Investigating soil microbial interactions of *Portulacaria afra*

By

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Dedication

I dedicate this to my late father hoping that if he was still alive, he would be proud of me and to my lovely family for their support, understanding and encouragement during my studies.

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My appreciation goes to my Supervisor, Professor J.F. Dames for her assistance, patience, love and tender care. Thank you for your endless source of inspiration and for that I developed a deep respect for you. I thank NRF and CTHB for funding this study with Prof J.F. Dames as the grant holder, and Rhodes University for the facilities placed at my disposal.

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I would like to thank the Lord for giving me time, strength, passion and love to keep on with my project; also for giving me such a lovely mother (Nonkonzo Virginia Fulmaka) who has been patient enough to give me education even in the difficult financial situation. Her phone calls during time of stress meant a lot to me including those of my family members.

Abstract

Portulacaria afra commonly known as Spekboom contributes significantly to carbon sequestration and has been widely planted in degraded areas of the Eastern Cape. Approximately 50% of planted cuttings do not survive although the cause of this decline is unknown. Like many indigenous plants, Spekboom forms a symbiotic relationship with mycorrhizal fungi and the interaction with rhizobacteria may enhance and improve plant growth and establishment. This study aims to investigate these relationships which will include a survey of the arbuscular mycorrhizal (AM) fungal populations associated with Spekboom, determination of the causal agent of Spekboom decline, isolation and identification of the associated rhizobacteria and investigation of their plant growth promotion properties and assessing the ability of arbuscular mycorrhizal fungi and selected rhizobacteria to enhance establishment and growth of Spekboom. Soil and root samples from selected trial sites were used to assess AM fungal spore abundance and colonisation; isolation, characterization, and identification of rhizobacteria and determine the interaction of the microbes on Spekboom growth and tolerance to *Fusarium*. AM spore abundance and percentage root colonisation did not differ between the three Spekboom plots. Molecular analyses of the SSU region from the plots showed 4 families of AM fungi and were identified as Ambisporaceae, Glomeraceae, Claroideoglomeraceae and Paraglomeraceae. A suspected *Fusarium* pathogen was isolated and molecularly identified. Pathogenicity tests indicated reduced Spekboom growth with poor root development. Thirty four rhizobacterial isolates were tested for various plant growth promoting abilities. Of these, 6 were able to produce IAA which may promote plant root growth, 27 siderophores and 23 were phosphate solubilisers. Bacterial isolates were molecularly identified to be from various species of *Bacillus*, with some *Arthrobacter*, *Enterobacter*, *Pseudomonas* and *Microbacterium.* Inoculation of Spekboom cuttings with mycorrhizal fungi and selected rhizobacterial isolates significantly improved shoot height. Spekboom cuttings challenged with *Fusarium* and inoculated with mycorrhizal fungi and two rhizobacterial isolates significantly improved growth. The inoculation of cuttings in the nursery with mycorrhizal fungi and selected rhizobacteria is recommended prior to establishing Spekboom in the field.

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LIST OF ABBREVIATIONS

ACC-deaminase = Aminocyclopropane-1-carboxylate

AENP = Addo Elephant National Park

AM = Arbuscular mycorrhizal

ANOVA = Analysis of variance

ATP = Adenosine triphosphate

BLAST = Basic Local Alignment Search Tools

BNPRA = Benomyl Nystation Potato Refampicin Agar

CAM = Crassulacean Acid Metabolism

CAS = Chrome Azurol Sulfonate

CO_2 = Carbon dioxide

CSS = Conservation Support Services

DNA = Deoxyribonucleic acid

ECM = Ectomycorrhizal

ERM = Ericoid mycorrhizal

GIS = Geographic Information System

GPS = Global Positioning System

HCL = Hydrogen chloride

IAA = Indole acetic acid

ITS = Internal transcribed spacer

INVAM = International Collection of (vesicular) Arbuscular Mycorrhizal fungi

KOH = Potassium hydroxide

NA = Nutrient agar

NBRI = National Botanical Research Institute

NCBI = National Centre for Biotechnology Information

OTU = Operational taxonomic unit

PCR = Polymerase chain reaction

PDA = Potato dextrose agar

PGPR = Plant growth promoting rhizobacteria

PVLG = Polyvinyl alcohol-lactic acid glycerol

rRNA = Ribosomal ribonucleic acid

SSU = Small subunit

TSA = Tryptone soy agar

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 MYCORRHIZAS

In 1885, Frank described the term mycorrhizas which mean fungus-root for non-pathogenic, mutually beneficial associations between roots and fungi (Smith and Read, 2008). Mycorrhizal fungi are common soil microorganisms which are well known for their symbiotic relationship established with most vascular plants, where both partners exchange nutrients and energy (Brundrett, 2002). A fungal symbiosis is mostly found under nutrient-limiting conditions. About 80-90% of plants are reportedly colonised by mycorrhizal fungi (Parniske, 2008; Smith *et al*., 2011). They are relevant members of the rhizospheric microbial populations known to carry out many critical ecosystem functions such as improving the nutrient status of their host, playing a major role in nutrient cycling, influencing growth and water absorption, as well as providing protection from root diseases. The host plant is essential for fungal growth and reproduction (Smith and Read, 2008). Mycorrhizal benefits such as drought tolerance result from a combination of physical, nutritional and cellular effects (Aroca *et al*., 2008; Miransari, 2010).

Mycorrhizal fungi colonise the root cortex and develop an extraradical mycelium which permeates the soil surrounding the plant roots. This mycelium forms a network specialised for water and mineral nutrient acquisition from the soil particularly those whose ionic forms have poor mobility or are present in low concentrations in the soil solution such as phosphate and ammonia (Barea *et al*., 2005). The fungi need carbon in the form of sugars in order to develop and complete their life cycle, and the high levels of sugars in mycorrhizal plants is a result of increased photosynthetic capacity (Sheng *et al*., 2008; Wu *et al*., 2009). The accumulation of sugars induced by the symbiosis is a positive reaction to salt stress since sugars can prevent structural changes in soluble protein, maintain the osmotic equilibrium in plant cells and protect membrane integrity (Abd-El Baki *et al*., 2000). Under arid and semi-arid conditions, mycorrhizal plants have the ability to grow better and appear healthier than their non-mycorrhizal counterparts (Auge, 2001; Subramanian *et al*., 2006). Jeffries *et al*. (2003) reported that mycorrhizal structures effectively enhance phosphorus uptake at lower concentrations in the soil than is normally required for plant roots.

1.2 TYPES OF MYCORRHIZAS

Endo- and ectomycorrhizas are the two main types of mycorrhizal associations which differ in their structure, host and physiological relationships (Barea and Honrubia, 2004; Smith and Read, 2008).

Endomycorrhizas occur in most ecosystems of the world and they are associated with most agricultural crops. They also provide biological protection against soil-borne diseases (Smith and Read, 2008). Endomycorrhizas form structures within the root cortex, penetrate the cortical cells and invaginate the cell membrane (Allen, 1991; Harley and Smith, 1983).

Ectomycorrhizas are mostly associated within trees (Shalini *et al*., 2000) and they form a 'Hartig' net within the intercellular spaces of the root cortex and a sheath or mantle around the feeder roots acting as an interface for channelling of nutrients (Kumar and Satyanarayana, 2002). They do not penetrate the cortical cells but surround them. Their extensive mycelium network in the soil serves in transferring nutrients directly from the decaying vegetation (Suverch *et al*., 1991).

1.2.1 Ectomycorrhizas

There are about 6000 ectomycorrhizal plant species in 145 genera and 26 families (approximately 5600 angiosperms and 285 gymosperms), most of which are trees or shrubs. The dominant type of association between roots of most long-living woody perennials and trees and members of Basidiomycota and Ascomycota is known as an ectomycorrhizal (ECM) association (Smith and Read, 2008). Ducousso *et al*. (2004; 2008) discovered new ECM hosts which belong to the *Sarcolaenaceae* (related to *Dipterocarpacea*e and *Cistaceae*) and *Asteropeiaceae*. In South Africa, plantation forest trees species such as *Pinus* and *Eucalyptus* are the main ectomycorrhizal hosts (Hawley *et al*., 2008). It was indicated that the most abundant ECM fungi in many temperate and tropical forests have low host specificity (Smith *et al*., 2009). Some ectomycorrhizal fungi form associations with many different plant species (Horton and Bruns, 2001), while others form associations with fewer plants. ECM symbioses benefits coniferous trees by enabling water and nutrient uptake, protecting roots from

pathogens and interconnecting single plants into common mycorrhizal networks (Selosse *et al*., 2006; Smith and Read, 2008). Their great diversity depends on abiotic and biotic factors such as host plant, soil quality and pollution (Ishida *et al*., 2007; Lilleskov *et al*., 2002; Morris *et al*., 2008; Tedersoo *et al*., 2008 a, b). The main features of an ectomycorrhizal association are the hyphal mantle on the root surface, the 'Hartig' net formed between the cells of the root closest to the root surface and the extension of hyphae into the soil. The hyphae of ectomycorrhizal fungi do not enter root cells in the same way as endomycorrhizal fungi. Rather, the 'Hartig' net is formed when the ectomycorrhizal fungi enter roots between epidermal cells and spread out to form a network of hyphae throughout the root.

1.2.2 Ericoid mycorrhizas

Ericoid mycorrhizal (ERM) fungi, an endomycorrhizal type; forms specialised associations with members of the plant family Ericaceae (Smith and Read, 2008). In South Africa the main group of ericoid plants belongs to the genus *Erica* which is the second most dominant plant in the Fynbos biome (Hawley *et al*., 2008). They are known for their ability to stimulate plant growth in nutrient poor soils and their function is to assist the host plants through the absorption and translocation of nutrients from different biogenic sources that are either limited or unavailable for plants (Cairney *et al*., 2000; Emmerton *et al*., 2001; Grelet *et al*., 2009; Kosola *et al*., 2007). ERM fungi have been shown in culture to produce a wide range of extracellular enzymes that contribute to decomposition and nutrient acquisition (Read *et al*., 2004). They colonise fine roots known as hair roots and produce hyphal complexes in epidermal cells (Smith and Read, 2008). The hyphal connections to the soil unite around the roots as a fine network. The hair roots colonised by ERM fungi are short lived. Therefore, ERM fungi need to continuously re-establish the association as roots grow.

1.2.3 Orchid mycorrhizas

Orchids are the largest plant family that is made up of 25 000 species (Cribb and Govaerts, 2005) that represent 40% of all monocotyledons and 10% of all angiosperms (Scotland and Wortley, 2003); the Orchid mycorrhizas, also endomycorrhizal, are referred to as the second most prevalent mycorrhizal type according to plant species (Arditti, 1992; Rasmussen, 1995; Smith and Read, 1997). Orchids are found on all continents and some Antarctic islands growing in a wide diversity of habitats (Swarts

and Dixon, 2009). They usually form mycorrhizal associations with the Basidiomycota (Currah *et al*., 1997; Rasmussen, 2002; Roberts, 1999; Shefferson *et al*., 2007; Taylor and Bruns, 1997). The plants are characterised by the production of minute seeds that have limited nutrient reserves (Arditti and Ghanni, 2000). During the initial developmental stage, orchids usually depend on mycorrhizal fungi for the supply of resources that are needed for seed germination and growth (Rasmussen and Rasmussen, 2009; Smith and Read, 2008). This fungus dependent mode of nutrition is known as mycoheterotrophy (Freudenstein and Barret, 2010; Leake, 1994). The majority of orchid species are photosynthetic during maturity. Generally, fully mycoheterotrophic orchids have high mycorrhizal specificity towards fungal clades in the Thelephoraceae, Sebacinales and Russulaceae (Abadie *et al*., 2006; Taylor *et al*., 2002). These fungi are also known to form ectomycorrhizas with adjacent tree roots which provide C-based energy sources to support the fungus and the orchid's germination and continued growth.

1.2.4 Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal (AM) fungi are endomycorrhizal soil fungi that have been placed in the phylum Glomeromycota (Schüβler, 2001) and they form symbiotic relationships with over 80% of all terrestrial plants (Brundrett, 2002). They are considered obligate symbiotic biotrophs since they cannot grow in the absence of a host plant providing them with carbohydrates (Hamel and Plenchette, 2007; Harrison, 2005; Martin *et al*., 2007; Muchovej, 2001). It is suggested that AM fungi are the ancestral and predominant form of mycorrhizas (Wang and Qiu, 2006), they exist in the soil as spores and other infective propagules such as hyphal and colonised root fragments (Martin *et al*., 2007).

The fungal hyphae colonise the plant roots by entering the root cortex and serve as an extension of the root system (Douds and Millner, 1999; Muchovej, 2001). This association is distinguished by the formation of arbuscules which are finely branched hyphal structures inside cortical cells that function as nutrient exchange sites between the symbionts (Gregory, 2006; Muchovej, 2001). The AM fungal hyphae also proliferate into the soil (Bethlenfalvay and Linderman, 1992) which helps plants to obtain mineral nutrients and water from the soil and also contribute in improving the soil structure (Javaid, 2009; Rillig and Mummey, 2006). They also are associated with

legumes in the soils with a limited supply of phosphorus and other minor nutrients which can increase plant nutrient uptake (Antunes *et al*., 2006; Javaid, 2010). Improved legume nutrition has been observed with AM fungi and *Rhizobium* as well as increased nitrogen fixation (Guo *et al*., 2010; Requena *et al*., 2001; Tavasolee *et al*., 2011). Two morphological types of root colonisation have been described for AM fungi by Gallaud (1906) that are known as *Arum* and *Paris* type. These two types are distinguished by the presence and absence of intercellular hyphae within the intercellular spaces of the root cortex (Smith and Smith, 1996, 1997). *Arum* colonisation is recognized by the presence of intercellular hyphae spread within the root cortex. Short side branches enter the root cortical cells and branch extensively forming arbuscules. In the *Paris* type extensive intracellular coiled hyphae are observed and spread directly from cell to cell (Smith and Read, 2008). The *Arum* type is commonly found in crop plants whereas *Paris* type is found in plants growing in natural ecosystems. These two types are believed to depend on the host plant species (Brundrett and Kendrick, 1988, 1990) and fungal species may also influence the colonisation strategy (Cavagnaro *et al*., 2001; Gerdemann, 1965). The *Paris* type is observed more often in ferns, gymosperms and many wild angiosperms (Smith and Smith, 1996, 1997). AM fungi of the Glomeraceae family normally form the *Arum* type (Burleigh *et al*., 2002; Cavagnaro *et al*., 2001; Dickson, 2004; Feddermann *et al*., 2008), other genera in the Gigasporaceae family form *Arum* type or an intermediate type with *Paris* type hyphal coils (Cavagnaro *et al*., 2001; Dickson, 2004; Dickson *et al*., 2004; Karandashov *et al*., 2004; Smith *et al*., 2004). These structures are illustrated in Figure 1.1. Environmental factors can influence the interaction between the plant and fungus in different ways (Brundrett, 1991). The degree of colonisation and the structure of associations can be influenced by biotic factors such as genetic identity between the partners (Cavagnaro *et al*., 2001), whereas the abiotic factors such as soil P and N availability and soil water content can identify the development of mycorrhizal association (Brundrett, 1991).

This study focussed on AM fungal associations.

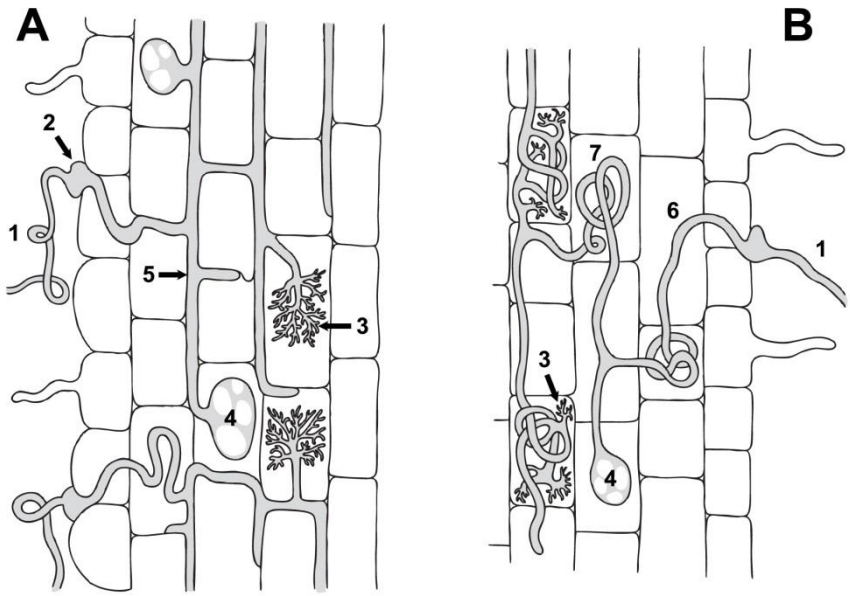

Figure 1.1: Two main types of root colonisation in arbuscular mycorrhizal associations. A: *Arum*-type B**:** *Paris*-type. 1: extraradical hyphae; 2: appressorium/hyphopodium; 3: arbuscule; 4: vesicle; 5: intercellular hyphae; 6: intracellular hyphae; 7: hyphal coils (Johnson *et al*., 2003).

1.3 DIVERSITY OF MYCORRHIZAL FUNGI

The diversity of AM fungi in ecosystems has significant ecological consequences, because individual species or isolates vary in their potential to promote plant growth and adaptation to biotic and abiotic factors (Jeffries *et al.,* 2003). Thus, the composition and dynamics of populations of AM fungi have a marked impact on the structure and diversity of the associated plant communities, both in natural and agricultural ecosystems (Gange *et al*., 1990; Grime *et al*., 1987; van der Heijden *et al*., 1999). Several studies reported that AM fungi increase plant diversity in European grasslands by as much as 30% (Grime *et al.,* 1987; Van der Heijden *et al.,* 1998). The fungi do this by promoting seedling establishment and enhancing competitive ability of subordinate plant species relative to dominant ones (Grime *et al.,* 1987; Van der Heijden *et al.,* 2006). In some cases, however, AM fungi can reduce plant diversity especially in ecosystems where the dominant plants have a high mycorrhizal dependency and obtain most benefit from AM fungi such as in tall grass prairie (Hartnett & Wilson 1999) or some annual plant communities in Australia (O'Connor *et al.,* 2002). The diversity of

AM fungi in the soil has been traditionally estimated on the basis of spore counts. This method can be problematic because fungal sporulation rates are governed by many factors (Bever *et al.*. 1996; Eom *et al*., 2000; Morton *et al*., 1995), and spores are not, therefore, a direct measure of diversity. Furthermore, the population of spores in the soil may bear little relation to the AM fungal population colonising roots (Clapp *et al*. 1995). Increasing plant diversity has been shown to increase AM fungal sporulation and community composition (Burrows and Pfleger, 2002).

1.4 CLASSIFICATION, DISTRIBUTION AND IDENTIFICATION OF MYCORRHIZAS

AM fungi are widely distributed in natural and agricultural soils, their distribution is affected by environmental factors such as soil type and texture, moisture, disturbance, temperature and nutrient availability (Hawkes *et al*., 2011; Lekberg *et al*., 2007; Pringle and Bever, 2002; Rillig *et al*., 2002). Other factors such as high selectivity between host plants and AM fungi may contribute to their distribution (Bever, 2002; Helgason *et al*., 2002; Zhang *et al*., 2010). However, their distribution is unaffected by latitude, elevation or plant species richness (Öpik *et al*., 2010). The classification and systematics of AM fungi has been recently revised (Oehl *et al*., 2011; Schüβler *et al*., 2001; Schüβler and Walker, 2010; Stürmer, 2012). There are currently 3 classes, 15 families and 31 genera in the phylum Glomeromycota (Goto *et al*., 2012; Oehl *et al*., 2011). Most AM fungal species belong to the families of Acaulosporaceae, Archaeosporaceae, Claroideoglomeraceae, Diversisporaceae and Gigasporaceae (Redecker *et al*., 2013). Some genera have increased in the number of known species such as *Acaulospora*, *Ambispora*, *Diversispora*, *Racocetra* and *Septoglomus* that include 10-40 species each (Blaszkowski *et al*., 2013; Estrada *et al*., 2011; Gamper *et al*., 2009; Oehl *et al*., 2012; Palenzuela *et al*., 2011; Lin and Yen, 2011). Their classification is based on the morphology of spores which are formed around or within root system, biochemical properties and molecular studies (Morton and Benny, 1990).

The AM fungi produce asexual spores that contain many nuclei (Hijri and Sanders, 2005; Sanders and Croll, 2010). Spores produced by AM fungi are extraradical and they can range in diameter of up to 500 µm also they consist of abundant storage lipids and some carbohydrate (Hosny *et al*., 1998; Smith and Read, 2008). Their spore walls are thick and resistant; they contain chitin and sometimes β-1,3 Glucan (Gianinazzi-

Pearson *et al*., 1994; Lemoine *et al*., 1995). The physical appearance of spores tends to vary among the species of AM fungi. The ability of spores to germinate is a prerequisite for the establishment of mycorrhizal symbiosis. The developmental process leading to the accumulation of nuclei within spores has been reported to occur by the transport of numerous nuclei from the coenocytic hyphae into the developing spore as observed in the AM fungus, *Glomus etunicatum.* Whether or not mitosis happens within spores is not known. There is no evidence of a sexual stage, nuclei in the hyphae and spores are haploid (Kuhn *et al*., 2001).

Molecular techniques that assess DNA sequences are the most powerful tools used to study the diversity of AM fungi (Jeffries *et al*., 2003). As a result these techniques have been used in combination with morphological or biochemical data to study the diversity of AM fungi. These methods provide greater resolution at intrageneric level than classical taxonomy based on morphology (Helgason *et al*., 1999). Recent studies have used polymerase chain reaction (PCR) techniques in combination with isolate or group specific primers to identify the AM taxa within roots and soil (Clapp *et al*., 1995; Helgason *et al*., 1995; Redecker, 2000; Wubet *et al*., 2003).

PCR is used to amplify the double stranded DNA template by using short single stranded DNA fragments that are synthesized to correspond to the beginning and ending of the DNA stretch to be copied in the presence of a polymerase. This technique has also been used to characterize fungi in mycorrhizal orchid roots thus evading the fungal isolation step (Taylor and Bruns, 1997, 1998). Most PCR methods normally amplify DNA fragments between 0.1 and 10 kilo base pairs (kb) while some techniques allow for amplification of fragments up to 40 kb in size (Cheng *et al*., 1994). The amount of amplified product is determined by the available substrates in the reaction, which become limiting as the reaction progresses (Carr and Moore, 2012). Modifications to the PCR are used to obtain DNA needed for other molecular techniques. Nested PCR is a type of modification of PCR; it is a two stage type of PCR which involves the initial amplification with selected primers (Martin and Rygiewicz, 2005). The specific primers have shorter products and their sequences are located within the sequence generated from the first PCR (Zeze *et al*., 1998).

