

Plant growth-promoting properties of fynbos rhizobia and their diversity

by

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Declaration

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Summary

Soil has a high diversity of microorganisms which differs from the bulk to the rhizosphere soil. Bulk soil often has a higher microbial diversity than the rhizosphere, whereas the rhizosphere has a higher microbial mass due the high levels of root exudates provided by the plant. Diazotrophs, free-living nitrogen fixing bacteria, play an important role in plant growth, particularly in the nutrient poor soil, a characteristic of fynbos soil. *Aspalathus linearis* (rooibos) and *Cyclopia* spp. (honeybush) are leguminous plants endemic to the fynbos region of South Africa. These plants thrive in the nutrient poor fynbos soils and have become popular as herbal teas. Leguminous plants in this region rely on rhizobia to fix nitrogen and supply plants with other important nutrients such as plant growth-promoting compounds in exchange for a carbon source. The aim of this study is to determine the diversity and function of diazotrophic bacteria and rhizobia associated with *A. linearis* and *Cyclopia* spp. plants.

The aim was achieved by determining the diversity of the diazotrophic community in the bulk and rhizosphere soils of commercial and natural rooibos and honeybush plants. Terminal restriction fragment length polymorphism (T-RFLP) was used to determine the diversity of the *nifH* gene in soil microbial DNA. Real-time Quantitative Polymerase Chain Reaction (qPCR) was also used to quantify the nitrogen fixing bacteria in soil. The copy number of *nifH* in the bulk soil was lower than that in the rhizosphere. The results indicated no significant differences between the diazotrophic communities of commercial and natural plants.

Rhizobia were isolated from rooibos and honeybush root nodules to determine the diversity of rhizobia that associate with *A. linearis* and *Cyclopia* plants. Sequence analysis of the rhizobial isolates was done on the 16S rRNA, *recA*, *atpD* housekeeping genes, as well as the *nodA* and *nifH* symbiosis genes. *Burkholderia* and *Rhizobium* species were isolated from the root nodules. *Aspalathus linearis* preferred to associate with α-proteobacteria, whereas *Cyclopia* spp. preferred *Burkholderia* (β-proteobacteria) species. Phylogenetic analysis showed that some of the isolates are closely related to isolates from other studies done on fynbos root nodules. Further studies have to be done on the isolates that are not closely related to known species to determine if they are novel species.

In the final part of the study, the rhizobial isolates were screened for the production of plant growth-promoting properties. Isolates were plated on specialised media and tested for the production of ammonia, hydrogen cyanide, phosphatase, 1-aminocyclopropane-1-carboxylate deaminase, and indole acetic acid. Results indicate that rhizobia provide the host plant with more than just available nitrogen. Rhizobia also produce indole acetic acid, HCN, and phosphatase. Some isolates produced more plant growth-promoting compounds than others, and considerable variation between strains was observed. These overproducing isolates could be used in potting trials to determine their effect on plant growth.

This study highlights the importance of symbiotic interactions between microorganisms and plants. Future studies should include sampling extensive areas, over different seasons, as well as screening, isolating, and identifying more rhizobia across various plant hosts.

Opsomming

Grond het 'n hoë diversiteit van mikroöorganismes wat verskil van die grootmaatgrond ("bulk soil") en die risosfeer. Grootmaatgrond het gewoonlik 'n hoër mikrobiële diversiteit as die risosfeer, maar die risosfeer het 'n hoër mikrobiële biomassa as gevolg van die wortel-ekssudaat-uitskeidings wat deur die plant vervaardig word. Diasotrofe is vrylewende stikstof-fikserende bakterieë en speel 'n belangrike rol in plantgroei, veral in voedingstof-arm grond wat 'n kenmerk van fynbosgrond is. *Aspalathus linearis* (rooibos) en *Cyclopia* spp. (heuningbos) is peulplante wat indemies is in die fynbos-area van Suid-Afrika. Die plante floreer in die voedingstof-arm fynbosgrond en is bekend as kruietee. Peulplante in die area is afhanklik van risobakterieë om stikstof te fikseer en verskaf ander belangrike voedingstowwe soos plantgroei bevorderingsverbindings in ruil vir 'n koolstofbron. Die doel van hierdie studie is om die diversiteit en funksie van diasotrofiese bakterieë asook die risobakterieë wat met *A. linearis* en *Cyclopia* spp. assosieer te bepaal.

Die doel van hierdie studie is bereik deur die diversiteit van die diasotrofiese gemeenskap in die grootmaatgrond en die risosfeer van kommersiële en natuurlike rooibos- en heuningbosplante, te bepaal. Terminale Restriksie Fragment Lengte Polimorfisme (T-RFLP) is gebruik om die diversiteit van die *nifH*-geen van die grondmikroörganisme DNA in die grond te bepaal. Die kopienommer van die *nifH*-geen in die grootmaatgrond was laer as in die risosfeer. Die resultate dui aan dat daar geen beduidende verskil tussen die diasotrofiese gemeenskappe van kommersiële en natuurlike plante is nie.

Risobakterieë is uit die wortelknoppies van rooibos- en heuningbosplante geïsoleer om die diversiteit van die risobakterieë wat met *A. linearis* en *Cyclopia* assosieer te bepaal. Volgorde bepalingsanalise van die 16S rRNA, *recA*, *atpD* huishouding-gene asook die *nodA* en *nifH* simbiose is gedoen om die isolate te identifiseer. *Burkholderia* en *Rhizobium* spesies is uit die wortelknoppies geïsoleer. *Aspalathus linearis* verkies om met α-proteobakterieë te assosieer, terwyl *Cyclopia* spp. verkies om met *Burkholderia* (β-proteobakterieë) te assosieer. Filogenetiese analise het getoon dat sommige isolate naby verwant is aan isolate wat tydens ander studies op fynbos-wortelknoppies geïsoleer is. Verdere studies moet nog op die isolate gedoen word wat nie naby verwant is aan bekende spesies nie om te bepaal of dit nuwe spesies is al dan nie.

In die laaste gedeelte van die studie is die isolate gekeur vir die produksie van plantgroei bevorderende eienskappe. Isolate is op gespesialiseerde media uitgeplaas en vir die produksie van ammoniak, waterstofsianied, 1-aminosiklopropaan-1-karboksilaat deaminase en indool asynsuur getoets. Die resultate dui aan dat die risobakterieë meer as net stikstof aan die plant verskaf. Sommige isolate het meer plantgroei bevorderende verbindings geproduseer as ander en daar was aansienlike variasie tussen stamme. Die stamme wat hoë vlakke geproduseer het, kan in potplantproewe gebruik word om hulle effek op plantgroei te bepaal.

Die studie lig die belang van simbiotiese interaksies tussen mikroörganismes en plante uit. Toekomstige studies kan uitgebreide areas van monsterneming, verskillende seisoene, keuring van meer isolate en die identifisering van meer risobia van verskillende plant-gashere insluit.

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Chapter 1:

Literature review

Rhizobial diversity and function in rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.) plants: a review

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Abstract

Aspalathus linearis (rooibos) and *Cyclopia* spp. (honeybush) are leguminous plants endemic to the fynbos region of South Africa. These plants have become popular for their use as herbal teas because of their health benefits as they are high in antioxidants. They thrive in the acidic, nutrient poor soils of the fynbos region that is known to have low and high rainfall regions as well as clay and sandy soil types. Majority of legumes in this region rely on rhizobia to fix nitrogen and supply plants with other important nutrients such as phosphorous, in exchange for a carbon source. This review focus on the species diversity of rhizobia associated with *A. linearis* and *Cyclopia* spp. plants. Genera that are regularly isolated from rooibos and honeybush root nodules include *Rhizobium*, *Burkholderia*, *Mesorhizobium* and *Bradyrhizobium*. These species produce bioactive compounds that directly or indirectly affect growth of leguminous plants.

1.1. Introduction

The Cape Floristic Region (CFR sensu Goldblatt & Manning 2000; also called the Core Cape Region sensu Manning & Goldblatt, 2012) is located to the south-western and western regions of South Africa and is characterized by a high floral diversity. Legumes in the CFR contribute ~10% of the endemic plant species (Manning & Goldblatt, 2012), and include two commercially important species, *Aspalathus linearis* (Burm.f.) R.Dahlgren (rooibos) (Figure 1.1a) and *Cyclopia* spp. (honeybush) (Figure 1.1b) (Garau et al., 2009; Howieson et al., 2013; Lemaire et al., 2015a). These two legumes belong to the family Fabaceae, where *Aspalathus* belongs to the tribe Crotalarieae and *Cyclopia* to the Podalyrieae (Joubert et al., 2008). *Aspalathus linearis* and some *Cyclopia* spp. (*C. subternata* Hofmeyr & E.Phillips., *C. intermedia* E.Mey., *C. sessifolia* Eckl. & Zeyh., *C. genistoides* (L.) Vent. and *C. longifolia* Vogel) are used to produce herbal teas known as Rooibos and Honeybush, respectively. One of the main reasons for the popularity of these teas is that it is free of caffeine and low in tannins (Joubert et al., 2007).

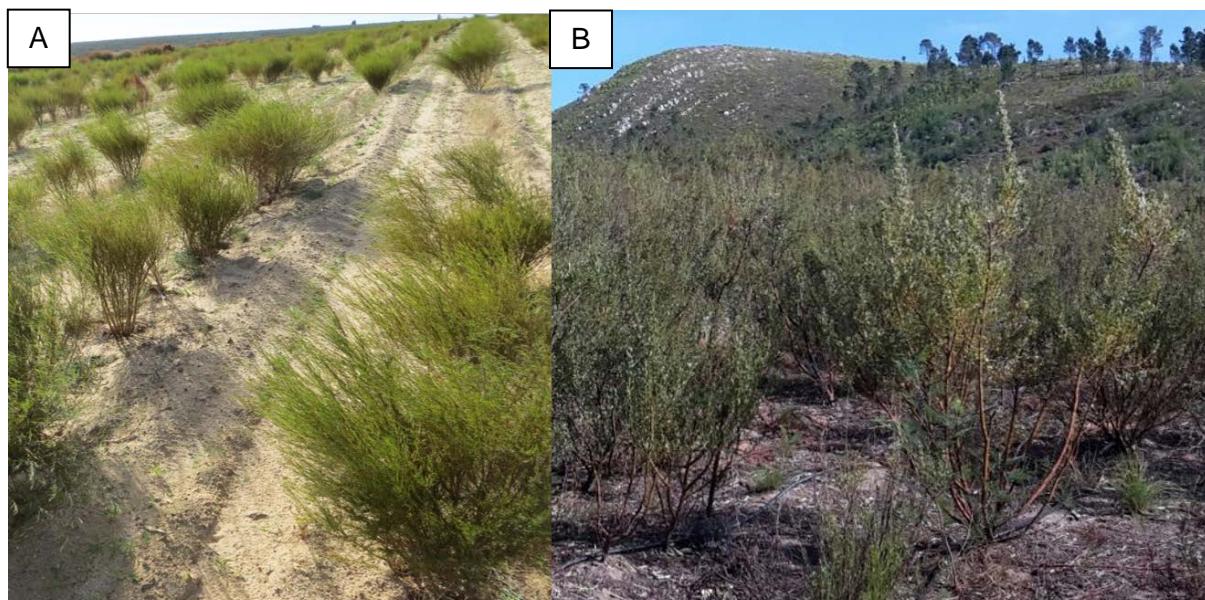


Figure 1.1. (A) *Aspalathus linearis* (rooibos) and (B) *Cyclopia subternata* (honeybush sp.) in plantations (photo credit: Jonathan Kriel and Armand van Wyk).

Aspalathus linearis is indigenous to the Cederberg region in the Western Cape of South Africa (Figure 1.2). This region has winter rainfall and only receive an average of 200 mm rain per year, and is, therefore, classified as an arid region (Hoffman et al., 2009). *Cyclopia* spp. can be found on the southern slopes of mountainous and coastal regions of the Eastern and Western Cape provinces of South Africa (Figure 1.2). Some honeybush species occur in a widespread region, while others can only be found in a small geographical area. Honeybush that prefer the mountainous region can be found on the wetter, cooler southern slopes. Honeybush plants have successfully adapted to the

natural occurring fires associated with the fynbos biome by developing two survival strategies, namely resprouters and seeders (Joubert et al., 2011). This differs from the commercially used Rooibos which is a seeder (Van Heerden et al., 2003). Power et al. (2011) suggests that the seeders are likely to have higher nutrient requirements than resprouters, due to more resources allocated for reproduction. The seeder plants have a hardened seed coat, consequently scarification or fire is usually required for effective germination to take place (Joubert et al., 2011).

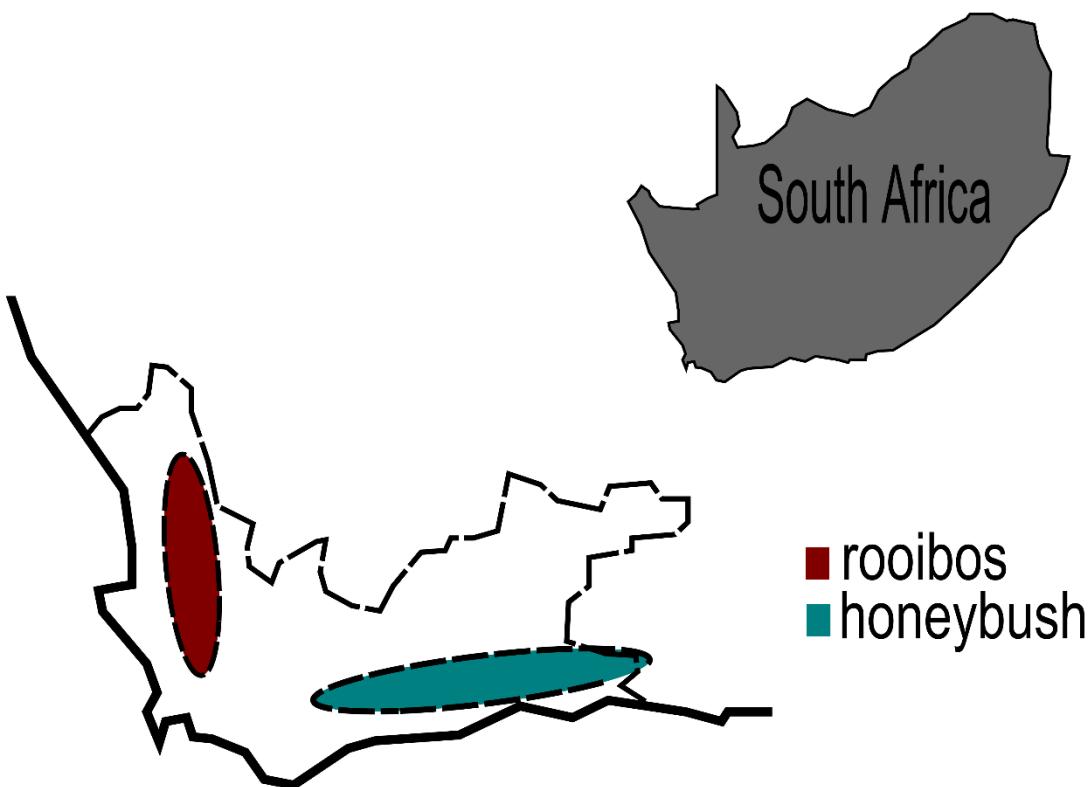


Figure 1.2. Map of geographical distribution of rooibos (red) and honeybush (green) of the Eastern and Western Cape.

Soils in which *Aspalathus* and *Cyclopia* species grow are acidic and low in nutrients, especially in nitrogen and phosphorus concentrations (Maistry et al., 2013; Muofhe and Dakora, 2000; Power et al., 2010; Richards et al., 1997). The availability of nutrients is one of the limiting factors for plant growth in this region (Vardien et al., 2014; Witkowski and Mitchell, 1987). To overcome this limitation, efficient nutrient exchange between plant roots and the soil environments as well as interactions with microorganisms occur in the rhizosphere (Dakora and Phillips, 2002). In this environment, microorganisms that form symbiotic interactions with plant roots play a critical role in nutrient uptake for plants (Richards et al., 1997; Vardien et al., 2014). Bacteria and fungi can provide plants with important nutrients such as nitrogen and phosphates (Sprent and James, 2007). Some

Gram-negative, rod shape bacteria, collectively known as rhizobia, have the ability to form root nodules with some legumes (Peix et al., 2015; Prell and Poole, 2006). These bacteria aid in nitrogen fixation for the plant in exchange for carbon sources (Ashraf et al., 2013). Apart from the positive effect rhizobia have on plant growth through nitrogen fixation, some members of this group of bacteria also produce plant growth-promoting compounds. Rhizobia often have a positive effect on plant growth and are known as plant growth-promoting rhizobacteria (PGPR). Although some PGPR form root nodules with the host plant, the majority are free-living soil bacteria and do not form root nodules (Hayat et al., 2010). In addition, some rhizobia are able to form root nodules, but do not produce plant growth-promoting compounds. There are many different species of PGPR that produce plant growth-promoting factors, albeit in different concentrations (Rosenblueth and Martínez-Romero, 2006). Consequently, rhizobia that usually produce the highest concentrations of plant growth-promoting factors, are often used in seed inoculations to improve growth of commercial crops (Akladious and Abbas, 2014; Bashan et al., 2014). Some rhizobia can have non-nodulating and often endophytic relationships with non-leguminous plants. These associations can have a positive effect on plant growth and nutrient uptake (Biswas et al., 2000; Mishra et al., 2012). However, the production of metabolites by rhizobia and their ability to colonization plant roots, are significantly influenced by various abiotic and biotic factors (Ciccillo et al., 2002).

The most abundant and frequently isolated rhizobial species in the CFR include members from the genera *Burkholderia* (Beukes et al., 2013; Garau et al., 2009; Gyaneshwar et al., 2011; Howieson et al., 2013; Lemaire et al., 2016a, 2016b), *Rhizobium* (Lemaire et al., 2015a) and *Mesorhizobium* (Lemaire et al., 2015a). Legume nodulating bacteria belong to the phylum Proteobacteria, although they do not form a monophyletic group (Peix et al., 2015; Williams et al., 2010; Williams and Kelly, 2013). Initially, rhizobia were only considered to belong to the subgroup α-Proteobacteria (Peix et al., 2015). This changed when Chen et al. (2001) detected nodule-like structures that did not fix nitrogen on the roots of *Macroptilium atropurpureum* and Moulin et al. (2001) confirmed that *Burkholderia* can fix nitrogen. This was followed by studies in 2005 that proved the ability of *Burkholderia* to form symbiotic relationships with legumes in their studies on *Mimosa* species (Chen et al., 2005a; 2005b). Some rhizobia species from subgroups α- and β-Proteobacteria have megaplasmids that contain the symbiosis genes. Phylogenetic analysis showed that there is a smaller difference in the *nod* genes of α- and β-Proteobacteria than the difference between the 16S rRNA genes from α- and β-Proteobacteria (Moulin et al., 2001). The presence of *nod* genes in α- and β-Proteobacteria suggests that it probably occurred through horizontal gene transfer. This is, however, only true for papilionoid-nodulating *Burkholderia* spp. In contrast, the *nod* genes from the *Mimosa* nodulating *Burkholderia* are different to the genes of α-rhizobia. This suggests that the symbiosis genes diverged over a long period of time within *Burkholderia* (Bontemps et al., 2010).

Agricultural practices usually result in a decline in microbial diversity and structure as observed in other crops (Griffiths et al., 2001). However, it was found that the bacterial diversity and species richness in the rhizosphere of commercial and wild honeybush plants are highly similar (Postma et al., 2016). This is most likely because land is not cleared for honeybush plantations as is the case with other cropping systems (Postma et al., 2016). A similar picture emerged from studies on the rooibos rhizosphere communities (Postma et al. submitted). On the other hand, a significant seasonal effect was found and the wet and dry seasons had different community structures in both the honeybush and rooibos systems (Postma et al., 2016). These seasonal changes in the microbial community suggest that bacteria react differently to environmental changes (Postma et al., 2016). In this review, we explore the current knowledge on the biodiversity and bioactivity of rhizobia that form root nodules with the two commercially important fynbos plants, rooibos and honeybush.

1.2. Biodiversity

Due to the high diversity of legumes that grow in the CFR, it is hypothesised that South Africa is one of the diversity hotspots. The legumes occupy highly heterogeneous soils of the CFR, which may have a high diversity of rhizobial species. Consequently, this diversity could then possibly be attributed to the high diversity of legumes that grow in different environmental conditions in the CFR (Lemaire et al., 2015a; Slabbert et al., 2010). Globally, the biodiversity of rhizobia species is enormous. However, identifying these microorganisms is a complicated process as the identification of rhizobia cannot be based solely on 16S rRNA sequence analysis. Housekeeping genes such as *recA* and *atpD*, as well as the symbiosis genes *nodA* and *nifH* needs to be analysed to accurately identify rhizobia (Lemaire et al., 2015a). Lemaire et al. (2015a, 2016a) found that the *nod* gene of *B. tuberum* STM678 is ubiquitous in *Burkholderia* and this strain is able to form root nodules with a wide range of legumes in the CFR, including with honeybush. These housekeeping genes are used to determine their phylogenetic relationships whereas symbiosis genes are used to study symbiotic traits and evolution (Lemaire et al., 2015a). The range of legumes that some rhizobia are associated with is also expanding as knowledge becomes available.

The most important genes that distinguish rhizobia species from other soil bacteria is the presence of *nod* and *nif* genes. The *nif* genes encode for the nitrogenase reductase enzyme that is involved in fixing atmospheric nitrogen. This gene is also found in free-living nitrogen fixing bacteria (Gaby and Buckley, 2012). The *nod* genes encode for Nod factors that are responsible for root nodule formation. The *nodA* gene is one of the most studied genes of rhizobia, because it determines the structure of the Nod factor and it can determine host specificity (Gerding et al., 2012). These genes can also be used for phylogenetic analysis to distinguish between the different bacterial species. It is found that most fast-growing *Rhizobium* spp. have megaplasmids (pSym)s that contain the *nod* and *nif* genes. However, not all rhizobia have these plasmids but carry the symbiotic genes on their

chromosome, such as the slow-growing *Bradyrhizobium* and *Mesorhizobium* (Nap and Bisseling, 1990; Van Rhijn and Vanderleyden, 1995). A study done by Martínez-Aguilar et al. (2008) found that some *Burkholderia* species have the *nifH* gene on plasmids, while other species have the gene on their chromosome. De Meyer et al. (2016) found that mimosoid-nodulating *Burkholderia* have *nod* genes on a symbiotic plasmid and that the *nod* genes differ from the papilionoid-nodulating *Burkholderia* which have the *nod* genes in the chromosome. This suggests that the mimosoid-nodulating and papilionoid-nodulating *Burkholderia* did not obtain the symbiosis gene from the same source which can also be observed in the *nod* gene sequences.

1.2.1. Free-living nitrogen fixing bacteria in fynbos soil

Some species of gamma- and delta-proteobacteria are known to be free-living diazotrophs and do not form root nodules with plants as they have no *nod* genes (Niederberger et al., 2012). Free-living nitrogen fixing bacteria tend to turn off nitrogen fixing when other nitrogen sources are available. Nitrogen fixing is an energy intensive process, but the nitrogen reductase enzyme complex cannot function in the presence of oxygen (Murphy, 2015). This is ironic as the process to make ATP requires oxygen. These are two conflicting demands on the organism. Forming a symbiotic relationship with plants solve this problem as plants provide bacteria with energy. These bacteria include: *Pseudomonas* (Hayat et al., 2010), *Azotobacter*, *Clostridium*, *Azospirillum* and *Klebsiella* (Murphy, 2015). Some *Paenibacillus* and *Bacillus* species are also able to fix nitrogen (Chauhan et al., 2015).

1.2.2. Fynbos rhizobia diversity

Leguminous tribes in the fynbos have a high preference to form symbiotic relationships with specific rhizobia. The tribe Psoraleeae has a preference for *Mesorhizobium*, while Podalyrieae has a preference for *Burkholderia* (Lemaire et al., 2015a). *Bradyrhizobium* and *Rhizobium* spp. have also been isolated from the fynbos legumes (Kock, 2004). A few novel *Burkholderia* species have recently been isolated from fynbos legumes. These novel species include: *B. rhynchosiae*, *B. sprentiae*, *B. dilworthii* and *B. aspalati* (De Meyer et al., 2014, 2013a, 2013b; Mavengere et al., 2014). However, an increased sampling effort is needed to fully determine the rhizobial diversity in the fynbos region.

1.2.2. Aspalathus rhizobia diversity

Most rhizobia associated with rooibos root nodules belongs to *Mesorhizobium* (Elliott et al., 2007). *Mesorhizobium* is also a PGPR that can produce indole acetic acid (IAA),

1-aminocyclopropane-1-carboxylic acid (ACC) deaminase and fix nitrogen for the plant (Lemaire et al., 2015b; Verma et al., 2013). Lemaire et al. (2015a) showed that *Mesorhizobium* spp. can co-exist with *Burkholderia* spp., because both genera occur in similar soil types with a low pH. Furthermore, Elliot et al. (2007) conducted a study to determine whether *Aspalathus* spp. and *Cyclopia* spp. can be nodulated by the same *B. tuberum* STM678 strain. The results showed that *Burkholderia* was only able to nodulate *Cyclopia* spp.

1.2.4. *Cyclopia rhizobia* diversity

In contrast to *Aspalathus* spp. that are associated with a diverse range of rhizobia, the tribe Podalyrieae (that include *Cyclopia* spp.) has a distinct preference to form root nodules with *Burkholderia* spp. (β -Proteobacteria) (Beukes et al., 2013; Elliott et al., 2007; Gyaneshwar et al., 2011; Lemaire et al., 2015a). Only a few α -Proteobacteria were isolated and identified as *Bradyrhizobium* species (Kock, 2004). A study done by Postma et al. (2016) also found that Proteobacteria dominated the rhizosphere of *Cyclopia* plants with Rhizobiales and Burkholderiales the most abundant orders.

Burkholderia is a rod shaped, Gram-negative, non-sporulating bacterium (Sessitsch et al., 2002). This genus contains pathogenic species that can infect humans, animals and plants. Other *Burkholderia* spp. are able to associate with plants and can be beneficial for plant growth (Suárez-Moreno et al., 2012). *Burkholderia* species can produce IAA, ACC deaminase and it is also able to solubilize phosphates (Palaniappan et al., 2010). One reason for the dominance of *Burkholderia* species in fynbos soil is most likely due to their adaption to nutrient poor and acidic soils (Garau et al., 2009). This finding was confirmed by Howieson et al. (2013) and Lemaire et al. (2015a).

A study done by Beukes et al. (2013) found that the *Burkholderia* that are associated with *Cyclopia* spp. have unique *nifH* and *nodA* genes. The *nodA* gene sequences from *Burkholderia* species isolated from South Africa, were closely related to those of the α -Proteobacteria, *Methylobacterium nodulans*. It is thought that the distribution of nodulating *Burkholderia* species is dependent on environmental factors rather than a specific host legume. *Burkholderia* spp. isolated from the fynbos had different *nod* genes than the *Burkholderia* spp. isolated from *Mimosa* spp. in South America (De Meyer et al., 2016; Garau et al., 2009; Howieson et al., 2013; Liu et al., 2014). Studies done by Lemaire et al. (2015a, b, 2016a, b) showed that *Burkholderia* nodulate a wide range of legumes in the fynbos. Different *Burkholderia* strains, however, do not have the same plant growth-promoting effect on the same *Cyclopia* species, but inoculation of *Cyclopia* seeds with *Burkholderia* strains, showed a significant increase in plant growth, compared to the un-inoculated plants (Spriggs and Dakora, 2009).

Different types of rhizobia are found in rooibos and honeybush plants, and it may seem that the diversity of rhizobia is higher for rooibos plants than honeybush plants. Either α- or β-Proteobacteria tend to nodulate *Aspalathus* spp (Lemaire et al., 2015a). However, *Cyclopia* spp. as well as most other members of Podalyriaceae, are exclusively nodulated by *Burkholderia* spp. (Beukes et al., 2013; Elliott et al., 2007; Gyaneshwar et al., 2011; Lemaire et al., 2016a, 2015a; Sprent et al., 2013). An increased sampling effort is needed to effectively determine the species and relative abundance of bacterial species that are able to form root nodules with rooibos and honeybush roots. More than one species of rhizobia can be isolated from a root nodule. Palaniappan et al. (2010) isolated two different colony types on yeast mannitol agar, from the same root nodule. Studies have shown that non-rhizobial bacteria have also been isolated from root nodules and may have a positive effect on plant growth (Bai et al., 2002; Rajendran et al., 2008). However, the extent and function of the non-rhizobial bacteria is still unknown.

Selected bacteria, that are isolated from rooibos and honeybush root nodules, can produce plant growth-promoting properties and are classified as PGPR (Palaniappan et al., 2010; Verma et al., 2013). Some of these bacteria are adapted to live in acidic and nutrient poor soils, that make them effective in infecting and forming root nodules in rooibos and honeybush plants (Lemaire et al., 2015a). This is however, another area of research that remains largely unexplored.

