Vachellia erioloba (camel thorn) and microbial interactions

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Abstract

Vachellia erioloba (camel thorn) is one of South Africa's economically important tree species and therefore requires further investigation to improve its health and growth. Beneficial soil microbes have positive effects on plants through various mechanisms such as nitrogen fixation, phosphate solubilisation, indole acetic acid and siderophore production and biofilm formation. These traits enhance plant growth and protect the host plant against parasitic organisms that are present in soil. The arbuscular mycorrhizal (AM) fungi are well known for their beneficial symbiotic effects on host plants. The objective of this study was to determine the role of AM fungi and associated beneficial rhizobacteria in improving the growth of *V. erioloba* seedlings. Soil and root samples were collected from a farm in the Northern Cape, South Africa. Fifty-seven bacterial cultures were isolated from the soil and tested for plant growth promoting characteristics. Fourteen isolates showing at least four beneficial traits were molecularly identified using the GenBank database. The AM fungal and bacterial populations in the soil samples were assessed using Illumina sequencing. Sequences were identified using the MaarJAM and GenBank databases, respectively. Three separate pot trials were conducted to determine; 1) the effects of cadmium (Cd) on seedling growth; 2) the individual effects of three selected bacterial isolates and AM fungi alone and combined on seedling growth, and 3) the combined effects of the selected bacteria on AM fungal inoculated and uninoculated seedlings. Of the fourteen isolates the *Enterobacter* genera was the dominant species identified, with *Acinetobacter*, *Pantoea* and *Bacillus* each having one isolate. All were described as plant growth promoting rhizobacteria. One isolate from each genus, excluding *Pantoea*, was used in the pot trials. Three genera were identified in the AM fungal population that was assessed, namely *Ambispora*, *Paraglomus* and *Glomus* with *Ambispora* being the dominant genus. The bacterial population assessed showed a high diversity of bacteria from the Actinobacteria phylum being the dominant group. The results of the heavy metal pot trial showed that the symbiotic relationship between the seedlings and AM fungi increased the seedlings' health and growth during heavy metal stress. The combination of bacteria and AM fungi increased growth parameters in all the inoculated seedlings, but not when compared to uninoculated seedlings indicating a possible competition for

nutrients. The results were influenced by the presence of a nematode, which was suspected to have been seed borne. Further investigations on these interactions are required. Inoculation of AM fungi and selected PGPR is recommended for *V. erioloba* seedling production.

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Chapter 1

Introduction

1.1 The Camel thorn tree

The Camel thorn tree, *Acacia erioloba*, is a tree that is native to the southern part of Africa (Orwa *et al.*, 2009). The genus *Acacia* is part of the Mimosoideae subfamily and in recent years the genus has become too large and reclassification of some species was needed. It has been estimated that more than 900 *Acacia* species are found in Australia, whereas in South Africa there are 100 *Acacia* species (Dlamini and Sisulu, 2005). In order to distinguish between Australian and African *Acacia* species, the African *Acacia* species were reclassified into the *Vachellia* and *Senegalia* genera. In 2008 *Acacia erioloba* was reclassified as *Vachellia erioloba* by P.J.H. Hurter and is internationally accepted as part of this genus (Kyalangalilwa *et al.*, 2013).

The Camel thorn tree forms part of two natural forests (Figure 1.1) situated near the town of Kathu in the Northern Cape (Powell, 2005). It is an indigenous tree of South Africa and is very important in semi-arid environments (Seymour and Milton, 2003). The benefits derived from the Camel thorn tree are only apparent when the tree has grown to a larger size (Barnes, 2001). The tree grows in sandy soil with a deep root system, penetrating up to 60 meters enabling access to groundwater along with dissolved nutrients (Barnes, 2001; Dlamini and Sisulu, 2005). The Camel thorn is a slow grower and natural regeneration is limited. These trees provide shade and shelter for livestock, birds and other animals, and the animals leave their dropping behind which provide nutrients for the tree (Barnes, 2001).

The Camel thorn tree is also economically important (Seymour and Milton, 2003). The wood of *V. erioloba* is very hard, heavy and durable, which is used for fence

posts, fuel wood for mining, and firewood (Barnes *et al.*, 1997). The tree gum is edible and high in protein and was used by the Koi-san people as a source of food. The seed pods, flowers, and young shoots are relished by livestock and wild animals because of their high nutritional value (Barnes *et al.*, 1997).

Since the early 1990s a decline in the Camel thorn population was observed and no cause could be identified. In 2008 the cause of the decline was investigated by the Centre for Tree Health Biotechnology, which found that Cerambycidae beetles and fungi were observed on dying trees (Internet 1). The beetle attacks and the removal of the trees due to mining and building processes, have contributed to the decline in the population. Seed pods are also removed and sold as fodder. Additionally, mining activities have led to the concern of heavy metal contamination and depletion of the groundwater table (Mans, 2011).

Figure 1.1: The Camel thorn forests located near Kathu (Mans, 2011).

During a rhizobial study Barnes and colleagues (1997) suggested that inoculating the tree with appropriate rhizobial strains could aid their development in the changing environment. In their study, they inoculated six *Acacia* species, including *Acacia erioloba* (from here on referred to as *Vachellia erioloba*), with different *Rhizobium* strains to test the competitiveness and effectiveness of the different strains. They

found that the *V. erioloba* seedlings were the only specie that did not nodulate under natural conditions (Barnes *et al.*, 1997; Barnes, 2001). They accessed *V. erioloba*'s ability to fix nitrogen by analysing the percentage leaf nitrogen and using the N^{15} method. Their results concluded that it obtained most of its nitrogen from groundwater, bringing it back into circulation on the surface, which might explain the rarity of nodules (Barnes *et al.*, 1997).

1.2 Mycorrhizal fungi

Most terrestrial plants are associated with mycorrhizal fungi, which form a symbiotic relationship with the roots of a plant host (Dell, 2002). Mycorrhizal fungi enable plants to overcome nutrient limitation, which can help to enhance growth and establishment of the plant (Dell, 2002). Different types of mycorrhizal associations can be formed, which differ in structure, involving different groups of fungi and host plants (Ruiz-Lozano and Azcón, 1995). There are two main plant associations with mycorrhizal fungi, namely ectomycorrhizas and endomycorrhizas (Dell, 2002). Ectomycorrhizas are more restricted in their host selection and form associations with mainly woody plants, whereas endomycorrhizas have a very broad host range. A specific group of endomycorrhizal fungi are relevant to this study.

1.2.1 Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal (AM) fungi are a group of endomycorrhizal fungi that naturally occur in different soil types (Ruiz-Lozano and Azcón, 1995). AM fungal symbiotic relationships with host plants are one of the most common interactions occurring with more than 80% of all terrestrial plants (Harrier, 2001). AM fungi belong to the class Glomeromycetes in the phylum Glomeromycota (Schüßler and Walker, 2010). There are four orders in which AM fungi are placed namely Archaeosporales, Diversisporales, Glomerales, and Paraglomerales, which comprise of thirteen genera. A total of 270 species have been described and identified, which is estimated to be less than 5% of the world's existing species (Krüger *et al.*, 2009; Schüßler and Walker, 2010).

AM fungal species are obligate biotrophs and need a photoautotrophic partner in order to complete its life cycle (Parniske, 2008). One plant host can be colonised by different AM fungal species within a single root. The AM fungal symbiosis shows little host specificity with colonisation of a diverse range of plants taking place. Being an endomycorrhizal association indicates the specific fungal structures are recognised within roots and root cells. The most characteristic feature of the AM association is the formation of tree-like hyphal branching that develops in the folds of the plasma membrane of root cells (Parniske, 2008). These intracellular structures are supported by a network of intercellular hyphae which run between cortical cells. Storage vesicles may also be present in roots. The fungal hyphae exit the root and extend into the surrounding soil. This extraradical network makes it possible for the fungus to access more nutrients and water from the soil environment. The colonisation of the AM fungi promotes the growth and development of the plant host and also enhances the plant's resistance against biotic and abiotic stresses (Harrier, 2001).

1.2.2 Development of arbuscular mycorrhizal fungi

There are several changes that take place in the AM fungal cycle, which includes: spore germination, hyphal differentiation, appressorium formation, root penetration, intercellular growth, arbuscule formation and nutrient uptake (Harrier, 2001). The morphological changes that AM fungi undergo before and after colonisation can be classified as host-dependant or host-independent (Harrier, 2001).

The spores of AM fungi can be seen as the beginning of the life cycle as well as the product of AM fungi. AM spores are formed during a process known as sporulation by the branched hyphae in soil (Bianciotto *et al.*, 2011). During sporulation, spore primordiums are formed by the intercalary swelling of the double-walled hyphae (Marleau *et al.*, 2011). After approximately 15 days the developing spores have subtending hyphae which are still attached to a septum of the mother hyphae. After 30 days differentiation of the spore wall becomes apparent. Fully developed AM fungal spores contain lipids, cytoplasm and a large number of haploid nuclei

(Giovannetti *et al.*, 2010). When the spores have reached maturity they will be ready to germinate. Although molecular techniques are the best way to identify AM fungal species, spores can also be identified by their morphological features although their characteristics are limited (Giovannetti *et al.*, 2010).

AM fungal spore germination can take place in three ways: (1) development of a germ tube from specific spore structures called germination shields, (2) germ tube growth through the spore wall and (3) regrowth of hyphae through the subtending hyphal attachments (Harrier, 2001). Compounds released from the root system into the rhizosphere are known as plant root exudates (Bücking *et al.*, 2008) and include enzymes, primary and secondary organic metabolites which contain flavonoids and polysaccharides. Components of these root exudates have been found to aid the development of AM fungi and ensure that the symbiotic relationship is established (Bücking *et al.*, 2008).

The presence of root exudates influences the hyphal development and branching during the pre-symbiotic stage (Singh and Prakash, 2012). Branching occurs in different directions, until it is in close proximity to the plant and then branching concentrates towards the plant root. Hyphal development can be 20 times slower when a host plant is not present and can cease if a host is not detected (Singh and Prakash, 2012). As the hyphae grow towards the root it encounters plant signals that are present in the root exudates. Signal molecules such as strigolactones stimulate branching towards the plant and increases the probability of direct contact between the fungi and plant host (Singh and Prakash, 2012; Foo *et al.*, 2013).

As the branching hyphae approach the host's root AM fungal exudates, such as signaling Myc factors, are released before penetration. These factors are identified by Myc factor receptors on the root surface, which enables the hyphae to penetrate through the root. This interaction causes a physical change in both the hyphae and root. The root cells start preparing an intracellular environment for the AM fungal hyphae. When finding an appropriate penetration site, the hyphae swells, flattens

and branches repeatedly leading to the development of the appressorium or infection cushion (Singh and Prakash, 2012).

Genre *et al.* (2005) found that plant cells develop a pre-penetration apparatus (PPA), formed through different chemical and mechanical stimuli just before the appressoria penetrate the epidermal cells. The PPA is seen as a cytoplasmic column that contains microfilaments and microtubule bundles, dense endoplasmic reticulum cisternae and a central membrane thread (Genre *et al.*, 2005). The fungi can only enter the cells after the column has been formed. Nagahashi and Douds (1997) found that appressoria form only in epidermal cells of roots and not on vascular or cortical cells. This might indicate that epidermal cells release biochemical signals that help hyphae recognise the penetration site and triggers appressorium development. It has also been observed that the cell wall of the epidermal cells thickens as the appressorium approaches the point of penetration. Even so, the thickened wall does not prevent the appressorium from penetrating the cell. As the appressorium penetrates the cell wall it bulges, indicating that mechanical force is applied (Freitag *et al.*, 2011).

As the intercellular space between cells is colonised by the appressorium changes take place in the middle lamella structure, indicating the involvement of fungal enzymes such as pectinases (Smith and Read, 1997). When the appressorium branch penetrates the cell wall it forms the trunk of the arbuscule. The plasma membrane is not penetrated but grows around the branching arbuscule. The arbuscule dichotomise repeatedly and the fungi are always outside the cytoplasm of the plant cell but in an apoplastic compartment. The peri-arbuscular membrane (PAM) that surrounds the arbuscules facilitates the transfer of nutrients, molecules and ions between the plant and its AM fungal partner (Smith and Read, 1997).

There are two types of AM fungal colonisation structures that can be formed in plants, namely *Arum* type and *Paris* type (Dickson, 2004). The *Arum* type is the most common type of colonisation that can be found in cultivated plants, which consist of

intercellular hyphae and arbuscules (Fig. 2-A). The *Paris* type (Fig. 2-B) is found in trees and forest herbs and forms intracellular hyphae, arbusculate coils and coils (Dickson, 2004). The *Paris* type grows slower than the *Arum* type and is also more compacted and dense (Smith and Read, 2008). Vesicles can be formed by both types as the colonisation ages, but AM fungal species from Gigasporaceae develop auxiliary cells (Smith and Read, 2008).

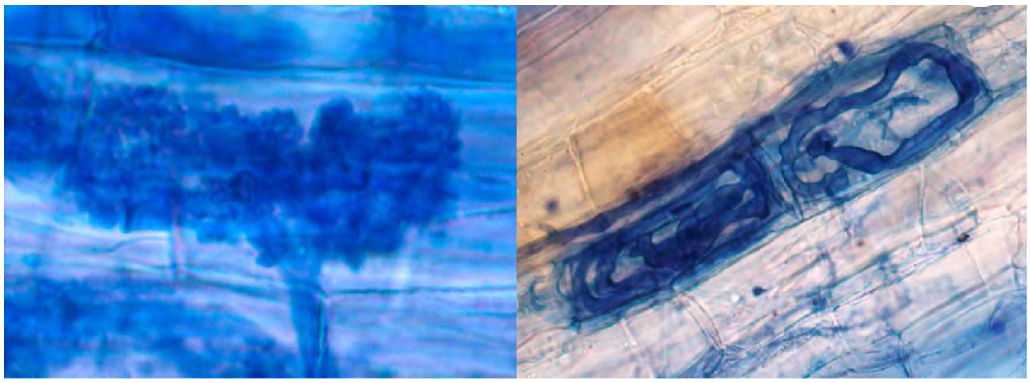


Figure 1.2: Micrographs of cells infected by AM fungi. (A) *Arum* type - trypan blue stained intracellular arbuscules. (B) *Paris* type - Hyphal coils in root cells. (De Vega *et al.*, 2011).

Smith and Smith (1997) found that some AM fungal families have either intermediate or both morphologies. Intermediate morphologies were assigned when the *Arum* and *Paris* type were found in the same plant species (Smith and Smith, 1997). Both morphologies were assigned when both types were observed in different AM fungal species of genera within a family. However, the formation of the two types depends on the plant host (Smith and Read, 2008).

The branching of arbuscules increases the contact surface area, making it easier for nutrient transfer between the host plant and the AM fungi. After a short time period the arbuscules reach maturity and start to progressively degenerate (Smith and Read, 1997). During degeneration the cytoplasm in the arbuscules are retracted and a septa forms, which separate the degrading arbuscule from the rest of the hyphae

(Javot *et al.*, 2011). As the arbuscule collapse it forms an amorphous clump and gradually disappears from the cortical cell, which remains undisturbed (Javot *et al.*, 2011).

After successful root penetration the external hyphae (extraradical hyphae) in the soil will start to grow more rapidly (Smith and Read, 1997). These hyphae aid nutrient uptake and transport to the plant exchanging these nutrients in the arbuscules for photosynthetically derived carbohydrates. The extraradical hyphae are very important for spore production, and for the translocation of carbohydrates into the spores. In some fungi-plant symbiosis an increase in spore production can be seen as the plant matures, while in other symbiotic relationships a decrease in spore production is observed as the plant matures (Smith and Read, 1997).

1.2.3 Benefits of arbuscular mycorrhizal fungi

1.2.3.1 Water acquisition

The symbiotic relationship with AM fungi has been shown to increase a plant's tolerance to drought conditions (Zhao *et al.*, 2015). This symbiotic trait is able to alter the rate and movement of water in, through and out of the plant, which can affect tissue hydration and physiology of the plant (Augé, 2001). Different mechanisms have been ascribed to this beneficial result of symbiosis. These mechanisms include direct water uptake through fungal hyphae, aquaporin regulation, improving hydraulic conductivity, increasing transpiration rate and leaf elasticity, lowering stomatal resistance and increased rooting depth and length (Augé, 2001; Khalvati *et al.*, 2005).

The direct uptake and transfer of water through the hyphae to the plant is considered to be one of the important mechanisms (Khalvati *et al.*, 2005). The hyphae penetrate the soil pores that are inaccessible to root hairs and absorb water that is unavailable to the plant (Khalvati *et al.*, 2005). Allen (1991) estimated the rate at which water was transported from the external hyphae to the roots to be 100 nanoliters water per

hour per hyphal infection point (Khalvati *et al.*, 2005). This was considered to be sufficient to change the plant water relations. Others based their predictions on the rate of water uptake by the number of hyphal entry points per root length unit, water potential gradient, hyphal cross-sectional areas and suggested that the water transport rates by hyphae were insignificant (Khalvati *et al.*, 2005).

The conflict between the studies may be due to a lack of a reliable split-root-hyphal system, which separates the water uptake contribution of the fungal hyphae and plant roots. The two-compartment system that Khalvati and colleagues (2005) made helped to minimize non-hyphal water transport between chambers. With this system they concluded that AM fungi do enhance drought tolerance, but also found that an increased water uptake in roots can be associated with leaf water relations, stomatal conductance, leaf growth, and net photosynthetic rate (Khalvati *et al.*, 2005).

Aquaporin regulation is another mechanism that is used by AM fungi to regulate water uptake (Aroca *et al.*, 2007). Aquaporins are transmembrane proteins that channel the passive movement of water molecules along a water potential gradient (Kruse *et al.*, 2006). Aquaporins transport other molecules such as glycerol, CO_2 and ammonium. These molecules can be divided into 5 subfamilies, including nodulin26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs), X intrinsic proteins (XIPs), plasma membrane intrinsic proteins (PIPs) and tonoplast intrinsic proteins (TIPs). The PIPs and TIPs play an important role in regulating the hydraulic conductivity of the root, and osmoregulating the cytoplasm of cells (Luu and Maurel, 2005). In order for a plant to increase its water uptake, the aquaporin genes must be upregulated to cope with the increased rate of the transcellular water flow (Javot and Maurel, 2002). Aroca *et al.* (2007) found that AM fungi help regulate the gene expression of aquaporins when the plant is under stress. It was also demonstrated that AM fungi are able to regulate the hydraulic properties of a host plant's root, which is linked to the regulation of the plant's aquaporins (Ruíz-Lozano and Aroca, 2010).

Subranmanian *et al.* (2006) found that mycorrhizal inoculated plants show more resistance to dry conditions than non-inoculated plants. AM fungi inoculated plants maintained a higher leaf water potential and transpiration rate, greater root surface area and proliferation and more effective root length (Subranmanian *et al.*, 2006). They found that as the drought stress continued, the stomata gradually closed, leading to a decrease in stomatal resistance. Even with the stomata closing they stayed open longer than the non-mycorrhizal plants, which allowed for the fixation of CO_2 more effectively leading to higher sugar levels in inoculated plants (Subranmanian *et al.*, 2006).

Subranmanian *et al.* (2006) suggested that the lower stomatal resistance could have been due to the increased sugar and amino acid levels in the plant. A higher green leaf area was also maintained during drought stress, which could be due to an enhanced nitrogen acquisition through the external hyphal transport of NO_3^- (Subranmanian *et al.*, 2006). The higher green leaf area maintained a higher photosynthetic rate during drought stress. Their study concluded that plants inoculated with AM fungi have higher tolerance under drought stress conditions and that an increased water uptake rate can aid nutrient uptake (Subranmanian *et al.*, 2006).

1.2.3.2 Nutrient acquisition

AM fungi have a significant impact on the plant host and can aid in nutrient uptake and plant health under normal and stressful growth conditions. There are two pathways that can be used by plants in order to acquire nutrients, namely the direct pathway (DP) and mycorrhizal pathway (MP) (Smith *et al.*, 2011). The direct pathway is where nutrients are taken up by phosphorus (P) and nitrogen (N) transporters located in the root epidermis and root hairs at the soil-root interface. The mycorrhizal pathway takes up nutrients at the fungal-soil interface by extraradical mycelium (ERM). The nutrients are translocated to the intraradical mycelium (IRM) and taken up by the P and N transporters in the peri-arbuscular membrane at the fungal-plant interface of the plant host (Harrison *et al.*, 2002; Guether *et al.*, 2009).

When the root is colonised by mycorrhizal fungi the DP nutrient transporters are down regulated due to the MP (Fellbaum *et al.*, 2012). The MP inducible transporters are expressed at the fungal-soil interface instead. The ERM situated at the fungal-soil interface take up inorganic N and convert it into arginine (Arg) via argininosuccinate synthase (ASS), glutamine synthetase (GS), carbamyol-phosphate synthase (CPS) glutamine chain and argininosuccinate lyase (AL). Arg is a basic amino acid that acts as a charge balance and is transported to the IRM along with negatively charged polyphosphates (polyP) that are synthesised within the ERM from P that was taken up from the soil (Fellbaum *et al.*, 2012).

The chemical composition of poly-P is restructured in the IRM and is released as inorganic phosphate (P_i) and Arg. The P_i and Arg is then converted into NH_4 by means of the urea cycle's catabolic arm along with urease and the activity of fungal arginase. The NH_4 molecules are then transported into the plant and are used for the different processes. In order for the mycorrhizal fungi to take up the nutrients from the surrounding environment and convert it, interactions with other bacteria in the soil are needed to optimise the processes (Fellbaum *et al.*, 2012).

In return for the nutrient acquisition from the AM fungi the plant host supplies the AM fungi with carbon (C) that is stored by the fungi and used for AM fungal growth and development (Wang *et al.*, 2015). The plants that are associated with AM fungi increase their photosynthetic rates in order to maintain the fungal-host interaction. The C cost of the interactioin is considerably high, up to 20% photosynthetically fixed C (Ashmelash *et al.*, 2016). When C is taken up by the AM fungi during colonisation the plant's C reserves are drained, which is used for fungal structure development and spore production. The plant host is only able to replenish its C reserves when colonisation has reached its plateau. If the AM fungi exceed the cost of the nutritional benefit, a negative growth response can occur where the AM fungi and plant host have decreased growth until the C reserves are replenished (Trouvelot *et al.*, 2015). Thus, plant development and C reserve mobilisation can significantly affect AM fungi and a balance must be maintained (Trouvelot *et al.*, 2015).

1.3 Beneficial microorganisms

Mycorrhizal fungi, being soil inhabitants, are associated with soil microbes that are beneficial to the fungi and the host plant (Miransari, 2010). Most of these interactions between soil microbes and mycorrhizal fungi have been described as synergistic and plant growth promoting (Fig. 1.3). Such interactions can lead to the modification of soil structural properties and enhanced nutrient availability (Miransari, 2010). Thus, understanding the interaction between mycorrhizal fungi, soil bacteria, and the plant host can lead to important implications in ecology and agriculture (Miransari, 2010). The recognition of different interactions between AM fungi and soil microbes can lead to the identification of genes that contribute to soil production (Barea *et al*., 2005). There are different soil microbes that interact with each other that can influence soil properties, thus influencing plant health and microbe activity (Zaidi *et al*., 2003). These bacteria include rhizobacteria in the soil and bacteria in the cytoplasm of some fungal species (Fig. 1.3) (Bonfante, 2003).

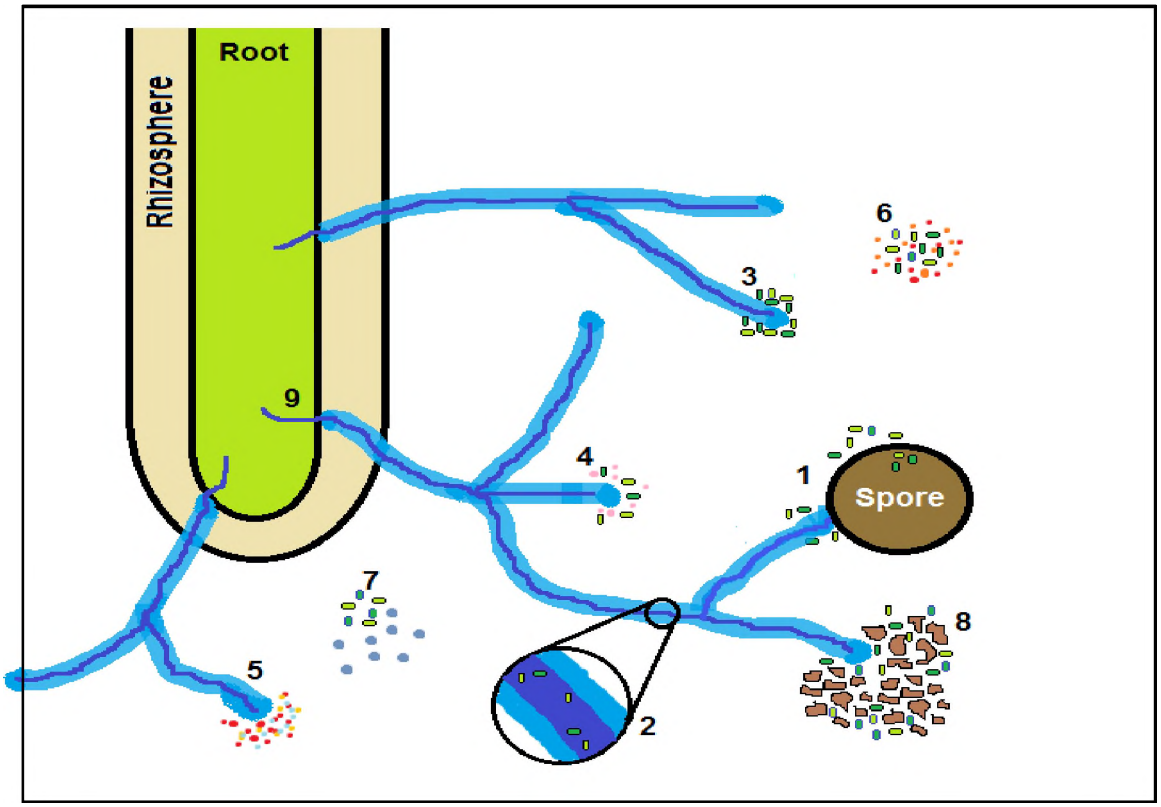


Figure 1.3: Schematic illustration of the interactions between rhizospheric bacteria and AM fungi. (1) Spore associated bacteria found in and around the spore. (2) Bacteria-like organisms (BLOs) move into the germinating hyphae as it emerges

from the spore. (3) Bacteria promoting the growth of hyphae, germination of fungal propagules and survival of hyphae. (4) Bacteria improving fungal nutrition by growth factor production and in return, the fungi secrete exudates that are nutrients for the bacteria. (5) Nutrients, minerals, and water are collected by the bacteria and transported to the hyphae or plant. (6) Nutrients, minerals, and water that are present in the soil are mobilised by the AM fungi, which is transferred to the roots. (7) Plant growth promoting rhizobacteria (PGPR) using direct and indirect mechanisms to increase plant growth. (8) Chelated soil aggregates that are broken down by bacteria and transferred to the hyphae. (9) Bacteria present in the hyphae travel into the roots and alter the architecture of the roots (Adapted from Frey-Klett and Garbaye, 2005; Frey-Klett *et al.*, 2007; Yan-de *et al.*, 2007; Bonfante and Anca, 2009).

There are different factors that can influence the interactions between AM fungi and other soil microbes (Artursson *et al.*, 2006). For example, the bacteria's ability to attach to the AM fungal hyphae differs between species and can be affected by the hyphae's physiological stage. Other enhanced associations include root colonisation by AM fungi, phosphate solubilisation, pathogenic suppression and germination and growth of spores and AM fungal hyphae, respectively (Artursson *et al.*, 2006). There are two groups, broadly referred to as mycorrhizal helper bacteria (MHB) and plant growth promoting rhizobacteria (PGPR), in which the microbes are placed according to their function and ability to interact with AM fungi and their host plant (Miransari, 2010).

1.3.1 Mycorrhizal helper bacteria

Mycorrhizal helper bacteria (MHB) are rhizobacteria that increase the ability of AM fungi to establish a relationship with the plant host (Garbaye, 1994). MHB can be categorised as: (1) bacteria that directly influences the formation of mycorrhizal fungi and (2) the bacteria that positively affect the mycorrhizal fungi that have already established their symbiosis (Rigamonte *et al.*, 2010). Even though there are different or overlapping microbial groups that can represent the two categories, both are

described as MHB. These bacteria are mainly closely associated with mycorrhizal fungi because they are fungal-specific but are not plant-specific (Garbaye, 1994). Some signaling pathways of fungal species have been found to be mutually regulated by different rhizobacteria, whereas other pathways are regulated by specific MHB (Rigamonte *et al.*, 2010).

Some MHB form an endosymbiotic relationship with AM fungi, where the bacteria live inside the mycorrhizal fungus (Toljander, 2006). Bacteria-like organisms (BLOs) have been found in the cytoplasm of some AM fungal species (Cruz, 2004; Lumini *et al.*, 2007), which can affect spore germination, colonisation and hyphal growth (Horii and Ishii, 2006; Horii *et al.*, 2008). These bacteria are also described as spore-associated bacteria (SAB) that are found in and around the spore and can also be attached to the germinating hyphae (Gopal *et al.*, 2012). During the early stages of hyphae development, the SAB protect the fungi from soil pathogens and transfers nutrients to help the fungi grow (Gopal *et al.*, 2012). These species are very hard to identify because most of them are unculturable. Technology has allowed us to identify most uncultivable organisms by combining molecular and morphological techniques (Bianciotto *et al.*, 2000).

These BLOs are vertically transmitted between fungal generations, which suggest that they produce endocellular components that are needed by the AM fungi (Bianciotto *et al.*, 2004). These bacteria seem to move into the germinating hyphae as it emerges from the spore (Tojander, 2006). The bacteria inside the hyphae enable the transfer of nutrients and minerals that have been collected by the hyphae and transfer it to the plant host. These bacteria also detoxify, removing unwanted molecules from the hyphae, releasing them back into the rhizosphere (Frey-Klett *et al*, 2007). Endocellular bacteria have relatively small genomes, which is common in bacteria permanently living within their host (Jargeat *et al.*, 2004).

Several MHB beneficial mechanisms have been proposed for example: (1) Producing endoglucanase and hydrolase enzymes that softens the middle lamella

and cell wall between the root cells, facilitating fungal penetration, (2) interfacing with the recognition mechanism between the mycorrhizal fungi and plant host by attaching either to the root or fungi, which improves cell wall properties and or facilitates the symbiosis establishment, (3) assisting the mycorrhizal fungi during the asymbiotic life stages and (4) through metabolic activity modifying the physico-chemical properties of the soil, which assists mycorrhizal colonisation (Garbaye, 1994).

There are also other mechanisms that have been described where MHB affect mycorrhizal fungi at different stages of fungal development (Kannan *et al.*, 2011). For example, MHB can enhance mycelial growth and spore germination by the production of growth factors or through the inhibition and or detoxification of antagonistic substrates and competitors. Previous studies found that the spores of many AM fungal species are not able to germinate without direct contact with bacteria (Frey-Klett *et al.*, 2007). Xavier and Germida (2003) found that the direct contact of bacteria was needed in order to induce spore germination of *Glomus clarum* (reclassified as *Rhizophagus clarus*), which indicated a ligand-receptor interaction.

The bacteria that stimulated spore germination were accompanied by rhizospheric bacteria that produced antagonistic volatiles, which suggest that a complex bacterial consortium is present on *R. clarus* spores that regulate spore germination. Tylka *et al.* (1991) found that species of *Streptomyces* produce volatile compounds that promote spore germination of *G. mosseae* (reclassified as *Funneliformis mosseae*). Hildebrandt *et al.* (2002) found that when *Glomus intraradices* (reclassified as *Rhizophagus intraradices*), interacted with *Paenibacillus validus*, it could sporulate and grow in a bacterium-fungus co-culture. A carbon source, raffinose, was detected in the bacterial cultures, which supported mycelial growth (Frey-Klett *et al.*, 2007).

These bacterial lineages of MHB fall mainly in the bacterial classes of Proteobacter (*Enterobacter*, *Pseudomonas*), Firmicutes (*Bacillus*, *Brevibacillus*) and

Actinomycetes (*Streptomyces*) (Rigamonte *et al.*, 2010). Many MHB have also been described as PGPR. Overlapping of species between MHB and PGPR is a result of the large number of pseudomonad and bacillus species that can be found in both groups. The distinction between the two groups is broadly based on functionality and confusion has occurred because many studies on PGPR have excluded mycorrhizal evaluations (Rigamonte *et al.*, 2010).

1.3.2 Plant growth promoting rhizobacteria

PGPR are some of the most important bacteria that can be found in soil that interact with AM fungi (Miransari, 2010). It has been found that *Bacillus* and *Pseudomonas* are the two predominant genera that are classified as PGPR, that interact with AM fungi (Beneduzi *et al.*, 2012; Frey-Klett *et al.*, 2007; Francis *et al.*, 2010). There are other Gram-negative bacteria that have been identified that are PGPR, but the interactions of Gram-positive bacteria with AM fungi are more prominent (Artursson *et al.*, 2006). These interactions can be positive, negative or neutral towards the plant, but the effect of a bacterial strain can vary under different soil conditions (Artursson *et al.*, 2006).

The different bacteria associated with AM fungi have different functions in the soil, which can affect the plant in different ways (Artursson *et al.*, 2006). PGPR can influence plant growth by using direct or indirect mechanisms (Ahemad and Kibret, 2014). The direct mechanisms include Indole Acetic Acid (IAA) production, nitrogen fixation, phosphate solubilisation, siderophore and 1-Aminocyclopropane-1-carboxylate (ACC) deaminase production. Indirect mechanisms are relevant when PGPR function as biocontrol agents in the rhizosphere (Ahemad and Kibret, 2014).

