C with beta- rhizobia exhibiting a higher tolerance for temperature stress. Majority (93%) of the symbionts showed significant potential growth under salinity stress with *Burkholderia* having the highest response to extreme salinity of up to 3%. *Mesorhizobium* had the highest tolerance towards low acidic conditions; contrary to the expectation that beta-Proteobacteria perform better in acidic soils.

Chapter 3

Distribution of nodule forming *Burkholderia* in Core Cape Subregion soils.

Introduction

The major phylum, Proteobacteria is made up of alpha, beta, gamma, delta, epsilon and zeta- proteobacteria which may either be pathogenic or free-living (Kersters et al. 2006; Spain et al. 2009). Alpha rhizobia consist of several genera, including *Azorhizobium*, *Bradyrhizobium*, *Ensifer, Mesorhizobium* and *Rhizobium.* There are capable of forming nitrogen fixing symbioses with CCR members of the subfamily Papilinoideae which includes tribes Astragaleae, Crotalarieae, Genisteae, Indigofereae, Millettieae, Phaseoleae, Podalyrieae, Psoraleeae, Sesbanieae and more. (Hassen et al. 2012; Kock 2004; Kanu & Dakora 2012; Lemaire et al. 2015a). Beta-Proteobacteria are chemolitrophic bacteria (genus *Nitrosomonas*), can be phototrophic (genera *Rhodocyclus* and *Rubrivivax)*, but more known for their role as animal and plant pathogens (Compant et al. 2008; Suárez-Moreno et al. 2012) as well as free living in the soil (da Silva et al. 2012) and their recognition as rhizobia has become prominent in the last two decades. For example, *Aspalathus carnosa* was previously known to be nodulated by *Bradyrhizobium* (Deschodt & Strijdom, 1976), but later Moulin et al. (2001) discovered that the species formed an association with a *Burkholderia*. This was the very first discovery of a species of legumes in South Africa that was found to be nodulated by members of the beta-Proteobacteria. *Burkholderia* are now known to form associations with a number of legume hosts, both in subfamilies Papillionoideae (Elliott et al. 2007) and Mimosoideae (Bontemps et al. 2010).

Recent studies in the CCR have shown that the *Burkholderia* form nitrogen fixing associations with members of, among other tribes, Crotalarieae, Podalyrieae and Indigofereae (Mavengere et al. 2014; Beukes et al. 2013). Furthermore, Lemaire et al. (2015a&b) reported that Podalyrieae was exclusively nodulated by *Burkholderia* whereas some species of Indigofereae (*Indigofera*) and Crotalarieae (*Aspalathus*) were nodulated by both alpha-and beta- Proteobacteria. The genus *Burkholderia* consists of over 90 species (http://www.bacterio.net/burkholderia.html), which are exceptionally versatile gram-. They are naturally found in soil, water, and abundantly in the rhizosphere of plants (Parke & Gurian-Sherman, 2001). *Burkholderia* species are generally known as nitrogen fixing soil bacteria and also as pathogens among plant and animal including humans (Table 3.1).

Table 3.1: The association of various known *Burkholderia* species with plants, animals and humans.

Role	**Habitat**	**Rhizobia species**	**Reference**
Nitrogen fixing	Rhizosphere	*B. ambifaria*	Coenye et al. 2001
		B. anthina	Vandammeet al. 2002b
		B. graminis	Viallard et al. 1998
		B. phenazinium	Viallard et al. 1998
		B. phytofirmans	Sessitsch et al. 2005
		B. xenovorans	Goris et al. 2004
	Rhizosphere (pathogenic)	*B. terrestris*	Vandammeet al. 2013
		B. udeis	Vandamme et al. 2013
		B. choica	Vandamme et al. 2013
		B. humi	Vandamme et al. 2013
		B. glathei	Vandamme et al. 2013
		B. telluris	Vandamme et al. 2013

		B. megalochromosomata	Baek et al. 2015
		B. jiangsuensis	Liu et al. 2014
		B. cordobensis	Draghi et al. 2014
	Rhizosphere, plant (root, stem)	*B. tropica*	Reis et al. 2004
		B. unamae	Caballero-Mellado et al. 2004
	Rhizosphere, plant (root, leaves)	*B. silvatlantica*	Perin et al. 2006
	Rhizosphere (pathogenic), plant (root, leaves)	*B. grimmiae*	Tian et al. 2013
	Rhizosphere, plant root	*B. vietnamiensis*	Gillis et al. 1995
	Rhizosphere, plant	*B. pyrrocinia*	Vandamme et al. 2002b
	Root nodules	*B. caribensis*	Achouak et al. 1999
		B. dilworthii	De Meyer et al. 2014
		B. dipogonis	Sheu et al. 2015
		B. mimosarum	Chen et al. 2006
		B. nodosa	Chen et al. 2007
		B. phymatum	Vandamme et al. 2002a
		B. rhynchosiae	De Meyer et al. 2013
		B. tuberum	Vandamme et al. 2002a
Nitrogen fixing		*B. gladioli*	Yabuuchi et al., 1992

Pathogenic			
Pathogenic	Plants roots	*B. glumae*	Uurakami et al. 1994
		B. caryophylli	Yabuuchi et al., 1992
		B. plantarii	Urakami et al. 1994
	Rhizosphere	*B. dolosa*	Vermis et al. 2004
	Rhizosphere, plant root	*B. cenocipacia*	Vandamme et al. 1997
	Plant (root, stem, leaves)	*B. andropogonis*	Gillis et al. 1995
	Human and animal	*B. mallei*	Ribot & Ulrich 2006
		B. pseudomallei	Brett et al. 1997
	Fungus	*B. sordidicola*	Lim et al. 2003
Transitional	Root nodules	*B. symbiotica*	Sheu et al., 2012
	Rhizosphere	*B. dabaoshanensis*	Zhu et al. 2012
		B. soli	Yoo et al. 2007
		B. rhizoxinica	Partida-Martinez et al. 2007
		B. endofungorum	Partida-Martinez et al. 2007

There is strong evidence to support the view that members of *Burkholderia* copiously live as plant commensals, an association between the plant and the rhizobia whereby the plant benefits and the rhizobia derives neither benefit nor harm, in ecological communities with roots (Ramette et al. 2005; Tabacchioni et al. 2002). Some bacteria, such as *Burkholderia plantarii, B. unamae, B. nodosa, B. caryophylli and B. cepacia* are not restricted to the rhizosphere surfaces of the roots but are capable of colonising the internal root tissue (Hebbar et al. 1992).

Globally, the distribution of *Burkholderia* extends beyond South Africa, having been isolated in North America (Reis et al. 2004), Asia and South America (Chen et al. 2006), Central America (Barrett & Parker, 2005), Martinique in the French West Indies (Vandamme et al. 2002) and Australia. *Burkholderia* have been isolated in mimosoid legumes (especially *Mimosa* species) from Central America (Colorado Island- Panama), South America (Cerrado and Caatinga- Brazil) and Asia (Dehong- southern China) (Barrett & Parker, 2005; Liu et al. 2012; Bueno dos Reis et al. 2010). In South African Papilionoid legumes, all known rhizobial *Burkholderia* are recorded only in the Core Cape Subregion (CCR), occurring in tribes Astragaleae (*Lessertia*); Crotalarieae (*Aspalathus, Crotalaria, Lebeckia, Rafnia*); Genisteae (*Argyrolobium);* Hypocalypteae (*Hypocalyptus*), Indigofereae (*Indigofera*); Millettieae (*Tephrosia);* Phaseoleae (*Bolusafra, Dipogon, Rhynchosia*); Podalyrieae (*Cyclopia, Podalyria, Virgilia*), and Psoraleeae (*Psoralea*). (Beukes et al. 2013; De Meyer et al. 2013; Mavengere et al. 2014; Lemaire et al. 2015a). In the CCR, *Burkholderia* distribution has been found in sites ranging from the Cederberg to Tsitsikama (Figure 3.1). The localities are diverse and consist of different edaphic factors, eco-regions and soil types which may be driving factors for plant distribution and therefore influence *Burkholderia* distribution in the biodiversity hotspot.

Aims and Rationale

The genus *Burkholderia* occupies diverse ecological niches. The habitats in the CCR are highly heterogeneous due to differences in edaphic factors, topography climatic conditions, hence the local distribution and occurrence of *Burkholderia* within the CCR is not yet well understood.

This study aims to investigate the distribution and relationship of nodule forming *Burkholderia* species in soils from different sites of the CCR. This was done by collecting soil from 13 localities of the CCR and conducting glasshouse trapping experiments using *Podalyria calyptrata* and *Indigofera filifolia* as host plants. The relationship was phylogenetically assessed. It is expected that *Burkholderia* will be isolated from soils from all CCR localities, but the *Burkholderia* strains will differ between the two legume hosts.

Materials and methods

Soil collection and chemical analysis

The soil samples were aseptically collected from 13 localities in the CCR (Fig. 3.1 Table 3.4) consisting of various soil types with diverse legume species (Table 3.2). A global positioning system (GPS) was used to record the coordinates of the localities for each sample.