Parts of the nuclear rRNA regions of AM fungi have also been amplified and sequenced using universal fungal primers (White *et al*., 1990) and genus specific primers (Clapp *et al*., 1995; Simon *et al*., 1992). These genus-specific primers were made to amplify glomalean nuclear rRNA genes straight from colonised roots while removing amplification of plant or non glomalean fungal DNA (Redecker, 2000; Simon *et al*., 1992; Wubet *et al*., 2003). On the other hand, most of the specific primers designed and used in these studies on AM diversity in plant roots only amplified representatives of a limited number of glomalean taxa and also have their limitations (Clapp *et al*., 1999; Daniell *et al*., 2001; Millner *et al*., 2001).

Internal Transcribed Spacer (ITS) is the most widely used region for diversity estimations, taxonomic identification of fungal isolates and uncultured taxa (Kelly *et al*., 2011; Landeweert *et al*., 2003; Wang *et al*., 2011). Also it was modified as the barcode region of fungi by the consortium for the barcode of life (Schoch *et al*., 2012). It is situated in eukaryotic rRNA genes between 18S and 5.8S (ITS1) coding regions and between 5.8S and 28S coding regions (ITS2) (Figure 1.2). The large subunit rRNA region found immediately downstream of ITS has been used for phylogenetic assignment of cultures (Arnold *et al*., 2009; James *et al*., 2006a, b), and also for environmental surveys (Lekberg *et al*., 2012; Weber *et al*., 2013). White *et al*. (1990) have reported that ITS length normally ranges from 600 and 800 bp and can be amplified with universal primers that are specific for the rRNA. The complete ITS region can separate almost all AM fungi species excluding *Glomus intraradices* and its close relatives (Stockinger *et al*., 2010). Large ITS sequences are kept in public databases and the online genus search tool known as Emerencia offers great convenience for extracting AM sequences from GenBank (Ryberg *et al*., 2009). The level of ITS sequence variation found between the individuals of the same species is almost the same as the one found in ITS repeats within individuals (Dallas *et al*., 2000; Gasser *et al*., 1998; Heise *et al*., 1999; Hugall *et al*., 1999; Nadler *et al*., 2000; Stevenson *et al*., 1995). ITS sequences placed in International Nucleotide Sequence Databases have provided an invaluable source for global scale studies in *Inocybe*s (Ryberg *et al*., 2008) and *Tuber* (Bonito *et al*., 2010), both ectomycorrhizal fungi.

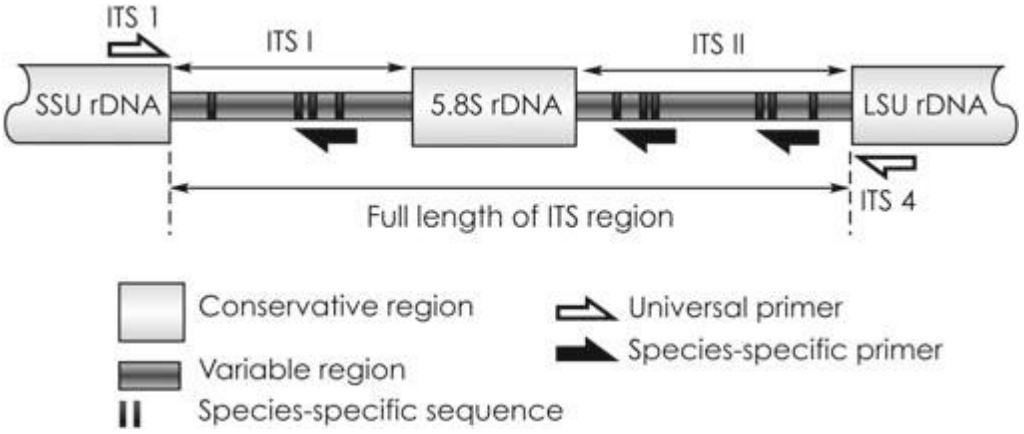

Figure 1.2: Schematic representation of internal transcribed spacer (ITS1-5.8S-ITS2) region (Ludwig *et al*., 2004)

Pyrosequencing (454) is a practical method that is used for increasing sequencing depth and coverage with lowered time, labour and cost (Huse *et al*., 2007; Margulies *et al*., 2005; Moore *et al*., 2006; Weber *et al*., 2007; Wicker *et al*., 2006). It was firstly used to study the composition of microbial communities found in the ocean (Sogin *et al*., 2006) and soil (Roesch *et al*., 2007). One of the critical advantages of this approach is its read length which is generally 400 bp (Shendure and Ji, 2008). Chances of finding sequencing error levels are low because of homopolymer runs (Huse *et al*., 2007; Margulies *et al*., 2005; Moore *et al*., 2006) but these errors tend to be determined in cases where there is enough coverage depth to allow assembly of overlapping reads thus it can be more accurate than traditional Sanger sequences (Goldberg *et al*., 2006; Moore *et al*., 2006; Wicker *et al*., 2006). Nevertheless, short sequences reads make assembly of overlapping sequences problematic (Trombetti *et al*., 2007). In a glacier foreland in the high arctic, Schütte *et al* (2010) conducted a study of bacterial diversity using 454 sequencing which showed a high species turnover along the chronosequence. It was observed that the sequence allow the analysis of a large amount of sequences simultaneously, making it more likely to detect infrequent taxa (Öpik *et al*., 2009). Schluter *et al.* (2008) and Szczepanowski *et al.* (2008) were the first to use pyrosequencing to study antibiotic resistance plasmids in waste water treatment systems. Due to the read length the 18S, small subunit (SSU) was recommended for use when studying AM fungal communities (Öpik *et al*., 2013).

1.5 BENEFITS OF MYCORRHIZAS

1.5.1 Increased uptake of elements

A range of sixteen micro and macronutrients are needed for plant growth and yield production (Marschner, 1995). The loss of nutrients in fertile agricultural ecosystems has become a major concern globally since it causes eutrophication of surface water with adverse consequences for human health, and diversity of downstream ecosystems (Matson *et al*., 1997). In infertile ecosystems, the loss of nutrients lessens plant productivity and ecosystem sustainability if there is no additional nutrient input to maintain plant productivity (Chadwick *et al*., 1999; Wardle *et al*., 2004). A number of factors have been shown to influence the rate at which nutrients leach from ecosystems. These include nutrient availability, land use practises, climate, plant species composition, soil type and structure. These factors influence nutrient cycling within an ecosystem community (Herzog *et al*., 2008; Hooper and Vitousek, 1998; Phoenix *et al*., 2008; Scherer-Lorenzen *et al*., 2003).

AM associations are important in nutrient cycling through their microbial activity and involvement in nutrient uptake (Barea, 1991; Bethlenfalvay and Schüepp, 1994). They enhance the uptake of nutrients for their host plants by development of an extensive extraradical mycelium that explores a larger volume of soil compared to the roots, especially for immobile ions such as phosphate. In addition to improved nutrient supply, AM symbiosis enhances tolerance to biotic and abiotic stress (Bonfante and Genre, 2010; Parniske, 2008; Smith and Smith, 2011). Mycorrhizal hyphae transport mineral nutrients over greater distances, extending the absorption area to beyond the nutrient depletion zone that develops around the roots (Cavagnaro *et al*., 2005; Hodge *et al*., 2001; Jakobsen, 1995). AM colonisation can cause changes to the rhizosphere thus affecting root/hyphae uptake of some nutrients (Amora-Lazcon and Azcón, 1997; Amora-Lazcon *et al*., 1998; Marschner, 1995). Mycorrhizal colonisation increases root length and architecture (Berta *et al*., 1995). They play a role in uptake of phosphorus (P) (Grace and Smith, 2009; Smith and Read, 2008) and a role of nitrogen capture has been demonstrated (Hodge and Fitter, 2010; Leigh *et al*., 2009). Plant micronutrient acquisition can be enhanced by AM fungi (Liu *et al*., 2000; Marschner and Dell, 1994). AM fungi are normally believed to improve the supply of mineral nutrients to the plants in salt stressed soil, more especially the supply of P as it tends to be precipitated by ions

such as Ca^{2+}, Mg^{2+} and Zn^{2+} (Azcon-Aguilar *et al*., 1979; AI-Karaki and Clark, 1998; AI-Karaki, 2000; AI-Karaki *et al*., 2001).

1.5.2 Improved uptake of soil phosphorus

Phosphorus (P) is an important macronutrient required for growth and development of plants which is involved in processes such as photosynthesis, nutrient uptake and cell division (IIImer and Schinner, 1992; Vance *et al*., 2003). The inorganic phosphorus supplied as a chemical fertilizer is used to boost crop production. Extensive use results in deterioration of soil quality (Tewari *et al*., 2004). In soil solution, inorganic P exists as orthophosphate ions depending on the soil pH. Normally, the concentration of soluble P in soil is very low (Goldstein, 1994) because it is fixed by free oxides and hydroxides of aluminium and iron in acidic soils. In alkaline soils, it is fixed by calcium (Goldstein, 1986, 1994; Jones and Smith, 1991). Mineralisation of most organic P substances is carried out by means of phosphatase enzymes and the phosphatase activity is significantly increased in the rhizosphere. The association between AM fungi and plant is mutualistic based on exchange of plant C for soil P and other nutrients including nitrogen (Smith and Read, 2008; Smith and Smith, 2011). High P increases root growth thus it lowers the ratio of colonised to non-colonised root length since there may be no beneficial effects of P uptake by the fungus *per se* (Marschner, 1995; Smith *et al*., 1992). However, very high P application can adapt features of root colonisation and decrease AM fungal biomass in roots and soil as well as alter recognition factors between the fungus and the host (Smith and Read, 2008).

Bruce *et al*. (1994) communicated that early reduction in cucumber colonisation with added P was mediated by slower growth of fungal infection units within the roots while Balzergue *et al*. (2011) enlarged this observation by showing marked reductions in pea roots at high P that were mediated by internal plant derived signals. Most studies have shown that mycorrhizal and non-mycorrhizal fungi use the same labile P sources (Bolan, 1991; Hernández *et al*., 2000) while other studies revealed that mycorrhizal fungi obtain P normally from unavailable sources (Bolan *et al*., 1987; Feng *et al*., 2003; Jayachandran *et al*., 1989, 1992; Koide and Kabir, 2000). Ness and Vlek (2000) noticed that only mycorrhizal maize took up P from hydroxy-apatite which in turn was transferred to maize. Studies that were conducted in Ontario have shown that corn grain yield was strongly affected by P supply and tissue P concentration in the L4 to L5 stage

instead of affected by P concentration at a later stage of growth (Barry and Miller, 1987; Lauzon and Miller, 1997).

Plants that are inoculated with AM fungi are normally more efficient in acquisition of nutrients leading to improved plant growth (Oseni *et al*., 2010). Colonisation of roots by AM fungi has been shown to enhance plant tolerance to various stress factors thus improving crop growth and productivity (Javaid and Riaz, 2008). Yamato *et al*. (2006) have indicated that Biochar, a soil amendment, can cause changes in physical and chemical properties to the soil thus resulting to increase nutrient availability in the soil and increase plant root colonisation by mycorrhizal fungi. At low concentrations in the soil solution, mycorrhizal plants can absorb more P compared to non-mycorrhizal plants as shown in soybeans (Plenchette and Morel, 1996). This has been attributed to the mycorrhizal hyphae that have a higher affinity for P than roots (Howlever *et al*., 1981). In neutral or calcareous soils, mycorrhizas can acidify the rhizosphere through increased proton efflux (Rigou and Mignard, 1994) which can mobilize P (Bago and Azcon-Aguilar, 1997). In acidic soil where P is mainly bound to iron or aluminium, excretion of chelating agents by mycorrhizal fungi can enhance the bioavailable P supply of the soil (Cress *et al*., 1986; Haselwandter, 1995).

1.5.3 Uptake of nitrogen

Nitrogen is the main element in plant development and a limiting factor in plant growth (Vitousek and Howarth, 1991). Atmospheric nitrogen can be used only by some prokaryotes through a process which is known as biological nitrogen fixation where it converts atmospheric N_2 to NH_3 in a form that can be used by plants (Franche *et al*., 2009; Lam *et al*., 1996). During nitrogen fixation, the conversion of N_2 to NH_3 is catalysed by nitrogenase enzyme which is sensitive to oxygen and it is irreversibly inactivated when extracted from cells in the presence of free energy (Robson and Postgate, 1980). The presence of *Rhizobium* or *Azospirillum* species can increase plants productivity and plant communities through the production of available nitrogen (Cleveland *et al*., 1999). Primarily, *Rhizobium* connects with legumes and its existence can increase total community nitrogen by direct supply to legumes (He *et al*., 2004; Johansen and Jensen, 1996; Paynel and Cliquet, 2003; Thomas and Asakawa, 1993). A shift community composition allows legumes to compete with dominant species (van der Heijden *et al*., 2006a; Wurst and van Beersum, 2009). Associative N_2 fixers that are

connected with the rhizosphere of plants may lead to significant levels of N_2 fixation (Montanez *et al*., 2009; Wickstrom and Garono, 2007). Studies have also revealed an increased plant growth as a result of growth hormones made by the bacteria or enhanced mineral uptake connected with colonisation by *Azospirillum* (Dobbelaere *et al*., 2003; Lin et al., 1983; Okon and Labandera-Gonzalez, 1994). AM fungi and nitrogen fixing bacteria have the ability to lessen phosphorus and nitrogen limitation on host plants (Larimer *et al*., 2010; Stanton, 2003). They can also aid establishment of plants on degraded sites such as mine spoils (Roy *et al*., 2007; Walker *et al*., 2004).

1.5.4 Water acquisition

Water is one of the important limiting factors that affect plant growth, development and yield in arid and semi-arid areas where plants are exposed to drought stress (Kramer and Boyer, 1997; Maggio *et al*., 2000). Drought limits crop productivity by lowering average yields by more than 50% (Wang *et al*., 2003). The reaction of plants to water stress relies on various factors like plant genetic resistance, developmental stage and duration of exposure to drought (Echave *et al*., 2005; Panozzo and Eagles, 1999; Song *et al*., 2011). Critical water stress in wheat during post-anthesis causes chlorophyll loss, leakage of cell solutes, and flag leaf yellowing (Beltrano *et al*., 1994, 1999). It also results in changes in cell membrane properties including selective permeability, fluidity and microscopic viscosity (Beltrano *et al*., 1994, 1999; Navarri-Izzo *et al*., 1993).

Symbiotic interactions of plants with mycorrhizal fungi are agriculturally and economically important (Indrasumunar, 2007). Inoculation by AM fungi has been found to improve water relations of many plants. Most plants benefit from mycorrhizal symbiosis under water stress environments by the improvement of the water status and uptake (Al-Karaki, 1998; Aliasgharzad *et al*., 2006; Auge 2001; Bolandnazar *et al*., 2007; Koch *et al*., 2006; Omirou *et al*., 2013; Smith *et al*., 2010; Smith and Read, 1997; Wu *et al*., 2013). Root colonisation by AM fungi has been shown to improve productivity of many crop plants in soils under drought stress (Al-Karaki and Al-Raddad, 1997; Al-Karaki and Clark, 1998; Faber *et al*., 1990; Sylvia *et al*., 1993). It has also been shown to increase drought resistance of wheat (Allen and Boosalis, 1983; Al-Karaki and Al-Raddad, 1997; Al-Karaki and Clark, 1998; Ellis *et al*., 1985; Sylvia *et al*., 1993) including other plant species (Davies *et al*., 1992; Ruiz-Lozano *et al*., 1995).

Leaf water potential in non-stressed plants are not affected by AM fungal symbioses (Goicoechea *et al*., 2005). Mycorrhizal plants enhance water-use efficiency and plants return to normal health after water stress as compared to non mycorrhizal plants (AI-Karaki *et al*., 2004). Porcel and Ruiz-Lozano (2004) have discovered that leaf water potential increased in stressed mycorrhizal plants than in non-mycorrhizal stressed plants. AM fungal colonisation has also been noted to affect stomatal sensitivity to atmospheric water status (Huang *et al*., 1985). Mycorrhizal lettuce plants showed increased superoxide dismutase activity under drought stress and this correlates to plant protection against drought (Ruiz-Lozano *et al*., 1996, 2001a). AM soybean plants undergoing drought had lower oxidative damage to lipids and proteins in nodules that non-AM plants, and this was connected to protection against nodule senescence (Porcel *et al*., 2003; Ruiz-Lozano *et al*., 2001b). The effect of AM fungal inoculation on water stress in wild strawberry was investigated by Borowicz (2010) and he reported that there were no strong effects of AM fungi on strawberry tolerance to drought. Yin *et al*. (2010) communicated that inoculation of AM fungi in drought stressed strawberry plants increased enzymes that are connected with the plants protective system to water stress. Strawberry plants can be highly responsive to AM fungal colonisation (Borkowska, 2002; Castellanos-Morales *et al*., 2010). These studies highlighted the complex nature of mycorrhizal interactions.

1.6 PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR)

Plant growth promoting rhizobacteria (PGPR) are important for increasing plant growth and development under non-stress and stress conditions by direct and indirect mechanisms (Glick *et al*., 2007; Nadeem *et al*., 2010b; Zahir *et al*., 2004). Direct mechanisms occur by supplying beneficial compounds to the host plant synthesized by the bacterium or facilitating uptake of nutrients from the soil (Kloepper *et al*., 1978; Zahir *et al*., 2004). Growth of host plants associated with PGPR is facilitated by fixation of atmospheric nitrogen, production of phytohormones and solubilisation of phosphate compounds increasing availability (Glick, 1995; Kloepper *et al*., 1989; Patten and Glick, 2002). Indirect mechanism occurs when PGPR prevent or reduce a number of harmful effects of plant pathogens (Glick and Bashan, 1997). PGPR produce metabolites which lessen pathogen populations and siderophores that sequesters iron making this less available for pathogens (Arora *et al*., 2001; Bhattacharyya and Jha, 2012; Kloepper, 1996). They also expand plant resistance against diseases by changing

the susceptibility of the host plant via induced systemic resistance providing protection against pathogen attack (Saravanakumar *et al*., 2007). They enhance plant growth and development by ACC-deaminase and chitinase enzymes also by the production of substances that help plants to resist stress conditions (Ashraf *et al*., 2004; Glick *et al*., 2007; Sandhya *et al*., 2009). Adverse effects of drought stress on growth of pea plants are reduced by PGPR having ACC-deaminase (Zahir *et al*., 2008). The following environmental factors such as climate, weather conditions, soil characteristics and interaction with other natural microbial flora in the soil determine the efficiency of PGPR (Giongo *et al*., 2008). PGPR may colonise the rhizosphere, the surface of the root or intercellular spaces (McCulley, 2001) and they will also join to the surface of the plant in these interactions (Andrews and Harris, 2000). The moisture content of soil affects the colonisation of the plant rhizosphere by the PGPR (Burret *et al*., 1978). The number of PGPR decreases in the rhizosphere after inoculation while their effects last through the growing season, indicating a possible occupation of intercellular spaces (Jacoud *et al*., 1998). Rhizobacterial strains can solubilise complexed inorganic P (Khan and Zaidi, 2007; Zaidi and Khan, 2005, 2007), mineralise organic P (Khan *et al*., 2007; Ponmurugan, 2006), improve plant stress tolerance to drought and metal toxicity, thereby resulting in increased plant growth. Some PGPR have the ability to alleviate salinity accumulation due to irrigation which negatively effects crop production (Hamaoui *et al*., 1996).

PGPR improve/enhance the development of mycosymbionts and facilitate the colonisation of plant roots by AM fungi (Hildebrandt *et al*., 2002; Jaderlund *et al*., 2008). The occurrence of PGPR supports the establishment of mycorrhiza and enhances their ability to carry out different functions for example inoculation with *Paenibacillus brasilensis* has been reported to increase the extent of root colonisation by *G. mosseae* on clover (Artursson, 2005). A study that was conducted by Constantino *et al*. (2008) observed that the combination of PGPR and mycorrhiza was effective for improving plant growth and nutrient content of habanero chilli. Plots inoculated with PGPR and or mycorrhizas alone or in combination had higher amount of N, P and K (Adesemoye *et al*., 2012). The positive effects of PGPR and mycorrhiza interactions with barley roots enhanced water and nutrition absorption (Najafi *et al*., 2012). Some endophytic PGPR may use other organisms as vectors to get access to apoplastic spaces in their host plant. AM fungi and the mealybug (*Saccharicoccus sacchari)* have been showed to aid

endophytic colonisation of host plants by *Gluconacetobacter diazotrophicus* (Franke *et al*., 2001; Isopi *et al*., 1995). Different types of bacterial species belonging to the genera *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, A*lcaligens*, *Athrobacter*, *Burkholderia*, *Bacillus* and *Serratia* have been reported to enhance plant growth (Patten and Glick, 2002). *Pseudomonas* and *Bacillus* have appeared as the largest and the most promising group of PGPR due to their rapid growth, mobility, nutritional requirements and ability to use different organic substrates (Defago *et al*., 2010).

1.6.1 *Bacillus*

Bacillus is the dominant genus in the rhizosphere and different metabolites that are released by these species affect the environment by increasing nutrient availability to plants (Charest *et al*., 2005). The secondary metabolites have antibacterial or antifungal activity against pathogens (Asaka and Shoda, 1996; Compant *et al*., 2005). The bacteria are in contact with higher plants and promote their growth (Garcia *et al*., 2004). When *B. licheniformis* was inoculated on tomato and pepper, colonisation was established and it was incorporated as a biofertilizer without changing the normal greenhouse management practices (Garcia *et al*., 2004). Phosphate solubilising *Bacillus* species stimulates plant growth by enhanced P nutrition and increasing the uptake of nitrogen, potassium and iron (Biswas *et al*., 2000). *Bacillus* species have been used as a commercial biofertilizer and biocontrol products to improve agricultural yields (Bano and Mussarat, 2009; Vassilev *et al*., 2006; Wani *et al*., 2007). These biofertilizers can have direct effects on plant growth by the synthesis of plant growth hormones (Amer and Utkhede, 2007). It has an ability to survive under unfavourable conditions by a trait of endospore formation (Emmart and Handelsman, 1999).

Selected *Bacillus* species can suppress plant diseases caused by root and foliar pathogens by inducing a resistance response in the host plant (Choudhary and Johri, 2009). *Bacillus megaterium* can reduce metals potentially increasing the bioavailability of iron (Valencia-Cantero *et al*., 2007). *Bacillus mucilaginous* has the ability to solubilise potassium (Wu *et al*., 2005) and phosphate (Idriss *et al*., 2002). The growth of soybean as well as increased levels of nodulation has been enhanced by the inoculation of *Bacillus* and *Bradyrhizobium japonicum* (Bai *et al*., 2001). It was shown that *Bacillus subtilis* assisted hydroponically grown tomato plants to overcome salinity

stress (Woitke *et al*., 2004). Jaizme-Vega *et al*. (2004) deduced that *Bacillus* species can enhance plant health and survival rates in nurseries after they assessed the effect of a rhizobacterial consortium of *Bacillus* species on first developmental stages of micropropagated banana. Under organic growing conditions, *Bacillus* species have the ability to increase the yield, growth and nutrition of raspberry plants (Orhan *et al*., 2006). In Mongolia, *Bacillus pulimus* has been used as a biofertilizer to increase the crop yield of the wheat variety Orkhon (Hafeez *et al*., 2006).

