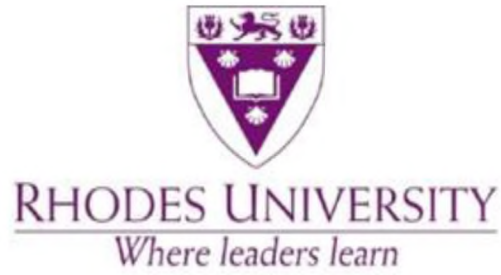

Assessing the potential role of microorganisms in the production of seedlings for the restoration of Albany Thicket

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Prepared

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ABSTRACT

The role of microorganisms in restoration of the Albany Thicket has not been well documented, although the benefits to plants of these various interactions has been well documented. Microorganisms are chief ecological engineers and assist in resolving environmental problems and act to restore degraded ecosystem function by forming mutual relationships with the roots of the plants. The aim of this study was to assess the potential of microorganisms for the improved biomass production of selected woody and succulent seedlings used in mesic thicket restoration. Three tree species were selected for propagation in this study namely; *Mystroxylon aethiopicum* *Scutia myrtina* and *Aloe ferox*. Soil samples were collected from a degraded and intact thicket site from Bathurst, South Africa. Soils were evaluated for number of arbuscular mycorrhizal (AM) spores, mycorrhizal infectivity potential and nutrient availability both before and after seedling propagation. Pasteurized soil from the degraded site was used in a pot trial. Ten replicates seedling for plant species were planted and subjected to four treatments which included inoculation with AM fungi and the rhizobacterium, *Enterobacter* sp., alone and in combination; the fourth treatment was an un-inouclated control. Plant growth parameters were recorded at regular intervals where appropriate and seedlings were harvested after 24 weeks for biomass measurements and AM colonisation assessments.

Although generally low (< 1 spore per gram) the density of AM fungal spores was significantly higher in soils from the intact site when compared with soils from the degraded site. The mycorrhizal potential of the soils was however not significantly different. *Mystroxylon aethiopicum* seedling shoot height, canopy diameter and shoot biomass showed a significant increase when inoculated with AM fungi while *S. myrtina* seedlings showed increased shoot height when inoculated with both AM fungi and *Enterobacter* sp. *Aloe ferox* seedlings did not respond to microbial inoculation. The concentration of soil P and Na increased in treatments with *Enterobacter* sp. alone and in combination with AM fungi. *Mystroxylon aethiopicum* and *S. myrtina* seedlings showed a dependency on microbial inoculants indicating the importance of inoculation in the nursery before planting out into the field. Overall AM fungal inoculants applied to seedlings can be used to compensate for nutrient deficiency in soils. Although the *Enterobacter* isolate used was known to have various plant growth promoting capabilities. It is recommended that other rhizobacterial isolates be investigated.

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ABBREVATIONS

% percentage

Al aluminium

AM arbuscular mycorrhizal fungi

ANOVA analysis of variance

ATP adenosine triphosphate

C carbon

Ca calcium

CKs cytokinin's

cm centimetre

cm^3 cubic centimetres

Cont. control

EC electrical conductivity

Ent. sp. *Enterobacter* sp.

Fe iron

g gram

H height

H_2O water

ha hectors

HCL hydrochloric acid

HCN hydrogen cyanide

hrs hours

IAA indole acetic acid

K potassium

KCl potassium chloride

kg kilograms

km^2 square kilometre

KOH potassium hydroxide

m meters

M molarity

m^2 square meters

MD mycorrhizal dependency

Mg magnesium

MHB mycorrhizal helper bacteria

Min(s) minutes

MIP mycorrhizal infective potential

ml millilitre

mm millimetre

N nitrogen

N_2 atmospheric N

Na sodium

NH_4 ammonium

NO_3 nitrate

^oC degrees celsius

OM organic matter

P phosphorous

PGPR plant growth promoting rhizobacteria

PO_4 phosphate

PSB phosphate soluble bacteria

SWH soil water holding capacity

UV ultra violet

µm micrometres

H^+ hydrogen ion

OH^- hydroxyl ion

CHAPTER ONE: INTRODUCTION

1.1 ALBANY THICKET

Albany Thicket is one of the biomes of South Africa (Mucina and Rutherford, 2006) but also a major component of the hotspot (Mittermeier *et al.* 2011). It is transitional between Nama-Karoo, Fynbos including Grassland biomes and includes the subtropical regions of the eastern seaboard of southern Africa (Palmer 2004; Hoare *et al.* 2006). The mean annual rainfall is between 200 mm and 950 mm (Palmer, 2004). Temperature varies with mean monthly maximum temperatures ranging between 29 - 32°C and minimum monthly temperatures between 4 - 6°C. The thicket supports a high diversity of animal and plant species, including high densities of indigenous herbivores, ranging from grey duiker, *Sylvicapra grimmia,* to elephants, *Loxondonta africana* (Palmer, 2004). The arid forms of the Albany Thicket Biome (ATB) have a dense formation of evergreen and weakly deciduous succulent shrubs such as *Portulacaria afra* and spinescent shrubs such as *Azima tetracantha, Gymnosporia polycantha, Putter lickia pyracantha* and *Searsia longispina*. Low-growing trees such as *Pappea capensis, Euclea undulate* and *Schotia afra* with a height of between 2–5 m is also prevalent (Hoare *et al.* 2006: Sigwela *et al.* 2006). In the mesic form in the Albany Thicket, woody taxa such as *Hippobromus pauciflorus, Olea europea, Buddleja saligna, Ptaeroxylon obliquum, Diospyros dichrophylla, Scutia myrtina* have dominated. In addition to this, emergent stem succulents such as *Euphorbia triangularis* and *E. tetragona* are also found (Weatherall-Thomas, 2009).

Mesic thicket vegetation is recognised as being the most vulnerable to degradation (Kerley and Landman, 2006; Weatherall-Thomas, 2009; Rutherford *et al.* 2014). According to Lechmere-Oertel *et al.* (2005), excessive browsing by goats has had a major impact on the structure of the thicket vegetation. Generally, the most vulnerable thicket is the drier Arid and Valley forms, which are dominated by the tree like *P. afra* also known as spekboom. The dense closed canopy shrubland species has been changed by goats to an open pseudo- savanna (Lechmere-Oertel *et al.* 2005b; Weatherall-Thomas, 2009; Van der Vyver *et al.* 2012). Goat's destroy the canopy skirt which alters the microclimate beneath the canopy leaving an open community (Van der Vyver *et al.* 2012, 2013). Consequently, with greater evaporation of moisture from the soil it reduces the mycorrhizal

fungal abundance (Allsopp *et al.* 1996 cited by Weatherall-Thomas, 2009) and general soil microbial activity (Jenkinson, 1981 cited by Mills *et al.* 2005) resulting in changes in the availability of nutrients in the soil (Mills *et al.* 2005).