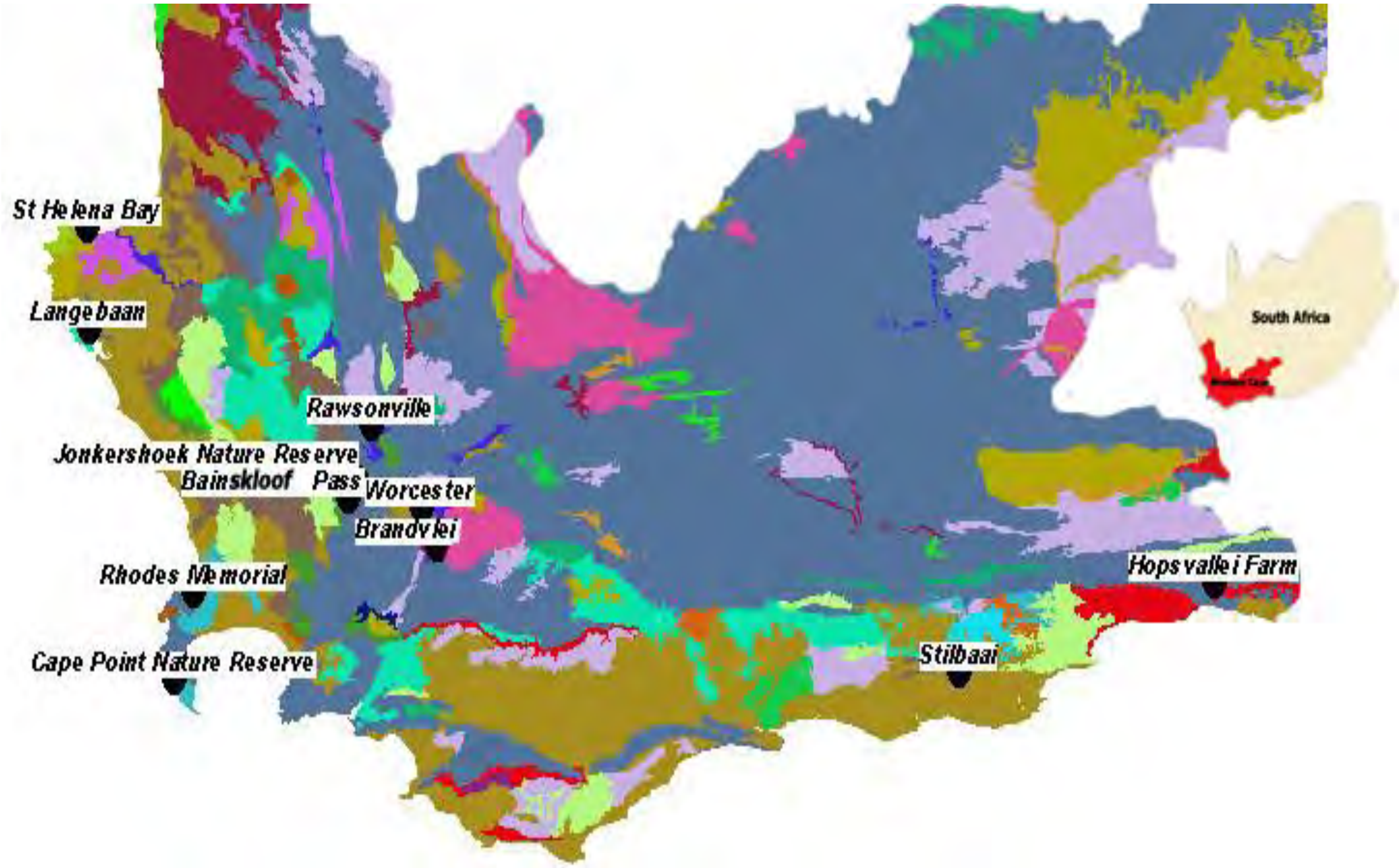

Figure 3.1: Sampling sites within diverse habitats of legumes in the CCR. The map was created in Diva GIS (R J Hijmans, Guarino, Cruz, & Rojas, 2001) and edited in Photoshop CS5.

Three replicate soil samples were collected at legume stands per site using an auger or a trowel to a depth of 15 cm. Each replicate soil sample was transferred into a sterile plastic bag, sealed tightly and allocated sample numbers. The soil samples were stored in a 10˚C refrigerator, to preserve the soils and the microbial communities harbouring the samples, until when the experiments begun.

For the chemical analysis of soil, a subsample of the soil initially stored in 10°C refrigerator were air-dried in a laboratory and sieved with a 1 mm mesh. To analyse for macronutrients and pH, three replicates from each locality were sent to the soil laboratory at Elsenburg (Department Agriculture, Western Cape). Soil was analysed for available phosphorus (P) by adding 25 mL of extracting solution, the mixture was centrifuged at 10,000 rpm for 10 minutes. The supernatant were filtered through no. 40 filter paper. Macronutrients, namely calcium (Ca), magnesium (Mg), potassium (K) and Sodium (Na) were measured by atomic absorption spectroscopy (AAS) in a tenfold diluted KCl extract (Carter et al., 2007). To determine soil pH, 2 g of soil was shaken in 20 ml 1 M KCI at 180 rpm for 60 min, centrifuged at 10 000g for 10 min and pH of the supernatant was measured with a pH meter.

Statistical analysis of the field soil nutrient data

The data were log-transformed to reduce inequality of variance before statistical analysis. To determine similarities and/or differences in the nutrient concentration of soil from the various sites, principal component analysis (PCA) was performed. In addition, the one-way analysis of variance (ANOVA) was adopted to test any significant differences between the means of the 13 independent and unrelated groups of soils. Both the PCA and ANOVA analyses were performed in Statistica software package (Statistica Ver. 12, StatSoft, Inc., Tulsa, OK, USA).

Table 3.2: Locality of soil samples, soil types and diversity of legumes found in the area.

Soil type	Locality	Co-ordinates	Altitude (m)	Legume assemblage
Acid sand	Brandvlei Dam (Worcester) 5440	33°48’24.2”S 19°27’00.3”E	229	*Aspalathus muraltoides, A. spicata* Thunb., *A. submissa* R.Dahlgren, *A. terbeculata, Indigofera heterophylla* C.Presl., *I. incana* Thunb.
Acid sand	Cape Point	34°14’21.9”S 18°22’49.5”E	10	*Aspalathus amphithalea, A. callosa* L., *A. juniperana subsp. Juniperana*
Acid sand	Worcester 5444	33°40’35.6”S 19°23’01.1”E	229	*Aspalathus spicata* Thunb., *A. terbeculata, Indigofera heterophylla* C.Presl., *I. incana* Thunb.
Acid sand	Worcester 5454	33°48’24.2”S 19°27”00.3”E	174	*Aspalathus spinosa L. subsp. spinosa*
Alluvium	Hopsvallei	33°53’13.7”S 22°21’00.6”E	716	*Aspalathus asparagoides L. subsp. rubrofusca, Psoralea azuroides*
Alluvium	Jonkershoek	33°58’39.0”S 18°56’37.4”E	272	*Aspalathus araneosa* L., *A. ciliaris, A. cordata* (L.) Dahlg., *A. ericifolia subsp. ericifolia, A. laricifolia* Berg. subsp. laricifolia, *Dipogon lignosus,* (L.) Verdc. *Otholobium virgatum* (Burm.f.) C.H. Stirt., *Psolarea asarina* (Berg.) Salter, *P.biflora* Harv., *P. usitata* C.H.Stirt.
Alluvium	Rawsonville	33°43’33.8”S 19°22’40.0”E	423	*Aspalathus acanthoclada* R.Dahlgren, *A. aurantiaca* R.Dahlgren, *A. hirta subsp. hirta* E.Mey., *A. pachyloba subsp. pachyloba, A. pigmentosa* R.Dahlgren, *A. turbeculata, Indigofera frutescens* L.f.
Granite	Langebaan	32°46’05.2”S 18°01’04.6”E	68	*Indigofera sp., Podalyria sericea* R.Br.
Granite	St Helena Bay	32°46’05.2”S 18°01’04.6”E	68	*Indigofera sp.*
Limestone	Stillbaai	34°20’59.0”S 21°45’14.7”E	100	*Aspalathus alopecurus* Burch. ex Benth., *A. capensis* R.Dahlgren, *Indigofera harvey, Psolarea muirii*

Sandstone	Bainskloof 5337	33˚38’32.10”S 19˚05’08.7”E	420	*Aspalathus divaricata* Thunb., *A. laricifolia* Lam., *Psolarea imbricata, P. fleta* C.H.Stirt., *P. ivumba* C.H.Stirt., *P. rigidula* C.H.Stirt., *P. usitata* C.H.Stirt.
Sandstone	Bainskloof 5346	33°36’47.4"S 19°06’04.8"E	769	*Psoralea fleta* C.H.Stirt., *P. rigidula* C.H.Stirt.
Shale	Rhodes Memorial	33˚57’01.1”S 18˚27’25.5”E	203	*Aspalathus ciliaris* L., *A. cordata* (L.) Dahlg., *A. hispida* Thunb., *A. macanthra, A. retroflexa* L., *Aspalathus sp., Otholobium fruticans* (L.) C.H.Stirt., *O. hirtum* (L.) C.H.Stirt., *O. virgatum* (Burm.f.) C.H. Stirt., *Psoralea asarina* (P.J.Bergius) T.M.Salter, *P. imbricata, P. pinnata* L., *Indigofera filiformis* Thunb.

Seed germination and plant growth

Two legume species representing tribes Indigofereae and Podalyrieae (*I. filifolia* and *P. calyptrata,* respectively) were selected as trapping hosts to represent CCR legumes with a high affinity for *Burkholderia* (Dlodlo, 2012; Lemaire et al. 2015a&b). Legume seeds were obtained from Silverhill Seed Company (Kenilworth, Cape Town, South Africa). Seeds sterilization and germination see methods section in Chapter 2 (Figure 3.2).

Figure 3.2: A tea strainer used to transfer seeds between solutions for efficient immersion in sterilization and water rinsing

The experimental pots were prepared by half-filling them with sterile acid washed sand, and topped with a layer of the field soil and another layer of sterile acid washed sand, and the whole pot covered with plastic bags until seed planting. At planting, a watering tube was inserted in the midpoint of the pot and a layer of polyvinyl chloride beads were added to cover the sand to minimize airborne contamination (Figure 3.3).