1.6.2. *Pseudomonas*

P. fluorescens strains have been used as seed inoculants on crop plants to promote growth and increase yields (Wachowska *et al*., 2006). They have been isolated from different areas of the world with metabolic activities such as production of siderophores and hydrogen cyanide that affects the availability of soil nutrients and protects against pathogens (Abbas-Zadeh *et al*., 2010; Glick *et al*., 1998; Jalili *et al*., 2009; Miransari, 2013). They are important PGPR used as biofertilizers and have the ability to enhance crop yield by direct and indirect mechanisms (Walsh *et al*., 2001). Some studies have revealed that predominance of *Pseudomonas* in the rhizosphere of different crops (Kumar and Sugitha, 2004) and strains of *Pseudomonas* have the ability to solubilise phosphorus in soil and increase its availability to plants (Sundara *et al*., 2002). In a study that was conducted with wheat and a pseudomonad, results indicated that the less fertile the soil, the greater the plant growth stimulation by the PGPR (De Freitas and Germida, 1990a). It was reported that these species are able to over-winter in sufficient quantities on the roots of winter wheat (De Freitas and Germida, 1990b).

P. fluorescens strain P13 has the ability to promote plant growth however in contaminated soils, it enhance plant growth due to its inability to reduce phytotoxicity (Li *et al*., 2005, 2010). *P. fluorescens* have been used to suppress wilts of various *Fusarium* plant pathogens (Lemanceau and Alabouvette, 1993) and the casual-agent of bacterial canker (Amkraz *et al*., 2010). A number of *Pseudomonas* strains that are used in-biocontrol can produce one or more antibiotic compounds with antifungal abilities *in-vitro* (Raaijmakers *et al*., 2002). Inoculation of canola seeds with *P. fluorescens* and *P. putida* lead to alleviation of salinity stress on canola seed germination and seedling growth (Miransari, 2011a, b, c). *P. fluorescens* showed strong antifungal activity

against *Rhizoctonia bataticola* and *Fusarium oxysporum* found in sugarcane and rice by the production of antifungal metabolites (Kumar *et al*., 2004).

1.7 SPEKBOOM

Spekboom also known as *Portulacaria afra* (*P. afra*) is a small succulent tree (Figure 1.3) which reaches a height of 2,5 m to 4,5 m with a trunk which averages 20 cm in diameter and can live up to 200 years (Guralnick, 1984b). It is a member of the Didiereaceae family as well as dominant species which grows well in areas with an annual rainfall of 250 to 375 mm and warm summers (Cowlings and Mills, 2010; Vlok *et al*., 2003).It is also known for its high leaf litter production (Lechmere-Oertel, 2008) and soil binding properties. Like many indigenous plants, Spekboom forms a symbiotic relationship with mycorrhizal fungi and the interaction with rhizobacteria may enhance growth and establishment. It plays a role in thicket restoration and is able to fix carbon in semi-arid environments (Lechmere-Oertel, 2003; Mills *et al*., 2006). It has a unique ability of absorbing more carbon from the atmosphere compared to other plants and Spekboom thicket stores carbon in excess of 200 tons per hectare (Mills *et al*., 2005a) which is an incredible aspect for semi-arid environment unlike that of mesic forest ecosystems (Mills *et al*., 2005). It is able to switch between crassulacean acid metabolism (CAM) and a C_3 photosynthetic pathway depending on the soil moisture (Guralnick *et al*., 1984; Guralnick and Ting, 1987). This allows *P. afra* to absorb carbon even in times of drought. CAM is adapted for increased water efficiency which is usually found in plants that grow under arid conditions (Herrera, 2008). At night, a plant that uses CAM opens its stomata to allow carbon dioxide to enter. It is secured as organic acids that are kept in vacuoles while during the day, stomata closes to avoid loss of water and carbon is released to the Calvin cycle for photosynthesis to occur (Herrera, 2008; Ting, 1985). This plant is rich in manganese, cobalt and more especially magnesium and large quantities of the micro elements, iodine and selenium (Milewsky, 2004). Degradation of the thicket has resulted primarily due to overgrazing by domestic livestock which strip the leaves from the bottom unlike indigenous browsers such as elephant and kudu which graze the shrubs from above (Stuart-Hill, 1992), and consequently destroyed the spekboom and the micro-climate under their protective thickets (Hoffman and Cowling, 1990; Lloyd *et al*., 2002).

Of the 16,942 km^2 of solid thicket, 46% has been heavily impacted, 36% of which has resulted from domestic herbivores whereas 1.8% and 0.5% have been changed by cropping and urbanization (Lloyd *et al*., 2002). Thicket degradation leads to loss of biodiversity (Lechmere-Oertel *et al*., 2005; Moolman and Cowling, 1994), great losses of ecosystem carbon stocks (Mills *et al*., 2005), a reduction in soil quality (Mills and Fey, 2004) and reduction in plant productivity (Stuart-Hill and Aucamp, 1993). In semi-arid environments, degraded thicket does not return to a normal state if the pressure of the livestock is lowered. *P. afra* grows from cuttings and can be used to return vegetation structure in many intact thicket types (Mills and Cowling, 2006). Spekboom thickets which have *P. afra* as the dominant canopy tree (Vlok *et al*., 2003) are mostly limited to the Eastern Cape (Lombard *et al*., 2003). The scale of degradation and the costs of restoration show that considerable financial investment will be needed to restore intact thicket (Lechmere-Oertel, 2003). It was suggested that *P. afra* could be one of the cost effective method of rehabilitating large areas of the subtropical thicket biome (Swart and Hobson, 1994). Restoration of the degraded Sundays Spekboom veld with *P. afra* cuttings sequester great amount of carbon for a semi-arid area which in turn makes the restoration of this plant species type financially attractive at current prices of carbon credits on international markets (Mills *et al*., 2007). Powell *et al*. (2004) recommended a joint approach of rehabilitating with *P. afra* truncheons and large specimens of *Aloe*, *Euphorbia*, *Crassula* and other succulent species to create instant bush structure. Planting *P. afra* truncheons into closely spaced parallel rows was regarded as the applicable restoration methodology for degraded spekboom thicket landscapes (Mills *et al*., 2007).

P. afra is a useful medicinal plant and traditional uses include the increase of breast milk by lactating mothers. The leaves are used to quench thirst while sucking a leaf is used for treating exhaustion, dehydration and heat stroke. Crushed leaves provide relief for blisters and corns. The leaves are chewed as a treatment for sore throat and mouth infections and the juice is used for soothing skin ailments such as pimples, rashes, insect stings and sunburn. The honey made from *P. afra* is unsurpassable in flavour and texture (Roberts, 1990).

Figure 1.3: Spekboom or jade plant, Eastern Cape, South Africa

1.8 PROBLEM STATEMENT

South African Subtropical thicket ranges from closed shrubland to low forest and it is situated on coastal forelands of the Eastern Cape. This vegetation supports natural diversity and large browsing mammals (Kerley *et al*., 1999; Skead, 1987). Mature crops are gathered from the veld by local people for wood and medicines (Cocks and Wiersum, 2003). It is a centre of a growing tourism industry and stores great amount of carbon (Mills *et al*., 2005). In South African drylands, desertification is mainly caused by livestock overgrazing (Hoffman and Ashwell, 2001; Milton *et al*., 1994) and subtropical thicket appears as one of the most livestock-degraded ecosystems in the country (Hoffman and Cowling, 1990; Kerley *et al*., 1995; Mills and Fey, 2004; Thompson *et al*., 2009). In Subtropical thicket, browsing by livestock lead to loss of shrub canopy (Lechmere-Oertel *et al*., 2005) whereas in savanna dryland, livestock induced degradation lead to shrub encroachment (Higgins *et al*., 1999; O'Connor, 1994). The loss of canopy cover causes changes in soil properties and water interactions connected to the supply of water-related ecosystem services like local erosion prevention, topsoil maintenance, catchment-scale flood prevention and maintenance of the river baseflow (Andreu *et al*., 1998; Bautista *et al*., 2007; Fernandez *et al*., 2012; O'Farrell *et al*., 2009). Subtropical thicket has been changed by unsustainable farming practices over the last century which has decreased benefits for

pastoralists, local communities, farmers, tourism operators and conservationists (Lechmere-Oertel *et al*., 2005; Mills *et al*., 2005). Spekboom as a dominant canopy species was the first to show decline due to browsing and does not show clear regeneration when the browsing pressure is removed. In serious cases of degradation it is absolutely eliminated (Lechmere-Oertel *et al*., 2005). Planting *P. afra* cuttings is one of the most cost-effective ways of addressing climate change as it plays an important role in controlling climate change since it absorbs CO_2 (Mills and Cowling, 2006; Swart and Hobson, 1994); and it can provide temporary employment to people by helping in the alleviation of rural poverty and the landscape value for land users would be increased (Clewell and Aronson, 2005; Cocks, 2006). Deforestation accounts for over 20% of the CO_2 humans produce, rivalling emissions from other sources. The capacity for sequestering carbon credits in South Africa from spekboom on the international carbon trading market is appreciable (Mills *et al*., 2007) because this plant has the ability to counteract harmful carbon emissions equal to that of moist, subtropical forest. In South Africa, this plant is used to restore habitats that have been over-used and stripped bare by the herds of goat and other livestock (Stuart-Hill, 1992).

Overall aim

The overall aim of this study was to investigate soil microbial interactions of *Portulacaria afra*.

Specific objectives

In order to achieve this aim, the following was addressed:

1. To conduct a survey of arbuscular mycorrhizal populations associated with Spekboom in selected areas of the Eastern Cape.
2. To isolate and identify the causal agent of Spekboom decline.
3. To isolate and identify the plant growth promoting rhizobacteria.
4. To assess the plant growth promotion properties of bacterial isolates.

5. To assess the interaction between arbuscular mycorrhizal fungi, PGPR and their biological control potential under greenhouse conditions.

Hypothesis

Arbuscular mycorrhizal fungi in combination with plant growth promoting bacteria improve growth of *Portulacaria afra*.

CHAPTER 2: METHODS AND MATERIALS

2.1 STUDY SITES AND SAMPLING

Initially, three soil and plant root samples were collected from Addo Elephant National Park (AENP) which is situated close to Port Elizabeth with GPS coordinates of 33°26′46′S, 25°44′45′E to isolate potential pathogens and verify if the suspected fungal pathogens affect the growth of spekboom. Soil and root samples were further collected at three different Spekboom trial plots at Echo, Helspoort and Hounslow to investigate plant growth promotion abilities of bacterial isolates and also assessing the ability of AM fungi and selected rhizobacteria to enhance establishment and growth of spekboom.

Spekboom (*Portulacaria afra*) is regarded as a suitable plant for restoring degraded semi-arid subtropical thicket in the Eastern Cape as well as being a valuable asset for farmers in term of browse value, soil retention, carbon sequestration and carbon offset credits. The Department of Water Affairs, through the Eastern Cape Restoration Programme, initiated an ambitious project to plant spekboom cuttings in over 300, 50 x 50 m (1/4 hectare) trial sites throughout areas of degraded semi-arid subtropical thicket. Subsequently, the trial has migrated with all the thicket restoration work to the Department of Environmental Affairs, the Natural Resource Management Directorate (Mike Powell, personal communication). The trial sites were planted in 2008 after selection of suitable propagation material. The growth and survivorship of cuttings were assessed by the Conservation Support Services (CSS)-Geographic Information Specialists (GIS), Grahamstown, South Africa in 2012. Trial sites were scored for percentage living stems giving an average of 26.3% survivorship (CSS-GIS, unpublished data). Plant condition, plant height, canopy diameter, stem diameter and mortality data was combined and plots were rated as high, medium or weak condition (Figure 2.1). Three sites close to Grahamstown representing one trial plot in each category were selected (Table 2.1, Figure 2.1 and Figure 2.2).

Soil and root samples were randomly collected within each plot at 10 points. Samples were placed in ziplock bag, taken to the laboratory and stored in the cold room at 4ºC. Samples for analysis were prepared within 2 days of collection. Composite samples were mixed using 100 g of each replicate for each plot and were sent for soil nutrient analysis to Eco- Analytica Laboratory, Potchefstroom, South Africa.

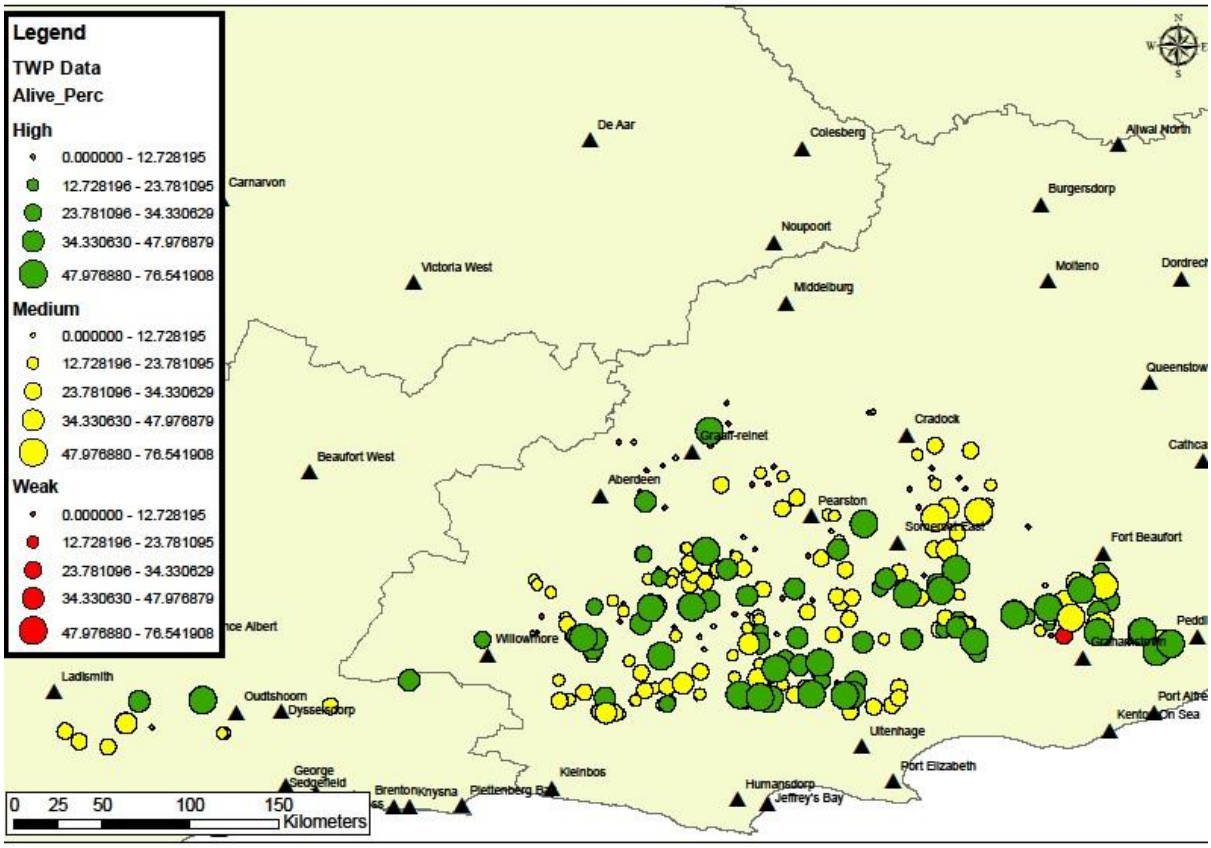

Figure 2.1: Spekboom trial plots indicating survivorship and plant condition (CSS-GIS, unpublished data).

Table 2.1: Spekboom plant trials used in the current study (CSS-GIS, unpublished data).

Site name	Latitude	Longitude	Elevation (m)	Plot number	Alive (%)	Plant condition
Echo	-33.05550315	26.35857732	426	S0027	55.11	High
Helspoort	-33.169537	26. 31622667	585	S0056	21.00	Medium
Hounslow	-33.19738082	26.43825013	533	S0017	27.30	Weak

Figure 2.2: Condition of the Spekboom at trial sites a) Echo, b) Helspoort and c) Hounslow.

2.2 AM FUNGAL SPORE ENUMERATION AND CHARACTERIZATION

Spores were extracted from soil samples using a wet sieving and decanting technique followed by sucrose centrifugation (Brundrett *et al*., 1996; Smith and Dickson, 1997) in order to determine spore abundance and separate morphotypes. One hundred grams of soil was weighed and suspended in 500 ml of water. The suspension was mixed to free spores from soil particles and allowed to settle for 45 s. The supernatant was decanted through a combination of sieves having mesh sizes of 425, 250, 125 and 45 µm to separate spores of interest. The sieves were then washed and the large debris was discarded. Debris from the remaining sieves was washed with water into 50 ml centrifuge tubes. Samples were centrifuged using Megafuge 1.0R at 1900 rpm for 5 min

and the supernatant was discarded without disturbing the pellet. The pellet was re-suspended in 60% sucrose solution and further centrifuged for 5 min. The supernatant was filtered using a Buchner funnel onto a gridded-filter paper (Whatman #1) and washed with water to remove sucrose. The filter paper was transferred to a clean Petri dish and spore abundance was examined microscopically using a Leica S4E dissecting microscope. Spores were selected using a moistened needle and were separated into morphotypes. They were mounted onto a microscope slide with a drop of polyvinyl alcohol-lactic acid-glycerol (PVLG) (Koske and Tessier, 1983) and another drop of PVLG mixed with Melzer's reagent (Brundrett *et al*., 1994). The spores were then examined and morphological characteristics such as spore shape, colour, spore wall characteristics and subtending hyphae were recorded. Images were captured using a compound microscope (Olympus BX50).

2.3 ANALYSIS OF AM FUNGAL POPULATIONS

A composite soil sample from each plot (equal quantity from each replicate sample, thoroughly mixed) was prepared. A composite 1 cm root sample from each replicate per plot was also prepared. DNA was isolated from a composite soil (1 g) and root sample from Echo, Hounslow and Helspoort trial plots using ZR Soil Microbe DNA MiniPrep™ (Catalog No: D6001) and ZR Plant/Seed DNA MiniPrep™ (Catalog No: D6020), respectively according to the manufacturer's instructions to identify the diversity of arbuscular mycorrhizal fungi. The roots were ground using a mortar and pestle. DNA extracted from soil and root samples was amplified using SSU primers which were AML2 (5'-GAACCCAAACACTTTGGTTTCC-3') and NS31 (5'-TTGGAGGGCAAGTCTGGTGCC-3') (Davison *et al*., 2012). The PCR reaction consisted of 12.5 µl of KAPA HiFi Hotstart ReadyMix (Catalogue No: KK2602), 1.5 µl of primers (AML2 and NS31), 3 µl of DNA (each of soil and root sample separately) and 6.5 µl of nuclease-free water. The reaction (25 µl) was carried out in a MJ Mini Personal Thermal Cycler under the following conditions; initial denaturation at 98ºC for 5 mins, 10 cycles of denaturation at 98ºC for 30 s, 49.7ºC at 45 s, 72ºC for 60 s. Followed by 15 cycles of the annealing step at 98ºC at 30 s, 65ºC at 45 s and 72ºC at 60 s then the final extension at 72ºC for 5 mins. The amplified PCR products from root and soil were assessed and quantified for purity of DNA using Thermo Scientific Nanodrop 2000 Spectrophotometer also analysed using 1% (1 g of agarose gel, 100 ml of TBE and 2 µl of ethidium bromide) agarose gel. The gel was loaded with 5 µl of the

100 bp marker (Promega, Catalogue No: G173A) and 10 µl of extracted DNA which was mixed with 5 µl of the loading dye (Promega, Catalogue No: G190A). The gel was then run at 100 V for 45 mins and viewed under UV transilluminator (UVIPRO Chemi) on Gel Doc, an image was obtained. The untagged PCR product from roots and soil samples of each pot trial was mixed (1:1) and amplified using MID tagged primers under the same cycling parameters and temperatures but with different cycles of denaturation (5 cycles) and annealing step (20 cycles). The amplified products were separated using agarose gel as previously described.

The PCR product was purified using a Promega Wizard SV Cleanup kit (Catalogue no: A9281) following the manufacturer's instructions. The tagged PCR product was purified by excising a DNA band from the gel which was placed in a 1.5 ml microcentrifuge tube. Membrane Binding Solution (300 µl) was added to each gel slice. This was vortexed and incubated at 50-65ºC until the gel slice was completely dissolved. An equal amount of Membrane Binding Solution was added to the amplified product which in turn was transferred to the SV Minicolumn into a collection tube and incubated at room temperature for 1 min. The minicolumn was centrifuged at 13, 000 x g for 1 min and the flow through was discarded. The minicolumn was reinserted into a new collection tube. Membrane Wash Solution (700 µl) was added and centrifuged at 13, 000 x g for 1 min then the flow through was discarded. This step was repeated with 500 µl of Membrane Wash Solution and centrifuged at 13, 000 x g for 5 mins. The collection tube was emptied and the column assembly was re-centrifuged for 1 min with the microcentrifuge lid open to allow evaporation of any residual ethanol. The minicolumn was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of nuclease-free water was added to the column then incubated at room temperature for 1 min. After centrifugation at 13, 000 x g for 1 min, the DNA was stored at 4ºC. The amplified products were separated using agarose gel as described previously. The 454-pyrosequencing was conducted. After the pyrosequencing data was received, it was analysed using Mothur (Schloss *et al*., 2009). Sequences were aligned and singletons were removed. Alignments were divided into operational taxonomic units; these were submitted to MaarjAM database (Öpik *et al*., 2010) to be used as a reference for environmental sequence identification. The criteria for identification were to select the first three sequences from the database within the highest percentage identity with the highest number of base pairs coverage was used.