1.3. Bioactivity

Rhizobia produce bioactive compounds that affect plant growth. Some rhizobia species isolated from rooibos and honeybush plants such as *Burkholderia*, *Mesorhizobium* and *Bradyrhizobium* are able to produce compounds with plant growth-promoting properties (Palaniappan et al., 2010; Verma et al., 2013).

1.3.1. Plant growth-promoting compounds

The relationship of rhizobia with plant hosts can be rhizospheric where they colonize the surface of the root, or endophytic when forming nodules in the roots of the plant (Ashraf et al., 2013). Rhizobia can influence plant growth directly, through the secretion of plant growth-promoting metabolites such as cytokinins and IAA (Ashraf et al., 2013). Indole acetic acid is part of the auxin plant hormones and the production of IAA changes the auxin/cytokinin balance, which is a prerequisite for nodule organogenesis. Auxin also stimulates the plant cell to elongate and results in plant growth (Casimiro et al., 2001; Glick, 2005). Indole acetic acid can act as a signalling molecule to control the expression of various bacterial genes. These genes are associated with virulence, bacterial adaptation, stress response and amino acid synthesis (Duca et al., 2014). Another direct mechanism is the production

of ACC deaminase. 1-aminocyclopropane-1-carboxylic acid deaminase lowers the plant ethylene level by metabolizing ACC, which is a precursor for ethylene, into α -ketobutyric acid and ammonia. Ethylene is produced by the plant under environmental stress to inhibit root growth and development (Arshad et al., 2007). The production of ACC deaminase, therefore, allows the plant to be more resistant to environmental stress (Glick, 2005). During infection of rhizobia in the root, the plant is under stress and increase the amount of ACC produced, resulting in higher levels of ethylene (Khandelwal and Sindhu, 2013). It has been reported that this increase in ethylene inhibit nodulation. Rhizobia that can produce enough ACC deaminase to lower the ethylene levels are able to effectively nodulate plants (Khandelwal and Sindhu, 2013). This might be a potential mechanism for plants to select for specific rhizobia.

Furthermore, rhizobia can also influence plant growth through indirect mechanisms to protect the plant against phytopathogens by the production of antibiotics, siderophores and hydrogen cyanide (Ashraf et al., 2013). Rhizobia can inhibit pathogens through competition for nutrients or the production of antibiotics. In addition, rhizobia can activate the immune system of the plant which is known as induced systemic resistance (Chauhan et al., 2015; Van Loon, 2007). The combination of hydrogen cyanide and siderophore production is known to inhibit fungal growth (Ahmad et al., 2008; Verma et al., 2013).

1.3.2. Nitrogen fixation and root nodules

Nitrogen fixing is a unique characteristic of rhizobia. Biological nitrogen fixation (BNF) is affected by abiotic factors, especially drought. During drought, there is reduced infection of the legume by rhizobia and the nodule development and growth is affected negatively. This directly affects nodule function (Arrese-igor et al., 2011) and the growth of rooibos and honeybush plants. Because the plants are rain-fed, it receives little water during the summer months. This corresponds with a study done by Lotter et al. (2014) where the rooibos growth rate declined by 50% during drought. This decline in growth is due to the negative effect on BNF and the plants have to rely on the available nitrogen in the soil. During drought, the C:N ratio increase in nutrient poor ecosystems such as the fynbos. The drought had direct effects on the functioning and activity of the root nodules (Lotter et al., 2014). The reason for the lower levels of BNF is that the water limitation decreases photosynthesis and lowers the level of photosynthates needed by the bacteria for nitrogen fixation.

Studies have been done to determine the percentage of nitrogen derived from atmospheric nitrogen, through nitrogen fixation, found in rooibos and honeybush plants. The amount of nitrogen fixed has also been determined (Table 1). Interestingly, the natural honeybush plants had more consistent values of nitrogen derived from atmospheric nitrogen compared to the managed plants. Some species had similar values in the managed plants and the natural sites, but there is variation of

values at different sites (Muofhe and Dakora, 1999; Spriggs and Dakora, 2009). Maseko and Dakora (2015) found that annual harvesting of the plants led to a decrease in N₂ fixation. Removal of the shoots affect the oxygen diffusion to the root nodules and leads to a decrease in nitrogenase activity (Maseko and Dakora, 2015).

Table 1. Amount of nitrogen fixed under field conditions and the % N derived from atmospheric nitrogen in rooibos and honeybush plants

Species	Amount of nitrogen fixed under field conditions (g.plant ⁻¹)	%N derived from atmospheric nitrogen		Reference
		Field trial	Natural sites	
<i>Aspalathus linearis</i>	3.80±0.3	52.0±3.0	N/A	Muofhe and Dakora, 1999
<i>Cyclopia subternata</i>	N/A	100±41.1	69.7±4.1	Spriggs and Dakora, 2009
<i>Cyclopia genestoides</i>	N/A	100±17.5	100±7	Spriggs and Dakora, 2009
<i>Cyclopia intermedia</i>	N/A	62.8±16.1	100±16.5	Spriggs and Dakora, 2009
<i>Cyclopia maculata</i>	N/A	89.0±3.2	100±42.4	Spriggs and Dakora, 2009
<i>Cyclopia sessiliflora</i>	N/A	100±27.7	100±17.1	Spriggs and Dakora, 2009

1.3.3. Root nodule formation

Plants also produce bioactive compounds in the form of root exudates that are secreted into the rhizosphere. The root exudates contain biologically active chemicals such as ions, oxygen, water, enzymes and different primary and secondary metabolites (Bais et al., 2006). Plants can interact positively or negatively with the microorganisms in the rhizosphere. Positive interactions include microorganisms that can act as biocontrol agents or form root nodules to fix nitrogen. Whereas, negative interactions include the secretion of antimicrobial compounds or where the microorganisms produce phytotoxins (Bais et al., 2006). The roots of leguminous plants secrete specific compounds to control nodulation via a signalling loop named autoregulation of nodulation. This signalling loop controls the number of nodules formed by controlling the secretions of compounds such as peptide hormones, small metabolites and receptor kinases (Ferguson et al., 2010). The rhizobia respond to the plant-derived metabolites in the rhizosphere by activating the *nod* genes and synthesizing a signalling molecule known as Nod factor. Nod factor is a lipochitooligosaccharide that causes root hair deformation in the plant as well as nodule organogenesis (Terpolilli et al., 2012). At first contact the plant protect itself from infection by the rhizobia. However, rhizobia produce exopolysaccharides (EPS), capsular polysaccharides (CPS) and lipopolysaccharides (LPS) to suppress the host's defence reactions during infection (Terpolilli et al., 2012).

As the plant is infected with the bacteria and root nodules are formed, the bacteria differentiate into bacteroids. The bacteroids change shape and size. During the differentiation of the bacteria, the induction of bacteroid genes that are critical to reduce N_2 to NH_4^+ are activated. The genes that are induced are known as the *nif* genes and are responsible for the production of the nitrogenase enzyme complex that reduce nitrogen to ammonium (Terpolilli et al., 2012). Figure 1.3 shows the carbon source from the plant and how it is used to fix nitrogen. The nitrogenase enzyme produced by bacteroids are used to catalyse the reduction of N_2 to ammonia. The reaction is as follows:

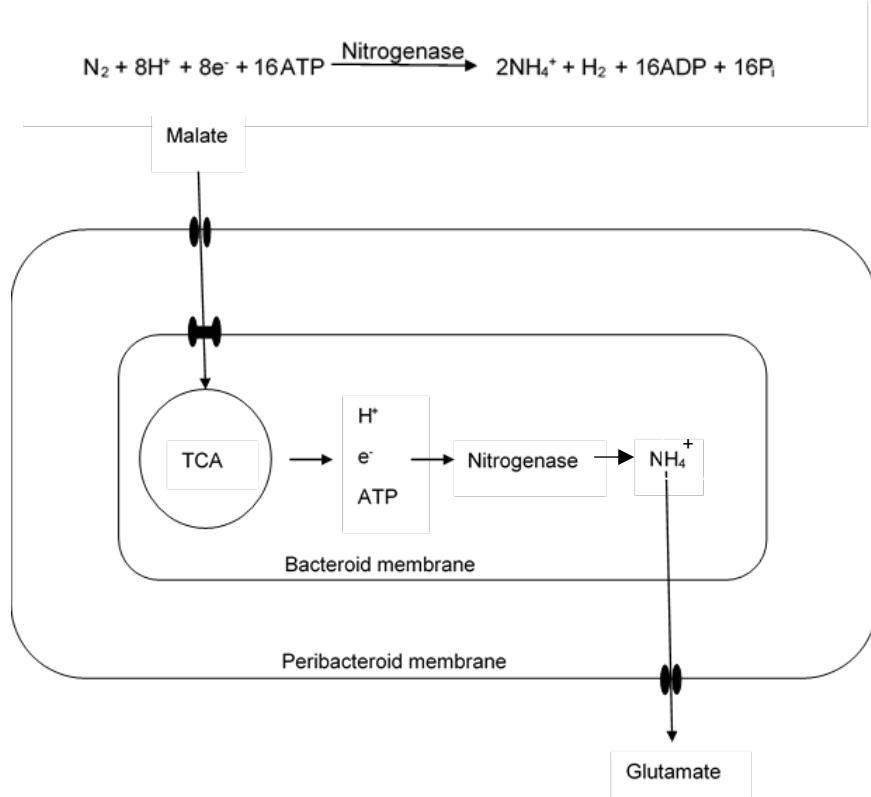


Figure 1.3. Classical model of the carbon and nitrogen exchange during the rhizobia-legume symbiosis with the tricarboxylic acid (TCA) cycle and nitrogenase enzyme (Lodwig and Poole, 2003; Prell and Poole, 2006).

1.3.4. Determinate and indeterminate root nodules

Root nodules differ in shape and size based on the bacteroid population and can be classified into two groups, indeterminate (Figure 1.4) or determinate nodules. Indeterminate nodules contain a heterogeneous population of bacteroids. These nodules undergo continuous cell division and this results in different developmental stages as the root nodules elongate. Determinate nodules contain a homogenous population of bacteroids as differentiation of the infected cell is synchronized and the nodules have a short live-span of only a few weeks. Determinate root nodules are often more rounded in shape than indeterminate root nodules (Ferguson et al., 2010). Although both rooibos and honeybush plants form indeterminate root nodules (Elliott et al., 2007; Gyaneshwar et al., 2011;

Lemaire et al., 2015a), rooibos root nodules are branched whereas honeybush root nodules are less branched (Sprent et al., 2013).

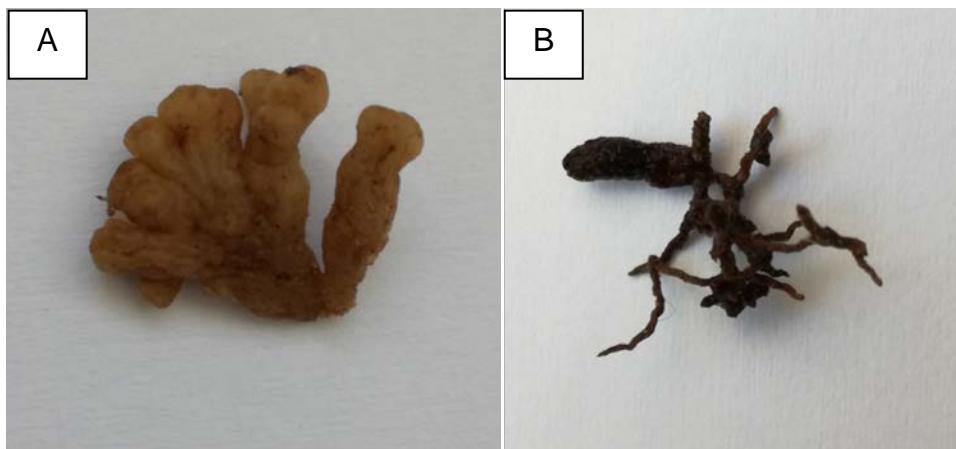


Figure 1.4. Different indeterminate rooibos (A) and honeybush (B) root nodules (photo credit: Casper Brink).

1.3.5. The effect of pH on rhizobia and plants

The soil pH also has an effect on root nodule functioning. Morón et al. (2005) found that an acidic environment lead to increased production of Nod factors by *R. tropici*. Higher soil pH is often associated with lower concentrations of Nod factors that are produced. At the optimum pH for rhizobia ($\text{pH} = 5.5$), rhizobia showed a significant increase in Nod factors and plant growth when compared to higher pH. Rhizobia strains that are sensitive to pH, might be unable to regulate their internal pH. The regulation of cytoplasmic pH might be a requirement for growth under acidic conditions. This may be beneficial for rhizobia that prefer an acid growth environment. Low pH affects the interaction between the micro-symbiont and the legume host, as well as growth of the plant (Dakora, 2012). Nitrogen fixing rhizobia isolated from the nutrient poor and acidic soils from fynbos are tolerant to low pH levels and this indicates that they have adapted to their environment.

Burkholderia and *Mesorhizobium* spp. have been isolated from acidic soils with the pH ranging from 3.18 to 6.7 (Lemaire et al., 2015a). Other studies also confirm that *Burkholderia* are able to grow over a pH range from 4.5 to 8 without the pH effecting growth (Howieson et al., 2013; Liu et al., 2014), while *Mesorhizobium* spp. did not grow at a pH below 5 (Howieson et al., 2013). The bacteria are tolerant to these environments and have adapted to survive in these conditions (Dakora, 2012).

Rooibos plants can control the pH of the rhizosphere and elevate the pH from 4 to 6.8 to overcome the growth inhibition of the symbiotic microorganisms (Muofhe and Dakora, 2000). During nitrogen fixation, the root nodules produce protons, resulting in a higher rhizosphere pH. At night, the pH lowers in the rhizosphere. This could be because nitrogen fixing is an energy demanding process and can be linked to the photosynthetic activity of the plant. As the photosynthetic activity decrease

at night, nitrogen fixation also decreases, resulting in the rhizosphere to acidify at night (Blossfeld et al., 2013). This acidification occurs through the release of soluble P from the inorganic or organic phosphorus source resulting in a significant drop in pH (Alikhani et al., 2006). During the day, soluble P may also be released, but it is consumed as well that results in no change in the soil pH (Maistry et al., 2015).

1.4. Challenges

To study rhizobial diversity remains a challenge due to the complex interactions between rhizobial species with each other, the host plants and their environment. In addition, most soil bacteria are unculturable and culture based methods often fall short to characterize the true diversity of this group of bacteria. This makes it difficult to use culture based methods to study rhizobial diversity. Studies on microbial diversity and community structures have moved towards using molecular based techniques such as next-generation sequencing (Mardis, 2008; Metzker, 2010; Van Dijk et al., 2014). Next-generation sequencing is used in metagenomic studies that is based on the genetic material from environmental samples (Handelsman, 2004). Metagenomics can be used to determine the microbial diversity and identify microorganisms to a certain extent (Neelakanta and Sultana, 2013). A disadvantage of metagenomics is that it is expensive and time consuming (Zhou et al., 2015)

Although molecular techniques are useful, there are also some challenges in studying rhizobia. Some rhizobial species have multiple copies of the *nodA* and *nifH* genes (Nap and Bisseling, 1990; Van Rijn and Vanderleyden, 1995). This makes it difficult to do phylogenetic analysis, especially as the 16S rRNA genes are not sufficient in identifying these species. Furthermore, the databases of the housekeeping genes (*recA* and *atpD*) are incomplete.

There is a lack of knowledge on the rhizobia that associate with rooibos and honeybush plants. More research is needed on the plant growth-promoting compounds, nodule functioning and nodulation kinetics of rhizobia that associate with rooibos and honeybush plants. For example, IAA and ACC deaminase production have been studied in other rhizobia and have been shown to have a positive effect on plant growth (Ashraf et al., 2013; Glick, 2005). Root nodule formation and the differentiation of rhizobia into bacteroids as well as all the other metabolic compounds that have an influence on these processes in rooibos and honeybush plant still have to be studied. This has been done on other leguminous plants as shown by Debrosses and Stougaard (2011) and Terpolilli et al. (2012). The pathway of how nitrogen is transported from the bacteroid to the plants cell also has to be determined for rooibos and honeybush.

Research in microbial inoculants for rooibos and honeybush plants, may be instrumental to increase the yield of the plants and increase plants resistance to environmental stress. However, the development of a microbial inoculant will take time. It will be necessary to screen and test different combinations of rhizobia, as some rhizobia can have a negative effect on each other and different strains may produce different amounts of plant growth-promoting metabolites.

1.5. Conclusion

Rooibos and honeybush plants are unique not only for their use as herbal teas, but because they are legumes that can host different species of rhizobia. The symbiotic relationship between rhizobia and legumes is complex and further studies are required to fully understand the mechanisms of nodulation and host specificity. Rhizobia are ecologically important microorganisms that provide nitrogen, phosphorous and other nutrients to plants. These bacteria also produce compounds that have a positive effect on plant growth. The metabolic characteristics and function of rhizobia associated with legumes might provide insight into some of the mechanisms that are still unknown.

1.6. Project aim

The identified challenges have led to the aim and objectives of this study. The aim is to determine the diversity of the nitrogen-fixing bacteria and plant growth-promoting properties of these bacteria that associate with rooibos and honeybush plants.

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Chapter 2:

Diversity of free-living nitrogen fixing
bacteria associated with rooibos and
honeybush plants

Abstract

There are differences in the diversity of microorganisms between the bulk and rhizosphere soil. Bulk soil often has a higher microbial diversity than the rhizosphere, whereas the rhizosphere has a higher microbial mass due the high levels of carbon root exudates provided by the plants. Diazotrophic bacteria play an important role in plant growth, particularly in fynbos soil which is nutrient poor, and the microbial community is influenced by environmental parameters. Community fingerprinting methods can be used to determine and to monitor effects of the various environmental factors on the diversity of diazotrophic bacteria. The aim of this study was to determine if there were any differences between the diazotrophic communities of commercial and natural rooibos and honeybush plants. Soil DNA was extracted from bulk and rhizosphere soil of rooibos and honeybush plants and the *nifH* genes were amplified using FAM-labelled primers. The amplicons were digested using *Hae*III restriction enzyme for 3h and run through an automated sequence analyser from which electropherograms were obtained. Statistical analysis was done to determine similarities and differences between microbial communities within the soil. Real-time quantitative polymerase chain reaction was used to determine the copy number of the *nifH* gene in these soils. The copy number of *nifH* in the bulk soil was lower than that in the rhizosphere. The results indicated that no significant differences were detected between the diazotrophic communities of commercial and natural plants. Differences between rooibos and honeybush diazotrophic communities were detected, with the α -proteobacteria associating more with rooibos plants and the β -proteobacteria associating with honeybush plants. The method used in this study can be used to monitor changes in the soil diversity of diazotrophic bacteria associated with commercial rooibos and honeybush plants. If a decrease in diversity is detected, crop rotation might be necessary to restore the diversity of the soil microbiome.

2.1. Introduction

Soil productivity and health depend on functional processes carried out by soil microbial communities (Pankhurst et al., 1996). Studying microbial diversity and processes contribute to the understanding of ecological theory of ecosystems (Bertin et al., 2008; Zhou et al., 2008). One of the microbial processes is biological nitrogen fixation that is a natural process that convert atmospheric nitrogen into ammonia that are usable by plants (Cleveland et al., 1999). Biological nitrogen is fixed by rhizobia, mainly belonging to the proteobacteria (Gyaneshwar et al., 2011), for plant use. Not all of these bacteria associate with plants and some are known as free-living nitrogen fixing bacteria (Hayat et al., 2010). There are many unidentified and unculturable diazotrophs found in soil, and several studies have suggested that the non-cultivated diazotrophs are dominant nitrogen organisms in soil ecosystems (Buckley et al., 2007; Hamelin et al., 2002; Poly et al., 2001).

Agricultural practices usually result in a decline in soil microbial diversity and structure as observed in crops (Griffiths et al., 2001). However, Postma et al. (2016) found highly similar patterns of bacterial diversity and species richness in the rhizosphere of commercial and wild honeybush plants. This is most likely because land is not cleared for honeybush plantations as is the case with other cropping systems (Postma et al., 2016). A very similar picture emerged from studies on the rooibos rhizosphere communities (Postma et al. unpublished). However, a significant seasonal effect was found, with wet and dry seasons representing different microbial community structures in both the honeybush and rooibos systems. These seasonal changes in the microbial community suggest that bacteria react to environmental changes by changing the community structure (Postma et al., 2016).

The soil type differs between rooibos and honeybush plants as rooibos grows in a sandy soil, whereas honeybush grow in a loam and clay soil. The soils in which these plants grow is nutrient poor where nitrogen is one of the limiting nutrients (Vardien et al., 2014; Witkowski and Mitchell, 1987). These plant species also prefer different proteobacteria to form root nodules, with honeybush selecting for β -proteobacteria and rooibos for α -proteobacteria (Elliott et al., 2007).

Diazotrophic communities have been studied in soil to determine their effects on agricultural crops. Nitrogen fixing bacteria contain the *nif* genes which encode for the nitrogenase complex used for nitrogen fixation (Gaby and Buckley, 2012). The *nifH* gene is a 400 base pair conserved region but the sequences differs between species. The *nifH* gene, encoding for the nitrogenase reductase subunit in the nitrogenase complex, is commonly used to study the diversity of diazotrophic communities and has been linked to function in soil ecosystems (Hsu and Buckley, 2009; Zehr et al., 2003). Similar studies also using the *nifH* have been done on rhizosphere communities of other plants (Gupta et al., 2014; Yukun et al., 2011), but not those of rooibos and honeybush plants.

It is mostly accepted that the higher the diversity of soil microorganisms, the healthier the soil. Magurran (1988) stated that diversity consists of three components namely richness, evenness and genetic diversity. These components are frequently related, but can vary independently. Differences in function and composition of the compared communities are used to determine differences in community structure (Hsu and Buckley, 2009). Comparing community structure and diversity can be done by using community fingerprinting methods. Denaturing Gradient Gel Electrophoresis (DGGE), Automated Ribosomal Intergenic Spacer Region Analysis (ARISA), and Terminal Restriction Fragment Length Polymorphism (T-RFLP) have been used to study microbial diversity in soil (Gros et al., 2006; Gupta et al., 2014; Mårtensson et al., 2009; Niederberger et al., 2012; Yukun et al., 2011; Zhan and Sun, 2012). ARISA and T-RFLP are two community fingerprinting methods that can be used for routine monitoring of soil microbial communities and provides high resolution diversity patterns (Hartmann et al., 2005). The results of these methods are reproducible, although T-RFLP is more demanding than ARISA but can be used to identify organisms with known RFLP profiles (Hartmann et al., 2005).

The abundance of the *nifH* gene in soil is used for assessing the nitrogen fixing potential of the soil. Quantitative real time PCR (qPCR) is often used in combination with community fingerprinting to determine the abundance of a specific gene in a community. Quantitative real time PCR can be used to quantify the *nifH* gene abundance in soils in order to assess the nitrogen fixing potential of the soil (Hayden et al., 2010; Huhe et al., 2014; Orr et al., 2011; Wakelin et al., 2010).

In this study, ARISA, T-RFLP, and qPCR were used to determine the abundance and diversity of free-living nitrogen fixing bacteria in the bulk soil and rhizosphere soil of rooibos and honeybush plants. It is hypothesised that the diversity of the nitrogen fixing bacteria will be higher in the bulk soil than the rhizosphere soil and that the copy number of the *nifH* gene will be higher in the rhizosphere soil than the bulk soil.

2.2. Materials and methods

2.2.1. Sample collection

The rooibos and honeybush samples were collected in March and April 2016 during the dry season from commercial and natural plants. Eight rhizosphere and eight bulk soil samples were collected in triplicate from the honeybush plants across the two honeybush farms. Seven rhizosphere and bulk soil samples were collected in triplicate from the rooibos farm. The three farms that were used for sampling were the Klipopmekaar farm ($S32^{\circ}02.874' E18^{\circ}59.500'$) in the Cederberg region, as well as Groendal ($S33^{\circ}47.443' E23^{\circ}34.272'$) and Guavajuice ($S34^{\circ}02.802' E24^{\circ}20.812'$) in the Langkloof (Figure 2.1). The Klipopmekaar soil samples had rooibos vegetation, while the Langkloof sites had honeybush plants. The samples collected from Groendal farm have natural vegetation of

Cyclopia longifolia, whereas the Guavajuice farm have natural *C. subternata* vegetation. Soil samples were also taken from two sites where a one year and two year old oats field was prepared for a new rooibos plantation at Klipopmekaar.

Bulk soil samples were collected at a depth of 10 cm, while the rhizosphere samples were collected at a depth of between 10 and 20 cm (Postma et al., 2016). Root fragments of 15 cm with closely surrounding soil were placed in sterile plastic bags. Soil samples were stored on ice until the DNA was extracted.



Figure 2.1. (A) Sampling site on the Klipopmekaar farm, (B) sampling site on the Groendal farm (photo credit: Jonathan Kriel and Armand van Wyk).

2.2.2. DNA extraction from samples

DNA was extracted from the bulk and rhizosphere soil in triplicate within 24 h of sample collection. The ZR Soil Microbe DNA kit (Zymo Research, California, USA) was used for genomic DNA extraction. The manufacturer's protocol was followed, and 0.25 g of soil was used with 100 µl of genomic DNA extracted per sample. After DNA extraction, samples were stored at -20°C until used for PCR.

2.2.3. Automated Ribosomal Intergenic Spacer Analysis (ARISA)

Fluorescently labelled primers were used to amplify the 16S rRNA region to determine the bacterial community structure by using ARISA. The amplicons of the samples were run on an ABI 3010XL Genetic analyser to obtain electropherograms containing fragment length and fluorescent intensity. The bacterial ARISA PCR samples were run with the LIZ1200 size standard. Genemapper 5 software converted the fluorescence data to peaks of different fragment lengths. These peaks of different fragment lengths are termed operational taxonomic units (OTU's). Peak heights are used to indicate the relative abundance of the fragments in the sample. The fragment lengths are calculated by using a best fit curve of the size standards (Slabbert et al., 2010). Only fragment sizes between 100 and 1000 base pairs and peak heights above 150 fluorescent units were used for analysis as OTU's. A bin size of 3 bp was used to minimize inaccuracies of the ARISA profile (Brown et al., 2005; Slabbert et al., 2010).

2.2.4. Terminal Restriction Fragment Length Polymorphism (T-RFLP)

The *nifH* gene primers, *nifH* F1 and *nifH* 439R (Boulygina et al., 2002; De Meyer et al., 2011) were used to study the diversity of the free-living nitrogen fixing bacteria in the bulk soil and the rhizosphere. FAM-labelled *nifH* F1/*nifH* 439R primers were used to amplify the *nifH* genes in the soil DNA. 10 µl PCR were set up as follows: 5 µl Kapa Robust 2G readymix, 4.1 µl PCR grade water, 0.2 mM of each primer and 0.5 µl DNA. After the PCR, the restriction enzyme digest was set up using 10 µl PCR product, 18 µl water, 2 µl 10x Buffer L, 1 U *Hae*III restriction enzyme (New England Biolabs, UK). The amplicons were digested for 3 h at 37°C followed by heat inactivation for 20 min at 80°C. The diversity of the *nifH* gene in the soil was determined by using T-RFLP analysis (Gupta et al., 2014). The samples were run on an ABI 3010XL Genetic analyser at CAF (Stellenbosch University). The peak heights from the raw data were considered for analysis obtained from GeneMapper 5. Fragments between 45 and 364 bp in length and 200 fluorescence were used to minimize the influence of the primer, primer dimers and background on the community profile (Ferrando and Fernández Scavino, 2015). This is similar to the analysis of ARISA. Alpha diversity indexes were determined (Blackwood et al., 2007; Yukun et al., 2011; Zhan and Sun, 2012) as well as the beta diversity were visualized using non-metric multidimensional scaling plot by using MS Excel, Statistica 13.2 and R Vegan package (Oksanen, 2017).

2.2.5. Frequency of operational taxonomic units

Histograms of the frequency of OTU's were constructed for ARISA and T-RFLP profiles. Sizes were represented as 0 or 1 depending on the presence or absence of peaks. The frequency of the different sizes was summated to construct a histogram.

2.2.6. Species richness

Species richness per sample were calculated as the number of unique OTU's per sample. This gives an indication of the relative number of species at each site.