IAA is a phytohormone auxin that is produced by more than 80% of rhizospheric bacteria and plays an essential role in the interactions between rhizospheric organisms and plants. IAA can affect a plant by: (1) increasing root and xylem development, (2) affecting photosynthesis and pigment formation, (3) increasing root

surface area and length, (4) initiating lateral and adventitious root formation, and (5) increasing resistance to soil pathogens and stressful conditions (Ahemad and Kibret, 2014). There are different metabolic pathways that can lead to the biosynthesis of IAA. The formation of IAA via indole-3-pyruvic acid and indole-3-acetic aldehyde is described as two of the main mechanisms used by bacteria with tryptophan, an amino acid, as the main precursor for IAA production (Ahemad and Kibret, 2014).

Nitrogen (N_2) fixing is one of the most described mechanisms in the soil environment that is used by different organisms (Ahemad and Kibret, 2014). More than 78% N_2 is present in the atmosphere and is unavailable to plants. The atmospheric N_2 is converted by nitrogen fixing bacteria by using nitrogenase, a complex enzyme system, into plant-utilisable forms such as ammonia (Ahemad and Kibret, 2014). Nitrogen fixing bacteria are categorised as: (1) symbiotic N_2 fixing bacteria such as *Rhizobium* that establish a relationship with the roots of the plant host forming nodules and (2) free-living N_2 fixing bacteria, such as Cyanobacteria, that are present in the rhizosphere (Ahemad and Kibret, 2014). Both symbiotic and free-living N_2 fixing bacteria possess the *nif* gene, from which nitrogenase is transcribed, enabling the bacteria to convert atmospheric N_2. Since *V. erioloba* do not form nodules, the trees must obtain some of their nitrogen from free-living bacteria (Lindemann and Glover, 1996). The bacteria convert the N_2 into ammonium, which is directly taken up by the plant for its different metabolic functions (Lindemann and Glover, 1996).

PGPR are able to solubilise phosphate that is found in organic and complex-inorganic forms (Ahemad and Kibret, 2014). Phosphorus (P) is also a plant growth-limiting nutrient that is just as essential as nitrogen. Even though there is a large P reservoir in soil, only a small amount is available to the plants at any given time. Plants can only absorb P in its monobasic dihydrogen phosphate ($H_2PO_4^-$) and diabasic hydrogen phosphate (HPO_4^{2-}) forms (Ahemad and Kibret, 2014). Insoluble P is present in different minerals such as apatite, soil phytate, phosphotriesters and phosphomonoesters. Rhizobacteria such as *Bacillus* and *Enterobacter* have been

described as the most significant phosphate solubilising bacteria in soil (Ahemad and Kibret, 2014).

Solubilisation of P occurs by the simultaneous production of low molecular weight organic acids, which are synthesised by the bacteria. On the other hand, mineralisation of organic P takes place through the synthesis of different phosphatase molecules, which catalyses the hydrolysis of phosphoric esters (Ahemad and Kibret, 2014). The mineralised phosphate can then be either taken up directly by the plant roots or be taken up, transported and transferred by the AM fungi (Artursson *et al*. 2006).

Siderophores are iron chelation molecules that are secreted by PGPR (Ahemad and Kibret, 2014). Bacteria have intra- and extracellular siderophores, which enable them to convert iron (Fe^{3+}) that is available in the environment, into Fe^{2+} that can be absorbed. Thus, siderophores are solubilising molecules for iron that help to collect iron from organic or mineral compounds in the rhizosphere during iron deficiency (Ahemad and Kibret, 2014). Siderophores are also able to bind to other heavy metals that are present in the soil and form stable complexes. Hence, siderophores produced by bacteria alleviate heavy metals stresses that are imposed on plants (Ahemad and Kibret, 2014). Crowley and Kreamer (2007) found the presence of a mediated iron transport system in oat plants and rhizosphere bacteria that produce siderophores, which delivered iron to the oats. These bacteria were able to form Fe-siderophore complexes when iron resources were limited (Crowley and Kreamer, 2007).

Bacteria also have another strategy, which helps to control the intracellular metal levels (Hall, 2002). An active efflux pump is found on the plasma membranes of the bacteria, which involves efflux transporters such as P-type adenosine triphosphatase (ATPases) and or cation/H^+ antiporters. These transporters are able to bind to the heavy metal ions and transport then into the plasma membrane where they are accumulated (Hall, 2002). Rhizospheric bacteria also produce trace element-

chelating organic acids such as citric, acetic and oxalic acids (Sessitsch *et al.*, 2013). These acids help to solubilise metal ions from the surrounding soil, which is then accumulated in the cell walls of the bacteria (Sessitsch *et al.*, 2013).

Plants are able to produce ethylene, a metabolite that is essential for plant development and growth (Ahemad and Kibret, 2014). This hormone is produced in all plants and can also be produced by biotic and abiotic processes in soil, which can induce different important physiological changes in the plant. Ethylene can have positive effects on a plant when the concentrations are low, but when the levels increase during stress conditions it negatively influences plant growth (Ahemad and Kibret, 2014). PGPR possessing the ACC deaminase enzyme are able to facilitate the growth and development of plants by decreasing the levels of ethylene, reducing drought stress and inducing salt tolerance (Ahemad and Kibret, 2014).

PGPR such as *Acinetobacter*, *Enterobacter* and *Bacillus* are a few genera of PGPR that exhibit ACC deaminase activity (Ahemad and Kibret, 2014). These bacteria are able to take up the ethylene precursor, ACC, which is converted into 2-oxobutanoate and ammonia that is used for other processes. Plants that are inoculated with PGPR that produce AAC deaminase enables increases in roots and shoot growth and higher resistance against ethylene-inducing stresses (Glick, 2014). These same bacteria can promote mycorrhizal colonisation in different crops (Ahemad and Kibret, 2014).

PGPR can also be used as an indirect method of biocontrol, where PGPR are able to compete with pathogens for nutrients, induce systematic resistance, and produce antimicrobial metabolites (Ahemad and Kibret, 2014). Induced systematic resistance (ISR) increases the plant's resistance against soil pathogens which is due to the interaction between plants and rhizobacteria. ISR involves hormonal signaling in the plant, which stimulates the plant's defense response against various plant pathogens (Ahemad and Kibret, 2014). The traits of PGPR on plants do not work independently. The combination of the direct and indirect mechanisms enables PGPR to enhance

plant growth (Ahemad and Kibret, 2014). A wide scale application of PGPR may ultimately decrease the global dependence on chemical methods. The combination of interactions between the plant host, mycorrhizal fungi and rhizospheric bacteria, offers an understanding of how the complexity of mycorrhizas works (Bonfante and Anca, 2009).

1.4 Mechanisms enabling heavy metal tolerance

Heavy metal contamination has been a major environmental problem that has increased with the development of different industries (Das *et al.*, 2008). There are heavy metals that occur naturally in soil, which can be tolerated by the soil environment but increased levels can be toxic (Chibuike and Obiora, 2014). Elevated levels of heavy metals in soil can affect plant development and growth (Bano and Ashfaq, 2013). Heavy metals can affect different biochemical, physiological and molecular processes. Photosynthesis is decreased and seed germination is dramatically reduced due to heavy metal stress (Bano and Ashfaq, 2013).

Previous studies have described different mechanisms that have been found to increase a plant host's tolerance to heavy metals (Bano and Ashfaq, 2013). Phytoremediation is a form of bioremediation where plants are used to remove environmental pollutants from soil, which includes other mechanisms such as phytoextraction and phytostabilisation (Glick, 2010; Chibuike and Obiora, 2014). Phytoextraction involves the accumulation of heavy metals in the roots and shoots of plants, which are later harvested and incinerated (Chibuike and Obiora, 2014). Phytostabilisation is a rapid method where plants are used to immobilise heavy metals by reducing the bioavailability of heavy metals that occur during leaching and erosion (Chibuike and Obiora, 2014).

The interactions between the plant host, AM fungi and rhizobacteria are known to increase the success of phytoextraction and phytostabilisation in stressed environments (Chibuike and Obiora, 2014). The plant itself also has its own defense

mechanisms in place where different molecules are produced that can defend it from possible damage, or repair damage caused by stress conditions. Plants have a series of antioxidant enzymes that activate a series of defense mechanisms that help alleviate the effects of different stresses in the environment (Bano and Ashfaq, 2013). Cadmium (Cd) and lead (Pb) can be tolerated by a plant to a certain extent, but when in high concentrations, the activities of the antioxidant enzymes are reduced. This happens due to reactive oxygen species (ROS), such as oxide (O^{2-}), hydrogen peroxide (H_2O_2), and hydroxide (OH^-) that are produced during heavy metal stress (Bano and Ashfaq, 2013). During the radical displacement reactions, different metallic ions are formed that inhibit the antioxidant enzyme activity. Thus, affecting the enzymatic antioxidant defense system and ultimately inhibiting plant productivity and growth (Bano and Ashfaq, 2013). However, ROS scavenger molecules such as catalase (CAT), superoxide dismutase (SOD) and ascorbate peroxidase (APX) are produced in a rapid response to the effects of ROS and counteracting and restoring the damage of ROS (Bailey-Serres and Mittler, 2006).

Mechanisms to tolerate heavy metals in plants differ between species which includes (1) immobilisation of heavy metals, (2) the role of the plasma membrane to expel heavy metals and (3) inhibition of transport and uptake of heavy metals (Bano and Ashfaq, 2013). A plant is able to accumulate heavy metals in the cell walls of the roots, shoots, and leafs. The ability of the plant to accumulate heavy metals in its biomass varies between plant species and the cultivars within a species (Lone *et al.*, 2008). Molecules such as metallothioneins (MTs), which are produced by AM fungi and plants, protect a plant against oxidative stress caused by high levels of heavy metals in soil (Bano and Ashfaq, 2013). MTs are involved in the nullifying of heavy metals through cellular sequestration, metal transport adjustment and homeostasis of intracellular metal ions (Emamverdian *et al.*, 2015). These molecules are also involved in the activation of ROS scavengers, maintaining the redox level, plasma membrane repair, cell proliferation, and repairing DNA damage (Emamverdian *et al.*, 2015).

Mycorrhizal fungi have also been used in different remediation studies in order to remove heavy metals from soil (Chibuike and Obiora, 2014). These studies have shown that mycorrhizal fungi employ different mechanisms for heavy metal remediation. Some studies showed that phytoextraction and phytostabilisation was enhanced when combined with AM fungi. The benefits of mycorrhizal fungi are their ability to increase a plant's health and growth during stress. AM fungi can remove heavy metals via bioaccumulation or biosorption processes (Gadd and White, 1993). During biosorption, the cell wall components of AM fungi bind to the heavy metals and remove the metals from the soil environment (Gadd and White, 1993; Volesky and Holan, 1995). AM fungi produce a glycoprotein known as glomalin, coating hyphae which prevent nutrient loss during transfer (Nichols, 2002). Glomalin is resistant to microbial decay and cannot dissolve in water, but is soluble at high temperatures.

Glomalin enables the hyphae to keep soil particles together forming aggregates (Nichols, 2002). These aggregates contain minerals and organic matter which help to improve the nutrient cycle in AM fungal hyphae, increase water retention and infiltration near roots and store nitrogen and or carbon in the rhizosphere (Nichols, 2002). Glomalin has also been efficient in sequestering copper, cadmium, lead and manganese (González-Chávez *et al.*, 2004). Stommel and colleagues (2001) found that the AM fungus, *Gigaspora rosea*, has a MT-like sequence that helps with cadmium detoxification and metal chelation. Lanfranco *et al.* (2002) identified and characterised the MT-encoding gene and showed that there is a difference in the gene expression during the pre-symbiotic and symbiotic stages. When the gene is expressed the immobilisation of heavy metals in the rhizosphere is increased (Bano and Ashfaq, 2013).

Bioremediation is another technique where plants or microbes are used to remove pollutants from soil (Chibuike and Obiora, 2014). The microbes degrade the pollutants by metabolising the pollutants, and using this as a source of carbon and energy while transforming the pollutants into their less hazardous, environmentally friendly compounds (Singh *et al.*, 2014). During bioremediation heavy metals are not

degraded, but undergo transformation and becomes less toxic. Due to the oxidation state of heavy metals they can become (1) less water soluble, allowing them to precipitate and be removed from the environment, (2) more water soluble, where heavy metals can be removed through leaching or (3) less bioavailable (Chibuike and Obiora, 2014).

Many of the rhizobacteria that have been identified as PGPR assist with the remediation of heavy metals from soil. Rhizobacteria such as *Bacillus subtilus*, *Pseudomonas putida* and *Enterobacter cloacae* can successfully reduce chromium (VI) to chromium (III) that is less toxic (Chibuike and Obiora, 2014). Microorganisms such as *B. cereus* and *B. thuringiensis* have also been shown to be able to increase the extraction of Cd from Cd-rich soil and soil polluted with waste from metal industries. The ability of the bacteria to produce siderophores facilitates the extraction of heavy metal molecules from soil (Chibuike and Obiora, 2014). It has also been reported that the presence of heavy metals in soil can increase the formation of siderophores. Thus, the activity of heavy metals can influence the production and activities of siderophores and in return, the bacteria's mobility and extraction of heavy metals from soil is increased (Chibuike and Obiora, 2014).

Overall, phytoremediation studies that included PGPR have shown that heavy metal stress levels are reduced in plants. Plants that are inoculated with *Bacillus* sp. have shown enhanced accumulation of Cd and nickel (Ni) in the plant's biomass (Chibuike and Obiora, 2014). Another study found that *Methylobacterium oryzae* and *Burkholderia* spp. can increase plant growth by reducing Cd and Ni accumulation in the roots and shoots of tomato plants. Thus, the mechanisms used by PGPR during phytoremediation can differ between PGPR species and plants. Different MHB have also been found to relieve plants from heavy metals stress (Frey-Klett *et al.*, 2007). During heavy metal stress, the MHB had a positive impact on presymbiotic growth and spore germination of the AM fungi. The bacterial inoculations were able to reduce the heavy metal damage to *R. mosseae* hyphae and increase mycorrhizal formation and mycelial growth (Frey-Klett *et al.*, 2007).

Motivation

Vachiella erioloba is an indigenous tree of southern Africa, which can survive harsh conditions (Hayward, 2004). The Camel thorn tree is able to grow in disturbed areas very quickly and can repair depleted soil by bringing nutrients, which are unavailable to other plants, back into circulation (Barnes *et al.*, 1997; Hayward, 2004). This study investigated the role of mycorrhizal fungi and associated beneficial microbes in improving growth and health of *Vachellia erioloba* seedlings. Mycorrhizal fungi such as AM fungi form the most common symbiotic relationship with terrestrial plants (Harrier, 2001). AM fungi form hyphae that branch out and develop in the folds of the plasma membrane of root cells, creating an interface where nutrients are exchanged (Parniske, 2008). AM fungi also develop an extra-radical network in the rhizosphere around the plant host, which enhances nutrient exchange and accessibility, water uptake and provides protection against heavy metals in soil (Parnike, 2008; Bothe *et al.*, 2010). The hyphae of AM fungi are coated with a glycoprotein, glomalin, which binds to soil particles assisting in the formation of aggregates (Nichols, 2002). These aggregates can contain nutrients, minerals and heavy metals molecules. Mycorrhizal fungi also associate with soil microbes that are beneficial to the fungus and the host plant, promoting plant growth and enhancing nutrient availability (Miransari, 2010). The soil microbes can produce compounds that, as a result, enhance root colonisation by mycorrhizal fungi, which increase the beneficial effect of mycorrhizal fungi to their host plant (Barea *et al.*, 2005). The causes of the decline in growth of *V. erioloba* are not well understood but may be related to lowering of the water table and increased levels of heavy metals due to anthropogenic activities. Mycorrhizal fungi and their associated microbes may improve the growth and health of *V. erioloba* seedlings.

Aim:

Determining the role of mycorrhizal fungi and associated beneficial microbes for improved growth of *Vachellia erioloba* seedlings.

Objectives:

- Determining the mycorrhizal interaction with *V. erioloba.*
- Determining the effects of Cd on *V. erioloba* seedling growth.
- Isolation, characterisation and identification of mycorrhizal associated beneficial microbes.
- Assessment of mycorrhizal and bacterial populations from *V. erioloba* rhizospheric soil.
- Evaluate the ability of mycorrhizal fungi and selected associated microbes to promote seedling growth and health.

Chapter 2

Materials and Methods

2.1 Sample collection

Soil and root samples were collected from a farm in the Northern Cape (27°91'26.7" S, 21°14'62.5" E), South Africa. Three samples were collected from under three healthy Camel thorn trees and were labelled as Camel thorn 1, Camel thorn 2 and Vaalwater. Soil and root samples were collected from under each tree and were placed in Ziploc™ bags separately. The samples were placed in a sealed container and sent to Rhodes University, Grahamstown, South Africa for further analysis. Seed pods were collected from the three Camel thorn trees, and care was taken to harvest only pods that were undamaged by insects.

2.2 Soil analysis

Soil was sent to Eco-Ananlytica Laboratories, Potchefstroom, South Africa for nutrient analysis. The soil was analysed for nutrients such as Calcium (Ca), Magnesium (Mg), Potassium (K), Phosphate (P) and Sodium (Na). The Walkley Black method was used to determine the percentage Carbon (C) and the LECO combustion method was used to determine the percentage nitrogen (N). The analysis also included Cation Exchange Capacity (CEC), the total amount of exchangeable cations (S-value), soil pH, base saturation (%) and electric conductivity (EC).

2.3 Arbuscular mycorrhizal fungal spore extraction

Spore extraction was done by using wet sieving and decanting method of Smith and Dickson (1997). This method is one of the most commonly used methods to study AM fungi in soil. However this method only detects fungi that produce spores and not all of the spores that are observed are viable. Non-viable spores are spores that are

empty shells, spores parasitized by other fungi or gas spores which float. Viable spores are filled with lipids and can also float, which makes it difficult to distinguish between viable and non-viable spores (Gaur and Varma, 2007).

The soil collected was used for AM spore extraction. Two hundred grams of soil was weighed and placed in separate brown bags, which were left open for a day to allow the sample to air dry. A 2 mm sieve was used to separate all the large debris from the soil. One hundred grams of soil was weighed in a 500 ml glass beaker and 200 ml water was added to the beaker and stirred. The soil suspension was agitated for 5 minutes using a magnetic stirrer and allowed to settle for 15 seconds. The supernatant was decanted through a nest of soil sieves (425 µm, 250 µm, 125 µm and 45 µm mesh size). The sieves where gently washed over a sink with water. The 425 µm sieve was examined under a dissection microscope for any large spores. The debris of the remaining sieves was washed into separate 50 ml centrifuge tubes. Each tube was labelled according to the sieve size and site, which was filled up to 50 ml with water and closed.

The 50 ml tubes were balanced and centrifuged for 5 min at 3500 rpm using an Alllsheng centrifuge. The supernatant was carefully discarded to ensure none of the sample was lost. The pellet was re-suspended in 60% sucrose solution and centrifuged for a further 5 min. The supernatant was decanted onto a 45 µm sieve and rinsed with water to remove the sucrose. A 9 cm grid filter paper (Watman #1) was placed into a Buchner funnel, attached to a water vacuum. The supernatant was decanted onto the filter paper and the spores were rinsed with water. The filter paper was then transferred to a petri dish and the spores were examined and counted under a dissecting microscope (Leica, Model: LED2500).

2.4 Mycorrhizal colonisation assessment

Roots from each site were separated from the soil and placed in McCartney bottles separately. Roots were stained by following a modified staining protocol as

described by Koske and Gemma (1989) and Smith and Dickson (1997). Roots from each site were carefully washed over a 120 µm sieve to prevent the loss of material and to remove excess soil particles that were attached to the roots. The roots were placed in McCartney bottles with 5% potassium hydroxide (KOH) (Appendix A) and autoclaved for 20 minutes at 121ºC. After the roots cooled down the KOH was discarded and the roots were rinsed with water over a 120 µm sieve to prevent the loss of material. The roots were bleached with alkaline hydrogen peroxide (H_2O_2) (Appendix A) for 45 min and rinsed with water. Root samples were then placed in 0.1 M hydrochloric acid (HCl) (Appendix A) for 3 hours. The HCl was discarded but the roots were not rinsed. The roots were then placed in a lactoglycerol (containing lactic acid, glycerol, and water in a ratio of 13:12:16) trypan blue (0.05 %) staining solution (Appendix A). The roots were left in the staining solution overnight. The staining solution was poured off and the roots were placed in a lactoglycerol destain solution (without trypan blue) and were left overnight to destain.

The roots were placed on microscope slides in destain and covered with a cover slip. Pressure was applied to the coverslip to flatten the roots. The slides were examined using a Leica (Model: CME) microscope and a total of 100 fields-of-view was examined for the presence of arbuscules, spores and hyphae. The percentage of colonisation was determined by how many fields-of-view out of a hundred contained AM fungal structures.

2.5 Effect of Cadmium on seedling growth

Pods were cracked open and seeds were removed. Seeds were placed in boiling water one day prior to planting and left to cool. River sand was steam pasteurised on two successive days for 2 hrs at 80°C (Jorgustin, 2011; Fox, 2015). Plastic torpedo tubes (200 ml) were filled with pasteurised sand and one Camel thorn seed was planted in each tube. Half of the seeds were inoculated with AM fungi and the other half were used as controls. Mycorrhizal fungi obtained from Mycoroot™, Grahamstown, South Africa was used to inoculate seeds. The Mycoroot™ product contains a selection of AM fungal species (most probable number of propagules is

80 per g inoculum), which included *Glomus clarum* (new name *Rhizophagus clarus*), *Gigaspora gigantea*, *Glomus mosseae* (new name *Funneliformis mosseae*), *Glomus etunicatum* (new name *Claroideoglomus etunicatum*) and *Paraglomus occulum*. Inoculated seeds received 5 g of the Mycoroot^{TM} inoculum.

A range of Cd standards (0, 25, 50 and 100 ppm) was prepared from a 1000 ppm Cd stock solution in sterile distilled water (Patil and Umadevi, 2014). Six inoculated and six un-inoculated seedlings were used for each of the concentrations. The Cd solutions were added after the seeds had germinated. A total of 48 seedlings were used for the experiment. The initial shoot height was measured and subsequencial measurements were taken every 2 weeks. Every four weeks 5 ml of each of the Cd solutions was added to the seedlings. The seedlings were grown in a Mycorrhizal research tunnel with natural light, min/max temperatures of 20-25/30-35°C and automatically irrigated daily with Ultraviolet (UV) treated water.

After three months the pot trial was harvested and the final shoot height was recorded. Other growth parameters such as the fresh and dry weight were measured (Vijayaragavan *et al.*, 2011; Subhashini *et al.*, 2013). Fresh weight of the plant was recorded by weighing the shoots and root separately and placed in individual brown paper bags. The plant material was then placed in a plant press and left to dry for 6 weeks. The dry roots and shoots were weighed separately and the weights were recorded. A subsample of the fresh roots was removed for assessment of AM fungal colonisation. The subsample weight was used to correct the root dry weight.

2.5.1 Cadmium analysis

After the dry weights were recorded the plant material was prepared for Cd analysis using a modified hotplate method as described by Chen and Ma (2001). The shoot and root samples were grounded separately using a mortar and pestle and placed in a 50 ml glass test tubes. A stock solution of 1 L was made by using 750 ml nitric acid (HNO_3) and 250 ml HCl (1:3) and 15 ml of the stock solution was added to each

sample. The samples were placed in a Labnet dry bath at 110°C for 3 hours. The samples were then transferred separately into 100 ml volumetric flasks and 85 ml double distilled water was added to make up a total volume of 100 ml and the extract was stored at 4°C until analysis.

Five Cadmium standards (0ppm, 25ppm, 50ppm, 100ppm, 150ppm) were prepared. All samples and standards were filtered through 0.45 µm filters (Millex®-HV Sterile Filter Unit with Durapore® PVDF Membrane) before analysis. The Cd concentrations for the samples were determined by Inductive Coupled Plasma with Optical Emission Spectroscopy (ICP-OES) analysis using a Thermo ICAP-6300 spectrometer (Chemistry Department, Rhodes University). The results obtained were quantified by using a standard curve that was prepared by using the Cd concentrations of the standards (Appendix B).

2.6 Isolations and characterisation of plant growth promoting rhizobacteria

Bacteria were isolated from the soil samples by conducting serial dilutions (10^{-2}-10^{-7}) in sterile distilled water and plating on nutrient agar. The plates were incubated at 28°C for 24 hrs. Single colonies from each plate were selected and grown in sterile nutrient broth for 24 hrs. One hundred µl of each broth were spread onto new nutrient agar plates. Single colonies were selected and discontinuously streaked on plates containing different selective agars (Appendix C) and nutrient agar, separately: Bennett media (Himedia, 2011), Streptomyces agar (Awad *et al.*, 2009), King's B agar (Scharlau, 2002) and Gould's modified S1 media (Tarnawski *et al.*, 2003). The Bennett and Streptomyces media are selective for *Streptomyces* species. Gould's modified S1 media and the King's B agar is selective for *Pseudomonas* species. All the media were incubated for 24 hrs except for Gould's modified S1 media, which were incubated for two weeks. Colonies were selected based on their different morphology and were discontinuously streaked on new nutrient agar plates and incubated as before.

2.6.1 Gram staining

Single colonies were selected and Gram stained (Willey *et al.*, 2011) to determine the Gram reaction and shape of the cells. A loopful of a colony was taken and placed in a droplet of sterile distilled water on a microscopic slide and heat fixed. The smear was stained with 1% crystal violet for 60 seconds and gently rinsed with water. The crystal violet turns the cells dark purple. The smear was flooded with iodine solution for 30 seconds, which fixes the crystal violet in the cells. The iodine solution was discarded and the slide was rinsed with water. The excess water was shaken off and the smear was decolorized with 80% alcohol until most of the colour was removed. Counterstaining was then performed with a safranin solution for 30 seconds. The slide was rinsed with water and left to dry. The bacterial slides were examined on a Nikon YS100 compound microscope under oil immersion.

Gram positive bacteria colour purple due to their thicker peptidoglycan layer, which consists of dissacharrides and amino acids (Willey *et al.*, 2011). The crystal violet and the iodine precipitates within the layer, and are not eluted by the alcohol. In contrast gram negative bacteria's outer lipopolysacharide layer is disrupted by the alcohol and the crystal violet is washed out through the thin peptidoglycan layer (Willey *et al.*, 2011). Due to this the gram negative cells become colourless and then retain a pink colour during the counterstaining with safranin. The cell's colour and shape was recorded. The colonies that were not pure were re-streaked on their respective agar and gram staining was performed again until pure cultures were obtained. The pure cultures were grown in nutrient broth and 500 µl of each culture were mixed with 500 µl of 50% glycerol and stored at -80ºC.

2.6.2 Siderophore production

A siderophore production assay was performed on the bacterial isolates using Chrome Azurol S (CAS) Agar (Tortora *et al.*, 2011) (Appendix D). An overlay CAS method (Pérez-Miranda *et al.*, 2007) was used where the isolates were grown on nutrient agar and CAS agar was prepared and poured over the cultures. The bacterial isolates from an overnight culture were streaked out in triplicate on the

nutrient agar and incubated at 28°C for 24 hrs. The CAS agar was prepared, left to cool until just before setting point and gently poured over the grown cultures. The plates were then sealed with Parafilm (Bemis Company, Inc.) and incubated at 28°C for 3 days. After incubation if an orange colour change or clear zone formed around the bacteria, the culture was then positive for siderophore production. The diameter of the reaction was also measured (mm).

2.6.3 Indole acetic acid production

A Salkowski assay was used to quantify the production of IAA (Rahman *et al.*, 2010). Isolates were grown in 5 ml nutrient broth for 48 hours at 28ºC. The bacterial cell concentration was measured at a wavelength of 660 nm and the final optical density OD was adjusted to 0.2 with sterile nutrient broth. One hundred µl of each isolate and sterile nutrient broth (control) was added to 5 ml of DEV- tryptophan broth and incubated at 28ºC for 5 days. This was done in triplicate. After the incubation period 1.5 ml of each isolate was transferred to sterile microcentrifuge tubes. The tubes were centrifuged (Hangzhou Allsheng super mini centrifuge) at 13 000 rpm for 1 minute. Two hundred microliters of Salkowski reagent (Appendix E) was added to each well of the 96 well Microtiter plate. One hundred microliters of each isolate was then added to a well (in triplicate) and left at room temperature for 25 minutes to undergo colour change, which was measured spectrophotometrically (UV mini-120 UV-VIS spectrophotometer) at 530 nm. The results obtained were quantified by using a standard curve that was prepared from known IAA concentrations (Appendix E).

2.6.4 Phosphate solubilisation

The ability of the isolates to solubilise phosphate was tested on National Botanical Research Institute's phosphate (NBRIP) medium that was supplemented with 1.5% bacteriological agar as described by Islam *et al.* (2007). The medium contained (per litre): Glucose, 10 g, Tricalcium phosphate ($Ca_3(PO_4)_2$), 5 g, Magnesium chloride hexahydrate ($MgCl_2.6H_2O$), 5 g, Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), 0.25 g, Potassium chloride (KCl), 0.2 g, Ammonium sulphate ($(NH_4)_2SO_4$), 0.1 g,

Bacteriological agar, 15 g. Each plate was stab-inoculated with an isolate, in triplicate, and incubated for two weeks at 28ºC. After the incubation period the solubilisation index was done by determining the ratio of the total diameter of the colony and halo zone to the diameter of the colony (Edi-Premono *et al.,* 1996).

2.6.5 Nitrogen fixation

The ability of the isolates to fix nitrogen was tested on Ashbys Mannitol agar as described by Mazinani *et al*. (2012) and Muthuselvan and Balagurunathan (2013). The agar contained (per litre): Mannitol, 20 g, Potassium dihydrogen phosphate (KH_2PO_4), 0.2 g, $MgSO_4.7H_2O$, 0.2 g, Sodium chloride (NaCl), 0.2 g, Potassium sulphate (KSO_4), 0.1 g, Calcium carbonate ($CaCO_3$), 5 g, Bacteriological agar, 15 g. Isolates were streaked on the agar and incubated at 28ºC for 5 days. After the incubation period if growth was observed, then the bacteria was able to fix nitrogen.

2.6.6 Biofilm quantification in a liquid medium

Isolates were tested for biofilm formation by using crystal violet method adapted from Morikawa *et al*. (2006). Isolates were grown in nutrient broth for 24 hrs at 28°C, 1.5 ml of each isolate were added in a microcentrifuge tube and centrifuged for 3 minutes at 13 400 rpm to form a pellet. The pellet was re-suspended in 500 µl of 0.85% NaCl aqueous solution. Two hundred microliters of each isolate was added to a well in a 96 well microtiter plate, which was done in triplicate. The OD of the cultures was measured at 600 nm and adjusted to 0.3 with 0.85% NaCl. Two hundred microliters of nutrient broth was added to each well of a clean 96 well microtiter plate and 20 µl of the adjusted inoculum was added to each well containing nutrient broth.

The open wells left on the microtiter plate were filled with sterile distilled water and covered with a lid to restrict evaporation. The plate was then incubated for 3 days at 28°C and afterwards the OD was measured at 600 nm to determine the growth units. The plates were only grown for 3 days, because if the plates were left longer fungi

started to grow in the wells. The formation of a "ring" and pellicle was observed at the zone of contact. The liquid culture was carefully removed with a pipette without touching the walls of the wells.

After incubation the liquid in the wells was removed without touching the walls. Crystal violet (1%) was added and left for 30 minutes, and after careful removal, the wells were rinsed with 96% ethanol to solubilise the crystal violet. The wells were then rinsed twice with sterile distilled water and the final OD reading was measured at 575 nm. The final biofilm production results were then defined as the cell density at 575 nm (Djordjevic *et al.*, 2002).

2.7 Molecular identification of plant growth promoting rhizobacteria

2.7.1 Deoxyribonucleic acid extraction

The Deoxyribonucleic acid (DNA) of the pure cultures was extracted by using a ZR Fungal/Bacterial DNA Mini Prep™ kit (Zymo Research Corp, D6005). Cultures were grown in nutrient broth overnight and centrifuged for 3 minutes at 10 000 rpm until a pellet of 0.1 g was obtained. The pellet was suspended in 200 µl sterile distilled water and added to a ZR BashingBead™ Lysis tube to which 750 µl of the lysis solution was added. The lysis solution and the beads aid in breaking and lysis the cells. The tube was vortexed for 7 min to assist with the lysis process and centrifuged for 1 min at 10 000 rpm. Four hundred microliters of the content was transferred to a Zymo-Spin™ IV Spin Filter in a collection tube and centrifuged for 1 min at 7000 rpm. This was done to remove the cell debris that was not removed during the first centrifuge step. One thousand two hundred microlitres of Fungal/Bacterial DNA Binding Buffer was added to the collection tube.

Eight hundred µl of the mixture was transferred to a Zymo-Spin™ IIC Column in a collection tube, which was centrifuged for 1 min at 10 000 rpm. Five hundred microliters of Fungal/Bacterial DNA Wash Buffer was added to the Zymo-Spin™ IIC Column and centrifuged again for 1 min at 10 000 rpm. The Zymo-Spin™ IIC Column

was placed in a clean 1.5 ml microcentrifuge tube, 100 µl of DNA Elution buffer was added to the column and centrifuged for 30 sec at 10 000 rpm. This step released the DNA from the column. The DNA was stored at 4°C until needed.

2.7.2 Polymerase Chain Reaction amplification

Polymerase Chain Reaction (PCR) is a technique that amplifies double stranded DNA templates, in the presence of a *Taq* polymerase enzyme, with the use of single stranded DNA fragments known as primers (Willey, 2011). The 16S rDNA bacterial gene was amplified in a reaction volume of 25 µl. The reaction volume contained 10 µl double distilled water, 1 µl rP2 primer (Table 2.1), 1 µl Fd1 primer (Table 2.1), 2.5 µl template DNA, and 12.5 µl of 2 x KAPA HiFi HotStart Ready Mix (Catalogue KM2605). KAPA HiFi HotStart contained DNA polymerase (1 U/50 µl reaction), 2.5 mM Magnesium chloride ($MgCl_2$) (1x) and 0.3 mM for each dNTP.

Table 2.1: Oligonucleotides used for 16SrDNA gene sequencing.

Primers	Sequences	References
Fd1	5'-AGAGTTTGATCCTGGCTCAG-'3	Weisburg *et al.*, 1991
rP2	5'ACGGCTACCTTGTTACGACTT 3'	Weisburg *et al.*, 1991

The amplification of the 16S rDNA gene was carried out by using a 2720 Thermal Cycler (Applied Biosystems) (Table 2.2). Denaturing is where the DNA strands are separated and annealing allows the primers to attach onto the DNA. The extension step is where the DNA polymerase enzyme elongates the DNA strand using dNTPs (Willey, 2011).