Mycorrhizal fungi are ubiquitous in all soils and forms a symbiotic relationship with the roots of the majority of plant species (*more details in section 1.2.1*) (Van Der Heijden *et al.* 2008).

Increasingly the landscape becomes covered by ephemeral species which eventually die off (Davis, 2007; Weatherall-Thomas, 2009; Van Luijk *et al.* 2013) with no new plant recruitment appearing (Davis, 2007; Van der Vyver *et al.* 2012), resulting in erosion. Van der Putten *et al.* (2007) recognized that degraded environments may lose mycorrhizal dependent plant species in favour of low or non-mycorrhizal dependent species. Mills *et al.* (2007) confirmed that the vegetation is reduced with an increase in umbrella-shaped species such as *P. capensis*, *E. undulatae* and *S. afra,* unpalatable trees such as *Atriplex lindleyi* subsp. *inflate,* herbs such as *Lobelia inflate* and grasses such as *Cynodon dactylon* (Lechmere-Oertel *et al.* 2008; Sigwela *et al.* 2009) resulting in a savanna like ecosystem (Moolman and Cowling, 1994).

Therefore, this environment requires restoration. The Thicket is distinctive; it produces seeds and has an important function in ecosystem services. The Thicket provides high levels of plant biomass in the form of bushclumps creating favourable conditions underneath the canopy resulting in a cooler, and nutrient rich soil environment (Lechmere-Oertel, 2003), which are essential for soil microorganisms and plant development. Powell, (2009) hypothesised that in a highly-degraded thicket environment soil microorganisms are essential and this requires further attention to improve ecological function and stability (Singh, 2015).

Ecosystem rehabilitation is achieved by applying correct management knowledge based on ecological principles (Singh, 2015) and understanding of soil-microbe-plant interactions (Wandeto, 2014; Singh, 2015). This view was further supported by Van der Heijden *et al.* (2008) who recognized the role played by microorganisms in plant production especially in degraded areas. The symbiotic mycorrhizal fungi supply limiting nutrients, such as phosphorous (P), nitrogen (N), sulphur (S) and iron (Fe) to their host plants stimulating plant productivity. Singh, (2015) acknowledged that the role played by different groups of microorganisms as chief

ecological engineers assists in resolving environmental problems. It is therefore important to harness these microbial tools in order to restore the degraded thicket environments.

The involvement of microorganisms in restoration of the Albany Thicket in the Eastern Cape has not been well documented. A study by Fabricius (1997), in the Xeric Succulent Thicket, on the influence of domestic herbivores on species richness and floristics of bush clumps examined arbuscular mycorrhizal (AM) spore density in soil. Soil samples were collected at 10 cm depth from microsites of 100 m^2 in the bush clumps. Mycorrhizal spores were separated using standard wet sieving and decanting methods and spores were counted under a stereomicroscope. Spore numbers did not differ inside and outside the bush clumps when undisturbed, but due to animal grazing inside the bush clumps, the mycorrhizal spores were more abundant in soils outside the bush clumps which also had higher organic matter content. Seedling germination of *P. afra* outside the bush clumps was up to eight *P. afra* seedlings/100 m^2 while no seedlings of any species inside the bush clump was recorded, possibly due to reduced mycorrhizal populations as *P. afra* is dependent on this relationship (Fulmaka, 2015). Ozaki *et al.* (2004) showed that soil organic matter content correlated with increased AM spore numbers and percentage root colonization which promoted plant growth. These results emphasised the relevance of mycorrhizal fungi for ecological restoration effort and improved understanding of plant functions (Birhane *et al.* 2015). Wandeto, (2014) highlighted the importance of understanding the dynamics of mycorrhizal symbiosis along the disturbance gradient of planned restoration ecosystems as being essential in improving restoration strategies.

Direct inoculation of microorganisms in degraded areas has been recognized as a requirement for restoration and successful re-establishment of seedlings (Koricheva *et al.* 2009). Re-establishment of these important relationships increases seedling tolerance to transplant shock maintaining growth in the long term. Van der Heijden *et al.* (2008) stated that at least 2 000 plants species require microbial symbionts to persist in natural environments especially when nutrients are limited.

Schizachyrium scoparium seedlings were planted in a greenhouse in soil collected from a site with *Smilax rotundifolia* thickets to investigate the role of microbial community. Soils were sterilised and inoculated with arbuscular mycorrhizal (AM) and bacterial fractions. This has resulted in

increased growth of *S. scoparium* seedlings (Rachel, 2009). A study by Birhane *et al.* (2013) showed that *Acacia etbaica* and *Boswellia papyrifera,* collected during a drought period from dry deciduous woodlands, and inoculated with *Glomus* species (an AM fungus) resulted in significantly increased biomass. Shoot biomass of *A. etbaica* seedlings was increased by 24% and in *B. papyrifera* seedlings the root biomass was increased by up to 78%. In a follow up study by Birhane *et al.* (2015) increased seedling biomass was attributed to enhanced absorption of water and nutrients as a result of the mycorrhizal relationship with *A. etbaica, A. senegal* and *B. papyrifera*. The growth rate of the *Acacia* species showed larger mycorrhizal benefit at high water supply and *Boswellia* species at lower water supply. It is clear that healthy soil abundant with microorganisms is crucial to producing healthy seedlings (Richardson and Simpson, 2011; Wu *et al.* 2014). Given the importance of microbial relationships this study seeks to expand existing knowledge on the role of selected microorganisms in producing seedlings for restoration of degraded mesic thicket in the ATB, in the Eastern Cape.

1.2 SOIL MICROORGANISMS

With an estimated abundance of one million to one billion cells per one gram of topsoil, microorganisms are by far the most abundant and diverse biotic group in the soil ecosystem (Pal and Pandey, 2014) which includes bacteria and mycorrhizal fungi (McCauley *et al.* 2005; Van der Haijden *et al.* 2008; Hazard *et al.* 2013). Van der Heijden *et al.* (2008) estimated that one gram of soil contains approximately 200 m of fungal hyphae. These microorganisms serve as both source and sink of nutrients by maintaining soil structure and unlocking nutrients in the soil (Hart *et al.* 2005). They also play a role in terrestrial biogeochemical cycles by supporting primary production through provision of mineral nutrients to plants (Pal and Pandey, 2014).