2.2.7. Alpha diversity

The Shannon-Weaver (H) index was calculated for every sample to determine diversity of each site. P_i represents the fraction of each peak of the total integrated area and S is the number of OTU's per sample (Slabbert 2008). P_i is the proportional abundance of species per sample. Shannon-Weaver index is not a linear measure of diversity.

$$H = - \sum_{i=1}^S p_i \ln p_i$$

Simpsons diversity was also calculated for each site. The larger the Simpson's index, the higher the chance that two randomly picked species will be the same species. The Simpson's index focuses more on abundant species and is not strongly influenced by minor species. P_i is the number of a specific species divided by the total number of OTU's observed (Simpson, 1949).

$$D = 1 - \sum_{i=1}^S p_i^2$$

Crosby and Criddle (2003) found that the Shannon-Weaver diversity index may be inaccurate up to 0.3 index units for diversity estimation of ARISA. This is due to multi-copy 16S rRNA fragments found in certain organisms and these fragments may overlap with same size fragments from other organisms. ARISA is, however, accurate estimating evenness and diversity when compared to other high throughput methods (Crosby and Criddle, 2003). The same data format is generated with T-RFLP and can also be used to estimate the diversity of nitrogen fixing bacteria by using the *nifH* gene, although some organisms have multiple copies of the gene.

2.2.8. Beta diversity

The Bray-Curtis method was used to determine the β -diversity of the samples as it focusses on variation in a community structure (Anderson et al., 2011; Bray and Curtis, 1957). Bray-Curtis dissimilarity between samples i and i' is calculated as follows:

$$b_{ii'} = \frac{\sum_{j=1}^j |n_{ij} - n_{i'j}|}{n_{i+} + n_{i'+}}$$

The values from this equation is between 0 and 1. The number 0 indicates the samples are identical between sites and the number 1 indicates the samples are completely different. n_{ij} is the sample row totals and n_{ij} is the counts of different species. The Bray-Curtis method have been used in numerous studies to determine the beta diversity of a microbial community (Danovaro et al., 2006; Wakelin et al., 2010; Wood et al., 2008; Zhao et al., 2016).

2.2.9. qPCR assay

The *nifH* PCR product from a *Burkholderia* strain was used for preparation of the standards as well as a positive control. The PCR product was purified using GeneJET PCR Purification Kit (Thermo Scientific). The PCR product size of 380 bp was verified by electrophoresis on a 1% agarose gel. The purified PCR products were quantified using a μ LITE (Biodrop, Cambridge, UK) and the *nifH* gene copy number was determined using the fragment length, molecular weight and Avogadro's number. The known concentration of the PCR product was used to prepare a standard curve, in triplicate, to measure *nifH* gene copy numbers. The *nifH* gene copy numbers of the soil samples was quantified by using quantitative PCR (qPCR) using the *nifH* F1 and *nifH* 438r primers (Boulygina et al., 2002; De Meyer et al., 2011). qPCR assays were performed using the LightCycler 96 (Roche) with a SYBR Green 1 fluoroprobe as the protocol suggested.

A standard curve was generated for every qPCR run ranging from 1×10^{10} to 1×10^0 gene copies/ μ L. The experiment was done in triplicate. Each run also included a positive control as well as a negative control. Each rooibos and honeybush soil sample was run in duplicate. The reaction volume contained 3 μ L nuclease free water, 2 μ L *nifH* F1 (100 nM) and *nifH* 438r (100 nM) primers, 10 μ L SYBR Green I Master Mix (2x) and 5 μ L sample DNA as described by manufacturers specifications. The same thermal cycle was used for qPCR as with the conventional PCR and consisted of 95°C for 5 min followed by 40 cycles of 94°C for 60 s, 60°C for 60 s and 72°C for 30 s. A melt curve analysis was done after the 40 cycles to verify specificity of amplicons. ANOVA was used to determine if there is a significant difference in copy number between sites. The results are expressed as copy number per gram soil and was calculated as follows:

$$\frac{x \text{ copy number } nifH \text{ gene}}{5\mu\text{l DNA}} \times \frac{100 \mu\text{l DNA}}{0.25 \text{ g soil}} = x \text{ copy number/gram soil}$$

2.3. Results

2.3.1 DNA extraction, PCR amplification of ARISA and T-RFLP products

The DNA was successfully extracted from all samples. DNA quantities were above 200 ng/ μ l. ARISA PCR products size ranged from 100 bp to 1000 bp and produced smears as the length of the amplified fragment varies in the soil community. The nifH F1/nifH 439R produced PCR products of the expected size (ie. 380 bp) from the nitrogen-fixing strains and soil DNA samples.

2.3.2. Rooibos ARISA

2.3.2.1 Frequency of OTU's generated with ARISA

The frequency of the bacterial OTU size data is represented in Figure 2.2. Fragments between 220 and 400 bp in length are dominant in the bacterial population.

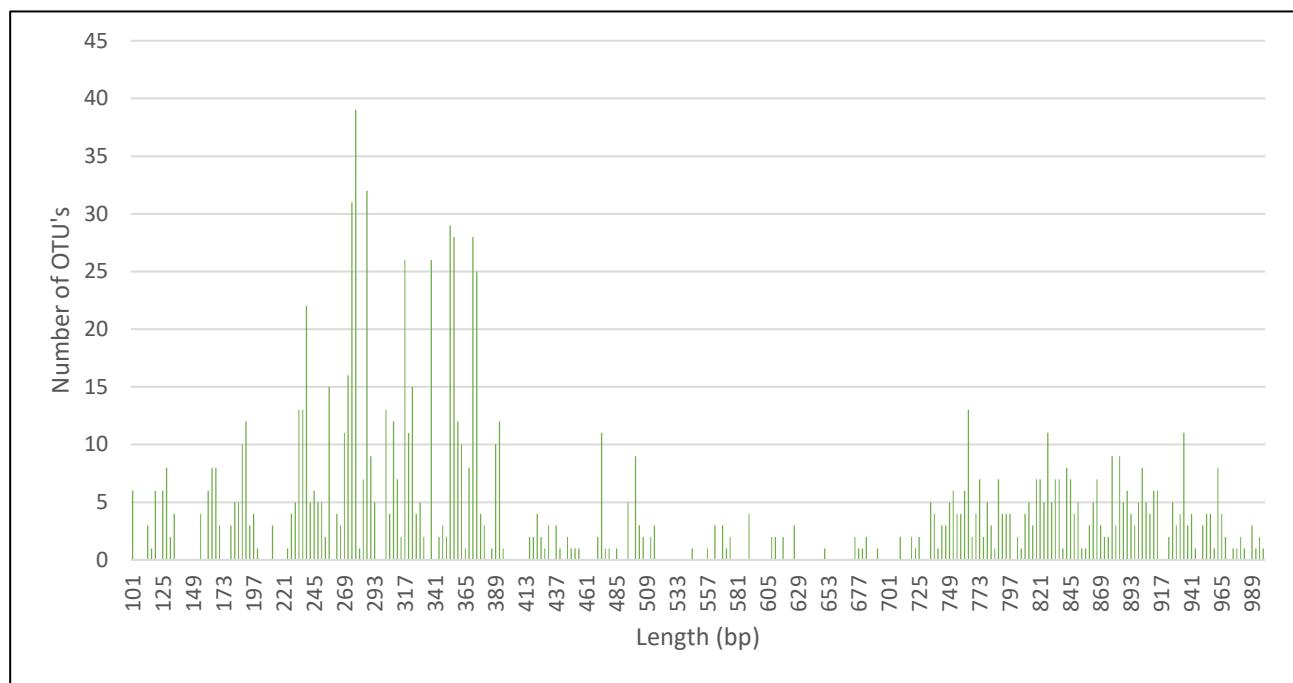


Figure 2.2. Frequency of OTU's of the rooibos bacterial community ranging from 100-1000 bp in length.

2.3.2.2 Species richness of the rooibos bacterial community

The number of OTU's ranged from 18 to 48. The natural bulk soil, oats prepared soil 1 and 2 had a significantly lower OTU count compared to the other samples (Figure 2.3). The rhizosphere soils had the highest number of OTU's compared to the bulk soil.

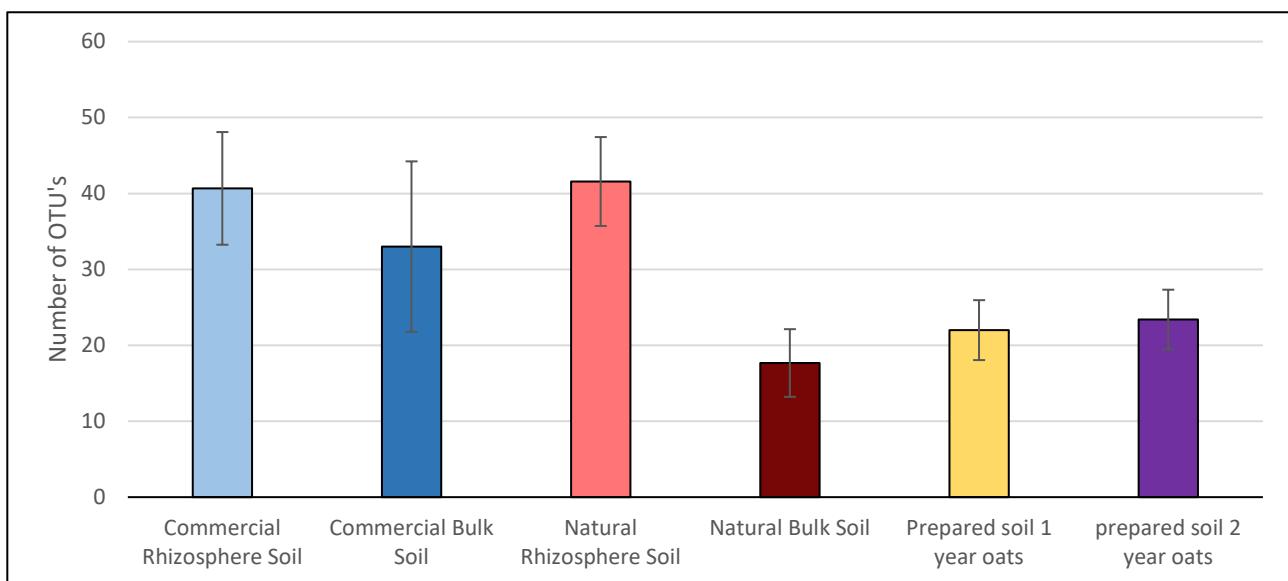


Figure 2.3. The number of different bacterial OTU's found at selected rooibos sites.

2.3.2.3 Species accumulation curve of the rooibos bacterial community

The species accumulation curve is shown in figure 2.4. The estimated number of species is between 250 and 300.

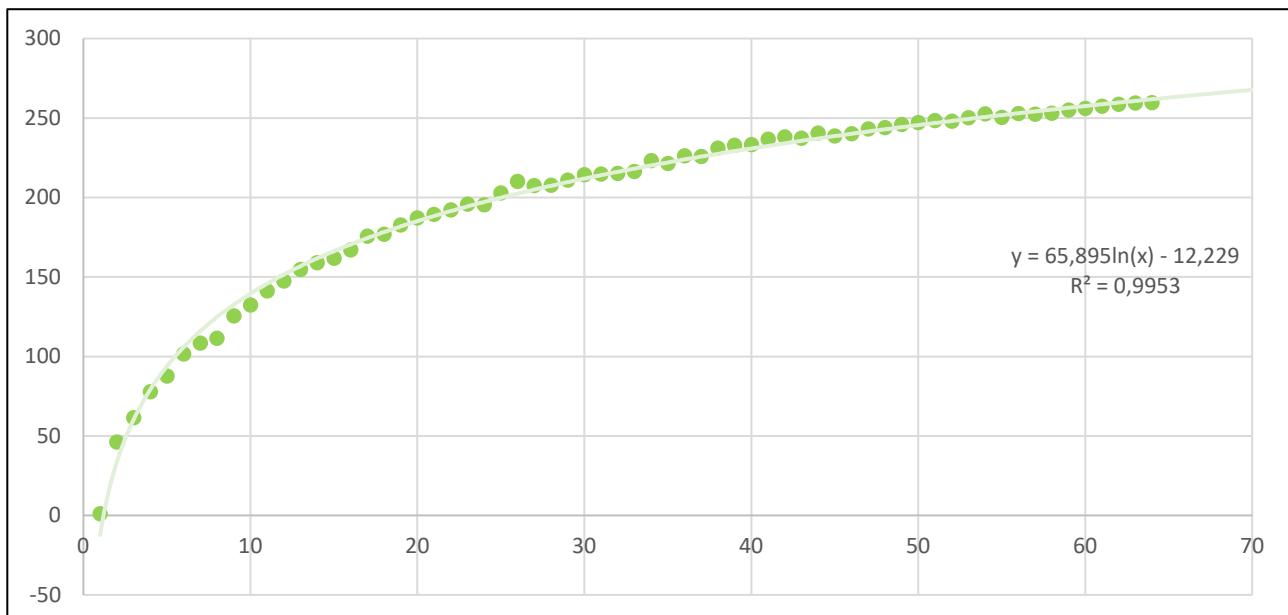


Figure 2.4. Species accumulation curve of bacteria of rooibos soil estimating 260 different bacterial species in rooibos soil.

2.3.2.4 Alpha diversity indexes of the rooibos bacterial community

The Shannon diversity calculates the number of species with the number of representatives of each species. The presence of dominant species is not visible. The natural bulk soil and prepared soil 1 and 2 had a significantly lower Shannon diversity indices compared to the other rooibos samples (Figure 2.5).

The Simpson's diversity index shows that the natural bulk soil, prepared soil 1 and 2 had a significantly lower Simpson's diversity index compared to the other samples (Figure 2.6).

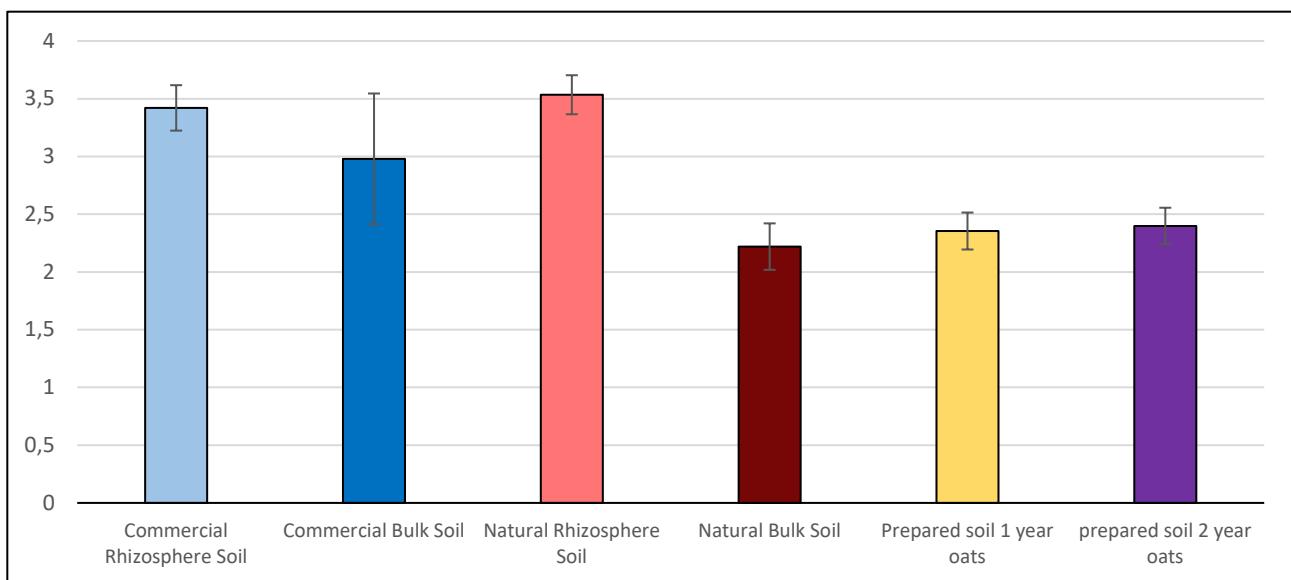


Figure 2.5. Shannon diversity index of the whole bacterial community of rooibos soil.

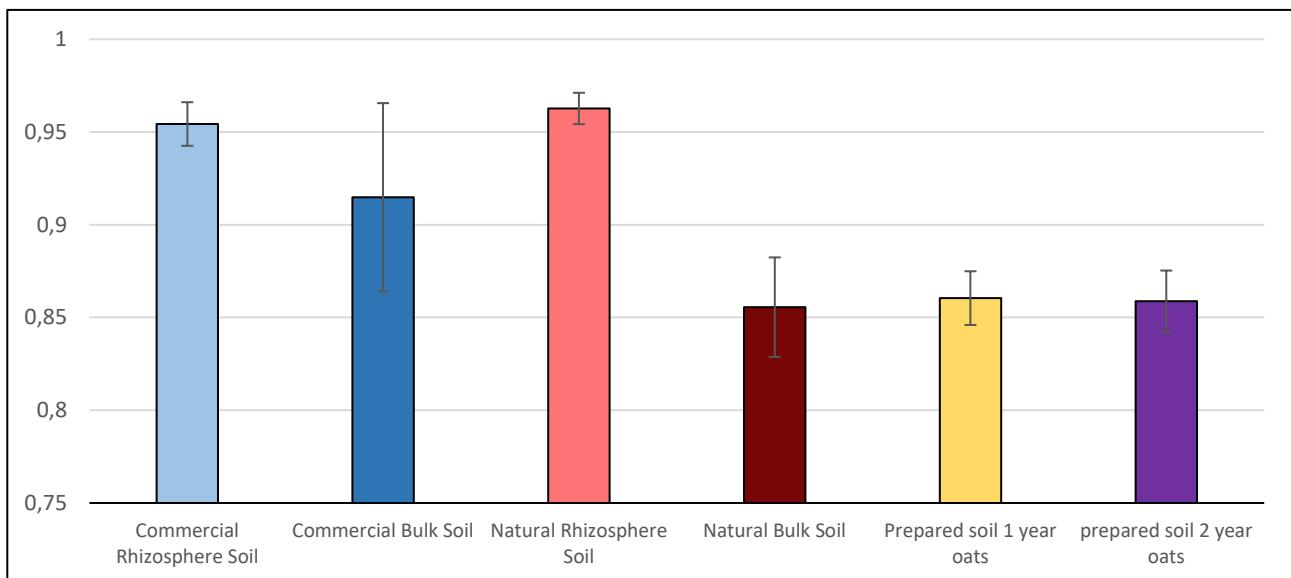


Figure 2.6. Simpson's diversity of the bacterial community of rooibos soil.

2.3.2.5 Beta diversity of the rooibos bacterial community

The results for beta diversity is shown in the NMDS plot (Figure 2.7). This graph with the ANOSIM analysis ($p<0.05$) indicate that there is a significant difference between the bulk and rhizosphere soil. No significant differences were detected between the natural and commercial sites.

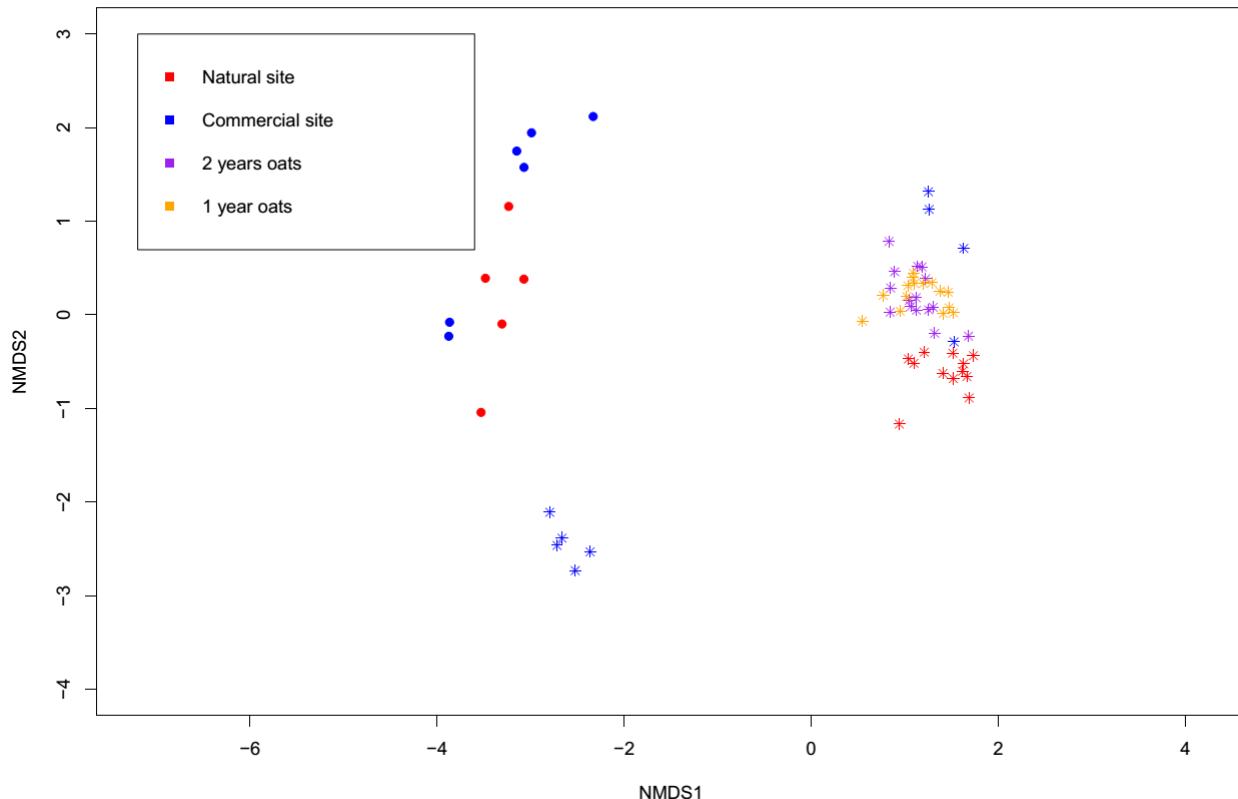


Figure 2.7. Non-metric multidimensional scaling plot of the beta diversity of rooibos soil. Bulk soil samples indicated by asterixes (*) and rhizosphere samples indicated by circles (●).

2.3.3 T-RFLP

2.3.3.1 OTU frequency

The fragment length's, 45-55 and 95-105 have higher frequencies than the other lengths for rooibos and honeybush (Figure 2.8 & 2.9). This is an indication that these organisms are dominant in the nitrogen fixing community of rooibos and honeybush soils.

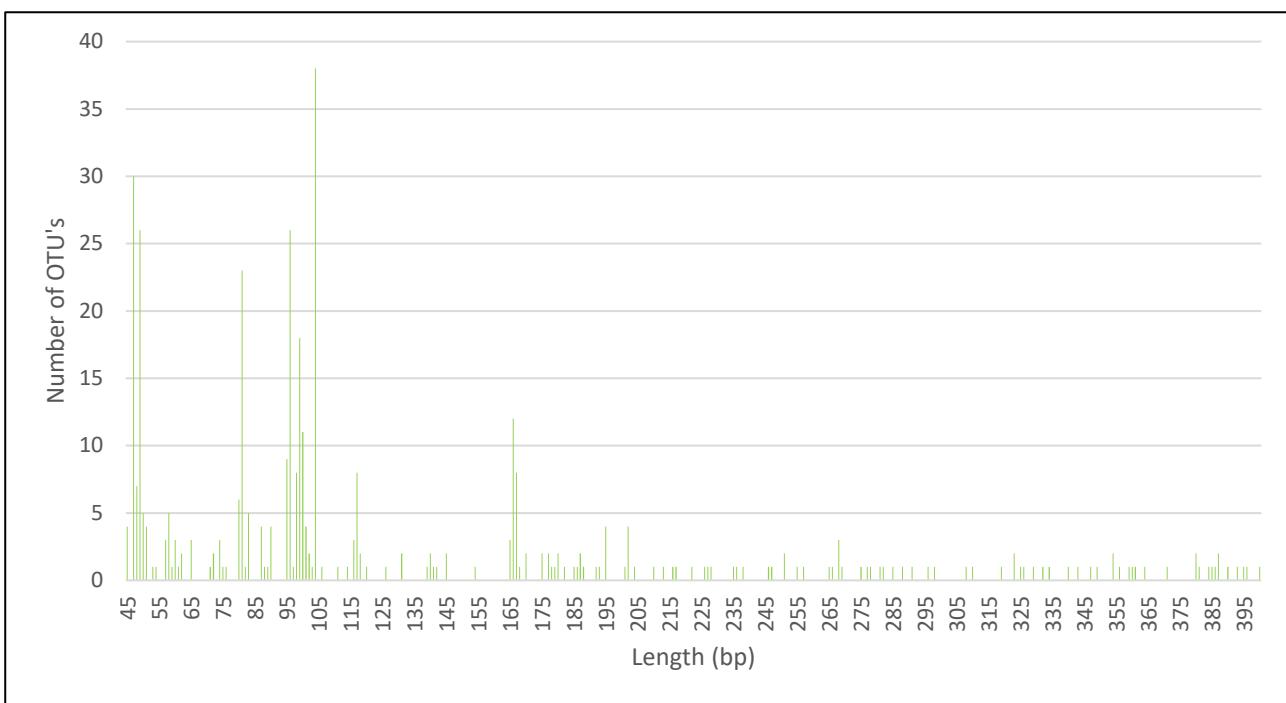


Figure 2.8. Frequency of OTU's of T-RFLP of the *nifH* gene from rooibos soil.

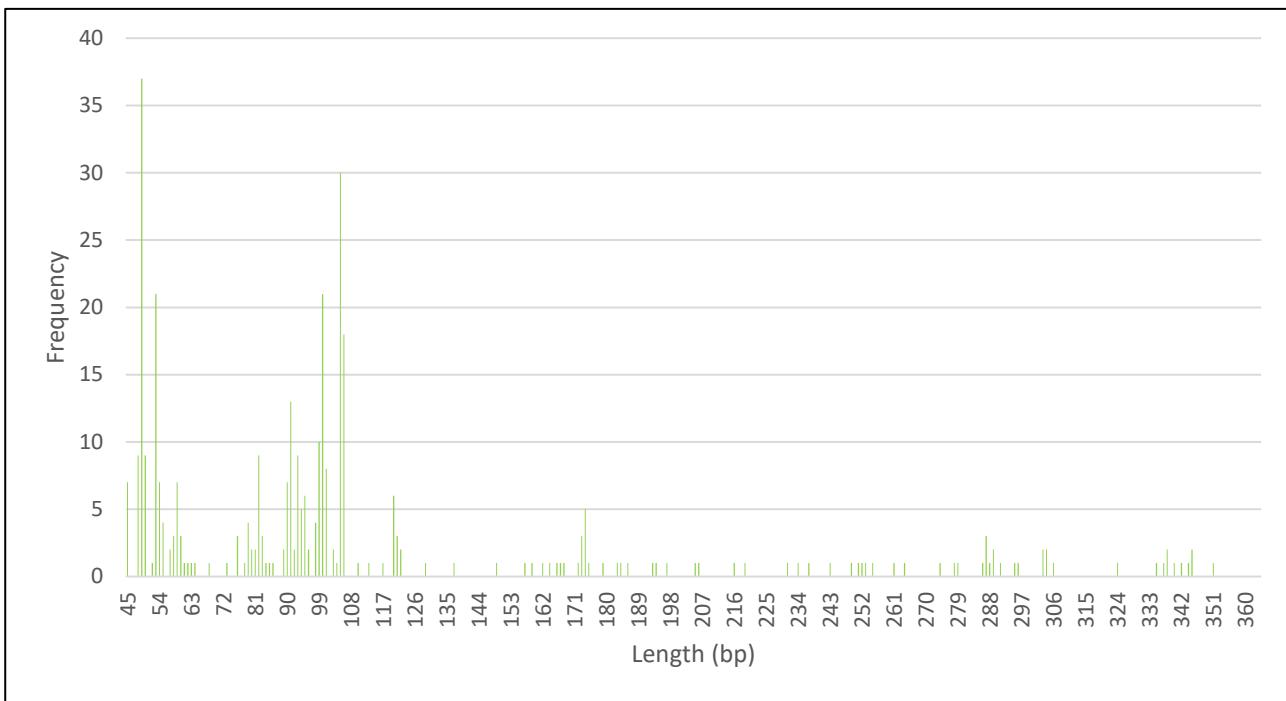


Figure 2.9. Frequency of OTU's of T-RFLP of the *nifH* gene from honeybush soil.

2.3.3.2. Species richness of the bacterial nitrogen fixing community

Based on the T-RFLP data, the rooibos samples had a slightly higher diversity than the honeybush samples, but this was not significant. The OTU's ranged from four to ten per sample. ANOVA ($p>0.05$) of rooibos nitrogen fixing species richness showed no significant difference in the number of OTU's between the bulk and rhizosphere soils (Figure 2.10). There were, however, significant differences in the honeybush species richness (Figure 2.11). The Groendal commercial bulk soil sample for *C. maculata*, commercial rhizosphere for *C. intermedia* and the bulk *C. intermedia* had significantly lower OTU counts than the other samples.