Table 2.2: Thermal cycling parameters used to amplify 16S rDNA.

Parameters	Temperature (°C)	Time (seconds)	Cycles
Initial Denaturation	98	300	1
Denaturation	98	45	30
Annealing	52	45	
Extension	72	60	
Final Extension	72	300	1

2.7.3 Agarose gel electrophoresis

The amplified DNA was evaluated after PCR through 1% (W/V) agarose gel electrophoresis. The gel contained 1x Tris-borate-ethylenediaminetetraacetic acid (TBE) and 2 µl ethidium bromide (concentration 0.5 µg/ml) and was run for 1 hr and 30 min at a current of 500 Amps and 100 volts (V). Two microliters of DNA template was added to the gel. A Promega Lambda/*EcoR1* + *HindII* (Catalogue No G1731) marker was used to determine the size of the DNA and an Uvitec gel doc (Moore *et al.*, 1987) was used to visualize the gel under UV fluorescence. Bands of approximately 1500 base pairs (bp) were expected.

2.7.4 Polymerase Chain Reaction clean-up

After PCR has been completed there are still some reaction components such as primers, enzymes and dNTP's in the mixture along with the DNA. These components were removed to avoid interference with the DNA sequencing process. The manufacturer's instructions of a Promega Wizard® SV PCR clean-up kit (Catalogue A9281/2/5) were followed in order to purify the PCR products. Gel electrophoresis was conducted to view the cleaned products. The piece of gel containing the band was excised. The gel was weighed and 10 µl of Membrane binding buffer was added to every 10 mg of gel. The mixture was then vortexed and incubated at 55ºC in a dry bath until the gel was dissolved. A SV Minicolumn was inserted into a collection tube. The mixture was added to the Minicolumn assembly and incubated at room

temperature for 1 min and centrifuged for 1 min after which it was 13 400 rpm. The flow-through was discarded.

Seven hundred microliters of the membrane wash solution was added into the Minicolum assembly and centrifuged for 1 min at 13 400 rpm. The flow-through was discarded and an additional 500 µl was added to the assembly which was centrifuged again for 5 min at 13 400 rpm. The column was then placed in a clean 1.5 ml microcentrifuge tube and 50 µl of Nuclease-free water was added and incubated at room temperature for 1 min. The tubes were then centrifuged for 1 min at 13 400 rpm to elute the DNA. The purified DNA was then stored at -20ºC. Gel electrophoresis was conducted to evaluate the purified DNA as described previously.

After the PCR clean-up was completed, selected isolates were sent to Inqaba Biotechnology, Pretoria, South Africa for Sanger sequencing. Sequences were analysed using FinchTV 1.4.0 (Geospiza software) and identified using the Basic Local Alignment Search Tool (BLAST) on the National Centre of Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). Sequences were then submitted to GenBank for comparative identification.

2.7.5 Differentiation of *Enterobacter* species

The DNA of the ten isolates identified as *Enterobacter* sp. were digested with the restriction enzyme Xbal in order to determine whether the isolates were different species. The Xbal restriction enzyme is one of the commonly used enzymes to characterise *Enterobacter* species (Iversen, 2014). Digestion reaction contained 2 µl Xbal, 9.5 µl double distilled water, 1 µl template DNA, and 12.5 µl of 2 x KAPA HiFi HotStart Ready Mix (Catalogue KM2605). KAPA HiFi HotStart contained DNA polymerase (1 U/50 µl reaction), 2.5 mM Magnesium chloride (MgCl2) (1x) and 0.3 mM for each dNTP. The reaction mix was left at 37°C for 1 hr, which is the optimal digestion temperature. In order to stop the digestion the temperature was adjusted to 65°C for 15 minutes. The digestion reaction was evaluated using agarose gel

electrophoresis and visualised using an Uvitec gel doc (Moore *et al.*, 1987) as previously described.

2.8 Assessment of mycorrhizal and bacterial populations from *Vachellia erioloba* rhizospheric soil.

2.8.1 Deoxyribonucleic acid extraction

DNA was extracted from rhizospheric soil by using a ZR Soil DNA Extraction Kit (Zymo Research, Catalogue number D6001). One soil sample from each of the three sites was used and 250 mg of each soil sample was weighed and added to a ZR BashingBead™ Lysis Tube. Seven hundred and fifty microliters of the Lysis solution was added to the tubes. The tubes were then secured on a bead beater fitted with a 2 ml tube holder assembly and processed for 5 min at maximum speed. The tubes were centrifuged for 1 min at 10 000 rpm. Four hundred microliters of the supernatant was transferred to a Zymo-Spin™ IV Spin Filter in a collection tube and centrifuged for 1 min at 7 000 rpm. One thousand two hundred microliters of the Soil Binding Buffer was added to the filtrate in the collection tube.

Eight hundred microliters was transferred to a Zymo-Spin™ IIC Column in a collection tube and centrifuged for 1 min at 10 000 rpm. This was repeated to transfer the entire sample to the filter. Two hundred microliters of the DNA Pre-Wash Buffer was added to the Zymo-Spin™ IIC Column in a collection tube and centrifuged for 1 min at 10 000 rpm. Five hundred microliters of the Soil DNA Wash Buffer was added to the column and centrifuged for 1 min at 10 000 rpm. The filter was then transferred to a clean 1.5 ml microcentrifuge tube and 100 µl of the DNA Elution Buffer was added directly onto the filter. The microcentrifuge tube with the filter, was centrifuged for 30 sec at 10 000 rpm to elute the DNA. The eluted DNA was then transferred to a Zymo-Spin™ IC-HRC Spin Filter in a clean 1.5 ml microcentrifuge tube and centrifuged for 1 min at 8 000 rpm. This was done to get suitable DNA for PCR.

2.8.2 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was conducted to amplify the extracted soil DNA. Amplification was done with a 2720 Thermal Cycler (Applied Biosystems). The extracted DNA was split and used to identify fungal and bacterial species present in the soil.

2.8.2.1 Amplification of 16S rDNA

The 16S rDNA of the bacteria were amplified by using MiSeq primers (Table 2.2). A 25 µl reaction mixture was set up as follows: 5 µl template DNA, 0.75 µl forward primer, 0.75 µl reverse primer, 5 µl $MgCl_2$, 0.75 µl dNTPs, 0.5 µl Bovine serum albumin (BSA), 0.5 µl Kapa HiFi Hotstart ready mix and 0.75 µl Nuclease-free water. Amplification was performed using a 2720 Thermal Cycler (Applied Biosystems) with the cycling parameters as described in Table 2.3. Gel electrophoresis was conducted to evaluate the PCR product followed by PCR gel clean-up as previously described. Adaptors were added to the sample during a secondary 8 cycle PCR that was done by Dr. Gwynneth Matcher at the sequencing facility at Rhodes University according to the MiSeq manufacturers' protocol.

Table 2.3: Nucleotide sequences of the 16S rDNA MiSeq primers.

Primer	Sequence
Forward primer	5' – CAGCAGCCGCGGTAA – 3'
Reverse primer	5' – GTAAGGTTCTTCGCGT – 3'

Table 2.4: Thermal Cycler parameters used in the amplification of the 16S DNA.

Parameters	Temperature (°C)	Time (sec)	Cycles
Initial Denaturation	98	300	1
Denaturation	98	45	
Annealing	45	30	5
Extension	72	60	
Denaturation	98	45	
Annealing	50	30	20
Extension	72	60	
Final Extension	72	300	1

2.8.2.2 Amplification of 18S rDNA

The 18S rDNA of the AM fungi was amplified using AM fungal specific untagged primers NS31 and AML2 (van Geel *et al.*, 2014). A 25 µl reaction mix was set up as follows: 5 µl template DNA, 1 µl NS31 primer (Table 2.4), 1 µl AML2 primer (Table 2.4), 5.5 µl Nuclease-free water and 12.5 µl KAPA HiFi Hotstart ReadyMix. The amplification of the 18S rDNA was done using the same Thermal Cycler as before with cycling parameters as described in Table 2.5. Gel electrophoresis was carried out on the PCR product as previously described.

Table 2.5: Nucleotide sequences of the AM fungal specific untagged primers.

Primer	Sequences
AML2	5'- GAACCCAAACACTTTGGTTTCC - 3'
NS31	5'- TTGGAGGGCAAGTCTGGTGCC - 3'

Table 2.6: Thermal Cycling parameters used to amplify the 18S rDNA.

Parameters	Temperature (ºC)	Time (seconds)	Cycles
Initial Denaturation	98	300	1
Denaturation	98	30	
Annealing	49.7	45	5
Extension	72	60	
Denaturation	98	30	
Annealing	65	45	25
Extension	72	60	
Final Extension	72	300	1

After the amplification with the untagged primers was completed, PCR was redone by amplifying the PCR product with MiSeq primers (Table 2.6). The 25 µl reaction mix was set up as follows: 1 µl forward primer, 1 µl reverse primer, 5 µl Nuclease-free water, 12.5 µl Kapa Hotstart ReadyMix, 5 µl PCR product, and 0.5 µl BSA. Amplification was done using the 2720 Thermal Cycler (Applied Biosystems) with the cycle parameters as described in Table 2.5. Gel electrophoresis was done to visualise the PCR product and PCR clean-up was performed as described before.

Table 2.7: Nucleotide sequences of the AM fungal specific MiSeq primers.

Primers	Sequences	References
NS31F - MiSeq	5' – TCGTCGGCAGCGTCAGATGTGTATAAGA GACAGTTGGAGGGCAAGTCTGGTGCC – 3'	IDT Cat. No. 208747347
AML2R - MiSeq	5' – GTCTCGTGGGCTCGGAGATGTGTATAA GAGACAGGAACCCAAACACTTTGGTTTCC – 3'	IDT Cat. No. 208739597

2.8.3 Agarose gel electrophoresis

The amplified DNA of the fungal (18S) and bacterial (16S) samples were evaluated after PCR through 1% (W/V) agarose gel electrophoresis. The gel contained 1x TBE

and 2 µl ethidium bromide (concentration 0.5 µg/ml) and was run for 2 hrs at a current of 500 Amps and 80V. For the fungal samples a 100 bp DNA molecular marker (Promega Catalogue no G210A) was used, and for the bacterial samples a Promega Lambda/*EcoR1* + *HindII* (Catalogue No G1731) marker was used to determine the size of the DNA. An Uvitec gel doc was used to visualise the gel under fluorescence (Moore *et al*., 1987). The band sizes of the bacterial and fungal DNA were expected to be approximately 500bp.

2.8.4 Illumina Sequencing

The PCR products were sent for Illumina sequencing at the University of Cape Town. MID tags were added using 5 µl of each relevant index from the Nextera XT Index kit (Illumina) with 25 µl 2X KAPA HiFi Hotstart Ready Mix, 5 µl template from the PCR products and made up to 50 µl with PCR grade water. Final amplification with MID tagged primers was done using the cycle parameters described in Table 2.7.

Table 2.8: Thermal cycler parameters for final annealing with MID tagged primers.

Parameters	Temperature (ºC)	Time (seconds)	Cycles
Initial Denaturation	95	180	1
Denaturation	95	30	
Annealing	55	30	8
Extension	72	30	
Final extension	72	300	1

PCR Clean-Up was performed by vortexing the AMPure XP beads for 30 seconds to evenly distribute the beads. Fifty six microliters of beads were added to each sample and gently pipetted up and down 10 times. The samples were left at room temperature for 5 minutes (without shaking) and then placed on a magnetic stand for 2 minutes or until the supernatant cleared and removed. The beads were washed twice with 200 µl freshly prepared ethanol (80%). The ethanol was prepared by incubating the ethanol for 30 seconds on the magnetic stand and then the

supernatant was removed. The beads was left to air dry for 10 minutes and 27.5 µl of 10 mM Tris (pH 8.5) was added and incubated at room temperature for 2 minutes. The plate was placed on the magnetic stand for 2 minutes or until the supernatant cleared. Twenty five microliters of the supernatant from the index PCR plate was transferred to a new 96-well PCR plate. The samples were then pooled for multiplex sequencing on a MiSeq Sequencing platform (Illumina), using a 300 bp paired end run.

After sequencing only the forward primer sequences were used, because the reverse primers were of poor quality and could not processed. Sequences were curated using Mothur (Schloss *et al.*, 2009), although two different protocols were used. During the curation, the AM fungal chimera sequences were removed using the Uchime algorithm (Edgar *et al.*, 2011), whereas the Vsearch algorithm (Rognes *et al.*, 2016) was used to remove the bacterial chimera sequences. The Bacterial and AM fungal sequences were assigned to OTUs using a 97% species identity threshold. The bacterial and AM fungal sequences from the twenty most dominant OTUs was blasted against the MaajAM database (Öpik *et al.*, 2010), for the AM fungi, and GenBank, for the bacteria and identified up to genus level. The bacterial and AM fungal sequences were then submitted to GenBank on the NCBI website (http://www.ncbi.nlm.nih.gov).

2.9 Evaluation of the ability of selected bacterial isolates and mycorrhizal fungi to promote seedling growth.

2.9.1 Individual abilities of the isolated bacteria and arbuscular mycorrhizal fungi to promote seedling growth.

Seeds of *V. erioloba* were germinated in a perlite:vermiculite mix (1:1). The seedlings were planted into pasteurised compost:vermiculite mix (1:1 ratio). Half of the seedlings were inoculated with 5 ml Kalahari AM crude inoculum (Moore, 2014) applied below the seedlings. Three bacterial isolates namely F4, F8 and F10 were selected and grown in nutrient broth for 48 hrs, their OD was adjusted to 0.3 at 590 nm. The seedlings treatments included the inoculation with three selected bacterial

isolates separately. The 14 seedlings received 5 ml of each of the isolate, of which seven were inoculated with the AM crude inoculum and seven uninoculated. A control treatment of seven inoculated and seven uninoculated seedlings were also set up and did not contain bacteria.

The seedlings were grown in the mycorrhizal research tunnel (minimum temperatures 20-25ºC, maximum 35ºC, irrigated daily with UV treated water and natural lighting) and 20 ml Long Ashton's low Phosphate nutrient solution (Appendix I) was applied every two weeks. The seedling's initial shoot height was recorded and measured every week thereafter. After 12 weeks the pot trial was harvested and plant parameters such as the shoot and root biomass and percentage mycorrhizal colonisation were measured as previously described.

2.9.2 Combined abilities of the isolated bacteria and arbuscular mycorrhizal fungi to promote *Vachellia erioloba* seedling growth and health.

V. erioloba seeds were collected, germinated and planted as previously described, using the same Kalahari AM crude inoculum. The same bacteria were selected and prepared as previously described and 2 ml of each isolate (combined) was added to fourteen seedlings, of which seven were inoculated with the AM crude inoculum and seven uninoculated. A control treatment of seven inoculated and seven uninoculated seedlings were also set up and did not contain bacteria.The seedlings were grown in the mycorrhizal research tunnel under the same conditions as previously described and 20 ml Long Ashton's low Phosphate nutrient solution (Appendix I) was applied every two week. The seedling's initial shoot height was recorded and measured every week thereafter. After 12 weeks the pot trial was harvested and plant parameters such as the shoot and root biomass and percentage mycorrhizal colonisation, were measured as previously described.

2.10 Statistical analysis

All experimental data was collected from all replicates, with n being equal to 3-7 depending on the experiment. Means and standard errors were calculated using Microsoft Excel 2010. The data recorded was statistically analysed by using one-way analysis of varaince (ANOVA) using SPSS software (Windows version 18). Duncan's multiple range tests were performed at $P < 0.05$ on each of the significant variables measured (Duncan, 1955).

Chapter 3

Results

3.1 Soil analysis

Soil nutrient analysis results are presented in Table 3.1 and the exchangeable cations present in the soil are presented in Table 3.2. Calcium (Ca) had the highest concentration of the nutrients analysed and sodium (Na) had the lowest concentration. The soil pH indicates that it was acidic, nitrogen was not detected and a low percentage carbon was determined.

Table 3.1: Nutrient status of the composite Kalahari soil sample.

Ca	Mg	K	Na	P	pH	pH	EC	N	C
		(mg/kg)			(H_2O)	(KCl)	(ms/m)	(%)	(%)
200.5	60	124.5	8	26.4	5.46	4.33	36	0	1.75

Table 3.2: The exchangeable cations present in the soil.

Ca	Mg	K	Na	CEC	S-value	Base saturation
			(cmol(+)/kg)			(%)
1.00	0.49	0.32	0.03	6.30	1.85	29.36

3.2 Arbuscular mycorrhizal fungal spore assessment

AM fungal spores were extracted using the wet sieving and decanting method. The spores were mainly oval shaped with subtending hyphae and yellow to dark brown in colour (Fig. 3.1). Spore diameters ranged from 80 µm to 180 µm. The spores were tentatively placed in the *Glomus* genus. The number of spores isolated from the soil from each site varied. The average number of spores was 42.3 spores/100 g soil, which is less than 1 spore per gram of soil. However, the total number of spores includes viable and non-viable spores.

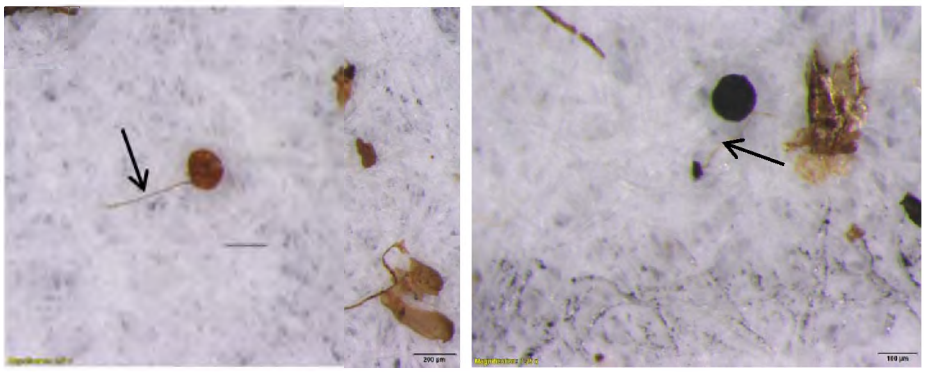


Figure 3.1: AM fungal spores extracted from the soil samples and observed using a dissecting microscope. The arrows show where the subtending hyphae can be seen still attached to the spores.

3.3 Mycorrhizal colonisation assessment

Examination of stained roots revealed AM fungal hyphae growing intercellularly between root cells (Fig. 3.2), arbuscules were not readily observed. Colonisation was confirmed in roots from all sites with an average of 38.3%.

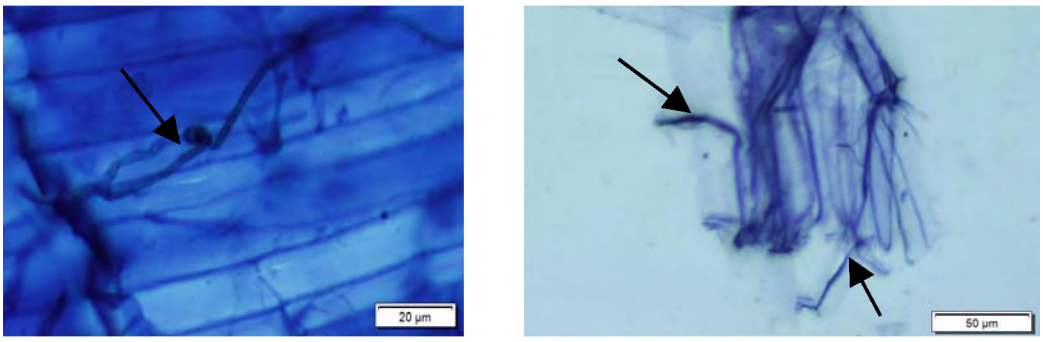


Figure 3.2: Roots of *Vachellia erioloba* stained with lactoglycerol trypan blue. The arrows show the intercellular hyphae present between the root cells.

3.4 Effect of Cadmium on seedling growth

After three month's exposure to Cd, Camel thorn seedlings were harvested. Final shoot height was recorded. Total plant mass was recorded, shoots and roots were

weighed separately and recorded (Fig. 3.5). Because of their fragile nature, the roots and shoots were placed in a plant press and left to dry. After drying, the root weight was recorded and corrected for the weight of root subsample removed for staining.

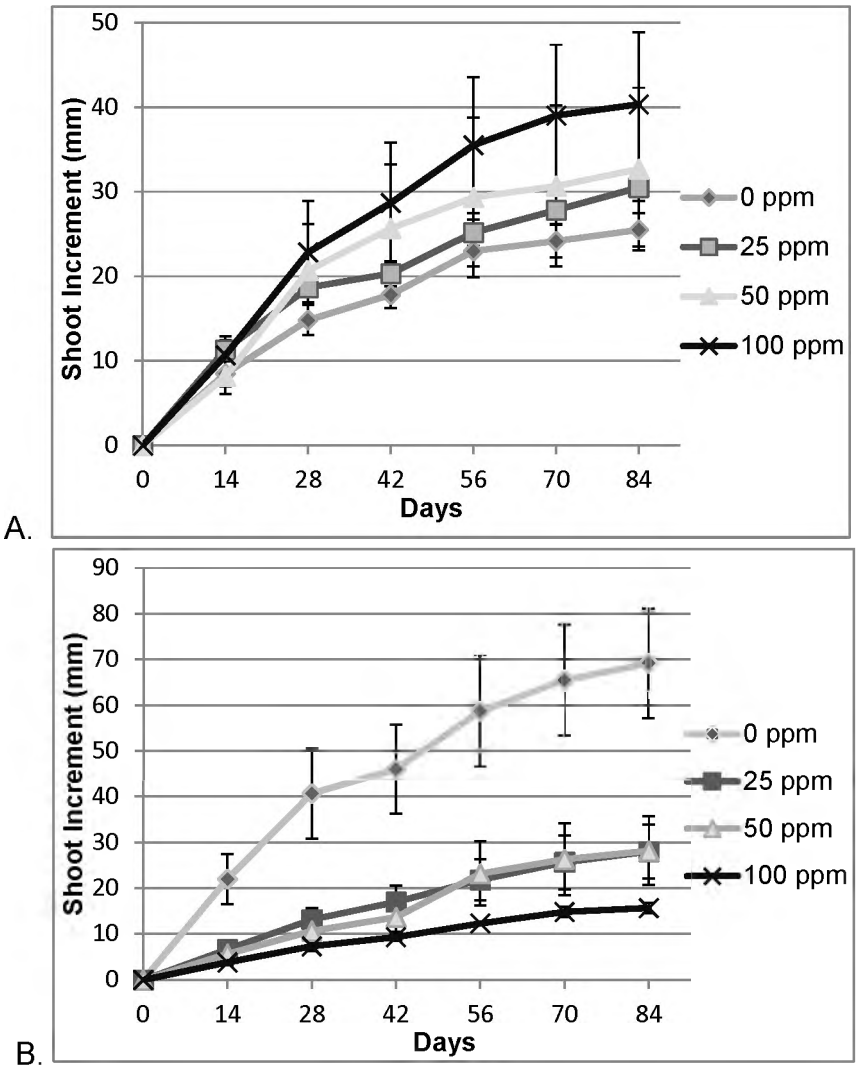


Figure 3.3: Shoot height increment of AM fungal (A) inoculated ($F_{(3, 120)}$ = 4.032, P = 0.009, n = 6) and (B) uninoculated ($F_{(3,120)}$ = 46.9, P < 0.001, n = 6), *Vachellia erioloba* seedlings exposed to Cd over a time period of three months. Points represent means ± standard errors.

The inoculated heavy metal treatments had an average growth rate of 0.38 mm/day and the uninoculated heavy metal treatments had an average growth rate of 0.42

mm/day. During the three month growth period the AM fungal inoculated 100 ppm treatment had the most growth, whereas the inoculated 0 ppm treatment had the lowest shoot growth (Fig. 3.3-A). The uninoculated control (0 ppm) had the highest growth rate and the 100 ppm uninoculated treatment had the lowest growth (Fig.3.3-B). A significant difference was found between the treatments of the inoculated ($P = 0.009$) and uninoculated ($P < 0.001$) treatments.

An increase in the overall shoot height of *V. erioloba* seedlings in the different treatments is shown in Figure 3.4. The uninoculated control seedlings grew an average of 69.17 mm compared to the 100 ppm uninoculated treatment that only grew 15.67 mm. The AM fungal inoculated control grew only 25.5 mm, while exposed to 100 ppm Cd the growth difference was 40.33 mm. The three inoculated Cd treatments had higher shoot growth than the uninoculated Cd treatments. Between the inoculated treatments, the shoot height increased as the Cd concentration increased. In both inoculated and uninoculated treatments the 50 ppm Cd treatments had a similar shoot increment to the 25 ppm Cd treatment. A significant difference was found between the different AM fungal inoculated and uninoculated treatments ($P < 0.001$).

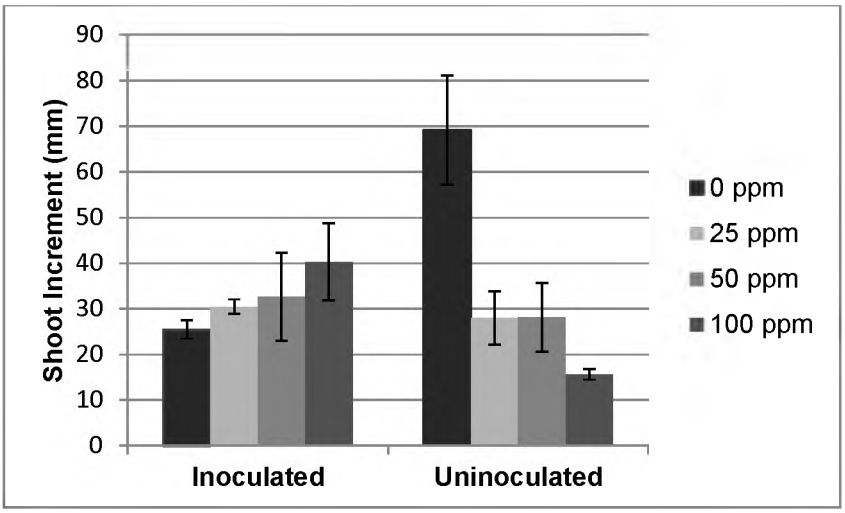
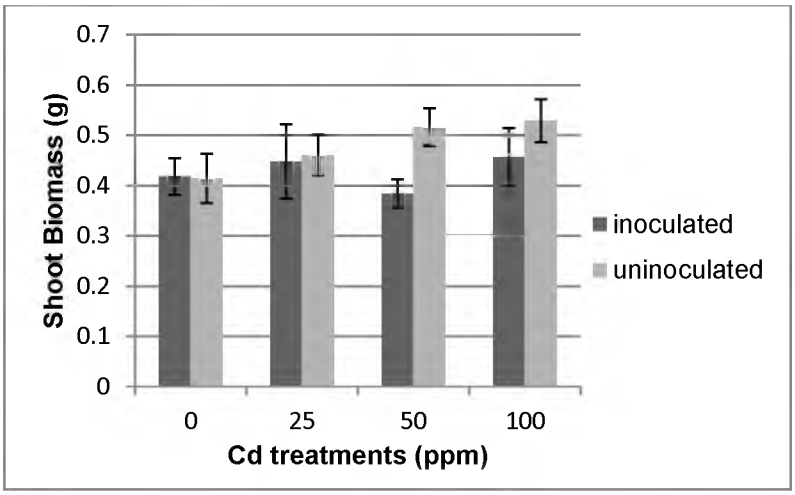


Figure 3.4: Shoot height increment of the AM fungal inoculated and uninoculated *Vachellia erioloba* seedlings at each Cd concentration ($F_{(7, 35)}$ = 49.253, P < 0.001, n = 6). Columns represent means ± standard errors.

Shoot biomass of inoculated and uninoculated controls were similar (Fig. 3.5-A). The uninoculated Cd treatments produced slightly more shoot biomass than the inoculated Cd treatments, although there was no significant difference (P = 0.404) between the treatments due to the high variability.

A.



B.

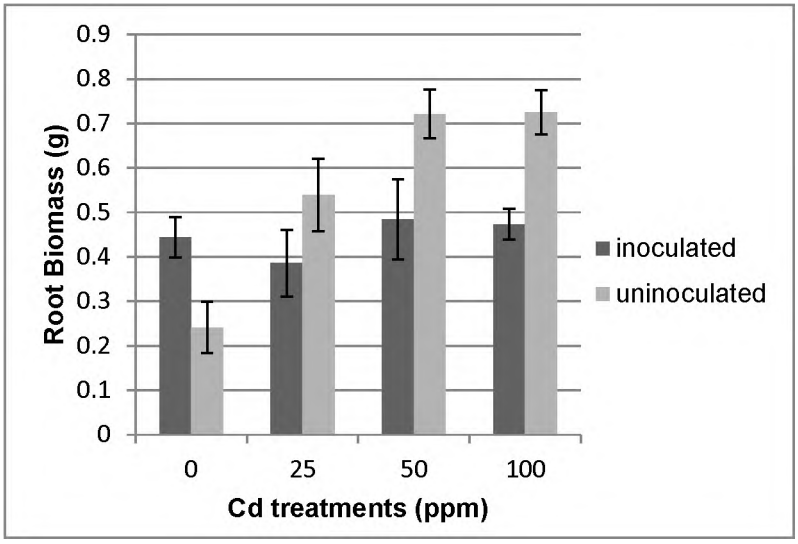


Figure 3.5: Biomass of *Vachellia erioloba* seedlings exposed to different cadmium (Cd) concentrations with and without AM fungal inoculum. A) Shoot biomass of *Vachellia erioloba* seedlings, $F_{(7, 40)} = 1.064$, $P = 0.404$, $n = 6$. B) Root dry biomass of *Vachellia erioloba*, $F_{(7, 40)} = 6.406$, $P < 0.001$, $n = 6$. Columns represent means ± standard error.

The root biomass of the inoculated control (0 ppm) (0.444 g) treatment was higher than the uninoculated control (0 ppm) treatment (0.241 g) (Fig. 3.5-B). The root biomass of the different uninoculated Cd treatments was higher than the inoculated Cd treatments. A significant difference ($P < 0.001$) was found between the root biomass production of the different treatments. The significant differences in root biomass are illustrated in Table 3.3.

Table 3.3: Least significant difference (P = 0.05) in root biomass between the different Cd treatments.

		Control	Control	25	25	50	50	100	100
		I	U	I	U	I	U	I	U
Control	**I**		X				X		X
Control	**U**	X			X	X	X	X	X
25	**I**						X		X
25	**U**		X						
50	**I**		X				X		X
50	**U**	X	X	X		X		X	
100	**I**		X				X		X
100	**U**	X	X	X		X		X	

I – Inoculated U – Uninoculated

X – Significant difference

The ability of the AM fungal inoculum to successfully colonise the roots decreased as the Cd concentration increased (Table 3.5). The colonisation ranged from 19.5% to 20% when the Cd concentration increased from 0 ppm to 25 ppm. The colonisation decreased from 20% to 5.83% with each doubling of the Cd concentration. A significant difference was found between the inoculated treatments (P = 0.007). Although reduced, colonisation was successful in the AM fungal inoculated seedlings (Fig. 3.6).

Table 3.4: AM fungal colonisation of *Vachellia erioloba* seedlings exposed to different Cd concentrations treatments, $F_{(3, 20)} = 5.289$, P = 0.007. Values represent means ± standard error.

Treatments	Colonisation (%)
0 ppm	19.5 ± 1.231 [a*]
25 ppm	20 ± 4.837 [a, b]
50 ppm	10.33 ± 4.386 [b]
100 ppm	5.83 ± 2.455 [a]

* Different letters indicate significant differences.

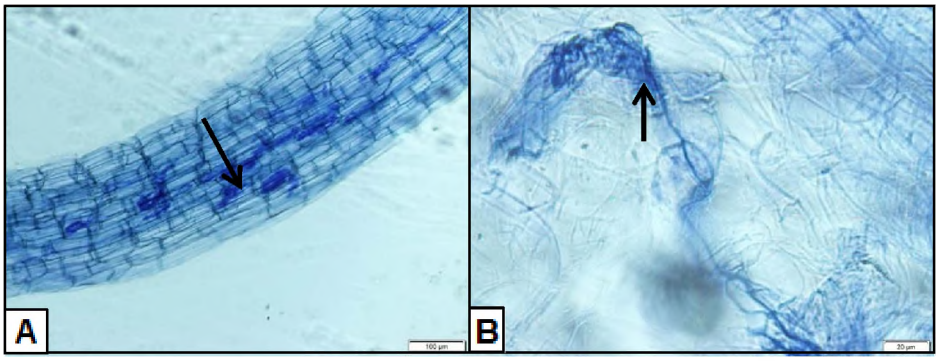


Figure 3.6: Intercellular hyphae (arrows, A and B) present in root cells indicate colonisation of *Vachellia erioloba* seedlings.

The shoots of the uninoculated treatments had higher Cd concentrations than the shoots of the AM fungal inoculated treatments (Fig. 3.7-A). The uninoculated 50 ppm Cd exposed treatment had the highest concentration (17.058 mg/g) between all the shoots from the different treatments. The shoots of the AM fungal inoculated 100 ppm Cd exposed treatment had the lowest Cd concentration (10.216 mg/g). No significant difference was found between the Cd concentrations of the different treatments. The roots of the uninoculated control had the highest Cd concentration (20.119 mg/g), with the roots of the inoculated 25 ppm treatment (20.025 mg/g) having the second highest concentration (Fig. 3.7-B).