1.2.1 Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal (AM) fungi are found in many types of soils in a wide range of ecosystems (Van Der Heijden *et al.* 2008). They have a symbiotic relationship with plant roots known as a mycorrhizal association (Pietikäinen, 2009; Hazard *et al.* 2013). Harrier and Watson, (2004) defined these fungi as being obligate biotrophs which develop a symbiosis with their host plants and cannot complete their lifecycle without a host. They are vital components of almost all terrestrial ecosystems - forming a mutually beneficial relationship with the roots of around 80 to

90% of vascular plants (Harrier and Watson, 2004; Morgan *et al.* 2005; Smith and Read, 2008; Hazard *et al.* 2013). Arbuscular mycorrhizas are the most common type of mycorrhizal relationship (Klironomos, 2003; Brundrett, 2009) particularly in South Africa which has a predominantly AM associated indigenous vegetation (Allsopp and Stock, 1993; Hawley and Dames, 2004). Despite their worldwide distribution, only about 250 species have been described and named. These fungi were placed in their own fungal phylum the Glomeromycota and recent revision of the taxonomy has placed them into two families, *Glomeraceae* based on *Glomus macrocarpum*, and *Claroidoglomeraceae* based on the former *Glomus claroideu* (Schüßler and Walker, 2010).

1.2.2 Role of mycorrhiza associations

Mycorrhizal fungi benefit their host by increasing their access to nutrients, improving water relations, soil structure and providing protection from pathogens (Pal and Pandey, 2014; Clark, 1997). Plants associated with AM fungi are able to access unavailable nutrients in nutrient poor soils (Pal and Pandey, 2014). Nutrients are supplied to the host plant via fungal structures within the root cortical cells (Van der Heijden *et al.* 2008; Wandeto, 2014) in exchange for photosynthetically derived carbon (C) compounds, such as sugars, required by the fungi for growth and energy (Fitter *et al.* 2000; Morgan *et al.* 2005). Fitter *et al.* (2000) recognised that plants provide approximately 5 to 25% of C to the fungus. Associated AM fungi cannot access C from other sources making them obligate biotrophs (Dames and Ridsdale, 2012; Rouphael *et al.* 2015).

Plants associated with AM fungi enables them to access unavailable nutrients in nutrient poor soils (Marschner and Dell, 1994; Pal and Pandey, 2014). The association is recognised by the development of interradical fungal structures, on entering the root fungal hyphae grow between cortical roots cells forming an intercellular hyphal network. Hyphae penetrate cortical root cells and develop specialized finely branched, intracellular tree-like structures known as arbuscules (Pietikäinen, 2009; Smith and Smith, 2011; Wandeto, 2014). Mirzakhani *et al.* (2014) described arbuscules as the exchanging interface for nutrients within the host plant roots. The extraradical phase is composed of spores and mycelium forming an absorptive hyphal network and runner hyphae (Pietikäinen, 2009; Miransari, 2014; Wandeto, 2014). The AM fungal hyphal network has three essential functions which includes the absorption of nutrients beyond the depletion zone surrounding roots in the soil thus increasing absorptive capacity of the roots. Secondly, the hyphae

form a physical bond or bridge between the root-surface and the rhizosphere which includes linking roots of different plants below ground. Thirdly, the extraradical hyphae spread around the root system and perform important ecosystem functions such as increasing the plant's ability to explore the soil for nutrients, especially P (Wandeto, 2014) from up to 10 cm away from the roots (Cavagnora *et al.* 2015).

The role of mycorrhizal fungi in improving mineral nutrition of the plant host has been well studied and is the recognized primary function of the association. Mycorrhizal fungi facilitate plant growth by promoting uptake of macronutrients and micronutrients at low soil mobility (Pal and Pandey, 2014). Marschner and Dell, (1994) and Morgan *et al.* (2005) reported the important role that mycorrhizal roots play in the uptake of P, N, calcium (Ca), and potassium (K) from soil, especially at low fertility levels. It has been estimated that AM fungi contribute approximately 80% of P, 10% of K and 25% of N uptake in plant (Marschner and Dell, 1994).

Nitrogen in the soil is unavailable for plant uptake because it is a component of dead organic matter (OM) (Koller *et al.* 2013). Atmospheric N (N_2) is also unavailable to plants (Maathuis, 2009). The highly efficient extraradical AM hyphal network can mineralize N from OM via various enzymatic activities. AM fungi uses their hyphal network to take up N in a form of inorganic ammonium (NH_4^+) and nitrate (NO_3^-) as well as organic amino acids (Cavagnaro *et al.* 2015). The network is therefore important in the acquisition and translocation of N to host plants in exchange for C rich photosynthesis (Koller *et al.* 2013). N is an essential macronutrient for plants required for protein synthesis and the formation of chlorophyll required for photosynthesis (Dames and Ridsdale, 2012). Mycorrhizal fungi enhance N absorption promoting plant growth rate and plant functioning. Mycorrhizal fungi however lack the ability to fix atmospheric N (Mikola, 1986).

Phosphorus is immobile in the soil and forms insoluble complexes with Fe, Al and Ca cations (Dames and Ridsdale, 2012; Urcoviche *et al.* 2015). Consequently, the movement of phosphate ions becomes slow in soils reducing the amount of P available for plant root absorption (Cavagnaro *et al.* 2015). Urcoviche *et al.* (2015) recognized that AM fungal hyphae are about 10 times thinner than plant roots and are able to exploit the soil environment more effectively in search of P. The hyphae release organic acids which solubilize complex inorganic P for further translocation to the plant. In addition to this, they can reach and capture P beyond the depletion zone formed by the

roots system in surrounding soil and make it available to plants to promote development (Yoneyama, 2007). P is essential for the formation of cell membranes, nuclei acids and energy rich molecules such as adenosine triphosphate (ATP) (Morgan et *al.* 2005).