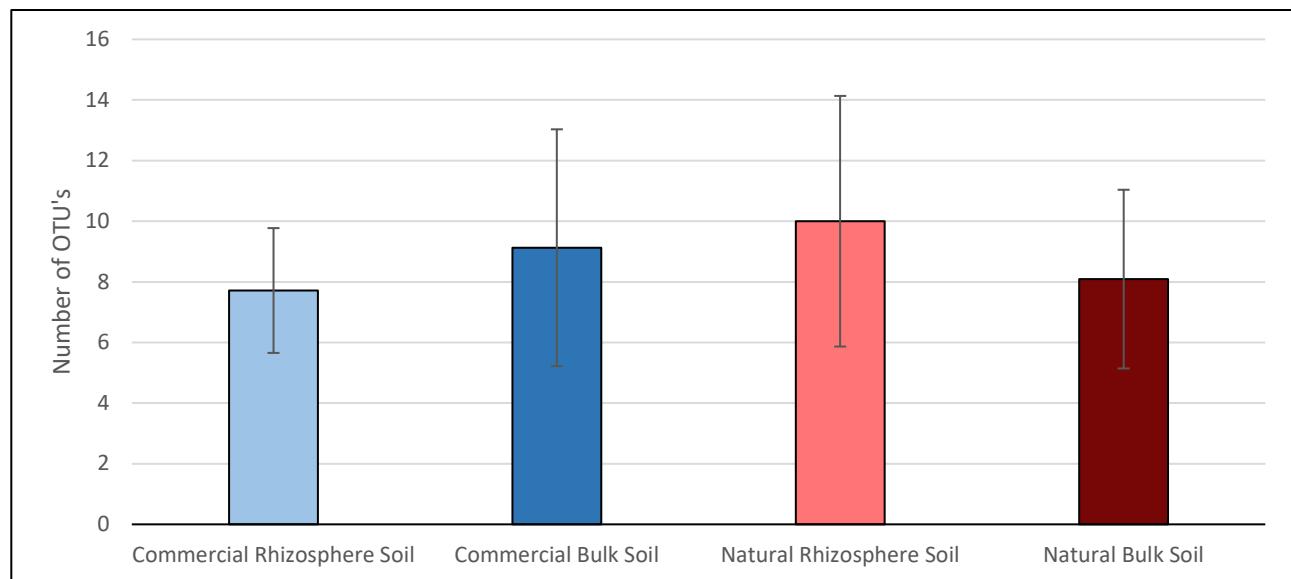


Figure 2.10. The number of unique species found in the different rooibos soil samples.

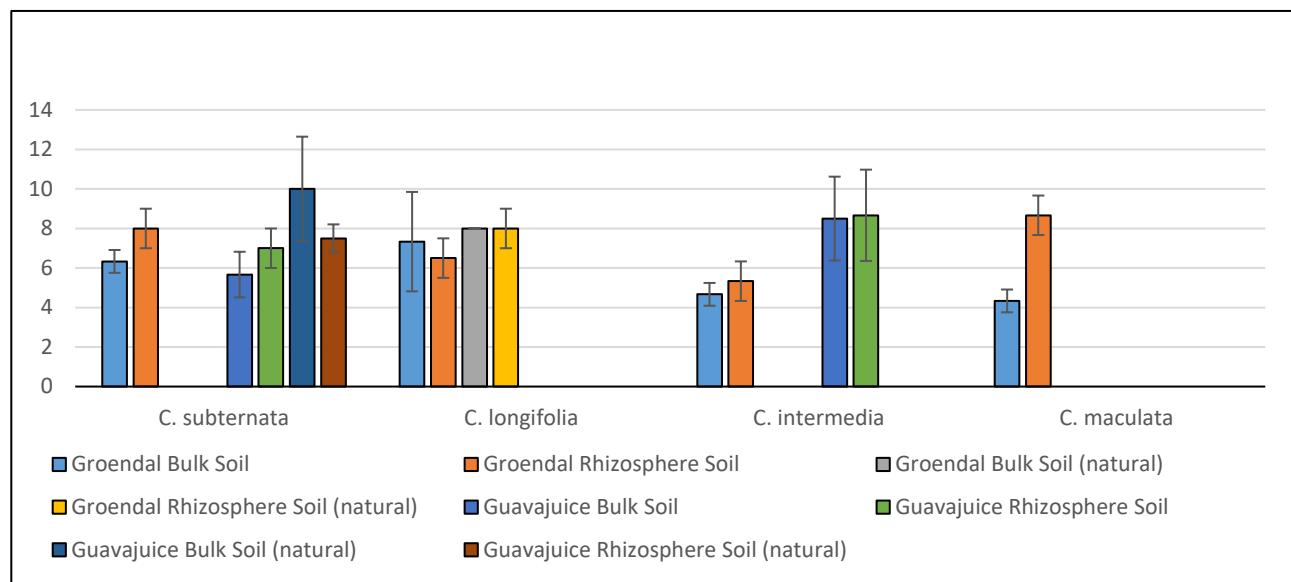


Figure 2.11. The number of unique species found in the different honeybush soil samples.

2.3.3.3 Species accumulation curve

The species accumulation curve for rooibos and honeybush are highly similar in shape and trend (Figure 2.12). An increased sampling effort is necessary to get a more accurate estimation of where the curve will plateau.

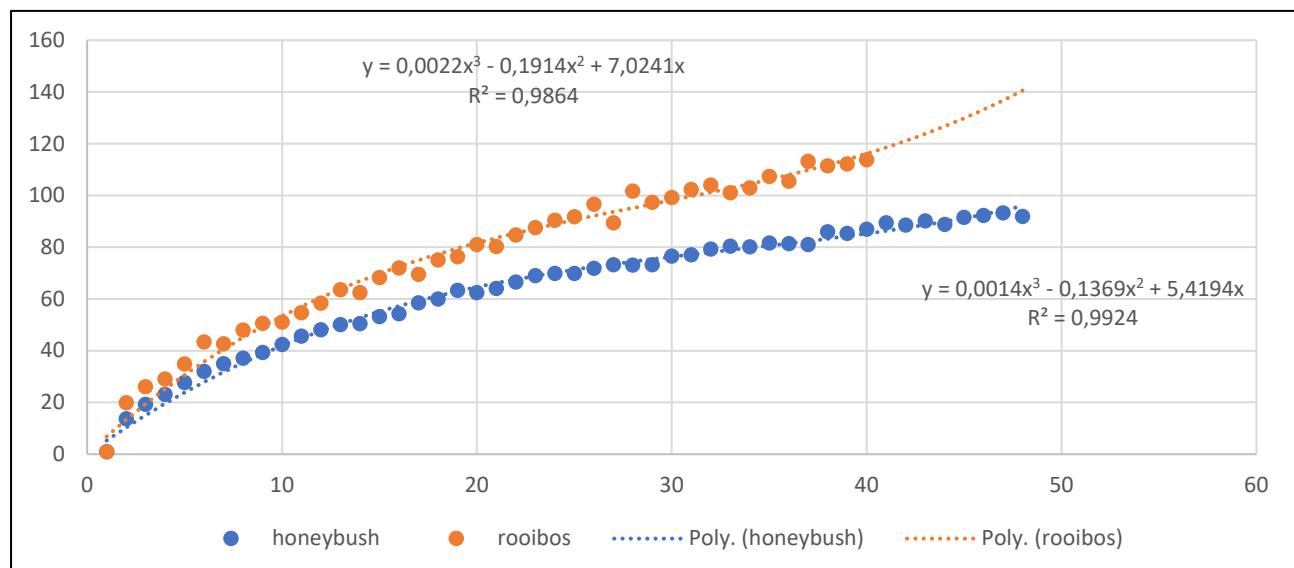


Figure 2.12. Species accumulation curve of the *nifH* gene in rooibos soil.

2.3.3.4 Alpha diversity indexes

The Shannon diversity for the whole community is higher than the nitrogen fixing community, which is expected. The same result can be seen with the number of OTU's per sampling site. No significant difference was detected in the Shannon diversity between the different rooibos (Figure 2.13) sampling sites ($p>0.05$). There were significant differences between the Groendal *C. intermedia* and *C. maculata* bulk soil and the rest of the honeybush samples as these samples had a significantly lower Shannon diversity (Figure 2.14). The honeybush samples had a higher Shannon diversity than the rooibos samples.

The Simpson's diversity indicates that there are dominant nitrogen fixing species in the rooibos soil as the Simpson's diversity values for the rooibos samples are between 0.5 and 0.6 (Figure 2.15). Most of the Simpson diversity values for honeybush samples were between 0.7 and 0.8 (Figure 2.16). There are, therefore, fewer dominant species in honeybush soil than rooibos soils. There is also a significant difference between Groendal *C. intermedia* commercial bulk soil when compared to the other samples.

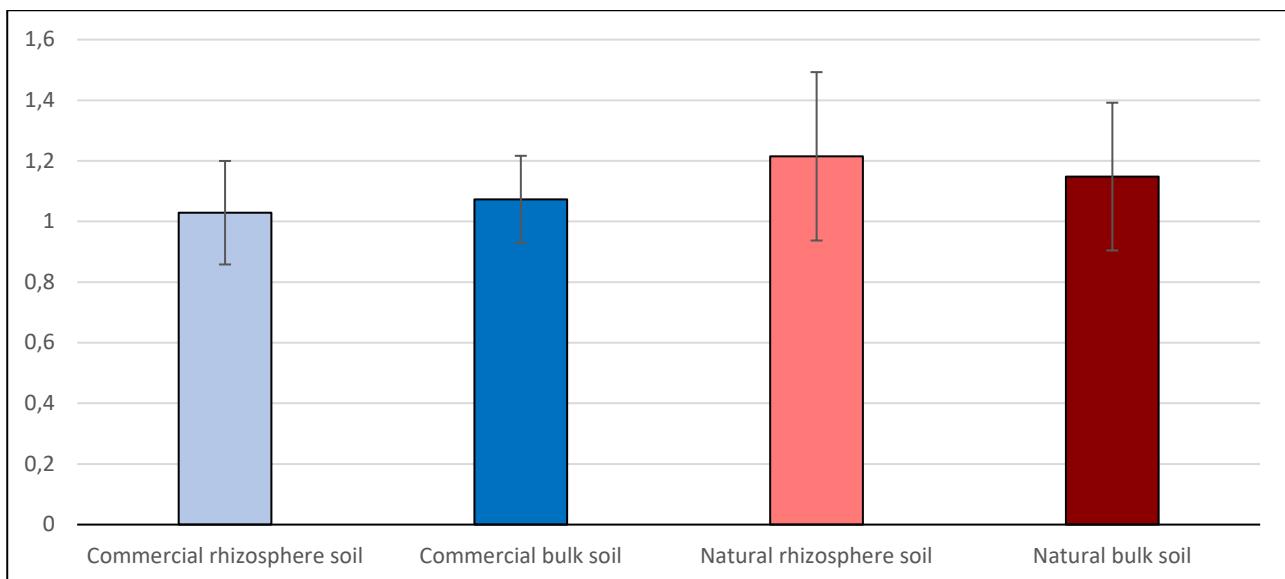


Figure 2.13. Shannon diversity of the nitrogen fixing community of rooibos soil.

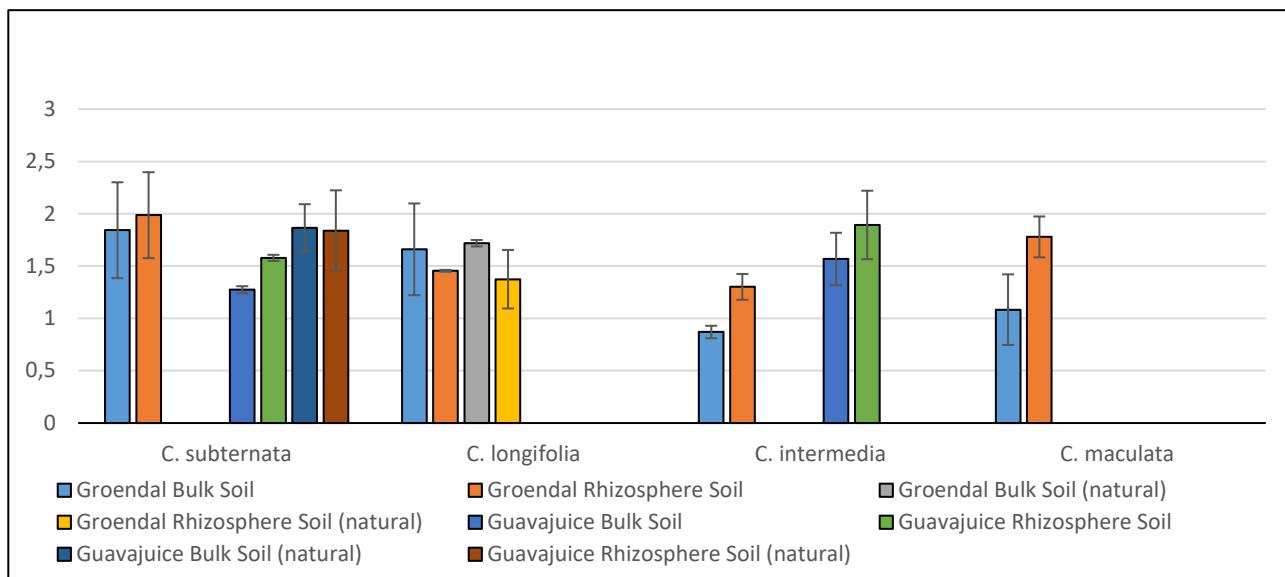


Figure 2.14. Shannon diversity of the nitrogen fixing community of honeybush soil.

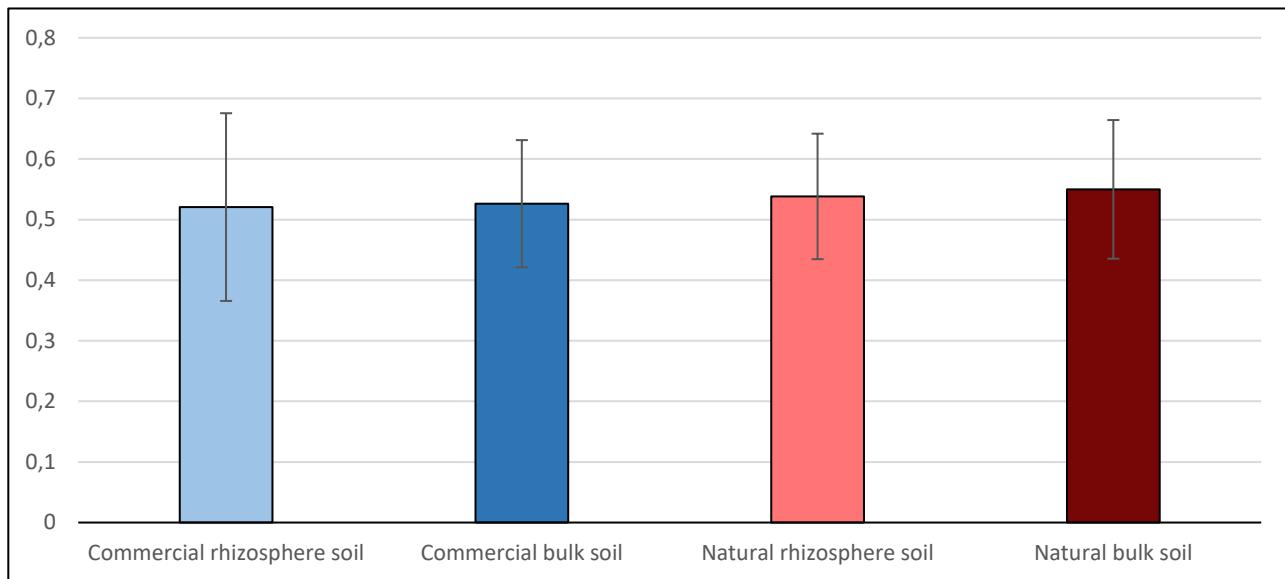


Figure 2.15. Simpson's diversity of the nitrogen fixing community of rooibos soil.

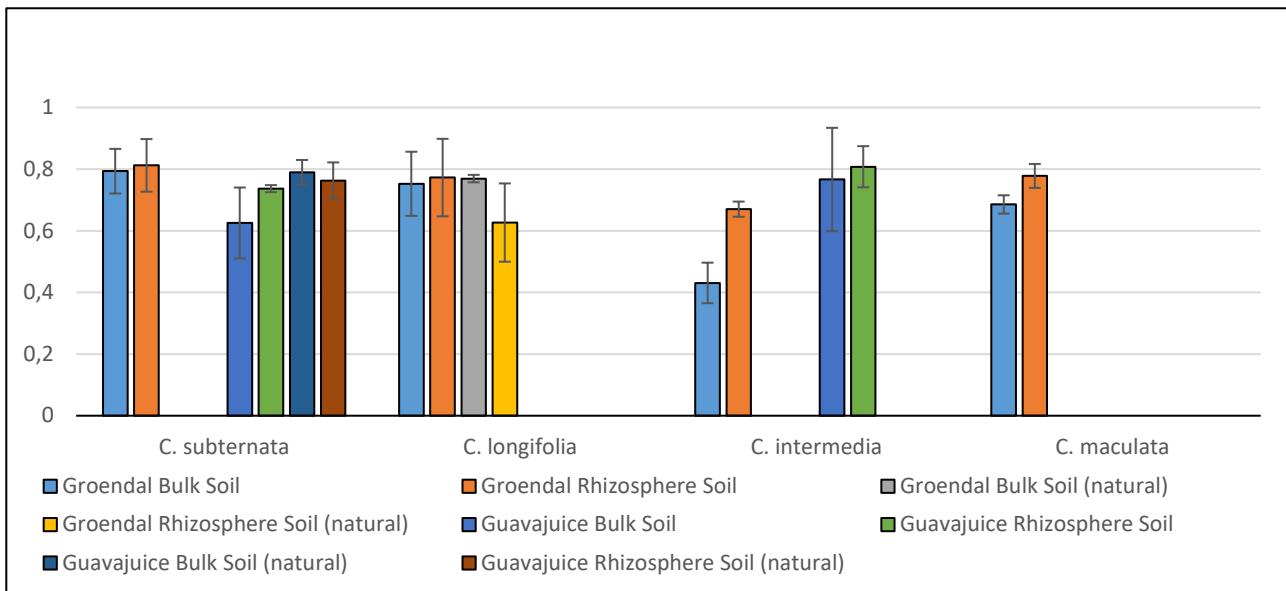


Figure 2.16. Simpson's diversity of the nitrogen fixing community of honeybush soil.

2.3.3.5 Beta diversity

The beta diversity is an indication of how similar different sites are. The rooibos samples statistically formed three clusters and there was no distinction between samples from different sites as the samples clustered randomly and not according to site. The ANOSIM R-value = 0.942 ($p < 0.05$) indicates a difference between the clustered groups, but when these samples are displayed on a NMDS plot, there are no clear difference between clusters (Figure 2.17).

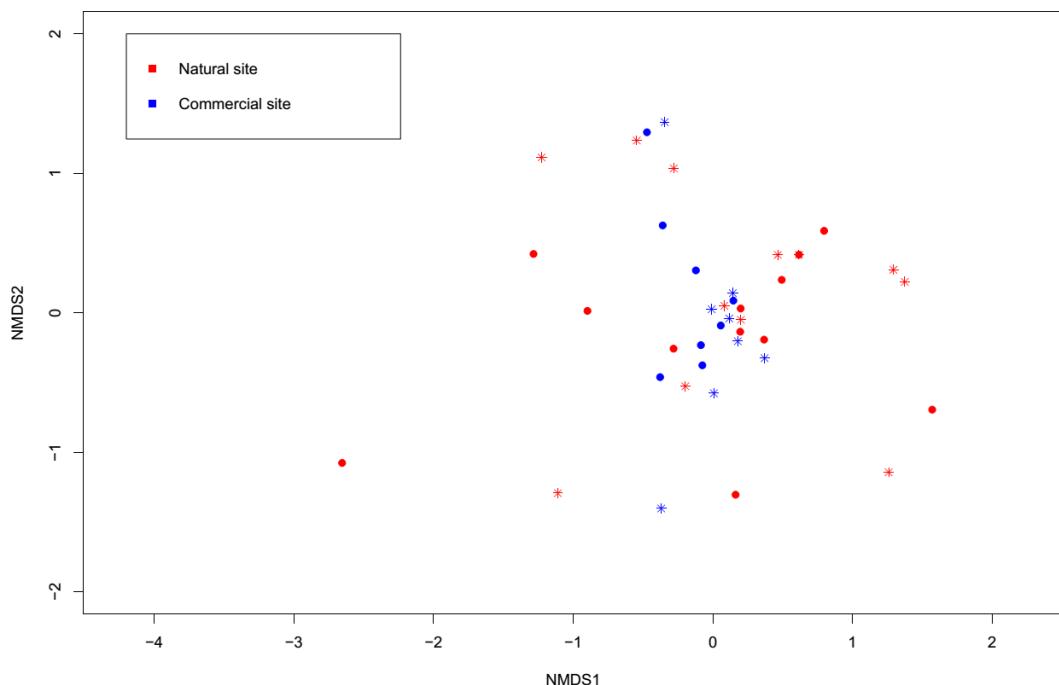


Figure 2.17. Non-metric multidimensional scaling plot of the nitrogen fixing community of rooibos soil. Bulk soil samples indicated by asterisks (*) and rhizosphere samples indicated by circles (●).

The honeybush samples grouped into two clusters and the ANOSIM analysis indicates a significant difference between the clusters (R -value = 0.877, $p=0.001$). These samples also did not group according to the different samples. The NMDS plot look similar to rooibos and there are no significant differences between different honeybush species (Figure 2.18).

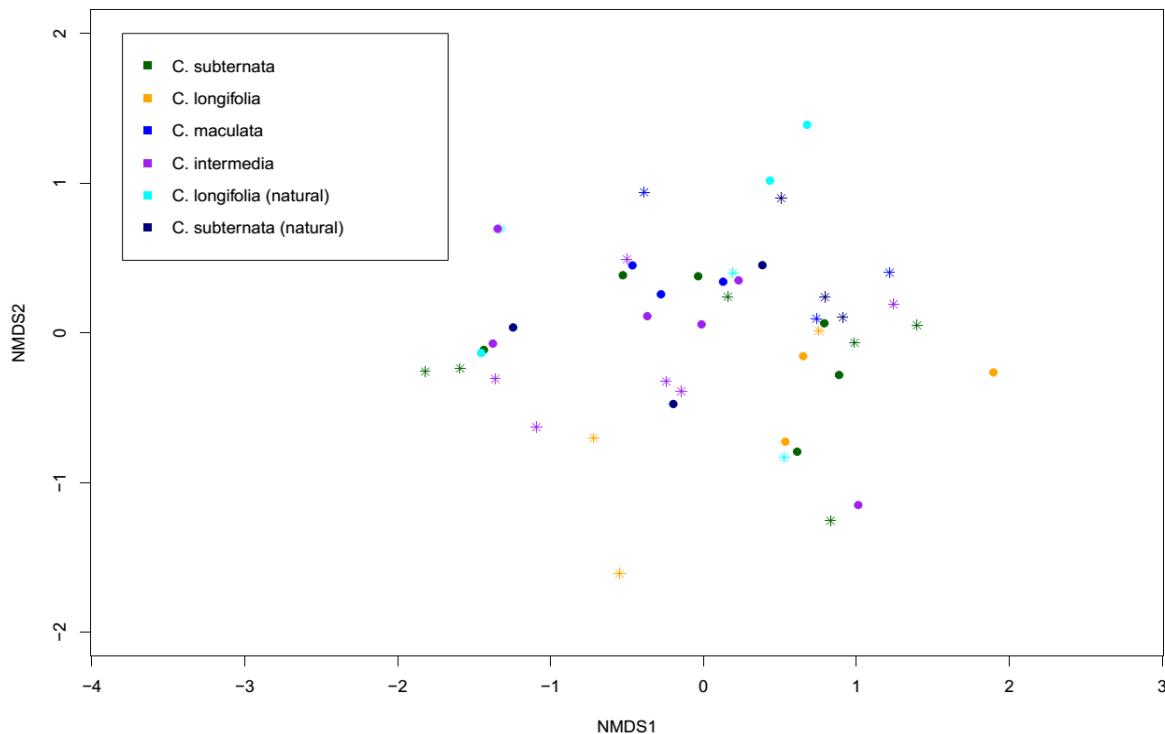


Figure 2.18. Non-metric multidimensional scaling plot of the nitrogen fixing community of honeybush soil. Bulk soil samples indicated by asterisks (*) and rhizosphere samples indicated by circles (●).

2.3.4. qPCR

The primer set used in this study are able to detect alpha-, beta- and gamma-proteobacterial nitrogen fixing bacteria in soil samples (Gaby and Buckley, 2012). An efficiency above 1.8 (90%) was achieved with the standard curve. The temperature for the melt curve analysis was 90°C. This was used to confirm that qPCR products are the correct size and that nonspecific binding did not occur. Detection limit was 8000 copies / gram soil.

The commercial rhizosphere and the natural bulk soil had significantly higher copy numbers than the other rooibos samples. Statistical analysis showed that the commercial bulk soil and natural rhizosphere soil had significantly lower copy numbers than the natural bulk soil and commercial rhizosphere soil. From figure 2.19 it can be seen that the commercial rhizosphere has the highest copy numbers. There was no correlation between the *nifH* diversity and the copy number as there was no significant difference in the diversity that could explain the differences in copy number.

The honeybush qPCR data were grouped according to species to determine differences between species (Figure 2.20). The honeybush species, *C. intermedia* and *C. longifolia*, had a significantly higher copy number of the *nifH* gene in the rhizosphere than in the bulk soil. This differs from *C. subternata* and *C. maculata* where the bulk soil samples had significantly more copy numbers than the rhizosphere samples.

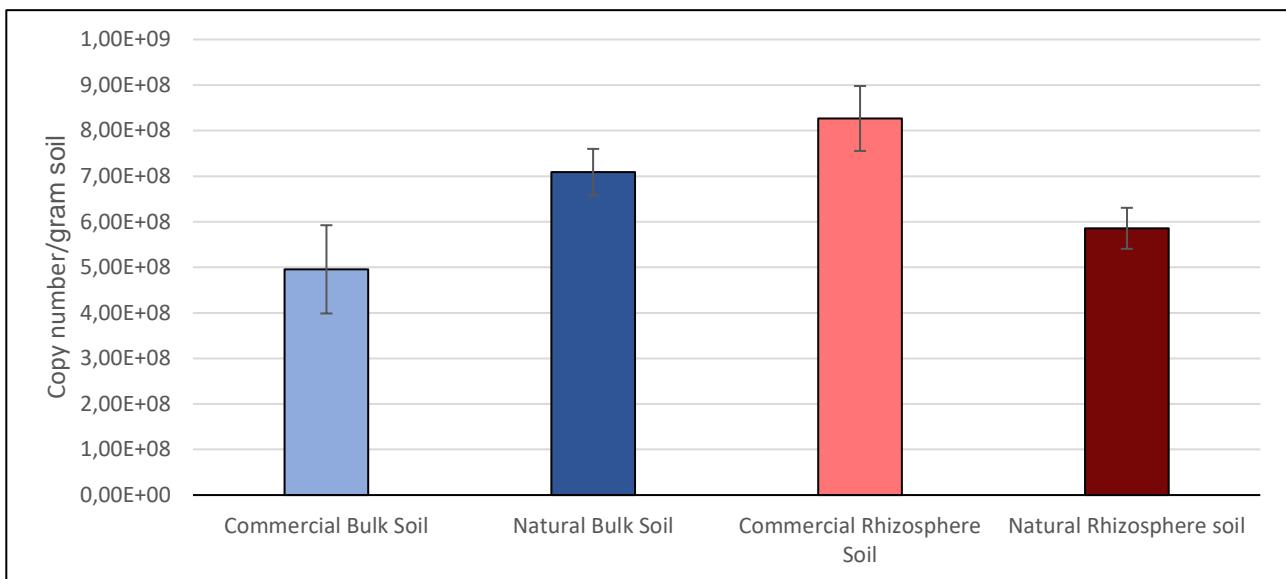


Figure 2.19. The *nifH* gene copy number of rooibos soil samples.