2.4 ASSESSMENT OF COLONISATION

Root samples were washed with water to remove soil and cut into sections of 1-3 cm. They were covered with 5% KOH to remove cytoplasm and left overnight at room temperature. The roots were rinsed with water to remove KOH. Roots were bleached using freshly prepared alkaline H_2O_2 solution for 30 mins. The bleach was discarded when roots were white and roots were rinsed with water. They were covered with 0.1 M HCl solution and left at room temperature overnight to acidify roots. The HCl was discarded and roots were stained with a mixture of lactic acid, glycerol, water solution in the ratio of 13: 12: 16 and 0.05% trypan blue (Sigma, Catalogue no: 23850) (Brundrett *et al*., 1996) overnight at room temperature. The stain was poured off and roots were destained with lactoglycerol solution for 12-24 hrs. Stained root samples were placed on a microscope slide with lactoglycerol and scanned for the presence of AM structures such as hyphae, arbuscules and vesicles at a magnification of up to 400X using an Olympus BX50 microscope. The colonisation was calculated as;

% = number of root segments with any colonization / total number of segments examined (Brundrett *et al*., 1996)

2.5 PATHOGEN ISOLATION, VERIFICATION AND IDENTIFICATION

2.5.1 Isolation

Root and soil samples from the declining Spekboom collected at AENP were used to isolate potential pathogens. Root pieces were washed with water and surface sterilized in 70% ethanol for 2 mins. Root pieces of approximately 1 cm were plated onto BNPRA (Downes *et al*., 2001; MacFaddin, 2000) and Potato Dextrose Agar (PDA, Merck, Catalogue no: HG00C100.500) plates. The plates were incubated at 28ºC and observed daily for 14 days. Any potential fungal pathogens found were sub-cultured onto new media to establish pure cultures.

2.5.2 Verification

Spekboom cuttings were planted into a pasteurized sand medium and allowed to establish for a month in the mycorrhizal tunnel (min/max 19/30ºC, natural lighting, irrigated daily with filtered UV treated water). Cuttings were selected for a trial to test the pathogenicity of the suspected fungal isolates and 10 replicates were used for each

treatment. Spore suspensions (150 ml) of each of the potential fungal isolates were made using sterile saline solution (0.2% saline with 10 µl of Tween 20 added to the suspected fungal pathogen and mixed using glass spreader); 10 ml was drawn from the suspensions and inoculated onto surface of the soil. Controls were inoculated with sterile saline. After the pot trial was removed, biomass and shoot to root ratio was recorded. This experiment was repeated with pre-rooted cuttings. The cuttings were placed in water until they rooted and water was changed regularly.

2.5.3 Molecular Identification

2.5.3.1 DNA extraction

Genomic DNA was extracted from potential fungal strains isolated on PDA medium by scraping the mycelia straight from the plate using ZR Fungal/Bacterial DNA MiniPrep kit (Catalogue No: D6005) following the manufacturer's protocol. The scraped mycelia were transferred to ZR BashingBead Lysis Tubes and 750 µl of Lysis Solution was added to these tubes for lysing cells. Then these tubes were processed in a bead beater at maximum speed for 5 min to disrupt cells. After that they were centrifuged in a Super mini centrifuge at 10, 000 x g for 1 min, 400 µl of supernatant was transferred to a Zymo-Spin IV Spin filter in a collection tube for clarifying solutions including crude cell lysates and centrifuged at 7, 000 x g for 1 min. Fungal/Bacterial DNA Binding Buffer (1200 µl) was added to the filtrate and centrifuged, 800 µl of the mixture was taken and transferred to a Zymo-Spin IIC Column in a collection tube for the purification of DNA and then centrifuged at 10, 000 x g for 1 min. The flow through from this collection tube was discarded and the above step was repeated. DNA Pre-Wash Buffer (200 µl) was added to the Zymo-Spin IIC Column in a new collection tube and centrifuged at 10, 000 x g for 1 min, then the supernatant was discarded. After that, 500 µl of Fungal/Bacterial DNA Wash Buffer was added to the Zymo Spin to remove any remaining impurities and centrifuged at 10, 000 x g for 1 min. The Zymo-Spin IIC Column was transferred to a clean 1.5 ml microcentrifuge tube and 100 µl of DNA Elution Buffer was added directly to the column matrix to elute DNA. This was centrifuged at 10, 000 x g for 30 sec. Thereafter DNA was ready to use and 1% of agarose gel was prepared (1 g of agarose gel, 100 ml of TBE buffer and 2 µl of ethidium bromide). The gel was loaded with 5 µl of the 100 bp marker (Promega, catalogue no: G173A) and 10 µl of extracted DNA which was mixed with 5 µl of the

loading dye (Promega, catalogue no: G190A). The gel was then run at 100 V for 45 min and viewed under UV transilluminator (UVIPRO Chemi) on Gel Doc after that an image was obtained.

2.5.3.2 PCR conditions

The highly conserved fungal ITS region was selected for PCR amplification with rDNA primers which were ITS1-F (5'-CTTGGTCATTTAGAGGAAGAAGTAA-3') and ITS 4 (5'-TCCTCCGCTTATTGATAT-3') (White *et al.*, 1990). The PCR amplification was carried out in a total volume of 50 µl and the reactions contained 25 µl of KAPA HiFi Hotstart ReadyMix (Catalogue No: KK2602), 2 µl of each primer, 5 µl of template DNA and 16 µl of sterile water. PCR reactions for fungal DNA were carried out in an automated Applied Biosystems 2720 Thermo Cycler using the following parameters; initial denaturation at 95ºC for 2 min, followed by 30 cycles of denaturation at 94ºC for 30 sec, annealing at 49.7ºC for 45 sec and extension at 72ºC at 60 sec. The amplification was terminated by a final extension at 72ºC for 5 min. The amplified PCR products were assessed and quantified for purity of DNA using Thermo Scientific Nanodrop 2000 Spectrophotometer and visualised on 1% of agarose gel electrophoresis to determine the quality of amplification as described previously.

2.5.3.3 Clean-up

PCR products were purified using a Promega Wizard SV Clean up kit (Catalogue no: A9281) following the manufacturer's instructions. An equal amount of Membrane Binding Solution was added to the amplified product which in turn was transferred to the SV Minicolumn into a collection tube and incubated at room temperature for 1 min. The minicolumn was centrifuged at 13, 000 x g for 1 min and the flow through was discarded. The minicolumn was reinserted into a new collection tube. Membrane Wash Solution (700 µl) was added and centrifuged at 13, 000 x g for 1 min then the flow through was discarded. This step was repeated with 500 µl of Membrane Wash Solution and centrifuged at 13, 000 x g for 5 min. The collection tube was emptied and the column assembly was re-centrifuged for 1 min with the microcentrifuge lid open to allow evaporation of any residual ethanol. The minicolumn was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of nuclease-free water was added to the column then incubated at room temperature for 1 min. After centrifugation at 13, 000 x g for 1

min the DNA was stored at 4ºC. The amplified products were separated using agarose gel as described previously.

2.5.3.4 Sequencing

The purified fungal PCR products were sent to Inqaba Biotechnology in Pretoria, South Africa for sequencing. The obtained chromatograms were edited using Chromas Lite for editing the chromatograms and were identified by BLAST nucleotide searches in the NCBI website which in turn were submitted to GenBank for comparative identification (Altschul *et al*., 1990). The criteria for identification were to select the sequences from BLAST having low E-value with the highest percentage identity over the highest number of base pairs coverage.

2.6 RHIZOBACTERIAL ISOLATION, CHARACTERIZATION AND IDENTIFICATION

2.6.1 Isolation

One gram of soil from AENP and Hounslow was transferred into a McCartney bottle (10^{-1}) with 9 ml of sterile saline solution (0.2%). A series of 10 fold serial dilutions were prepared and 100 µl was spread plated onto Nutrient Agar (Merck, Catalogue no: HG0000C1.500) and Tryptone Soy Agar (TSA) (Merck, Catalogue no: HG000C17.500) plates using a sterile glass spreader. The plates were incubated at 37ºC for 24-48 hrs and after incubation single colonies were selected to establish pure bacterial cultures.

A randomly selected 1 cm section of root was selected from each sample from Echo, Hounslow and Helspoort plots and placed into 20 ml of sterile saline and Tween 20. The samples were vortexed and allowed to stand at room temperature for an hour. Six 10 fold dilutions were prepared and 100 µl from each dilution was spread plated onto TSA plates and incubated at 37ºC. Bacterial colonies were selected and streaked to establish pure cultures.

2.6.2 Gram staining

Gram staining was conducted to determine the shape of bacteria, presence of endospores, cell wall type and to establish purity of the cultures (Bergey *et al*., 1994).

Bacteria were placed on a drop of water on a slide and heat fixed over a flame. The smear on the glass slide was then covered with drops of Crystal violet. The slide was washed with water after a minute then a few drops of Gram's iodine was applied which served as a mordant and allowed to react for a minute (Llewellyn, 2005). After the slide was washed with water again, the smear was decolorized with ethanol for 30 sec. After decolorization, it was washed with water then counterstained with few drops of Safranin for 1 min. The excess was washed off with water and the slide was air-dried. Slides were examined under a microscope (Olympus BX50) and Gram-positive cells stained purple while Gram-negative cells stained pink (Madigan *et al*., 2004). Observations were recorded. Glycerol stocks of all isolates were prepared and stored at -80ºC.

2.6.3 Characterization

2.6.3.1 In-vitro inhibitory test

Thirteen rhizobacterial isolates, as well as three known bacterial isolates, *Burkholderia*, *Pseudomonas fluorescens* and *P. putida* (previously isolated) by Moore, (2012) were grown in Nutrient Broth (Merck, Catalogue no: HG000C24.500) overnight and incubated at 37ºC. *Burkholderia* and *Pseudomonas* species were included as they had shown activity against *Alternaria* in a previous study (Moore, 2012). The broth cultures of the selected isolates were adjusted to an OD_{600} of 0.5 using an UV-VIS Spectrophotometer and used to determine if any of the isolates had an inhibitory effect against the suspected fungal pathogens. Bacterial isolates (50 µl) were co-inoculated and screened against the suspected fungal pathogens on PDA. Five replicates of each bacterial isolate were used and the un-inoculated broth was used as controls. The suspected fungal pathogen was centrally inoculated in dual culture (Figure 2.3). Fungal radial growth was recorded after 5 days.

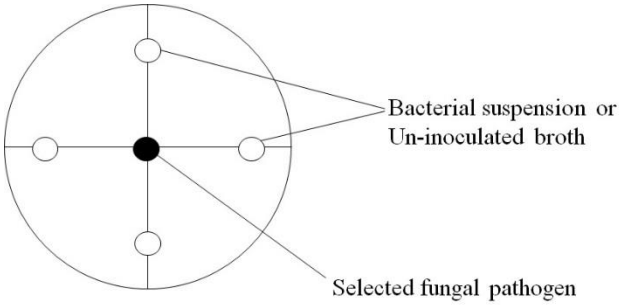

Figure 2.3: Dual culture plate design to determine antifungal activities.

2.6.3.2 Indole Acetic Acid

The bacterial isolates were grown in DEV Tryptophan Broth (Merck, Catalogue no: 1092930100) for 3 days at 37ºC to determine if any of the isolates had an ability to produce Indole Acetic Acid (IAA). This medium contained L-tryptophan which is an amino acid precursor required in the formation of IAA. For each bacterial isolate, two replicates were used and the un-inoculated broth was used as a control. After the period of incubation, cells were centrifuged at 4500 rpm for 20 min and 250 µl of Salkowski reagent (2 ml 0.5 $FeCl_3$, 49 ml of water and 49 ml of 70% perchloric acid) was added to 500 µl of the supernatant. A colour change to red/pink was an indication of IAA (Cappuccino and Sherman, 2008).

2.6.3.3 Siderophore production

Bacterial isolates were streaked on NA plates and incubated at 37ºC. Chrome Azurol Sulfonate (CAS) medium was prepared and sterilized. Also it was used as an overlay on the NA plates after incubation for 2 days to determine if the bacterial isolates had any ability to chelate iron from the medium. The change of colour from blue to orange/yellow determined the presence of siderophores (Schwyn and Neilands, 1997; Suneja *et al*., 1994).

2.6.3.4 Phosphate solubilising production

Qualitative measurements of phosphorus solubilisation by bacterial isolates were determined using a modified NBRI medium (Mehta and Nautiyal, 2001) containing insoluble $Ca_3(PO_4)_2$. The medium was prepared separately from bromophenol blue (0.1 g of bromophenol blue and 20 ml of water) which was added as a pH indicator. The pH of the medium was adjusted to 7.0. The medium and bromophenol blue solution were autoclaved at 121°C for 15 min. When the media had cooled, it was mixed with the bromophenol blue solution and poured into the plates. Individual bacterial isolates (50 µl) were inoculated into wells made in the plates and incubated at 28ºC for 7 days. Isolates that showed clearing zones were scored as positive for phosphate solubilisation. Un-inoculated broth was used as controls.

2.6.4 Molecular identification of selected bacterial isolates

2.6.4.1 DNA extraction

A single colony of 31 bacterial isolates was inoculated into NB and incubated for 24-48 hrs at 37ºC. Genomic DNA was extracted from these isolates using ZR Fungal/Bacterial DNA MiniPrep kit following the manufacturer's protocol as described previously. The quality of the isolated DNA was assessed through 1% agarose gel as previously described. PCR amplification of the bacterial isolates was conducted as described previously using 16S forward and reverse primers fd1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rp2 (5'-ACGGCTACCTTGTTACGACTT-3') (Weisburg *et al*., 1991) at different cycling parameters; initial denaturing at 95ºC for 5 min followed by 30 cycles of denaturation at 98ºC for 20 sec, annealing at 54.5ºC for 15 sec and extension at 72ºC for 30 sec. The final extension took place at 72ºC for 5 min. The amplified PCR products were separated using agarose gel and purified before sequencing using Promega Wizard SV Clean up kit following the manufacturer's instructions as described previously. The purified bacterial PCR products were sent to Inqaba Biotechnology in Pretoria for sequencing. The fd1 primer was sent with DNA samples. The obtained sequences were identified by BLAST nucleotide searches in the NCBI website which in turn were submitted to GenBank for comparative identification (Altschul *et al*., 1990). The criteria used for identification were as previously described.

2.7 AM FUNGAL RHIZOBACTERIAL INTERACTIONS

Spekboom cuttings were planted into pasteurized compost sand (mixed 1:1) in 8 cm pots to assess the ability of selected bacteria and AM fungi to promote Spekboom growth. AM fungal inoculum was obtained from Mycoroot (Pty) Ltd, Grahamstown, South Africa and contained a combination of arbuscular mycorrhizal isolates from South African soils - *Glomus Clarum* (new name *Rhizophagus clarus*), *Gigaspora gigantea*, *Glomus mosseae* (new name *Funneliformis mosseae*), *Glomus etunicatum* (new name *Claroideoglomus etunicatum*) and *Paraglomus occulum* (identifications are molecular determinations). The inoculum has a minimum of 10 spores/g with a most probable number of propagules of 100/g. Initial measurements of cutting height and weight were recorded prior to inoculation. Spekboom cuttings were inoculated on planting with sterile saline (control), rooting powder (RP), selected bacterial isolates (B1-B6) and arbuscular mycorrhizal (AM) fungi separately and in combination; there

were 10 replicates for each treatment (Table 2.2). Plants were harvested 3 months after planting. The roots were washed from the soil with tap water. The roots and shoots were separated and fresh weights were determined. A weighed subsample of root material was removed for colonisation assessment as described previously. The remainder of the roots and shoots were dried in an oven at 60°C for 2 days. The plant growth parameters such as biomass and mycorrhizal colonisation were recorded.

Table 2.2: AM fungal, rhizobacterial interaction with Spekboom.

Saline (control)	**RP**	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**	**AM fungi**
+								
	+							
								+
		+						+
			+					+
				+				+
					+			+
						+		+
							+	+

RP=rooting powder; B1=M1; B2=*Burkholderia*; B3=HB2; B4=HB5; B5=HP5; B6=HP6

A second pot trial was established for determine potential biological control of selected bacterial isolates and AM fungi against the suspected fungal pathogen. Initial measurements of the cutting height and weight were recorded prior to inoculation. There were 10 replicates for each treatment and treatments include inoculations with potential fungal isolate alone (*Fusarium*, F2 isolate), and in combination with AM fungi and rhizobacterial isolates (BC1-BC4) (Table 2.3). After 3 months, plants were

harvested and the same process that was described previously was followed. A visual assessment of disease was also recorded.

Table 2.3: Interaction against potential fungal pathogen (F2 isolate)

***Fusarium* isolate**	**AM fungi**	**BC1**	**BC2**	**BC3**	**BC4**
+					
+	+				
+	+	+			
+	+		+		
+	+			+	
+	+				+

BC1=*Burkholderia*; BC2= *P. fluorescens*; BC3=*P. putida* and BC4= HB9

2.8 STATISTICAL ANALYSIS

Statistical analysis refers to a collection of methods used to process large amounts of data and report overall trends. All experiments were conducted with suitable controls and quantitative data was statistically analysed using Statistica. ANOVA was used for to analyse data. Significant differences were further investigated using Fischer Least Significant Differences ($p \leq 0.05$) (Hayter, 1986).

CHAPTER 3: RESULTS

3.1 SOIL NUTRIENT ANALYSIS

Nutrient analysis of soil from the three plots indicated that Echo soil had the lowest nutrient levels (Table 3.1) but had the highest EC value of 83 (mS/m). The soil pH (H_2O) ranged between 4.67 to 8.04 with Hounslow and Helspoort having an alkaline pH of 8.04 and 7.59, respectively. Helspoort had the lowest EC value of 64 (mS/m) while Hounslow had the highest Ca, Na and P (mg/kg) levels. The CEC value ranged from 19.63 to 21.03 cmol(+)/kg

Table 3.1: Soil nutrient analysis of the selected Spekboom plots

Sample name	**Ca (mg/kg)**	**Mg (mg/kg)**	**K (mg/kg)**	**Na (mg/kg)**	**P (mg/kg)**	**pH (H_2O)**	**pH (KCI)**	**EC (mS/m)**	**CEC cmol(+)/kg**
Echo	610.0	170.5	279.0	17.0	37.4	5.69	4.67	83	20.67
Helspoort	3400.0	370.0	665.5	62.5	60.0	7.59	6.84	64	21.03
Hounslow	3464.5	297.0	410.0	92.5	69.6	8.04	7.35	75	19.63

Ca = Calcium; Mg = Magnesium; K = Potassium; Na = Sodium; P = available phosphorus; EC = Electrical conductivity and CEC = Cation Exchange Capacity.

3.2 AM FUNGAL SPORE ENUMERATION AND CHARACTERIZATION

Spore abundance within the three plots showed that Helspoort had the highest mean number of spores (112.7) followed by Echo farm (80.7). Hounslow which was considered as the weak trial site had the lowest mean number of spores (77.2). There were no significant differences between spores extracted from the 3 plots (Figure 3.1).

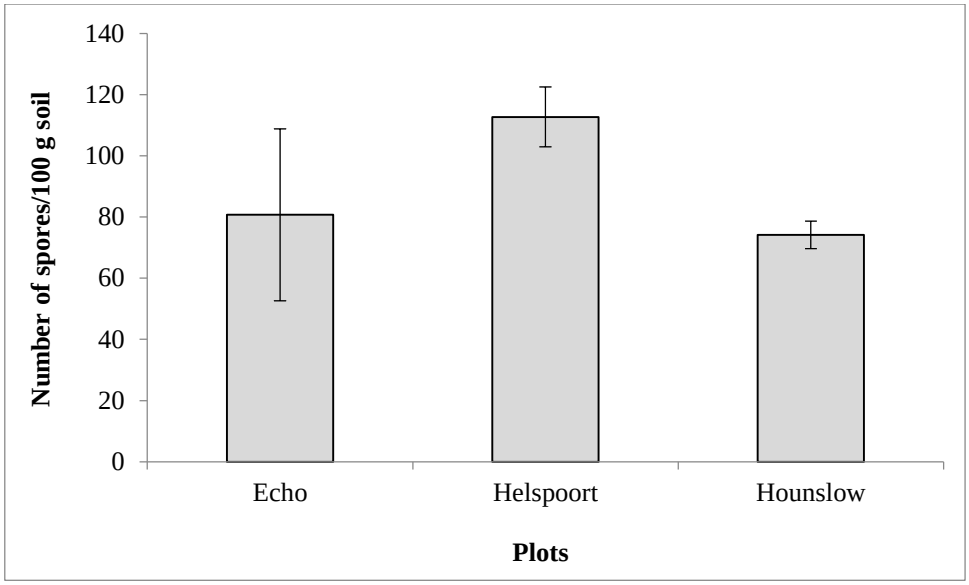

Figure 3.1: AM fungal spore abundance of soil at Spekboom plots. Columns are mean spore numbers ± standard errors. Spore abundance was not significantly different (n=10; p=0.27).

3.2.1 Spore characterization

Dominant AM spore morphotypes extracted from the soil samples were microscopically examined; yellow brown to brown spores were globose, ovoid and round in shape and some had subtending hyphae. Tentative identifications were made on limited spore morphological characteristics. Different genera compromising *Glomus* (Figure 3.2 A and B), Entrophospora (Figure 3.2 C and D), *Funneliformis* (Figure 3.2 E), *Diversispora* (Figure 3.2 F) and *Gigaspora* (Figure 3.2 G and H) were found in three plots of which the genus *Glomus* was dominant. Spore diameters ranged from 80 to 540 µm. *Glomus* spores (family Glomeraceae) were glomoid in shape, with a cylindrical subtending hyphae, the spore wall consisted of a single layer (Figure 3.2 A and B). Entrophospora (family Entrophosporaceae) lacked a subtending hyphae and had two wall layers (Figure 3.2 C and D). *Funneliformis* also a member of the Glomeraceae has a typical funnel shaped subtending hypha at the point of attachment and single wall layer (Figure 3.2 E). *Diversispora* (Family Diversisporaceae) has an irregular subtending hyphal attachment (Figure 3.2 F) while *Gigaspora* were large spores with a diameter over 300 µm with a flared subtending hypha and single cell wall layer (Figure 3.2 G and H) (Oehl *et al*., 2011; Walker *et al.*, 2007a; Walker *et al.*, 2007b; INVAM)

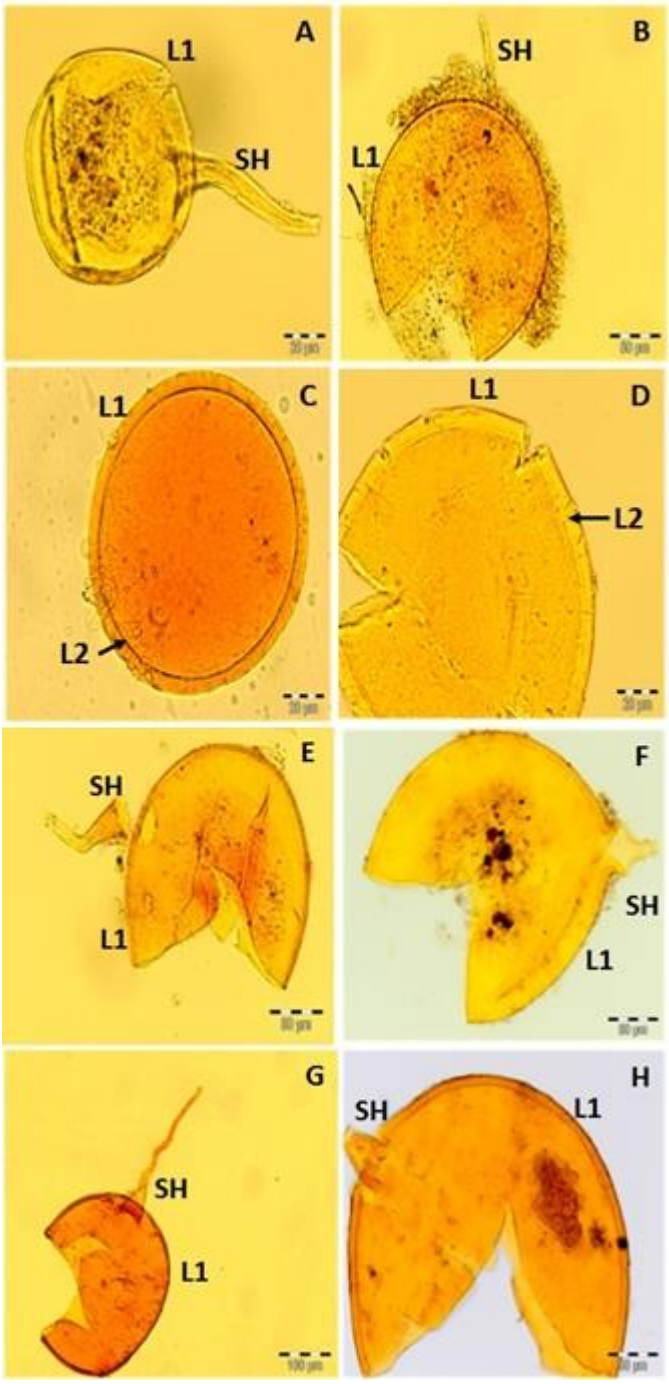

Figure 3.2: Representative spores of Glomeraceae, *Glomus* species (A and B), Entrophosporaceae, *Entrophospora* species (C and D), Glomeraceae, *Funneliformis* species (E), Diversisporaceae, *Diversispora* species (F), Gigasporaceae, *Gigaspora* species (G and H). SH – subtending hyphae, L1 – Cell wall layer 1; L2 Cell wall layer 2 as indicated by arrow.