Arbuscular mycorrhizal fungi enhance nutrient uptake from different soils, for example in soils with high and low pH (Clark and Zeto, 2000). Weathering and leaching are the major causes of Ca deficiency in acid soils (Maathuis, 2009) resulting in concentrations of cationic bases (K, Ca, Mg, Na) limiting plant growth (Clark and Zeto, 2000). The AM association assists plants in absorbing more of these nutrients under acidic environmental conditions, thus alleviating deficiencies. Absorption capacity may vary for example K acquisition compared to Ca and Mg was especially enhanced in AM colonised switch grass grown in soils having a pH of 4.0 (Clark and Zeto, 2000).

Infiltration and water storage capacity of the soil is crucial where uneven distribution of precipitation is the limiting factor for plant production. The structure of soil controls fluxes of water and nutrients (Rillig and Mummey, 2002). Colonization by AM fungi may improve the water relations of plants (Baum *et al.* 2015). The extensive hyphal network grows into the soil medium absorbing water and nutrients. This is beneficial for host plants under water stress, because enhanced water and nutrients uptake increase plant growth (Miransari, 2014). Improved water uptake by the extraradical phase of the mycorrhizal root system results from direct mycorrhizal effects (Wu *et al.* 2008).

Arbuscular mycorrhizal fungi are important in macro-aggregate formation and long-term soil stabilization (Pal and Pandey, 2014). Soil aggregation is defined as when soil particles stick together allowing for favourable environmental conditions (Leifheit *et al.* 2014). Arbuscular mycorrhizal fungi bind these soil particles with hyphae to build a macroporous structure of soil to allow penetration of water and air (Soka and Ritchie, 2014). In addition to this, the production of glomalin, a glycoprotein has the ability to bind soil particles (Pal and Pandey, 2014). These attributes as shown by Duchicela *et al.* (2012) in semiarid shrublands contributed to soil stability, subsequently resulting in improved soil water movement and holding capacity which promoted plant growth and prevented erosion. Leifheit *et al.* (2014) discovered that AM fungal hyphae stabilizes macroaggregates (>2.5 cm) and may stabilize soils up to a period of five months. However, the presence of AM fungi in soil ensures that glomalin is continually released into the soil environmental.

Arbuscular mycorrhizal fungi play an important role in protecting plants from fungal pathogens and disease. This is based on root morphology changes (Baum *et al.* 2015). When AM fungi colonize the root system of the host plant substantial morphological changes occur. These include increased root branching, and cell wall lignifications which aids in bioprotection of the plant against penetration by pathogens (Johnson *et al.* 1997). Furthermore, biochemical analyses of different plant defence-related enzymes showed a local induction of mycorrhiza-related new isoforms. For example, superoxide dismutase is an enzyme that is involved in cell protection against oxidative stress (Morgan *et al.* 2005).

1.3 MYCORRHIZAL DYNAMICS

The distribution and composition of AM fungal taxa in agroecosystems can be explained by many factors. Bashan *et al.* (2007) observed that soil physical and chemical characteristics such as soil organic matter and nutrient content, in particular the availability of P can affect the mycorrhizal population in the soil. AM fungal spores can be extracted from soil with wide pH ranges. a wide range of soil pH ranges. Several AM fungal species can be isolated from either acid or alkaline soils, while others have been isolated from both. Most AM fungi appear to be adapted to soil pH conditions close to those from which they are extracted for example soil pH 2.7 to 9.2 (Clark, 1997 cited Siqueira *et al.* 1984). This indicates that when investigating restoration of acidic soils, AM fungal isolates extracted from soils of similar pH should be considered.

Nutrient uptake by mycorrhizal plants is dependent on the development of both internal and external mycorrhizal structures. Colonisation of roots is reliant on the presence of viable AM fungal spores and other infective propagules. These propagules are stimulated to germinate in response to various host plant signals such as components which comprises of the root exudates. The presence of these propagules in soil form the asymbiotic phase of the life cycle (Figure 1.1). Germinated spores found in moist soil environments in the absences of host plants will cease germination (Giovannetti and Sbrana, 1998). After several unsuccessful spore germination attempts, C reserves will be depleted resulting in loss of spore viability (Giovannetti and Sbrana, 1998). In the presence of a host plant, the germinating spore will develop a branched hyphal system and grow towards the root (presymbiotic phase) and attach to the plant root initiating root colonization (symbiotic phase) (Giovannetti, 2000) (Figure 1.1). Although regarded as non-specific in their host preference different densities of colonization occur. Plants with coarse

unbranched roots and short root hairs are more dependent and respond quickly to mycorrhizal colonisation than plants with a more fibrous root system. Birhane *et al.* (2015) reported this when comparing plants with fine branched roots having numerous long root hairs and more sparsely rooted plants. Arbuscular mycorrhizal fungi also stimulate root branching patterns and alter architecture of host plant roots (Hetrick, 1991). Arbuscular mycorrhizal fungal colonization of adventitious roots resulted in larger diameters and lower specific root lengths (Harrier and Watson, 2004). This trait was recognized in plant species which were grown in soil low in P and with reduced ability to absorb P from the surrounding soil environment (Hetrick, 1991; Wandeto, 2014).

Smith *et al.* (2009, 2011) and Klironomos (2003) observed that rate of colonization or aggressiveness is not the same in different host plants, even when colonized by the same AM fungal species.This also held true for naturally co-occurring plants and fungi in the same site (Klironomos, 2003).