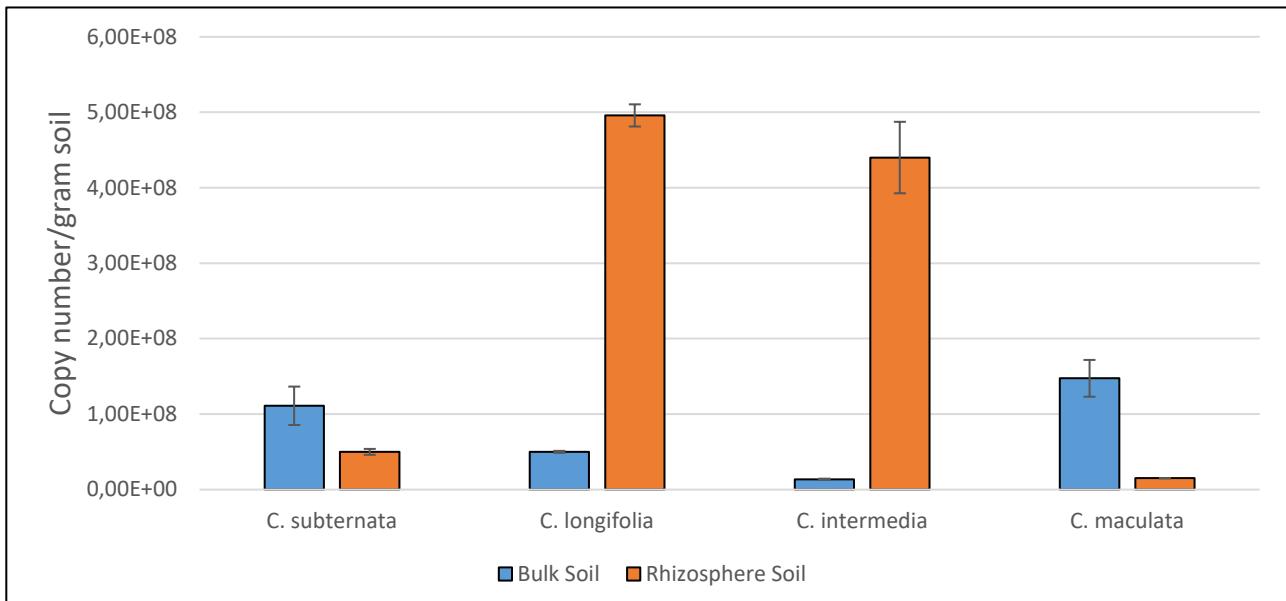


Figure 2.20. The *nifH* gene copy numbers of the soil from different honeybush species.

2.4. Discussion

2.4.1. *Rooibos ARISA*

2.4.1.1. OTU frequency generated with ARISA

In figure 2.2 the Gram-positive bacteria are most likely presented in the smaller fragments as they have no tRNA in the spacer region (Gurtler and Stanisich, 1996). This is expected as Gram positive bacteria are known to be dominant in soils (Smalla et al., 2001). Nitrogen fixing bacteria are Gram-negative and have longer fragments than the Gram-positive bacteria.

2.4.1.2. Species richness of the rooibos bacterial community

The OTU's of oats prepared soil 1 and 2 are below the range of the OTU counts detected in other studies (Meier and Wehrli, 2008; Slabbert et al., 2010). The commercial and natural rhizosphere soil had higher OTU counts than the bulk soil (Figure 2.3). This is possibly due to more nutrients released by the plant in the rhizosphere (Maistry et al., 2015).

The species richness observed for the rooibos bacterial community was at the lower end of the range when compared to other studies. Other studies observed between 40 and 68 OTU's per sample (Meier and Wehrli, 2008; Slabbert et al., 2010). It is expected for the prepared and bulk soils to have lower OTU counts as there are less organic nutrients available in the bulk soil. The rhizosphere OTU's of commercial and natural soil were highly similar and confirm the findings of Postma et al (unpublished). Species richness have been correlated to the nutritional status of soil (Van Der Heijden et al., 2008). The higher the microbial diversity, the healthier the soil. Microbial diversity can thus be a good indicator for soil health.

2.4.1.3. Species accumulation curve

The initial steep increase of the accumulation curve indicates a dominant bacterial species and is an estimation of Simpson's diversity (Lande et al., 1971). The flatter tail of the curve indicates a lack of rare species. This is used to determine the number of sampling efforts to isolate the corresponding number of unique species. The species accumulation curve did not plateau completely as this is not a closed system (Scheiner, 2004).

2.4.1.4. Alpha diversity

The results from the Shannon diversity index (Figure 2.5) was not expected as it is thought that the rhizosphere soil would have a lower diversity than the bulk soil as the plants indirectly create a micro-habitat favouring a subset of bacteria within the ecosystem bacteria for the rhizosphere (Bonkowski et al., 2000).

The Simpson's diversity index (Figure 2.6) indicates that there are dominant species in the rhizosphere and is a more accurate representation of the diversity in the soil than the Shannon diversity index. The Shannon diversity does not account for relative abundance such as Simpson's diversity. The low Simpson's diversity of the natural bulk and prepared oats soil samples can be linked to the Shannon diversity index and indicates that there are fewer species present and there are more abundant species present than the rhizosphere samples and the commercial bulk soil samples.

2.4.1.5. Beta diversity ARISA

Rooibos farming practices are mostly organic and can be the reason that there were no significant differences between the commercial and natural soil samples (Figure 2.7). The rooibos is planted in the indigenous area and fynbos is cleared before rooibos is planted. With the ARISA profiles generated from the soil prepared for rooibos seedlings that had one year and two years' crop rotation with oats, it is visible from the NMDS plot that one year is enough to change the diversity of the soil. This means that it is not necessary to plant oats on old rooibos field for two years, as one year will be sufficient to restore microbial diversity. From the multidimensional scaling plot (Figure 2.7), it is visible that the bacterial communities in the rhizosphere of the natural and commercial plants are similar. This differs from the bulk soil where some commercial bulk soil samples differ from the other commercial and natural bulk soil samples. The commercial bulk soil samples that differs from the others are collected from 2 and 3-year-old rooibos plantations. These plantations will be replaced within the next two years as the rooibos plantation cycle is between four and six years.

The oats prepared soil and the natural bulk soil showed similar alpha and beta diversity values. The low diversity of these samples could indicate competition between species as the nutrients are limited. This is in contrast to literature where the bacterial rhizosphere communities are known to be less diverse and more abundant compared to bulk soil (Dennis et al., 2010; Uroz et al., 2010).

2.4.2. T-RFLP

ARISA was not used to study the *nifH* gene, as the *nifH* gene is a conserved length and ARISA is based on differences in lengths of the same gene. T-RFLP provided the ideal technique as it is more sensitive to lower concentrations of DNA, more reproducible and automated (Smalla et al., 2007).

Initial screening included IGK and DVV primers (Gaby and Buckley, 2012), but these were found unsuitable as nonspecific binding occurred. After testing the *nifH* primers on the soil samples, FAM-labelled primers were used. The Readymix Taq did not bind to the FAM-labelled primers and Robust 2G Taq (Kapa Biosystems) was used to amplify the *nifH* genes.

Different digestion enzymes have been used in other studies, but *HaeIII* provided the best differentiation between *nifH* genes from different bacteria (Poly et al., 2001). Other restriction enzymes such as *AluI*, *MnuI* and *NdeII* have been used, but did not give the desired results. Certain restriction enzymes were not able to distinguish between two species from the same genus, while others distinguished between two strains from the same species (Poly et al., 2001; Singh et al., 2010; Widmer et al., 1999). Some studies have used restriction enzymes in combination to obtain the desired resolution between different diazotrophic communities (Poly et al., 2001; Singh et al., 2010).

2.4.2.1 Frequency of OTU's generated with T-RFLP

Analysing the T-RFLP of a soil community is different from analysing the T-RFLP from a pure culture. A T-RFLP from a pure culture will produce a specific fingerprint, whereas a T-RFLP from a community will have a combined fingerprint of all the diazotrophic bacteria (Poly et al., 2001). This makes it difficult to identify specific species within a community profile. It is not possible to estimate the group of bacteria at a specific fragment length as the species from the genus *Burkholderia* produce fragments of variable length. The frequency of the *nifH* gene size after digestion were similar for rooibos (Figure 2.8) and honeybush (Figure 2.9). Although the profiles are similar, it does not necessarily indicate that the same species are present in both the rooibos and honeybush samples as T-RFLP is not based on the whole sequence of the gene but only the fragment sizes generated with restriction sites. High throughput sequencing of the *nifH* gene can be a solution to determine the different genera present in the soil samples obtained from rooibos and honeybush plants.

2.4.2.2. Species richness

The number of OTU's from the rooibos T-RFLP analysis is lower than the number of OTU's obtained from ARISA. This is expected as ARISA includes the whole bacterial community and the *nifH* T-RFLP only includes the nitrogen fixing bacteria. The number of OTU's for most of the rooibos and

honeybush samples were between 6 and 14 OTU's per sample (Figure 2.10 & 2.11). This is a high variation and an increased sampling effort may decrease this variation.

The *C. intermedia* and *C. maculata* bulk soil samples also had significantly lower OTU counts than the other samples (Figure 2.11). This confirms that species richness and genetic diversity is related (Magurran, 1988). The higher diversity is preferred as it implies that there is functional redundancy. It is suggested that functional redundancy help to maintain bacterial processes after environmental changes (Kennedy and Smith, 1995).

2.4.2.3. Species accumulation

The initial increase of species accumulation for the nitrogen fixing bacteria (Figure 2.12) differ from the whole bacterial community (Figure 2.4). This is an indication that there is a better evenness and not a single species which dominate (Lande et al., 1971). The rooibos species accumulation curve did not plateau after 40 samples and the honeybush samples species accumulation curve did not plateau after 48 samples. The tail of the honeybush species accumulation curve is flatter than the rooibos species accumulation curve. This is an indication that there are more rare species present in the rooibos samples than the honeybush samples as the tail of the species accumulation curve indicates rare species.

2.4.2.4. Alpha diversity

No significant differences were detected between the rooibos samples for Shannon (Figure 2.13) and Simpson's diversity (Figure 2.15). Most of the honeybush samples had a higher Shannon (Figure 2.14) and Simpson's diversity index (Figure 2.16) than the rooibos samples. The low dominance of species may indicate that the honeybush soil is more resilient than the rooibos soil. The hypothesis is however rejected as there is no significant difference in the diversity of the nitrogen fixing bacteria between the bulk and rhizosphere soils. The similarity between the bulk and rhizosphere soil were not expected as studies have shown that the plants select for bacteria and no significant difference in dominance were found between the bulk and rhizosphere samples (Bürgmann et al., 2005; Shu and Perez, 2012).

2.4.2.5. Beta diversity T-RFLP

The beta diversity of the *nifH* gene produced unexpected results. No separate clusters between the bulk and rhizosphere samples were detected for either rooibos or honeybush sites. A previous study has found that the total bacterial community differ between the bulk and rhizosphere soil, but the nitrogen fixing community of the bulk and rhizosphere soil was highly similar (Li et al., 2016). This

is true for the rooibos data as there were differences between the bulk and rhizosphere samples for whole bacterial community (Figure 2.7) and bulk and rhizosphere samples of the nitrogen-fixing bacterial community were similar (Figure 2.17). The absence of rhizosphere and bulk soil clusters for both rooibos (Figure 2.17) and honeybush (Figure 2.18) samples can be an indication that there is no clear selection of the nitrogen fixing bacteria in the rhizosphere, but there are selection for other soil bacteria.

2.4.3. qPCR

The *nifH* gene copy number for commercial rooibos soil corresponds with the hypothesis that the rhizosphere has a higher *nifH* copy number than the bulk soil, while the natural soil samples showed the opposite (Figure 2.19). This might be due to the monoculture of the rooibos plantation and the plants are further away from each other than with the natural plants.

This is preferred, as higher copy number indicates a higher nitrogen fixing capacity and this is beneficial for plant growth. The low copy numbers of the natural rhizosphere might be due to variation in bioavailability of nutrients such as organic carbon and trace elements (Yousuf et al., 2014). Differences in soil management may also influence the *nifH* gene copy numbers in the soil samples (Orr et al., 2011).

Some of the honeybush copy number results did not correspond with the hypothesis (Figure 2.20). *Cyclopia longifolia* and *C. intermedia* occur naturally on the Groendal farm and can be the reason that these plants have the highest *nifH* copy number as they are in their natural environment whereas the other honeybush species are planted in suboptimal habitats.

The rooibos samples had higher *nifH* copy numbers than the honeybush samples. This might be due to the different soil types these plants grow in and the differences in plant-bacteria interactions (Joubert et al., 2008). Further studies can be done on the seasonal variation on the copy number of nitrogen fixing communities of the plant species.

2.5. Conclusion

The changes in diazotrophic community structure are related to N-fixation rates (Hsu and Buckley, 2009). Although it is not clear if the N-fixation can be explained by the change in the community diversity or from changes in soil structure affecting the community structure and function. T-RFLP focus on the diversity of the nitrogen fixing community, whereas qPCR quantify the genes and provides a better indication of the nitrogen fixing potential of the soil. The overall diversity of bacterial communities might differ significantly between bulk and rhizosphere samples, but it does not always indicate that the diversity of nitrogen fixing community will differ significantly.

The similarity of diversity between the bulk and rhizosphere soil of the nitrogen fixing communities may be an indication that the legume does not select for specific free-living nitrogen fixing bacteria from the bulk soil to dominate the rhizosphere, but the qPCR results confirm that the abundance of free-living nitrogen fixing bacteria is higher in the rhizosphere than the bulk soil for the rooibos samples. Some results from qPCR produced unexpected results and may be due to other environmental factors such as the variation in bioavailability of nutrients for example, organic carbon and trace elements.

Future studies may include RNA extractions of the soil to study the number of *nifH* genes that are actively expressed using qPCR and the diversity of the expressed genes using T-RFLP. The effect of seasonal variation and different farming practices on nitrogen fixing bacteria can also be included in future studies. T-RFLP can be used to monitor changes in the soil diversity of diazotrophic bacteria associated with commercial rooibos and honeybush plants and can be used as an additional indicator to assess soil health.

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Chapter 3:

Biodiversity of rhizobia symbionts
associated with root nodules from
rooibos and honeybush plants

Abstract

The fynbos region has a high diversity of native legumes as well as rhizobial species. These legumes form symbiotic relationships with rhizobia that fix nitrogen for the plant. Recently there were studies that described novel species of rhizobia isolated from the root nodules of fynbos legumes. In this study, rhizobia were isolated from *Aspalathus linearis* and *Cyclopia* spp. root nodules to determine the diversity of rhizobia associated with *Aspalathus linearis* and *Cyclopia* plants. Sequence analysis of the rhizobial isolates were done on the 16S rRNA, *recA*, *atpD* housekeeping genes as well as the *nodA* and *nifH* symbiosis genes. Both *Burkholderia* and *Rhizobium* species were isolated from the root nodules. *Aspalathus linearis* preferred to associate with α-proteobacteria (*Rhizobium*), whereas *Cyclopia* spp. associates with *Burkholderia* (β-proteobacteria) species. Some of these are closely related to isolates from previous studies done on fynbos root nodules. Further sequencing and experiments have to be done to identify novel isolates and to provide a more complete reference database.

3.1. Introduction

The Cape Floristic Region of South Africa has a rich diversity of leguminous plants, with over 1700 species (Manning & Goldblatt, 2012). Examples of these leguminous plants are *Aspalathus linearis* (Rooibos) and *Cyclopia* sp. (Honeybush) that are endemic to the fynbos biome (Manning & Goldblatt, 2012). Legumes in the fynbos, form root nodules with nitrogen fixing rhizobia, and play an important role in the nutrient poor ecosystem (Sprent et al., 2013, 2010; Sprent and Gehlot, 2010). The fynbos biome has diverse soil types and together with the high plant diversity, creates microhabitats for diverse microorganisms (Garau et al., 2009; Howieson et al., 2013; Lemaire et al., 2015). This results in fynbos legumes that associate with different *Mesorhizobium*, *Rhizobium*, *Bradyrhizobium* and *Burkholderia* species (Lemaire et al., 2015).

Recently, there has been an increased exploration of rhizobia associated with indigenous fynbos legumes (De Meyer et al., 2014; Elliott et al., 2007; Howieson et al., 2013; Lemaire et al., 2016; Mishra et al., 2012). A wide range of *Burkholderia* spp. including novel species have recently been isolated from fynbos legumes. The novel species include *B. rhynchosiae*, *B. sprentiae*, *B. kirstenboschensis*, *B. dilworthii* and *B. aspalati* (De Meyer et al., 2014, 2013a, 2013b; Mavengere et al., 2014; Steenkamp et al., 2015). There is a dominance of *Burkholderia* species in fynbos soil and they are able to form root nodules with a wide variety of fynbos legumes (Beukes et al., 2013; Lemaire et al., 2016, 2015). *Cyclopia* spp. prefer to form root nodules with *Burkholderia* which is part of betaproteobacteria whereas *A. linearis* is nodulated by mostly *Mesorhizobium* spp. (Beukes et al., 2013; Elliott et al., 2007).

With bacteria, the 16S rRNA gene is not enough to accurately group different bacterial groups as the resolution is too low (Janda and Abbot, 2007). Other housekeeping genes such as *recA* and *atpD* are studied with the nitrogen fixation (*nifH*) and nodulation (*nodA*) genes. The *nod* and *nif* genes is also known as the symbiosis genes (Lemaire et al., 2015). By using multigene phylogeny, the diversity and evolution of rhizobia can be determined (Beukes et al., 2013; Lemaire et al., 2015). All rhizobia belong to the Proteobacteria, but the different species do not form a monophyletic group (Peix et al., 2015; Williams et al., 2010; Williams and Kelly, 2013). Until recently, only α-Proteobacteria was considered to be rhizobia and it was not until 2005 when Chen et al. (2005) showed that *Burkholderia* species are able to form root nodules.

The *nifH* gene encode for the nitrogenase enzyme complex, that is responsible for nitrogen fixation while the *nod* genes encode for the Nod factors that is responsible for root nodule formation. The *nodA* gene determine host specificity and the structure of the Nod factor (Gerding et al., 2012). The symbiosis genes are often located close together on plasmids (Flores et al., 2005; Giraud et al., 2007; Young et al., 2006).

Not all bacteria isolated from root nodules are rhizobia. Other bacteria frequently isolated from root nodules include species from the genera *Bacillus* and *Paenibacillus*. These are gram-positive, sporulating, rod-shaped endophytes, whereas rhizobia are gram-negative non-sporulating, rod-shaped endophytes (Deng et al., 2011; Li et al., 2008; Shiraishi et al., 2010; Zakhia et al., 2006).

The Genbank database for the *recA*, *atpD*, *nodA* and *nifH* genes is incomplete. This has an effect on species identification using phylogenetic analysis. The hypothesis of this study is that a high diversity of rhizobial species will be isolated from rooibos and honeybush root nodules. The aim of this study is to determine the diversity of the different bacterial species isolated from the root nodules of rooibos and honeybush plants.

3.2. Materials and methods

3.2.1. Isolation of microorganisms

Root nodules were sampled from Rooibos plants on the Klipopmekaar farm (S32°02.874' E18°59.500') in the Cederberg region where the plants range from one to four years old. The Honeybush root nodules were sampled from the farms Guavajuice (S34°02.802' E24°20.812') and Groenkloof in the Langkloof. Root nodules were removed from the plant and placed in McCartney bottles containing cotton wool and calcium chloride pellets (Prévost and Antoun, 2006). Nodules were surface sterilized by placing them in a 20% bleach solution for 60 seconds; the nodules were removed from the bleach solution and placed in 70% ethanol for 30 seconds. The root nodules were then washed three times in milliQ water (Prévost and Antoun, 2006) and plated on Yeast Mannitol Agar (10g mannitol, 0.5g K₂HPO₄, 0.2g MgSO₄.7H₂O, 0.1g NaCl, 1g yeast extract, 15g Agar, 1L H₂O, 10 mL 0.25% Congo red, 2 mL 2% cycloheximide). Congo red was added to differentiate between rhizobia and other bacteria, as rhizobia do not absorb Congo red and it also inhibits penicillin susceptible bacteria. Cycloheximide was added as an antifungal agent. The rhizobia were incubated at 30°C for 5 days (Beukes et al., 2013). The bacteria were isolated from the YMA plates and re-streaked to obtain pure colonies. The strain information is shown in table S1.

3.2.2. DNA extractions, PCR and sequencing

DNA was extracted from pure cultures using ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research, USA). The rhizobia that produce excessive exopolysaccharide (EPS) when cultured on YMA, might result in the EPS inhibiting reagents in the DNA extraction. These rhizobia were cultivated in tryptone yeast broth (TSB). This reduced the amount of EPS produced by the bacteria (Beukes et al., 2013) and the DNA extractions were performed using the ZR Fungal/Bacterial DNA MiniPrep kit. Bacteria were identified using the 16S rRNA, *atpD*, *recA* for amplifying the housekeeping genes and *nodA* and *nifH* for amplifying the symbioses genes (Lemaire et al., 2015; Palaniappan et al., 2010). The

primer pairs with their sequences are shown in table S2. All amplification reactions were done using GeneAMP PCR System 9700 (Applied Biosystems, Foster City, CA, USA) with PCR parameters as described by authors (table S1). Amplified DNA were visualized on an ethidium bromide stained 1% (w/v) agarose gel under UV-light. The PCR amplicons were sent to Central Analytical Facilities (Stellenbosch) for Sanger sequencing using the same sequencing primers as the initial PCR.

3.2.3 Phylogenetic analysis

Sequences were trimmed using Chromas and compared to other sequences on the GenBank database by using BLASTN. The sequences together with sequences obtained from Genbank and sequences obtained from Lemaire et al. (2015, 2016) and Beukes et al. (2013) were aligned using Muscle alignment in Mega 7. Mega 7 were also used for constructing the neighbour joining tree (Saitou and Nei, 1987) with 1000 permutations for bootstrap confidence analysis to determine reliability of the tree topologies (Felsenstein, 1985).

3.3. Results

3.3.1 Identification of rhizobia

The majority of isolates were initially classified into rhizobial genera by comparing the 16S rRNA sequence to the GenBank database by using BLASTN. The 16S rRNA BLAST results showed that the rhizobial strains belonged to the genera *Burkholderia* and *Rhizobium*. The similarity of the strains varied from 94-100% with known bacterial species (table S3). Some of the isolates were similar to other isolates from fynbos root nodules. The *recA*, *atpD*, *nifH* and *nodA* gene sequences were also compared to the Genbank sequence database. Phylogenetic analysis was done with reference strains obtained from accession numbers published in studies done by Kock (2004), Lemaire et al. (2015; 2016) and Beukes et al. (2013).

The 16S rRNA BLAST results of strains H1Ci1.1 and H1Cm10.3 gave different results when compared to their *nifH* and *nodA* BLAST results. There were also differences between the different blast results for strains H1WCI2.1, H1WCI2.1, R3C1.10 and R3C3.3 (table S3). The resolution of the 16S rRNA gene is not sufficient to identify the species (Figure 3.1). Some of the rooibos isolates identified as potential *Rhizobium* spp, showed significant variation based on the 16S rRNA results and did not group as a monophyletic group in *Rhizobium*. (table S3).

The *nifH* genes of isolated strains showed relation to the reference strains, but not to identified species (Figure 3.2). The *nodA* genes of these strains showed little relation to the reference strains or identified species (Figure 3.3). Most of the strains isolated from *C. maculata* formed a monophyletic group with strains isolated in a study done by Beukes et al. 2013. The rooibos isolates

formed a strongly supported clade with *R. tropici* and *R. multihospitium* species for the *recA* genes (Figure 3.4). The *atpD* genes formed a stongly supported cluster on their own with no known isolates (Figure 3.5). From the 16S rRNA, *nodA* and *nifH* multigene phylogenetic tree, strains R3C2, H1Cm1.5, H1Ci1.1 and H1Cm4.1 formed a clade with strains CB2, RAU2k, RAU2i and RAU2f (Figure 3.6). The GenBank database for the *recA*, *atpD*, *nodA* and *nifH* genes are incomplete for most of the reference strains and multigene phylogeny cannot be done, but it can be analysed seperately. Other non-rhizobial isolates such as *Bacillus* and *Serratia* spp. were also isolated from the root nodules. The BLAST results are shown in table S3.