3.2.2 Identification

Molecular analyses of the SSU region from the Spekboom plots indicated that 4 families of AM fungi were prevalent. These were identified as Ambisporaceae, Glomeraceae, Claroideoglomeraceae and Paraglomeraceae. Sequences with the highest similarity of 98% were from Glomeraceae. A total of 464 OTUs were obtained from Echo plot and these OTUs were assigned to 2 AM fungal families with 5 species of *Ambisporaceae* and 5 species of *Glomeraceae*; 307 OTUs from Helspoort were assigned to 3 families with 4 species of *Glomeraceae*, 1 species of *Claroideoglomus* and 5 species of *Ambispora*; and lastly 270 OTUs in Hounslow were assigned to 4 families with 4 species of *Glomeraceae*, 1 species of *Claroideoglomus*, 1 species of *Paraglomus* and 4 species of *Ambispora*. Among the OTUs obtained from the three plots, 10 OTUs representing the highest number of sequences were selected from each plot and were aligned with sequences from MaarjAM database (Table 3.2). A total of 22 OTUs did not match any reference sequence with greater than 97% similarity. Within the Glomeromycotan OTUs, those belonging to the *Glomerales* and *Ambisporaceae* were dominant in all plots. *Paraglomus* and *Claroideoglomu*s were less frequent in Helspoort and Hounslow.

Table 3.2: MaarjAM analysis of AM fungal SSU from the three Spekboom plots.

OTU number	**Accession no.**	**Closest match**	**Similarity/Bp coverage (%)**	**E-value**	**N**
Echo 1	AB047302	Ambisporaceae *Ambispora leptoticha* VTX00242	91/97	0.0	530
Echo 2	AJ301861	Ambisporaceae *Ambispora leptoticha* VTX00242	90/99	0.0	499
Echo 3	GU353953	Glomeraceae *Glomus* NES31 VTX00151	93/76	0.0	184

OTU number	Accession no.	Closest match	Similarity/Bp coverage (%)	E-value	N
Echo 4	AJ301861	Ambisporaceae *Ambispora leptoticha* VTX00242	90/99	0.0	129
Echo 5	AB047302	Ambisporaceae *Ambispora leptoticha* VTX00242	90/96	0.0	112
Echo 6	GU353953	Glomeraceae *Glomus* NES31 VTX00151	98/79	0.0	103
Echo 7	JN009133	Glomeraceae *Glomus* Liu2012b Phylo-25 VTX00092	91/77	0.0	103
Echo 8	GQ140598	Ambisporaceae *Ambispora sp*. VTX00242	91/97	0.0	82
Echo 9	JN009133	Glomeraceae *Glomus* Liu2012b Phylo-25 VTX00092	98/56	0.0	53
Echo 10	AB698596	Glomeraceae *Glomus* *Goomaral*13a Glo10 VTX00156	95/77	0.0	51
Helspoort 11	HE614986	Claroideoglomeraceae *Claroideoglomus* *torrecillas*12b Glo G1 VTX00193	93/51	5,00E-79	1475
Helspoort 12	DQ371663	Glomeraceae *Glomus* *Franke* A3 VTX00092	92/32	7,00E-32	796
Helspoort 13	JN009133	Glomeraceae *Glomus* Liu2012b Phylo-25	98/56	0.0	793

OTU number	Accession no.	Closest match	Similarity/Bp coverage (%)	E-value	N
		VTX00092			
Helspoort 14	JN009133	Glomeraceae *Glomus* Liu2012b Phylo-25 VTX00092	98/56	0.0	265
Helspoort 15	GQ140598	Ambisporaceae *Ambispora sp*. VTX00242	91/97	0.0	159
Helspoort 16	GU353953	Glomeraceae *Glomus* NES31 VTX00151	98/79	0.0	74
Helspoort 17	AJ301861	Ambisporaceae *Ambispora leptoticha* VTX00242	91/97	0.0	61
Helspoort 18	AJ006466	Ambisporaceae *Ambispora leptoticha* VTX00242	90/97	0.0	49
Helspoort 19	JX999461	Ambisporaceae *Ambispora Shi1*4a Phy-15 VTX00283	91/98	0.0	43
Helspoort 20	AJ006793	Ambisporaceae *Ambispora leptoticha* VTX00242	90/97	0.0	41
Hounslow 21	JN009367	Glomeraceae *Glomus* Liu2012b Phylo-31 VTX00067	95/56	6,00E-97	1781
Hounslow 22	JN009133	Glomeraceae *Glomus* Liu2012b Phylo-25 VTX00092	98/56	0.0	667
Hounslow 23	HE614986	Claroideoglomeraceae	93/51	5,00E-79	422

OTU number	Accession no.	Closest match	Similarity/Bp coverage (%)	E-value	N
		*Claroideoglomus Torrecillas*12b Glo G1 VTX00193			
Hounslow 24	JN009133	Glomeraceae *Glomus* Liu2012b Phylo-25 VTX00092	98/56	0.0	287
Hounslow 25	HF566780	Paraglomeraceae *Paraglomus sp.* VTX00001	93/51	4,00E-73	161
Hounslow 26	AM268193	Ambisporaceae *Ambispora fennica* VTX00283	90/96	0.0	83
Hounslow 27	AJ301861	Ambisporaceae *Ambispora leptoticha* VTX00242	90/99	0.0	74
Hounslow 28	GU353953	Glomeraceae *Glomus* NES31 VTX00151	98/79	0.0	68
Hounslow 29	AB015052	Ambisporaceae *Ambispora leptoticha* VTX00242	91/97	0.0	41
Hounslow 30	AJ301861	Ambisporaceae *Ambispora leptoticha* VTX00242	91/97	0.0	41

N=number of sequences assigned to each OTU

3.3 ASSESSMENT OF COLONISATION

Characteristic of AM fungal structures were observed in roots of Spekboom sampled at the 3 plots, confirming that Spekboom associate with AM fungi with typical *Arum* colonisation strategy (Figure 3.3). Mean percentage colonisation was 41.81 in all sites. No significant differences were recorded in colonisation (Table 3.3).

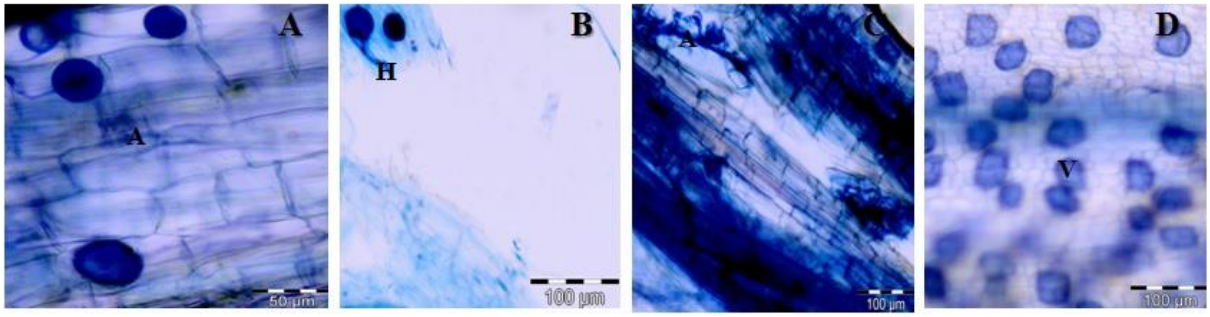

Figure 3.3: Characteristic of AM fungal colonisation structure in Spekboom roots.

Table 3.3: Percentage of AM fungal root colonisation.

Soil sample	Hyphal (%)	Arbuscules (%)	Vesicles (%)	Total colonisation (%)
Echo	27.68±7.51	3.54±2.36	55.19±6.52	37.04±4.76
Helspoort	43.45±4.14	2.73±1.44	61.97±4.00	26.33±5.62
Hounslow	48.31±5.84	2.2±1.55	71.6±5.21	62.08±12.61
F value (2,27)	3.2409	0.87365	2.3793	4.7337
P<0.05	NS	NS	NS	NS

Means ±SD. Means represents replicates from each site (n=10). NS=not significant

3.4 PATHOGEN ISOLATION, VERIFICATION AND IDENTIFICATION

Two potential fungal pathogens were isolated from root material on PDA and no fungal growth was observed on BNPRA medium indicating the absence of *Phytophthora*. The isolates were microscopically examined and tentatively identified as different *Fusarium* isolates (Figure 3.4).

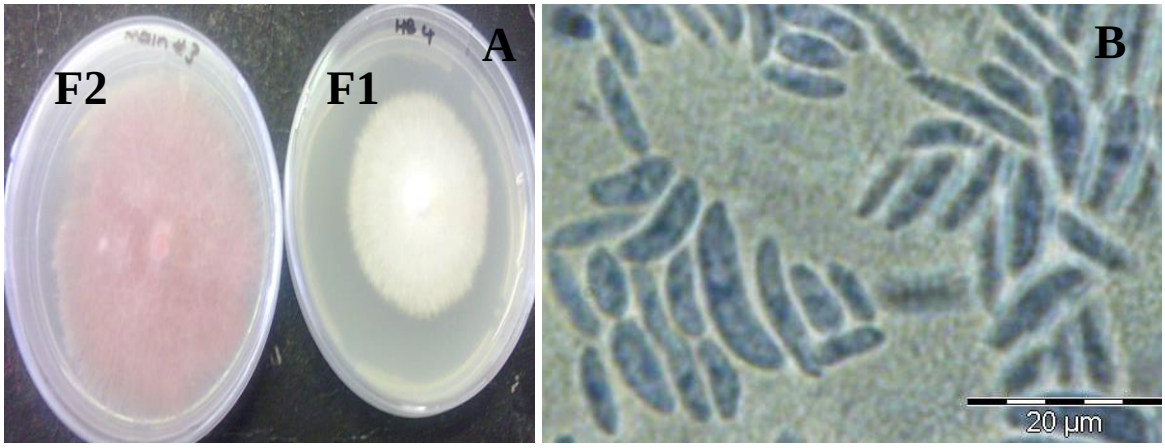

Figure 3.4: Two potential fungal pathogens (Isolates F1 and F2) growing on PDA (A) and macroconidia of F2 isolate (B).

3.4.1 Pathogen verification

Spekboom cuttings grew relatively well, forming new leaves and shoots despite inoculation with the potential fungal pathogen. Poor root development of some cuttings was observed. Some Spekboom cuttings inoculated with the two suspected pathogen isolates produced visual disease symptoms. These included yellowing, leaf drop and shoot cracking (Figure 3.5).

Figure 3.5: Spekboom cuttings inoculated with two suspected fungal pathogens (F1 and F2 isolate) after 1 month. A and B) control plants and roots; C and D) plants and roots inoculated with F1 isolate; E and F) plants and roots inoculated with F2 isolate.

Although shoot: root was not significant, the inoculation of the pathogens tended to depress growth (Figure 3.6).

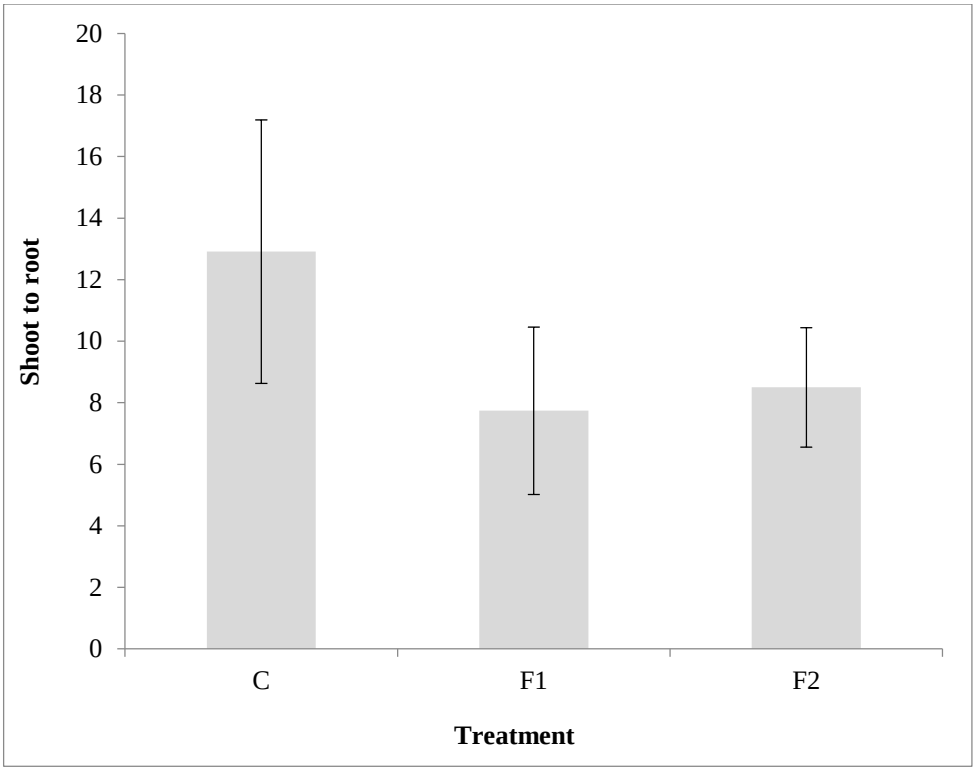

Figure 3.6: Shoot to root ratio of Spekboom when inoculated with two potential fungal pathogens (F1 and F2 isolate). Columns are means ± standard error. Ratios are not significantly different (n=10; p=0.81).

In a follow-up trial, cuttings were rooted prior to inoculation. When plants were inoculated with the two suspected fungal isolates, some of the cuttings withered and died as shown in Figure 3.7 below B and C. This was more noticeable in the F2 treatment D and E and the shoot turned brown.

Figure 3.7: Pathogenicity test with pre-rooted cuttings. A) un-inoculated controls; B and C) plants that were inoculated with F1; D and E) plants that were inoculated with F2; F) shows the plantlets after they were harvested, C=controls; F1 and F2=fungal isolates.

Although no significant differences were found there was a general trend of depressed growth with introduction of the pathogen particularly the F2 isolate (Figure 3.8).

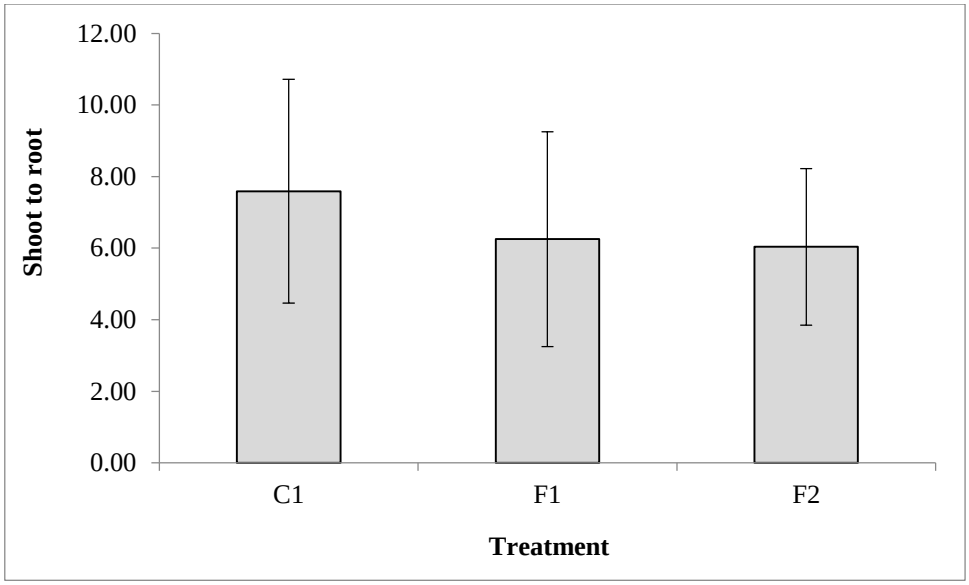

Figure 3.8: Shoot to root biomass ratio of the Spekboom when treated with the two suspected fungal pathogens. Columns are means ± standard errors. Ratio were not significantly different (n=10).

3.4.2 Pathogen identification

The suspected fungal isolates were further identified using molecular techniques and the extracted DNA was amplified by PCR using ITS1 and ITS4 primers. Figure 3.9 shows the agarose gel of amplified PCR product after clean-up. The size of the band was approximately 550 bp. Sequence analysis identified two *Fusarium* species (Table 3.4).

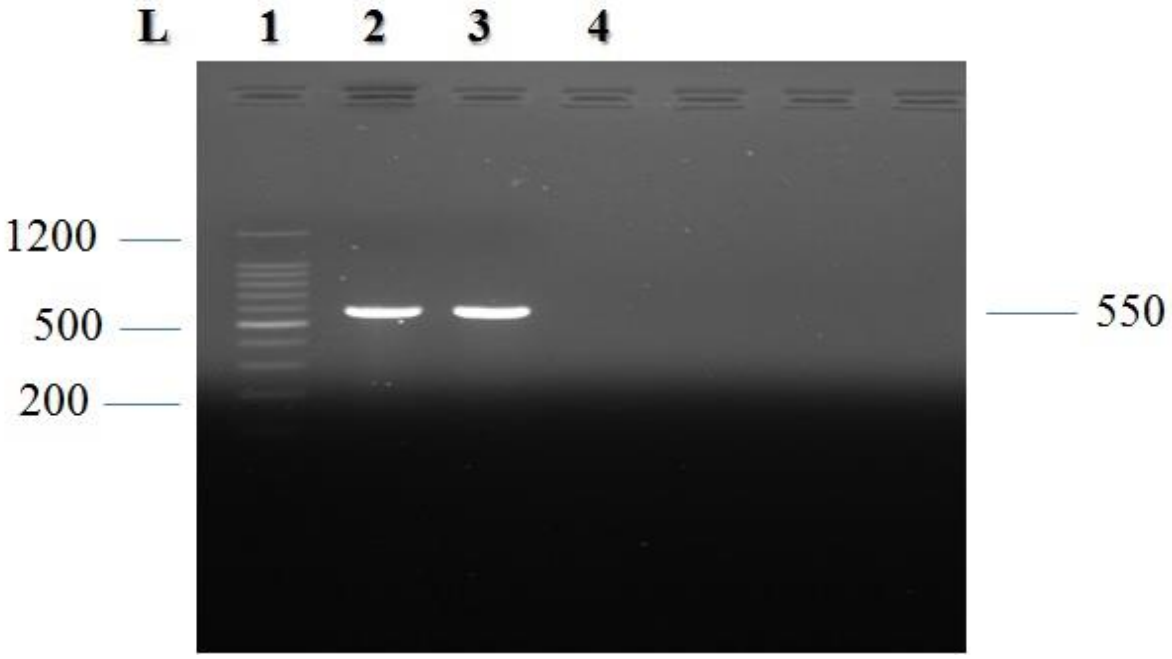

Figure 3.9: PCR product of fungal isolates as visualized on 1% agarose gel stained with 2 µl of ethidium bromide. Lane 1 represents a marker (100 bp), lane 2 (F1), lane 3 (F2) and lane 4 (negative control).

Table 3.4: Molecular identification of suspected fungal pathogens from Spekboom root samples

Isolate code	**Accession number**	**Closest match**	**Similarity (%)**	**Query cover (%)**	**E-value**
F1	HQ674657.1	*Fusarium*.sp RGT-S4	100	100	0.0
F2	KF367514.1	*Fusarium*.sp 2 BRO-2013	100	100	0.0

3.5 RHIZOBACTERIAL ISOLATION, CHARACTERIZATION AND IDENTIFICATION

The majority of the bacterial isolates selected were Gram positive with two isolates being Gram negative. All the Gram positive and Gram negative bacteria were rod-shaped. The isolates were tested for plant growth promoting abilities such as IAA, siderophore production and phosphate solubilisation (Table 3.5).

3.5.1 In-vitro inhibitory test

The bacterial isolates were tested to determine their ability to reduce growth of the fungal pathogen isolates. Several bacterial isolates were able to reduce the growth of the pathogens in dual culture. After initial screening, six bacterial isolates were tested for activity against the F1 fungal pathogen (Figure 3.10) and 4 were tested against F2 pathogen (Figure 3.11). *Pseudomonas fluorescens* significantly reduced mycelial growth of both the F1 and F2 isolates as compared to the other bacteria.