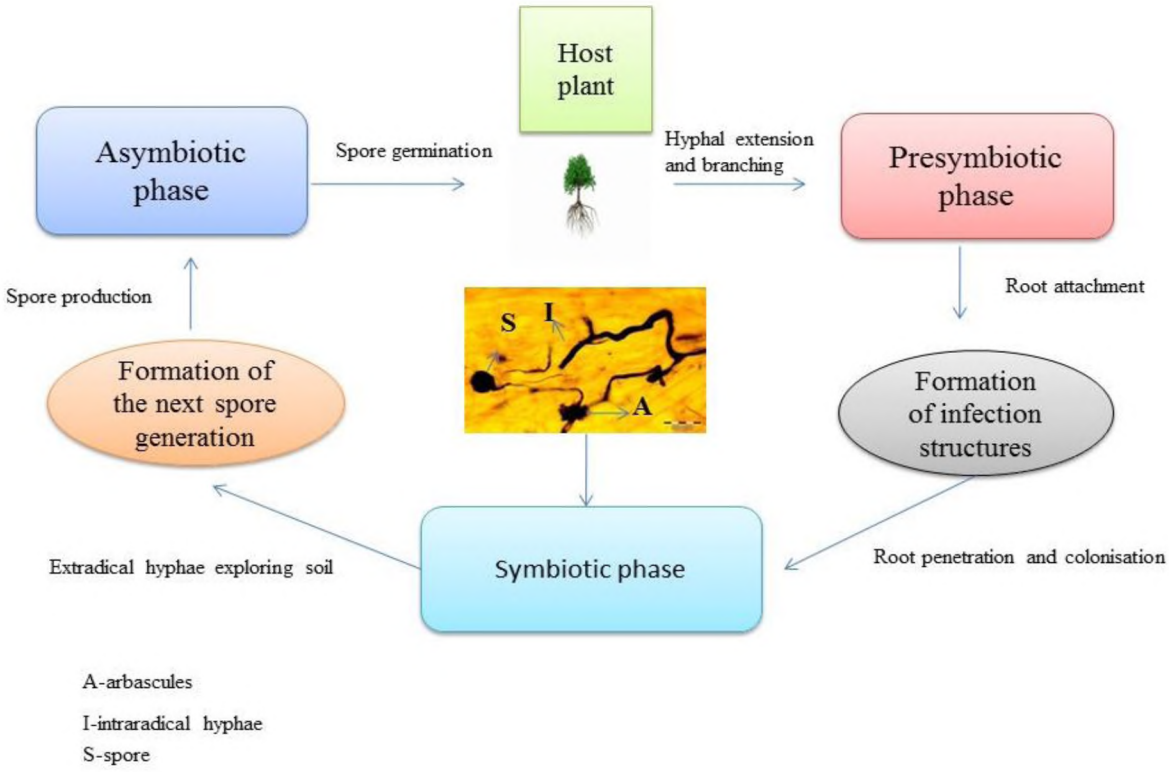

Figure 1.1: The life cycle of arbuscular mycorrhizal fungi

1.4 RHIZOBACTERIA

Bacteria are microscopic soil microorganisms found in a variety of environments (Hoorman and Islam, 2010), they propagate quickly and exist in large quantities (McCauley *et al.* 2005). The plant growth promoting rhizobacteria (PGPR) are a functional group of bacteria which are found in soil adjacent to plant roots (rhizosphere) and are associated with almost all plant species (Bashan and de-Bashan, 2005). Barea *et al.* (2005) and Miransari, (2010) defined the 'rhizosphere as soil which is influenced by the roots affecting microbial activities'. The rhizoplane is the root surface, with strong adhering soil particles. The PGPR exert an influence on plants that can benefit growth and plant development (Nadeem *et al.* 2014; Miransari, 2010). McCauley *et al.* (2005) reported that legume species with root-nodulating bacteria such as rhizobia provide about 90% of N to the host plants through atmospheric N fixation. As a result, this improves the supply of a renewable source of N required to sustain plant growth and in return the plants supplies simple sugars to the bacteria (Hoorman, 2011). Rhizobia are regarded as PGPR. Functionally PGPR provide many advantageous to plant growth.

1.4.1 Influence of PGPR on plants

Plant growth promoting rhizobacteria can benefit plant growth through different mechanisms either directly or indirectly (Mayak *et al.* 1999; Bashan and de-Bashan, 2005; Beneduze *et al.* 2012; Glick and Bashan, 1997). This stimulation is due to production of bio-active compounds (Singh, 2015). Directly, they affect plant metabolism by providing substances that are usually in short supply such as N, Fe and P. Plant growth promoting rhizobacteria can also produce hormones, such as auxins, gibberellins and cytokinins required for plant development (Mayak *et al.* 2004a, b; Bashan and de-Bashan, 2005). Indirectly PGPR act as biocontrol agents preventing damage caused by phytopathogenic microorganisms (Bashan and de-Bashan, 2005).

Direct stimulation provides plants with available N, phytohormones, Fe^{+3} which have been sequestered by bacterial siderophores and soluble P (Mayak *et al.* 1999; Mayak *et al.* 2004a; Bashan and de-Bashan, 2005). N-fixing bacteria known as rhizobia belong to the genera *Rhizobium, Sinorhizobium, Bradyrhizobium, Mesorhizobium,* and *Azorhizobium* and interact with roots of leguminous plants. This symbiotic relationship forms N fixing root nodules which regulate plant productivity because plants cannot fix atmospheric N (Van der Heijden *et al.* 2008). Nitrogen

fixing bacteria convert N from the atmosphere into ammonium (NH_4), a form of N form which is available for plant use (Hoorman, 2011; Miransari, 2010). The contribution of nitrogen-fixation to plant N can be as much as 20% as has been shown in tropical savanna and tropical forests which are dominated by legumes (Van der Heijden *et al.* 2008; Hoorman, 2011). Many soil bacteria are free living N fixers these include the genera *Enterobacter, Azobacter,* and *Azosprillum* (Glick, 2012)

Phytohormones such as indole-3-acetic acid (IAA), gibberellins and cytokinins are produced by plants and PGPR (Glick and Bashan, 1997; Ma *et al.* 2011; Dames and Ridsdale, 2012). Indole-3-acetic acid is produced in the shoots of the plant and transported basipetally to the root tips where it is associated with cell elongation and division, contributing to plant growth and defence (Ma *et al.*2011). Dames and Ridsdale, (2012) concluded that IAA produced by PGPR have beneficial influences on plant growth by altering root development. Numerous bacteria such as *Azotobacter* and *Pseudomonas* spp. have been recognized as producers of phytohormones such as IAA (Kurepin *et al.* 2015).

Gibberellins are diterpenoid compounds which regulating plant growth (Miransari and Smith, 2014). They are involved in developmental and physiological processes such as seed germination, seedling emergence, stem, and leaf growth (Dames and Ridsdale, 2012; Miransari and Smith, 2014), breaking of seed dormancy and embryo expansion (Miransari and Smith, 2014). Kurepin *et al.* (2015) confirmed that several bacterial species such as *Bacillus pumilus. B. licheniformis*, *Acetobacter* sp., other *Bacillus* sp., *Azospirillium* sp., *Herbaspirillum seropedicae* and *Rhizobium* can produce gibberellins.