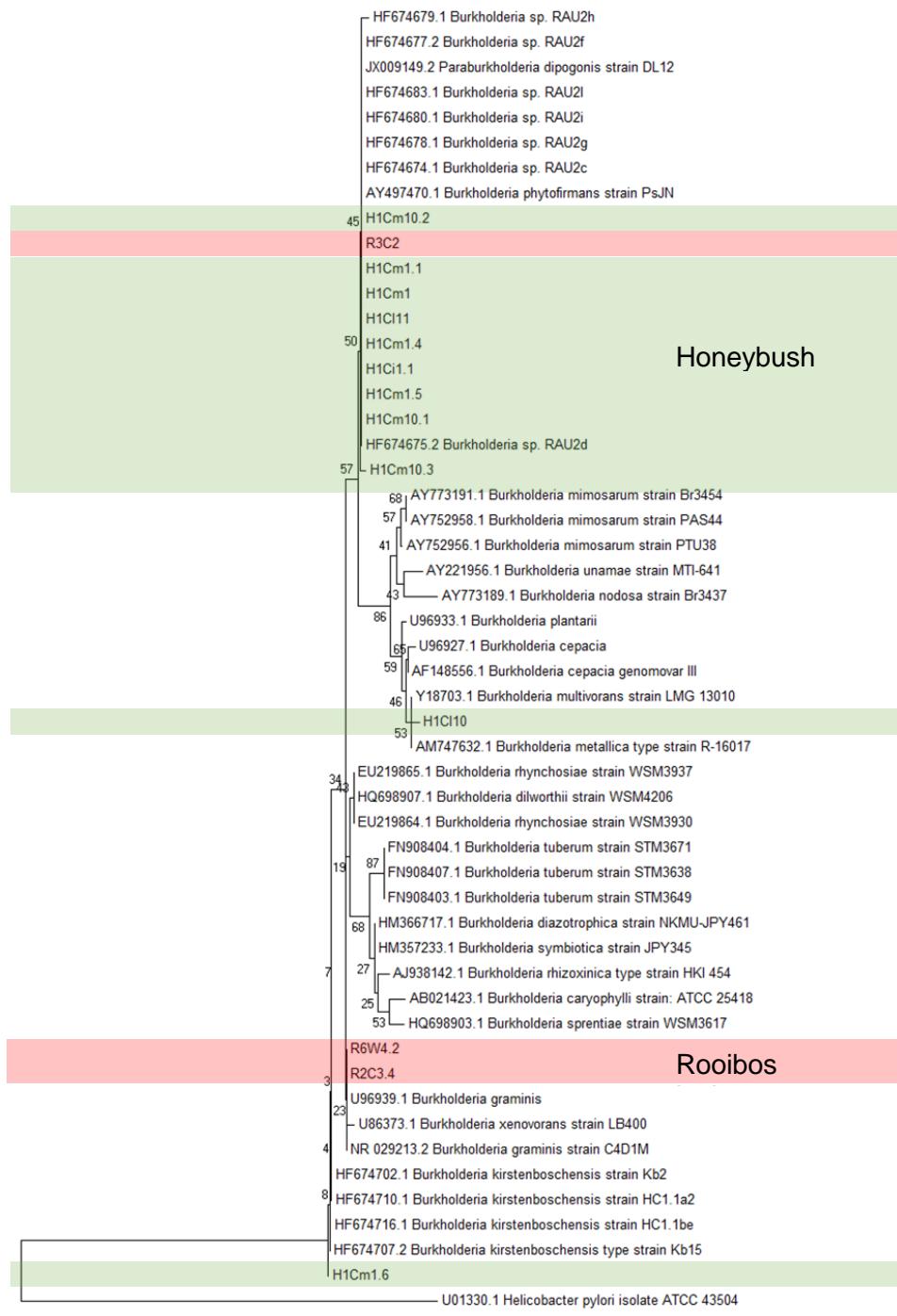
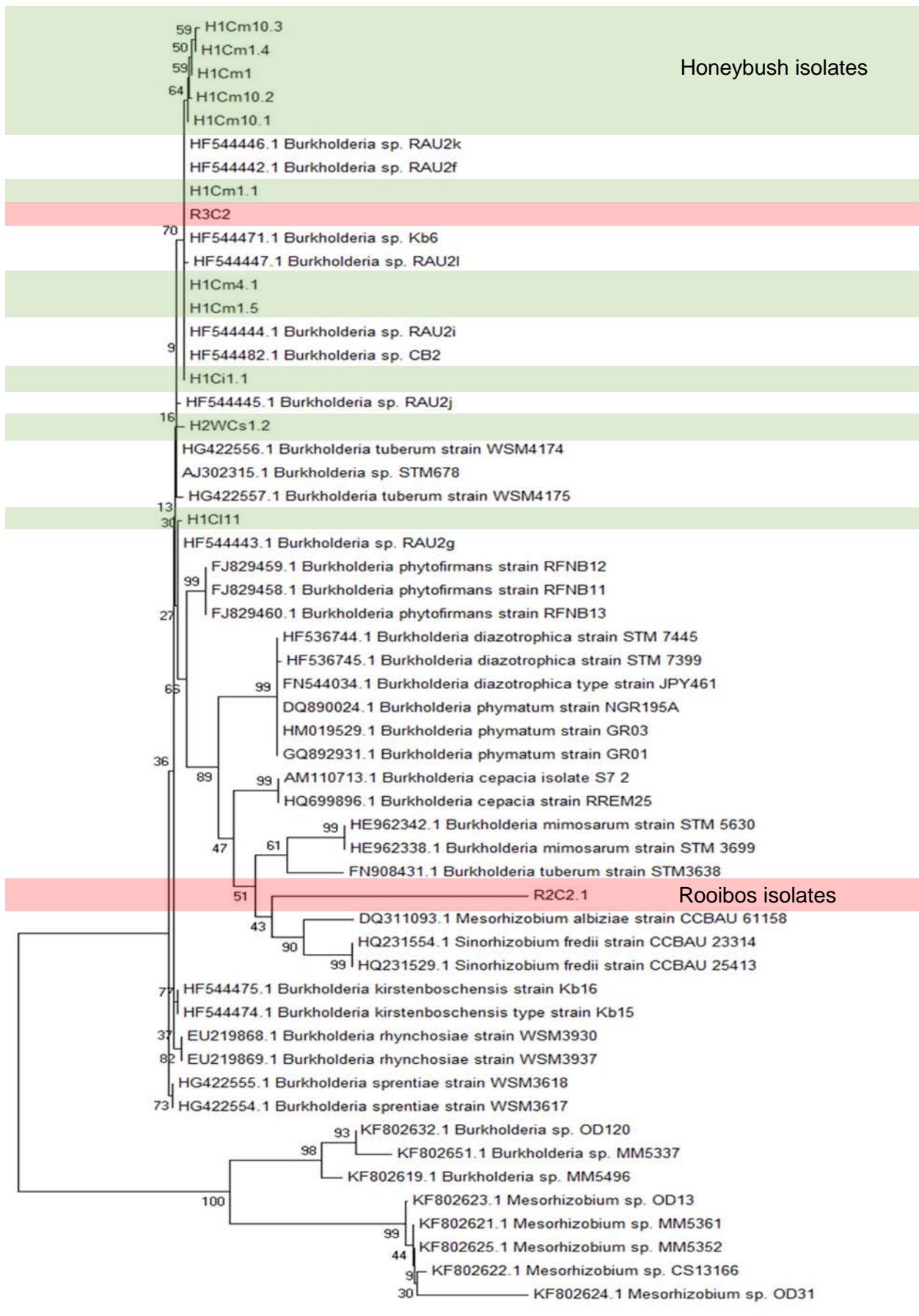
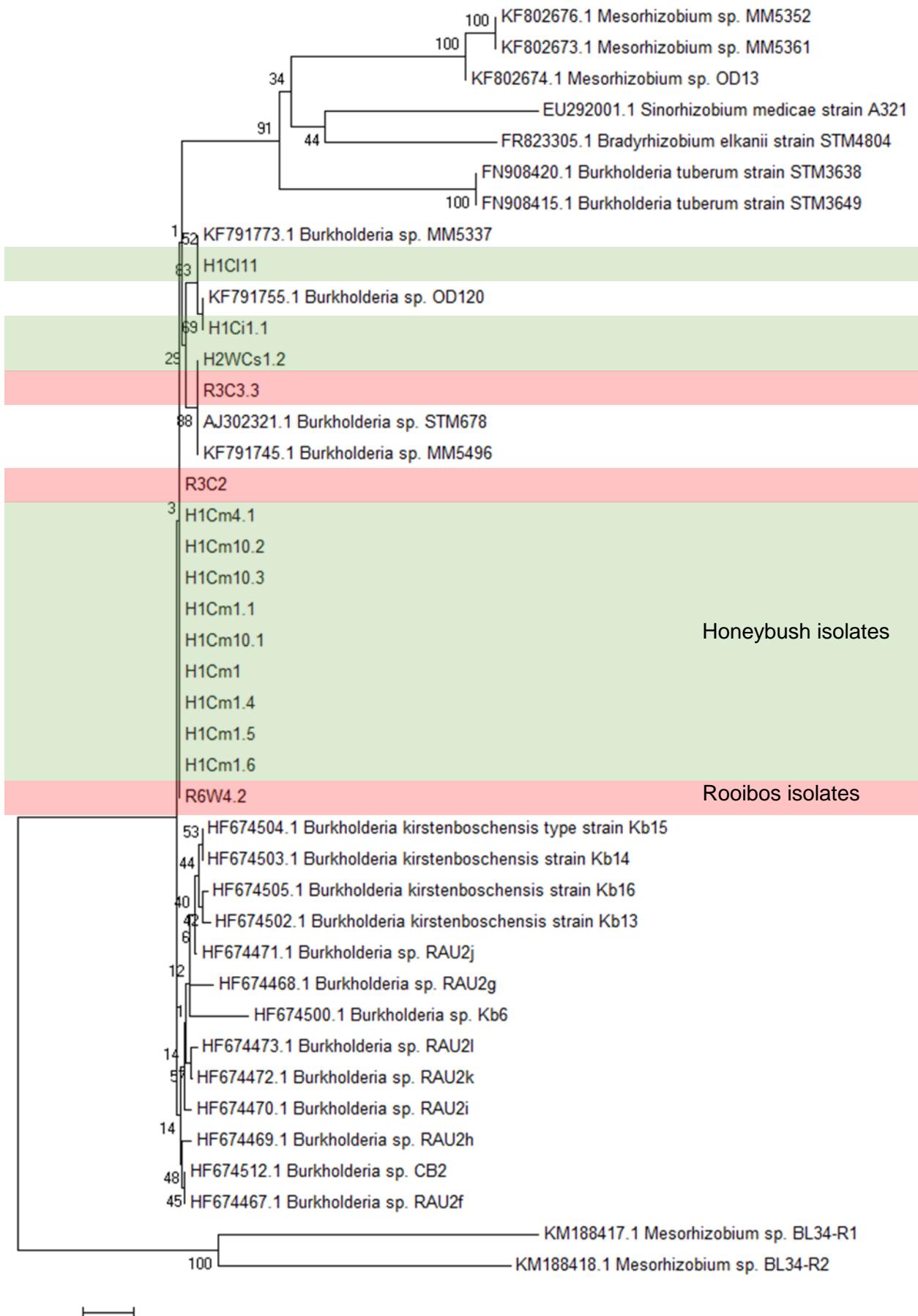


Figure 3.1. Phylogenetic tree based on the partial 16S rRNA sequences of the *Burkholderia* isolates.

Figure 3.2. Phylogenetic tree based on the partial *nifH* sequences of the isolates in *Burkholderia*.

Figure 3.3. Phylogenetic tree based on the partial *nodA* sequences of the isolates.

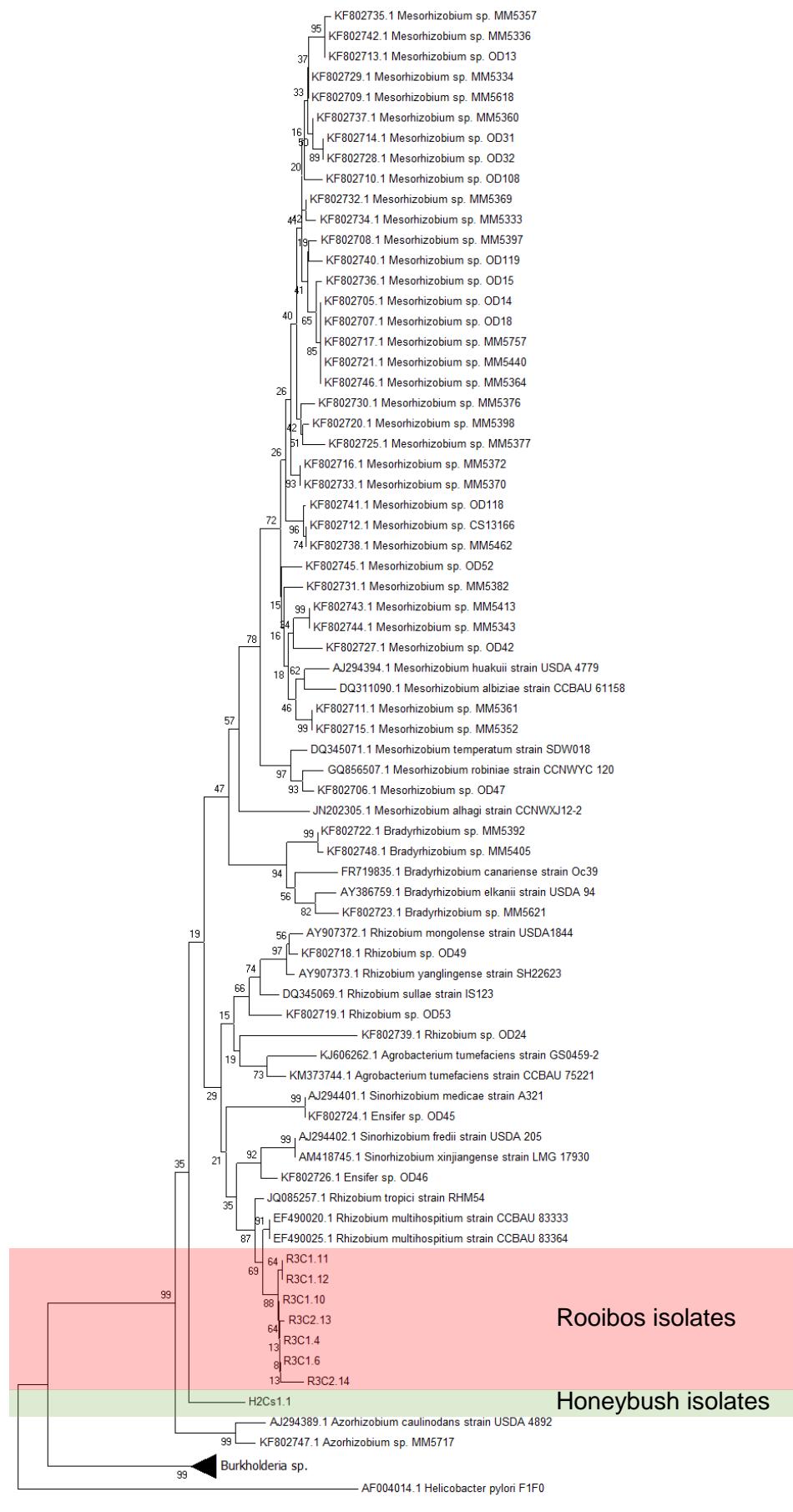


Figure 3.4. Phylogenetic tree based on the partial *recA* sequences of the isolates.

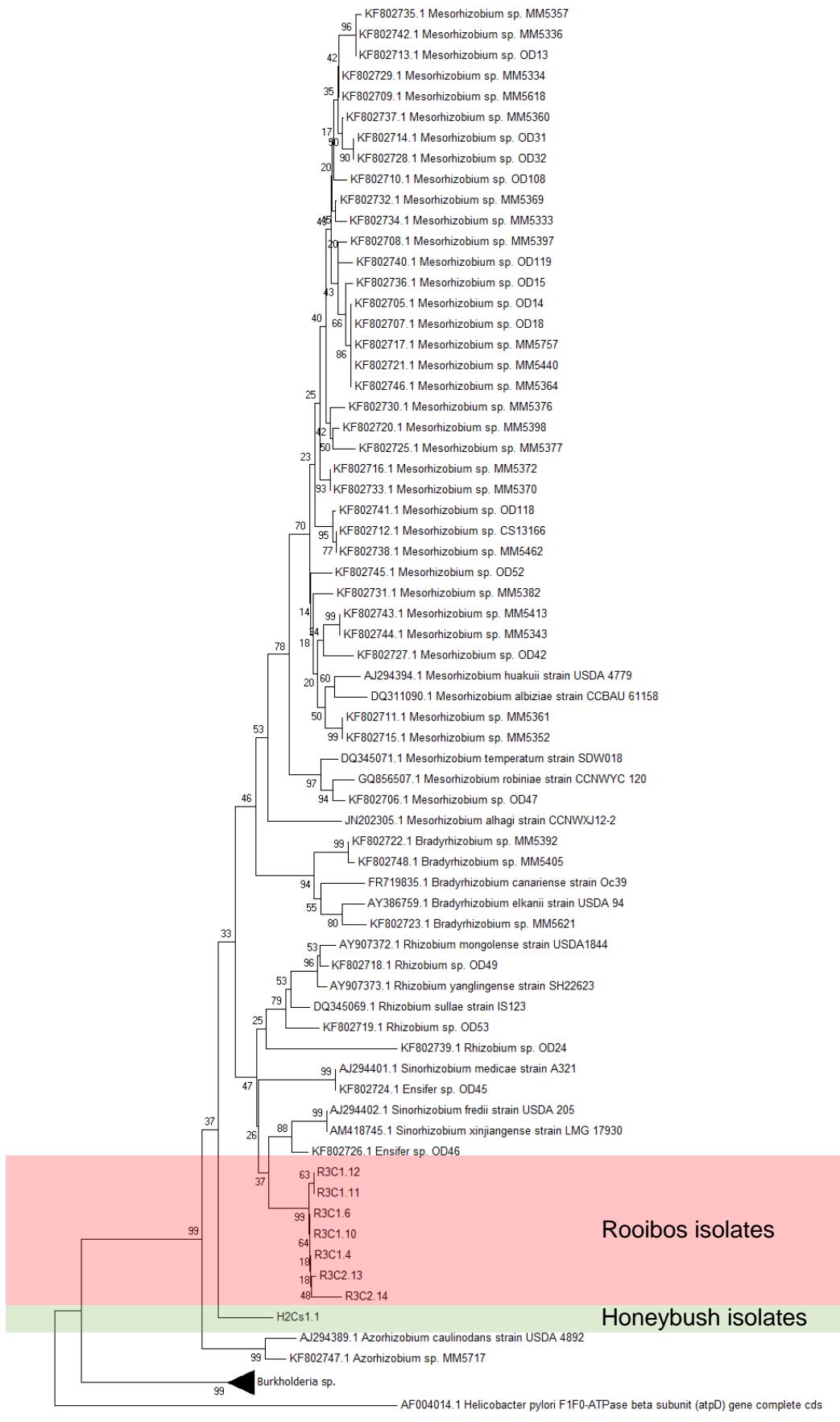


Figure 3.5. Phylogenetic tree based on the partial *atpD* sequences of the isolates that produced the correct PCR product.

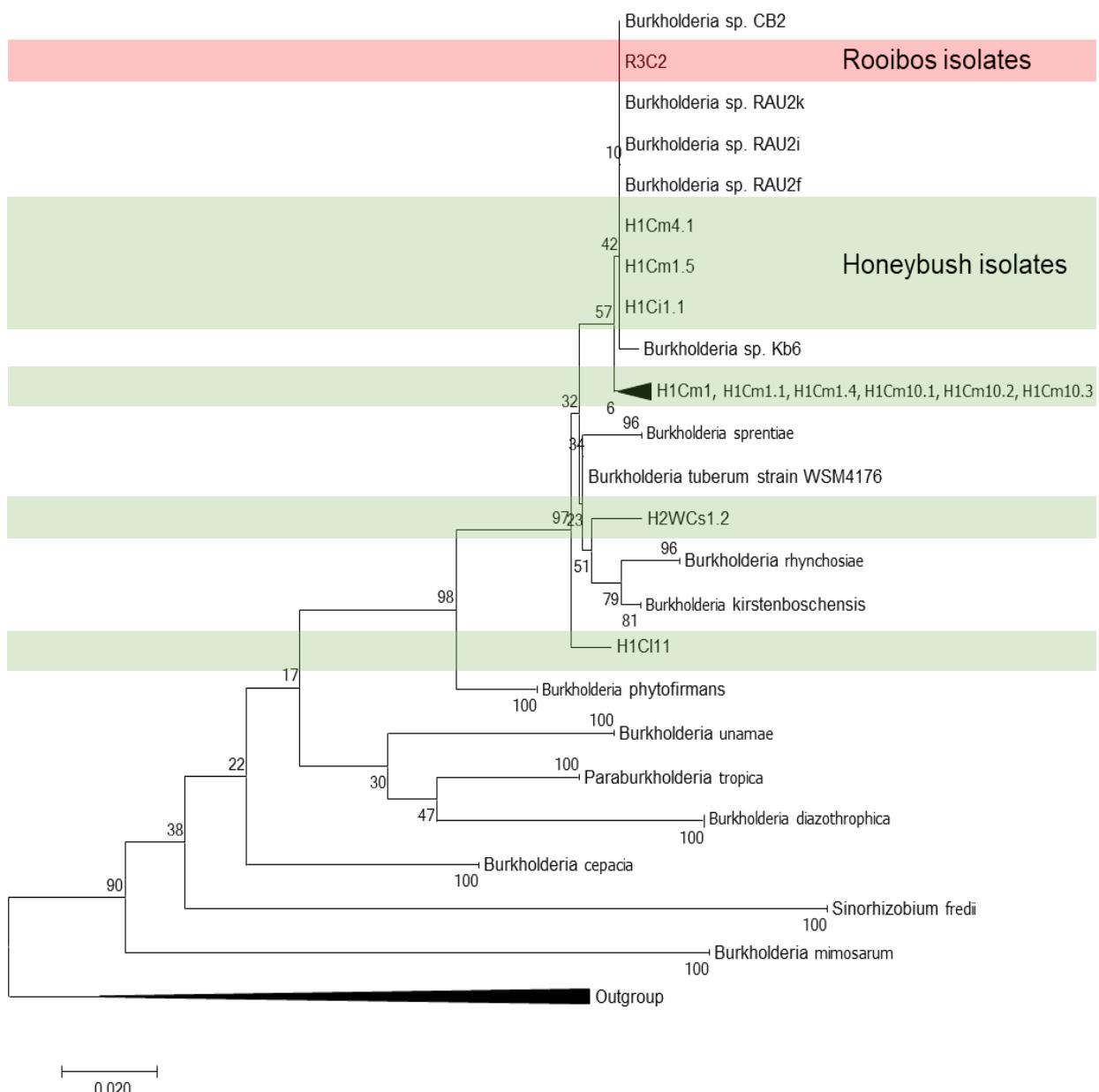


Figure 3.6. Phylogenetic tree based on the partial 16S rRNA, *nodA* and *nifH* sequences of the *Burkholderia* isolates.

3.4. Discussion

Rhizobium and *Burkholderia* are common symbionts of fynbos legumes (Kock, 2004; Lemaire et al., 2015). *Burkholderia* adapted to the acidic fynbos soil and is commonly found in fynbos soil (Garau et al., 2009). Plant associated *Burkholderia*, also known as *Paraburkholderia* (Sawana et al., 2014), have lower G+C content than the opportunistic human pathogen *Burkholderia* spp. (Gyaneshwar et al., 2011).

Several *Burkholderia* strains has been isolated from *Cyclopia* root nodules (Figure 3.2 & 3.3). Several isolates, mostly from *C. maculata* showed high similarity with reference strains RAU2f, RAU2k and RAU2l, isolated by Beukes et al. (2013) in South Africa. The strains isolated by Beukes et al. (2013) were isolated from *Hypocalyptus* spp in the Eastern Cape of South Africa. The strains isolated from *Cyclopia* root nodules by Kock (2004) did not group with the isolates from *Cyclopia* root nodules from this study. This suggest that *Burkholderia* spp. may have a wide host range and are not very host specific. This was also found by Lemaire et al. (2015). The 16S rRNA phylogenetic tree did not have sufficient resolution to distinguish between different species of closely related strains.

The isolates from the *A. linearis* root nodules had low similarity when they were compared to the GenBank database and formed their own monophyletic group with a bootstrap confidence value of 94 (Figure 3.4 & 3.5). None of the strains isolated from *A. linearis* were *Mesorhizobium* or *Bradyrhizobium* spp. as Elliot et al. (2007) had found. Strain H2Cs1.1 did not group with the *Burkholderia* strains as expected (Figure 3.4 & 3.5). Kock (2004) isolated *Rhizobium* strains from *Cyclopia* root nodules. It is possible that α-proteobacteria can form root nodules with *Cyclopia* plants.

The isolates that did not belong to α- or β-proteobacteria, belonged to *Bacillus*, *Paenibacillus* and *Serratia* genera. Studies have isolated these non-rhizobial genera from root nodules and found that some of these strains can be plant growth-promoting bacteria (Deng et al., 2011; Li et al., 2008; Shiraishi et al., 2010; Zakhia et al., 2006).

The *atpD* and *recA* genes showed similar phylogenetic analysis compared to the 16S rRNA phylogenetic analysis (table S3). A study done by Gaunt et al. (2001) showed that *atpD* and *recA* phylogeny support the 16S rRNA classification of rhizobia. The *atpD* and *recA* blast results were similar for both genes and some varied with the 16S rRNA BLAST results (table S3). This might also be due to the fact the sequences on GenBank are not reviewed and could lead to inaccurate identifications. There was difficulty sequencing some of the five genes of the isolates, for example there were difficulty sequencing the *atpD* and *recA* genes of *Burkholderia* strains and the *nifH* and *nodA* genes of the *Rhizobium* strains. Futher optimization is required to obtain the missing gene sequences.

The multigene phylogenetic analysis showed that strains H1Cm1, H1Cm1.4, H1Cm10.1, H1Cm10.2 and H1Cm10.3 formed their own clade whereas strains R3C2, H1Cm1.5, H1Cm4.1 and H1Cm1.1 formed a clade with strains isolated from other studies (Figure 3.6). These strains formed different clades when the genes were analysed separately when compared to the multigene analysis, but the bootstrap values are not significant and cannot be used to distinguish between the different strains.

The strains that have no close relative in the phylogenetic trees could be possible new species as the fynbos soil is a biodiversity hotspot for rhizobia (Gyaneshwar et al., 2011). However, the gene database for rhizobia in the fynbos is incomplete and this makes it difficult to identify every isolated strain. Some of the *Burkholderia* strains that are sequenced and available on GenBank do not have sequences for all the genes needed to identify them phylogenetically. Recently, studies done by Lemaire et al. (2015, 2016) and Beukes et al. (2013) have provided complete sequencing records and provides a good reference database for fynbos isolated *Burkholderia* species. An increased sampling and sequencing effort is needed to complete the rhizobia database. Variation in the genome within species also adds to the difficulty to accurately identify bacteria or determine if it is a new species. The symbiosis genes that are located on a plasmid can be transferred to other non-rhizobial species through horizontal gene transfer (Barcellos et al., 2007).

3.5. Conclusion

A variety of different rhizobia has been isolated from the root nodules of *Cyclopia* spp. and *A. linearis* plants. This study has confirmed that *Cyclopia* spp. prefers *Burkholderia* spp. and *A. linearis*, *Rhizobium* spp. as symbionts. Some isolates grouped with known strains, while other strains had little relation to known strains and grouped separately in phylogenetic analysis. Future studies can include complete genome sequencing and DNA hybridization to determine if some of these species are novel species. Root nodule forming authentication can be done to confirm that the isolates can form root nodules with *Cyclopia* and *A. linearis* plants. An increased sampling effort is needed to determine the diversity of rhizobia that associates with *Cyclopia* and *Aspalathus* plants.

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Supplementary information

Table S1. Isolate strain information

A. linearis	Strain number	Locality	Cyclopia	Strain number	Locality
	R3C1.11	Klipopmekaar	<i>C. maculata</i>	H1Cm1	Groendal
	R1C1.1	Klipopmekaar		H1Cm1.1	Groendal
	R1C1.2	Klipopmekaar		H1Cm1.2	Groendal
	R1C1.5	Klipopmekaar		H1Cm1.3	Groendal
	R1C4.1	Klipopmekaar		H1Cm1.4	Groendal
	R2C1	Klipopmekaar		H1Cm1.5	Groendal
	R2C1.2	Klipopmekaar		H1Cm1.6	Groendal
	R2C1.3	Klipopmekaar		H1Cm10.1	Groendal
	R2C1.6	Klipopmekaar		H1Cm10.2	Groendal
	R2C2.1	Klipopmekaar		H1Cm10.3	Groendal
	R2C2.4	Klipopmekaar		H1Cm11.3	Groendal
	R2C3.1	Klipopmekaar		H1Cm11.4	Groendal
	R2C3.4	Klipopmekaar		H1Cm11.5	Groendal
	R3C1.10	Klipopmekaar		H1Cm13.2	Groendal
	R3C1.12	Klipopmekaar		H1Cm13.3	Groendal
	R3C1.2	Klipopmekaar		H1Cm4.1	Groendal
	R3C1.4	Klipopmekaar		H1Cm4.2	Groendal
	R3C1.6	Klipopmekaar			
	R3C1.8	Klipopmekaar	<i>C. longifolia</i>	H1C12.1	Groendal
	R3C2	Klipopmekaar		H1Cl1.1	Groendal
	R3C2.13	Klipopmekaar		H1Cl1.2	Groendal
	R3C2.14	Klipopmekaar		H1Cl1.3	Groendal
	R3C2.15	Klipopmekaar		H1Cl10	Groendal

R3C2.21	Klipopmekaar		H1CI11	Groendal
R3C2.3	Klipopmekaar		H1CI2.1	Groendal
R3C20	Klipopmekaar		H1WCI2	Groendal
R3C3.2	Klipopmekaar		H1WCI2.1	Groendal
R3C3.3	Klipopmekaar		H1WCI2.2	Groendal
R4C3.1	Klipopmekaar		H1WCI2.3	Groendal
R4W3	Klipopmekaar			
R5W2	Klipopmekaar	C. <i>intermedia</i>	H1Ci1.1	Groendal
R6W1	Klipopmekaar		H1Ci1.4	Groendal
R6W1.1	Klipopmekaar		H1Ci3.1	Groendal
R6W3.3	Klipopmekaar		H2Ci1.1	Guavajuice
R6W3.4	Klipopmekaar			
R6W4.2	Klipopmekaar	C. <i>subternata</i>	H1Cs1.1	Groendal
R6W4.5	Klipopmekaar		H1Cs1.2	Groendal
R6W5.1	Klipopmekaar		H1Cs1.3	Groendal
R6W5.2	Klipopmekaar		H1Cs1.4	Groendal
R7W1.1	Klipopmekaar		H1Cs3.1	Groendal
R7W2	Klipopmekaar		H1Cs4.1	Groendal
			H2Cs1.1	Guavajuice
			H2Cs1.2	Guavajuice
			H2Cs1.5	Guavajuice
			H2Cs3	Guavajuice
			H2Cs3.4	Guavajuice
			H2WCs1.2	Guavajuice

Table S2. Primer pairs used in this study with annealing temperatures.

Primer pair	Primer sequence 5'-3'	Annealing temperature	Reference
16S 27F	AGAGTTGATCCTGGCTCAG	52°C	Weisburg et al., 1991
16S 1492R	GGTTACCTTGTACGACTT		Weisburg et al., 1991
nifH F1	TAYGGNAARGGNGGNATYGGNAARTC	60°C	Boulygina et al., 2002
nifH 439R	GGCATNGCRAANCCDCCRCA		De Meyer et al., 2011
recA 63F	ATCGAGCGGTCGTTCGGAAGGG	60°C	Gaunt et al., 2001
recA 504R	TTGCGCAGCGCCTGGCTCAT		Gaunt et al., 2001
nodA 1F	TGCRGTGGAARNTRNNCTGGAAA	60°C	Haukka et al., 1998
nodA 2R	GGNCCGTCRTCRAAWGTCARGTA		Haukka et al., 1998
atpD 237F	SCTGGGSCGYATCMTGAACGT	60°C	Gaunt et al., 2001
atpD 771R	GCCGACACTTCCGAACCNGCCTG		Gaunt et al., 2001

The degeneracies used are described by the International Union of Pure and Applied Chemistry Conventions as follows:

Y = C/T; D = G/A/T; S = G/C; R = A/G; B = C/G/T; H = T/C/A; N = A/G/C/T; W = A/T; I = inosine.

Table S3. BLASTN searches for 16S rRNA, *nodA*, *nifH*, *recA* and *atpD* sequences of rhizobial isolates obtained from rooibos and honeybush plants. Blank spaces are where no amplification or not the correct fragment size were observed.