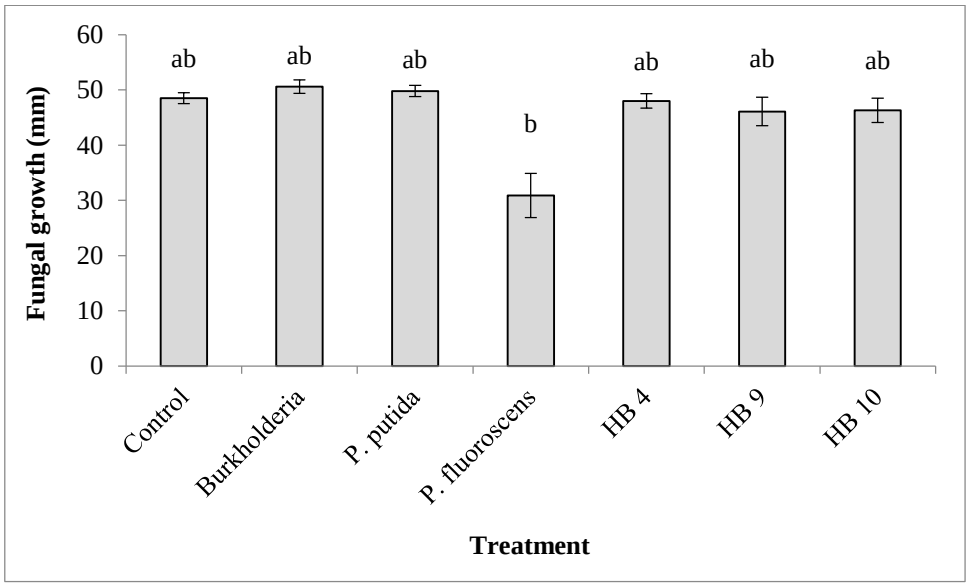

Figure 3.10: Antifungal activity of bacterial isolates against potential pathogen F1. Columns are means ± standard errors. $F_{(12,52)}=25.894$, $p<0.001$.

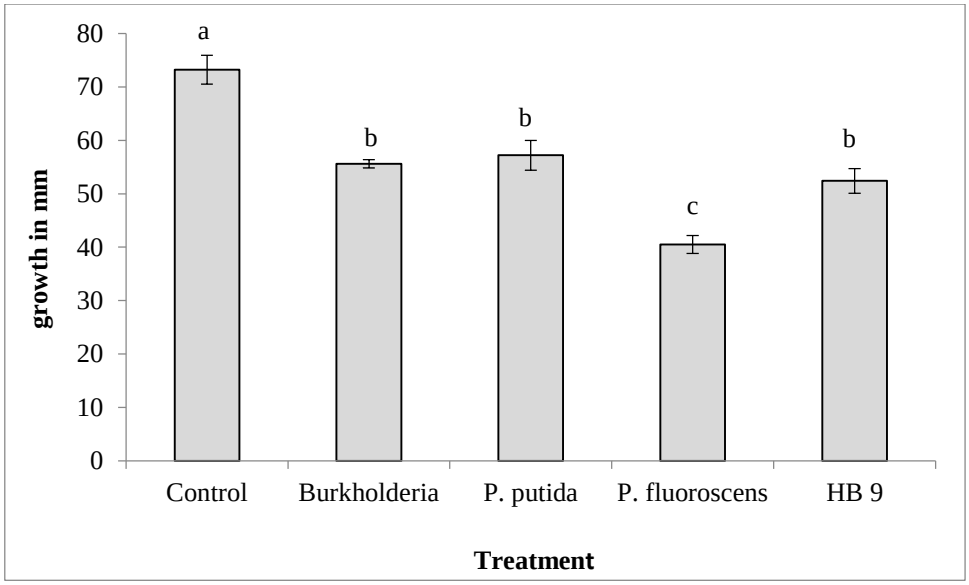

Figure 3.11: Antifungal activity of bacterial isolates against potential pathogen F2. Columns are means ± standard deviations. $F_{(4,20)}=114.19$ $p<0.001$.

3.5.2 Rhizobacterial characterization

3.5.2.1 Indole Acetic Acid

Of the 34 bacterial isolates tested for IAA production, 6 isolates were positive as indicated by a colour change to pink (Figure 3.12). The positive isolates were M1, HB2, HB5, HP5, HP6 and *Burkholderia* (Table 3.5). Controls did not change colour.

Figure 3.12: IAA production indicated by a colour change to pink

3.5.2.2 Siderophore production

A total of 27 bacterial isolates were found to have the siderophore activity on the CAS agar medium (Table 3.5). HB5 showed distinct siderophore production based on visual appearance (Figure 3.13).

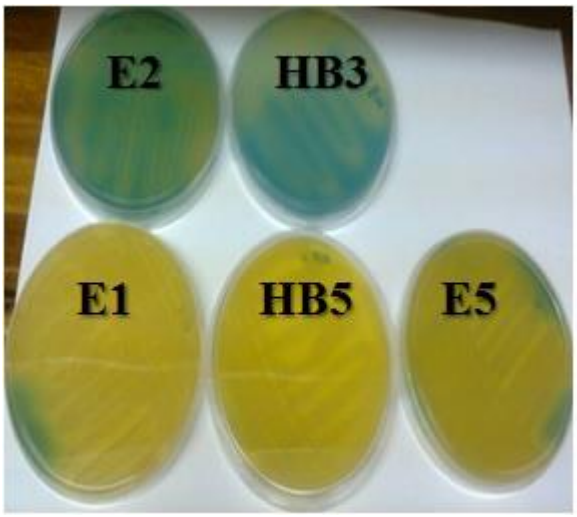

Figure 3.13: Siderophore production on CAS medium. The colour change to yellow indicates a positive reaction. Controls remained blue.

3.5.2.3 Phosphate solubilisation

Among the 34 bacterial isolates screened for phosphate solubilisation only 23 isolates were able to form a zone of clearance around the inoculated wells (Table 3.5) (Figure 3.14).

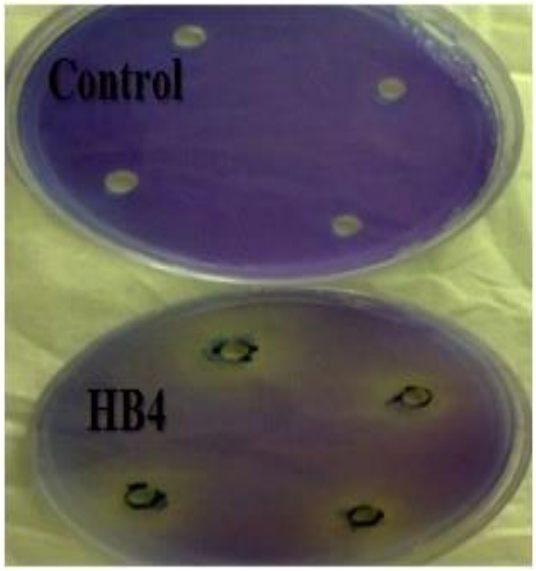

Figure 3.14: Phosphate solubilisation as indicated by a zone of clearance and a pH change.

Table 3.5: Plant growth-promoting abilities and morphological characteristics of rhizobacterial isolates.

Isolate	**IAA**	**Siderophore production**	**Phosphate solubilisation**	**Gram reaction**	**Shape**
M1	+	+	-	-	Rod
M2	-	+	+	+	Rod
M3	-	+	+	+	Rod
Burkholderia	+	+	+	-	Rod
P. fluoroscens	-	+	-	-	Rod
P. putida	-	+	-	-	Rod
HB1	-	+	+	+	Rod
HB2	+	+	+	+	Rod
HB3	-	-	-	+	Rod
HB4	-	+	+	+	Rod
HB5	+	+	+	+	Rod
HB6	-	+	+	+	Rod
HB7	-	+	+	+	Rod
HB8	-	+	-	+	Rod
HB9	-	+	+	+	Rod
HB10	-	-	+	-	Rod
E1	-	+	+	+	Rod
E2	-	-	+	+	Rod
E3	-	-	-	+	Rod

Isolate	IAA	Siderophore production	Phosphate solubilisation	Gram reaction	Shape
E4	-	+	-	+	Rod
E5	-	+	+	+	Rod
E6	-	-	+	+	Rod
HL1	-	+	-	+	Rod
HL2	-	+	-	+	Rod
HL3	-	-	+	+	Rod
HL4	-	+	+	+	Rod
HL5	-	+	+	+	Rod
HL6	-	+	-	+	Rod
HP1	-	-	+	+	Rod
HP2	-	+	-	+	Rod
HP3	-	+	+	+	Rod
HP4	-	+	+	+	Rod
HP5	+	+	+	+	Rod
HP6	+	+	+	+	Rod

M1-M3=initial isolates collected from AENP; HB1-HB10= initial samples collected from Hounslow; E1-E6= from Echo; HP1-HP6=samples collected from Helspoort and HL1-HL6= samples collected from Hounslow pot trial.

3.5.4 Identification of the selected bacterial isolates

DNA was extracted from 31 bacterial isolates. Only 30 species were successfully amplified and sequenced and produced clean DNA sequences that could be aligned. The bacterial species that failed to amplify was HL 2. Among all the bacterial isolates that were identified, 2 isolates were identified to be from the genus *Enterobacter*, 23 from *Bacillus*, 2 from *Arthrobacter*, 1 from *Pseudomonas*, 1 from *Microbacterium* and 1 from uncultured bacterium (Table 3.6).

Table 3.6: Identification of rhizobacterial isolates from Spekboom soil samples

Isolate	**Accession number**	**Closest match**	**Similarity (%)**	**Query cover (%)**	**E-value**
M1	JQ682638.1	*Enterobacter aerogenes* strain KNUC5012	100	100	0.0
M2	HM480350.1	*Bacillus megaterium* strain EIF 43	99	100	0.0
M3	KM517569.1	*Bacillus* sp. R-NB-5	100	100	0.0
HB1, HB5, HB6, HB7 & HB9	KM251578.1	*Bacillus cereus* strain SM-SR14	99	100	0.0
HB2 & HB4	AM291029.1	*Bacillus thuringiensis*	99	100	0.0
HB3	AP007209.1	*Bacillus* NC7401	91	100	0.0
HB8	KF318384.1	*Arthrobacter nicotianae* strain P-8	99	100	0.0
HB10	KJ482869.1	*Pseudomonas* CV7NOV	99	100	0.0
E1 & E3	KJ186075.1	*Bacillus aryabhattai* strain CMB-12	99	100	0.0

Isolate	Accession number	Closest match	Similarity (%)	Query cover (%)	E-value
E2	KF956573.1	*Enterobacter* sp. S20112	99	100	0.0
E4	NR114342.1	*Microbacterium saccharophilum* strain K-1	99	100	0.0
E5, HL1 & HL5	KM015444.1	*Bacillus anthracis* B-48	99	100	0.0
E6	JF768712.1	*Bacillus sp*. PKSW 3	93	97	0.0
HL3	KF956568.1	*Arthrobacter globiforms* strain S11402	99	100	0.0
HL4	GU086810.1	*Bacillus* strain SRS-30	89	99	0.0
HL6	DQ643061.1	*Bacillus* sp. CO46	99	100	0.0
HP1	KJ524507.1	*Bacillus simplex* strain BF9	99	100	0.0
HP2	KF863852.1	*Bacillus* sp. hb55	99	100	0.0
HP3	DQ302161.1	*Bacillus* sp. Y17	89	99	0.0
HP4	FM213585.1	Uncultured bacterium	91	7	9e-17
HP5	KF419128.1	*Bacillus subtilis* strain QAP12	99	100	0.0
HP6	DQ105975.1	*Bacillus sp*	86	99	0.0

M1-M3=initial samples collected from AENP; HB1-HB10= initial samples collected from Hounslow; E1-E6= from Echo; HP1-HP6=samples collected from Helspoort and HL1-HL6= samples collected from Hounslow plot.

3.6 AM FUNGAL RHIZOBACTERIAL INTERACTION

Plants from the bacterial and AM fungal pot trial grew with the development of new green fleshy leaves and no sign of disease (Figure 3.15).

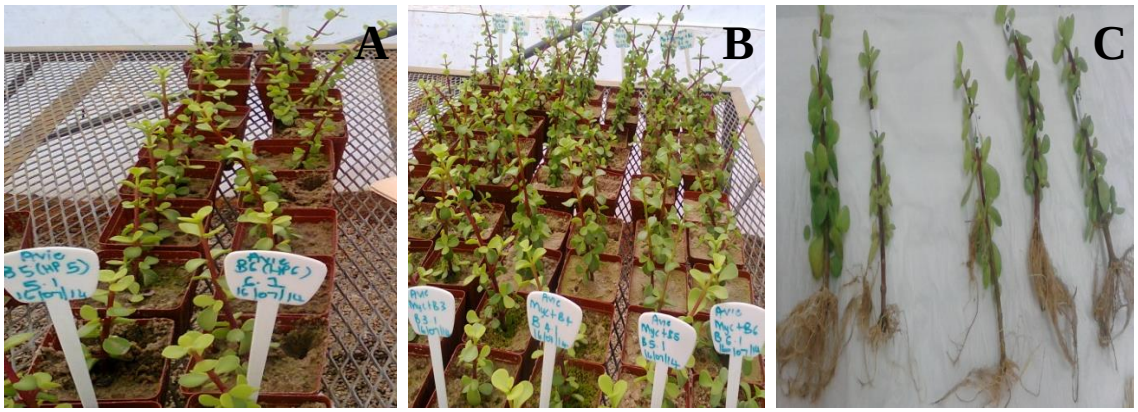

Figure 3.15: Spekboom plants that were inoculated with bacteria alone and in combination with AM fungi. A) plants that were inoculated with bacteria; B) plants that were inoculated with bacteria alone and in combination with AM fungi; C) shows plantlets after they were harvested.

All controls, bacterial and AM fungal treatments applied alone were generally significantly different (Table 3.7) showing less growth in shoot height when compared to combined inoculation of AM fungi and bacteria (Figure 3.16).

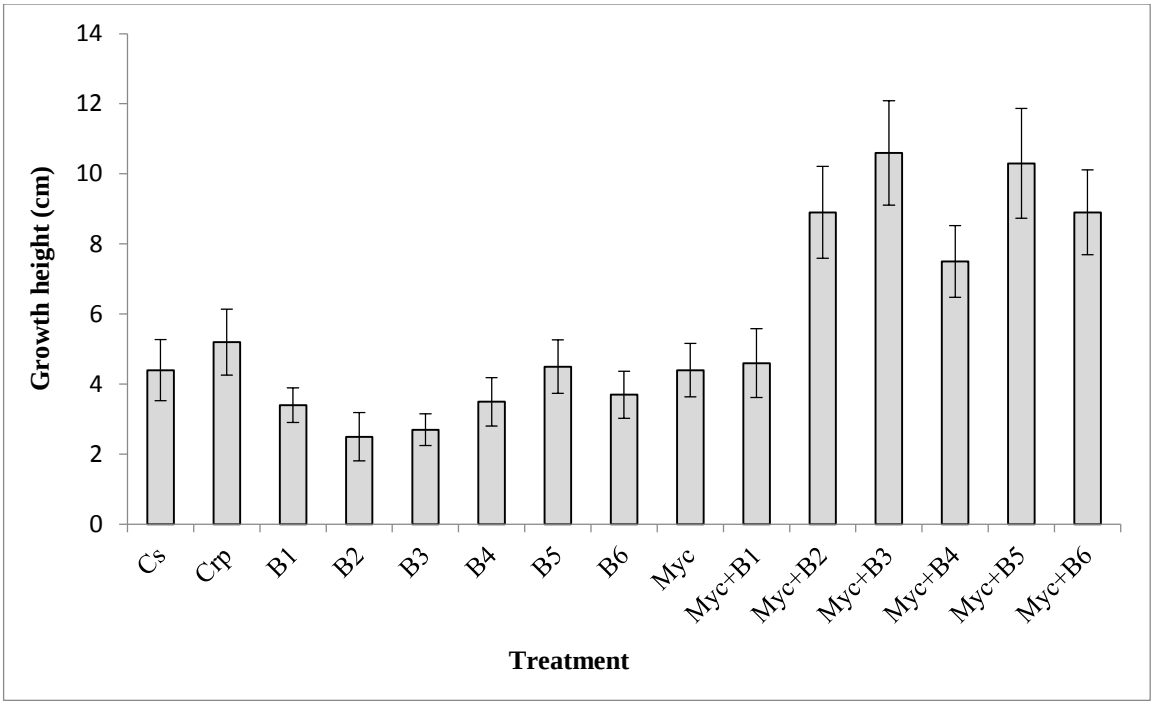

Figure 3.16: Growth in height (cm) of Spekboom when treated with selected bacteria alone or in combination with AM fungi. Columns are means ± standard errors (n=10).

Table 3.7: Growth in shoot height of Spekboom inoculated alone or in combination with selected bacteria and AM fungi.

	Cs	**Crp**	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**	**AM**	**AM+B1**	**AM+B2**	**AM+B3**	**AM+B4**	**AM+B5**	**AM+B6**
Cs		NS	NS	NS	NS	NS	NS	NS	NS	NS	*	*	*	*	*
Crp			NS	NS	NS	NS	NS	NS	NS	NS	*	*	NS	*	*
B1	NS	NS		NS	NS	NS	NS	NS	NS	NS	*	*	*	*	*
B2	NS	NS	NS		NS	NS	NS	NS	NS	NS	*	*	*	*	*
B3	NS	NS	NS	NS		NS	NS	NS	NS	NS	*	*	*	*	*
B4	NS	NS	NS	NS	NS		NS	NS	NS	NS	*	*	*	*	*
B5	NS	NS	NS	NS	NS	NS		NS	NS	NS	*	*	*	*	*
B6	NS	NS	NS	NS	NS	NS	NS		NS	NS	*	*	*	*	*
AM	NS	NS	NS	NS	NS	NS	NS	NS		NS	*	*	*	*	*
AM+B1	NS	NS	NS	NS	NS	NS	NS	NS	NS		*	*	*	*	*
AM+B2	*	*	*	*	*	*	*	*	*	*		NS	NS	NS	NS
AM+B3	*	*	*	*	*	*	*	*	*	*	NS		*	NS	NS
AM+B4	*	NS	*	*	*	*	*	*	*	*	NS	*		*	NS
AM+B5	*	*	*	*	*	*	*	*	*	*	NS	NS	*		NS
AM+B6	*	*	*	*	*	*	*	*	*	*	NS	NS	NS	NS	

NS= not significant; *=significant difference, p<=0.05

AM fungal treatments especially (Myc+B2 and Myc+B3) and control (Crp, rooting powder) showed better shoot weight (g) when compared to other treatments (Figure 3.17).

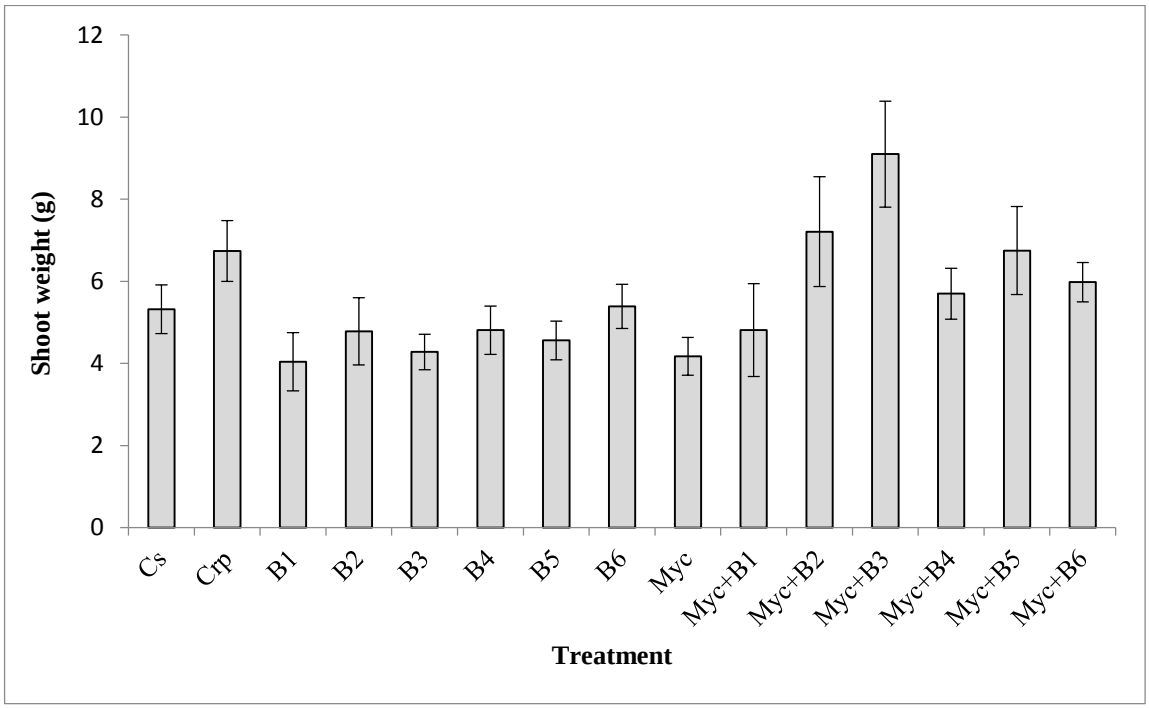

Figure 3.17: Shoot weight (g) of Spekboom when treated with selected bacteria alone or in combination with AM fungi. Columns are means ± standard errors (n=10).

Table 3.8: Shoot weight of Spekboom inoculated alone or in combination with selected bacteria and AM fungi.

	Cs	**Crp**	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**	**AM**	**AM+B1**	**AM+B2**	**AM+B3**	**AM+B4**	**AM+B5**	**AM+B6**
Cs		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	NS	NS
Crp	NS		*	NS	*	NS	NS	NS	*	NS	NS	*	NS	NS	NS
B1	NS	*		NS	NS	NS	NS	NS	NS	NS	*	*	NS	*	NS
B2	NS	NS	NS		NS	NS	NS	NS	NS	NS	*	*	NS	NS	NS
B3	NS	*	NS	NS		NS	NS	NS	NS	NS	*	*	NS	*	NS
B4	NS	NS	NS	NS	NS		NS	NS	NS	NS	*	*	NS	NS	NS
B5	NS	NS	NS	NS	NS	NS		NS	NS	NS	*	*	NS	NS	NS
B6	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS	*	NS	NS	NS
AM	NS	*	NS	NS	NS	NS	NS	NS		NS	*	*	NS	*	NS
AM+B1	NS	NS	NS	NS	NS	NS	NS	NS	NS		*	*	NS	NS	NS
AM+B2	NS	NS	*	*	*	*	*	NS	*	*		NS	NS	NS	NS
AM+B3	*	*	*	*	*	*	*	*	*	*	NS		*	*	*
AM+B4	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	*		NS	NS

	Cs	Crp	B1	B2	B3	B4	B5	B6	AM	AM+B1	AM+B2	AM+B3	AM+B4	AM+B5	AM+B6
AM+B5	NS	NS	*	NS	*	NS	NS	NS	*	NS	NS	*	NS		NS
AM+B6	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	NS	

All controls, bacterial and AM fungal treatments applied alone were not significantly different (Table 3.9) showing more root weight compared to inoculation of AM fungi and bacteria (Figure 3.18).