Cytokinins (CKs) are groups of natural compounds produced by the plants to control cell division and enlargement (Dames and Ridsdale, 2012) as well as differentiation processes in the meristematic tissues of plants (Miransari and Smith, 2014). Cytokinins are also required in physiological processes such as root development, stem initiation and leaf expansion (Miransari and Smith, 2014). *Rhizobium leguminosarum* has been shown to produce various CKs (Kurepin *et al.* 2015). Miransari and Smith, (2014) recognized their efficacy in nodule formation during the establishment of the N-fixing symbiosis.

Bashan and de-Bashan (2005) defined Fe^{+3} as an essential microelement for microbial growth and is unavailable in the soil because it is present in a hard-to-solubilize mineral form. However numerous microorganisms secrete binding molecules called siderophores (Morgan *et al.* 2005). This low-molecular-weight molecule binds iron in the rhizosphere (Rasouli-Sadaghiani *et al.* 2014). Beneduze *et al.* (2012) described siderophores as small peptidic molecules with few side chains and functional groups with high affinity ligands to bind Fe. Ma *et al.* (2011) reported that siderophores protect plants from microbial pathogens; they chelate Fe in the rhizosphere and therefore reduce its availability to pathogens. The plant benefits in two ways; PGPR sequestered Fe and make it unavailable for plant pathogens and also improve Fe nutrition which results in an increased plant growth (Morgan *et al.* 2005). Plants bind and release Fe from bacterial iron siderophore complexes increasing its availability for plants uptake (Bashan and de-Bashan 2005; Ma *et al.* 2011). Plants also use this iron for respiration and photosynthetic metabolic processes required for plant functioning (Rout and Sahoo, 2015).

Indirect stimulation by the bacteria includes the prevention of phytopathogens which inhibit the development of plants (Glick and Bashan, 1997; Mayak *et al.* 2004a, b). According to Glick and Bashan, (1997) PGPR produce six classes of antibiotics which play a role in reducing root diseases. They include diffusible antibiotics such as phenazines, phloroglucinols, pyoluteorin, pyrrolnitrin, cyclic lipopeptides and volatile hydrogen cyanide (HCN) (Hassan *et al.* 2011). Nihorimbere *et al.* (2010) showed that *B. subtilis* produced volatile compounds and antibiotics which resulted in an increase in plant growth due to their negative effect on plant pathogens. These bacteria also induce a systemic response in plants thus priming their defence mechanisms which act against plant pathogens.

1.4.2 Plant growth promoting rhizobacteria and mycorrhizal interactions

Smith *et al.* (2011) articulated that phosphate (PO_4^{3-}) makes up about 0.2% of dry weight in the soil; the majority is in the complex inorganic form and is unavailable to plant growth. Bacteria such as phosphate soluble bacteria (PSB) which are regarded as PGPR support the arbuscular mycorrhizal symbiosis. Phosphate soluble bacteria increase the bioavailable P in areas with low P through their ability to solubilize complexed inorganic forms of P (Barea, 1987 cited in Barea *et al.* 2005). They also release phosphate ions from unavailable insoluble inorganic and organic P compounds in the soil through the production of organic acids and phosphatase enzymes (Barea *et*

al. 2005). Therefore, this enables the extraradical AM fungal hyphae to increase the uptake of soil P for translocation to the plant.

Barea *et al.* (2005) and Dames and Ridsdale, (2012) described the mycorrhizosphere as the area under the influence of the AM fungal hyphal network which interacts with bacterial communities. Some of these bacteria have been reported to increase the extent of mycorrhizal colonization assisting as mycorrhizal helper bacteria (MHB). These MHB have the ability to produce enzymes required to soften root cell walls resulting in easier penetration of AM fungi into root cells (Bharadwaj *et al.* 2012). Mycorrhizal helper bacteria may also assist with initial AM spore germination through the supply of nutrients or the reduction of inhibitory compounds in the soil environments (Dames and Ridsdale, 2012). These many interacting factors indicate that PGPR and AM fungi can work in synergy when coinoculated on host plants (Bharadwaj *et al.* 2012; Miransari, 2010).

1.5 MOTIVATION

Mesic thicket is one of the thicket vegetation type found in the ATB. Most of mesic thicket vegetation is composed of succulent woodland and two sub-formations that include taller sub-succulent woodland and low succulent scrub (Vlok *et al.* 2003). This vegetation supports a high diversity of animal and plant species, including high densities of indigenous herbivores, ranging from duiker to elephants (Palmer, 2004). About 46% of the thicket has been heavily degraded and 36% moderately degraded by goat browsing (Lloyd *et al.* 2002). Degradation of the Thicket affects the biological components of the soil which play a vital role in plant growth and also in the soil ecosystem. Globally, restoration of degraded thicket environments using microorganisms is not well documented. In the mesic form of the ATB in the Eastern Cape Province, South Africa no detailed studies have been done on the role of microorganisms on thicket restoration. It is easy to see the appeal of microorganisms as tools for ecological studies. They are diverse and play an important part in ecological and ecosystem services (Singh, 2015). The influence of AM fungal associations with PGPR in promoting plant growth and mycorrhizal development has been well studied (reviewed in Dames and Ridsdale, 2012). So, it is very important to use these microorganisms to restore degraded thicket environments.

1.6 STUDY OBJECTIVE

The main objective of the study was to assess the potential of microorganisms for the improved biomass production of selected woody and succulent seedlings used in mesic thicket restoration.

Hypothesis:

1. Arbuscular mycorrhizal (AM) fungi can improve growth of seedlings.

2. *Enterobacter* sp. can improve growth rate of seedlings.

3. Synergistically AM fungi and *Enterobacter* sp. can improve growth of seedlings.

The specific aims and research questions of the study were to:

1. Assess mycorrhizal status of the soil

Research questions

- How many AM spores were found in the soil?

2. Determine mycorrhizal infectivity

Research question

- Can Thicket soil containing AM propagules colonize roots of a standard host plants?

3. Test the efficacy of microbial inoculation to improve plant growth of thicket seedlings

Research question

- Does microbial inoculation have an effect on the relative shoot growth and biomass production of *S. myrtina* and *M. aethiopicum* seedlings over time?
- Does microbial inoculation have an effect on biomass of *A. ferox* seedlings?

4. Assess mycorrhizal colonization of seedling roots

Research questions

- What is the percentage of mycorrhizal colonization in the roots of the seedlings?
- Does the PGPR selected aid as a MHB?

5. Determine the relative amount of nutrients available in the soil

Research questions

- What are the concentrations of nutrients available in the soil in the degraded state?
- Which nutrients were utilized/ increased by selected host seedlings?