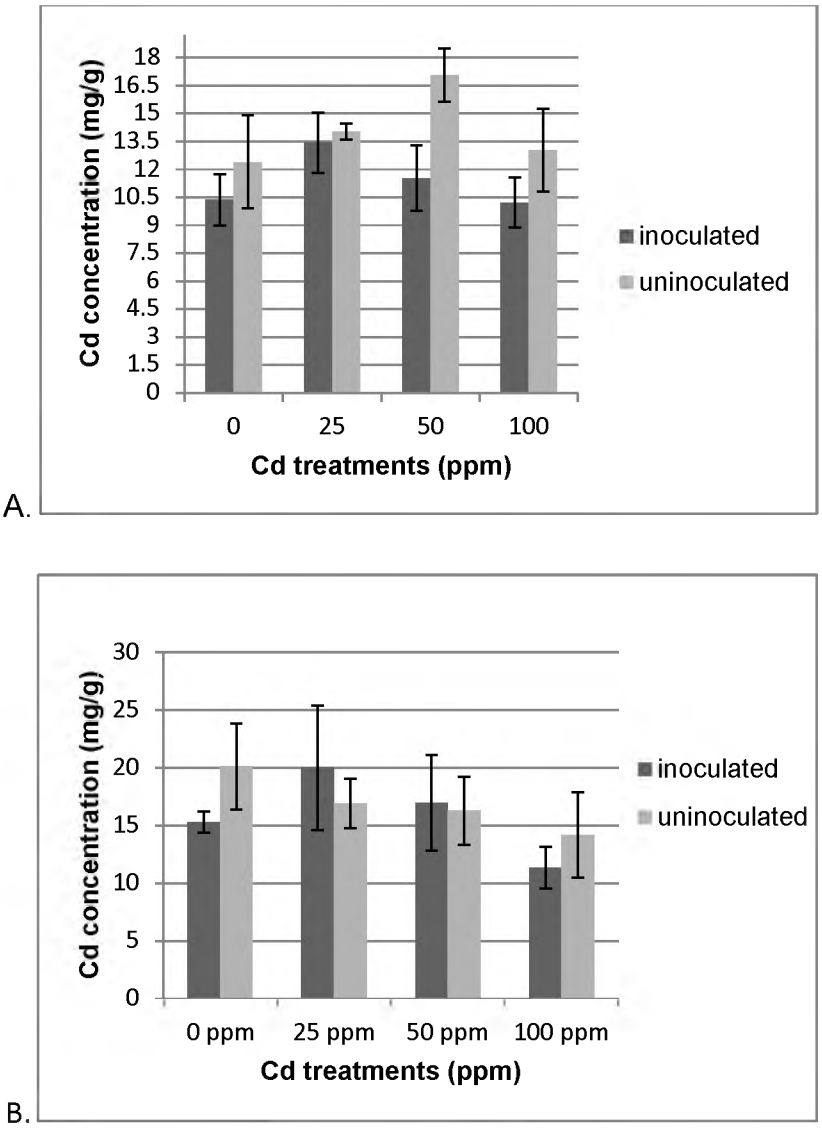


Figure 3.7: Cd concentrations (mg/g) as determined by ICP-EOS analysis of the (A) shoot, $F_{(7, 40)} = 1.733$, $P = 0.129$, $n = 7$, and (B) root, $F_{(7, 40)} = 0.736$, $P = 0.643$, $n = 7$, components of *Vachellia erioloba* seedlings. Columns represent means ± standard error.

The roots of the AM fungal inoculated 100 ppm Cd exposed treatment had the lowest Cd concentration (11.375 mg/g). The uninoculated control (0 ppm) and 100 ppm Cd exposed treatments had higher Cd concentrations than the inoculated control and 100 ppm treatments. Whereas, the inoculated 25 ppm and 50 ppm Cd exposed treatments had higher Cd concentrations than the uninoculated 25 ppm and

50 ppm treatments. No significant difference was found between the roots of the different treatments.

3.5 Isolation and characterisation of plant growth promoting rhizobacteria

A total of 57 pure bacterial cultures were isolated from the soils (Appendix F). Gram staining was performed on all the isolates to ensure pure cultures were obtained. All isolates were tested for nitrogen fixation, phosphate solubilisation, siderophore production, indole acetic acid production and biofilm formation. Of the 57 initial isolates, 14 were selected for identification based on a positive characterisation test, only 1 of these were gram positive and all were rod shaped (Table 3.1). All 14 isolates grew on the Ashby's Mannitol agar, indicating their ability to fix nitrogen. The range at which phosphate was solubilised on the NBRIP media was between 1.4 PSI and 6.132 PSI for the 14 isolates. Isolate F10 having highest solubilisation ability (Table 3.5^{d}). A significant difference was found between the 14 isolates ($P < 0.001$) for phosphate solubilisation. All the isolates produced siderophores on the CAS media (Fig. 3.8), which ranged between 28.94% (F4) and 66.66% (F11) siderophores (Table3.5_{b}). A significant difference in siderophore production between isolates was recorded ($P < 0.001$).

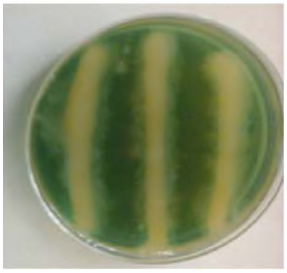


Figure 3.8: An overlay CAS media plate after incubation, inoculated with F6. Clear zones around the streaks can be clearly seen indicating a positive result for siderophore production.

Table 3.5: PGPR characterisation of the selected bacterial isolates. Values represent means ± standard error.

Sample	Gram stain (+/-)	Cell Shape	N_2 Fixation	Phosphate solubilisation (PSI) [a]	Siderophore test (%) [b]	IAA (µg/ml) [c]	Biofilm Production (OD_{575nm}) [d]
F1	-	rod	+	2.97 ± 0.22	53.33 ± 3.33	7.56 ± 0.6	1.06 ± 0.03
F2	-	rod	+	5.49 ± 0.6	43.33 ± 3.33	6.97 ± 0.74	1.2 ± 0.24
F3	-	rod	+	5.9 ± 0.97	35.71 ± 7.14	6.57 ± 2.93	0.26 ± 0.1
F4	+	rod	+	4.43 ± 0.42	41.83 ± 11.04	12.83 ± 4.31	0.96 ± 0.04
F5	-	rod	+	4.53 ± 0.47	44.84 ± 12.07	12.32 ± 3.57	1.00 ± 0.03
F6	-	rod	+	5.56 ± 0.53	51.59 ± 11.02	4.62 ± 1.06	1.82 ± 0.75
F7	-	rod	+	4.63 ± 0.45	55.56 ± 5.55	2.38 ± 0.34	2.2 ± 0.6
F8	-	rod	+	3.92 ± 0.39	51.11 ± 8.89	6.42 ± 3.08	0.3 ± 0.3
F9	-	rod	+	5.74 ± 0.38	63.69 ± 12.39	3.26 ± 1.19	0.88 ± 0.21
F10	-	rod	+	6.31 ± 0.85	71.08 ± 6.3	2.54 ± 1.69	1.85 ± 0.74
F11	-	rod	+	1.41 ± 0.05	20 ± 0	4.62 ± 1.06	0.25 ± 0.07
F12	-	rod	+	1.63 ± 0.19	45.56 ± 13.65	5.00 ± 1.27	2.14 ± 0.48
F13	-	rod	+	2.19 ± 0.27	46.67 ± 3.33	7.16 ± 3.53	1.58 ± 0.34
F14	-	rod	+	4.25 ± 0.0	36.11 ± 7.35	8.33 ± 2.03	0.57 ± 0.13
Significant Differences				$F_{(13, 28)}$ = 10.687 P < 0.001 n = 3	$F_{(13, 28)}$ = 4.14 P < 0.001 n = 3	$F_{(13, 28)}$ = 1.383 P = 0.228 n = 3	$F_{(13,28)}$ = 3.302 P = 0.004 n = 3

Isolates F4 (12.83 µg/ml) and F5 (12.29 µg/ml) produced the highest IAA concentrations of the 14 isolates (Table 3.5c). Figure 3.9 shows the IAA production of the bacterial isolates. No significant difference was found between the IAA concentrations of the 14 isolates. Biofilms on the walls of the microtiter plate (Figure 3.10) and floating biofilm or pellicles were also observed before the wells were washed. Isolate F7 had the highest biofilm production between all the isolates. A significant difference for biofilm production was found (P = 0.004) between the isolates.

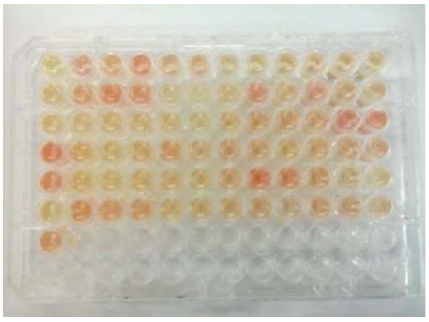


Figure 3.9: IAA production of 24 of the 57 bacterial isolates that are indicated by a colour change, isolates are replicated.

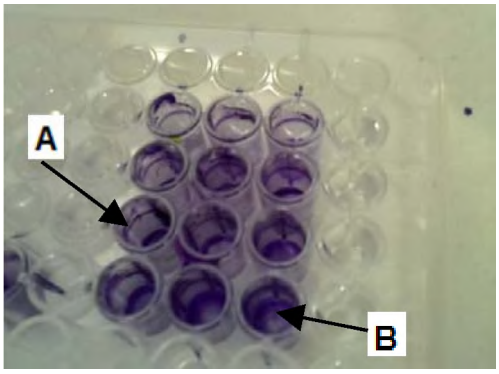


Figure 3.10: Biofilm formation as indicated by 4 isolates. The (A) rings formed in wells indicates biofilm formation. Some of the pellicles (B) are still present even after the liquid was removed.

3.6 Molecular identification of plant growth promoting rhizobacteria

Sequencing of the 16S rDNA was conducted to identify the genus and species of the isolates. Figure 3.11 shows the agarose gel of the 14 isolates after PCR amplification and gel clean-up. The band size was approximately 1500 bp prior to sequencing.

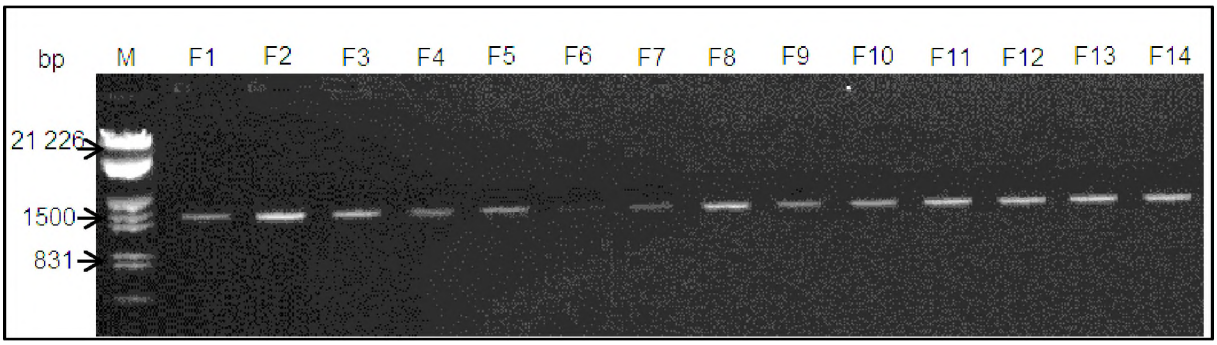


Figure 3.11: The PCR products visualised on a 1% agarose gel stained with ethidium bromide (0.5 µg/ml). The Promega Lambda/*EcoR1* + *HindII* DNA marker (M) is shown in the first lane followed by the 14 isolates, which indicates successful PCR amplification. The DNA size of the isolates was approximately 1500 bp.

Sequences received from Inqaba Biotechnologies were analysed using FinchTV 1.4.0 (Geospiza software). The isolates were identified by using BLAST on the NCBI website (http://www.ncbi.nlm.nih.gov). The sequences were submitted to GenBank and accession numbers were obtained. All of the isolates were identified up to at least genus level. Of the 14 isolates 11 were identified as *Enterobacter* species and the remaining three isolates were identified as *Bacillus cereus*, *Pantoea septica* and *Acinetobacter calcoaceticus* (Table 3.6).

Table 3.6: Molecular identification of the bacterial isolates.

Isolate	Accession number	Identification	Aligned sequence	E-value	% Query coverage	% Identity
F1	KU667098	*Pantoea septica*	KJ534279.1	0	100	100
F2	KU667099	*Enterobacter* sp.	AB673459.1	0	100	99
F3	KU667100	*Enterobacter* sp.	AB673459.1	0	100	99
F4	KU667101	*Bacillus cereus*	KU512628.1	0	100	99
F5	KU667102	*Enterobacter* sp.	AB673459.1	0	98	99
F6	KU667103	*Enterobacter* sp.	AB673459.1	0	97	96
F7	KU667104	*Enterobacter* sp.	AB673459.1	0	100	99
F8	KU667105	*Acinetobacter calcoaceticus*	AB862147.1	0	99	100
F9	KU667106	*Enterobacter* sp.	AB673459.1	0	100	99
F10	KU667107	*Enterobacter* sp.	AB673459.1	0	97	99
F11	KU667108	*Enterobacter* sp.	AB673459.1	0	100	99
F12	KU667109	*Enterobacter* sp.	AB673459.1	0	100	99
F13	KU667110	*Enterobacter* sp.	AB673459.1	0	100	99
F14	KU667111	*Enterobacter* sp.	AB673459.1	0	100	99

3.6.1 Differentiation of *Enterobacter* species

After the DNA restriction digestion of the eleven *Enterobacter* isolates it was found that all eleven isolates were the same *Enterobacter* species.

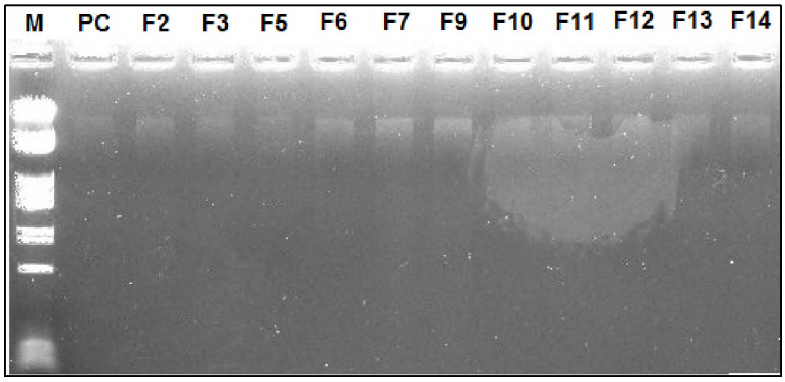


Figure 3.12: Products formed after the DNA digestion of eleven Enterobacter sp. isolates with the Xbal restriction enzyme. The promega Lambda/*EcoR1* + *HindII* DNA marker (M) is shown in the first lane followed by a positive control and the eleven isolates.

3.7 Assessment of mycorrhizal and bacterial populations from *Vachellia erioloba* rhizospheric soil.

The extracted soil DNA was divided in order to be used for the amplification of bacterial and AM fungal DNA. The bacterial DNA was amplified and visualised on an agarose gel (Figure 3.13 - A) with an approximate base pair size of 500 bp. The fungal DNA was amplified and visualised on an agarose gel (Figure 3.13 - B), which had a band size of approximately 550 bp (tagged primers included).

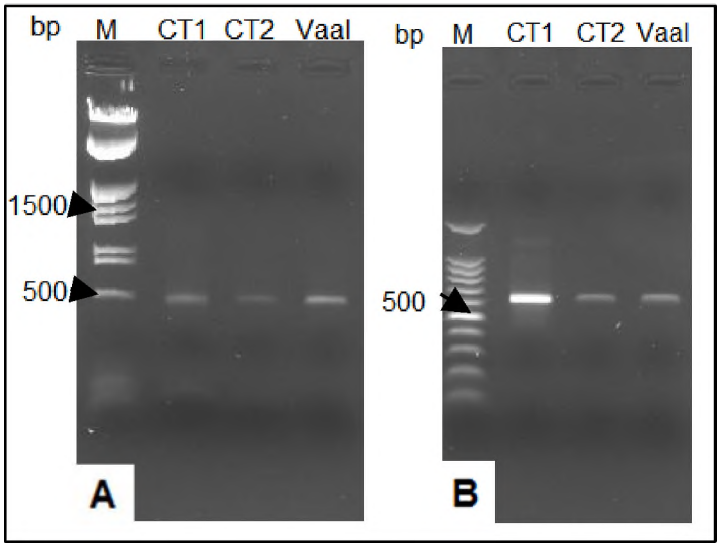


Figure 3.13: (A) The PCR products were visualised on a 1% agarose gel stained with ethidium bromide (0.5 µg/ml) and using the Lambda/EcoR1 + HindII DNA marker (M) for (A) bacterial PCR products and the 100 bp DNA molecular marker for (B) the AM fungal PCR products.

3.7.1 Illumina sequencing analysis of the arbuscular mycorrhizal fungal data

Only two sites, namely Camel thorn 2 and Vaal, were successfully sequenced. The remaining sequences after curation were separated into Operational Taxonomic Units (OTU) with a species identity threshold of 97% (Appendix H). A summary of the curation steps is shown in Table 3.8.

A total of 131 824 sequences were assigned to 105 376 OTUs of which, 4434 OTUs had two or more sequences assigned (Table 3.7). A total of 100 942 singletons were found during the curation of the sequences. There were only 10 OTUs that had overlapping sequences. The twenty OTUs which had the highest number of sequences, were selected and identified by performing a stand-alone BLAST using the MaarJAM database (Öpik *et al.*, 2010). The sequences were submitted to GenBank on the NCBI website (http://www.ncbi.nlm.nih.gov) to obtain accession numbers.

Table 3.7: Summary of the arbuscular mycorrhizal fungal Illumina sequencing data curation steps.

Curation steps		Number of reads	
		Camel thorn 2	Vaal
Summary at the start		81707	94927
Trim all sequences <300 bp		74718	90176
Merge files	Total	164894	
	Unique	147767	
Align sequences	Total	164887	
	Unique	147760	
Precluster and trim	Total	134362	
	Unique	107407	
Remove chimeras (Uchime)	Total	131824	
	Unique	105376	
Final number of sequences		58059	73765
Number of OTUs (singletons excluded)		2051	2383

The sequences were grouped into phyla (Figure 3.14). The Glomeromycota phylum was not identified during the analysis and was assigned to the unknown phylum. Most of the sequences were classified under the Ascomycota phylum, which makes it the dominant phylum between all the sequences.

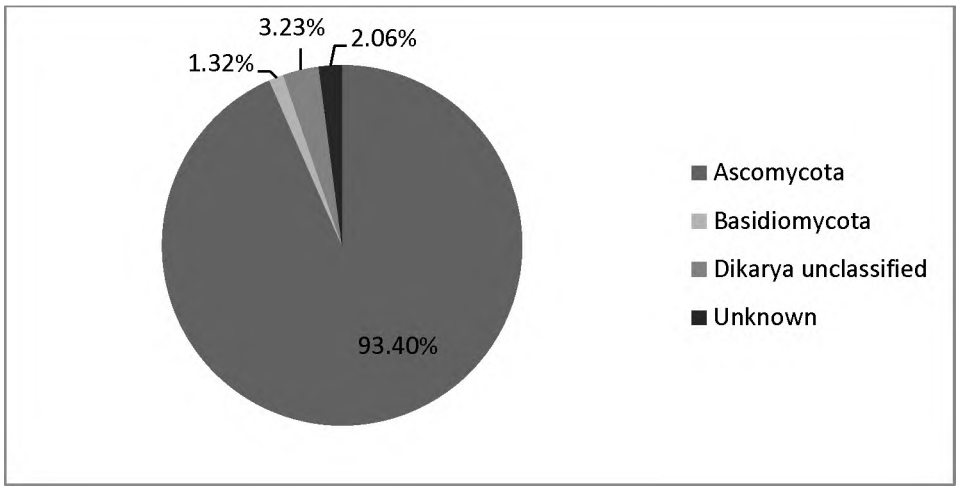


Figure 3.14: Proportions of the 18S fungal OTUs that were assigned to the different phyla. The arbuscular mycorrhizal fungal phylum (Glomeromycota) was assigned to Unknown.

Table 3.8: Identification of the arbuscular mycorrhizal fungal sequences.

OTU	GenBank Accession number	Identification		MaarJAM Aligned sequence	E	% Qc	% ID
		Family	Genus				
1	KY617872	Ambisporaceae	*Ambispora*	AB047302	0.0	100	88.0
2	KY617873	Ambisporaceae	*Ambispora*	AB047302	0.0	100	87.8
3	KY617874	Ambisporaceae	*Ambispora*	AJ301861	0.0	100	89.3
4	KY617875	Ambisporaceae	*Ambispora*	JX999461	0.0	100	88.7
5	KY617876	Ambisporaceae	*Ambispora*	AB047308	0.0	100	88.0
6	KY617877	Paraglomeraceae	*Paraglomus*	JX144113	0.0	100	86.8
7	KY617853	Ambisporaceae	*Ambispora*	AB015052	0.0	100	88.1
8	KY617878	Ambisporaceae	*Ambispora*	JX999461	0.0	100	87.5
9	KY617892	Ambisporaceae	*Ambispora*	AB047305	0.0	100	87.7
10	KY617854	Paraglomeraceae	*Paraglomus*	HE576912	0.0	93.1	88.1
11	KY617855	Ambisporaceae	*Ambispora*	JX999461	0.0	100	88.2
12	KY617879	Ambisporaceae	*Ambispora*	JX999461	0.0	100	88.2
13	KY617880	Ambisporaceae	*Ambispora*	FN820274	0.0	100	88.2
14	KY617857	Glomeraceae	*Glomus*	KF467296	0.0	31.1	100
15	KY617881	Ambisporaceae	*Ambispora*	GU238387	0.0	100	87.8
16	KY617856	Paraglomeraceae	*Paraglomus*	EU123462	0.0	99.7	91.0
17	KY617882	Ambisporaceae	*Ambispora*	JX999461	0.0	100	87.5
18	KY617883	Paraglomeraceae	*Paraglomus*	HE613456	0.0	100	88.1
19	KY617884	Ambisporaceae	*Ambispora*	JX999461	0.0	100	87.7
20	KY617858	Ambisporaceae	*Ambispora*	JX999461	0.0	100	88.2

E – E-value

Qc – percentage Query coverage

ID – percentage identification

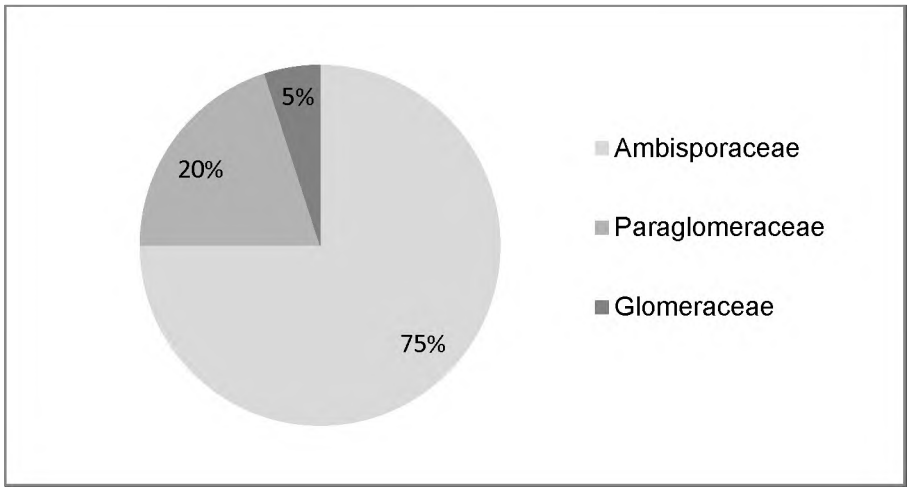


Figure 3.15: Proportions of the arbuscular mycorrhizal fungal families identified that are classified under the Glomeromycota phylum.

Only three AM fungal families were identified, namely Ambisporaceae, Paraglomeraceae, and Glomeraceae. The genera in Table 3.8 are given as tentative identifications. Ambisporaceae was the dominant AM fungal family, followed by Paraglomeraceae and Glomeraceae (Fig. 3.15).

3.7.2 Illumina sequencing analysis of bacterial data

All three sites were successfully sequenced and curated using Mothur (Appendix G). The sequences were separated into OTUs using a species identity threshold of 97%. The bacterial sequences showed high diversity after the curation was completed (Fig. 3.16). A summary of the curation steps is shown in Table 3.9.

Table 3.9: Summary of the bacterial Illumina sequencing data curation steps.

Curation steps		**Number of reads**		
		Camel thorn 1	Camel thorn 2	Vaal
Summary at the start		49 932	121 412	178194
Trim all sequences <350 bp		49 215	119 788	175 031
Merge files	Total		344 034	
	Unique		323 904	
Align sequences	Total		344 034	
	Unique		323 034	
Precluster and trim	Total		329 336	
	Unique		268 461	
Remove chimeras (Vsearch)	Total		306 271	
	Unique		246 359	
Final number of sequences		44 145	107 480	149 519
Number of OTUs (including simpletons)			132 432	
Number of dominant OUT's (excluding singletons)			26 046	

A total of 349 538 bacterial sequences were obtained after sequencing was completed. The Vaal site had the most number of sequences and the Camel thorn 1 site had the least. After the sequences were curated, they were assigned to 132 432 OTUs, of which 26 046 OTUs had two or more sequences assigned to it. The twenty OTUs having the most sequences assigned, were identified and submitted to GenBank (Table 3.10).

Eight sequences were identified as being uncultured bacteria and 12 were identified to either phylum or genus level (Table 3.10). The 12 species identified, belong to three phyla namely, Actinobacteria (10 sequences), Bacteroidetes (1 sequences), and Firmicutes (1 sequence). The Actinobacteria phylum was the dominant bacterial phylum amongst all the known sequences followed by, Bacteroidetes, Proteobacter, and Firmicutes (Fig. 3.16).

Table 3.10: Identification of the bacterial sequences.

	Accession number	Identification	Phylum	Aligned sequence	E-value	% Qc	% ID
1	KY675300	*Mycobacterium sp.*	Actinobacteria	KX607311.1	0.0	100	100
2	KY675302	Uncultured bacterium		JN693762.1	0.0	100	100
3	KY684818	Uncultured Bacterium		JF437533.1	0.0	100	100
4	KY684821	Uncultured *Actinobacterium*	Actinobacteria	KJ849433.1	0.0	100	100
5	KY675303	Uncultured *Blastococcus* sp.	Actinobacteria	KF956776.1	0.0	100	99
6	KY675301	Uncultured Bacteroidetes bacterium	Bacteroidetes	KC172337.1	0.0	100	100
7	KY675304	*Bacillus* sp.	Firmicutes	KX419199.1	0.0	100	100
8	KY684823	*Streptomyces* sp.	Actinobacteria	LC212885.1	0.0	99	100
9	KY684825	Uncultured Conexibacteraceae bacterium	Actinobacteria	JX505168.1	0.0	100	100
10	KY684826	Uncultured bacterium		LC026878.1	0.0	100	99
11	KY684822	Uncultured bacterium		KT460422.1	0.0	100	100
12	KY684828	Uncultured *Tetrasphaera* sp.	Actinobacteria	JN693944.1	0.0	100	99
13	KY684827	*Microbacterium* sp.	Actinobacteria	KY381889.1	0.0	100	100
14	KY684824	*Actinomycetospora* sp	Actinobacteria	KP126372.1	0.0	100	99
15	KY684820	*Streptomyces* sp.	Actinobacteria	LC212905.1	0.0	100	100
16	KY675312	Uncultured bacterium		JF178796.1	0.0	99	99
17	KY675318	*Arthrobacter* sp.	Actinobacteria	KC160921.1	0.0	100	100
18	KY684819	Uncultured bacterium		HM125317.1	0.0	99	100
19	KY684829	Uncultured bacterium		KJ661885.1	0.0	99	99
20	KY675308	Uncultured bacterium		EU372381.1	0.0	100	99

Qc – Query coverage

ID – Maximim identified

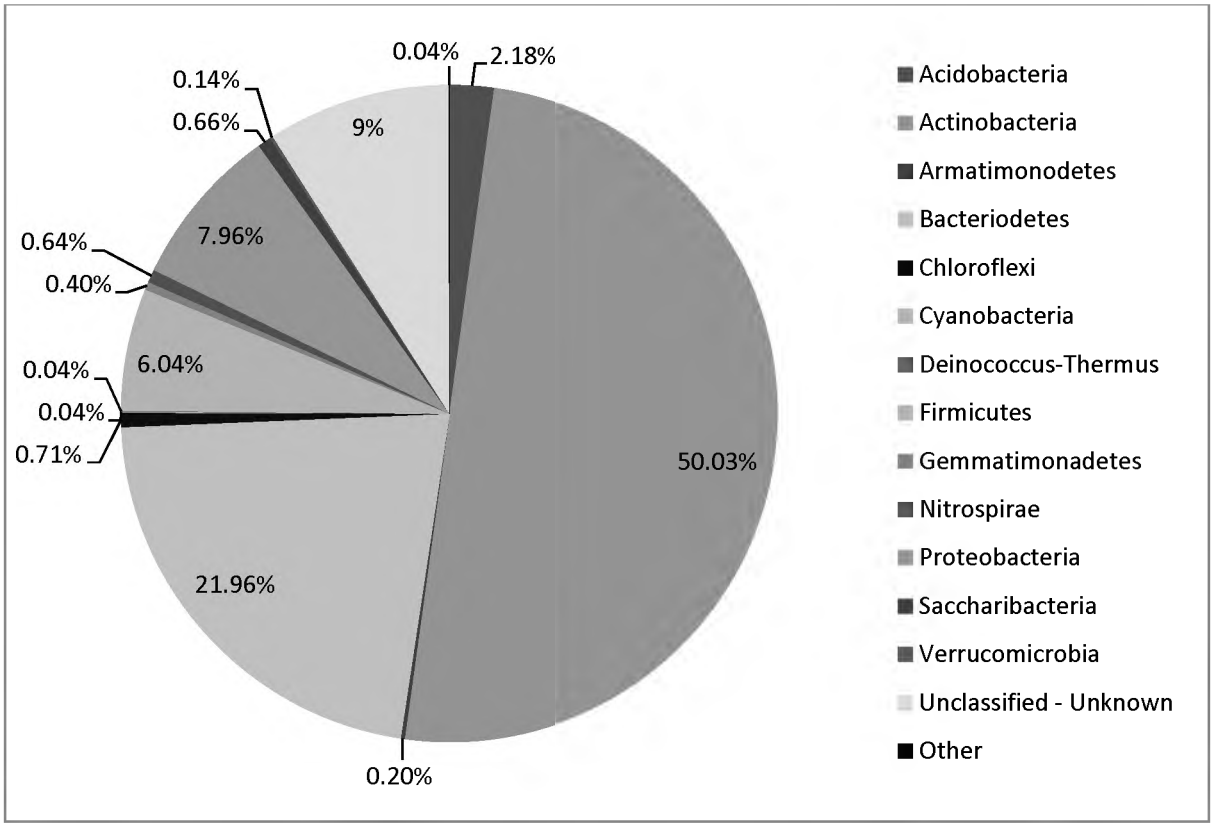


Figure 3.16: Proportions of the 16S bacterial OTUs that were assigned to different phyla.

3.8 Evaluation of the ability of selected bacterial isolates and mycorrhizal fungi to promote seedling growth.

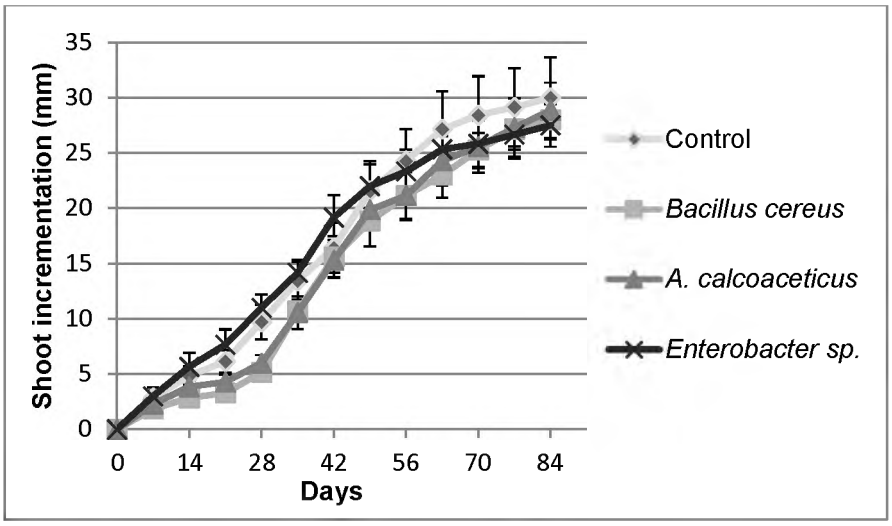
The abilities of the bacteria were tested in two pot trials. The first pot trial tested the abilities of the three selected individual bacterial isolates to promote *V. erioloba* seedling growth which were inoculated and uninoculated with AM fungi. The second pot trial was a combination trial of the three bacterial isolates, inoculated, and uninoculated with AM fungi to test their combined abilities to promote *V. erioloba* seedling growth.

3.8.1 Individual abilities of the isolated bacteria and arbuscular mycorrhizal fungi to promote seedling growth.

The Camel thorn seedlings were harvested after three months and the final shoot height was recorded. Plant biomass of the shoots and roots were recorded separately and placed in brown bags to dry. Shoot and root biomass was recorded after drying and the final root weight was corrected for the subsample removed for staining.

The inoculated treatments had an average growth rate of 0.34 mm/day and the uninoculated treatments had an average growth rate of 0.43 mm/day. The inoculated control treatments had the highest (30 mm) shoot incrementation and the inoculated *Enterobacter* sp. treatment had the lowest (27.5 mm) (Fig. 3.17-A). The inoculated *A. calcoaceticus* treatment had the highest shoot (28.86 mm) increment amongst the bacterial treatments. A significant difference (P = 0.001) was found between the inoculated treatments. The uninoculated *B. cereus* treatment had the highest (42.8 mm) shoot increase and the uninoculated *Enterobacter* sp. treatment had the lowest (30.28 mm) shoot increase (Fig. 3.17-B). No significant difference (P = 0.245) was found between the uninoculated treatments.

A.



B.