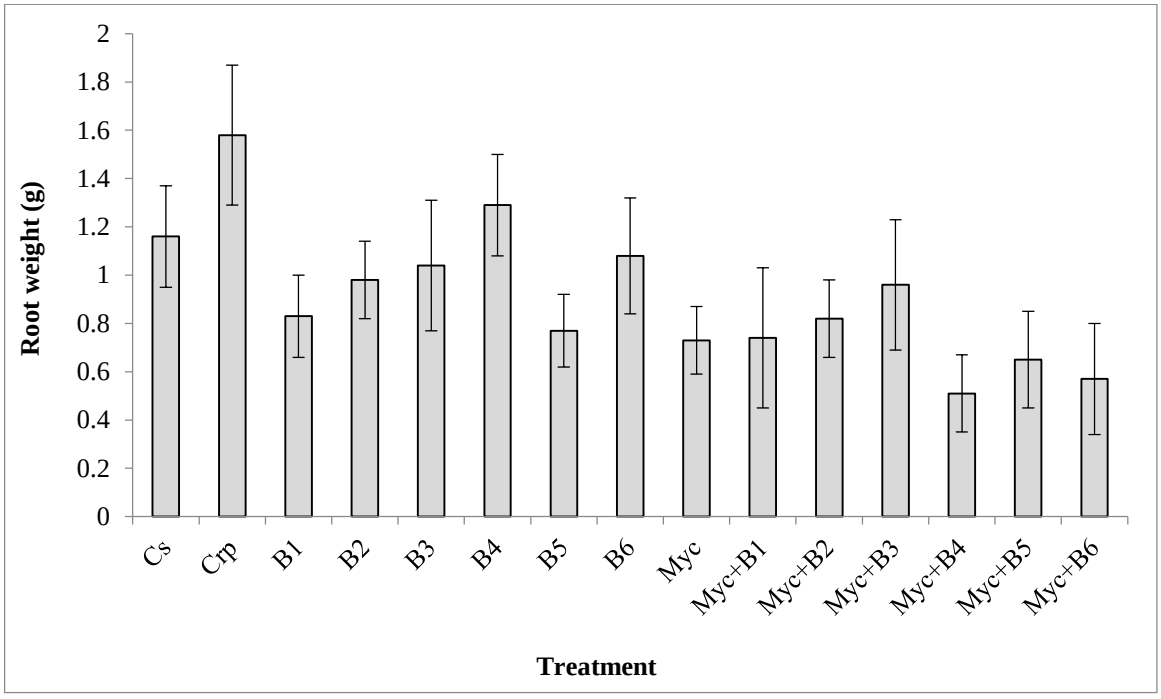

Figure 3.18: Root weight (g) of Spekboom when treated with selected bacteria alone or in combination with AM fungi. Columns are means ± standard errors (n=10). Root weights are not significant.

Table 3.9: Root weight of Spekboom inoculated alone or in combination with selected bacteria and AM fungi.

	Cs	Crp	B1	B2	B3	B4	B5	B6	AM	AM+B1	AM+B2	AM+B3	AM+B4	AM+B5	AM+B6
Cs	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Crp	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	NS
B1	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
B2	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
B3	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
B4	NS	NS	NS	NS	NS		NS	NS	*	NS	NS	NS	*	NS	*

	Cs	Crp	B1	B2	B3	B4	B5	B6	AM	AM+B1	AM+B2	AM+B3	AM+B4	AM+B5	AM+B6
B5	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS
B6	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS	NS	NS
AM	NS	NS	NS	NS	NS	*	NS	NS		NS	NS	NS	NS	NS	NS
AM+B1	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS	NS
AM+B2	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS	NS
AM+B3	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS
AM+B4	NS	*	NS	NS	NS	*	NS	NS	NS	NS	NS	NS		NS	NS
AM+B5	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS
AM+B6	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	NS	NS	

Controls and plants that were inoculated with bacteria alone showed traces (less than 5%) of mycorrhizal colonisation, resulting from splashing during irrigation. Combined inoculation of AM fungi and bacteria showed successfully colonisation (Table 3.10).

Table 3.10: Percentage of AM fungal root colonisation in inoculated spekboom plantlets.

Isolate	**Hyphae (%)**	**Arbuscules (%)**	**Vesicles (%)**	**Total colonisation (%)**
Myc	25.78±4.93	31.07±5.41	70.64±8.55	78.62±9.14
Myc+B1	11.15±4.81	1.97±1.58	44.33±6.15	43.06±6.95
Myc+B2	19.85±2.58	0.0±0.0	49.45±4.23	56.62±3.82
Myc+B3	24.24±2.37	4.4±1.63	48.43±4.21	57.35±4.68
Myc+B4	27.47±3.82	7.36±1.81	57.26±5.05	68.62±4.66
Myc+B5	24.85±4.07	26.32±3.57	60.04±3.62	75.64±2.81
Myc+B6	23.5±3.77	23.54±3.69	61.8±4.27	79.51±1.55
F value (14.135)	6.154	6.289	16.908	23.421
P<0.05	NS	NS	NS	NS

Values represents means from each treatment ± standard deviations (n=10).

Interaction between the suspected fungal pathogen, AM fungi and bacteria

Growth of Spekboom was significantly reduced in the presence of F2 pathogen. However the application of mycorrhizal fungi and bacterial isolates B2 and B4 significantly improved growth (Figure 3.19; 3.20).

Figure 3.19: Plants that were inoculated with the suspected fungal pathogen. A) shows plantlets that were inoculated with F2 and bacterial isolate and in combination with AM fungi; B) plants that were inoculated with F2 isolate alone and C) plants that were inoculated with F2 isolate and AM fungi.

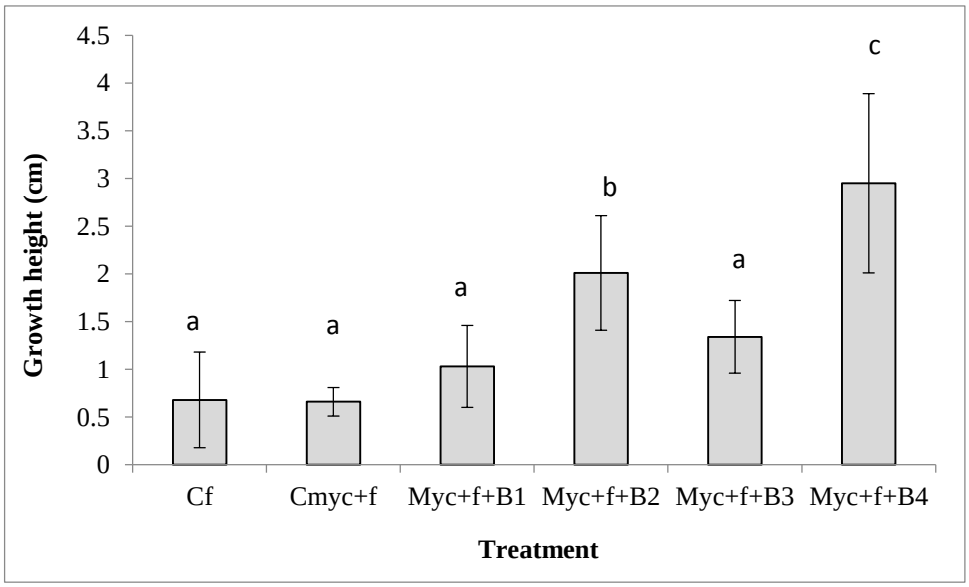

Figure 3.20: Growth in height (cm) of the Spekboom when treated with F2 isolate. $F_{(5,54)}$=2.61, p=0.035. Columns are means ± standard errors. Different letters indicates significance (n=10).

The treatment Myc+f+B4 had the highest shoot weight compared to other treatments and the control (Figure 3.21).

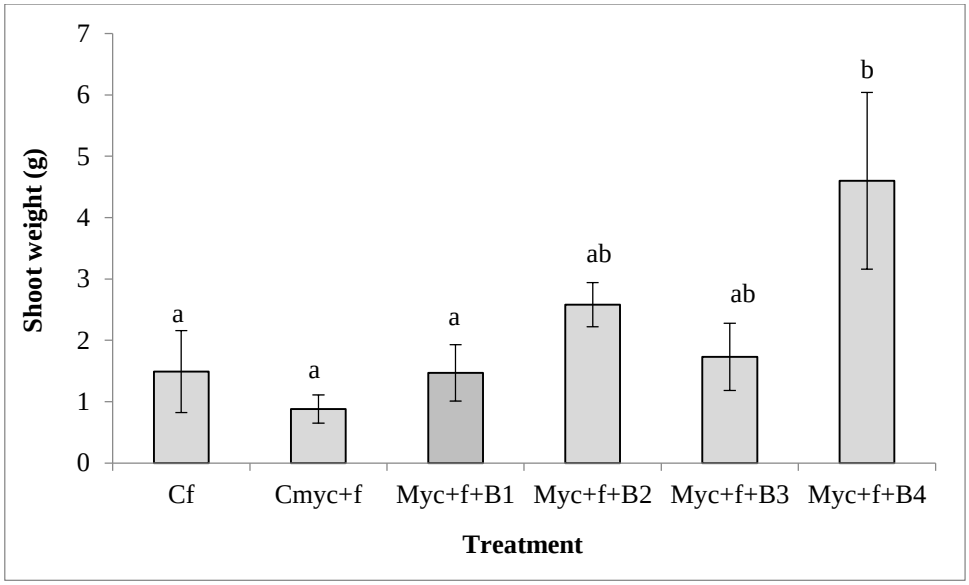

Figure 3.21: Shoot weight (g) of the Spekboom when treated with F2 isolate. $F_{(5,54)}$=3.31, p=0.011. Columns are mean shoot weight after 3 months ± standard errors.

Root weight was not significantly different between treatments (Figure 3.22). Although highest weights were recorded for myc+f+B2/B3 and B4 combinations.

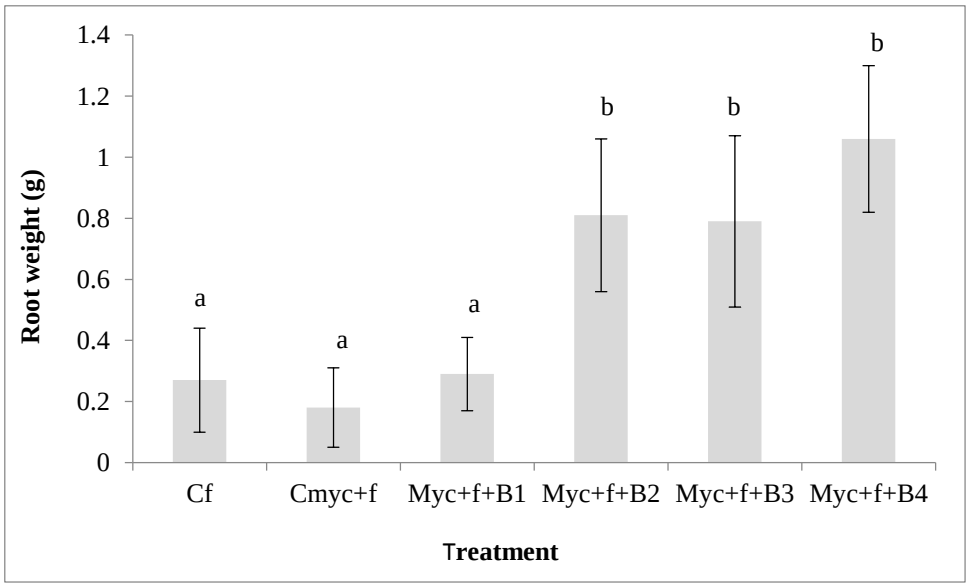

Figure 3.22: Root weight (g) of the Spekboom when treated with the suspected fungal pathogen, F2. $F_{(5,54)}$=3.69, p=0.01. Columns are means ± standard errors. Different letters indicates significance (n=10).

Control plant showed traces of mycorrhizal colonisation while other treatments where successfully colonised (Table 3.11).

Table 3.11: Percentage of AM fungal root colonisation. Means represents replicates from each treatment (n=10).

Isolate	**Hyphae %**	**Arbuscules %**	**Vesicles %**	**Total colonisation %**
Cmyc+f	3.65±2.02	2.78±1.58	8.9±4.82	8.91±5.14
Myc+f+B1	4.95±1.65	5.89±1.87	15.2±4.92	24.51±7.32
Myc+f+B2	4.11±1.34	10.38±3.08	23.32±5.51	28.56±8.26
Myc+f+B3	10.69±4.88	7.69±2.26	31.39±7.22	25.21±7.52
Myc+f+B4	2.5±1.37	7.01±1.78	25.89±3.39	11.93±6.33
F value (5.54)	2.22	3.47	5.08	7.00
P<0.05	NS	NS	NS	NS

CHAPTER 4: DISCUSSION

4.1 AM FUNGAL ASSOCIATION

4.1.1 Spore densities

Thicket shrub is dominated by Spekboom and receives 250-300 nm rainfall annually, falling mainly in autumn and spring. Temperatures are moderate although extremes may be experienced for short periods (Low and Kebelo, 1996). AM fungal spores were extracted from the three Spekboom plots and abundance was less than 1 spore/g soil. Spores are the major reproduction form that exists in soil providing a source of inoculum over a look period of time. Sporulation is seasonal and depends on identity and physiological status of the fungus and plant (Mangan *et al*., 2010; Sanders, 2004; Siguenza *et al*., 1996). Seasonality was demonstrated in a study of natural vegetation in regions of the Eastern Cape indicating that spore densities increased in August, which is late winter (Mambo, 2008; Ngonyama, 2009). This supported earlier investigations on coastal dunes in the region which demonstrated that spore densities increased in winter (Haller, 2000). Other studies have reported that sporulation potential of AM fungi is increased by higher temperatures and light intensities during the summer season (Cardoso *et al*., 2003; Gamage *et al*., 2004; Guadarrama and Alvarez-Sanchez, 1999; Hetrick, 1984). Sampling of rhizosphere soil may yield low spore numbers in natural soils, viability is also questionable as spores may be dead, parasitized or appear as spore cases (Muthukumar and Udaiyan, 1999). The Spekboom plots were sampled only once in March which may account for the low abundance. In natural environments dominated by perennial shrubs, low spore densities are generally recorded when compared to environments dominated by annual grass or crops (Abbot and Robson, 1977; Mosse and Bowen, 1968).

Unlike other fungi, which sporulate in spring, AM fungi produce spores towards the end of summer (Schultz *et al*., 1999). Increased spore numbers are expected when sampling is conducted in the dry season (Verbruggen and Kiers, 2010). The Eastern Cape region, particularly around Grahamstown has a transitional climate falling between summer and winter rainfall regions. Castillo *et al*. (2006) recorded 46 spores/g soil in a Southern Chile deciduous forest. High AM spore densities were reported in Brazilian Atlantic forests during summer and spring (Aidar *et al*., 2004; Bononi and

Trufem, 1983; Pagano *et al*., 2009; Silva *et al*., 2006; Trufem and Viriato, 1990), while low AM spore densities (8-57 per g/soil) were observed under *Acacia* shrubs (Ingebly *et al*., 1997). Maja (2009) reported low AM fungal spore density of *Cordeauxia edulis* (leguminous shrub) in Ethiopia. Variation in sporulation can be attributed to climatic factors, variation in vegetation, host specificity, age of plants, disturbance and sporulation ability of different AM fungal taxa (Husband *et al*., 2002; Muthukumar and Udaiyan, 2002). AM fungal spore development depends on successful colonisation of roots in order to access plant carbohydrates and convert them into fatty acids and other compounds (Solaiman *et al*., 1999; Trepanier *et al*., 2005). Although, AM fungal spores are considered as important propagules ensuring the survival of the symbiosis their numbers are not correlated with mycorrhizal formation in soil, as colonisation can occur from a single viable spore or through contact with mycorrhizal hyphae in the soil (Abbott and Robson, 1984, 1991; Ebbers *et al*., 1987; McGee, 1989; Mukerji and Kapoor, 1986; Schmidt and Reeves, 1984).

4.1.2 Colonisation

Cardoso *et al*. (2001) reported that environmental factors such as temperature, light intensity and soil moisture determine the morphological type of arbuscular mycorrhizal colonisation. The Spekboom root segments analysed were confirmed to be colonised by typical *Arum* AM fungal structures with the presence of hyphae, vesicles and arbuscules. Arbuscules may be difficult to observe and are often not recorded (Meney *et al*., 2003; Muthukumar *et al*., 1996; Sengupta and Chaudhuri, 2002) since they are the structures responsible for the bidirectional flow of nutrients between the symbionts (Smith and Read, 2008). Arbuscles are short lived with a life span of only about 7 days. Slower growing plants can have longer lived arbuscles or have a *Paris* type colonisation strategy (Smith and Read, 1997). Their presence confirms an active growth of host and its dependence on mycorrhizal fungi (Jasper *et al*., 1989). Hounslow had the lowest number of spores but it had the highest mean percentage of AM fungal colonisation of hyphae and vesicles as compared to other plots. This agrees with report by Fontenla *et al*. (1998) and Zhao *et al*. (2000) indicating that lack of correlation between spore numbers and colonisation percentage. Lack of correlation may be due to their reliance on an extensive hyphal network (Biermann and Linderman, 1983). Percentage colonisation was recorded in this study to be between 26-62%, which is fairly moderate. As with spores densities colonisation varies seasonally with other

studies reporting high percentages (up to 92%) in the winter months in the Eastern Cape (Haller, 2000).

Low AM fungal colonisation has been recognised as a common trait in the natural plant symbiosis in toxic metal environments (Oryowska *et al*., 2005). Straker *et al*. (2007) conducted a study during late summer on South African mine tailings and recorded that the colonisation levels of selected plant species was low to moderate dependant on the host. AM fungal colonisation levels were low on *T. usneoides* on mine tailings and contaminated sites (Buck, 2011; Spruyt, 2010). Muthukumar and Udaiyan (2000) reported low AM fungal colonisation in herbaceous species in Southern India. Mycorrhizal colonisation is influenced by host plant, productivity, foliar quality (Goverde *et al*., 2000), clonal morphology (Streitwolf-Engel *et al*., 1997), fitness and availability of soil P (Xiaohong and Koide, 1994). It is well known that mycorrhizal symbiosis is adversely affected by high P (Smith and Read, 2008). The Hounslow and Helspoort plots, rated medium and weak plant condition, respectively, had similar soil nutrient concentrations with double the amount of P (60-69.6 mg/Kg) and higher pH (H_2O) of between 7-8.0 when compared to the Echo plot soil, which was low in P (37.4 mg/Kg) with a more acidic pH of 5.69. Despite the higher P concentration Hounslow had the highest percentage colonisation, indicating other unknown factors (biotic or abiotic) influenced successful colonisation.

4.1.3 Identification

Identification using only spore morphology of field extracted spores can be difficult as the spores are often old or damaged and when spore numbers are low diversity can be underestimated due to insufficient spores showing identifying characteristics. Molecular techniques are being more readily applied to solve this problem and as shown in this study the use of 454 Pyrosequencing yielded 464 OTU. Analysis of the sequence data was challenging as a direct Bioinformatics pipeline was not available. AM fungal sequences obtained in this study were aligned with other Glomeromycotan sequences using the MaarjAM database (Öpik *et al*., 2010). Only the first 10 OTU groupings in each plot were identified with these indicating differences between the three Spekboom trial plots. Soils from Echo were dominated by Ambisporaceae with majority of sequences (based on Number of sequence reads) aligning with *Ambispora*

leptoticha. Helspoort and Hounslow soils appeared more diverse with members of the Glomeraceae being more prevalent.

In *Acacia* shrubs, the AM fungal species found in earlier studies were predominantly from the genera *Glomus* and *Gigaspora* (Michelson, 1993; Yohannes and Assefa, 2009). The genera *Glomus* and *Acaulospora* were found to dominate in an Atlantic rainforest biome which is in accordance with other findings for tropical rainforest (Guadarrama and Alvarez-Sanchez, 1999; Louis and Lim, 1987; Trufem and Viriato, 1990). AM fungal species belonging to *Glomus* and *Acaulospora* are commonly recorded in disturbed soil since their spores are resistant to the effects of the modified ecosystems (Picone, 2000; Silva *et al*., 2006; Zhango and Moreira, 2010). *Glomus* species are common in many ecologically different environments; from natural woodlands to conventionally managed agro-ecosystems (Helgason *et al*., 2002; Hijri *et al*., 2006; Oehl *et al*., 2005). *Glomus* species are believed to produce more intraradical hyphae compared to external hyphae thus being less dependent on the mycelial network which is dislodged when soils are tilled (Dodd *et al*., 2000; Hart and Reader, 2002; Maherali and Klironomos, 2007). Studies in South Africa have shown that the rhizosphere of cassava (wooden shrub) in Limpopo contain different genera comprising *Acaulospora*, *Glomus* (dominant) and *Gigaspora* whereas the soil in Mpumalanga had *Acaulospora*, *Glomus*, *Gigaspora* and *Scutellospora* species (Straker *et al*., 2010). Cassava in Nigeria was associated with *Acaulospora*, *Ambispora*, *Claroideoglomus*, *Glomus*, *Gigaspora*, *Pacispora* and *Scutellospora*. Sieverding (1989) reported that cassava was associated with predominantly *Glomus* species indicating their remarkable adaptation to tropical forests. This AM fungal diversity reflects other results from areas such as Benin (Tchabi, 2008), India (Muthukumar and Udaiyan, 2000) and East Africa (Mathimaran *et al*., 2007).

Under organic farming management practices, diversity of AM fungi was shown to increase and included the genera *Acaulospora*, *Scutellospora* and *Entrophospora* (Jansa *et al*., 2002). The analysis of a variety of plants growing on coal overburden mine dumps in India showed five genera comprising *Glomus*, *Acaulospora*, *Gigaspora*, *Scutellospora* and *Entrophospora* (Hazarika *et al*., 2014). In Southern Chile, 14 species of AM fungi from deciduous forest were identified as *Acaulospora*, *Archaeospora*, *Diversispora*, *Entrophospora*, *Glomus*, *Pacispora*, *Paraglomus* and *Scutellospora*

(Castillo *et al*., 2006). These studies indicated that AM fungi are ubiquitous in nature and that soil may harbour diverse populations.