6. Observe role played by inoculants in improving soil water holding capacity

Research questions

- What is the water holding capacity of the soil prior to plant establishment and growth?
- After propagation, which treatments affected soil water holding capacity?

CHAPTER TWO: ASSESSMENT OF MYCORRHIZAL STATUS OF ALBANY THICKET SOIL

2.1 INTRODUCTION

Mycorrhizal fungi are ubiquitous in all soils and form a symbiotic relationship with the roots of most plant species (Van Der Heijden *et al.* 2008). As critical soil microorganisms, examination of the impact of land use changes on mycorrhizal fungal communities is important (Chelius and Triplett, 1999; Trejo *et al.* 2016) when determining measures for biodiversity conservation (Trejo *et al.* 2015). Arbuscular mycorrhizal (AM) fungi produce different propagules, such as spores, hyphae or colonised root fragments which can interact with host plant roots (Duponnois *et al.* 2001; Schalamuk and Cabello, 2010). Assessment of these propagules provides information about AM fungal activity in the soil ecosystem indicating the potential for symbiosis development (Trejo *et al.* 2015).

The ability of the fungi determines mycorrhizal activity of the soil by colonizing the root system of the host plant and this is referred to as the mycorrhizal infective potential (MIP) (Smith and Smith, 2011). This is determined by planting a selected host plant with high mycotrophic tolerance (Duponnois *et al.* 2001) such as *Sorghum* in the soil under investigation. For example, in undisturbed soil ecosystems, high mycorrhizal colonization is expected due to the presence of the extra-radical hyphae network resulting from adjacent colonized plants and the presence of viable mycorrhizal spores (Schalamuk and Cabello, 2010). In disturbed soils AM fungal propagules are reduced (Schalamuk and Cabello, 2010) and extraradical hyphae networks are broken which subsequently affects the mycorrhizal population and the MIP.

The impact that disturbance of natural ecosystems has on mycorrhizal activity is not well understood hence the need for further investigation (Trejo *et al.* 2015) especially in Thicket biome of South Africa.

The aim of this study was to determine the mycorrhizal status and potential of soils collected from a degraded and an intact ATB site.

2.2 STUDY AREA

Bathurst is a small agricultural town located 15 km inland from the Indian Ocean coastline of the Eastern Cape Province, South Africa (Puttick *et al.* 2011; Stickler and Shackleton, 2015). Fairy Glen Private Nature Reserve (FGPNR) lies at 33°32′ S and 26°49′ E and is located 2.5 km south of Bathurst and 12 km inland from the coastal town of Port Alfred (Figure 2.1). Before the early 1950's FGPNR was used to farm chicory, but due to change in land use and ownership there was an increase of thicket clearing for cattle grazing (Lunderstedt, 2012). Since the early 1980's land ownership continued to change and the fields were abandoned and left to regenerate. However, currently these old lands are represented by 15 ha of the 200 ha reserve that has been replaced by thicket species and a decline in vegetation cover is still evident (Lunderstedt, 2012).

2.2.1 Climate

The altitude of FGPNR extends from the river at 10 m to 160-180 m above sea level. It receives a mean annual rainfall of 717 mm that has bimodal peaks in October/November and in February/March as shown (SAWS 2005 cited by Stickler and Shackleton, 2015) (Figure 2.2). The area has a mild subtropical climate, with winter (June to September) temperatures ranging from 10°C to 21°C and summer temperatures ranging from 17 and 26°C (Puttick *et al.* 2011; Stickler and Shackleton, 2015).

2.2.2 Vegetation

FGPNR vegetation is classified under the Albany Thicket vegetation type (Hoare *et al.* 2006). It has a wide range of growth forms and a high diversity of plant species. It is divided into two vegetation units, Kowie Thicket, and the Albany Coastal Belt (Hoare *et al.* 2006). In the Kowie Thicket closed canopy thickets are dominated by evergreen trees, with a dense understory of thorny shrubs and a poorly developed herbaceous layer (Hoare *et al.* 2006). The Albany Coastal Belt has short grasslands with scattered bush clumps and trees such as *Vachellia karroo* and forming a savanna type vegetation. The Albany Coastal belt vegetation has arisen from the original thicket vegetation which was cleared by herbivores (Puttick *et al.* 2011).

2.2.3 Geology

Bathurst formation is give rise to different types of soil, with marine invertebrate fossils traces found. One of the most common soils is derived from conglomerate limestones, calcareous sandstones (often indurated and crystalline) and conglomerate (Johnson & Le Roux 1994).