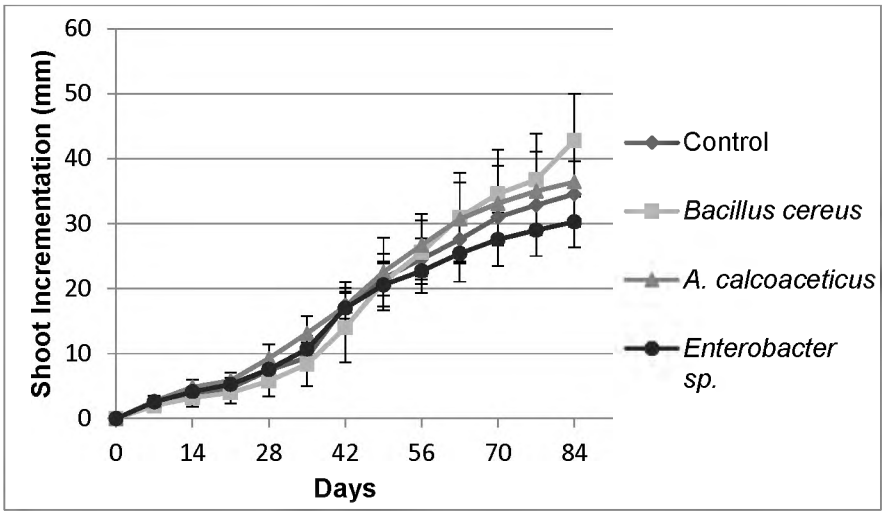


Figure 3.17: Shoot incrementation of (A) AM fungal inoculated, $F_{(3, 276)}$ = 5.309, P = 0.001, n = 7 and (B) uninoculated, $F_{(3, 264)}$ = 1.393, P = 0.245, n = 7, Camel thorn seedlings that were inoculated with bacterial isolates separately. Points represent means ± standard errors.

The shoot incrementation shown in Figure 3.18 correlates with the shoot incrementation of Figure 3.17. The inoculated and uninoculated *Enterobacter* sp. treatments had the lowest shoot incrementation. The uninoculated treatments had higher shoot incrementation than the inoculated treatments (Fig. 3.18). A significant difference (P < 0.001) was found between the inoculated and uninoculated treatments.

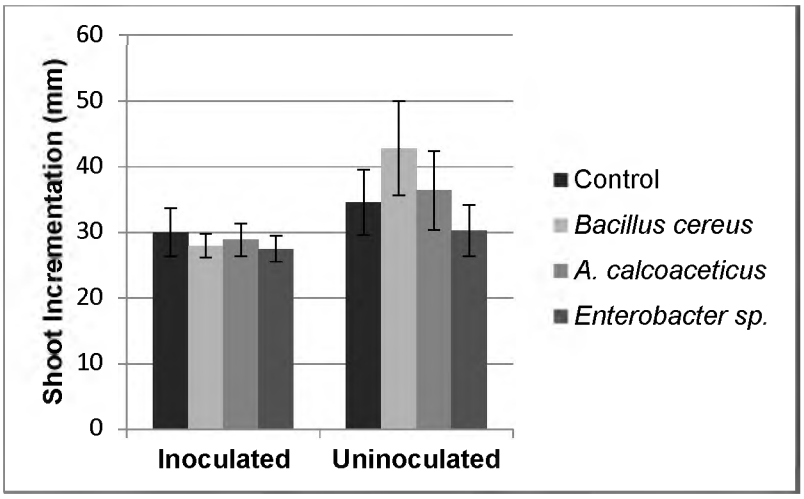


Figure 3.18: Shoot height incrementation of the AM fungal inoculated and uninoculated *Vachellia erioloba* seedlings treated with the different bacteria ($F_{(7, 77)}$ = 5.743, P < 0.001, n = 7). Columns represent means ± standard error.

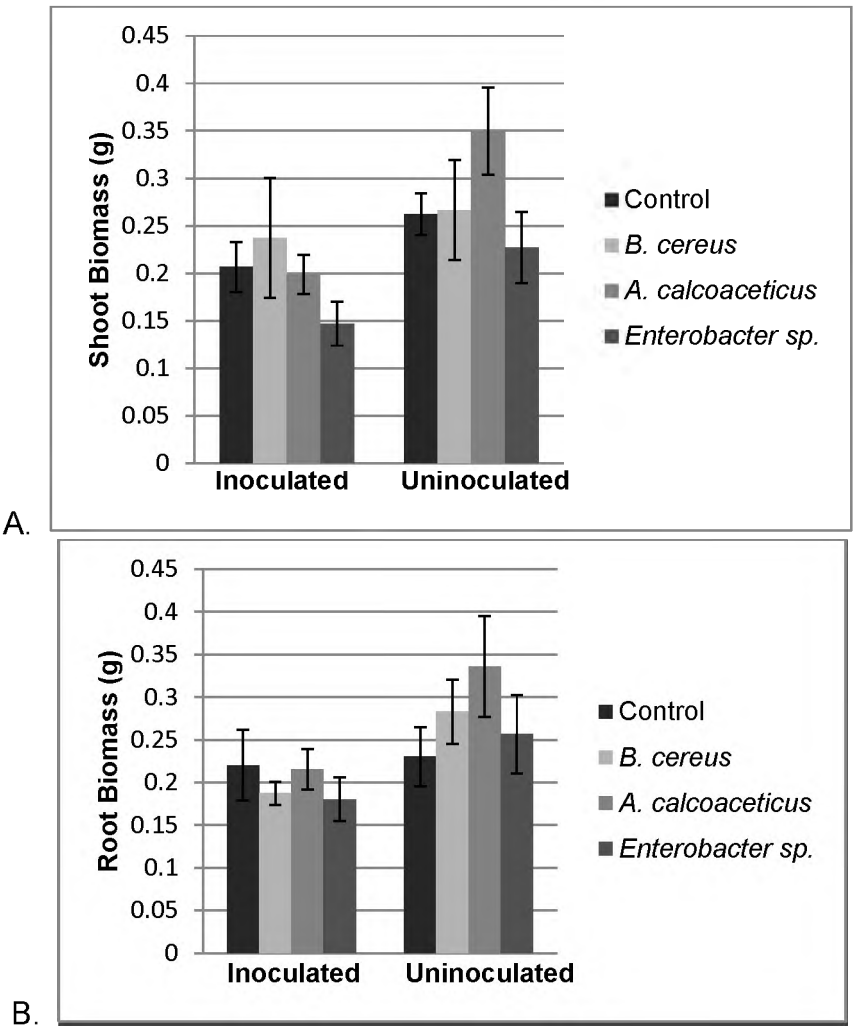


Figure 3.19: Biomass of the *Vachellia erioloba* seedlings exposed to individual bacteria with and without AM fungi. A) Shoot biomass of *Vachellia erioloba seedlings*

($F_{(7, 45)}$ = 3.065, P = 0.01, n = 7). B) Root biomass of *Vachellia erioloba* seedlings ($F_{(7, 45)}$ = 1.831, P = 0.104, n = 7). Columns are means ± standard error.

The shoot biomass of *B. cereus* treatment was the highest (0.24 g) between the inoculated treatments, and *A. calcoaceticus* had the highest shoot biomass (0.35 g) between the uninoculated treatments. The *Enterobacter* sp. treatments had the lowest, inoculated (0.15 g) and uninoculated (0.23 g), shoot biomass (Fig. 3.19-A). A significant difference was found between the shoot biomass of the inoculated and uninoculated treatments (P = 0.01). The root biomass of the inoculated control treatment was the highest (0.22 g) and the inoculated *Enterobacter* sp. treatment had the lowest (0.18 g) root biomass (Fig 3.19-B). The root biomass of *A. calcoaceticus* uninoculated treatments was the highest (0.34 g) and the uninoculated control had the lowest (0.23 g) root biomass. No significant difference was found between the root biomass of the different treatments.

Table 3.11: Percentage colonisation of the AM fungal inoculated *Vachellia erioloba* seedlings treated with individual PGPR ($F_{(3, 24)}$ = 0.648, P = 0.592, n = 7).

Treatments	Colonisation (%)
Control	16.71 ± 3.234
Enterobacter sp.	22.16 ± 2.561
B. cereus	18.42 ± 5.154
A. calcoaceticus	17.57 ± 3.257

The influence of PGPR on AM fungal colonisation is shown in Table 3.11. The inoculated control had the lowest colonisation and the *Enterobacter* sp. treatment had the highest colonisation. The inoculated bacterial treatments had an average colonisation of 18.71% and no significant difference was found between the treatments.

Nematodes were found attached and around the roots after staining (Fig. 3.20 C, D). Their presence was not related to any specific treatment. No clear identification of

the nematodes could be made due to the staining process that washed out any internal structures that could aid identification. Figure 3.20-D shows a nematode trapped by fungi that have colonised the root cells.

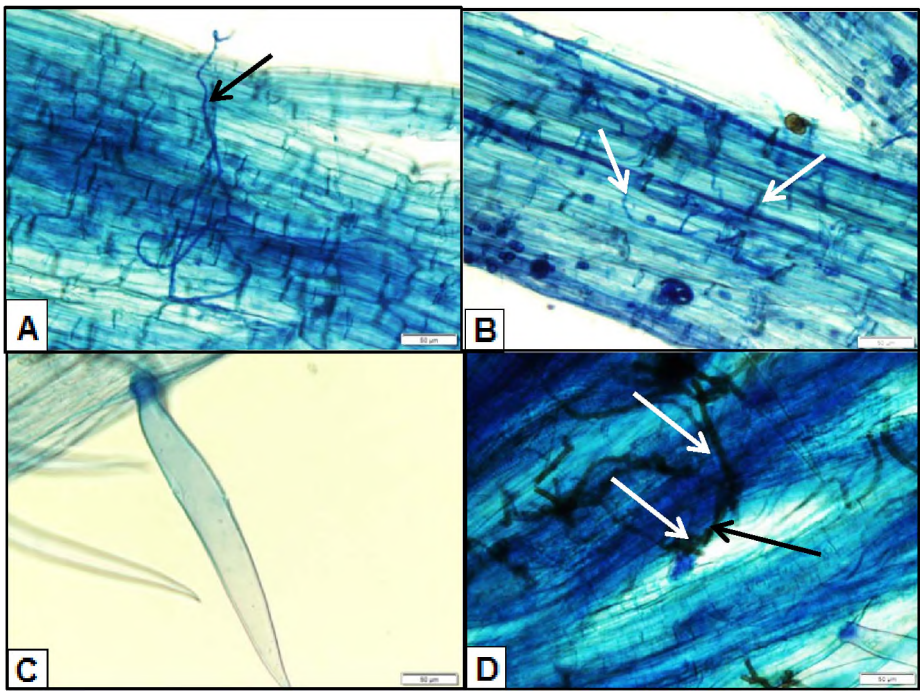
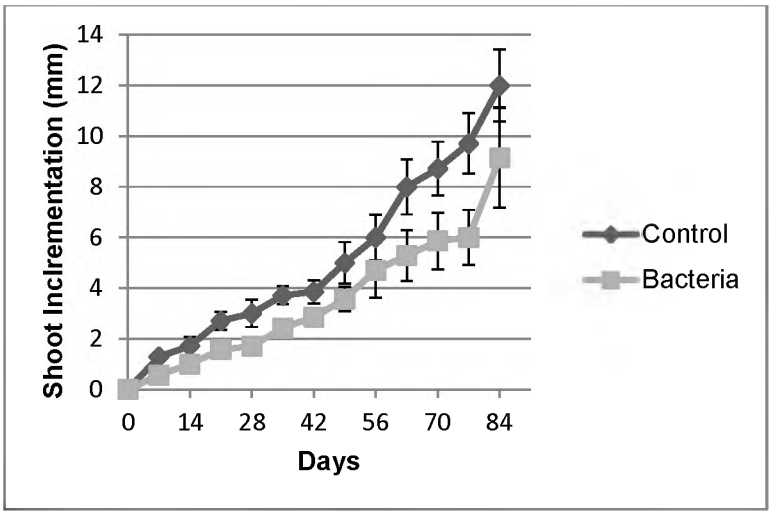


Figure 3.20: Intracellular AM fungal hyphae (arrows A and B) that indicate successful colonisation of *Vachellia erioloba* roots. Nematodes attached to the roots (C). Nematode entangled by fungi (D – arrows).

3.8.2 Combined abilities of the isolated bacteria and arbuscular mycorrhizal fungi to promote *Vachellia erioloba* seedling growth and health.

After harvesting the *V. erioloba* seedlings the final shoot height was recorded. The total plant biomass was recorded and the weights of the roots and shoot were recorded separately. After drying the roots and shoots the dry biomass was recorded and root final weight was corrected for the subsample that was taken.

A.



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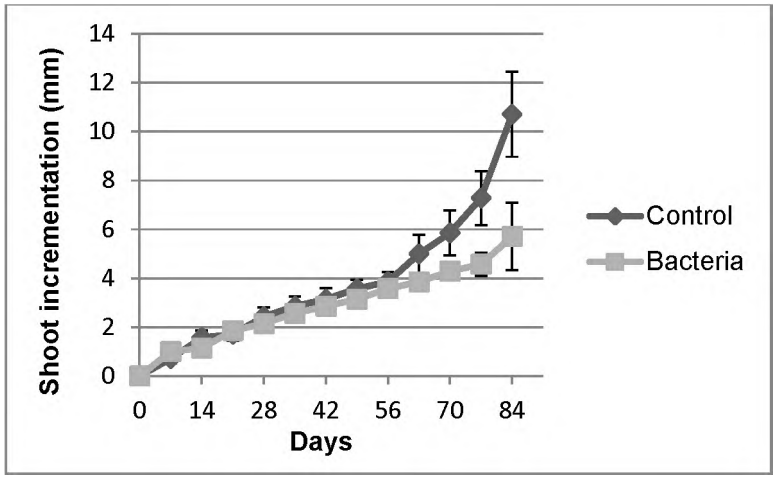

Figure 3.21: Shoot incrementation of (A) AM fungal inoculated ($F_{(1, 144)}$ = 25.472, P < 0.001, n = 7) and (B) uninoculated treatments ($F_{(1, 144)}$ = 15.373, P < 0.001, n = 7) that were inoculated with combined PGPR. Points represent means ± standard errors.

The inoculated treatments had an average growth rate of 0.13 mm/day and the uninoculated treatments had an average growth rate of 0.1 mm/day. In both inoculated and uninoculated treatments, the controls had higher shoot growth (Fig.3.21). A significant difference was found between the control and bacterial treatments for the inoculated (P < 0.001) and uninoculated (P < 0.001) treatments. Shoot growth rate of the bacterial combination treatment (Fig. 3.21) was lower than when individually applied (Fig. 3.17). The AM fungal inoculated treatments had a

higher growth than the uninoculated treatments (Fig. 3.21). A significant difference ($P < 0.001$) was found between the inoculated and uninoculated treatments.

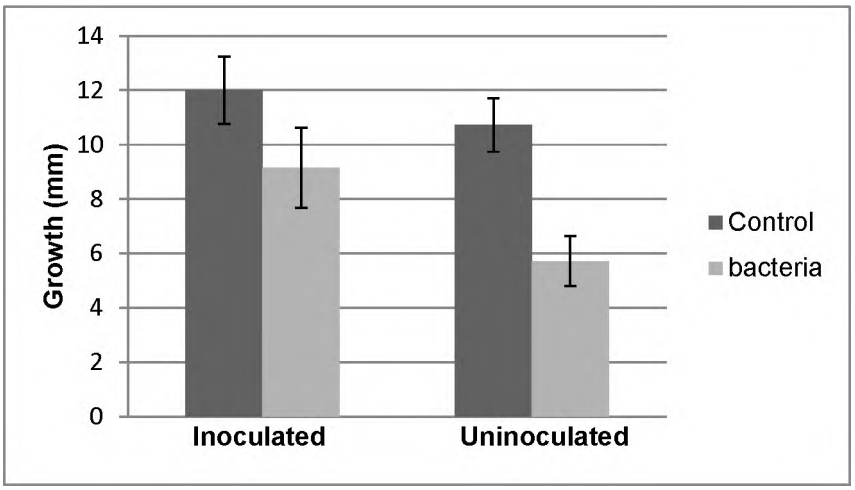

Figure 3.22: Shoot height incrementation of the AM fungal inoculated and uninoculated *Vachellia erioloba* seedlings treated with PGPR ($F_{(3, 33)} = 14.682$, $P < 0.001$, $n = 7$). Columns represent means ± standard error.

The biomass of the combined treated seedlings (Fig. 3.23) was lower than the individual treated seedlings (Fig. 3.18). The root biomass was higher than the shoot biomass (Fig. 3.23). The uninoculated control treatment had higher biomass than the inoculated control treatment, whereas the AM fungal inoculated bacterial treatments had a higher biomass than the uninoculated bacterial treatment. No significant difference was found between the biomass production of the roots and shoots.

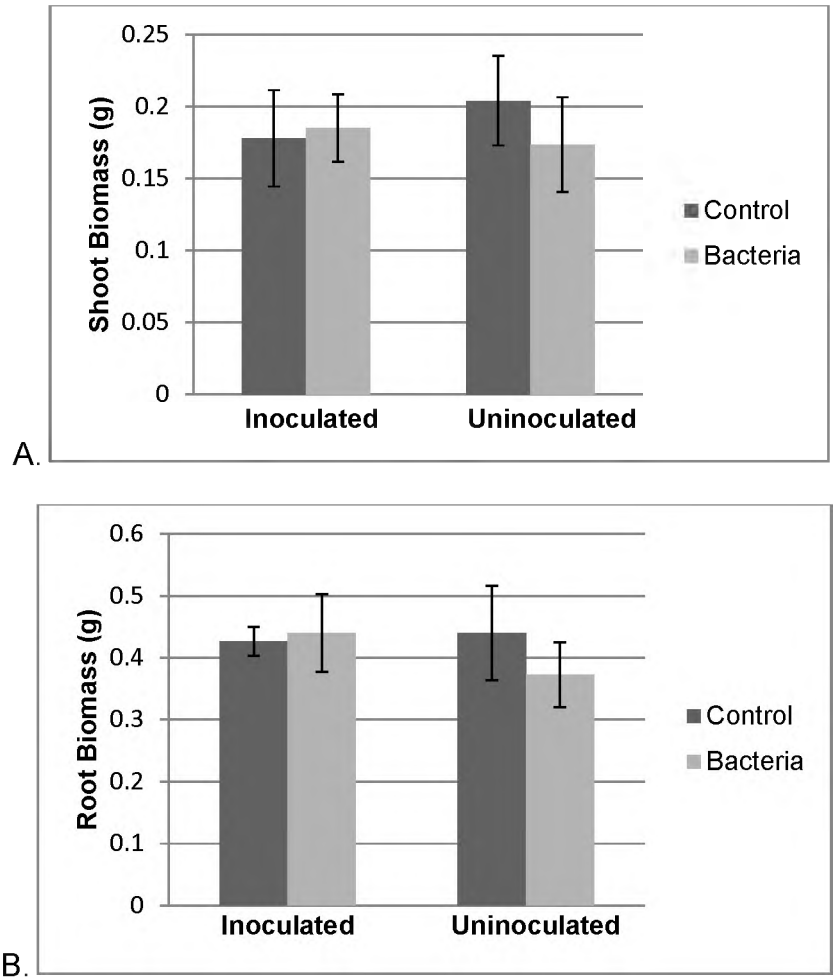

Figure 3.23: Biomass of the *Vachellia erioloba* seedlings treated with combined PGPR that were inoculated with AM fungi and the three bacteria. A) The shoot biomass of the seedlings ($F_{(3, 24)}$ = 0.263, P = 0.851, n = 7). B) The root biomass of the seedlings ($F_{(3, 24)}$ = 0.315, P = 0.814, n = 7). Columns represent means ± standard error.

The AM fungal inoculum successfully colonised the root cells of the *V. erioloba* seedlings (Fig 3.24 A-B). The AM fungal inoculated treatments had an average colonisation of 25.14% (Table 3.12). An increase of 3.14% was found between the colonisation of the inoculated control treatments and the bacterial treatment. No significant difference was found between the inoculated control and bacterial treatments.

Table 3.12: Percentage colonisation of the inoculated *Vachellia erioloba* seedlings in the combination pot trial, ($F_{(1, 10)}$ = 0.002, P = 0.968, n = 7).

Treatments	Colonisation (%)
Control	23.57 ± 5.042
Bacteria	26.71 ± 8.473

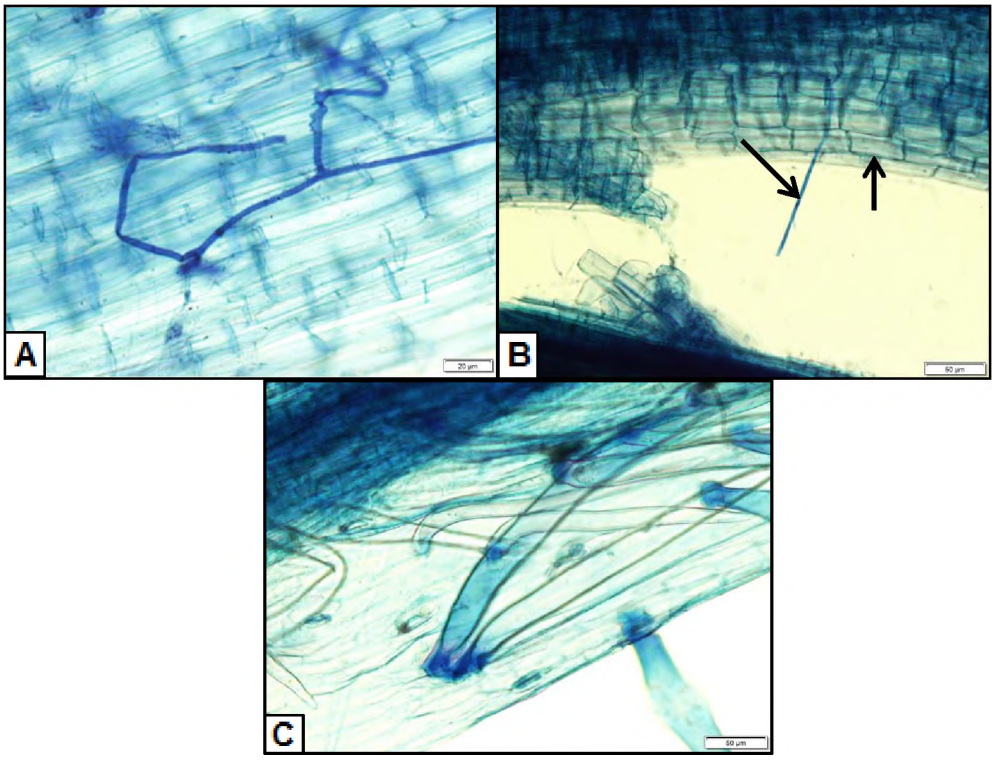

Figure 3.24: (A and B) Intercellular hyphae indication of successful colonisation of *Vachellia erioloba* roots. (C) Nematodes attached to the root cells.

Nematodes were also present amongst the roots (Fig. 3.24 C), they appear to be the same nematodes found in the individual PGPR treated seedlings (Fig. 3.20 C-D). Nematodes were found in all the treatments, inoculated and uninoculated.

Chapter 4

Discussion

4.1 Soil nutrient analysis

The soil is only considered to be nutrient rich if soil pH is above 7, the CEC is above 20 cmol/kg^{-1} and the base saturation is high (100%)(Skarpe *et al.*, 2014). The low concentrations of nutrients and exchangeable cations in the soil are characteristic of semi-arid soil environments. Thus, the soil collected from the Kalahari is nutrient poor and has low carbon content as seen in Table 3.1 and 3.2. The water pH was lower than the potassium chloride (KCl) pH due to the soil analysis that includes the reserved acidity in the colloids of the soil (Follett and Follett, 1983). The soil was moderately acidic, which is characteristic of Kalahari soil (Mphinyane, 2001).

Organic matter impacts on the availability of soil nutrients, structure, water holding capacity, plasticity and cation adsorption capacity (Mphinyane, 2001). Crusts have been found to form on the surface, which consist of organic matter, soil particles cyanobacteria and nitrogen-fixing bacteria (Mphinyane, 2001; Thomas and Dougill, 2007). These crusts protect against wind erosion and nutrient loss. However, there was no presence of a soil crust during sampling, which could be the reason why no nitrogen was detected during the soil analysis. The absence of crusts can be due to grazing disturbances of livestock and wild animals in the area (Thomas and Dougill, 2007).

AM fungi depend on C that is photosynthetically produced by the plant host that is needed for AM fungal growth and development (Wang *et al.*, 2016). Under normal conditions the photosynthetically produced C is distributed through the plant for its own metabolic processes (Jansa *et al.*, 2013). The excess C is transferred to the AM fungi where it is incorporated into the hyphal structures, respired or gradually transferred to the rhizosphere as root exudates where soil bacteria (MHB or PGPR)

can utilise it (Jansa *et al.*, 2013). Under extreme conditions AM fungi are able to adapt to biotic and abiotic stress, however this depends on the AM fungal specie (Millar and Bennett, 2016). In this study the C content in the rhizospheric soil of *A. erioloba* was very low (1.75%) and the AM fungal colonisation was also not very high. This could be due to the generally high temperatures that are experienced in this semi-arid environment. Despite these conditions *V. erioloba* trees from which the rhizospheric soil was collected were still thriving. It is possible that the nutrient exchange despite the limited colonisation was still occurring. Püschel *et al.* (2016) reported that under nutrient deficient conditions competition between the plant host and AM fungi could negate mycorrhizal growth benefits.

4.2 Arbuscular mycorrhizal fungal assessment of Kalahari soil

AM fungi are obligated biotrophs and cannot be cultured without a plant host (Gadkar *et al.*, 2001). It is known that AM fungi can improve growth and nutrient uptake, promoting plant health and sustainability in the environment in exchange for carbon resources from the plant (Bago *et al.*, 2002; Torrecilllas *et al.*, 2012). Different biotic and abiotic factors have been found to affect AM fungal colonisation (Symanczik *et al.*, 2015). Biotic factors such as the introduction of non-native AM fungal species and human activities can change the structure of the native AM fungal species communities. Abiotic factors such as soil characteristics, pollutants, environmental factors, seasonal changes and host age can influence the AM fungal diversity and colonisation rate (Ingleby and Dick, 2006; Symanczik *et al.*, 2015).

Albornoz *et al.* (2016) studied AM fungal and ectomycorrhizal colonisation on *Acacia rostellifera* and *Melaleuca systena* growing on sand dunes in South Western Australia in three areas in which soils had different types of limitations, with the main focus on nitrogen and phosphorus limiting factors. In both trees they found that the AM fungal colonisation declined as the phosphorus levels increased in the soil, which enabled the ectomycorrhizal species to increase their colonisation (Albornoz *et al.*, 2016). They concluded that AM fungi have increased colonisation in nitrogen rich soil. This could be possible because there was no nitrogen in the soil and the

phosphate was high (Table 3.1). AM fungal colonisation was present in the roots, but the number of AM fungal species present in the soil was very low. The study of Albornoz *et al.* (2016) showed that even though ectomycorrhizal species can be associated with *Acacia* species, this association was not observed in *V. erioloba*. This could be due to mycorrhizal interactions that differ between *Acacia* species.

Diagne *et al.* (2006) found that when AM fungi colonise *Acacia laeta* and *Acacia mellifera*, the spore numbers were low during dry seasons and high during rain seasons. Diop *et al.* (1994) also found that the total viable number of spores were lower around an adult *Acacia albida* tree than around a young tree. This correlates with the results of this study where the low number of spores may be related to the sampling around adult *V. erioloba* trees during the dry season.

Vegetation acts as reservoirs for AM fungi when conditions are unfavourable for fungal growth (Ingleby and Dick, 2006). When soil is disturbed it can affect the viability of AM fungal spores and hyphae which can degrade if separated from a plant host (Tahat and Sijam, 2012). Areas used for agricultural purposes are known to have a lower AM fungal population. This is due to the removal of vegetation by livestock which leads to soil erosion and degradation. Ingleby and Dick (2006) showed that soil from undisturbed areas has a higher number of AM fungal propagules, which include spores, extraradical hyphae, and infected roots, than soil from areas where livestock were grazing. Livestock grazing may have also contributed to the low spore numbers that were found in the soil, which were located on a stock and game farm.

The colonisation of the roots was not considerably high, which could be due to the nitrogen limitations and high levels of phosphorus present in the soil. AM fungal symbiosis is reduced in nitrogen limited soil environments with high phosphorus levels (Saia *et al.*, 2015). However, AM fungi are able to adapt to environmental changes and biological functions, but their ability to adapt varies between AM fungal species (Berutti *et al.*, 2015). No arbuscules were found in the root cells because

after a short time period the arbuscules reach maturity and start to progressively degenerate without killing the root cell (Smith and Read, 1997). It has been suggested that *V. erioloba* is colonised by the *Paris* type AM fungi because *Paris* type colonisation is found in trees (Dickson, 2004). However, this would only be confirmed through more extensive sampling. The extensive interradical hyphae observed in root sections does however suggest an *Arum* type colonisation strategy.

4.3 Effect of heavy metal accumulation on seedling health and growth

Cadmium is a trace element that is present in the environment but is known to be toxic when present in high concentration (Haneef *et al.*, 2014). Cd is naturally emitted into the air via environmental (volcanic activities, forest fires, and sea-salt aerosols) and anthropogenic sources (metal mining, burning fossil fuel) (ATSDR, 2012). Air emitted Cd can be deposited (wet or dry) on soil and into water, leading to Cd in remote areas through the movements of water currents and winds (ATSDR, 2012). Cadmium is known to be a mobile element that can more easily be absorbed by plants in acidic soil when Cd binds to organic matter (Sękara *et al.*, 2005; ATSDR, 2012). This could account for the detected presence of Cd in the untreated seedlings (Fig. 3.7), suggesting that the metal was present in seeds at the time of harvesting. Seeds were not analysed for Cd in this study.

As the heavy metal is absorbed by the plant, the concentration can decrease from roots > shoots > leaves > seeds (Sękara *et al.*, 2005). Previous studies have shown *Acacia mangium* and *Acacia tortilis* are able to accumulate heavy metals in the biomass (AL-Farraj and Al-Wabel, 2007; Majid *et al.*, 2012). Majid *et al.* (2012) found that *Acacia mangium* accumulated Cd in all of its biomass, but was the highest in the stems. This contradicts the results that were found in this study and proves that different species accumulate heavy metals into the different parts of the plant. Liu *et al.* (2013) found that growth conditions of trees can differ due to interactions with the soil microbial populations they harbour, which can affect translocation and metal uptake. Heavy metal accumulation can also be affected by soil heavy metal speciation, CEC, soil fertility and soil pH (Liu *et al.*, 2013).

Tan *et al.* (2015) studied the effects of Cd accumulation by the AM fungus, *Glomus versiforme*, on the growth of *Solanum photeinocarpum*. They found that Cd concentration in the uninoculated shoots of the Cd-treated plants was higher than that in the AM fungal inoculated shoots. They also found that the Cd concentration in the roots was higher than in the shoots. In Figure 7-A the Cd-treated uninoculated shoot had higher Cd concentrations than that of the AM fungal inoculated shoot. Figure 3.5 correlated with the study of Tan *et al.* (2015), where the roots had higher Cd concentrations than the shoots. Al-Ghamdi and Jais (2012) found that even though AM fungi can increase heavy metal relocation in plant tissue, the type of heavy metal can influence the colonisation rate of AM fungi. During their study, they found that AM fungal colonisation rate decreased as the concentrations of cadmium, uranium, zinc, copper and cobalt increased (Al-Ghamdi and Jais, 2012). This correlates with Table 3.5 where the colonisation rate decreased as the Cd concentration increased.

According to the results of Liao and colleagues (2003) exposure to 50 ppm Cd concentration increased the weight of the uninoculated *Zea mays* seedlings. They also found that the uninoculated seedling at the different Cd concentrations had the highest growth than any of their inoculated seedlings (Liao *et al.*, 2003). This could be that the hyphae of the mycorrhizal fungi are not needed to mobilise and absorb nutrients to improve the growth of the plant host. It is also possible that the fungi consume a portion of the photosynthetic products, which could decrease the seedling's growth (Liao *et al.*, 2003).

4.4 Isolation and characterisation of plant growth promoting rhizobacteria

Levels of plant growth promoting mechanisms that are expressed, vary between bacterial species, environmental conditions and plant host species (Ahemad and Kibret, 2013; Vacheron *et al.*, 2013). The four identified bacterial species (Table 3.6) are known as PGPR, but can also be grouped as MHB depending on the strain present (Gopal *et al.*, 2012; Vacheron *et al.*, 2013). The PGPR abilities of IAA production, siderophore production, nitrogen fixing, phosphate solubilisation and

biofilm formation were present in all 14 isolates. Three of the four bacterial species identified, namely *Enterobacter* sp., *Pantoea septica* and *Acinetobacter calcoaceticus*, are classified in the Gammaproteobacter class that represents the Proteobacter phylum (NCBI, 2016). The forth bacterial isolate, namely *Bacillus cereus*, is classified in the Firmicutes phylum (NCBI, 2016).

Bacillus cereus produced the highest IAA concentration amongst the 14 isolates. All 14 isolates were able to fix nitrogen. Sivasankari and Anandharaj (2014) found that *Bacillus cereus* has the ability to fix high concentrations of atmospheric nitrogen and produce high concentrations of IAA. The ability of PGPR to fix nitrogen increases nitrogen availability for the plant and interacting microorganisms (Ahemad and Kibret, 2014). This is important as *V. erioloba* does not nodulate (Barnes *et al.*, 1997). Ogbo and Okonkwo (2012) found that *Enterobacter* sp. enhanced nitrogen availability around the plant host, which helped increase plant growth. The PSI of the 14 isolates indicates their ability to convert phosphate reservoirs in soil into the forms that the plant can absorb (Table 3.5). Khan *et al.* (2009) described *Bacillus* and *Enterobacter* sp. as important phosphate solubilising bacteria in desert environments.

A. calcoaceticus is known to enhance phosphate solubilisation and nitrogen fixation in plant hosts (Khan *et al.*, 2015). *A. calcoaceticus* have been associated with *Acacia tortilis* subsp. *raddiana* and is considered to be a non-symbiotic, endophytic bacterium that is associated with root nodules (Fterich *et al.*, 2012). *A. calcoaceticus* have been described as a PGPR, but the ability of the bacteria to act as a PGPR depends on the strain (Rokhbakhsh-Zamin, 2011). Maindad *et al.* (2014) found that *A. calcoaceticus* produced a type of acinetobactin-like siderophore that acts as a defense against pathogenic fungi. However, as an endophytic bacterium, the abilities of *A. calcoaceticus* as a PGPR is not fully understood (Lacava and Azevedo, 2013).