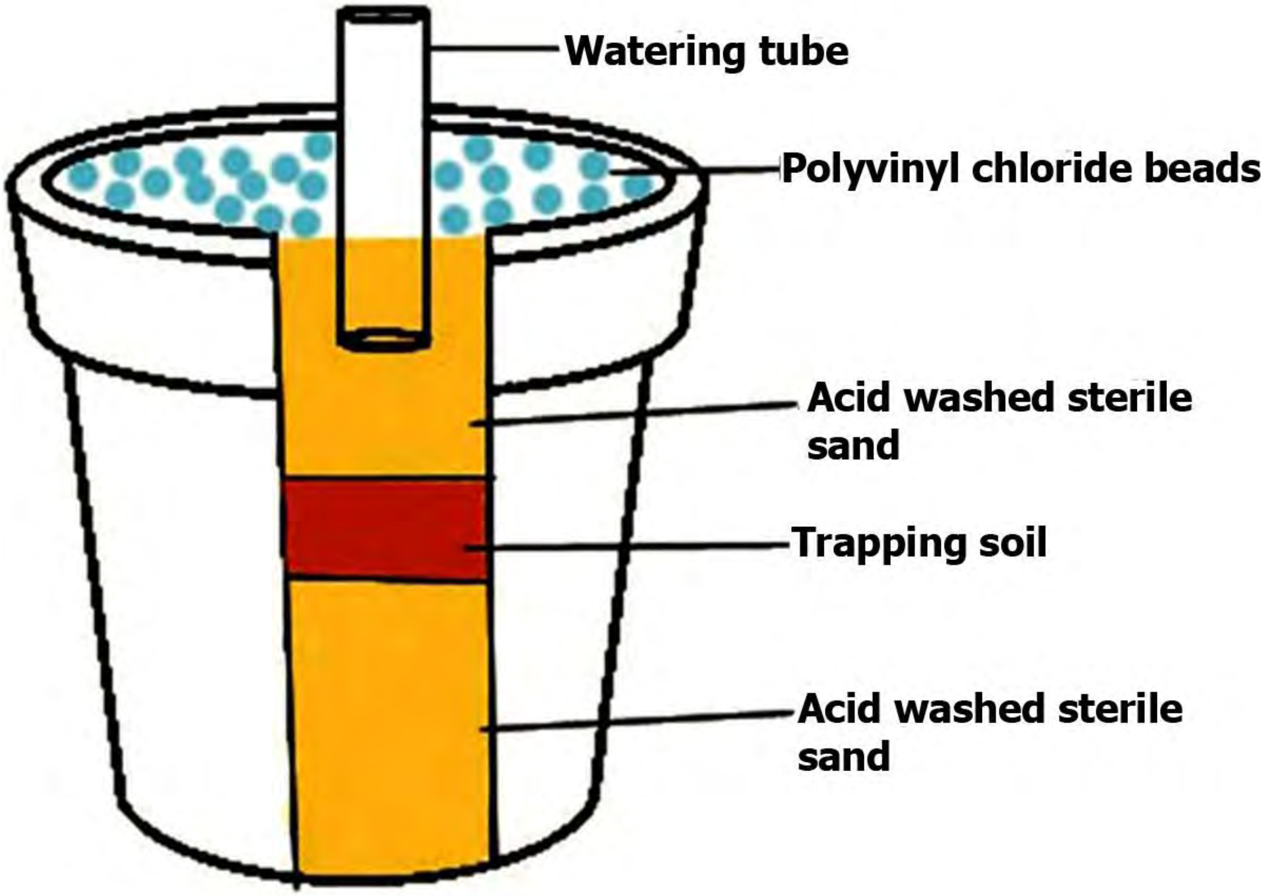

Figure 3.3: Cross section of the sterile pot for growth of germinated seedlings. (Created in Photoshop CS5 Extended).

Two replicate pots were planted with six plants, three plants in both pots from each one of the two host species (*I. filifolia* and *P. calyptrata*). Plants were watered with sterile distilled water every second day through the watering tube. The pots were arranged in a completely randomised design on tables in the glasshouse at the Department of Biological Sciences, University of Cape Town. The plants were grown aseptically and harvested approximately 4 months later (Figure 3.4).

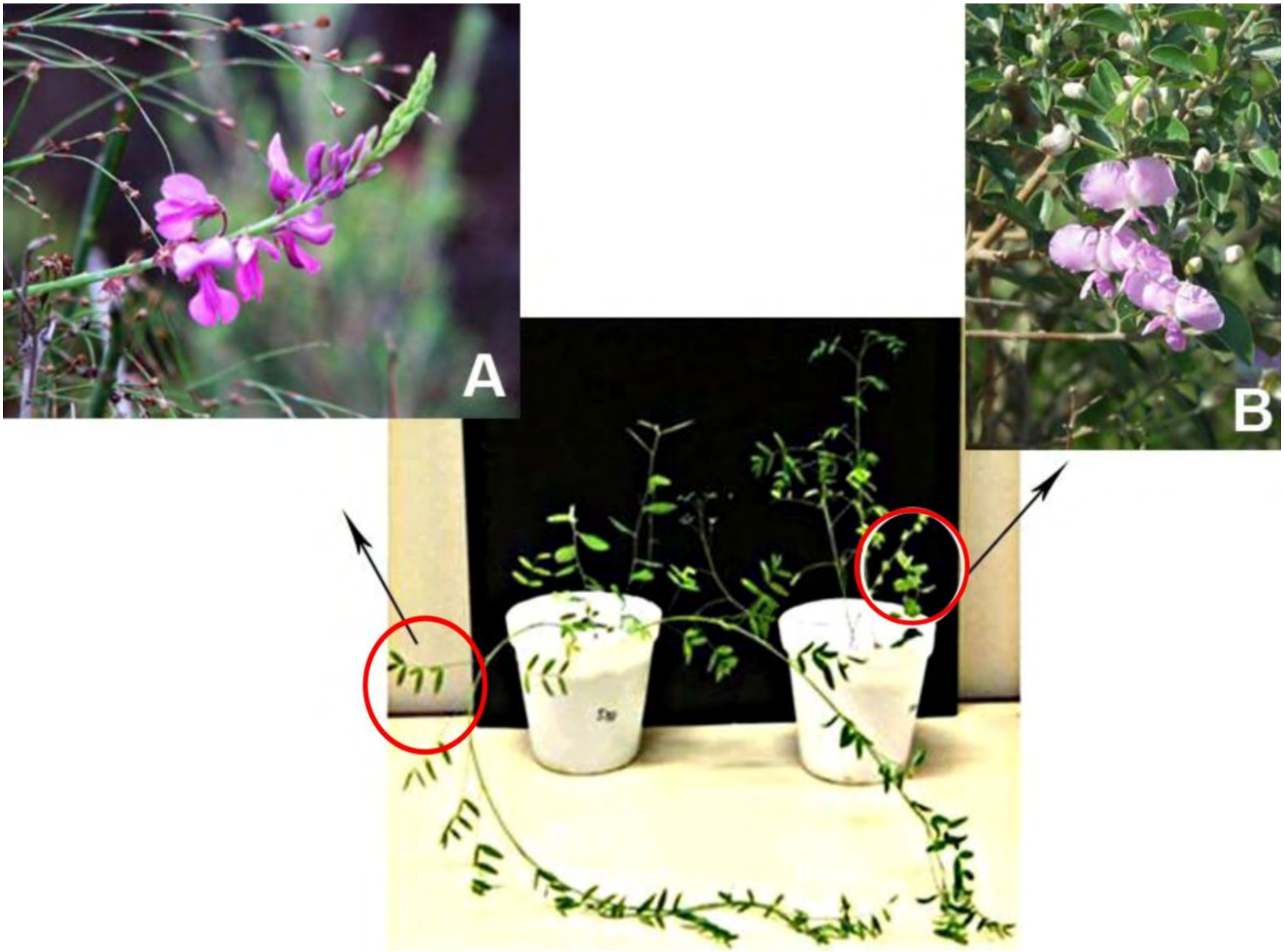

Figure 3.4: Experimental setup for *I. filifolia* (A) and *P. calyptrata* (B) in trapping pots before harvest.

Nodule collection at harvest

During nodule collection, roots were immersed into a bucket of water and the roots were gently freed while under water for ease of removing the soil and sand around the roots and preserving the nodules onto roots. Nodules were separated from the roots using a pair of forceps. Figure 3.5 shows plant roots of both *P. calyptrata* and *I. filifolia* (bearing a nodule) and shoots of both species after harvest.