Strain	Closest 16S rRNA BLASTN match with similarity	Closest recA rRNA BLASTN match with similarity	Closest atpD rRNA BLASTN match with similarity	Closest nifH rRNA BLASTN match with similarity	Closest nodA rRNA BLASTN match with similarity
H2WCs1.2				<i>Burkholderia</i> sp. MM5384 (KF802652.1), 99%	<i>Paraburkholderia sprentiae</i> WSM5005 (CP017562.1), 99%
H1Ci1.1	<i>Pseudomonas</i> sp. O-NR7 (JN613472.1), 94%			<i>Burkholderia</i> sp. WC7.3a (HF544476.1), 100%	<i>Burkholderia</i> sp. OD120 (KF791755.1), 99%
H1Cm4.1				<i>Burkholderia</i> sp. WC7.3a (HF544476.1), 99%	<i>Paraburkholderia sprentiae</i> WSM5005 (CP017562.1), 98%
H1Cm10.2	<i>Burkholderia</i> sp. BL25 I1R1 (KR154605.1), 100%			<i>Burkholderia</i> sp. WC7.3a (HF544476.1), 98%	<i>Burkholderia</i> sp. BL21-ind5-R2 (KM188387.1), 99%
H1Cm10.3	<i>Phyllobacterium myrsinacearum</i> B18 (EU169173.1), 99%			<i>Burkholderia</i> sp. WC7.3a (HF544476.1), 97%	<i>Paraburkholderia sprentiae</i> WSM5005 (CP017562.1), 98%
R3C2	<i>Burkholderia</i> sp. BL29 I6R2 (KR154610.1), 99%			<i>Burkholderia</i> sp. WC7.3a (HF544476.1), 100%	<i>Paraburkholderia sprentiae</i> WSM5005 (CP017562.1), 98%
H1Cm1.1	<i>Burkholderia</i> sp. BL29 I6R2 (KR154610.1), 99%			<i>Burkholderia</i> sp. WC7.3a (HF544476.1), 99%	<i>Paraburkholderia sprentiae</i> WSM5005 (CP017562.1), 98%
H1Cm10.1	<i>Burkholderia</i> sp. BL25I1R1 (KR154605), 99%			<i>Burkholderia</i> sp. WC7.3a (HF544476.1), 99%	<i>Paraburkholderia sprentiae</i> WSM5005 (CP017562.1), 98%
H1Cl11	<i>Paraburkholderia phytofirmans</i> PSB48 (KX881479.1), 99%			<i>Burkholderia</i> sp. UCT43 (HF544498.1), 99%	<i>Burkholderia</i> sp. BL28-ind1-R3 (KM188428.1), 100%
H1Cm1	<i>Burkholderia</i> sp. BL29 I6R2 (KR154610.1), 99%			<i>Burkholderia</i> sp. WC7.3a (HF544476.1), 98%	<i>Paraburkholderia sprentiae</i> WSM5005 (CP017562.1), 98%
H1Cm1.4	<i>Burkholderia</i> sp. BL29 I6R2 (KR154610.1), 100%			<i>Burkholderia</i> sp. WC7.3a (HF544476.1), 96%	<i>Paraburkholderia sprentiae</i> WSM5005 (CP017562.1), 98%
H1Cm1.5	<i>Burkholderia</i> sp. Kb12 (HF674704.1), 99%			<i>Burkholderia</i> sp. WC7.3a (HF544476.1), 100%	<i>Burkholderia</i> sp. BL21-ind5-R2 (KM188387.1), 99%

H1Cm1.6	<i>Burkholderia</i> sp. HC87 (KJ123806.1), 99%				<i>Paraburkholderia sprentiae</i> WSM5005 (CP017562.1), 98%
R3C2.14		<i>Rhizobium</i> sp. 8213 (HM064003.1), 94%	<i>Rhizobium multihospitium</i> CCBAU 83333 (EF490020.1), 98%		<i>Paenibacillus</i> sp. Y412MC10 (CP001793.1), 83%
R3C3.3	<i>Pseudomonas</i> sp. O-NR7 (JN613472.1), 94%	<i>Rhizobium</i> sp. 8213 (HM064003.1), 94%			<i>Paraburkholderia sprentiae</i> WSM5005 (CP017562.1), 99%
R6W4.2					<i>Paraburkholderia sprentiae</i> WSM5005 (CP017562.1), 98%
R2C1.6		<i>Rhizobium freirei</i> PRF 81 (EU488827.1), 92%			
R6W5.2	<i>Bacillus megaterium</i> Ns3 (MG544100), 100%	<i>Rhizobium</i> sp. 8213 (HM064003.1), 95%			
H1WCI2	<i>Bacillus</i> sp. WYT039 (JQ807864.1), 100%	<i>Rhizobium</i> sp. 8213 (HM064003.1), 92%			
H2Cs1.1	<i>Serratia</i> sp. FILDES001 (KX434589), 99%	<i>Agrobacterium tumefaciens</i> C58 (FM164330.1), 96%	<i>Agrobacterium tumefaciens</i> C58 (AE007869.2), 95%		
H1Cm1.2		<i>Rhizobium</i> sp. R-46234-t495 (FR772684.1), 99%			
R3C1.11		<i>Rhizobium</i> sp. 8213 (HM064003.1), 94%	<i>Rhizobium multihospitium</i> CCBAU 83333 (EF490020.1), 98%		
R3C1.10	<i>Bacillus wiedmannii</i> TSS6 (MF620066.1), 98%	<i>Rhizobium</i> sp. 54-3-1 (KM378432.1), 94%	<i>Rhizobium multihospitium</i> CCBAU 83333 (EF490020.1), 98%		
R3C1.12		<i>Rhizobium</i> sp. 8213 (HM064003.1), 94%	<i>Rhizobium multihospitium</i> CCBAU 83333 (EF490020.1), 99%		
H1WCI2.1	<i>Bacillus thuringiensis</i> FJAT-hcl-33 (KY653105.1), 99%	<i>Rhizobium tropici</i> NCSU 2459 (KJ535983.1), 97%			
R3C2.21	<i>Phyllobacterium myrsinacearum</i> B18 (EU169173), 98%	<i>Rhizobium</i> sp. 8213 (HM064003.1), 94%			
R3C1.2		<i>Rhizobium</i> sp. CA3b_15, (KR400891.1), 95%			

R3C1.5			<i>Rhizobium multihospitium</i> CCBAU 83333 (EF490020.1), 96%		
R3C2.13			<i>Rhizobium multihospitium</i> CCBAU 83333 (EF490020.1), 98%		
R2C2.1	<i>Paenibacillus jamilae</i> Sco-A16 (FN386708.1), 99%		<i>Paenibacillus kribbensis</i> AM49 (CP020028.1), 96%	<i>Paenibacillus terrae</i> HPL-003 (CP003107.1), 96%	
R3C1.4			<i>Rhizobium multihospitium</i> CCBAU 83333 (EF490020.1), 98%		
H1Cs1.1	<i>Bacillus wiedmannii</i> TSS6 (JF495463.1), 96%				
H2Cs1.2	<i>Pseudomonas</i> sp. O-NR7 (JN613472.1), 94%				
H1Cs1.2	<i>Bacillus cereus</i> strain FJAT-46427 (KY849413.1), 99%				
H1Cm1.3	<i>Bacillus</i> sp. DU185(2010) (HM567067.1), 99%				
H1Cl2.1	<i>Burkholderia stabilis</i> YHNG13 (MG516201), 99%				
R6W4.2	<i>Burkholderia graminis</i> G2Bd5 (KT37810), 99%				
H2Ci1.1	<i>Bacillus</i> sp. sMM46 (KX527684), 99%				
H1Cl10	<i>Burkholderia stabilis</i> YHNG13 (MG516201), 99%				
H1Cm13.3	<i>Bacillus simplex</i> LMTK25 (KY614179), 99%				
R3C1.6	<i>Rhizobium</i> sp. TUTVU50 (KY941260), 96%		<i>Rhizobium multihospitium</i> CCBAU 83333 (EF490020), 96%		
R2C1	<i>Bacillus</i> sp. MRF-37 (MG266368), 99%				

R3C20	<i>Phyllobacterium myrsinacearum</i> B18 (EU169173), 99%				
H1Cm4.2	<i>Bacillus</i> sp. Z10 (KF295438), 100%				
R1C1.5	<i>Serratia liquefaciens</i> Noth 8 (MF716555), 100%				
H1Cm11.3	<i>Burkholderia phytofirmans</i> 11620960 (KF981567), 99%				
R6W5.1		<i>Rhizobium</i> sp. 8211 (HM064003), 95%			
R7W2	<i>Pseudomonas</i> sp. R84 (KT890305), 99%				

Chapter 4:

Metabolic properties of rhizobia isolated from rooibos and honeybush plants

Abstract

The fynbos region is known as one of the plant biodiversity hotspots in the world and is characterised by nutrient poor and acidic soils. Legumes in the fynbos play an important role in nutrient cycling and form symbiotic relationships with rhizobia. The plants provide the bacteria with a carbon source through root exudates and in return the bacteria provide the plants with nitrogen. Some rhizobia species can produce plant growth-promoting compounds that can promote plant growth directly or indirectly. The aim of this study is to determine the plant growth-promoting properties of rhizobia strains isolated from rooibos and honeybush root nodules. Isolated strains were plated on specialised media and tested for ammonia, HCN, phosphatase, 1-aminocyclopropane-1-carboxylate deaminase and indole acetic acid production. Results indicate that fixing nitrogen for the plant is not the only function of rhizobia. Rhizobia also produce indole acetic acid, HCN and phosphatase. Some species produced more plant growth-promoting compounds than others, and there was considerable variation between strains. The study highlights the importance of symbiotic interactions between microorganisms and plants. Knowledge about these interactions will result in more efficient farming practices of these plants, releasing stress on the harvesting of natural vegetation.

4.1. Introduction

The rhizosphere of plants provides a niche for certain soil bacteria as it is high in root exudates. The densities of bacteria are higher in the rhizosphere than in the bulk soil and up to 15% of the roots may be covered in bacteria (Van Loon, 2007). While the plants provide the bacteria with nutrients through root exudates, the bacteria also secrete metabolites into the rhizosphere that can have an effect on the plant (Van Loon, 2007). Signalling molecules are used by the plants and bacteria to interact with each other, which may be positive or negative. The most studied plant-bacterial interaction is the symbiosis of legumes and rhizobia, and varies from non-specific to specific interactions (Van Loon, 2007).

Rooibos (*Aspalathus linearis*) and Honeybush (*Cyclopia* spp.) rely on microbial interactions to survive in the nutrient poor fynbos soil (Sprent et al., 2013, 2010; Sprent and Gehlot, 2010). Many rhizobia produce plant growth-promoting compounds that have a positive effect on plant growth. Plant growth can be improved by direct or indirect mechanisms. Direct mechanisms such as phytohormones have a direct impact on plant growth, whereas indirect mechanisms such as biocontrol protect the plant from phytopathogens. Rhizobia that produce plant growth-promoting compounds are known as plant growth-promoting rhizobacteria (PGPR) (Andrews and Harris, 2000). Some species of *Burkholderia*, *Mesorhizobium* and *Bradyrhizobium* that associates with honeybush and rooibos have been found to produce plant growth-promoting compounds (Palaniappan et al., 2010; Verma et al., 2013). Other bacterial spp. such as *Bacillus* (Bai et al., 2002), *Pseudomonas* (Chanway et al., 1989), *Azotobacter* (Burns et al., 1981), *Serratia* (Zhang et al., 1997) and *Azospirillum* (Baldani et al., 1997) can also produce plant growth-promoting compounds and are known as plant growth-promoting bacteria (PGPB). These species are often used as microbial inoculants. Co-inoculation of PGPR and PGPB resulted in a greater increase in yield than with just PGPR alone (Bai et al., 2002). There are commercial rhizobial inoculants available for plants, but these inoculants are specific for plant species and one inoculant will not work for all the species (Spriggs and Dakora, 2007). *Aspalathus* spp. can be nodulated by either α- or β-proteobacteria, whereas *Cyclopia* species are exclusively nodulated by the betaproteobacteria, *Burkholderia* spp. (Elliott et al., 2007; Lemaire et al., 2015).

Plant growth-promoting bacteria that are used in inoculants can produce a variety of plant growth-promoting compounds such as indole acetic acid (IAA), 1-aminocyclopropane-1-carboxylate (ACC) deaminase, phosphatase, siderophores and hydrogen cyanide (HCN). The production of plant growth-promoting compounds is strain specific and sometimes differs in the amount produced (Spriggs and Dakora, 2009). Indole acetic acid is part of the auxin plant growth hormones and stimulates root development (Duca et al., 2014). The IAA pathway is shown in figure 4.1 and the interaction with the plant is shown in figure 4.2. 1-aminocyclopropane-1-carboxylate deaminase reduces the levels of ACC. ACC is a precursor for ethylene which is a plant stress hormone (Glick,

2005). The plant-bacteria interaction of ACC deaminase is shown in figure 4.2. Phosphates is an essential nutrient that is limited to most plants, as most phosphates are in an unavailable form that cannot be used by the plant. Phosphatase converts the unavailable (compound-bound) phosphates in the soil that makes it available to the plant (Alikhani et al., 2006; Nguyen et al., 1992).

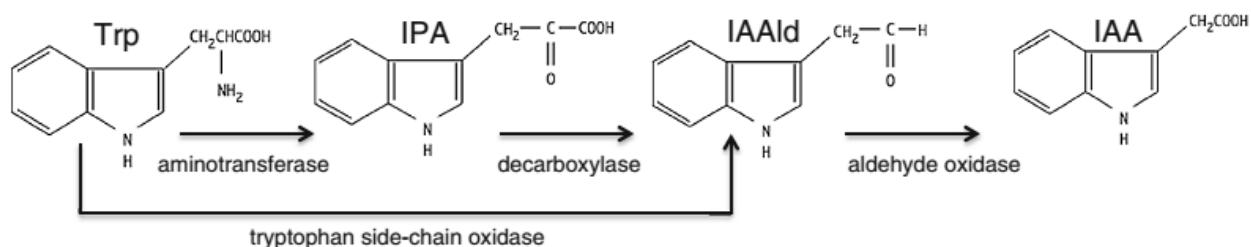


Figure 4.1. Indole acetic acid production pathway of bacteria. Adopted from Duca et al. 2014.

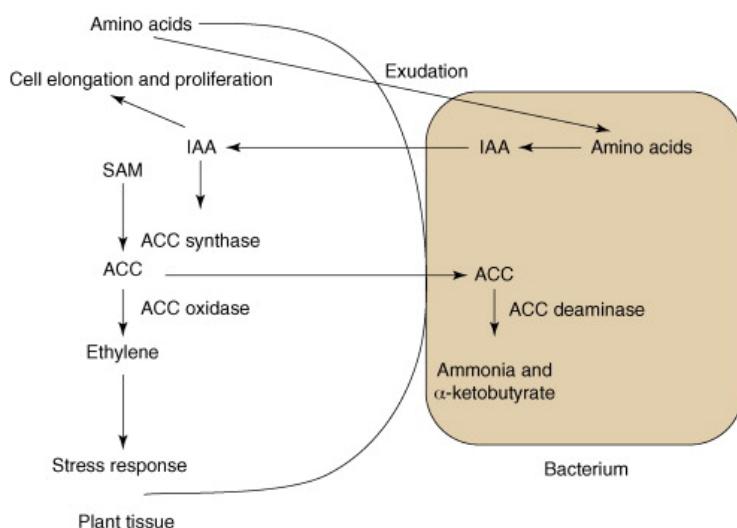


Figure 4.2. ACC deaminase and IAA interaction between plants and bacteria. Adopted from Arshad et al. 2007.

Siderophores can be used by both PGPR and PGPB to inhibit growth of phytopathogens or to prevent other bacteria access the available iron in the soil. Siderophores are often used in combination with hydrogen cyanide (HCN) to inhibit growth of phytopathogens (Verma et al., 2013).

The production of plant growth-promoting compounds is not limited to specific genera or species of soil bacteria and the exact mechanisms of the production of plant growth-promoting properties is not fully understood (Ahmad et al., 2008). The hypothesis of this study is that the bacteria isolated from rooibos and honeybush root nodules produce plant growth-promoting compounds and do not only

fix nitrogen for the plant. The aim of this study is to determine the plant growth-promoting properties of rhizobia strains isolated from rooibos and honeybush root nodules. The rhizobia that produced the most plant growth-promoting compounds together with nodule forming capabilities might be a good inoculant for rooibos and honeybush, depending on the compatibility of the bacteria with the host.

4.2. Materials and Methods

4.2.1. Isolation of rhizobia

The rhizobial strains used in this chapter are the same strains that were isolated in Chapter 3.

4.2.2. Screening for nitrogen fixing

Nitrogen-fixing organisms grow well on a nitrogen-free medium. These bacteria utilize atmospheric nitrogen gas for their cell protein synthesis. The composition of the nitrogen free media is as follows: Sucrose, 20.0 g; dipotassium phosphate, 1.0 g; magnesium sulphate, 0.5 g; sodium chloride, 0.5 g; ferrous sulphate, 0.1 g; sodium molybdate, 0.005 g; calcium carbonate, 2.0 g and agar, 15.0 g. No or little growth, indicates non-nitrogen fixer. Luxurious growth indicates a nitrogen fixer. Sucrose acts as the energy source, while sodium molybdate in the media increases the fixation of nitrogen. Sodium chloride maintains osmotic equilibrium of the media and calcium stimulates nodulation when present as chloride or sulphate. Strains were incubated at 26°C for 4 days (HIMEDIA technical data, 2015).

4.2.3. Phosphate solubilisation capabilities

The rhizobia were plated out on basal Sperber (1958) medium. The medium contains 10 g glucose, 0.5 g yeast extract, 0.1 g CaCl_2 , 0.25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 15 g agar, 1 L of dH₂O and 2.5 g $\text{Ca}_3(\text{PO}_4)_2$ (TCP) as an inorganic phosphate source. The pH was adjusted with NaOH to 7.2 before autoclaving. The rhizobia were inoculated in duplicate and incubated at 26°C for 7 days (Alikhani et al., 2006). The bacteria that produce phosphatase enzymes that can solubilize phosphate will produce a clear zone around the bacterial colonies. The diameter of the colony and the diameter of the clear zone was measured to calculate the solubilizing efficiency of the rhizobia (Nguyen et al., 1992). The efficiency of the phosphatase activity is calculated as follows:

$$E = \frac{\text{Solubilization diameter (S)}}{\text{Growth diameter (G)}} \times 100$$

4.2.4. Screening for siderophore production

Isolated strains were inoculated on chromeazurol S (CAS) agar plates. The protocol used was developed by Shwyn and Neilands (1987). Distilled water (750 mL), 100 mL 10X MM9 salts, 30.24 g Pipes, 12 g 50% (w/w) NaOH and 15 g agar were autoclaved and allowed to cool. CAS (60.5 mg) were dissolved in 50 mL water and mixed with 10 mL iron (III) solution (1 mM FeCl₃.6H₂O, 10 mM HCl). While stirring, 72.9 mg HDTMA (CTAB) dissolved in 40 mL water was added to the CAS mixture. The CAS mixture, 30 mL casamino acids (10%), 10 mL glucose (20%), 5 mL L-glutamic acid (10%, neutralized) and 2.5 mL (+)-biotin (0.02%) were filter sterilized and added to the agar. The rhizobia were inoculated on the plates for 4 days at 26°C (Schwyn and Neilands, 1987). Siderophore activity is determined by the production of a yellow to orange halo around the colony.

4.2.5. Hydrogen cyanide production assay

All the isolates were screened for the production of hydrogen cyanide by adapting the method of Lorck (1948). The media consisted of 20 g nutrient broth, 15 g agar, 4.4 g glycine and 1 L dH₂O. A Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed in the top of the plate. Plates were sealed with parafilm and incubated at 26°C for 4 days. Development of orange to red colour indicated HCN production (Ahmad et al., 2008).

4.2.6. ACC deaminase production assay

The rhizobia were plated onto Dworkin Foster (1958) minimal medium supplemented with ACC (Nain et al., 2012). No growth indicates that the microorganism is not able to break down ACC. The strains were incubated at 26°C for 3-5 days. Cultures capable of growing on ACC supplemented plates are capable of using ACC as a nitrogen source (Khandelwal and Sindhu, 2013). The composition of Dworkin Foster minimal medium is: (NH₄)₂SO₄, 2 g; KH₂PO₄, 4.0 g; Na₂HPO₄, 6.0 g; MgSO₄.7H₂O, 0.2 g; FeSO₄.7H₂O, 1.0 mg; H₃BO₃, 10 µg; MnSO₄, 10.0 µg; ZnSO₄, 70 µg; CuSO₄, 50 µg; MoO₃, 10 µg; distilled water, 1 L and agar, 20.0 g (Dworkin & Foster, 1958). 5 µl of each strain were plated in triplicate on the media. Three variations of this media were used. The first one was the minimal media as described, the second one was without (NH₄)₂SO₄, and the third variation contained 0.3 g/LI⁻¹ ACC (Penrose and Glick, 2003).

4.2.7. IAA production assay

Rhizobial cultures were grown in Luria Bertani (LB) broth containing 5 g/L tryptone and one without tryptone for 48 h. After 48 h of incubation, Salkowski's method were used to quantify the IAA produced (Ryu and Patten, 2008). Cultures were centrifuged for 30 min at 3000 rpm (845 RCF). 160 µL of Salkowski's reagent (150 mL concentrated H₂SO₄, 250 mL dH₂O), 7.5 mL 0.5 M

$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) were added to 40 μL of the cell free supernatant (Parsons et al., 2015). These mixtures were loaded on a sterile flat bottom 96-well microtiter plate and allowed to incubate for 20 min at room temperature in the dark. Development of pink colour will indicate IAA production. The absorbance was read at 535 nm using a Bio Rad xMark Microplate Spectrophotometer. Known quantities of pure IAA in LB broth were used to generate a standard curve. The standard curve concentrations ranged from 10-100 mg/mL. Each sample was loaded in triplicate.

4.2.8. Potting trial experiment

The four strains that produced the highest quantities of plant growth-promoting compounds were used in a potting trial experiment to determine if the strains will be able to form root nodules and improve plant growth of *C. subterranea*. The strains also contained the *nifH* and *nodA* genes which code for the enzymes to fix nitrogen and form root nodules. Seeds were surface disinfected by treating the seeds with 95% ethanol for 30 s (Prévost and Antoun, 2006). The seeds were scarified by treating the seeds with concentrated hydrochloric acid for 3 min and rinsed three times with sterile distilled water. The seeds were then soaked overnight in sterile water containing smoke primer and incubated at 26°C for 2 days.

A mixture of peat and sand were used as soil for the potting experiment. The soil was autoclaved and placed into seed trays. The seeds were planted 1.5 cm below the surface. After the seeds were planted, they were treated with 30 mL of a rhizobia solution (2×10^{10} CFUs/mL). The plants were placed in a greenhouse and watered once every two days with 10 mL of water. The temperature ranged between 15 – 30°C. The plants' root development, root nodule formation, number of leaves and overall growth were analysed after four months in the greenhouse.

4.3. Results

4.3.1. Nitrogen free assay

In figure 4.3.a the bacterial growth can be seen and EPS are also produced as it is a high sugar media. In figure 4.3.b very little growth is visible as the strain is unable to fix nitrogen. The results of the strains are shown in table 4.1. There is a correlation between strains that grew well on the nitrogen free media and the *nifH* gene.

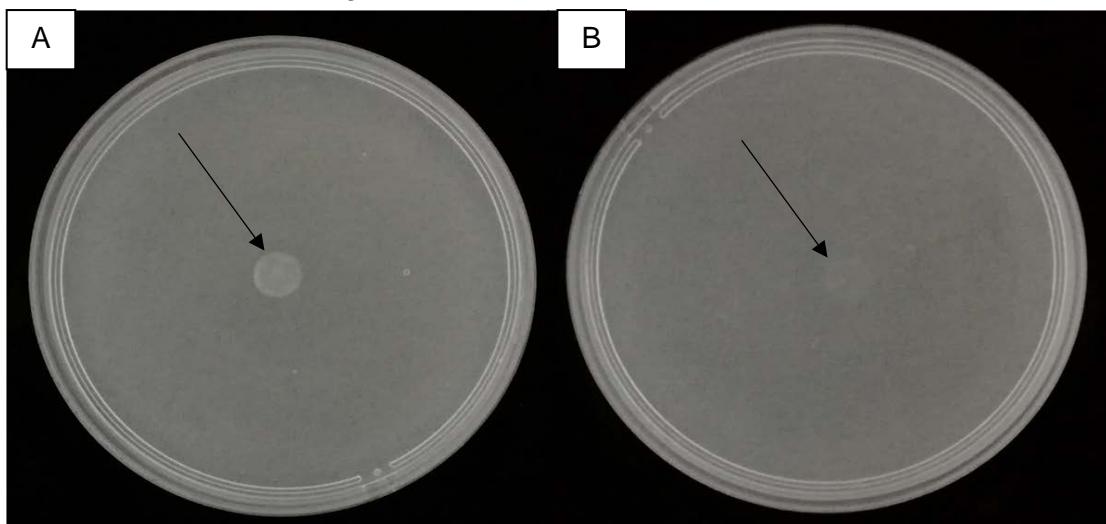


Figure 4.3 (A) Positive result on the nitrogen free media. (B) Negative result on nitrogen free media.

4.3.2. Phosphatase production assay

Some strains grow fast with little phosphatase activity while other strains grow slower, but have higher phosphatase activity. In figure 4.4, the positive and the negative results for phosphatase are shown. The positive strain produced a clear zone around the colony, whereas the negative strain produced no clear zone. The efficiency of the phosphatase of the different strains are shown in table 1. Strain H1C10 had the highest phosphatase efficiency of 440 compared to all the isolated strains.

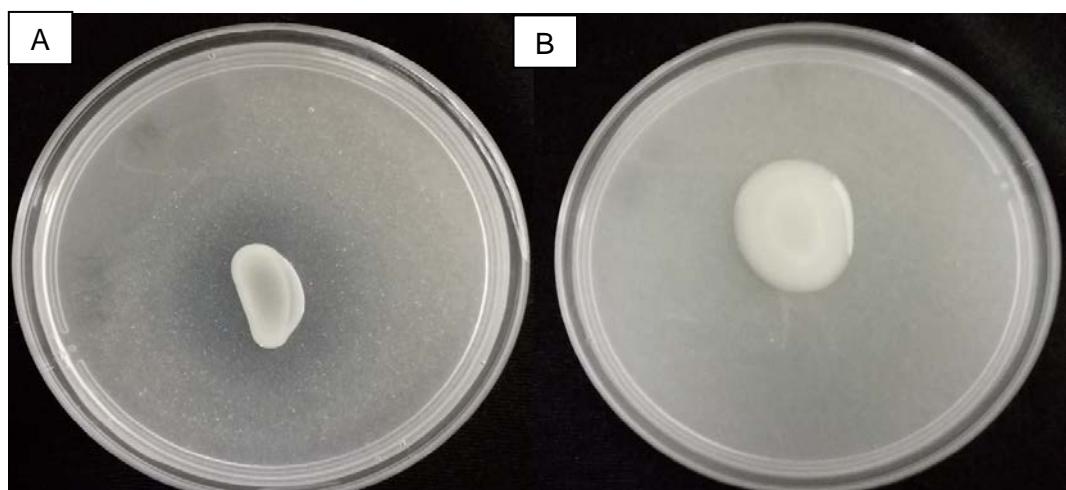


Figure 4.4. (A) Positive result for phosphatase activity. (B) Negative result for phosphatase activity.

4.3.3. Siderophore production assay

Most isolated strains were able to produce siderophores. Some strains were able to produce larger yellow zones than others (Table 4.1, Figure 4.5).

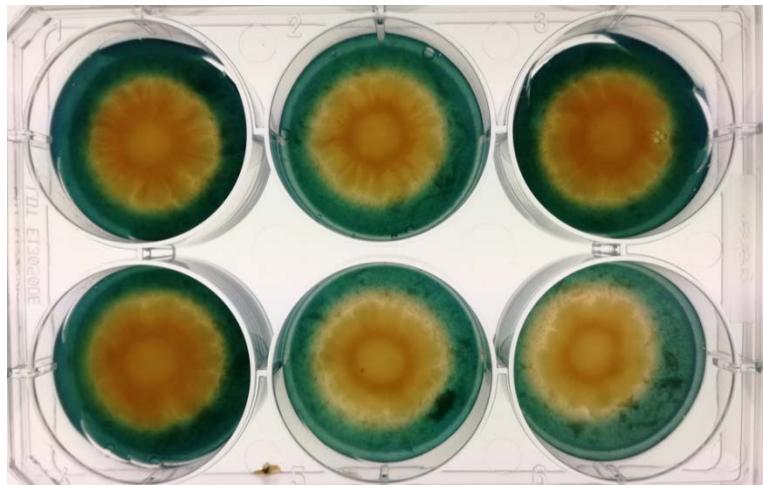


Figure 4.5. Siderophore production assay. The yellow zones around the colony indicates siderophore activity.

4.3.4. HCN production assay

Strain R2C1 could produce low quantities of HCN as the filter paper turned slightly orange and not completely orange as with strain R7W2 (Figure 4.6a).