The *Pantoea* genera are mainly studied for their biological control properties and some species are known human pathogens, such as *Pantoea septica* (Dastager *et*

al., 2009). *Pantoea septica* is a human pathogen that has been found in environmental samples where plants have been infected (Nadarasah and Stavrinides, 2014). The presence of *Pantoea septica* in the Kalahari soil could be due to environmental contamination. Species of *Pantoea* can adapt to different hosts in different environments (Dutkiewicz *et al.*, 2016). *Pantoea* sp. has been described as a PGPR, but this depends on the strain present (Dutkiewicz *et al.*, 2016). In this study, *P. septica* did prove to have plant growth promoting abilities (Table 3.5 – F1). Li et al. (2016) found that *Pantoea* sp. produced a higher siderophore concentration than *Bacillus* sp., correlating with this study.

When PGPR form biofilms it increases nutrient uptake, enables the bacteria to adapt quicker to changes in environmental conditions and inhibits competing soil organisms (Seneviratne *et al.*, 2010). Rafique *et al.* (2015) described the bacterial genera of *Pantoea* and *Bacillus* as having potential biocontrol activities when forming biofilms on the surfaces of plant roots. These biocontrol activities include the production of antibiotics, protecting against plant pathogens and harmful bacteria (Rafique *et al.*, 2015). However, the biocontrol activity of PGPR was not evaluated in this study.

PGPR are able to produce biofilms and the interactions with AM fungi allow biofilms to be produced on external hyphae and spores of AM fungi and roots of plant hosts (Bending *et al.*, 2006; Seneviratne *et al.*, 2010; Cruz and Ishii, 2016). Table 3.5 and Table 3 (Appendix F) show the cell density of the biofilms formed by the bacterial isolates. Not all the isolates formed thick biofilms, which had low cell density. Cell-to-cell communication is important to establish a strong surface community and further biofilm development (Davey and O'toole, 2000). The cell density of a biofilm is dependent on how strong that communication is between cells, which can differ among bacterial species and strains (Davey and O'toole, 2000). However, only the ability of the bacterial isolates to form biofilms was the only aspect tested in this study.

4.5 Assessment of mycorrhizal and bacterial populations from *Vachellia erioloba* rhizospheric soil.

Morphological identification has been the standard approach to identifying microorganisms (Lindeque, 2013). However, morphology is limited to what can be seen and many characteristics are shared by different species. This makes it difficult to distinguish between organisms, which can lead to incorrect identifications (Tshikhudo, 2013). Microbial species are difficult to culture and some are unculturable, which means that some species are overlooked (Tshikhudo, 2013). In the last two decades, molecular techniques have evolved to a point where organisms can be identified directly from environmental samples (Durmaz *et al.*, 2015).

Next generation sequencing, such as Illumina sequencing, have been very successful in correctly identifying organisms both cultured and uncultured (Peay *et al.*, 2016). The use of molecular methods has led to the correction of three main misconceptions namely, skewed determination of species abundances in populations, inaccurate identification of species based on morphology, and underestimation of the scale of fungal diversity in an environment (Peay *et al.*, 2016). In this study, Illumina sequencing was used to quantify the species diversity of AM fungal and bacterial species that were present at the three sites. Illumina sequencing has a high throughput, is more cost effective than 454 sequencing, and can analyse more sequences faster and produce large numbers of short reads (Degnan and Ochman, 2012).

4.5.1 Assessment of arbuscular mycorrhizal fungal data

In recent years it has been discovered that AM fungal genetic diversity is an important factor that can influence the productivity of ecosystems and the biodiversity in that specific ecosystem (Lee *et al.*, 2013). It was believed that low species diversity was a characteristic of AM fungal populations. This perception was due to the inability to culture this group of fungi and their limited spore morphology, resulting in species being overlooked (Peay *et al.*, 2016). However, next generation sequencing methods now used in AM fungal studies have enabled their identification

in environmental samples (Lee *et al.*, 2013). This has revealed that the AM fungal species diversity is much higher than originally expected. Illumina sequencing has been adopted as a successful sequencing platform but has not been widely used due to the short read lengths (Lindahl *et al.*, 2013).

In this study Illumina sequencing, using AM fungal primers, resulted in a total of 131 824 sequences (Table 3.8). Sequences were dominated by Ascomycota fungi which are the largest phylum. Ascomycota are monophyletic and is the largest fungal phylum containing more than 64 000 known species, which accounts for roughly 75% of described fungal species (Blackwell *et al.*, 2012; Aislabie and Deslippe, 2013). Ascomycota includes commercial yeasts, truffles, fungi that form lichen in combination with algae, antibiotic producing fungi and some toxic fungi that can cause food poisoning and human diseases (Blackwell *et al.*, 2012). This phylum has been found in deserts all over the world and has been described as the dominant phylum in soil (Makhalanyane *et al.*, 2015). The Glomeromycota phylum only has 240 described species, which makes it one of the smallest phylum in the fungal kingdom (Lee *et al.* 2013). Given the dominance of Ascomycota fungi identified, the use of the AM fungal primers selected must be reconsidered.

The read length of the sequences obtained was 300 bp and it is possible that these may have been too short to correctly identify the AM fungal sequences up to genus level. The reverse sequences were also of poor quality and could not be used. The results being the low percentage identities obtained for the AM fungal sequences. In this study, three families were identified, namely Ambisporaceae (75%), Paraglomeraceae (20%) and Glomeraceae (5%) that were classified under the Glomeromycota phylum (Fig. 3.14). In a previous study, using 454 Pyrosequencing, the AM fungal population in rhizospheric soil collected under *Stipgrostis cilata* var *capensis* in the Kalahari was assessed (Moore, 2015). Moore (2015) also identified *Ambispora*, *Paraglomus* and *Glomus* species, but *Paraglomus* was the dominant species followed by *Glomus* and *Ambispora*. Other AM fungal species such as *Archaeaspora*, *Redekera* and *Geosiphon* were also present (Moore, 2015).

The soil for Moore's (2015) study was collected from an area which is approximately 98 km away from where the soil was sampled for this study. The composition of microbial communities can differ between environments due to the interactions of soil microbes and plant hosts, which can result in positive or negative feedback from the plant host (Abbott *et al.*, 2015). Plant-microbe feedback, including bacteria and mycorrhiza, is a result of the species-specificity of plant-microbe associations. Thus, the soil communities and plant species respond differently to each other, which can be affected by positive or negative feedback (Reynolds and Haubensak, 2008). Positive feedback from a plant host is when the host accumulates beneficial soil microbes, which lead to the loss of soil microbial diversity in the rhizosphere and an increase in certain species (Reynolds and Haubensak, 2008).

Negative feedback from a plant host is the reduction of competitive dominance amongst soil microbial species, which increases the community diversity and species replacement over a period of time (Reynolds and Haubensak, 2008). A variation in how a plant responds to a mutualistic microbe was also found. For example, early succession plants, *Stipgrostis cilata* var *capensis*, have a low response to AM fungal species, whereas late succession plants, *V. erioloba*, can have a high response to AM fungal species (Abbott *et al.*, 2015). This would explain the difference between the AM fungal communities in the rhizospheric soils of the two different sites.

Millar and Bennette (2016) presented two hypotheses for how AM fungi are able to adapt to abiotic stresses over a long time period and whether AM fungi can adapt to sudden ecological impacts on the community structure over a short time period. They found that AM fungi are able to adapt to abiotic stresses such as temperature, CO_2 levels and heavy metals. Their study showed that different species flourish in different conditions. In their results, *A. leptoticha* had elevated colonisation in warm conditions, whereas *Glomus sp.* had increased sporulation in cold conditions (Millar and Bennette, 2016). This could be the reason why the Ambisporaceae family was the most abundant AM fungal family because it adapts well to higher temperatures.

Van der Heyde (2013) found that all three genera are dominant in areas where grazing takes place. Torrecillas *et al*. (2012) found that *Glomus* species are normally the abundant genera in semi-arid environments. Belay *et al*. (2015) found that AM fungal populations differ between environments and host species. One AM fungal species can be dominant in one area and can be the least abundant in another area. Their results showed that *Glomus* and *Paraglomus* species successfully colonised *Acacia seyal* and *Acacia nilotica* in an agricultural area. *Ambispora* was only present in a mixed fruit crop and *Zea may* (Belay *et al*., 2015). This shows that AM fungal species can adapt to changes in the environment.

4.5.2 Assessment of Bacterial data

Since the development of molecular identification techniques, it has become easier to identify unculturable bacterial species (Schloss *et al*., 2009). Next-generation sequencing has made it possible to identify species quicker and with less effort. In this study, Illumina sequencing was successfully performed with the bacterial sequences and a total of 26 046 dominant OTUs were obtained after the curation was completed (Table 3.10). The twenty OTUs that had the most sequences assigned to them were identified and belonged to three phyla, namely Actinobacteria, Bacteroidetes and Firmicutes (NCBI, 2016).

Five species within the Actinobacteria phylum were identified, namely *Blastococcus, Actinomycetospora*, *Streptomyces*, *Mycobacterium* and *Arthobacter* (Table 3.10). It was also the dominant phylum that had the most sequences assigned to it (Figure 3.14). Species from Actinobacteria are often identified from soil samples collected in extreme ecosystems that can survive and even flourish in environments with high chemical and physical parameters (Mohammadipanah and Wink, 2016). Parameters such as water content, temperature, salinity and pH play a vital role in the environment and can affect the microbial populations by either, limiting the functionality of a population, or allowing the microbes to flourish. Mohammadipanah and Wink (2016) found that Actinobacteria are the dominant bacterial phylum in arid and semi-arid deserts due to their adaptability. They discussed that *Blastococcus*

species are able to survive in environments having low water and nutrient availability, whereas *Streptomyces* species are described as being moderately thermophilic (Mohammadipanah and Wink, 2016). This would explain the dominance Actinobacteria in the Kalahari environment.

Actinobacteria have been described as PGPR and are one of the major components in rhizospheric soil (Franco-Correa and Chavarro-Anzola, 2016). Their PGPR characteristics include IAA production, nitrogen fixing, siderophore production and phosphate solubilisation. Franco-Correa and Chavarro-Anzola (2016) also found that Actinobacteria have MHB characteristics, where the microbes improved *Glomus* sporulation. A previous study isolated *Streptomyces* from *Acacia auriculiformis*, which improved nitrogen fixing and transfer to the plant host (Trujillo *et al.*, 2015).

Aislabie and Deslippe (2013) described the major phyla present in grassland, forest, and arid woodland soils from around the world of which Actinobacteria makes up 13%, Bacteroidetes 5% and Firmicutes 2% of the known bacteria. Bacteroidetes was the second largest phylum identified amongst all the sequences, but only two OTUs of the top 20 OTUs that were identified, belonged to this phylum (Table 3.8). Kavamura *et al.* (2013) isolated bacteria from the rhizospheric soil around *Cereus jamacaru*, located in a semi-arid environment. They found that during the dry season Actinobacteria is the dominant phylum followed by Proteobacteria, Firmicutes and Bacteroidetes being very small. In the rainy season, the Proteobacteria phylum is the dominant phylum followed by Actinobacteria, Bacteroidetes, and Firmicutes having the least number of isolates (Kavamura *et al.*, 2013). This correlates with Mohammadipanah and Wink (2016) where Actinobacteria were dominant under extreme environmental conditions.

The majority of the bacteria identified in this experiment are different from the bacteria cultured and identified in the PGPR identification experiment, in which the Proteobacter phylum was dominant. While isolating the bacteria, the *Streptomyces* selective media described by Awad *et al.* (2009) was used, but no Actinobacteria

bacteria were isolated from it. It is possible that the media or culture onditions were not selective enough for *Streptomyces* species.

4.6 Evaluation of the ability of selected bacterial isolates and mycorrhizal fungi to promote seedling growth.

V. erioloba is known as a slow growing tree species particularly during the first three to four years (Seymour and Milton, 2003). Figure 3.13 and Figure 3.17 shows the growth rate of the *V. erioloba* seedlings, which had a gradual growth rate. The *V. erioloba* seedlings of the individual PGPR treatments were younger than the *V. erioloba* seedlings of the PGPR combination treatments, although all were from the same batch of seeds. The shoot incrementation and biomass production of the combination treatments were much lower than the individual treatments. This shows that during the first three to five months from the point of seed germination *V. erioloba* seedlings growth is naturally fast, and then slows down.

When seedlings germinate more energy is allocated to the roots to increase root-surface contact zones to enable more nutrient translocation from the surrounding soil environment (Seymour, 2016). When seedlings are inoculated with AM fungi not a lot of energy is needed to produce more plant biomass (Moser, 2006). Moser (2006) found that more energy is allocated to the roots of *V. erioloba*, enabling the roots to grow deeper in soil where more water is available. Thus, the shoots appear to have less biomass than the roots. In the combination bacterial pot trial (Fig. 3.19) the root biomass was higher than the shoot biomass. In the individual bacterial pot trial (Fig. 3.15) five of the 8 treatments had higher root biomass than the shoot biomass.

The addition of AM fungi increases the amount of nutrients and water that can be transferred to the root cells directly (Moser, 2006). The colonisation of AM fungi can be influenced by plant species, soil microorganisms, soil properties, organic matter and phosphorus levels (Hodge, 2000; Carrenho, *et al.*, 2007). In both combination and individual pot trials the AM fungal inoculated controls without bacteria (Fig. 3.14

and Fig. 3.18) showed higher growth rates that the AM fungal inoculated bacterial treatments. However, this differs with the biomass production of the different treatments. The inoculated bacterial combination treatment had higher biomass than the inoculated control (Fig. 3.19). Whereas in the inoculated individual bacterial treatments, the biomass production varied between roots and shoots when comparing inoculated treatments (Fig. 3.15).

Soil bacteria can influence AM fungal colonisation and development either with negative, positive or neutral interactions (Hodge, 2000). An interaction can be influenced by the AM fungal species, plant species, PGPR species and the timing of when the microbes are added (Hodge, 2000). Co-inoculations of PGPR and AM fungi have been found to increase growth and dry biomass of the plant host considerably (Hodge, 2000; Dames and Ridsdale, 2012). PGPR have also shown to initially increase root colonisation, although after some time the colonisation percentage was similar regardless of the presence of PGPR (Hodge, 2000). The addition of individual (Table 3.7) and combined (Table 3.8) PGPR with AM fungi to *V. erioloba* seedling did show an increase in colonisation. However, the colonisation rate between the different treatments did not differ considerably from each other. It is possible that over a longer period of time the AM fungal colonisation would increase (Rai and Varma, 2005).

The inoculated *Enterobacter* sp. treatment had the highest colonisation rate, but the lowest growth rate and biomass production of all the inoculated treatments. Thus, the *Enterobacter* sp. used in this study showed more mycorrhizal helper abilities than plant growth promoting abilities. Some bacterial species can be described as MHB and PGPR, depending on the environmental and growth conditions of the plant host (Rigamonte *et al.*, 2010).

Hashem *et al.* (2016) studied the effects of *Bacillus subtilis* and an AM fungal inoculum on the growth of *Acacia gerrardii*. The AM fungal inoculum contained 2 species that were also used in this study namely *Claroideoglomus etunicatum* and

Funneliformis mosseae. Their results showed that the AM fungal inoculated *B. subtilis* had the highest shoot incrementation and biomass and the uninoculated control had the lowest shoot growth and biomass. The *B. subtilis* treatment had a higher growth rate and biomass production than the AM fungal treatment (Hashem *et al.*, 2016). The root biomass for all four treatments was higher than the shoot biomass (Hashem *et al.*, 2016). In this study, most of the treatments had higher root biomass than shoot biomass, except for the AM fungal inoculated *B. cereus* treatments that had higher shoot biomass than root biomass. Martínez-Viveros *et al.* (2010) described PGPR as having numerous interactions with the plant host and surrounding rhizosphere. Different bacterial species from the same genera can have various effects on plant growth and biomass production (Martínez-Viveros *et al.*, 2010).

AM fungi have been previously described as biocontrol agents that protect the plant host against nematodes and other soil pathogens (Schouteden *et al.*, 2015). Previous studies have shown that the effectiveness of AM fungi ranges from reducing pathogen reproduction and infection to enhancing tolerance against the pathogens. However, AM fungi are still not used on a regular basis as biocontrol agents. This is due to their variability in performance, which depends on the AM fungal isolate, environmental conditions, pathogen and plant species (Schouteden *et al.*, 2015). During this study, nematodes were found attached to the roots of *V. erioloba* seedlings that had reduced growth and colonisation.

The addition of nutrients seemed to improve the health and growth of the seedlings. However, Carrenho and colleagues (2007) found that the addition of nutrients can limit AM fungal development without affecting the growth and health of the plant host. During the colonisation assessment of the PGPR pot trials nematodes were found attached to young roots of some *V. erioloba* seedlings (Fig. 3.16 C-D) (Fig. 3.20 C). Marias and Swart (2001) found five nematode families associated with *V. erioloba* in the Douglas area, Northern Cape, South Africa. Families Belonolaimidae, Hoplolaimidae (*Helicotylenchus digonicus*, *Helicotylenchus vulgaris*, *Rotylenchulus parvus*), Longidoridae (*Longidorus pisi*, *Xiphinema vanderlindei*), Trichodoridae

(*Paratrichodorus minor*) and Tylenchulidae (*Paratylenchus* spp.) were the nematode species that were present on *V. erioloba* (Marias and Swart, 2001). Khan (2012) also described nematodes from the *Meloidogyne* genera, known as the root-knot nematode, associated with *Acacia* species.

All of these nematode species are known parasites that are described as endo-, ecto- and semi-endoparasites that damage plant roots (Krall, 1990; Subbotin *et al.*, 2010; Kahn, 2012; Archidona-Yuste *et al.*, 2016). These nematodes feed on root hairs, epidermal and cortical cells of young root close to the root tip, which leads to suppressed root growth and plant virus transmission and ultimately plant death (Decraemer, 1995; Subbotin *et al.*, 2011; Ghaderi *et al.*, 2014; Marias and Swart, 2014; Archidona-Yuste *et al.*, 2016). Nematodes from the Belonolaimidae family are only species commonly found in South Africa (Marais and Swart, 2014). The species from the other four families have only recently been found in South Africa (Marais and Swart, 2001). Nematodes were not observed on the roots from the Cd pot trial but were present in the PGPR pot trial. This may be due to the seeds used in the Cd pot trial which were from a different batch of seeds than the PGPR pot trials. The seedlings for the Cd pot trial were inoculated with AM fungi at germination and not after, which may explain the absence of nematodes in the Cd pot trial. The nematodes found in this study were not identified because characteristic features were not observed due to the staining procedure of the roots.

It is possible for nematodes to kill young seedlings if their defensive mechanisms are not strong enough to fend off or minimize damaging effects of plant parasites (Olsen, 1999). This causes only a few seedlings to mature, depending on the nematode population size. Plant-parasitic nematodes can be transferred via seeds and can survive long periods of dormancy until adequate growing conditions are present (Decker, 1989). Nematode populations are decreased by boiling seeds, but most species in their juvenile or eggs stage, such as *Paratylenchus* spp. and *Meloidogyne* spp., are tolerant of high temperatures (Warton, 2010; Riekert, 1996). Thus, the nematodes found in this study could possibly have been transferred via the seeds collected from the sites.

Siddiqui and Akhtar (2009) found that when plants are co-inoculated with AM fungi and PGPR the nematode activity and infection on the plant host is dramatically reduced. Saharan and Nehra (2011) found that when *Rhizobuim* is inoculated together with AM fungi and PGPR, plant growth is increased even more. However, the effectiveness of the *Rhizobium* strains depends on the interactions with other beneficial microbiota. Thus, strain selection is important to have the best effect on plant growth (Saharan and Nehra, 2011). In this study, nematodes were present on the roots of *V. erioloba* seedling that were co-inoculated AM fungi (with and without) and PGPR. In the individual PGPR treatments only one *Enterobacter* sp., AM fungal inoculated replicate died, whereas two uninoculated *B. cereus* seedlings died. Many other replicates had nematodes present on their roots but were still able to grow and AM fungal colonisation was properly established. Thus, the presence of AM fungi and PGPR could potentially act as biocontrol agents.

Conclusion

The interactions between beneficial soil bacteria and AM fungi have improved the health and growth of *V. erioloba*. AM fungal colonisation was established in the roots of *V. erioloba* that were collected in the Kalahari. AM fungi were also tested for their ability to keep a plant host heathy while exposed to different cadmium concentration. The *V. erioloba* seedling showed increased tolerance to cadmium proving that the AM fungi are able to increase health and growth while the plant host is under heavy metal stress. Soil bacteria were successfully isolated, characterised, and identified as PGPR. The bacteria were able to fix nitrogen, solubilise phosphorus, produce siderophores and IAA, and form biofilms. The bacteria were identified as being part of the *Enterobacter*, *Bacillus*, and *Acinetobacter* genera, which are known PGPR.

The health and growth of the seedlings in the PGPR pot trials with AM fungi, alone and combined, varied between the different treatments. The individually tested isolates showed better results than when the three isolates were combined. The health and growth of the *V. erioloba* seedlings was improved and successful colonisation was found in the roots of the seedlings. However, the presence of

nematodes could have influenced the outcome of the PGPR pot trials. PGPR and AM fungi were identified after the Kalahari soil was analysed by Illumina sequencing. Proteobacteria was the dominant phylum of the isolated bacteria and Actinobacteria was the dominant phylum of the bacteria directly sequenced from the soil. To my knowledge, no clear distinction could be made why there was a difference in the bacterial population between the two experiments. However, it proves that there are PGPR along with AM fungi present in the rhizospheric soil of *V. erioloba* trees. The results found in this study prove that the objectives were met.

Further research is needed in order to investigate the interactions between AM fungi and PGPR. Longer pot trials can be conducted to determine the long term effects of AM fungi and selected PGPR on *V. erioloba* seedlings. The nematode species associated with *V. erioloba* and their presence in seeds also needs to be investigated in order to prevent contamination in nurseries. More selective media needs to be used in order to isolate more bacterial diversity. Spore extractions and sequencing can also be done to determine the AM fungal populations in the soils from the different areas. The bacterial and AM fungal species can then be tested with the *V. erioloba* host in the field, to evaluate their interactions under natural conditions. This would allow the evaluation of the interactions between plant host, bacteria and AM fungi species. This can also be used to determine the dominant species that interact with the plant host. *V. erioloba* seedlings that are used in the field trial should be allowed to grow for longer time periods during different seasons. It can then be determined whether there is a difference between seasons and what species are more tolerant to the environmental conditions.

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Appendices

A. Root staining solutions used in preparation for mycorrhizal colonization assessment.

Please cite:

Koske, R.E, Gemma, J.N. (1989) A modified procedure for staining roots to detect VA mycorrhizas. *Mycological Research*, 92: 48-505.

Smith, S., Dickson, S. (1997) VA Mycorrhizas: Basic research techniques. Co-operative Research Centre for Soil and Land Management. Adelaide, Australia.

5% KOH:

KOH	100 g
Distilled water	2 L

Alkaline H_2O_2:

Ammonia (NH_4OH)	3 ml
10% H_2O_2	30 ml
Distilled water	567 ml

0.1M HCl: (32% MW 36.46)

HCl	22.79 ml
Distilled water	2 L

Lactoglycerol Trypan Blue Stain:

Lactic acid	520 ml
Glycerol	480 ml
Trypan blue	0.82 g
Distilled water	640 ml

Lactoglycerol destain:

Lactic acid	520 ml
Glycerol	480 ml
Distilled water	640 ml

B. Heavy metal analysis

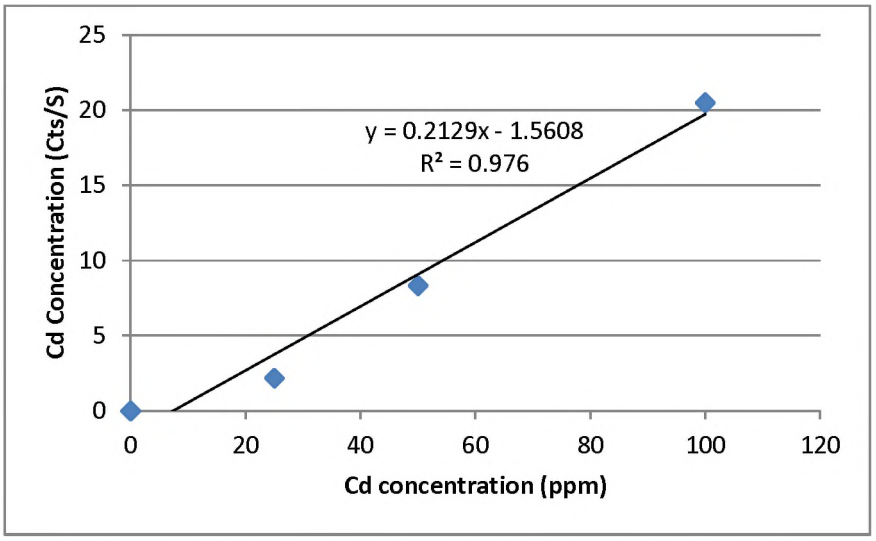

Figure 1: Standard curve prepared from the know cadmium (Cd) standards.

Table 1: The metal concentration (mg/L) returned by R^2=0.976

Cd concentration (ppm)	Average concentration (Cts/S)	Metal concentration (mg/L)
0	0	0
25	2.183	17.58
50	8.33	46.46
100	20.5	103.62

C. Isolation of Bacterial cultures.

1. Selective media used to isolate soil bacteria

Bennett media (Himedia, 2011) 1 L:

Yeast extract	1 g
Beef extract	1 g
Casein enzymatic hydrolysate	2 g
Dextrose	10 g
Bacteriological agar	15 g

Streptomyces agar (Awad *et al.*, 2009) 1 L:

Glucose	5 g
L-glutamic acid	4 g
KH2PO4	1 g
MgSO4.7H2O	0.7 g
NaCl	1 g
FeSO4.7H2O	0.003 g
Bacteriological agar	25 g

King's B agar (Scharlab, 2002) 1L:

Peptone powder	10 g
Casein digest	10 g
KH2PO4	1.5 g
MgSO4	1.5 g
Bacteriological agar	15 g

Gould's mS1 media (Gould *et al.*, 1985) 1 L:

Sucrose	20 g
Glycerol	10 ml
Casamino acid	5 g
NaHCO3	1 g
MgSO4.7H2O	1 g
K2HPO4	2.3 g
Sodium lauroyl sarcosine	1.2 g

D. Preparation of CAS media.

Minimal Medium/PIPES (900 ml dH_2O):

Malic acid	- 5 g
K_2HPO_4	- 0.5 g
$MgSO_4.7H_2O$	- 0.2 g
NaCl	- 0.1 g
$CaCl_2.2H_2O$	- 0.02 g
KOH	- 4.5 g
NH_4Cl	- 1 g

Adjust pH to 6.8 (adding NaOH). It is important to adjust pH in this step because it will be very difficult to adjust after PIPEs addition.

Add 30 g of PIPES. (This will make the liquid look turbid)

Add NaOH pellet by pellet until pH is 6.8 while stirring. Once the solution reaches the correct pH, it will look transparent.

When the pH is at 6.8 add 15 g agar and autoclave as normal.

CAS solution Blue dye (prepare in dark bottle):

Solution 1 (50 ml dH_2O):

Chrome Azurol S	- 0.121 g

Solution 2 (1mMFeCl3·6H2O, 10mMHCl):

$FeCl_3.6H_2O$ - 0.003 g

Mix the $FeCl_3.6H_2O$ in 10mL H2O with 10uL HCl (32%)

Solution 3 (use a bottle with enough room to add the other solutions):

Hexadecyltrimethylammonium (HDTMA) - 0.146 g

Mix in 40 ml dH2O

Add solution 1 and 2 to solution 3 and autoclave with the Minimal medium

Preparing the medium:

Once the solutions are sterile wait for it to cool down and then add the CAS solution to the Minimal Medium. Work in the Laminar flux to avoid contamination. Gently pour the media over the grown isolates and let it solidify. When the media is hot it will be a reddish color but as soon as it has solidified it will turn blue. If it does not turn blue then something was not done right.

E. Preparations of Indole acetic acid standards

The IAA standards were prepared using Tryptone broth (TB) (Merch, catalogue no. 1106940500) that was supplemented with 0, 5, 10, 20, 50 and 100 µg/ml IAA.

Salkowski reagent: 2 ml 0.5M $FeCl_3$; 49 ml 70% $HClO_4$, 49 ml d.H_2O

1) 10 mg IAA in 10 ml Acetone – Gives 1000 µg/ml stock solution
2) 1ml of the 1000 µg/ml stock was added to 9 ml TB – 100 µg/ml standard
3) 5 ml of 100 µg/ml standard was added to 5 ml TB – 50 µg/ml standard
4) 1 ml of 100 µg/ml standard was added to 9 ml TB – 10 µg/ml standard
5) 2 ml of 100 µg/ml standard was added to 8 ml TB – 20 µg/ml standard
6) 1 ml of 50 µg/ml standard was added to 9 ml TB – 5 µg/ml standard
7) 2 ml of Salkowski reagent was transferred to glass vials labelled with each standard.

8) 1 ml of each standard was added to a vial, including a control with only TB.

9) The vials were incubated at room temperature for 25 minutes and the colour development was measured at 530 nm.

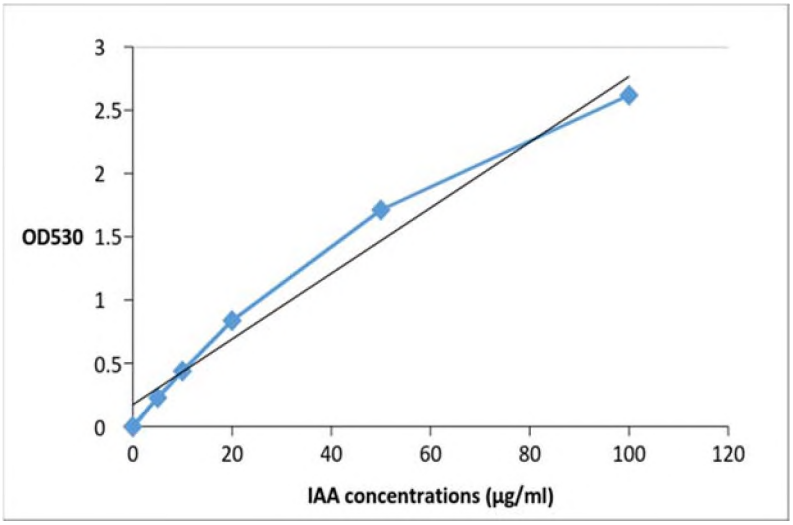

Figure 2: Standard curve prepared from known standards of IAA in tryptone broth.

10. The equation (y=0.0259x + 0.1716) provided by the graph (Figure 2) was used to calculate the IAA concentrations of the isolates.

Table 2: IAA standards concentration returned by equation R^2=0.973

All concentrations (µg/ml)	OD530	IAA calculation
0	0	-6.625
5	0.226	2.1
10	0.437	10.247
20	0.836	25.652
50	1.711	59.43
100	2.618	94.455

F. Bacterial isolates from the Kalahari soil.

Table 3: Characterisation tests of the fifty seven bacterial isolates (mean ± SE).