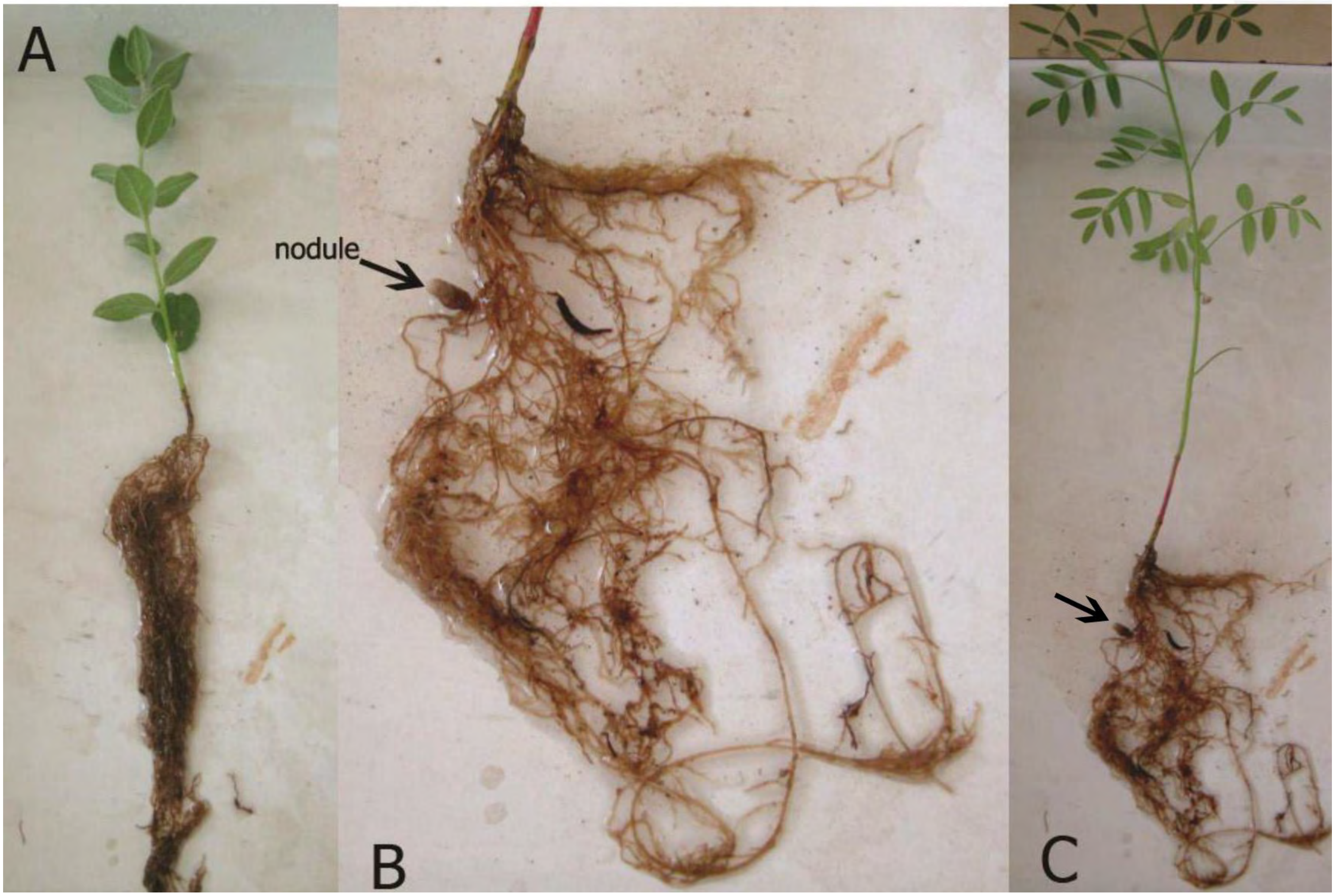

Figure. 3.5: (A) *Podalyria calyptrata* plant and (B) *Indigofera filifolia* roots bearing a nodule, (C) *Indigofera filifolia* root and plant after harvest.

Rhizobia isolation and culturing

Two nodules were picked from each plant that nodulated for rhizobia isolation and culturing. The nodules were stored in McCartney bottles at 4˚C until rhizobia isolation. The Vincent (1970) protocol was employed to prepare nodules for rhizobia strain isolation. Nodules were washed five times in sterile distilled water to remove excess soil. They were surface sterilized by dipping in 70% ethanol for 2 minutes to remove microorganisms on the surface and thereafter immersed for 3 min. in 0.1% acidified mercuric chloride ($HgCl_2$). The nodules were rinsed eight times with fresh changes of sterile distilled water to get rid of excess mercuric chloride.

The protocol outlined by Vincent (1970) was used in culturing of the soil bacteria. Each of the two nodules from a plant was aseptically crushed into a drop of sterile water. A loop full of squashed nodule exudate was aseptically streaked onto yeast extract Mannitol agar (YEMA) plates. The dilution streaking pattern was used to isolate single colonies. Plates were incubated at 25˚C for up to 4 days as bacteria were observed to be fast growing during preliminary studies. The cultured rhizobia growth was observed daily for rhizobial growth monitoring and possible contamination.

Deoxyribonucleic acid (DNA) extraction

A loop full of pre-grown rhizobia was aseptically added to 700 µl of Cetyltrimethylammonium Bromide (CTAB) solution in a tube. The CTAB simultaneously solubilises the plant cell wall, the lipid membranes of internal organelles and denatures proteins (enzymes). The tubes were briefly vortexed and incubated for approximately 1 hour at 68˚C. Into the lysate, 800 µl SEVAG (24ml chloroform and 1 ml isoamyl alcohol) was added and the mixture was vortexed. The tubes were centrifuged at room temperature for about 10 minutes at 10 000 rpm and the supernatant were transferred into new tubes with 450 µl isopropanol. The mixture was vortexed and incubated overnight at -20°C.

The tubes were centrifuged at room temperature for 20 minutes at 18 000 rpm and the supernatant were removed to leave the pellet at the bottom. To the pellet, 500 µl of 75% ethanol was added to clean and loosen the pellet. The pellet was centrifuged at room temperature for 10 minutes at 18 000 rpm and the supernatant were removed. To the pellet, 500 µl of 75% ethanol was added to clean and loosen the pellet. The pellet was centrifuged at room temperature for 10 minutes at 16 000 rpm and the supernatant were discarded. The pellet was air dried for 15 minutes while incubated at 37°C and 50-100 µl PCR water was added. The solution was vortexed and dissolved by incubation at 45°C.

Polymerase chain reaction (PCR) Amplification

All amplification reactions were completed in a 25 µl reaction mix (Kapa Biosystems), following manufacturer recommendations. All amplification reactions of the housekeeping genes, rec A, were performed using GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA) with PCR parameters as described by the authors: rec A (Gaunt, Turner, Rigottier-Gois, Lloyd-Macgilp, & Young, 2001). Amplification of the rec A gene of the selected *Burkholderia* symbionts was accomplished with the forward primer recA_63F ATC GAG CGG TCG TTC GGC AAG GG and reverse primer recA_504R TTG CGC AGC GCC TGG CTC AT. PCR reactions were prepared to a volume of 25 µl, containing 20 µl water, 2 µl buffer, 0.4 µl dNTP mix, 0.8 µl forward primer, 0.8 µl reverse primer, 0.08 µl taq and 1 µl DNA template. PCR thermal cycling profile cycle was set at an initial denaturing at 94°C for 3 seconds, followed by annealing at 55°C for 30 seconds and 1 minute of extension at 72°C. Approximately 1 µl aliquot of the amplified DNA was separated by 1% agarose gel electrophoresis in the presence of 1 X TAE buffer at 160 V for 13 minutes. DNA was visualised using UV light and recorded using a CCD camera. Amplified products were purified using a modification of the Exo/Sap enzyme cleaning protocol (Werle et al. 1994). Purified PCR amplicons were sent to Macrogen for sequencing (Macrogen, the Netherlands) using the same sequencing primers as for the initial PCR.

Phylogenetic analyses

The sequences acquired in the DNA analysis were edited using Bio Edit version 7.0.9.1. DNA sequences were aligned together with the sequences downloaded from GenBank in Mega 6.06 (Table 3.3) and manually adjusted in MacClade v.4.04 (Maddison & Maddison, 2000) to adjust ambiguously aligned sequences. Phylogenetic analyses were performed by Bayesian interference (BI) using CIPRES web portal (http://www.phylog.org). The GTR model was conducted with MrModeltest v.3.06 (Posada & Crandall, 1998). The analysis was run with four simultaneous Metropolis-coupled chains for 50 million generations, sampling a tree every 1000 generations (Drummond & Rambaut, 2007).

Table 3.3: Reference sequences (rec A) obtained from the GenBank .

Class	**GenBank accession number**	**Strain name**
Betaproteobacteria	MC406	*Burkholderia ambifaria*
Betaproteobacteria	LMG24066	*Burkholderia arboris*
Betaproteobacteria	MBA4	*Burkholderia caribensis*
Betaproteobacteria	MC03	*Burkholderia cenocepacia*
Betaproteobacteria	BR4802	*Burkholderia diazotrophica*
Betaproteobacteria	WSM3556	*Burkholderia dilworthii*
Betaproteobacteria	ICMP 19866	*Burkholderia dipogonensis*
Betaproteobacteria	ICMP 19430	*Burkholderia dipogonensis*
Betaproteobacteria	C4D1M	*Burkholderia graminis*
Betaproteobacteria	NCTC 10229	*Burkholderia mallei*
Betaproteobacteria	LMG23256	*Burkholderia mimosarum*
Betaproteobacteria	ATCC 17616	*Burkholderia multivorans*
Betaproteobacteria	C6786	*Burkholderia oklahomensis*
Betaproteobacteria	STM815	*Burkholderia phymatum*
Betaproteobacteria	PsJN	*Burkholderia phytofirmans*
Betaproteobacteria	1106a	*Burkholderia pseudomallei*
Betaproteobacteria	WSM3930	*Burkholderia rhynchosiae*
Betaproteobacteria	Br3407	*Burkholderia sabiae*
Betaproteobacteria	WSM5005	*Burkholderia sprentiae*
Betaproteobacteria	E264	*Burkholderia thailandensis*
Betaproteobacteria	STM678	*Burkholderia tuberum*
Betaproteobacteria	Bu	*Burkholderia ubonensis*
Betaproteobacteria	Brues C202	*Burkholderia unamae*

Betaproteobacteria	G4	*Burkholderia vietnamiensis*
Betaproteobacteria	LB400	*Burkholderia xenovorans*
Epsilonproteobacteria	ATCC43504	*Helicobacter pylori*
Alphaproteobacteria	MadridE	*Rickettsia prowazeki*

Results

Variation of the soil nutritional status of the locations

Nutrient analyses were conducted for 6 of the 13 soil sample locations because DNA amplification was successful only from those locations. These cover geographical spread and can be taken to be representative of localities. The soils were mostly acidic, with pH ranging from 3.7 (Cape Point) to 6.3 (Rhodes Memorial). Worcester soils had a pH of 3.9, Hopsvallei and Rawsonville soils had a pH of 4.1, St Helena Bay: 4.3, Bainskloof: 4.4, Jonkershoek: 4.7, Stilbaai: 4.7 and Langebaan soils had a pH of 5.3. The univariate analysis, which tested the equality of means of each nutrient element and pH, revealed that the sites had significantly different nutrients ($P<0.05$) (Table 3.4). Rhodes Memorial soil had the highest content for six of the eight nutrients tested, while the seven lowest nutrient concentrations were observed in soil from Jonkershoek.