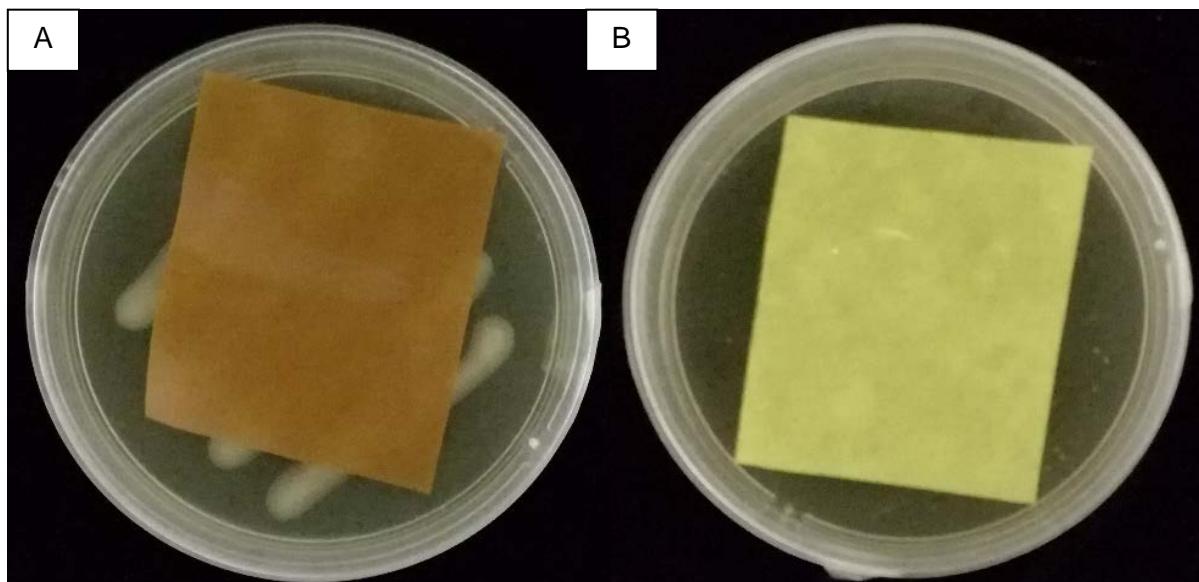


Figure 4.6. (A) Positive result for HCN production. (B) Negative result for HCN production.

4.3.5. ACC deaminase production

The results are shown in table 1. Positive and negative results are shown in figure 4.7. Some strains showed light growth on the plates without nitrogen. Most strains were able to grow on the plates containing $(\text{NH}_4)_2\text{SO}_4$ except for strain H1Cm1. Some of the strains were able to grow on the plates containing ACC. Strain H1Cl11 showed the best growth on the plates with only ACC as nitrogen source.

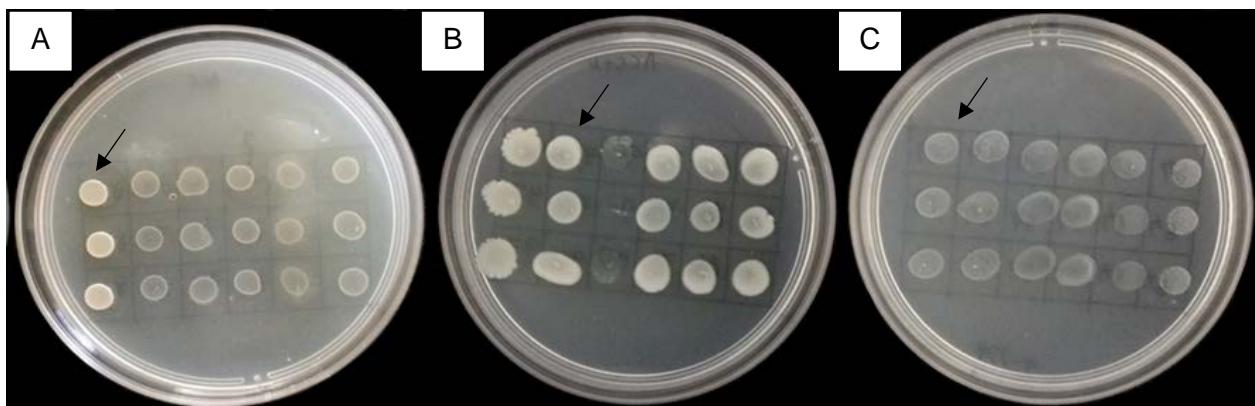


Figure 4.7. ACC deaminase assay. (A) The arrow indicates a positive result in the first column on media supplemented with ACC. (B) ACC media with $(\text{NH}_4)_2\text{SO}_4$ as a nitrogen source. Positive result is indicated with the arrow. (C) ACC media without nitrogen source. All the strains showed no growth as indicated by the arrow.

4.3.6. Indole acetic acid production

The amount of IAA produced vary from strain to strain. Non-rhizobial strains are also able to produce IAA and in many cases produced higher quantities of IAA. Strain R3C20 produced the highest concentration with $22.22 \mu\text{g.ml}^{-1}$.



Figure 4.8. IAA production assay of isolated strains. The arrows indicates the pink colour that is a positive result for the production of IAA.

Table 4.1. Plant growth-promoting compounds produced by isolated rhizobial strains

Strain number	Strain isolated								IAA (LB + tryp) (uM)	IAA (LB - tryp) (uM)
	from legume	Phosphatase activity	N-free	HCN production	Siderophore production	ACC-N	ACC+N	ACC		
R3C1.11	<i>A. linearis</i>	171,43	+	-	+++	-	+	+	6,67	1,11
H1Cs1.1	<i>C. subternata</i>	0	-	-	++	-	+	+	12,96	6,30
R3C1.10	<i>A. linearis</i>	0	+	-	+	-	+	+	2,96	0,37
R3C1.12	<i>A. linearis</i>	0	+	-	+++	-	+	+	1,48	0
R6W5.1	<i>A. linearis</i>	0	-	-	++	-	+	+	3,70	0,74
H1WCI2	<i>C. longifolia</i>	0	-	-	+++	-	+	+	3,70	0
R2C1.3	<i>A. linearis</i>	162,5	-	-	+++	-	+	+	7,78	4,81
H1WCI2.1	<i>C. longifolia</i>	100	-	-	++	-	+	+	0	0
R2C2.4	<i>A. linearis</i>	0	-	-	+++	-	+	+	5,19	0,74
R3C2.13	<i>A. linearis</i>	0	+	-	++	-	+	+	11,11	4,81
R3C2.14	<i>A. linearis</i>	126,67	+	-	+++	-	+	+	8,52	2,96
H2Cs1.1	<i>C. subternata</i>	123,53	+	-	+++	-	+	+	5,56	0
H1WCI2.2	<i>C. longifolia</i>	177,78	-	-	+++	-	+	+	7,78	0,37
H1WCI2.3	<i>C. longifolia</i>	0	-	-	+++	-	+	+	1,48	1,85
R3C1.4	<i>A. linearis</i>	0	+	-	+++	-	+	+	0	3,70
R3C2.15	<i>A. linearis</i>	0	-	-	++	-	+	+	2,22	0
R3C1.6	<i>A. linearis</i>	0	+	-	+++	-	+	+	4,44	0
H1Cs1.2	<i>C. subternata</i>	0	-	-	+++	-	+	+	7,78	0,74
H1Ci1.4	<i>C. intermedia</i>	0	-	-	+++	-	+	+	0	1,48
H1Cm1.3	<i>C. maculata</i>	0	-	-	++	-	+	+	7,41	0,74
H1Cm1.4	<i>C. maculata</i>	0	-	-	++	-	+	+	5,19	1,85
R1C1.1	<i>A. linearis</i>	0	-	-	+	-	+	+	5,93	2,22
R2C1	<i>A. linearis</i>	0	-	+	++	-	+	+	0	0,37
H1Cs1.3	<i>C. subternata</i>	0	-	-	++	-	+	+	7,41	1,85
R2C1.2	<i>A. linearis</i>	0	-	-	+++	-	+	+	5,19	1,11
R2C2.1	<i>A. linearis</i>	0	+	-	++	-	+	+	6,30	0
H1Ci3.1	<i>C. intermedia</i>	0	-	-	++	-	+	+	15,19	0
H2WCS1.2	<i>C. subternata</i>	0	+	-	+	-	+	+	5,93	0
R1C1.5	<i>A. linearis</i>	122,22	-	-	+	-	+	+	1,85	0
R3C3.3	<i>A. linearis</i>	113,64	+	-	+	-	+	+	2,59	0
R2C3.1	<i>A. linearis</i>	150	-	-	+	-	+	+	11,11	0
R7W1.1	<i>A. linearis</i>	130	-	-	+++	-	+	+	12,22	0
R2C1.6	<i>A. linearis</i>	119,05	-	-	+++	-	+	+	5,19	0
H1Ci1.1	<i>C. intermedia</i>	160	+	-	+	-	+	+	5,56	0
R3C1.8	<i>A. linearis</i>	129,41	-	-	+++	-	+	+	6,30	5,93
R1C1.2	<i>A. linearis</i>	383,33	-	-	+	-	+	+	4,07	1,48
H1Cm1.2	<i>C. maculata</i>	0	-	-	+++	-	+	+	5,93	5,93
H1Cm1.6	<i>C. maculata</i>	333,33	+	-	++	-	+	+	16,67	7,78
H1C12.1	<i>C. longifolia</i>	0	-	-	+	-	+	+	8,15	0
H1Cs3.1	<i>C. subternata</i>	115,79	-	-	+	+	+	+	9,26	0
H1Cm4.1	<i>C. maculata</i>	0	+	-	+	-	+	+	6,67	0
H1Cm4.2	<i>C. maculata</i>	350	-	-	++	-	+	+	5,56	0,74
H1Cs4.4	<i>C. subternata</i>	155,56	-	-	++	-	+	+	11,48	0
H1Cs4.1	<i>C. subternata</i>	337,5	-	-	+	-	+	+	12,59	0

R3C20	<i>A. linearis</i>	0	-	-	+++	-	+	+	22,22	2,59
R3C2.21	<i>A. linearis</i>	0	-	-	+++	-	+	+	2,96	4,44
R3C1.2	<i>A. linearis</i>	200	-	-	+	+	+	+++	7,04	4,07
H1Cm11.3	<i>C. maculata</i>	0	+	-	++	+	+	+++	5,93	5,93
H1Cm11.5	<i>C. maculata</i>	0	-	-	++	-	+	+	3,70	0
R6W3.4	<i>A. linearis</i>	100	-	-	+	+	+	+++	7,04	0
H1Cm13.3	<i>C. maculata</i>	0	-	-	+++	+	+	+++	11,85	0
H1Cm10.2	<i>C. maculata</i>	0	+	-	+	-	+	+	17,41	1,48
H1Cm10.3	<i>C. maculata</i>	0	+	-	+	-	+	+	10,74	0
R3C2	<i>A. linearis</i>	200	+	-	+	-	+	+	2,96	0
R6W1.1	<i>A. linearis</i>	0	-	-	+	-	+	+	5,93	0
R6W4.2	<i>A. linearis</i>	0	+	-	+	+	+	+++	8,52	3,33
H1Cl2.1	<i>C. longifolia</i>	180	+	-	-	+	+	+++	17,41	9,63
H1Cm10.1	<i>C. maculata</i>	0	+	-	++	-	+	+	0	9,63
H1Cm1.1	<i>C. maculata</i>	0	+	-	++	-	+	+	12,22	15,56
H1Cm1.5	<i>C. maculata</i>	0	+	-	+	-	+	+	0	4,81
H1Cm11.4	<i>C. maculata</i>	0	-	-	+	-	+	+	5,56	5,19
H2Cs3.4	<i>C. subternata</i>	120	-	-	++	-	+	+	7,04	8,15
R4C3.1	<i>A. linearis</i>	0	-	-	+	-	+	+	4,44	2,59
H1Cm1.4	<i>C. maculata</i>	100	-	-	++	-	+	+	22,96	7,04
H2Cs1.5	<i>C. subternata</i>	100	+	-	++	+	+	+++	3,70	4,81
R6W3.3	<i>A. linearis</i>	0	-	-	+++	-	+	+	0	2,59
R3C3.2	<i>A. linearis</i>	0	-	-	-	-	+	+	2,96	0
H1Cl1.2	<i>C. longifolia</i>	400	+	-	+	+	+	+++	1,11	2,96
R6W4.5	<i>A. linearis</i>	0	+	-	+	-	+	+++	2,22	9,63
H2Ci1.1	<i>C. intermedia</i>	0	+	-	+	-	+	-	0	0
H1Cm1	<i>C. maculata</i>	157,14	+	-	++	-	-	+	0,74	0
H1Cl10	<i>C. longifolia</i>	440	+	-	+	-	+	+	0,37	0,74
H1Cl1.1	<i>C. longifolia</i>	400	+	-	-	-	+	+	2,59	5,19
H1Cl1.3	<i>C. longifolia</i>	0	+	-	+++	-	+	+++	9,26	0,74
H2Cs1.2	<i>C. subternata</i>	0	+	-	+	-	+	+	5,93	2,96
H2Cs3	<i>C. subternata</i>	0	+	-	-	-	+	+	4,81	3,70
R7W2	<i>A. linearis</i>	200	-	+	+++	-	+	+++	0	0,74
R1C4.1	<i>A. linearis</i>	144,44	-	-	-	-	+	+	3,33	0,37
R2C3.4	<i>A. linearis</i>	162,5	+	-	+	-	+	+	0	0
R3C2.3	<i>A. linearis</i>	0	-	-	+	-	+	+		
H1Cm13.2	<i>C. maculata</i>	0	-	-	-	+	+	+++	7,41	5,19
R4W3	<i>A. linearis</i>	0	+	-	+	-	+	+	0,75	0,37
R5W2	<i>A. linearis</i>	0	+	-	-	+	+	+++	5,56	3,33
R6W1	<i>A. linearis</i>	140	-	-	+++	+	+	+++	0	2,59
R6W5.2	<i>A. linearis</i>	0	-	-	-	+	+	+++	4,07	1,48

The results of the nitrogen free assay and the HCN production assay are interpreted as follows: growth (+), no growth (-)

The results of the ACC deaminase and siderophore production are interpreted as follows: no growth / zone (-), growth / small zone (+), good growth / medium zone (++) , excellent growth / large zone (+++).

4.3.7. Preliminary results of the potting trial

All the rhizobial treatments resulted in the formation of root nodules. A root section with root nodules attached is shown in figure 4.9a. In figure 4.9b, a cross-section of a root nodule with the red leghaemoglobin is visible. All the treatments contained root nodules and had a red centre.

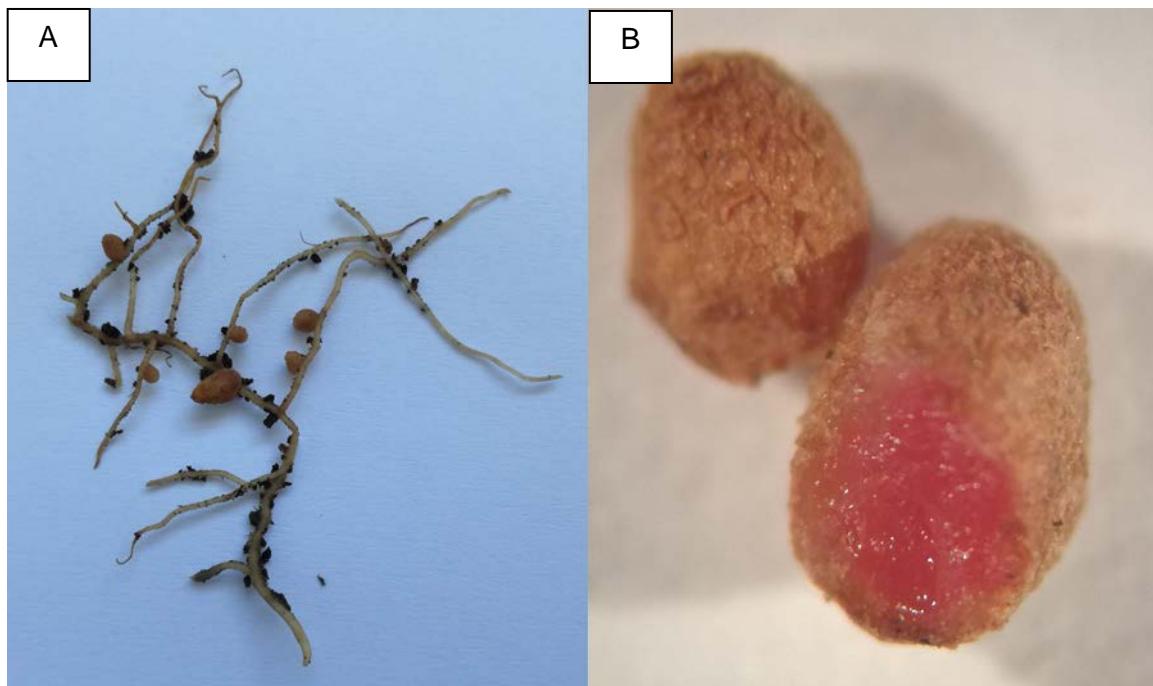


Figure 4.9. (A) Root nodules on the roots of one of the treated plants. (B) Cross-section of one of the root nodules.

The effect on growth by the treatments is shown in figure 4.10. All treatments showed a significant increase in plant growth, number of leaves formed and root development compared the uninoculated control plants. Plants treated with strains H1Cm1.4 and H1Cm1.6 showed the most plant growth, root development and number of leaves compared to the other treatments (Table 4.2).

30 cm





Figure 4.10. The treatments for the plants in this figure are as follows: (A) untreated control, (B) strain H1Cm1.6, (C) strain R7W2, (D) strain H1Cm1.4 and (E) strain H1Ci1.1.

Table 4.2. The effect of rhizobial treatments on plant growth.

Treatments	Shoot length (mm)	Root volume (mm)	Number of leaves	Dry weight (g)
control	115,67 ± 31,88	4,33 ± 1,15	55,67 ± 13,80	0,63 ± 0,26
H1Cm1.6	200 ± 26,46	17,5 ± 3,54	186 ± 25,46	5,67 ± 0,57
R7W2	196,67 ± 24,66	8,33 ± 2,89	102,67 ± 29,48	1,48 ± 0,63
H1Cm1.4	225 ± 42,72	17,5 ± 3,54	152 ± 30,51	5,64 ± 1,61
H1Ci1.1	175 ± 27,84	6,67 ± 2,89	86 ± 14,80	1,21 ± 0,01

4.4. Discussion

4.4.1 Nitrogen free assay

Studies have used nitrogen-free media to screen rhizobia isolated from root nodules for nitrogen fixation (Gerding et al., 2012; Kayasth et al., 2014; Li et al., 2008). The presence of the *nifH* gene in the rhizobial strains were confirmed with this assay as only strains capable of fixing nitrogen can grow on the nitrogen free media (Kayasth et al., 2014).

The nitrogen free media can be used to screen for nitrogen fixing bacteria from an environmental sample. There are sometimes other bacteria that associate with the rhizobia, but can be purified using the nitrogen free media instead of yeast mannitol agar (YMA) with congo red that is usually used to culture rhizobia. This method will be easier to isolate and purify rhizobia than just using YMA with congo red, as the nitrogen free media with the environmental sample will have less growth.

The nitrogen free assay differs from the ammonia production assay and can be used for screening rhizobia. Ammonia is the form in which the rhizobia supply the nitrogen to the plant (Chen et al., 2012). Other bacteria may also produce ammonia without fixing nitrogen, resulting in a false positive. Growth on the nitrogen free media is an indication that the rhizobia are able to fix nitrogen.

4.4.2 Phosphatase production assay

The phosphatase efficiency of the strains differed. The isolates that are able to fix nitrogen did not have high phosphatase activity compared to the non-nitrogen fixing strains. Useable phosphates in the soil are limiting, but a number of plants in the CFR including legumes such as rooibos are able to produce phosphatase to mobilize inorganic phosphate (Maseko and Dakora, 2013). Most agricultural crops do not produce their own phosphatases and by providing the soil with bacteria that uses phosphatases to make phosphates available to the plant can lower the costs of fertilization input.

4.4.3 Siderophore production assay

The majority of the isolates were able to produce siderophores as siderophores are crucial for survival of bacteria and without it, it will not be able to get the necessary iron for its metabolic functions. Rhizobia use siderophores to obtain the iron it requires for the nitrogenase iron protein to function optimally (Mishra et al., 2012). Another function of siderophores is an indirect mechanism of plant growth-promotion as the siderophores are used to prevent other organisms from obtaining iron from the environment. Some studies have linked high production of siderophores to antimicrobial function (Ahmad et al., 2008; Verma et al., 2013).

4.4.4 HCN production assay

Strain R7W2 (*Pseudomonas* sp.) was the only strain that produced HCN (Figure 4.6a) and strain R2C1 showed little production of HCN. The other strains were not able to produce HCN (Table 1). HCN inhibits growth of fungi as it affects the respiratory system (Verma et al., 2013). When HCN production is combined with siderophore production it showed antifungal activity (Ahmad et al.,

2008). The combination of HCN and siderophores can inhibit the growth of phytopathogens such as *Fusarium oxysporum* and *Rhizoctonia solani* (Verma et al., 2013). A strain that can inhibit the growth of common phytopathogens will be a good candidate in a microbial inoculant. Strain R7W2 and R2C1 still needs to be screened for antifungal activity.

4.4.5 ACC deaminase production

Strain H1CI11 (*Paraburkholderia* sp.) showed the best growth on the media supplemented with ACC (Figure 4.7a). Studies have shown that ACC deaminase promote plant growth as it breaks down the ACC into ammonia and α-ketobutyrate. ACC is a precursor for ethylene that is a known plant stress hormone (Glick, 2005). The *acdS* gene is linked to ACC deaminase production as rhizobial strains with a *acdS* gene mutation were impaired in their plant growth-promotion of tomato seedlings (Onofre-Lemus et al., 2009; Sun et al., 2009). Future studies should include screening rhizobial isolates for the *acdS* gene to determine that the plate assay result is not a false positive as it sometimes can be difficult to distinguish between a positive and a negative result.

4.4.6 IAA production assay

Strain R3C20 (*Phyllobacterium* sp.) produced the highest concentration of IAA from all the isolated strains. This strain was not able to grow on the nitrogen-free media and is not able to fix nitrogen. Strain R3C20 is not classified as rhizobia, but as a PGPB. This strain may have potential use in a microbial inoculum. Although some phytopathogens may also produce IAA, it is important to identify the strains and screening it in a potting trial before using it as an inoculum. HPLC can be used to quantify the IAA produced more accurately, but is more expensive than the colour metric assay if many samples need to be screened. By using the strains that produced IAA as an inoculant can lower the costs of spraying plant growth hormone. This will be more cost effective as the hormone will not leach into the soil and contaminate the surrounding soil.

4.4.7. Potting trial

All the rhizobial treatments, except the control, were able to form root nodules (Figure 4.9a). This confirms that the rhizobia contain the *nodA* gene that is involved in root nodule formation. The red colour on the inside of the sampled root nodule is an indication of leghaemoglobin that is found in efficient root nodules (Figure 4.9b). Leghaemoglobin is found in efficient root nodules and is necessary to lower the oxygen levels so that the nitrogenase enzyme is active. The nitrogenase enzyme is sensitive to atmospheric concentration of oxygen, and the nodules keep the oxygen concentration as low as possible (Terpolilli et al., 2012).

The root nodules were able to supply the plants with nitrogen and plant growth-promoting compounds as the plants treated with rhizobia showed a significant increase in plant growth and root development. Strains H1Cm1.6 (Figure 4.10a) and H1Cm1.4 (Figure 4.10d) showed the most growth compared to the other strains. These strains produced the highest quantities of the IAA from the selected strains. This may be the reason that these strains showed the most growth. The result of other plant growth-promoting properties may become visible during a field trial, when the plants are not in an artificial environment.

These results are very preliminary and the dry weight, the root and shoot length still needs to be determined. The rhizobia in the root nodules also need to be re-isolated and identified to confirm that the rhizobia in the root nodules are the same strain as the original treatment. Future studies should include a field trial on a honeybush farm to determine if the microbial inoculum will still have the same effect on plant growth.

4.5. Conclusion

The results showed that the plant growth-promoting compounds produced by the rhizobia are strain specific and varies in concentration. Correlation between the *nifH* gene and growth on the nitrogen free media is a method to isolate nitrogen fixing bacteria from the beginning. Studies suggest that there is also a correlation between siderophore and HCN production because of its antifungal properties. ACC deaminase is produced by the rhizobia to lower ethylene levels during infection of the root and to lower plant stress. The strains that produced IAA will have a positive effect on plant growth. The isolated strains can be used in combination to provide plants with a wide range of plant growth-promoting compounds. Further isolation and screening can be done to isolate organisms that produce more plant growth-promoting compounds. Future studies should include field trials to determine if these strains can be used as microbial inoculants in rooibos and honeybush nurseries. These strains can be inoculated with other plant species to determine if they will also improve plant growth.

4.6. References

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General conclusions and recommendations

General conclusions and recommendations

Rooibos and honeybush are leguminous plants that are endemic to the fynbos region of South Africa and are used for herbal tea production (Joubert et al., 2007). These plants host various rhizobial bacteria in symbiotic relationships by providing the bacteria with a carbon source while receiving nitrogen from the bacteria in return. The plants create a favourable environment for the rhizobia in the rhizosphere. The rhizobia then interact with the host plant through signalling molecules to form root nodules (Terpolilli et al., 2012). These bacteria play an important role in the ecology as they do not only fix nitrogen for the plant, but also produce plant growth-promoting compounds (Ashraf et al., 2013). The aim of this study is to determine the diversity of the nitrogen-fixing bacteria that associate with rooibos and honeybush plants by doing sequencing and community fingerprinting.

Microbial diversity can be used as an indicator for microbial soil health (Magurran, 1988). Automated Ribosomal Intergenic Spacer Analysis (ARISA) is useful technique to determine the diversity of a microbial community as it is not as expensive as high throughput sequencing, but more reliable than Denaturing Gradient Gel Electrophoreses (DGGE) and less labour intensive than Terminal Restriction Fragment Length polymorphism (T-RFLP) (Hartmann et al., 2005). Realtime Quantitative Polymerase Chain Reaction (qPCR) are also often used quantify the *nifH* gene of nitrogen fixing bacteria in soil (Hsu and Buckley, 2009; Zehr et al., 2003). Results obtained from ARISA showed a clear shift between the beta diversity of the bulk soil and rhizosphere soil of rooibos plants. The T-RFLP analysis of the *nifH* gene showed that the diversity of the free-living nitrogen fixing bacteria that associate with rooibos and honeybush plants differ. The rooibos plants have a lower diversity than the honeybush plants. No difference in beta diversity of the *nifH* were observed between the bulk and rhizosphere soil samples of rooibos and honeybush plants. There was no significant difference between the commercial and natural communities. Realtime Quantitative Polymerase Chain Reaction (qPCR) was used to determine the copy numbers of the *nifH* gene in the bulk and rhizosphere soil of rooibos and honeybush plants. There was a significant difference in copy number between the bulk and rhizosphere soil of both plants, although no significant difference in diversity between bulk soil and rhizosphere soil were detected. The quantification of the *nifH* gene in soil can be a useful tool to quantify the abundance of organisms that can fix nitrogen in the soil. This determines the nitrogen fixing capacity of the bacteria in the soil.

The rhizobia isolated from the root nodules of rooibos and honeybush plants are useful to determine the rhizobial species that the plants select for to form root nodules. This study found that honeybush prefers to form root nodules with beta-proteobacteria, while rooibos prefers alpha-proteobacteria. These results are supported by Elliott et al. (2007) and Kock (2004). These isolated strains were then used to screen for plant growth-promoting properties.

The methods in this research can be used to screen the rhizobia before potting and field trials to determine if the isolates will improve plant growth. By using high performance liquid chromatography (HPLC), plant growth-promoting compounds can be accurately quantified. Some isolates from this study produced increased amounts of plant growth-promoting compounds compared to the other isolates from this study. The strains that produced the highest quantities of plant growth-promoting compounds can be used in a potting trial to determine their effect on plant growth. These strains can then be used in nurseries to inoculate seedlings before planting them out into the field.

The key results in this study showed that there is no significant difference in the diversity of the free-living nitrogen fixing bacteria between the bulk and rhizosphere of rooibos and honeybush plants, but there was a higher copy number of the *nifH* gene present in the rhizosphere soil than the bulk soil in certain soil samples. This could indicate that the rhizosphere has a higher microbial biomass for nitrogen fixing bacteria for these samples, but edaphic may have had an influence on the other samples. Some of the rhizobial root nodule isolates may be novel species as they did not group phylogenetically with any known isolates from the GenBank database. This indicates a high diversity of rhizobia with some species that is still unknown. The screening of the isolated rhizobia showed that they also produce a variety of plant growth-promoting compounds that can be beneficial for plant growth.

Recommendations include sampling over seasons and a wider sampling area. Soil chemical analysis can be done to determine correlations with abiotic factors and the diversity of the nitrogen fixing bacteria. A trial with different rhizobial treatments can be done to determine the effect of the treatment on the diversity of the diazotrophic community. By using RNA from soil microorganisms, the actively expressed genes can be analysed and will provide the diversity of the active diazotrophs and the amount of nitrogen fixed by diazotrophs in the soil. This can be used to determine the number of active nitrogen fixing bacteria and can be useful when comparing different farming practices. T-RFLP and qPCR can also be used to determine the diversity and to quantify other functional genes in addition to the *nifH* gene such as phosphatase. Nitrogen free media can be used for isolations of rhizobia from root nodules. This method isolates rhizobia and screens for nitrogen fixing capability simultaneously. Whole genome sequencing can be done instead of sequencing the 16S rRNA, *recA*, *atpD*, *nifH* and *nodA* genes separately. Although it is more expensive, other gene sequences can be accessed as well and can be used to determine other gene functions of the bacteria. Future research could also focus on isolating, identifying, and screening more rhizobial isolates for plant growth-promoting properties for the use of microbial inoculants.

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