No.	Description	gram (+/-)	Form	N_2 test	Phosphorus test (PSI) [a]	CAS test (%) [b]	IAA test (µg/ml) [c]	Biofilm formation (cell dencity 575nm) [d]
1	*na vaal -5 sub 1	+	rod	+	-	46.67 ± 3.33	2.58 ± 1.26	0.19 ± 0.05
2	*strep vaal -3 sub 3	+	rod	+	-	50.79 ± 7.94	-1.39 ± 1.07	0.33 ± 0.08
3	strep vaal -3 sub 1	+	rod	+	1.24 ± 0.05	60.32 ± 8.84	2.91 ± 1.46	0.39 ± 0.09
4	*b vaal -2 sub 1	+	rod	+	-	33.33 ± 0	0.27 ± 0.39	0.45 ± 0.21
5 (F1)	strep vaal -2 sub 1	-	rod	+	2.97 ± 0.22	53.55 ± 3.33	7.56 ± 0.6	1.061 ± 0.03
6 (F2)	strep vaal -2 sub 2	-	rod	+	5.49 ± 0.6	43.33 ± 3.33	6.97 ± 0.74	1.204 ± 0.24
7	na vaal -4 sub 1	+	rod	+	-	42.33 ± 8.89	7.75 ± 3.1	0.22 ± 0.09
8	strep vaal -6 sub 2	+	rod	+	-	51.11 ± 8.89	0.99 ± 1.2	0.24 ± 0.08
9	na vaal -5 sub 2	-	rod	+	-	31.11 ± 5.88	1.17 ± 1.56	0.24 ± 0.04
10 (F3)	*ms1 vaal -2 sub 1	-	rod	+	5.9 ± 0.97	35.71 ± 7.14	6.57 ± 2.93	0.26 ± 0.1
11	strep vaal -3 sub 2	+	rod	+	-	64.45 ± 2.22	5.49 ± 2.09	0.44 ± 0.09
12	strep vaal -2 sub 3	+	rod	+	1.36 ± 0.07	56.67 ± 3.33	-1.79 ± 0.95	0.49 ± 0.26
13	strep vaal -7 sub 1	+	rod	+	-	58.73 ± 7.94	4.01 ± 1.08	0.24 ± 0.4
14	strep vaal -6 sub 1	-	rod	+	-	40 ± 10	-1.4 ± 0.45	0.23 ± 0.04
15 (F4)	strep ct1 -3 sub 1	+	rod	+	4.43 ± 0.42	41.83 ± 11.04	12.83 ± 4.31	0.96 ± 0.04
16	ms1 ct1 -2 sub 4	-	rod	+	4.29 ± 0.29	60 ± 0	0.61 ± 1.15	0.14 ± 0.02
17	strep ct1 -2 sub 1	+	rod	-	-	40 ± 10	5.97 ± 2.83	0.21 ± 0.06
18 (F5)	strep ct1 -4 sub 1	-	rod	+	4.53 ± 0.47	44.84 ± 12.07	12.32 ± 3.57	1.00 ± 0.03
19	strep ct1 -2 sub 3	+	rod	+	-	43.33 ± 3.33	8.93 ± 1.16	0.19 ± 0.02
20	b ct1 -7 sub 2	+	rod	+	-	38.33 ± 18.33	-0.73 ± 1.16	0.49 ± 0.23
21	b ct1 -4 sub 2	+	rod	+	-	38.89 ± 5.55	6.22 ± 3.86	0.29 ± 0.02
22	na ct2 -2 sub 2	+	rod	+	2.72 ± 0.15	33.33 ± 0	0.67 ± 1.43	0.186 ± 0.05

Table 3: continued

No.	Description	gram (+/-)	For m	N_2 test	Phosphorus test (PSI) [a]	CAS test (%) [b]	IAA test (µg/ml) [c]	Biofilm formation (cell dencity 575nm) [d]
23	b ct2 -6 sub 1	+	rod	+	-	20 ± 0	-3.63 ± 0.32	0.41 ± 0.09
24	strep ct2 -5 sub 1	+	rod	+	-	40.71 ± 12.73	-1.1 ± 0.53	0.36 ± 0.16
25	na ct2 -2 sub 1	-	rod	+	1.52 ± 0.02	33.67 ± 4.05	4.84 ± 2.84	0.4 ± 0.04
26	strep ct2 -4 sub 1	+	rod	+	-	33.33 ± 3.33	3.89 ± 1.13	0.25 ± 0.02
27	*ben ct2 -2 sub 1	-	rod	+	3.58 ± 0.22	50 ± 0.0	0.13 ± 1.65	0.19 ± 0.03
28	strep ct2 -3 sub 2	+	rod	+	-	33.33 ± 0.0	2.44 ± 1.45	0.21 ± 0.02
29 (F6)	strep ct2-3 sub 1	-	rod	+	5.56 ± 0.53	51.59 ± 11.03	4.62 ± 1.06	1.82 ± 0.75
30	b ct2 -3 sub 2	-	rod	+	-	61.11 ± 14.7	1.87 ± 1.45	0.27 ± 0.06
31 (F7)	strep ct2 -2 sub 3	-	rod	+	4.63 ± 0.45	55.56 ± 5.55	2.38 ± 0.34	2.2 ± 0.6
32	b ct2 -4 sub 1	+	rod	+	-	27.27 ± 0.0	5.69 ± 5.34	0.19 ± 0.06
33	strep ct2 -2 sub 1	+	rod	+	-	62.7 ± 6.5	-0.32 ± 0.39	0.39 ± 0.26
34 (F8)	strep ct2 -2 sub 2	-	rod	+	3.92 ± 0.39	51.11 ± 8.89	6.42 ± 1.08	0.3 ± 0.3
35 (F9)	ben ct2 -2 sub 2	-	rod	+	5.74 ± 0.38	63.69 ± 12.39	3.26 ± 1.92	0.88 ± 0.21
36	na vaal -5 sub 3	-	rod	+	-	32.75 ± 2.27	0.59 ± 1.54	0.24 ± 0.06
37	na vaal -3 sub 2	+	rod	+	-	46.67 ± 3.33	5.85 ± 1.37	0.31 ± 0.06
38 (F10)	na vaal -5 sub 4	-	rod	+	6.31 ± 0.85	71.08 ± 6.3	2.54 ± 1.69	1.85 ± 0.74
39	*king b vaal -4 sub 1	+	rod	+	-	35.65 ± 5.69	-3 ± 0.41	0.41 ± 0.08
40	king b vaal -5 sub 2	-	rod	+	-	66.67 ± 0.0	4.64 ± 0.48	0.22 ± 0.02
41	king b vaal -2 sub 3	-	rod	+	-	32.14 ± 4.92	4.88 ± 3.57	0.2 ± 0.03
42	king b vaal -3 sub 2	-	rod	+	-	51.67 ± 11.67	-0.14 ± 0.91	0.23 ± 0.04

Table 3: continued

No.	Description	gram (+/-)	Form	N_2 test	Phosphorus test (PSI) [a]	CAS test (%) [b]	IAA test (µg/ml) [c]	Biofilm formation (cell dencity 575nm) [d]
43	king b vaal -3 sub 1	+	rod	+	-	26.13 ± 5.97	-1.55 ± 0.91	0.29 ± 0.05
44	king b vaal -6 sub 1	-	rod	+	-	46.67 ± 3.33	0.79 ± 0.93	0.19 ± 0.04
45	king b vaal -4 sub 2	+	rod	+	-	33.33 ± 0.0	-3.45 ± 0.55	0.39 ± 0.04
46	king b vaal -2 sub 1	+	rod	+	-	66.67 ± 0.0	-2.43 ± 0.85	0.27 ± 0.01
47	king b vaal -5 sub 1	+	rod	+	-	30.04 ± 4.5	-2.62 ± 0.29	0.26 ± 0.09
48	king b ct1 -2 sub 1	+	rod	+	-	55.55 ± 11.11	-0.78 ± 0.44	0.52 ± 0.07
49 (F11)	king b ct1 -3 sub 3	-	rod	+	1.41 ± 0.05	20 ± 0.0	4.62 ± 1.06	0.25 ± 0.07
50 (F12)	king b ct1 -3 sub 1	-	rod	+	1.63 ± 0.19	45.56 ± 13.65	5 ± 1.27	2.14 ± 0.48
51	king b ct2 -2 sub 4	-	rod	+	2.09 ± 0.24	44.44 ± 5.55	-1.41 ± 0.64	0.4 ± 0.01
52	king b ct2 -2 sub 3	-	rod	+	-	62.22 ± 2.22	-1.86 ± 0.42	0.49 ± 0.32
53	king b vaal -2 sub 2	+	round	-	-	32.14 ± 4.92	1.26 ± 1.22	0.47 ± 0.07
54 (F13)	king b ct1 -2 sub 2	-	rod	+	2.19 ± 0.27	46.67 ± 3.33	7.16 ± 3.53	1.58 ± 0.34
55	king b ct1 -3 sub 2	-	rod	+	1.44 ± 0.09	43.33 ± 12.02	-0.55 ± 0.71	0.16 ± 0.02
56	king b ct2 -2 sub 2	-	rod	+	2.86 ± 0.42	42.22 ± 8.89	-1.5 ± 0.72	0.12 ± 0.05
57 (F14)	king b ct1 -2 sub 3	-	rod	+	4.25 ± 0.0	36.11 ± 7.35	8.33 ± 2.03	0.57 ± 0.13

*b – Bacillus media

*ben – Bennett media

*king b – King's B agar

*ms1 – Gould's modified S1 media

*na – Nutrient agar

*strep – Streptomyces agar

G. Mothur curation steps of the bacterial Illumina sequences.

Windows version

Running 64Bit Version

mothur v.1.36.1

Last updated: 7/27/2015

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When using, please cite:

Schloss, P.D., P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B, Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F. (2009) Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, 75(23):7537-41.

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Type 'help()' for information on the commands that are available

Type 'quit()' to exit program

Interactive Mode

mothur >

fastq.info(fastq=Sunet_1_bact_S37_L001_R1_001.fastq)

10000

20000

30000

40000

49932

Output File Names:

Sunet_1_bact_S37_L001_R1_001.fasta

Sunet_1_bact_S37_L001_R1_001.qual

[WARNING]: your sequence names contained ':'. I changed them to '_' to avoid problems in your downstream analysis.

mothur >

summary.seqs(fasta=Sunet_1_bact_S37_L001_R1_001.fasta)

Using 1 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	35	35	0	3	1
2.5%-tile:	1	300	300	0	3	1249
25%-tile:	1	301	301	0	4	12484
Median:	1	301	301	0	4	24967
75%-tile:	1	301	301	0	5	37450
97.5%-tile:	1	301	301	0	6	48684
Maximum:	1	301	301	35	35	49932
Mean: 1	300.846	300.846	0.000761035	4.5155		
# of Seqs:	49932					

Output File Names:

Sunet_1_bact_S37_L001_R1_001.summary

It took 4 secs to summarize 49932 sequences.

mothur > trim.seqs(fasta=Sunet_1_bact_S37_L001_R1_001.fasta, minlength=300, maxambig=0, processors=2)

Using 2 processors.

Appending files from process 1

Output File Names:

Sunet_1_bact_S37_L001_R1_001.trim.fasta

Sunet_1_bact_S37_L001_R1_001.scrap.fasta

mothur >

summary.seqs(fasta=Sunet_1_bact_S37_L001_R1_001.trim.fasta)

Using 2 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	300	300	0	3	1
2.5%-tile:	1	300	300	0	3	1231
25%-tile:	1	301	301	0	4	12304
Median:	1	301	301	0	4	24608
75%-tile:	1	301	301	0	5	36912
97.5%-tile:	1	301	301	0	6	47985
Maximum:	1	301	301	0	21	49215
Mean: 1	300.975		300.975	0	4.5147	
# of Seqs:	49215					

Output File Names:

Sunet_1_bact_S37_L001_R1_001.trim.summary

It took 3 secs to summarize 49215 sequences.

mothur > make.group(fasta=Sunet_1_bact_S37_L001_R1_001.trim.fasta, groups=Sunet_1_bact_S37_L001_R1_001)

Output File Names: Sunet_1_bact_S37_L001_R1_001.trim.groups

mothur >

fastq.info(fastq=Sunet_2_bact_S49_L001_R1_001.fastq)

10000

20000

30000

40000

50000

60000

70000

80000

90000

100000

110000

120000

121412

Output File Names:

Sunet_2_bact_S49_L001_R1_001.fasta

Sunet_2_bact_S49_L001_R1_001.qual

[WARNING]: your sequence names contained ':'. I changed them to '_' to avoid problems in your downstream analysis.

mothur > summary.seqs(fasta=Sunet_2_bact_S49_L001_R1_001.fasta)

Using 2 processors.

	Start	End	NBases		Ambigs	Polymer	NumSeqs
Minimum:	1	35	35	0	3	1	
2.5%-tile:	1	300	300	0	3	3036	
25%-tile:	1	301	301	0	4	30354	
Median:	1	301	301	0	4	60707	
75%-tile:	1	301	301	0	5	91060	
97.5%-tile:	1	301	301	0	6	118377	
Maximum:	1	301	301	35	35	121412	

Mean: 1 300.881 300.881 0.00260271 4.44763

# of Seqs: 121412

Output File Names:

Sunet_2_bact_S49_L001_R1_001.summary

It took 5 secs to summarize 121412 sequences.

mothur > trim.seqs(fasta=Sunet_2_bact_S49_L001_R1_001.fasta, minlength=300, maxambig=0, processors=2)

Using 2 processors.

Appending files from process 1

Output File Names:

Sunet_2_bact_S49_L001_R1_001.trim.fasta

Sunet_2_bact_S49_L001_R1_001.scrap.fasta

mothur > summary.seqs(fasta=Sunet_2_bact_S49_L001_R1_001.trim.fasta)

Using 2 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	300	300	0	3	1
2.5%-tile:	1	300	300	0	3	2995
25%-tile:	1	301	301	0	4	29948
Median:	1	301	301	0	4	59895
75%-tile:	1	301	301	0	5	89842
97.5%-tile:	1	301	301	0	6	116794
Maximum:	1	301	301	0	21	119788
Mean: 1	300.972		300.972	0	4.4443	

# of Seqs: 119788

Output File Names:

Sunet_2_bact_S49_L001_R1_001.trim.summary

It took 5 secs to summarize 119788 sequences.

mothur > make.group(fasta=Sunet_2_bact_S49_L001_R1_001.trim.fasta, groups=Sunet_2_bact_S49_L001_R1_001)

Output File Names: Sunet_2_bact_S49_L001_R1_001.trim.groups

mothur > fastq.info(fastq=Sunet-v-bact_S61_L001_R1_001.fastq)

10000

20000

30000

40000

50000

60000

70000

80000

90000

100000

110000

120000

130000

140000

150000

160000

170000

178194

Output File Names:

Sunet-v-bact_S61_L001_R1_001.fasta

Sunet-v-bact_S61_L001_R1_001.qual

[WARNING]: your sequence names contained ':'. I changed them to '_' to avoid problems in your downstream analysis.

mothur > summary.seqs(fasta=Sunet-v-bact_S61_L001_R1_001.fasta)

Using 2 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	35	35	0	2	1
2.5%-tile:	1	300	300	0	3	4455
25%-tile:	1	301	301	0	4	44549
Median:	1	301	301	0	4	89098
75%-tile:	1	301	301	0	5	133646
97.5%-tile:	1	301	301	0	7	173740
Maximum:	1	301	301	35	52	178194
Mean: 1	300.854	300.854	0.00245238	4.47004		
# of Seqs:	178194					

Output File Names:

Sunet-v-bact_S61_L001_R1_001.summary

It took 6 secs to summarize 178194 sequences.

mothur > trim.seqs(fasta=Sunet-v-bact_S61_L001_R1_001.fasta, minlength=300, maxambig=0, processors=2)

Using 2 processors.

Appending files from process 1

Output File Names:

Sunet-v-bact_S61_L001_R1_001.trim.fasta

Sunet-v-bact_S61_L001_R1_001.scrap.fasta

mothur > summary.seqs(fasta=Sunet-v-bact_S61_L001_R1_001.trim.fasta)

Using 2 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	300	300	0	3	1
2.5%-tile:	1	300	300	0	3	4376
25%-tile:	1	301	301	0	4	43758
Median:	1	301	301	0	4	87516
75%-tile:	1	301	301	0	5	131274
97.5%-tile:	1	301	301	0	6	170656
Maximum:	1	301	301	0	52	175031
Mean: 1	300.969		300.969	0	4.46666	
# of Seqs:	175031					

Output File Names:

Sunet-v-bact_S61_L001_R1_001.trim.summary

It took 6 secs to summarize 175031 sequences.

mothur > make.group(fasta=Sunet_v_bact_S61_L001_R1_001.trim.fasta, groups=Sunet_v_bact_S61_L001_R1_001)

Output File Names: Sunet_v_bact_S61_L001_R1_001.trim.groups

mothur > merge.files(input=Sunet_1_bact_S37_L001_R1_001.trim.groups-Sunet_2_bact_S49_L001_R1_001.trim.groups-Sunet_v_bact_S61_L001_R1_001.trim.groups, output=Bac.trim.groups)

Output File Names:

Bac.trim.groups

mothur > summary.seqs(fasta=Bac.trim.fasta)

Using 2 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	300	300	0	3	1
2.5%-tile:	1	300	300	0	3	8601
25%-tile:	1	301	301	0	4	86009
Median:	1	301	301	0	4	172018
75%-tile:	1	301	301	0	5	258026
97.5%-tile:	1	301	301	0	6	335434
Maximum:	1	301	301	0	52	344034
Mean: 1	300.971		300.971	0	4.46575	

# of Seqs: 344034

Output File Names:

Bac.trim.summary

It took 11 secs to summarize 344034 sequences.

mothur > summary.seqs(fasta=Bac.fasta, processors=8)

Using 8 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	300	300	0	3	1
2.5%-tile:	1	300	300	0	3	8601
25%-tile:	1	301	301	0	4	86009
Median:	1	301	301	0	4	172018
75%-tile:	1	301	301	0	5	258026
97.5%-tile:	1	301	301	0	6	335434
Maximum:	1	301	301	0	52	344034
Mean:	1	300.971	300.971	0	4.46575	

# of Seqs: 344034

Output File Names:
Bac.summary

It took 31 secs to summarize 344034 sequences.

mothur > screen.seqs(fasta=Bac.fasta, group=Bac.groups, maxambig=0, maxlength=350, minlength=250)

Using 8 processors.

Output File Names:
Bac.good.fasta
Bac.bad.accnos
Bac.good.groups

It took 10 secs to screen 344034 sequences.

mothur > count.groups()
Using Bac.good.groups as input file for the group parameter.
Sunet_1_bact_S37_L001_R1_001 contains 49215.
Sunet_2_bact_S49_L001_R1_001 contains 119788.
Sunet_v_bact_S61_L001_R1_001 contains 175031.

Total seqs: 344034.

Output File Names:
Bac.good.count.summary

mothur > unique.seqs(fasta=Bac.good.fasta)
344034 323904

Output File Names:
Bac.good.names
Bac.good.unique.fasta

mothur > summary.seqs(fasta=Bac.good.unique.fasta, name=Bac.good.names)

Using 8 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	300	300	0	3	1
2.5%-tile:	1	300	300	0	3	8601
25%-tile:	1	301	301	0	4	86009
Median:	1	301	301	0	4	172018
75%-tile:	1	301	301	0	5	258026
97.5%-tile:	1	301	301	0	6	335434
Maximum:	1	301	301	0	52	344034
Mean:	1	300.971	300.971	0	4.46575	

# of unique seqs: 323904
total # of seqs: 344034

Output File Names:
Bac.good.unique.summary

It took 4 secs to summarize 344034 sequences.

mothur > count.seqs(name=Bac.good.names, group=Bac.good.groups)

Using 8 processors.
It took 4 secs to create a table for 344034 sequences.

Total number of sequences: 344034

Output File Names:
Bac.good.count_table

mothur > summary.seqs(count=Bac.good.count_table)
Using Bac.good.unique.fasta as input file for the fasta parameter.

Using 8 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	300	300	0	3	1
2.5%-tile:	1	300	300	0	3	8601
25%-tile:	1	301	301	0	4	86009
Median:	1	301	301	0	4	172018
75%-tile:	1	301	301	0	5	258026
97.5%-tile:	1	301	301	0	6	335434
Maximum:	1	301	301	0	52	344034
Mean:	1	300.971	300.971	0	4.46575	

# of unique seqs: 323904
total # of seqs: 344034

Output File Names:
Bac.good.unique.summary

It took 4 secs to summarize 344034 sequences.

mothur > summary.seqs(fasta=Bac.good.unique.fasta, name=Bac.good.names)

Using 8 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	300	300	0	3	1
2.5%-tile:	1	300	300	0	3	8601
25%-tile:	1	301	301	0	4	86009
Median:	1	301	301	0	4	172018
75%-tile:	1	301	301	0	5	258026
97.5%-tile:	1	301	301	0	6	335434
Maximum:	1	301	301	0	52	344034
Mean:	1	300.971	300.971	0	4.46575	

# of unique seqs: 323904
total # of seqs: 344034

Output File Names:
Bac.good.unique.summary

It took 6 secs to summarize 344034 sequences.

mothur > align.seqs(fasta=Bac.good.unique.fasta, reference=silva.gold.align, flip=T)

Using 8 processors.

Reading in the silva.gold.align template sequences... DONE.
It took 12 to read 5181 sequences.
Aligning sequences from Bac.good.unique.fasta ...
[WARNING]: Some of your sequences generated alignments that eliminated too many bases, a list is provided in Bac.good.unique.flip.accnos. If the reverse compliment proved to be better it was reported.
It took 1449 secs to align 323904 sequences.

Output File Names:
Bac.good.unique.align
Bac.good.unique.align.report
Bac.good.unique.flip.accnos

mothur > summary.seqs(fasta=Bac.good.unique.align, count=Bac.good.count_table)

Using 8 processors.
[ERROR]: Your count file contains 323904 unique sequences, but your fasta file contains 311014. File mismatch detected, quitting command.

mothur > unique.seqs(fasta=Bac.good.fasta, group=Bac.good.groups)
group is not a valid parameter.
mothur > summary.seqs(fasta=Bac.good.unique.fasta, name=Bac.good.names)

Using 8 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	300	300	0	3	1
2.5%-tile:	1	300	300	0	3	8601
25%-tile:	1	301	301	0	4	86009
Median:	1	301	301	0	4	172018
75%-tile:	1	301	301	0	5	258026
97.5%-tile:	1	301	301	0	6	335434
Maximum:	1	301	301	0	52	344034
Mean:	1	300.971	300.971	0	4.46575	

# of unique seqs: 323904
total # of seqs: 344034

Output File Names:
Bac.good.unique.summary

It took 4 secs to summarize 344034 sequences.

mothur > summary.seqs(fasta=Bac.good.unique.fasta, count=Bac.good.count_table)

Using 8 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	300	300	0	3	1
2.5%-tile:	1	300	300	0	3	8601
25%-tile:	1	301	301	0	4	86009
Median:	1	301	301	0	4	172018
75%-tile:	1	301	301	0	5	258026
97.5%-tile:	1	301	301	0	6	335434
Maximum:	1	301	301	0	52	344034
Mean:	1	300.971	300.971	0	4.46575	

# of unique seqs: 323904
total # of seqs: 344034

Output File Names:
Bac.good.unique.summary

It took 3 secs to summarize 344034 sequences.
mothur > align.seqs(fasta=Bac.good.unique.fasta, reference=silva.gold.align, flip=T)

Using 8 processors.

Reading in the silva.gold.align template sequences... DONE.
It took 10 to read 5181 sequences.
Aligning sequences from Bac.good.unique.fasta ...
[WARNING]: Some of your sequences generated alignments that eliminated too many bases, a list is provided in Bac.good.unique.flip.accnos. If the reverse compliment proved to be better it was reported.
It took 897 secs to align 323904 sequences.

Output File Names:
Bac.good.unique.align
Bac.good.unique.align.report
Bac.good.unique.flip.accnos

mothur > summary.seqs(fasta=Bac.good.unique.align, count=Bac.good.count_table)

Using 8 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	0	0	0	0	1	1
2.5%-tile:	13129	25450	300	0	3	8601
25%-tile:	13129	25495	301	0	4	86009
Median:	13129	25495	301	0	4	172018
75%-tile:	13129	25495	301	0	5	258026
97.5%-tile:	13129	25497	301	0	6	335434

Maximum:	43116	43116	301	0	52	344034
Mean:	13266.8	25344.3	294.396	0	4.39644	
# of unique seqs:	323904					
total # of seqs:	344034					

Output File Names:
Bac.good.unique.summary

It took 446 secs to summarize 344034 sequences.

mothur > screen.seqs(fasta=Bac.good.unique.align, count=Bac.good.count_table, summary=Bac.good.unique.summary, start=13129, optimize=end, criteria=95)

Using 8 processors.
Optimizing end to 25452.

Output File Names:
Bac.good.unique.good.summary
Bac.good.unique.good.align
Bac.good.unique.bad.accnos
Bac.good.good.count_table

It took 1599 secs to screen 323904 sequences.

mothur > summary.seqs(fasta=current, count=current)
Using Bac.good.good.count_table as input file for the count parameter.
Using Bac.good.unique.good.align as input file for the fasta parameter.

Using 8 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	13125	25452	300	0	3	1
2.5%-tile:	13129	25452	301	0	3	8234
25%-tile:	13129	25495	301	0	4	82335
Median:	13129	25495	301	0	4	164669
75%-tile:	13129	25495	301	0	5	247003
97.5%-tile:	13129	25497	301	0	6	321103
Maximum:	13129	26782	301	0	52	329336
Mean:	13129	25490.3	300.981	0	4.43618	
# of unique seqs:	309558					
total # of seqs:	329336					

Output File Names:
Bac.good.unique.good.summary

It took 375 secs to summarize 329336 sequences.

mothur > filter.seqs(fasta=current, vertical=T, trump=.)
Using Bac.good.unique.good.align as input file for the fasta parameter.

Using 8 processors.
Creating Filter...

Running Filter...

Length of filtered alignment: 570
Number of columns removed: 49430
Length of the original alignment: 50000
Number of sequences used to construct filter: 309558

Output File Names:
Bac.filter
Bac.good.unique.good.filter.fasta

mothur > summary.seqs(fasta=current, count=current)
Using Bac.good.good.count_table as input file for the count parameter.
Using Bac.good.unique.good.filter.fasta as input file for the fasta parameter.
Using 8 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	568	272	0	3	1
2.5%-tile:	1	570	299	0	3	8234
25%-tile:	1	570	300	0	4	82335
Median:	1	570	300	0	4	164669
75%-tile:	1	570	300	0	5	247003
97.5%-tile:	1	570	301	0	6	321103
Maximum:	1	570	301	0	52	329336
Mean:	1	570	300.062	0	4.43547	

# of unique seqs: 309558
total # of seqs: 329336

Output File Names:
Bac.good.unique.good.filter.summary

It took 4 secs to summarize 329336 sequences.

mothur > unique.seqs(fasta=current, count=current)
Using Bac.good.good.count_table as input file for the count parameter.
Using Bac.good.unique.good.filter.fasta as input file for the fasta parameter.
309558
	307444

Output File Names:
Bac.good.unique.good.filter.count_table
Bac.good.unique.good.filter.unique.fasta

mothur > summary.seqs(fasta=current, count=current)
Using Bac.good.unique.good.filter.count_table as input file for the count parameter.

Using Bac.good.unique.good.filter.unique.fasta as input file for the fasta parameter.

Using 8 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	568	272	0	3	1
2.5%-tile:	1	570	299	0	3	8234
25%-tile:	1	570	300	0	4	82335
Median:	1	570	300	0	4	164669
75%-tile:	1	570	300	0	5	247003
97.5%-tile:	1	570	301	0	6	321103
Maximum:	1	570	301	0	52	329336
Mean:	1	570	300.062	0	4.43547	

# of unique seqs: 307444
total # of seqs: 329336

Output File Names:
Bac.good.unique.good.filter.unique.summary

It took 8 secs to summarize 329336 sequences.

mothur > pre.cluster(fasta=current, count=current, diffs=2)
Using Bac.good.unique.good.filter.count_table as input file for the count parameter.
Using Bac.good.unique.good.filter.unique.fasta as input file for the fasta parameter.

Using 8 processors.

Processing group Sunet_1_bact_S37_L001_R1_001:

Using 8 processors.

Processing group Sunet_2_bact_S49_L001_R1_001:

Using 8 processors.

Processing group Sunet_v_bact_S61_L001_R1_001:
45617 40069 5548
Total number of sequences before pre.cluster was 45617.
pre.cluster removed 5548 sequences.

It took 545 secs to cluster 45617 sequences.
109530 93775 15755
Total number of sequences before pre.cluster was 109530.
pre.cluster removed 15755 sequences.

It took 3093 secs to cluster 109530 sequences.
156108 135270 20838
Total number of sequences before pre.cluster was 156108.
pre.cluster removed 20838 sequences.

It took 6401 secs to cluster 156108 sequences.
It took 6433 secs to run pre.cluster.

Output File Names:
Bac.good.unique.good.filter.unique.precluster.fasta
Bac.good.unique.good.filter.unique.precluster.count_table
Bac.good.unique.good.filter.unique.precluster.Sunet_1_bact_S37_L001_R1_001.map
Bac.good.unique.good.filter.unique.precluster.Sunet_2_bact_S49_L001_R1_001.map
Bac.good.unique.good.filter.unique.precluster.Sunet_v_bact_S61_L001_R1_001.map

mothur > summary.seqs(fasta=Bac.good.unique.good.filter.unique.precluster.fasta, count=current)
Using Bac.good.unique.good.filter.unique.precluster.count_table as input file for the count parameter.
Using 8 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	568	272	0	3	1
2.5%-tile:	1	570	300	0	3	8234
25%-tile:	1	570	300	0	4	82335
Median:	1	570	300	0	4	164669
75%-tile:	1	570	300	0	5	247003
97.5%-tile:	1	570	301	0	6	321103
Maximum:	1	570	301	0	52	329336
Mean:	1	570	300.063	0	4.43306	

# of unique seqs: 268461
total # of seqs: 329336

Output File Names:
Bac.good.unique.good.filter.unique.precluster.summary

It took 7 secs to summarize 329336 sequences.

mothur > chimera.vsearch(fasta=Bac.good.unique.good.filter.unique.precluster.fasta, count=Bac.good.unique.good.filter.unique.precluster.count_table, dereplicate=t, processors=8)

Using 8 processors.
Checking sequences from Bac.good.unique.good.filter.unique.precluster.fasta ...

It took 95 secs to check 40069 sequences from group Sunet_1_bact_S37_L001_R1_001.

It took 328 secs to check 93775 sequences from group Sunet_2_bact_S49_L001_R1_001.

It took 596 secs to check 135270 sequences from group Sunet_v_bact_S61_L001_R1_001.

Output File Names:
Bac.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.count_table
Bac.good.unique.good.filter.unique.precluster.denovo.vsearch.chimeras
Bac.good.unique.good.filter.unique.precluster.denovo.vsearch.accnos

mothur > remove.seqs(fasta=Bac.good.unique.good.filter.unique.precluster.fasta, accnos=Bac.good.unique.good.filter.unique.precluster.denovo.vsearch.accnos)
[WARNING]: This command can take a namefile and you did not provide one. The current namefile is Bac.good.names which seems to match Bac.good.unique.good.filter.unique.precluster.fasta.
Removed 22102 sequences from your fasta file.

Output File Names:
Bac.good.unique.good.filter.unique.precluster.pick.fasta

mothur > summary.seqs(fasta=current, count=current, processors=8)
Using Bac.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.count_table as input file for the count parameter.
Using Bac.good.unique.good.filter.unique.precluster.pick.fasta as input file for the fasta parameter.

Using 8 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	568	275	0	3	1
2.5%-tile:	1	570	300	0	3	7657
25%-tile:	1	570	300	0	4	76568
Median:	1	570	300	0	4	153136
75%-tile:	1	570	300	0	5	229704
97.5%-tile:	1	570	301	0	6	298615
Maximum:	1	570	301	0	52	306271
Mean:	1	570	300.066	0	4.43519	

# of unique seqs: 246359
total # of seqs: 306271

Output File Names:
Bac.good.unique.good.filter.unique.precluster.pick.summary

It took 3 secs to summarize 306271 sequences.

Mother > summary.seqs(fasta=Bac.good.unique.good.filter.unique.precluster.pick.fasta, count=Bac.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.count_table, processors=8)

Using 8 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	568	275	0	3	1
2.5%-tile:	1	570	300	0	3	7657
25%-tile:	1	570	300	0	4	76568
Median:	1	570	300	0	4	153136
75%-tile:	1	570	300	0	5	229704
97.5%-tile:	1	570	301	0	6	298615
Maximum:	1	570	301	0	52	306271
Mean:	1	570	300.066	0	4.43519	

# of unique seqs: 246359
total # of seqs: 306271

Output File Names:
Bac.good.unique.good.filter.unique.precluster.pick.summary

It took 3 secs to summarize 306271 sequences.

mothur > classify.seqs(fasta=current, count=current, reference=silva.nr_v123.align, taxonomy=silva.nr_v123.tax, cutoff=80)

mothur > remove.lineage(fasta=current, count=current, taxonomy=current, taxon=Chloroplast-Mitochondria-unknown-Archaea-Eukaryota)
Using Bac.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.count_table as input file for the count parameter.
Using Bac.good.unique.good.filter.unique.precluster.pick.fasta as input file for the fasta parameter.
Using Bac.good.unique.good.filter.unique.precluster.pick.nr_v123.wang.taxonomy as input file for the taxonomy parameter.

[NOTE]: The count file should contain only unique names, so mothur assumes your fasta, list and taxonomy files also contain only uniques.

Output File Names:
Bac.good.unique.good.filter.unique.precluster.pick.nr_v123.wang.pick.taxonomy
Bac.good.unique.good.filter.unique.precluster.pick.pick.fasta
Bac.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.count_table

mothur > dist.seqs(fasta=current, cutoff=0.20)
Using Bac.good.unique.good.filter.unique.precluster.pick.pick.fasta as input file for the fasta parameter.

Using 8 processors.

Output File Names:

Bac.good.unique.good.filter.unique.precluster.pick.pick.dist

It took 45309 seconds to calculate the distances for 241582 sequences.

mothur > cluster(column=current, count=current)

Subsampling:

mothur > cluster(column=Bac.good.unique.good.filter.unique.precluster.pick.pick.dist, count= Bac.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.count_table)
********************#****#****#****#****#****#****#****#****#****#****#
Reading matrix: ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
****
changed cutoff to 0.03

Output File Names:
Bac.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.list

It took 12582 seconds to cluster

mothur > make.shared(list=current, count=current, label=0.03)
Using Bac.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.count_table as input file for the count parameter.
Using Bac good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.list as input file for the list parameter.
0.02

Output File Names:
Bac.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.shared

mothur > count.groups(shared=current)
Using Bac.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.shared as input file for the shared parameter.
Sunet_1_bact_S37_L001_R1_001 contains 44145.
Sunet_2_bact_S49_L001_R1_001 contains 107480.
Sunet_v_bact_S61_L001_R1_001 contains 149519.

Total seqs: 301144.

Output File Names:
Bac.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.count.summar y

mothur > sub.sample(shared=current, size=44145)
Using Bac.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.shared

as input file for the shared parameter.
Sampling 44145 from each group.
0.02

Output File Names:
Bac.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.0.02.subsampl
e.shared

mothur > venn(groups=Sunet_1_bact_S37_L001_R1_001-
Sunet_2_bact_S49_L001_R1_001-Sunet_v_bact_S61_L001_R1_001)
Using
Bac.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.0.02.subsampl
e.shared as input file for the shared parameter.
Sunet_v_bact_S61_L001_R1_001 contains 149519 is not a valid group, and will be
disregarded.
0.02

Output File Names:
Bac.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.0.02.subsampl
e.0.02.sharedsobs.Sunet_1_bact_S37_L001_R1_001-
Sunet_2_bact_S49_L001_R1_001.svg
Bac.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.0.02.subsampl
e.0.02.sharedsobs.Sunet_1_bact_S37_L001_R1_001-
Sunet_2_bact_S49_L001_R1_001.sharedotus

mothur > summary.seqs(fasta=
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.fasta, count=
Bac.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.subsample.c
ount_table, processors=8)

Using 8 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	568	275	0	3	1
2.5%-tile:	1	570	300	0	3	3311
25%-tile:	1	570	300	0	4	33109
Median:	1	570	300	0	4	66217
75%-tile:	1	570	300	0	5	99325
97.5%-tile:	1	570	301	0	6	129122
Maximum:	1	570	301	0	23	132432
Mean:	1	570	300.069	0	4.44	

# of unique seqs: 108272
total # of seqs: 132432

Output File Names:
/Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.summary

```
It took 1 secs to summarize 132432 sequences.

mothur > count.groups()
Using /Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.subsample.c
ount_table as input file for the count parameter.
Sunet_1_bact_S37_L001_R1_001 contains 44144.
Sunet_2_bact_S49_L001_R1_001 contains 44144.
Sunet_v_bact_S61_L001_R1_001 contains 44144.

Total seqs: 132432.

Output File Names:
/Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.subsample.c
ount.summary


mothur > dist.seqs(fasta=current, cutoff=0.20)
Using /Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.fasta as input file
for the fasta parameter.
Using 8 processors.

Output File Names:
/Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.dist

It took 13179 seconds to calculate the distances for 108272 sequences.

mothur > cluster(column=current, count=current)
Using /Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.dist as input file
for the column parameter.
Using /Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.subsample.c
ount_table as input file for the count parameter.
********************#****#****#****#****#****#****#****#****#****#
Reading matrix:     |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
***************************************************************************

Output File Names:
/Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.an.unique_list.list

It took 11 seconds to cluster

mothur > make.shared(list=current, count=current, label=0.03)
Using /Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.subsample.c
```

ount_table as input file for the count parameter.
Using /Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.an.unique_list.list as input file for the list parameter.
unique

Output File Names:
/Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.an.unique_list.shared

mothur > count.groups(shared=current)
Using /Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.an.unique_list.shared as input file for the shared parameter.
Sunet_1_bact_S37_L001_R1_001 contains 44144.
Sunet_2_bact_S49_L001_R1_001 contains 44144.
Sunet_v_bact_S61_L001_R1_001 contains 44144.