Table 3.4: Nutrient concentration and pH of soils from studied localities and Mean ± SE (standard error) shown.

Locality	**Mean ± Standard error**							
	Total P (mg/kg)	Calcium cmol(+)/kg	Potassium mg/kg	Magnesium cmol(+)/kg	Sodium mg/kg	Ammonium Nitrogen %	Sulphur mg/kg	pH
Bainskloof	47.33 ± 6.12b	0.8 ± 0.1a	163 ± 26.06c	0.48 ± 0.04b	18.67 ± 0.33a	0.15 ± 0.02a	8.3 ± 1.31a	4.43 ± 0.09a
Hopsvallei	18.33 ± 0.33e	0.43 ± 0.01a	62 ± 0.00bc	0.28 ± 0.01ab	26.67 ± 0.67a	0.24 ± 0a	11.33 ± 0.67a	4.1 ± 0a
Jonkershoek	5.00 ± 0.00c	0.38 ± 0.17a	10.67 ± 0.67a	0.17 ± 0.01a	7.67 ± 0.33a	0.03 ± 0.02b	3.43 ± 0.35a	4.67 ± 0.07a
Rhodes Memorial	120.33 ± 0.33a	30.46 ± 1.24b	526.33 ± 10.04d	7.5 ± 0.09e	127 ± 1.53d	0.59 ± 0.01d	19.33 ± 0.67b	6.27 ± 0.03b
St Helena Bay	86.00 ± 3.06d	1.35 ± 0.13b	65.67 ± 1.45b	1.16 ± 0.12c	276.33 ± 14.68b	0.1 ± 0a	71 ± 17.21a	4.3 ± 0.3a
Stilbaai	12.00 ± 0.00ab	1.88 ± 0.01a	189 ± 1.00c	1.6 ± 0.01d	30 ± 0b	0.15 ± 0c	4.83 ± 0.03a	4.7 ± 0a

The PCA showed that the localities were separated by soil nutritional status (Figure 3.5) where soil from Jonkershoek, Rhodes Memorial and St Helena Bay soils clearly separated from each other. However, the soils collected from Bainskloof, Hopsvallei, and Stilbaai showed similarities as they were in close proximity in figure 3.5.

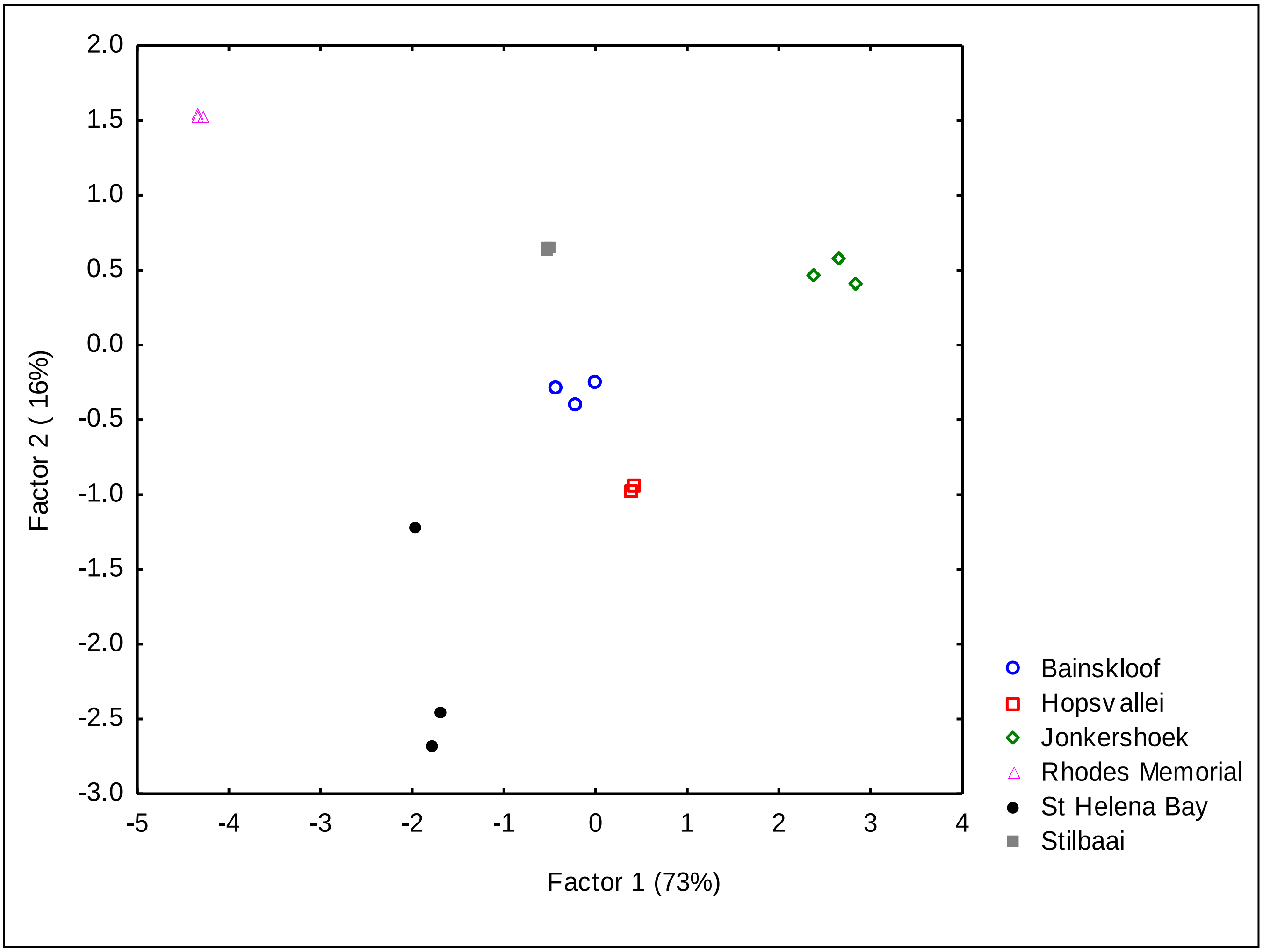

Figure 3.5: Scatter plot of locality by factors 1 and 2 that contributes 73% and 16% respectively to the multivariate separation of the sites

All the nutrients measured contributed markedly to the separation of the sites with Mg showing the greatest contribution followed by total P, K and Ca, Na and NH_4^+ (Table 3.5) along Factor 1. Along Factor 2, pH and S were the nutrient elements with the greatest influence on the separation of the soil locations.

Table 3.5: Variable contribution of each nutrient element to the separation of the study sites, and Eigenvalue of each factor from a Principal Component Analaysis. at $P<0.05$.

Variable	**Factor 1**	**Factor 2**
Total P (mg/kg)	-0.90	-0.25
Calcium cmol(+)/kg	-0.90	0.35
Potassium mg/kg	-0.90	0.09
Magnesium cmol(+)/kg	-0.97	0.18
Sodium mg/kg	-0.88	-0.38
Ammonium Nitrogen %	-0.84	0.022
Sulphur mg/kg	-0.76	-0.59
pH	-0.57	0.75
Eigenvalue	**5.80**	**1.27**

Rhizobia nodulation and amplification

Nodulation for *I. filifolia* was observed in 11 of the localities (85%) while for *P. calyptrata* nodulation was detected in 10 of the localities (77%). *I. filifolia* failed to nodulate when grown on soils from Hopsvallei, *P. calyptrata* failed to nodulate with soils from Rawsonville and St Helena Bay, and soils from Bainskloof (5346) failed to nodule both host species. Endosymbionts were purified from all the nodules, but among these several did not amplify, and soils from 6 localities (46%) produced nodules that had positive rec A amplification (Table 3.6). The presence of rec A in the six isolates fulfils Koch's postulates, thereby indicating the nodulation ability of the isolates.

Table 3.6: Representation plant nodulation and successful rec A DNA analyses of rhizobia trapped from the sampled rhizosphere soils.

Rhizosphere collection site	**Nodulation**		**Sequenced rhizobia isolates** (-) no amplification, (+) amplification	
	I. filifolia	***P. calyptrata***	***I. filifolia***	***P. calyptrata***
Bainskloof 5337	+	+	+	+
Bainskloof 5346	-	-	-	-
Brandvlei Dam (Worcester) 5440	+	+	-	-
Cape Point Nature Reserve	+	+	-	-
Hopsvallei	-	+	-	+
Jonkershoek Nature Reserve	+	+	+	+
Langebaan	+	+	-	-
Rawsonville	+	-	-	-
Rhodes Memorial	+	+	+	+
St Helena Bay	+	-	+	+
Stillbaai	+	+	+	+
Worcester 5444	+	+	-	-
Worcester 5454	+	+	-	-

Isolate phylogeny

The isolates from both *I. filifolia* and *P. calyptrata* from all 6 locations were *Burkholderia*, and closely matched with reference strains from the GenBank (Figure

3.6). The amplified isolates from 6 locations were distributed in 5 different clades (Figure 3.6, Table 3.7) of the Bayesian consensus tree based on recombinase A (Rec A). These clades had moderate phylogenetic support, as shown by the posterior probabilities (PP) between 0.83 to 0.94. Clade A (PP=0.88) comprised the reference strains *B. phytofirmans* and *B. dipogonensis*, together with *I. filifolia* endosymbionts (Bainskloof, Jonkershoek, Rhodes Memorial, St Helena Bay) and *P. calyptrata* endosymbionts (Jonkershoek, Rhodes Memorial). Clade B (PP=0.89) comprised the reference strains *B. tuberum*, together with *I. filifolia* endosymbionts (Jonkershoek, St Helena Bay, Stillbaai) and *P. calyptrata* endosymbionts (Bainskloof, Jonkershoek, Hopsvallei). Clade C (PP=0.88) had no named reference strains, and comprised *I. filifolia* endosymbionts (Rhodes Memorial) and no *Podalyria* endosymbionts from trapping study even though a previous field collected sample (Lemaire et al. 2015a) is part of this clade. Clade D (PP=0.83) comprised the reference strains *B. phytomatum, B. caribensis* and *B. diazotrophica*, together with *P.calyptrata* endosymbionts (Jonkershoek). Clade E (PP=0.94) had no named reference strains, and comprised *I. filifolia* endosymbionts (Bainskloof) and *P. calyptrata* endosymbionts (Jonkershoek).