4.2 SPEKBOOM DECLINE

The genus *Fusarium* represents the most important group of fungal pathogens that causes diseases such as crown rot, wilting, head blight and scab on economically important plant species (Agrios, 2005; Nelson *et al*., 1994). Wilting is regarded as critical in the agricultural area as even with sufficient rainfall or irrigation plants are stressed due to reduced uptake (Ploetz, 2006). This pathogen is soilborne disease causing on subterranean and aerial plant parts, the pathogen survives on plant debris and other organic substrates (Nelson *et al*., 1994). Although, this pathogen can be found in many environments, the development of the disease is notable under high temperatures and warm moist soils. On artificial media, the optimum temperature for growth is between 25-30°C, whereas the optimum soil temperature for root infection is 30°C and above (Goss, 1936). The disease caused by this pathogen is characterized by yellowing leaves, which at times can be mistaken with the lack of potassium and those symptoms caused by root rotting fungi, vascular browning and wilting are symptoms of the disease which may result in the death of the whole plant (Agrios, 2005). The wilt pathogens have a more specialised host range classified as forma speciales and are adapted to grow in the vascular system of their host as compared to other soil borne fungal pathogens (Michielse and Rep, 2009). Spekboom cuttings used to test pathogenicity of the suspected *Fusarium* isolates were harvested after a month and symptoms of internal discoloration of some roots were observed as well as some leaf yellowing. Due to the poor root development of the cuttings, a second trial using pre-rooted cuttings was also analysed. Re-isolation of *Fusarium* was not conducted and would be necessary to prove Koch's postulates. A greater vegetative development was seen in plants that were not inoculated with suspected fungal pathogen, although the shoots to root ratios were not significantly different.

In South Korea, *Fusarium* the wilt is the most serious soilborne disease of strawberry resulting in losses of up to 30% when transplanting (Nam *et al*., 2005). Discoloration symptoms of the vascular system may differ with host plant in Cavendish banana, red to brown discoloration was recorded while the same isolate caused dark or reddish

brown discoloration when inoculated on alfalfa roots (Frosheiser and Barnes, 1978). *Fusarium* infects plants mostly through roots, once inside the xylem; it rapidly spread in the vascular vessels by means of conidia. The invasion of the conidia within the vascular plant system, together with host defence responses, eventually blocks the plants vascular system causing it to wilt and die (Beckman *et al*., 1961, 1962; Beckman and Roberts, 1995; Stover, 1962).

4.3 RHIZOBACTERIAL ANALYSIS

4.3.1 In-vitro inhibitory test

Of the 16 rhizobacterial isolates screened for inhibitory effect against the suspected fungal pathogens, isolates showed antifungal activity were considered antagonists. Antagonists are a good source of biocontrol agents which can reduce the activity of pathogen (Whipps and Lumsden, 2001). Bacteria such as *Pseudomonas fluorescens*, *Pseudomonas putida*, other *Pseudomonas* species and *Bacillus* species have been reported as suitable antagonists against *Fusarium* (Georgakopoulos *et al*., 2002; Ikotun and Adekunle, 1990; Scher and Baker, 1982; Thomashow and Weller, 1990; Whipps and Lumsden, 2001). Bacterial species which live in the rhizosphere competent are able to provide protection to plant roots from soilborne pathogens as well as improve plant growth (Glick *et al*., 2001; Harish *et al*., 2009; Singh *et al*., 2011). The results obtained in this study are similar to previous studies by O'Sullivan and O'Gara (1992) and Kumar *et al*. (2004) who reported that *Pseudomonas* was antagonistic towards plant fungal pathogens including *Fusarium* and *Rhizoctonia* species. *P. fluorescens* are known to suppress soil-borne fungal pathogens by producing antifungal metabolites (Dwivedi and Johri, 2003; Kumar *et al*., 2002). Several research have been conducted on *pseudomonads* for their potential as biocontrols due to their superior root colonising abilities and their ability to produce a wide range of antimicrobial substances (Walsh *et al*., 2001). *P. fluorescens* produced significant disease suppression under greenhouse conditions (Ji *et al*., 2006). Many antifungal metabolites have been reported and shown to be effective *in-vitro*. These metabolites include ammonia, butyrolactones, 2-4-diacetylphloroglucinol, HCN, kanosamine, oligomycin A, oomycin aphenazine-1-carboxylic acid, pyoluterin, pyrrolnitrin, viscosinamide, xanthobaccin and zwittermycin A (Milner *et al*., 1996; Whipps 2001).

Among the PGPR, *Pseudomonas* and *Bacillus* are found to have a great potential as biocontrols since they are dominant in the rhizosphere (Mahaffe and Kloepper, 1997). *Bacillus sp*. is known to produce enzymes such as cellulase that have degrading properties against some pathogenic fungal cell wall components (Aktugano *et al*., 2003; Jarnagin and Ferrari, 1992; Pukall *et al*., 2005). Species of *Bacillus* are attractive as biocontrol agents because they produce endospores that are tolerant to heat and desiccation and are easily formulated (Weller, 1988). Some of *Bacillus* strains suppress plant pathogens by producing antibiotic metabolites whereas others stimulate plant host defense prior to pathogen infection (van Loon, 2007) which indirectly contributes to increase plant growth. Other bacteria such as *Burkholderia* have been shown to effectively control plant pathogenic fungi, yeast and protozoa due to the production of a broad range of antimicrobial compounds (Cain *et al*., 2000; Compant *et al*., 2005; Ji *et al*., 2006; Mao *et al*., 1998).

4.3.2 Indole acetic acid

Indole Acetic Acid (IAA) is the most common auxin produced by bacteria, fungi and plants which controls plant growth and development (Grossmann, 2010; Halliday *et al*., 2009). It is synthesized by bacteria to disturb physiological processes for their own benefit (Shih-Yung, 2010). It has been reported that 80% of microorganisms isolated from the rhizosphere of different cultivated plants have the ability to synthesize auxins as secondary metabolites due to a rich supply of substrates (Patten and Glick, 1996). IAA contributes to the production of longer roots increasing number of root hairs and enhancing lateral root formation which are involved in nutrient uptake (Datta and Basu, 2000). In IAA producing and non-producing species, IAA can act as a signal molecule even though a role for IAA as a signalling molecule in rhizobial species has not yet been described (Spaepen *et al*., 2007; Spaepen and Vanderleyden, 2011). In plants, it participates in cell division and elongation by modifying the osmotic contents of the cell and increasing permeability of water. It also inhibits or delays the removal of leaves and induces flowering as well as fruiting (Zhao, 2010). The amino acid tryptophan is the main precursor of IAA and it participates mostly in the regulation of IAA concentration (Zaidi *et al*., 2009). In legume plants, the levels of auxin are essential for nodule formation (Glick, 2012; Spaepen *et al*., 2007). Environmental stress factors such as acidic pH, osmotic stress and carbon limitation control the biosynthesis of IAA in different bacteria (Spaepen *et al*., 2007).

The ability to synthesize IAA is considered as an important tool for screening beneficial microorganisms suggesting that IAA production correlates with plant growth promotion (Wahyudi *et al*., 2011). Only 6 rhizobacterial isolates were positive for IAA production in this study. Four isolates were *Bacillus species*, *Enterobacter* species and *Burkholderia* species. Farah *et al*. (2008) had recorded the production of IAA by members of genera like *Pseudomonas, Bacillus, Azotobacter, Azomonas* and *Mesorhizobium*. *Pseudomonas* species especially *P. fluorescens* and *P. putida* are important group of PGPR due to their ability to produce auxin and promote yield (Bending *et al*., 2002; Founoune *et al*., 2001; Frey-Klett *et al*., 1996; Garbaye, 1994; Sbrana *et al*., 2002). In the current study, isolates of *Pseudomonas* were IAA negative and it has been shown that some of the *Pseudomonas* species have the ability to degrade IAA instead of producing it thus providing protection against pathogenic bacteria that over produce IAA and cause gall formation (Leveau and Lindow, 2004). Earlier studies recorded production of IAA in *Bacillus* species (Loper and Henkels, 1997; Xie *et al*., 1996) which supports results obtained in this study. Production of IAA by bacteria varies greatly among different species and the ability of bacteria to produce IAA in the rhizosphere depends on the availability of precursors and uptake of microbial IAA by plants (Flaishman *et al*., 2006; Vijila, 2000). In the current study, IAA production was detected less frequently compared to other plant growth promotion abilities and it is believed that the decrease in the level of IAA might be caused by the release of IAA degrading enzymes such as IAA oxidase and peroxidase as it has been reported in some bacteria (Datta and Basu, 2000), however this was not investigated.

4.3.3 Siderophore production

Iron is an important micronutrient utilised by bacteria for their metabolism. It is a cofactor for enzymes and a component of proteins (Bazylinski and Frankel, 2004). Under aerobic conditions, iron occurs as Fe^{3+} and it can form insoluble hydroxides and oxyhydroxides which are unavailable to plants and microorganisms thus it is not accessible (Rajkumar *et al*., 2010). Bacteria obtain iron through the secretion of low-molecular weight iron chelators known as siderophores which are important in sensing and uptake of iron (Rachid and Ahmed, 2005). Siderophores obtain iron from the environment making the mineral available to the microbial cell (Neilands, 1995). Aerobic microorganisms require iron for reduction of oxygen, ATP synthesis, reduction of nucleotide precursors of DNA, and for heme formation. Most siderophores are

water-soluble and they can be divided into two groups; hydroxamates and catecholates based on their structural features (Neilands, 1995; Winkelmann *et al*., 1987; Winkelmann, 1991). Hydroxamates are produced by both bacteria and fungi whereas catecholates are produced only by bacteria. Iron (Fe^{3+}) is reduced to Fe^{2+} on the bacterial membrane which in turn is released into the cell from siderophore through a gating mechanism (Drechsel *et al*., 1991).

Siderophores may be reduced or recycled during this reduction step (Neilands, 1995; Rajkumar *et al*., 2010) thus they serve as a solubilising agent for iron from minerals or organic compounds under iron limiting environment (Indiragandhi *et al*., 2008). Plants obtain iron required as a micronutrient through the direct uptake of siderophore-Fe complexes or by exchange reaction of ligand (Schmidt, 1999). Several reports have stated that microbial siderophores have a positive correlation with plant growth promotion and should be considered in PGPR screening programs (Crowley *et al*., 1991; de Weger *et al*., 1986; Tokala *et al*., 2002; Wang *et al*., 1993). CAS medium was used to detect siderophore production and also to indicate the ability of the compound to remove iron bound to the dye complex. Lopers and Henkels (1996) and Xie *et al*. (1996) recorded the production of siderophores in *Bacillus* species. Tian *et al*. (2009) indicated that most of the isolates belonging to the genera *Pseudomonas* and *Enterobacter* (Gram negative bacteria), *Bacillus* and *Rhodococcus* (Gram positive bacteria) produced siderophores. *P. putida* can use the siderophores produced by other rhizosphere microorganisms to enhance the level of iron available to it in the environment (Loper and Henkels, 1999). Many bacteria use siderophores to help the process of ferric iron uptake in the environment. Inability to show production of siderophores by some bacterial isolate maybe because the compounds were too dilute to react with the reagent. Microorganisms that have the ability to produce siderophores can protect themselves by binding toxic metals such as Al, Pb and Cd. At high levels, these metals can damage cell membranes, alter enzyme specificity, disrupt cellular functions, damage the structure of DNA (Bruins *et al*., 2000, Canovasa *et al*., 2003; Teitzelet *et al*., 2006), reduce crop yields and soil fertility (Stuczynskiet *et al*., 2003). Competition for iron in the environment between rhizobacteria and soil borne pathogens can also contribute to disease suppression (Tokala *et al*., 2002).

4.3.4 Phosphate solubilisation

Phosphorus is an important macronutrient needed for growth and development of plants (Illmer and Schinner, 1992) which in turn plays a role in processes such as photosynthesis, nutrient uptake, energy transfer and respiration (Khan *et al*., 2010) and nitrogen fixation in legumes (Saber *et al*., 2005). It is mostly found in soils in inorganic and organic forms which quickly become unavailable for root uptake (Khan *et al*., 2009). In plants, the amount of phosphorus is low due to the fact most of soil P occurs in insoluble form whereas plants can only absorb P in the form of soluble monobasic ($H_2PO_4^-$) and the diabasic (HPO_4^{2-}) ions (Bhattacharyya and Jha, 2012). Insoluble P is present in the form of inorganic minerals such as apatite or organic forms such as inositol phosphate, phosphomonesters and phosphotriesters (Glick, 2012). The application of P fertilizers only provides plants with limited P as most is converted into insoluble complexes (Mckenzie and Robert, 1990). Phosphate solubilising microorganisms such as *Azotobacter*, *Bacillus, Burkholderia, Microbacterium* and *Pseudomonas* play a crucial role in providing access to these inaccessible forms of P (Bhattacharyya and Jha, 2012; Khan *et al*., 2006).

Mineralization of organic phosphorus substances is carried out by means of phosphatase enzymes and the phosphatase activity is significantly increased in the rhizosphere (Glick, 2012). Phosphate solubilisation and mineralization can occur in the same bacterial strain (Tao *et al*., 2008). The activity of P-solubilisation is enhanced by the production and secretion of organic acids which in turn release the P converting them to soluble (Kpomblekou and Tabatabai, 1994). The majority of phosphate solubilisers in this study were Gram positive *Bacillus sp*. This is supported by Tilak *et al*. (2005) who reported that *Bacillus* and *Pseudomonas* are important phosphate solubilisers. Saidi *et al*. (2009) isolated and tested 20 species of *Bacillus* but only 5 species were able solubilise phosphate indicating variability within the genus. In this study, not all of *Bacillus* species have the ability to solubilise phosphate. Some of *Burkholderia* strains have been previously described as phosphate solubilisers (Caballero-Mellado *et al*., 2007; Khalimi *et al*., 2012; Peix *et al*., 2001; Silin-Cherif, 2012; Viruel *et al*., 2011). Phosphate solubilising bacteria are generally regarded plant growth promoters (Hafeez, 2004; Hilda Rodriguez and Fraga, 1999; Katiyar and Goel, 2003).

Isolates were predominantly identified as various *Bacillus* species which could possibly be a result of the persistence of endospores in the soil and prolific growth on media (Mobberley *et al*., 2010). No particular enrichment strategy was applied. Other isolates were identified as species of *Enterobacter, Arthrobacter* and *Microbacterium*. Only one *Pseudomonas* was isolated from this study, the isolates used in pot trial were available in the Research laboratory from a previous study (Moore, 2012).

4.4 AM FUNGAL RHIZOBACTERIAL INTERACTIONS

The use of microbial agents alone or combined as multiagents to improve plant growth and enhance biological control is not a new concept and has been investigated, the key is identifying the best microorganisms to use (Duffy *et al*., 1996; Guetsky *et al*., 2002). Kloepper *et al*. (1989) reported increase in yield after PGPR inoculation of a range of crops; however reduced yields were reported in some crops. Treatment of cuttings with *Bacillus* and *Pseudomonas* induced root formation (Esitken *et al*., 2003). In this study the use of AM fungi and selected rhizobacterial isolates alone as well as in combination were assessed for their ability to promote growth of Spekboom cuttings. Plants inoculated with AM fungi alone were not significantly different from controls and single rhizobacterial inoculation with regards to shoot height. Inoculation of AM fungi with 5 of the selected PGPR improved growth indicating enhanced activity of symbiosis, similar results were obtained by Artursson *et al*., 2006. The plant growth promoting bacteria identified and used in combination with the mycorrhizal fungi in this study were *Burkholderia* sp. (Treatment AM + B2, Moore, 2012), *Bacillus thuringiensis* (Treatment AM + B3), *B. cereus* (Treatment AM + B4), *B. subtilis* (Treatment AM + B5) and *Bacillus* species (Treatment AM + B6). These bacteria were selected for their ability to produce IAA and siderophores, and solubilise P. The most significant interaction promoting shoot weight was recorded with the AM + *Bacillus thuringiensis* (B3) treatment. *B. thuringiensis* is a well-known biological control agent used against insect pests (Madigan *et al*., 2005), this results indicates the multifunctionality of the bacterial species. Because of the poor root ability observed a rooting powder treatment was included. This treatment did not significantly affect Spekboom growth. Co-inoculation with PGPR can negatively affect plant growth because of the production of antibiotics and competition for attachment sites on root

surfaces (Gull *et al*., 2004; Mirza *et al*., 2007). This was not observed in this study but some bacterial isolates on their own can be regarded as neutral, having neither and positive not negative effect.

Synergistic beneficial effects on plant development have been observed with co-inoculation of AM fungi and PGPR association (Dhillion, 1992; Singh and Kappor, 1998). Grace *et al*. (2009) and Martin *et al*. (2012) indicated that P absorption and availability is enhanced with AM fungi but the symbiosis would benefit from interaction with P solubilising rhizobacteria (Singh and Kappor, 1998) making P more available for translocation through the mycorrhizal network. Root weight of Spekboom cuttings was not significantly affected by any treatments. It is likely that establishment of PGPR in the rhizosphere can be influenced by AM fungi as has been reported in several studies (Bianciotto and Bonfante, 2002; Bianciotto *et al*., 2002; Ravinskov *et al*., 1999). In general PGPR are attractive agents for increasing plant yield, growth and nutrition as was shown by Orhan *et al*. (2006) who reported the beneficial interaction of *Bacillus* with raspberry. Kloepper *et al*. (1980) reported similar results using *P. fluorescens* as a seed inoculant of selected crops.

Plants associated with AM fungi are believed to receive protection from pathogens relative to non-mycorrhizal plants in experimental studies (Akthar and Siddiqui, 2008). AM fungal taxa vary in their ability to protect host plants against pathogens (Sikes *et al*., 2009). In a natural soil environment, a diversity of AM fungi occurs as shown in the sequence analysis. There is a greater potential to protect host plants against pathogens when species are combined taking advantage of different function properties of individual species (Maherali and Klironomos, 2007). The Mycoroot inoculum used is a collection of indigenous South Africa isolates of AM fungi (Dames, personal communication). The bacterial isolates selected for testing control potential in pot trials were those that show antagonistic ability *in-vitro* dual culture. *Burkholderia* sp. (Treatment Myc+F+BC1); *Pseudomonas fluorescens* (Treatment Myc+F+BC2); *P. putida* (Treatment Myc+F+BC3) were obtained from Moore (2012) and *Bacillus cereus* (Treatment Myc+F+BC4) all produced siderophores and important biocontrol characteristic. Various studies have shown that bacteria such as *Pseudomonas* or *Bacillus* species may play a role in the reduction of disease. The combination of *P. fluorescens* and AM fungi showed reduction in disease intensity in wheat plants (Behn, 2008). *Pseudomonads* are known as good root colonisers because they can stimulate the

root colonisation of host plants by AM fungi (Meyer and Linderman, 1986). In the presence of the *Fusarium* pathogen, shoot height and weight was low whereas the addition of mycorrhizal fungi alone did not significantly improve growth. This was unexpected as AM fungi are known to inhibit pathogen attack or decrease the damage that is caused by the deleterious organisms (Abdalla and Abel-Fattah, 2000; Chandanie *et al*., 2009; Linderman, 1994; Sikora, 1995; Yao *et al*., 2002). The growth of these plants was suppressed by pathogenic infection and indicated suppressive growth. One of the known mechanisms of mycorrhizal protection is through the promotion of biomass and increased photosynthetic rates because of the enhanced P supply due to symbiotic association (Jia *et al*., 2004). AM fungi enhance uptake of other nutrients required by plants such as nitrogen, potassium, calcium, copper, manganese, magnesium, iron and zinc (Clark and Zeto, 2000; Karagiannidis *et al*., 2002). It is believed that mycorrhizal fungi obtain N from organic substances by enhancing decomposition (Goussous and Mohammad, 2009; Hodge *et al*., 2001). Root colonisation with AM fungi has been reported to either stimulate or inhibit plant growth depending on environmental conditions (Sanders, 1993). Poor root development of the Spekboom cuttings was again noted in this pot trial and hence this suggested that the presence of *Fusarium* was hampering root production. The paucity of roots would also influence mycorrhizal establishment and may have contributed to the results recorded in the Myc + F control treatment and as a results colonisation levels were low and difficult to assess statistically. Because of this lack of root development differences in root weight were not significant. The most significant interaction in terms of growth and shoot weight was treatment Myc+F+B4, which was the combination with *B. cereus*.

CHAPTER 5: CONCLUSION

Portulacaria afra, commonly referred to as Spekboom is an important component of the thicket vegetation in the Eastern Cape. Widely known for its grazing potential and ability to fix in excess of 200 tons/ha carbon under semi-arid condition. Re-establishing Spekboom is used to rehabilitate degraded thicket as well contribute to the international carbon trade market. Poor survival and plant condition in extensive trials monitored by the Department of Environmental Affairs, the Natural Resource Management Directorate (Mike Powell, personal communication) instigated this investigation to determine the interaction between mycorrhizal fungi, plant growth promoting rhizobacteria and Spekboom. The objectives and outcomes of this study were to:

Conduct a survey of arbuscular mycorrhizal populations associated with Spekboom in selected areas of the Eastern Cape.

Firstly, Spekboom was confirmed to associate with AM fungi showing typical Arum colonisation strategy. Identification of field extracted spores was problematic due to the low spores densities and poor spore quality. AM fungi are obligate biotrophs and cannot be grown in axenic culture which excludes this means of identification. The use of pyrosequencing proved to be very useful indicating a diverse AM fungal population. The analysis of this data present here is very preliminary as a suitable Bioinformatics pipeline is not currently available is the topic on a MSc Bioinformatics student project, once completed this data derived from Spekboom soils will be analysed in more depth and contribute to a diversity list of AM fungi in South African soils. Further studies can establish trap cultures of dominant AM fungal species which will aid in the development to a targeted inoculum for Spekboom.

Isolate and identify the causal agent of Spekboom decline

Two different *Fusarium* isolates were identified from soil and Spekboom root samples. Only identification to genus level was possible on submission on GenBANK. Sequencing of other fungal genes could have assisted in more detailed identification. Although, pathogenicity tests were not significant a decline in growth was certainly the trend. This coupled with poor root development of cutting could substantially affect Spekboom survival.

Isolate and identify the plant growth promoting rhizobacteria

Rhizobacteria were isolated using general media and subsequently molecularly identified, and shown to be predominantly members of the genus, *Bacillus*. Samples were not specifically enriched or plated onto selected media, which may be useful if particular rhizobacterial isolates were of interest such as *Pseudomonas* or *Streptomyces*.

Assess the plant growth promotion properties of bacterial isolates

Rhizobacteria were isolated and characterised based on their ability to produce IAA and siderophores as well as solubilise P. Not all isolates were positive for all three characteristics. The characteristics investigated do provide an indication of potential as plant growth promoters, although several other characteristics such as HCN, ammonium production and enzymatic activity could also be tested.

Assess the interaction between arbuscular mycorrhizal fungi, PGPR and their biological control potential under greenhouse conditions.

The interaction between AM fungi, *Burkholderia* and several *Bacillus* sp. did promote growth of Spekboom cuttings and the combination with *B. cereus* did promote growth when challenged with *Fusarium*. It is recommended that cuttings should be initially inoculated with these beneficial microorganisms under nursery conditions before transplanting in the field. Further trials investigating stimulation of rooting are also required.

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