Total seqs: 132432.

Output File Names:
/Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.an.unique_list.count.summary

mothur > venn(groups=Sunet_1_bact_S37_L001_R1_001-Sunet_2_bact_S49_L001_R1_001-Sunet_v_bact_S61_L001_R1_001)
Using /Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.an.unique_list.shared as input file for the shared parameter.
unique

Output File Names:
/Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.an.unique_list.unique.sharedsobs.Sunet_1_bact_S37_L001_R1_001-Sunet_2_bact_S49_L001_R1_001-Sunet_v_bact_S61_L001_R1_001.svg
/Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.an.unique_list.unique.sharedsobs.Sunet_1_bact_S37_L001_R1_001-Sunet_2_bact_S49_L001_R1_001-Sunet_v_bact_S61_L001_R1_001.sharedotus

To get dominant OTUs (removed rare sequences):

mothur > split.abund(fasta=/Users/gwynnethmatcher/Gwynneth/

Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.fasta, count=/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.subsample.count_table, cutoff=1)

Output File Names:
Bac.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.subsample.rare.count_table
Bac.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.subsample.abund.count_table
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.rare.fasta
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.abund.fasta

mothur > summary.seqs(fasta=current, count=current)
Using Bac.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.subsample.rare.count_table as input file for the count parameter.
Using Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.rare.fasta as input file for the fasta parameter.

Using 8 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	568	275	0	3	1
2.5%-tile:	1	570	299	0	3	2660
25%-tile:	1	570	300	0	4	26597
Median:	1	570	300	0	4	53194
75%-tile:	1	570	300	0	5	79790
97.5%-tile:	1	570	301	0	6	103727
Maximum:	1	570	301	0	23	106386
Mean:	1	570	300.069	0	4.48672	
# of unique seqs:	106386					
total # of seqs:	106386					

Output File Names:
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.rare.summary

It took 2 secs to summarize 106386 sequences.

mothur > summary.seqs(fasta=/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.abund.fasta, count=/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.subsample.abund.count_table)

Using 8 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	570	275	0	3	1
2.5%-tile:	1	570	300	0	4	652

```
25%-tile:	1	570	300	0	4	6512
Median: 	1	570	300	0	4	13024
75%-tile:	1	570	300	0	4	19535
97.5%-tile:	1	570	301	0	6	25395
Maximum:	1	570	301	0	8	26046
Mean:	1	570	300.072	0	4.24917
# of unique seqs:	1886
total # of seqs:	26046

Output File Names:
/Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.abund.summary

It took 0 secs to summarize 26046 sequences.

mothur > dist.seqs(fasta=/Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.abund.fasta,
cutoff=0.20)

Using 8 processors.

Output File Names:
/Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.abund.dist

It took 4 seconds to calculate the distances for 1886 sequences.

mothur > cluster(column=/Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.abund.dist,
count=/Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.subsample.a
bund.count_table)
********************#****#****#****#****#****#****#****#****#****#
Reading matrix:     ||||||||||||||||||||||||||||||||||||||||||||||||||
***********************************************************************
changed cutoff to 0.130583

Output File Names:
/Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.abund.an.unique
_list.list

It took 4 seconds to cluster

mothur > make.shared(list=/Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.abund.an.unique
_list.list, count=/Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.subsample.a
bund.count_table, label=0.03)
0.03
```

Output File Names:
/Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.abund.an.unique
_list.shared

mothur > count.groups(shared=current)
Using /Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.abund.an.unique
_list.shared as input file for the shared parameter.
Sunet_1_bact_S37_L001_R1_001 contains 8027.
Sunet_2_bact_S49_L001_R1_001 contains 9370.
Sunet_v_bact_S61_L001_R1_001 contains 8649.

Total seqs: 26046.
Output File Names:
/Users/gwynnethmatcher/Gwynneth/
Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.abund.an.unique
_list.count.summary
mothur >
get.oturep(column=/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.
unique.precluster.pick.pick.subsample.abund.dist,
count=/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.preclu
ster.denovo.vsearch.pick.pick.subsample.abund.count_table,
list=/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.preclust
er.pick.pick.subsample.abund.an.unique_list.list,
fasta=/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.preclu
ster.pick.pick.subsample.abund.fasta, sorted=group)
**********#****#****#****#****#****#****#****#****#****#****#
Reading matrix: ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
****

unique 1886
0.01 1286
0.02 727
0.03 555
0.04 435
0.05 349
0.06 281
0.07 238
0.08 190
0.09 166
0.10 141
0.11 116
0.12 104
0.13 94

Output File Names:
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.unique.rep.count_table
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi

ck.pick.subsample.abund.an.unique_list.0.01.rep.count_table
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.02.rep.count_table
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.03.rep.count_table
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.04.rep.count_table
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.05.rep.count_table
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.06.rep.count_table
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.07.rep.count_table
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.08.rep.count_table
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.09.rep.count_table
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.10.rep.count_table
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.11.rep.count_table
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.12.rep.count_table
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.13.rep.count_table
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.01.rep.fasta
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.02.rep.fasta
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.03.rep.fasta
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.04.rep.fasta
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.05.rep.fasta
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.06.rep.fasta
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.07.rep.fasta
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.08.rep.fasta
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.09.rep.fasta
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.10.rep.fasta
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.11.rep.fasta
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi
ck.pick.subsample.abund.an.unique_list.0.12.rep.fasta
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pi

ck.pick.subsample.abund.an.unique_list.0.13.rep.fasta
/Users/gwynnethmatcher/Gwynneth/Bac.good.unique.good.filter.unique.precluster.pick.pick.subsample.abund.an.unique_list.unique.rep.fasta

H. Mothur curation steps of the AM fungal Illumina sequences.

Windows version

Running 64Bit Version

mothur v.1.38.1

Last updated: 8/9/2016

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http://www.mothur.org

When using, please cite:

Schloss, P.D., P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B, Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F. (2009) Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, 75(23):7537-41.

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Type 'help()' for information on the commands that are available

Type 'quit()' to exit program

Interactive Mode

mothur > fastq.info(fastq=C:\Gwynneth\mothur\Sunet-v-amf_S25_L001_R1_001.fastq)

10000

20000

30000

40000

50000

60000

70000

80000

90000

94927

Output File Names:

C:\Gwynneth\mothur\Sunet-v-amf_S25_L001_R1_001.fasta

C:\Gwynneth\mothur\Sunet-v-amf_S25_L001_R1_001.qual

[WARNING]: your sequence names contained ':'. I changed them to '_' to avoid problems in your downstream analysis.

mothur > fastq.info(fastq=C:\Gwynneth\mothur\Sune-2amf_S13_L001_R1_001.fastq)

10000

20000

30000

40000

50000

60000

70000

80000

81707

Output File Names:

C:\Gwynneth\mothur\Sune-2amf_S13_L001_R1_001.fasta

C:\Gwynneth\mothur\Sune-2amf_S13_L001_R1_001.qual

[WARNING]: your sequence names contained ':'. I changed them to '_' to avoid problems in your downstream analysis.

mothur > system(copy C:\Gwynneth\mothur\Sunet-v-amf_S25_L001_R1_001.fasta amfV.fasta)

1 file(s) copied.

mothur > system(copy C:\Gwynneth\mothur\Sune-2amf_S13_L001_R1_001.fasta amf2.fasta)

1 file(s) copied.

mothur > make.group(fasta=amfV.fasta, groups=amfV.groups)

Output File Names: amfV.groups

mothur > make.group(fasta=amf2.fasta, groups=amf2.groups)

Output File Names: amf2.groups

mothur > merge.files(input=amfV.fasta-amf2.fasta, output=amf.fasta)

Output File Names:

amf.fasta

mothur > merge.files(input=amfV.groups-amf2.groups, output=amf.groups)

Output File Names:

amf.groups

mothur > count.groups()

Using amf2.groups as input file for the group parameter.

amf2.groups contains 81707.

Total seqs: 81707.

Output File Names:

amf2.count.summary

mothur > count.groups(group=amf.groups)

amf2.groups contains 81707.

amfV.groups contains 94927.

Total seqs: 176634.

Output File Names:

amf.count.summary

mothur > trim.seqs(fasta=amfV.fasta, minlength=300, maxlength=350, maxambig=0, processors=2)

Using 2 processors.

Appending files from process 1

Output File Names:

amfV.trim.fasta

amfV.scrap.fasta

mothur > trim.seqs(fasta=amf2.fasta, minlength=300, maxlength=350, maxambig=0, processors=2)

Using 2 processors.

Appending files from process 1

Output File Names:

amf2.trim.fasta

amf2.scrap.fasta

mothur > summary.seqs(fasta=amf2.trim.fasta)

Using 2 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	300	300	0	3	1
2.5%-tile:	1	301	301	0	5	1868
25%-tile:	1	301	301	0	5	18680
Median:	1	301	301	0	6	37360
75%-tile:	1	301	301	0	6	56039
97.5%-tile:	1	301	301	0	8	72851
Maximum:	1	301	301	0	42	74718
Mean: 1	300.98		300.98	0	5.86169	
# of Seqs:	74718					

Output File Names:

amf2.trim.summary

It took 2 secs to summarize 74718 sequences.

mothur > summary.seqs(fasta=amfV.trim.fasta)

Using 2 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	300	300	0	3	1
2.5%-tile:	1	301	301	0	4	2255
25%-tile:	1	301	301	0	5	22545
Median:	1	301	301	0	6	45089
75%-tile:	1	301	301	0	6	67633
97.5%-tile:	1	301	301	0	7	87922
Maximum:	1	301	301	0	27	90176

Mean: 1 300.98 300.98 0 5.8031

# of Seqs: 90176

Output File Names:

amfV.trim.summary

It took 2 secs to summarize 90176 sequences.

mothur > make.group(fasta=amfV.trim.fasta, groups=amfV.trim.groups)

Output File Names: amfV.trim.groups

mothur > make.group(fasta=amf2.trim.fasta, groups=amf2.trim.groups)

Output File Names: amf2.trim.groups

mothur > merge.files(input=amfV.trim.groups-amf2.trim.groups, output=amf.trim.groups)

Output File Names:

amf.trim.groups

mothur > merge.files(input=amfV.trim.fasta-amf2.trim.fasta, output=amf.trim.fasta)

Output File Names:

amf.trim.fasta

mothur > count.groups(group=amf.trim.groups)

amf2.trim.groups contains 74718.

amfV.trim.groups contains 90176.

Total seqs: 164894.

Output File Names:

amf.trim.count.summary

mothur > unique.seqs(fasta=amf.trim.fasta)

164894 147767

Output File Names:

amf.trim.names

amf.trim.unique.fasta

mothur > summary.seqs(fasta=amf.trim.unique.fasta, name=amf.trim.names)

Using 2 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	300	300	0	3	1
2.5%-tile:	1	301	301	0	4	4123
25%-tile:	1	301	301	0	5	41224
Median:	1	301	301	0	6	82448
75%-tile:	1	301	301	0	6	123671
97.5%-tile:	1	301	301	0	7	160772
Maximum:	1	301	301	0	42	164894
Mean: 1	300.98		300.98	0	5.82965	

# of unique seqs: 147767

total # of seqs: 164894

Output File Names:

amf.trim.unique.summary

It took 5 secs to summarize 164894 sequences.

mothur > classify.seqs(fasta=amf.trim.unique.fasta, name=amf.trim.names, group=amf.trim.groups, taxonomy=silva.eukarya.silva.tax, template=silva.eukarya.fasta, cutoff=80)

Using 2 processors.

Reading template taxonomy... DONE.

Reading template probabilities... DONE.

It took 15 seconds get probabilities.

Classifying sequences from amf.trim.unique.fasta ...

Reading template taxonomy... DONE.

Reading template probabilities... DONE.

It took 18 seconds get probabilities.

[WARNING]: M00792_51_000000000-ARA9L_1_2118_20554_11554 could not be classified. You can use the remove.lineage command with taxon=unknown; to remove such sequences.

[WARNING]: M00792_51_000000000-ARA9L_1_1106_8369_7590 could not be classified. You can use the remove.lineage command with taxon=unknown; to remove such sequences.

[WARNING]: M00792_51_000000000-ARA9L_1_1106_8356_7603 could not be classified. You can use the remove.lineage command with taxon=unknown; to remove such sequences.

[WARNING]: M00792_51_000000000-ARA9L_1_1113_20655_11469 could not be classified. You can use the remove.lineage command with taxon=unknown; to remove such sequences.

[WARNING]: M00792_51_000000000-ARA9L_1_1115_24020_18787 could not be classified. You can use the remove.lineage command with taxon=unknown; to remove such sequences.

[WARNING]: M00792_51_000000000-ARA9L_1_1117_18670_22096 could not be classified. You can use the remove.lineage command with taxon=unknown; to remove such sequences.

[WARNING]: M00792_51_000000000-ARA9L_1_2101_21049_4050 could not be classified. You can use the remove.lineage command with taxon=unknown; to remove such sequences.

[WARNING]: mothur reversed some your sequences for a better classification. If you would like to take a closer look, please check amf.trim.unique.silva.wang.flip.accnos for the list of the sequences.

It took 1104 secs to classify 147767 sequences.

Reading amf.trim.names... Done.

It took 22 secs to create the summary file for 147767 sequences.

Output File Names:

amf.trim.unique.silva.wang.taxonomy

amf.trim.unique.silva.wang.tax.summary

amf.trim.unique.silva.wang.flip.accnos

mothur > remove.lineage(fasta=amf.trim.unique.fasta, name=amf.trim.names, group=amf.trim.groups, taxonomy=amf.trim.unique.silva.wang.taxonomy, taxon=unknown)

Output File Names:

amf.trim.unique.silva.wang.pick.taxonomy

amf.trim.pick.names

amf.trim.unique.pick.fasta

amf.trim.pick.groups

mothur > summary.seqs(fasta=amf.trim.unique.fasta, name=amf.trim.names)

Using 2 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	300	300	0	3	1
2.5%-tile:	1	301	301	0	4	4123
25%-tile:	1	301	301	0	5	41224
Median:	1	301	301	0	6	82448
75%-tile:	1	301	301	0	6	123671
97.5%-tile:	1	301	301	0	7	160772
Maximum:	1	301	301	0	42	164894
Mean: 1	300.98		300.98	0	5.82965	

# of unique seqs: 147767

total # of seqs: 164894

Output File Names:

amf.trim.unique.summary

It took 5 secs to summarize 164894 sequences.

mothur > summary.seqs(fasta=amf.trim.unique.pick.fasta, name=amf.trim.pick.names)

Using 2 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	300	300	0	3	1
2.5%-tile:	1	301	301	0	4	4123
25%-tile:	1	301	301	0	5	41222
Median:	1	301	301	0	6	82444
75%-tile:	1	301	301	0	6	123666
97.5%-tile:	1	301	301	0	7	160765
Maximum:	1	301	301	0	42	164887
Mean: 1	300.98		300.98	0	5.82966	

# of unique seqs: 147760

total # of seqs: 164887

Output File Names:

amf.trim.unique.pick.summary

It took 5 secs to summarize 164887 sequences.

mothur > unique.seqs(fasta=amf.trim.unique.pick.fasta)

[WARNING]: This command can take a namefile and you did not provide one. The current namefile is amf.trim.pick.names which seems to match amf.trim.unique.pick.fasta.

147760 147760

Output File Names:

amf.trim.unique.pick.names

amf.trim.unique.pick.unique.fasta

mothur > summary.seqs(fasta=amf.trim.unique.pick.unique.fasta, name=amf.trim.unique.pick.names)

Using 2 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	300	300	0	3	1
2.5%-tile:	1	301	301	0	4	3695
25%-tile:	1	301	301	0	5	36941
Median:	1	301	301	0	6	73881
75%-tile:	1	301	301	0	6	110821
97.5%-tile:	1	301	301	0	8	144067
Maximum:	1	301	301	0	42	147760
Mean: 1	300.978		300.978	0	5.81517	

# of unique seqs: 147760

total # of seqs: 147760

Output File Names:

amf.trim.unique.pick.unique.summary

It took 5 secs to summarize 147760 sequences.

mothur > align.seqs(fasta=amf.trim.unique.pick.fasta, reference=silva.eukarya.fasta, flip=T, processors=2)

Using 2 processors.

Reading in the silva.eukarya.fasta template sequences... DONE.

It took 3 to read 1238 sequences.

Aligning sequences from amf.trim.unique.pick.fasta ...

Reading in the silva.eukarya.fasta template sequences... DONE.

It took 4 to read 1238 sequences.

[WARNING]: Some of your sequences generated alignments that eliminated too many bases, a list is provided in amf.trim.unique.pick.flip.accnos. If the reverse compliment proved to be better it was reported.

It took 1828 secs to align 147760 sequences.

Output File Names:

amf.trim.unique.pick.align

amf.trim.unique.pick.align.report

amf.trim.unique.pick.flip.accnos

mothur > summary.seqs(fasta=amf.trim.unique.pick.align, name=amf.trim.pick.names)

Using 2 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	0	0	0	0	1	1
2.5%-tile:	10351	19810	14	0	3	4123
25%-tile:	10351	20167	301	0	5	41222
Median:	10351	20167	301	0	6	82444
75%-tile:	10351	20168	301	0	6	123666
97.5%-tile:	43056	43116	301	0	7	160765
Maximum:	43116	43116	301	0	27	164887
Mean:	11536.7	20786.5	284.006	0	5.66645	
# of unique seqs:	147760					
total # of seqs:	164887					

Output File Names:

amf.trim.unique.pick.summary

It took 896 secs to summarize 164887 sequences.

mothur > screen.seqs(fasta=amf.trim.unique.pick.align, name=amf.trim.pick.names, group=amf.trim.pick.groups, start=10351, optimize=end, criteria=95, processors=2)

Using 2 processors.

Optimizing end to 20165.

Output File Names:

amf.trim.unique.pick.good.align

amf.trim.unique.pick.bad.accnos

amf.trim.pick.good.names

amf.trim.pick.good.groups

It took 1874 secs to screen 147760 sequences.

mothur > summary.seqs(fasta=amf.trim.unique.pick.good.align, name=amf.trim.pick.good.names)

Using 2 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	10307	20165	300	0	3	1
2.5%-tile:	10351	20165	301	0	5	3784
25%-tile:	10351	20167	301	0	5	37839
Median:	10351	20167	301	0	6	75678
75%-tile:	10351	20168	301	0	6	113516
97.5%-tile:	10351	20224	301	0	7	147571
Maximum:	10351	22551	301	0	27	151354
Mean:	10351	20187.3	300.996	0	5.84982	

# of unique seqs: 134362

total # of seqs: 151354

Output File Names:

amf.trim.unique.pick.good.summary

It took 794 secs to summarize 151354 sequences.

mothur > filter.seqs(fasta=amf.trim.unique.pick.good.align, vertical=T, trump=.)

Using 2 processors.

Creating Filter...

Running Filter...

Length of filtered alignment: 676

Number of columns removed: 49324

Length of the original alignment: 50000

Number of sequences used to construct filter: 134362

Output File Names:

amf.filter

amf.trim.unique.pick.good.filter.fasta

mothur > summary.seqs(fasta=amf.trim.unique.pick.good.filter.fasta)

Using 2 processors.

	Start	End	NBases		Ambigs	Polymer	NumSeqs
Minimum:	1	661	176	0	3	1	
2.5%-tile:	1	676	292	0	5	3360	
25%-tile:	1	676	299	0	5	33591	
Median:	1	676	300	0	6	67182	
75%-tile:	1	676	300	0	6	100772	
97.5%-tile:	1	676	301	0	7	131003	
Maximum:	1	676	301	0	27	134362	
Mean: 1	675.961		298.938		0	5.833	
# of Seqs:	134362						

Output File Names:

amf.trim.unique.pick.good.filter.summary

It took 9 secs to summarize 134362 sequences.

mothur > unique.seqs(fasta=amf.trim.unique.pick.good.filter.fasta)

134362 133517

Output File Names:

amf.trim.unique.pick.good.filter.names

amf.trim.unique.pick.good.filter.unique.fasta

mothur > summary.seqs(fasta=amf.trim.unique.pick.good.filter.unique.fasta, name=amf.trim.unique.pick.good.filter.names)

Using 2 processors.

	Start	End	NBases		Ambigs	Polymer	NumSeqs
Minimum:	1	661	176	0	3	1	
2.5%-tile:	1	676	292	0	5	3360	
25%-tile:	1	676	299	0	5	33591	
Median:	1	676	300	0	6	67182	
75%-tile:	1	676	300	0	6	100772	
97.5%-tile:	1	676	301	0	7	131003	
Maximum:	1	676	301	0	27	134362	
Mean: 1	675.961		298.938		0	5.833	

# of unique seqs: 133517

total # of seqs: 134362

Output File Names:

amf.trim.unique.pick.good.filter.unique.summary

It took 9 secs to summarize 134362 sequences.

mothur > system(copy amf.trim.unique.pick.good.filter.names AMF.names)

1 file(s) copied.

mothur > system(copy amf.trim.unique.pick.good.filter.unique.fasta AMF.fasta)

1 file(s) copied.

mothur > system(copy amf.trim.pick.good.groups AMF.groups)

1 file(s) copied.

mothur > count.seqs(name=AMF.names, group=AMF.groups)

Using 2 processors.

[ERROR]: processes reported processing 134362 sequences, but group file indicates you have 151354 sequences. Could you have a file mismatch?

It took 3 secs to create a table for 134362 sequences.

Total number of sequences: 134362

Output File Names:

AMF.count_table

mothur > pre.cluster(fasta=AMF.fasta, count=AMF.count_table, diffs=3)

Using 2 processors.

Processing group amf2.trim.groups:

Processing group amfV.trim.groups:

60204 49405 10799

Total number of sequences before pre.cluster was 60204.

pre.cluster removed 10799 sequences.

It took 2163 secs to cluster 60204 sequences.

73513 58013 15500

Total number of sequences before pre.cluster was 73513.

pre.cluster removed 15500 sequences.

It took 170210 secs to cluster 73513 sequences.

It took 170246 secs to run pre.cluster.

Output File Names:

AMF.precluster.fasta

AMF.precluster.count_table

AMF.precluster.amf2.trim.groups.map

AMF.precluster.amfV.trim.groups.map

mothur > summary.seqs(fasta=AMF.precluster.fasta)

Using 2 processors.

[WARNING]: This command can take a namefile and you did not provide one. The current namefile is AMF.names which seems to match AMF.precluster.fasta.

	Start	End	NBases		Ambigs	Polymer	NumSeqs
Minimum:	1	661	176	0	3	1	
2.5%-tile:	1	676	293	0	5	2686	
25%-tile:	1	676	299	0	5	26852	
Median:	1	676	300	0	6	53704	
75%-tile:	1	676	300	0	6	80556	
97.5%-tile:	1	676	301	0	8	104722	
Maximum:	1	676	301	0	27	107407	
Mean: 1	675.983		299.12		0	5.8153	
# of Seqs:	107407						

Output File Names:

AMF.precluster.summary

It took 5 secs to summarize 107407 sequences.

mothur > summary.seqs(fasta=AMF.precluster.fasta, count=AMF.precluster.count_table)

Using 2 processors.

	Start	End	NBases		Ambigs	Polymer	NumSeqs
Minimum:	1	661	176	0	3	1	
2.5%-tile:	1	676	292	0	5	3360	
25%-tile:	1	676	299	0	5	33591	
Median:	1	676	300	0	6	67182	
75%-tile:	1	676	300	0	6	100772	
97.5%-tile:	1	676	301	0	7	131003	
Maximum:	1	676	301	0	27	134362	
Mean: 1	675.961		298.936		0	5.8337	

# of unique seqs: 107407

total # of seqs: 134362

Output File Names:

AMF.precluster.summary

It took 6 secs to summarize 134362 sequences.

mothur > chimera.uchime(fasta=AMF.precluster.fasta, count=AMF.precluster.count_table, dereplicate=t, processors=2)

Using 2 processors.

uchime by Robert C. Edgar

http://drive5.com/uchime

This code is donated to the public domain.

Checking sequences from AMF.precluster.fasta ...

It took 25392 secs to check 49405 sequences from group amf2.trim.groups.

It took 31140 secs to check 58013 sequences from group amfV.trim.groups.

mothur > remove.seqs(fasta=AMF.precluster.fasta, accnos=AMF.precluster.denovo.uchime.accnos, count=AMF.precluster.count_table)

[NOTE]: The count file should contain only unique names, so mothur assumes your fasta, list and taxonomy files also contain only uniques.

Removed 2031 sequences from your fasta file.

Removed 2538 sequences from your count file.

Output File Names:

AMF.precluster.pick.fasta

AMF.precluster.pick.count_table

mothur > summary.seqs(fasta=AMF.precluster.pick.fasta, count=current)

Using AMF.precluster.pick.count_table as input file for the count parameter.

Using 1 processors.

	Start	End	NBases		Ambigs	Polymer	NumSeqs
Minimum:	1	661	176	0	3	1	
2.5%-tile:	1	676	292	0	5	3296	
25%-tile:	1	676	299	0	5	32957	
Median:	1	676	300	0	6	65913	
75%-tile:	1	676	300	0	6	98869	
97.5%-tile:	1	676	301	0	7	128529	
Maximum:	1	676	301	0	27	131824	
Mean: 1	675.961		298.931		0	5.83271	

# of unique seqs: 105376

total # of seqs: 131824

Output File Names:

AMF.precluster.pick.summary

It took 8 secs to summarize 131824 sequences.

mothur > classify.seqs(fasta=AMF.precluster.pick.fasta, count=AMF.precluster.pick.count_table, cutoff=80, reference=silva.eukarya.fasta, taxonomy=silva.eukarya.ncbi.tax, processors=2)

Using 2 processors.

Generating search database... DONE.

It took 4 seconds generate search database.

Reading in the silva.eukarya.ncbi.tax taxonomy... DONE.

Calculating template taxonomy tree... DONE.

Calculating template probabilities... DONE.

It took 15 seconds get probabilities.

Classifying sequences from AMF.precluster.pick.fasta ...

Reading template taxonomy... DONE.

Reading template probabilities... DONE.

It took 15 seconds get probabilities.

It took 626 secs to classify 105376 sequences.

It took 15 secs to create the summary file for 105376 sequences.

Output File Names:

AMF.precluster.pick.ncbi.wang.taxonomy

AMF.precluster.pick.ncbi.wang.tax.summary

mothur > unique.seqs(fasta=AMF.precluster.pick.fasta, count=AMF.precluster.pick.count_table)

105376 105376

Output File Names:

AMF.precluster.pick.unique.count_table

AMF.precluster.pick.unique.fasta

mothur > summary.seqs(fasta=AMF.precluster.pick.unique.fasta, count=AMF.precluster.pick.unique.count_table)

Using 2 processors.

	Start	End	NBases		Ambigs	Polymer	NumSeqs
Minimum:	1	661	176	0	3	1	
2.5%-tile:	1	676	292	0	5	3296	
25%-tile:	1	676	299	0	5	32957	
Median:	1	676	300	0	6	65913	
75%-tile:	1	676	300	0	6	98869	
97.5%-tile:	1	676	301	0	7	128529	
Maximum:	1	676	301	0	27	131824	
Mean: 1	675.961		298.931		0	5.83271	

# of unique seqs: 105376

total # of seqs: 131824

Output File Names:

AMF.precluster.pick.unique.summary

It took 5 secs to summarize 131824 sequences.

mothur > dist.seqs(fasta=AMF.precluster.pick.unique.fasta, cutoff=0.2)

Using 2 processors.

Output File Names:

AMF.precluster.pick.unique.dist

It took 35993 seconds to calculate the distances for 105376 sequences.

mothur > summary.seqs(fasta=AMF.fasta, processors=2)

Using 2 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	661	176	0	3	1
2.5%-tile:	1	676	293	0	5	3338
25%-tile:	1	676	299	0	5	33380
Median:	1	676	300	0	6	66759
75%-tile:	1	676	300	0	6	100138
97.5%-tile:	1	676	301	0	7	130180
Maximum:	1	676	301	0	27	133517
Mean: 1	675.973		299.045	0	5.83314	
# of Seqs:	133517					

Output File Names:

AMF.summary

It took 7 secs to summarize 133517 sequences.

mothur > cluster(column=AMF.precluster.pick.unique.dist,
count=AMF.precluster.pick.unique.count_table)

**********#****#****#****#****#****#****#****#****#****#****#

Reading matrix: ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

****

changed cutoff to 0.01

It took 1203 seconds to cluster

Output File Names:

AMF.precluster.pick.unique.an.unique_list.list

mothur > make.shared(list=AMF.precluster.pick.unique.an.unique_list.list,
count=AMF.precluster.pick.unique.count_table, label=0.03)

unique

Output File Names:

AMF.precluster.pick.unique.an.unique_list.shared

mothur > count.groups()

Using AMF.precluster.pick.unique.an.unique_list.shared as input file for the shared parameter.

amf2.trim.groups contains 58059.

amfV.trim.groups contains 73765.

Total seqs: 131824.

Output File Names:

AMF.precluster.pick.unique.an.unique_list.count.summary

mothur > sub.sample(shared=AMF.precluster.pick.unique.an.unique_list.shared, size=58059)

Sampling 58059 from each group.

unique

Output File Names:

AMF.precluster.pick.unique.an.unique_list.unique.subsample.shared

mothur > count.groups()

Using AMF.precluster.pick.unique.an.unique_list.unique.subsample.shared as input file for the shared parameter.

amf2.trim.groups contains 58059.

amfV.trim.groups contains 58059.

Total seqs: 116118.

Output File Names:

AMF.precluster.pick.unique.an.unique_list.unique.subsample.count.summary

mothur >
collect.single(shared=AMF.precluster.pick.unique.an.unique_list.unique.subsample.shared, calc=chao-invsimpson, freq=100)

Processing group amf2.trim.groups

unique

Processing group amfV.trim.groups

unique

Output File Names:

AMF.precluster.pick.unique.an.unique_list.unique.subsample.amf2.trim.groups.chao

AMF.precluster.pick.unique.an.unique_list.unique.subsample.amf2.trim.groups.invsimpson

AMF.precluster.pick.unique.an.unique_list.unique.subsample.amfV.trim.groups.chao

AMF.precluster.pick.unique.an.unique_list.unique.subsample.amfV.trim.groups.invsimpson

mothur >
rarefaction.single(shared=AMF.precluster.pick.unique.an.unique_list.unique.subsample.shared, calc=sobs, freq=100)

Using 2 processors.

Processing group amf2.trim.groups

unique

Processing group amfV.trim.groups

unique

Output File Names:

AMF.precluster.pick.unique.an.unique_list.unique.subsample.groups.rarefaction

mothur >
summary.single(shared=AMF.precluster.pick.unique.an.unique_list.unique.subsample.shared, calc=nseqs-coverage-sobs-invsimpson, subsample=58059)

Processing group amf2.trim.groups

unique

Processing group amfV.trim.groups

unique

Output File Names:

AMF.precluster.pick.unique.an.unique_list.unique.subsample.groups.ave-std.summary

AMF.precluster.pick.unique.an.unique_list.unique.subsample.groups.summary

mothur > classify.otu(list=AMF.precluster.pick.unique.an.unique_list.list, count=AMF.precluster.pick.unique.count_table, taxonomy=AMF.precluster.pick.NCBI.wang.taxonomy, label=0.03)

Your file does not include the label 0.03. I will use unique.

unique 105376

Output File Names:

AMF.precluster.pick.unique.an.unique_list.unique.cons.taxonomy

AMF.precluster.pick.unique.an.unique_list.unique.cons.tax.summary

mothur > make.biom(shared=AMF.precluster.pick.unique.an.unique_list.shared, constaxonomy=AMF.precluster.pick.unique.an.unique_list.unique.cons.taxonomy)

unique

Output File Names:

AMF.precluster.pick.unique.an.unique_list.unique.biom

I. Preparing Long Ashton's nutrient solution

Please cite:

Hewitt, E.J. (1966) Sand and water culture methods used in the study of plant nutrition. Commonwealth Agricultural Bureaux, Technical Communication No. 22, Second Edition. Cambridge University Press, Cambrigde.

Six stock solutions were prepared in one litre bottles.

Solution A:

$MgSO_4.7H_2O$	-	36.9 g
$MnSO_4.H_2O$	-	0.223 g
$CuSO_4.5H_2O$	-	0.24 g
$ZnSO_4.7H_2O$	-	0.0296 g
H_3BO_3	-	0.186 g
$(NH_4)_6Mo_7O_{24}.4H_2O$	-	0.0035 g
$CoSO_4.7H_2O$	-	0.0028 g
NaCl	-	0.585 g

Solution B:

FeEDTA	-	3 g

Solution C:

$CaCl_2$	-	50 g

Solution D:

K_2SO_4	-	21.74 g

Solution E

$(NH_4)_2SO_4$	-	105 g

Solution F:

$NaH_2PO_4.2H_2O$	-	2.5 g

Ten ml of each stock solution was added to a bottle, which was filled up to 1 liter to make up the Long Ashton's nutrient solution.