Isolates which formed associations with reference *Burkholderia* strains from GenBank showed that the strains in the 5 clades were of various *Burkholderia* species. Studied plant hosts specifically formed symbioses with *Burkholderia* strains belonging to a particular clade, at times picking similar rhizobia regardless of the bio-geographic origin of the soils (Table 3.7).

Table 3.7: Annotated representation of the rhizobial clades as they appear on the phylogenetic tree.

Rhizobia clade	**Host**	**Locality**	**Reference Strains**
Clade A	*Indigofera filifolia*	Bainskloof	*Burkholderia phytofirmans* PsJN
		Jonkershoek	*Burkholderia dipogonensis* ICMP 19866
		Rhodes Memorial	*Burkholderia dipogonensis* ICMP 19430
		St Helena Bay	
	Podalyria calyptrata	Jonkershoek	
		Rhodes Memorial	
Clade B	*Indigofera filifolia*	Stilbaai	*Burkholderia sprentiae* WSM5005
		Jonkershoek	
		St Helena Bay	
	Podalyria calyptrata	Hopsvallei	
		Bainskloof	
		Jonkershoek	
Clade C	*Indigofera filifolia*	Rhodes Memorial	
Clade D	*Podalyria calyptrata*	Jonkershoek	*Burkholderia sabiae* Br3407 *Burkholderia phymatum* STM815 *Burkholderia caribensis* MBA4 *Burkholderia diazotrophica* BR4802
Clade E	*Indigofera filifolia*	Bainskloof	
	Podalyria calyptrata	Jonkershoek	

There was a combination of diverse isolates in the different localities. In clade A, for example, *I. filifolia* formed symbiotic associations with *Burkholderia* species from localities which formed molecular relationships with *I. filifolia* symbionts from shared localities in clades B and E. *I. filifolia*, in clades A and E have different species of symbionts sharing Bainskloof, while Jonkershoek consisted of isolates in clades A and B. St Helena Bay also shared different species of *Burkholderia* symbionts which occurred in clades A and B. Endosymbionts from *P. calyptrata* in 4 (clades A, B, D and E) clades shared a locality (Jonkershoek). Rhodes Memorial consists of isolates species which were found in clades A and C.

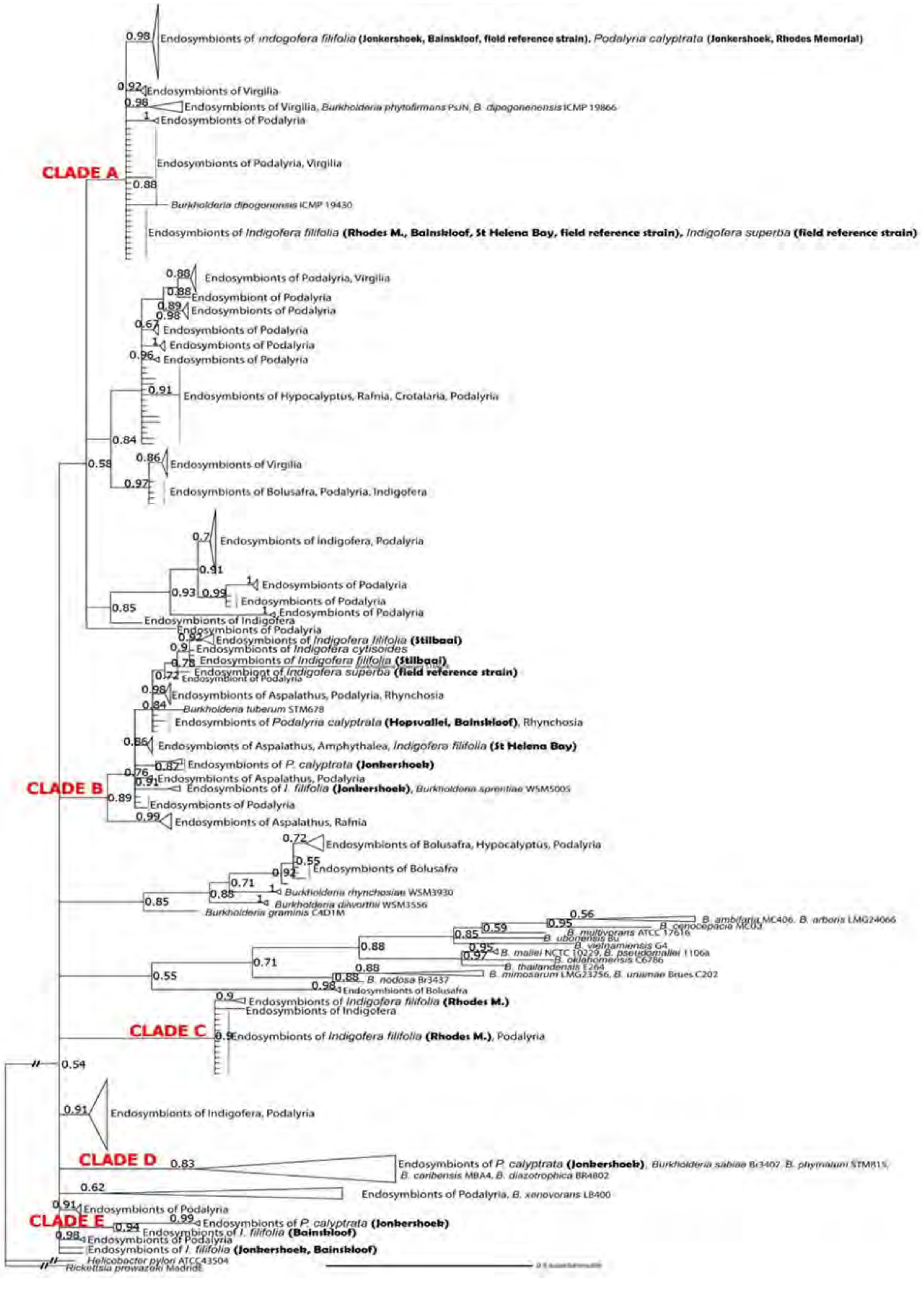
0.98 Endosymbionts of Indogofera filifolia (Jonkershoek, Bainskloof, field reference strain), Podalyria calyptrata (Jonkershoek, Rhodes Memorial)
0.92 Endosymbionts of Virgilia
0.98 Endosymbionts of Virgilia, Burkholderia phytofirmans PsJN, B. dipogonenensis ICMP 19866
1 Endosymbionts of Podalyria
Endosymbionts of Podalyria, Virgilia
CLADE A
0.88
Burkholderia dipogonensis ICMP 19430
Endosymbionts of Indigofera filifolia (Rhodes M., Bainskloof, St Helena Bay, field reference strain), Indigofera superba (field reference strain)
0.88 Endosymbionts of Podalyria, Virgilia
0.88 Endosymbiont of Podalyria
0.89
0.98 Endosymbionts of Podalyria
0.67 Endosymbionts of Podalyria
1 Endosymbionts of Podalyria
0.96 Endosymbionts of Podalyria
0.91 Endosymbionts of Hypocalyptus, Rafnia, Crotalaria, Podalyria
0.84
0.58
0.86 Endosymbionts of Virgilia
0.97 Endosymbionts of Bolusafra, Podalyria, Indigofera
0.7 Endosymbionts of Indigofera, Podalyria
0.91
1 Endosymbionts of Podalyria
0.93
0.99 Endosymbionts of Podalyria
1 Endosymbionts of Podalyria
0.85
Endosymbionts of Indigofera
Endosymbionts of Podalyria
0.92 Endosymbionts of Indigofera filifolia (Stilbaai)
0.9 Endosymbionts of Indigofera cytisoides
0.78 Endosymbionts of Indigofera filifolia (Stilbaai)
Endosymbiont of Indigofera superba (field reference strain)
0.72 Endosymbiont of Podalyria
0.98 Endosymbionts of Aspalathus, Podalyria, Rhynchosia
0.84 Burkholderia tuberum STM678
Endosymbionts of Podalyria calyptrata (Hopsvallei, Bainskloof), Rhynchosia
0.86 Endosymbionts of Aspalathus, Amphythalea, Indigofera filifolia (St Helena Bay)
0.87 Endosymbionts of P. calyptrata (Jonkershoek)
0.76 Endosymbionts of Aspalathus, Podalyria
0.91 Endosymbionts of I. filifolia (Jonkershoek), Burkholderia sprentiae WSM5005
CLADE B
0.89
Endosymbionts of Podalyria
0.99 Endosymbionts of Aspalathus, Rafnia
0.72 Endosymbionts of Bolusafra, Hypocalyptus, Podalyria
0.55
0.92 Endosymbionts of Bolusafra
0.71
1 Burkholderia rhynchosiae WSM3930
0.88
1 Burkholderia dilworthii WSM3556
0.85
Burkholderia graminis C4D1M
0.56 B. ambifaria MC406, B. arboris LMG24066
0.95 B. cenocepacia MC03
0.59 B. multivorans ATCC 17616
0.85 B. ubonensis Bu
B. vietnamiensis G4
0.88
0.95 B. mallei NCTC 10229, B. pseudomallei 1106a
0.97 B. oklahomensis C6786
B. thailandensis E264
0.71
0.88 B. mimosarum LMG23256, B. unamae Brues C202
0.55
0.88 B. nodosa Br3437
0.98 Endosymbionts of Bolusafra
0.9 Endosymbionts of Indigofera filifolia (Rhodes M.)
Endosymbionts of Indigofera
CLADE C
0.9 Endosymbionts of Indigofera filifolia (Rhodes M.), Podalyria
0.54
0.91 Endosymbionts of Indigofera, Podalyria
CLADE D
0.83 Endosymbionts of P. calyptrata (Jonkershoek), Burkholderia sabiae Br3407, B. phymatum STM815, B. caribensis MBA4, B. diazotrophica BR4802
0.62 Endosymbionts of Podalyria, B. xenovorans LB400
0.91 Endosymbionts of Podalyria
CLADE E
0.99 Endosymbionts of P. calyptrata (Jonkershoek)
0.94 Endosymbionts of I. filifolia (Bainskloof)
0.98 Endosymbionts of Podalyria
Endosymbionts of I. filifolia (Jonkershoek, Bainskloof)
Helicobacter pylori ATCC43504
Rickettsia prowazeki MadridE

Figure. 3.6: Bayesian consensus tree based on recombinase A (rec A) showing the relationships between the studied isolates in relation to GenBank reference strains. Isolated and field reference strains are illustrated in bold with reference to the trapping host and geographic origin of field sample.

Discussion

Statistical analysis of the sites which positively sequence for *Burkholderia* revealed that there were significant differences between the chemical composition and pH of soils, a characteristic of heterogeneity in the studied soils. Mutualistic associations with the host legumes may have facilitated ecological advantage of the *Burkholderia* to colonise diverse habitats with no biogeographical restrictions in the region. A study by Dlodlo (2012) supports the findings whereby *Burkholderia spp*. were isolated from predominantly Podalyrieae and Crotalarieae (specifically *Aspalathus* and *Rafnia*) collected from 20 localities in five phytogeographically different regions of the CCR (North-west, South-west, South-east, Langebaan and the Agulhas plain; Dlodlo, 2012). It was reported that *Dipogon lignosus* (Phaseoleae) in 3 different sites (Dinsdale, Hamilton, Jesmond Park, Hamilton and Mokau, Taranaki) of diverse soil types in New Zealand was nodulated by *Burkholderia spp*. isolates (Liu et al. 2014). In a study by Mishra et al. (2012) it was found that *Burkholderia spp.* were isolated from *Mimosa pudica* hosts in 8 different localities of diverse soil types in the French Guiana. This supports the findings in the current study that isolates are not structured by the geographic origin of the trapping soils, indicating that *Burkholderia* are widespread in the heterogeneous soils of the region.

The positive nodulation observed in the CCR soils may be a true indication of a widespread distribution of the rhizobia in the soil of the Southern tip of Africa. Bayesian consensus tree based on rec A patterns of the sequenced CCR isolates showed convincingly that the strains belong to the genus *Burkholderia* and are closely related to other nodulating *Burkholderia* species, such as *B. diazotrophica* Br 4802, *B. caribensis* MBA4, *B. dipogonenensis* ICMP 19866, *B. tuberum* STM678, *B. sprentiae* WSM 5005, *B. sabiae* Br3407 and *B. phymatum* STM815. The occurrence of *Burkholderia*, largely in soils of low nutrient content and low pH ranging from pH 4.7 to 6.3 from the region is in line with the view that *Burkholderia* are known to thrive in acidic conditions as they have been repeatedly isolated from low pH soils (Stopnisek et al. 2013; Garau et al. 2009). For instance, in the Cerrado and Caatinga biomes in South America, *Burkholderia* were reported to be the predominant symbionts of *Mimosa* plants (Bueno dos Reis et al. 2010) that grow in soil with pH ranging from 4.7 to 4.9. The wide distribution of *Burkholderia* in the region might be associated with the presence of the appropriate legume hosts in the different geographic localities. This is supported by previous studies (Lemaire et al. 2015a; Beukes et al. 2013; Mavengere et al. 2014; Garau et al. 2009) in which *Burkholderia* symbionts have been isolated from diverse legume tribes including Astragaleae, Crotalarieae, Hypocalypteae Indigofereae, Milletieae, Phaseoleae, Podalyrieae, Sesbanieae distributed across different localities (e.g. Cederberg Wilderness Area, St Helena Bay, Rhodes Memorial Jonkershoek and Tsitsikama) in the CCR. Globally, *Burkholderia* have been isolated from legume hosts including *Mimosa scabrella*, *Mimosa bimucronata, Mimosa pudica*, *Abarema macradenia* (Mimosoideae Tribe Mimoseae), *Machaerium lunatum* (Papilionoideae Tribe Dalbergieae) isolated from soil in Brazil, Martinique (the French West Indies), French Guiana, Taiwan, Venezuela and Caribbean islands, to name a few (Barrett & Parker, 2005; Chen et al. 2006, 2007; Mishra et al. 2012; Perin et al. 2006; Vandamme et al. 2002b).

On the other hand, there may have been limited assemblage of *Burkholderia* rhizobia in the soil from Hopsvallei and Rawsonville where only *I. filifolia* and *P. calyptrata*, respectively, nodulated. Furthermore, the failure of the soils from Bainskloof 5346 to nodulate both *I. filifolia* and *P. calyptrata* may be due to lacking *Burkholderia* symbionts. The soil from Bainskloof 5346 and Hopsvallei were from the higher altitude (769 m and 714 m, respectively) compared to the other sites (Table 3.2). According to Lemaire et al., (2015) and Bontemps et al., (2010), altitude plays an essential role in geographic patterning of *Burkholderia*. This may be an indication that *Burkholderia* distribution in the CCR may be influenced by altitude and some pockets of the region have lower or no symbionts available for effective plant-*Burkholderia* symbiosis.

Phylogenetic characterization of the rhizobia isolates revealed that the isolates consisted of different strains because they were located in different clades and some were closely related to already identified strains (e.g. *B. caribensis, B. diazotrophica, B. phymatum* and *B. sabiae*) obtained from GenBank. This shows great diversity of *Burkholderia* in the region, consistent with the report by Lemaire et al. (2015) who showed that the genus is able to explicitly colonize species in diverse legume tribes, namely: Crotalarieae, Hypocalypteae, Indigofereae, Phaseoleae and Podalyrieae showing a broad adaptation of *Burkholderia* to the CCR. The high diversity of *Burkholderia* observed may be associated with rich species diversity of legumes in the studied locations (Table 3.2). These results corroborate previous reports (Beukes et al. 2013; Garau et al. 2009a; Gyaneshwar et al. 2011; Howieson et al. 2013) that isolated different strains of *Burkholderia* from Fynbos legumes. Therefore, *Burkholderia* species associated with fynbos legumes are highly diverse and support the statement by Gyaneshwar et al. (2011) that CCR is a global biodiversity hotspot for *Burkholderia*.

The host legumes *P. calyptrata* and *I. filifolia* were colonized by *Burkholderia*, in spite of the soil sample being collected from locations (namely Bainskloof, Jonkershoek, Rhodes Memorial, St, Helena Bay, Stilbaai and Hopsvallei) that are known to associate with other rhizobia genera including *Mesorhizobium*, *Bradyrhizobium* or *Rhizobium*. This demonstrated that *P. calyptrata* and *I. folifolia* have specificity to *Burkholderia* species, a result that is consistent with previous reports (Beukes et al. 2013; Dlodlo, 2012; Lemaire et al. 2015a). The specificity of *P. calyptrata* for *Burkholderia* was not surprising because the genus *Podalyria* and tribe Podalyrieae are reported to be exclusively nodulated by *Burkholderia*, while species in the genus *Indigofera* can be nodulated by a range of rhizobia genera including *Burkholderia*, *Mesorhizobium*, *Bradyrhizobium* or *Ensifer* (Lemaire et al. 2015a). Furthermore, a study by Lemaire et al. 2015b suggests that there is high *Burkholderia* interaction with rhizobial lineages from South African isolates, and amongst those they found novel strains. The high *Burkholderia* diversity observed was evident from up to 12 clades formed, which included already identified strains and the potentially new rhizobia species. This may be the case in the current study whereby in clades C and E, the endosymbionts did not form any close relatedness with any strains already described in science. It is highly possible that these isolates may be novel to the science world.

Conclusion

This study revealed that *Burkholderia* symbionts cover entire geographic localities and are not restricted by soil type or legume species. Both *I. filifolia* and *P. calyptrata* formed a symbiosis with various species of *Burkholderia* isolates, an indication that the *Burkholderia* are common and have a high degree of diversity in CCR soils. The study overall showed that *Burkholderia* are common, diverse and *I. filifolia* and *P. calyptrata* have a substantial preference for *Burkholderia* in the Core Cape Subregion.

Chapter 4

General Discussion and Synthesis

In a recent study of rhizobia from Cape legumes (Lemaire, et al. 2015), nodule isolates were collected from 65 native Fynbos legumes of the papilionoid tribes and phylogenetically characterised. The analysis revealed great diversity of rhizobia in the region belonging to both beta- (*Burkholderia*) and alpha-rhizobial (*Azorhizobium*, *Bradyrhizobium*, *Ensifer, Mesorhizobium* and *Rhizobium*) lineages. Furthermore, the study showed that ecological factors such as site elevation correlated positively with genetic variation within *Burkholderia* and soil acidity with *Mesorhizobium*. As a follow-up to the study by Lemaire et al. (2015a), selected *Burkholderia* and *Mesorhizobium* isolates were phenotypically characterized to determine their growth period and response to edapho-climatic conditions such soil acidity and salinity, carbon sources, and temperatures changes. It was hypothesized that the characterized rhizobia isolates will form distinct patterns under the different abiotic growth conditions in relation to the beta- and alpha –rhizobia. The distribution of *Burkholderia* in CCR was also determined by inoculating the plants of *Podalyria calyptrata* and *Indigofera filifolia*, known to be nodulated by *Burkholderia*, with different soil collected from 13 locations in the CCR.

Majority (86%) of the isolates were fast growing, consistent with previous reports that isolates from acidic environments, like the CCR, are often fast growers (Dowdle & Bohlool, 1985; Hungria 2001; Odee et al. 1997). Contrary to the expectation that *Burkholderia* will grow better at low pH conditions, *Mesorhizobium* dominated growth in the more acidic media at pH 3 and 4 than *Burkholderia* in this study. This is an indication that in Cape soils, *Mesorhizobium* are more acid tolerant, enlightening why the genus was the more dominant in nodulating majority of legume species than the *Burkholderia* in the CCR (Lemaire et al. 2015a). The correlation of soil pH as an ecological factor and tolerance in low pH media could imply that the CCR rhizobia has gone through a natural selection process (Rai et al. 2012) enabling the low pH adapted rhizobia to dominate in the soil. Such specific adaptation to environmental stress could be explored for rhizobial selection for inoculant production for compatible legume species grown in similar soil conditions elsewhere. Isolates that grow well above 1.5% NaCl concentration are considered to show high level of salinity tolerance (Rai et al., 2012). At this level (1.5%) of NaCl concentration in the media, 93% of the isolates grew normally implying that the majority of the tested symbionts were highly tolerant to salinity with a minority of only *Indigofera* strains (*Bradyrhizobium* isolated from *I. frutescens* in Rawsonville Farm, *Burkholderia* isolated from *I. superba* in Hermanus and *Burkholderia* isolated from *Indigofera sp*. in Cederberg) showing tolerance to concentrations of up to 3%. This may suggest that *Indigofera* species may have evolved in highly saline soils and harbour rhizobia with high NaCl tolerance. Similarly, temperature tolerance was observed to be positively high, with normal growth up to 40˚C, showing that the studied isolates have mesophilic traits for tolerating extreme temperatures. The salinity (NaCl) levels in the CCR soil is relatively high (Flugel, 1993; Flügel & Kienzle, 1989; Owojori et al. 2009) and the region experiences hot and dry summers. It is known that high salt and temperature tolerance are important characteristics for survival under hot and dry conditions (Zerharil et al. 2000), but the actual mechanisms are not yet reported.

All isolates grew well when Mannitol was used in the medium as carbon source, followed by glucose, sucrose and considerable decline was observed in starch. This may be a reassurance of why Mannitol, amongst all the above mentioned carbon sources, is still universally preferred for culturing rhizobia in laboratories. However, several strains (66%) grew normally in all carbon sources, implying that they have amylase and cellulase enzymes (Rasul et al. 2012) to hydrolyse starch and cellulose, respectively, into simple sugars for rhizobial usage. Such rhizobia have potential to be used in food manufacturing industry as biocatalyst for industrial production of enzymes. Four rhizobia isolates grew well in all the carbon sources including *Burkholderia* from three *Indigofera* species (voucher No. 44, 5392 and 5419; Table 2.4) and *Mesorhizobium* from *Psorelea rigidula* (voucher No. 5343), representing the two dominant rhizobia genera in the CCR (Lemaire et al. 2015a). In addition to growth in all the carbon sources tested, these isolates tolerated temperature of up to 40°C and salinity of 2% and above, indicating that isolate nos. 44, 5392, 5419 and 5343 (Table 2.4) have distinctive characteristics with potential for biotechnological application after further investigations.

Isolates were highly (100%) tolerant of Mannitol medium. However *Burkholderia* isolates were also more tolerant (100% growth) of glucose unlike *Mesorhizobium* strains which had 92% growth in glucose media. *Burkholderia* isolates were the most (58%) tolerant in extreme (40°C) temperature stress, whereas *Mesorhizobium* had 15% growth. Symbionts could be physiologically flexible to adjust due to their temperature stress tolerance due to heat inducible protein regions (Münchbach et al. 1999; Riezman 2004; Parsell & Lindquist 1993). *Burkholderia* showed better growth in high salinity stress of 3%, this may be attributed to natural selection for NaCl stress tolerance (Rai et al. 2012).

The assessment of the distribution of *Burkholderia* in the CCR revealed that they are widespread in the area and that isolates consisted of different strains based on the rec A sequences on a Bayesian consensus tree. The different strains of the *Burkholderia* were not structured by the geographic origin of the soils, or the nutrient and pH levels of the soil. This is because the isolates from the same location were included in different clades of the Bayesian consensus tree, and isolates from diverse locations were found in the same clade. *Podalyria calyptra* and *I. folifolia* were colonized by *Burkholderia*, regardless of the soil sample being collected from location with multiple legume species that are also known to be associated with other rhizobia genera including *Mesorhizobium*, *Bradyrhizobium* or *Rhizobium*. This confirmed that *P. calyptrata* and *I. filifolia* are specifically nodulated by *Burkholderia* species.

Conclusion

It is clear from the results obtained that indeed there are phenotypic diversities among the strains studied. The isolates were fast growing, grew well in mannitol as the carbon source and had high tolerance to temperatures of up to 40°C, salinity of up to 3% NaCl and acidity of pH 3. Apart from Mannitol as the carbon source, *Burkholderia* grew better in glucose, while *Mesorhizobium* had a higher tolerance for starch and sucrose. *Burkholderia* showed the highest tolerance to extreme salinity of 3% whilst *Mesorhizobium* had the highest tolerance towards high acidic conditions; conflicting with expectations that beta- Proteobacteria are better survivors in acidic niches. The study also revealed that *Burkholderia* symbionts are common, and covering wide range of geographic localities in the CCR and are not restricted by soil type or legume species. *Indigofera filifolia* and *P. calyptrata* have exclusive preference for *Burkholderia* in the Core Cape Subregion.

Recommendations

Studied isolates are ideal for inoculation studies which may involve cross inoculation to determine host range and specificity of rhizobia strains. The isolated should also be assessed for their ability to fix nitrogen in competition experiments. This is essential for future agricultural purposes in improving plant yields by enhancing nitrogen fixation through rhizobial inoculation.

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Appendixes

Appendix A- Nutrient concentration and pH of soils from all 13 studied localities and Mean ± SE (standard error) shown.

Soil locality	**Mean standard error**							
	Total P (mg/kg)	**Calcium cmol (+)/kg**	**Potassium mg/kg**	**Magnesium cmol(+)/kg**	**Na mg/kg**	**Ammonium Nitrogen %**	**Sulphur mg/kg**	**pH**
Stilbaai	12 ± 0 ab	1.88 ± 0.01 a	189 ± 1c	1.6 ± 0.01 d	30 ± 0 b	0.15 ± 0 c	4.83 ± 0.03 a	4.7 ± 0 a
Hopsvallei	18.33 ± 0.33e	0.43 ± 0.01a	62 ± 0.00bc	0.28 ± 0.01ab	26.67 ± 0.67a	0.24 ± 0a	11.33 ± 0.67a	4.1 ± 0a
Rhodes Memorial	120.33 ± 0.33 a	30.46 ± 1.24 b	526.33 ± 10.04 d	7.5 ± 0.09 e	127 ± 1.53 d	0.59 ± 0.01 d	19.33 ± 0.67 b	6.27 ± 0.03 b
Jonkershoek	5.00 ± 0.00c	0.38 ± 0.17a	10.67 ± 0.67a	0.17 ± 0.01a	7.67 ± 0.33a	0.03 ± 0.02b	3.43 ± 0.35a	4.67 ± 0.07a
St Helena Bay	86 ± 3.05 d	1.35 ± 0.13 b	65.67 ± 1.45 b	1.16 ± 0.12 c	276.33 ± 14.68 b	0.1 ± 0 a	71 ± 17.21 a	4.3 ± 0.3 a
Langebaan	89 ± 2.52 d	1.47 ± 0.23 a	62 ± 3.06 bc	3.45 ± 0.29 f	74.67 ± 1.33 c	0.4 ± 0.03 d	19.33 ± 0.67 a	5.3 ± 0 g
Rawsonville	11.33 ± 2.4 ab	1.59 ± 0.55 a	44 ± 1.53 abc	0.37 ± 0.02 ab	11.33 ± 0.33 ab	0.07 ± 0.01 a	5.43 ± 0.49 a	4.2 ± 0 bcd
Cape Point	4.33 ± 0.33 a	1.69 ± 0.07 a	16.67 ± 0.67 ab	0.79 ± 0.06 bc	23 ± 0.58 ab	0.05 ± 0.01 a	4.77 ± 0.15 a	4.7 ± 0.06 ef
Worcester 5440	8.33 ± 1.45 ab	0.49 ± 0.02 a	41 ± 2.52 abc	0.27 ± 0.01 a	12.67 ± 0.33 ab	0.06 ± 0.01 a	3.67 ± 0.37 a	3.9 ± 0 ab
Worcester 5444	11.66 ± 1.76 ab	2.22 ± 0.08 a	185.67 ± 14.38 d	1.81 ± 0.07 e	30 ± 0 b	0.16 ± 0 b	6.37 ± 0.43 a	4.8 ± 0 f
Worcester 5454	6.33 ± 0.88 a	1.07 ± 0.19 a	39.33 ± 3.71 abc	0.61 ± 0.03 ab	22 ± 0 ab	0.04 ± 0.01 a	3.67 ± 0.27 a	4.77 ± 0.03 ef
Bainskloof 5337	47.33 ± 6.12b	0.8 ± 0.1a	163 ± 26.06c	0.48 ± 0.04b	18.67 ± 0.33a	0.15 ± 0.02a	8.3 ± 1.31a	4.43 ± 0.09a
Bainskloof 5346	12.67 ± 0.33 ab	0.56 ± 0.1 a	20.67 ± 0.33 abc	0.36 ± 0.06 ab	17.67 ± 0.33 ab	0.06 ± 0.03 a	6.57 ± 0.44 a	3.57 ± 0.03 a