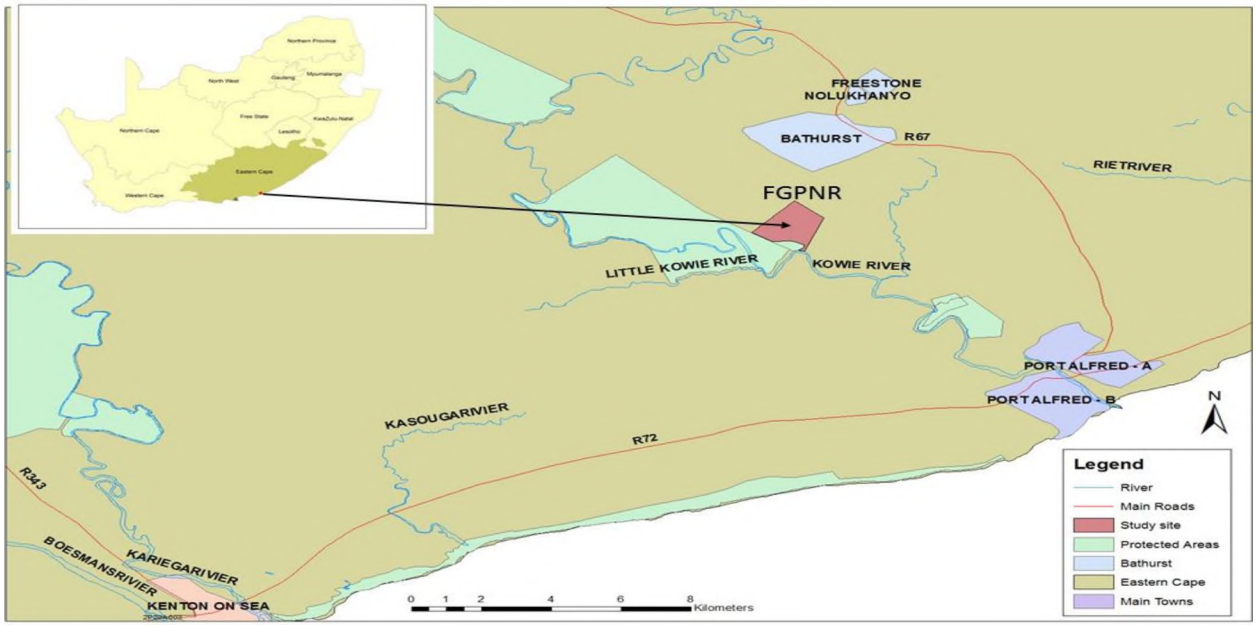

Figure 2.1: Map of the study area in relation to Bathurst and Port Alfred

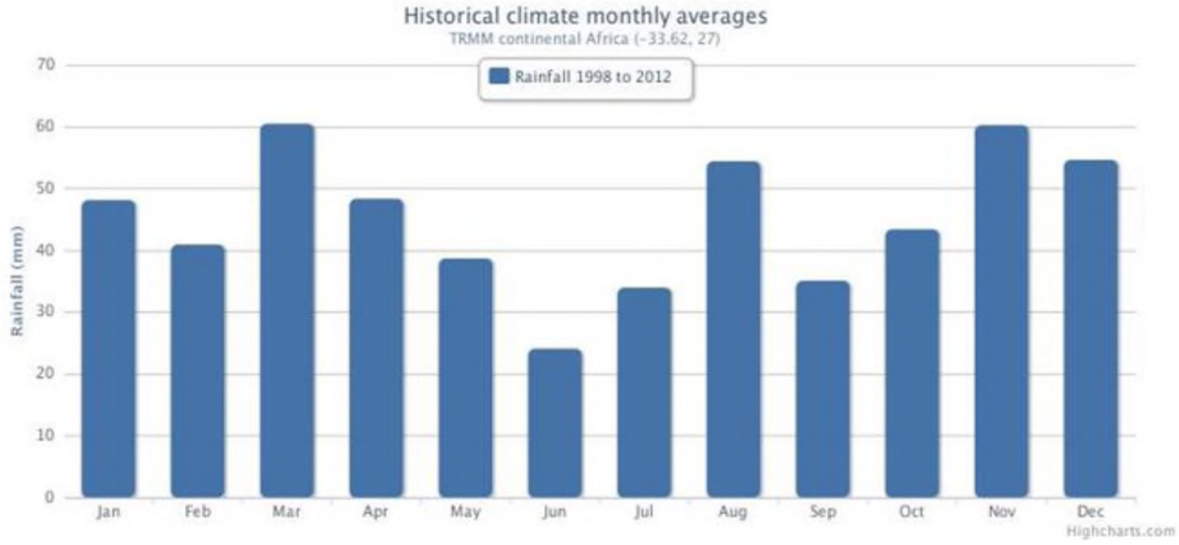

Figure 2.2: The average rainfall recorded at Bathurst, from 1998 to 2012 Eastern Cape, South Africa (www.cip.csag.uct.ac.za).

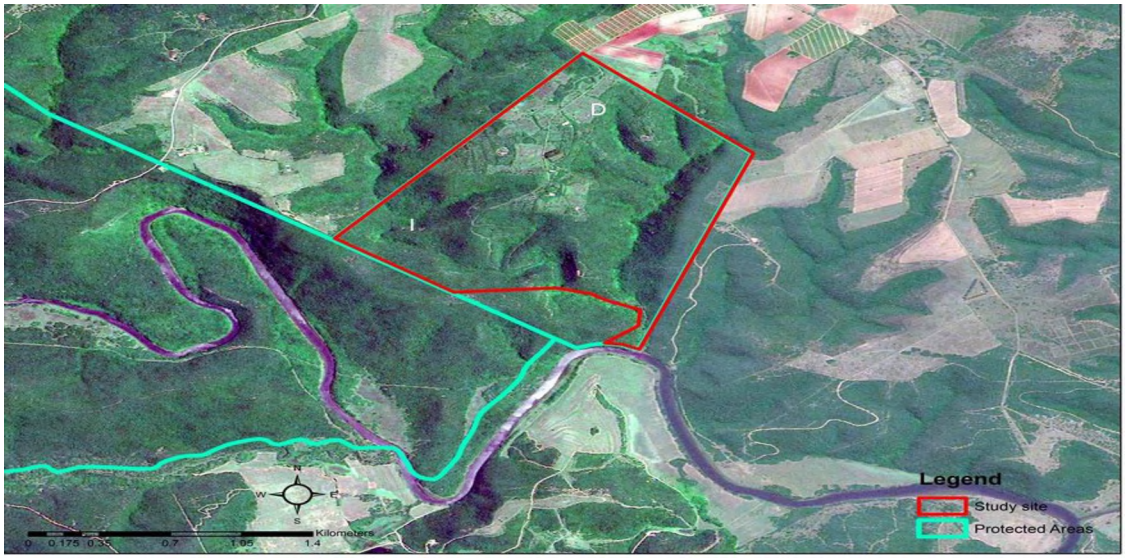

Figure 2.3: Google Earth image showing the vegetation cover of the study area, I-intact thicket, and D-degraded thicket site.

2.3 MATERIALS AND METHODS

2.3.1 Soil collection and preparation

Herbaceous vegetation and grass were carefully removed, from selected degraded and intact thicket sites at the FGPNR (Figure 2.3). The degraded thicket site had sparse vegetation, the soil was dry with a dark brown colour and the ground vegetation was mostly grass. The intact thicket site had dense vegetation, the soil was moist with a light brown colour and the cover was mostly tree species. At the intact site, approximately 10 kg of topsoil (up to a depth of 15-20 cm) was collected from a 1 m^2 area while approximately 600 kg was collected from a 2.7 m^2 area from the degraded site. After collection, soil was transported to Rhodes University, South Africa and sifted through a 2 mm mesh sieve to remove large debris and stones.

2.2.2 Nutrient analysis

Soil sub samples of approximately 500 g from the intact and degraded sites were sent to Eco-Analytica Laboratories, Potchefstroom, South Africa, for nutrient analysis. The nutrient analysis included quantification of Calcium (Ca), Magnesium (Mg), Potassium (K), Sodium (Na) and Phosphorus (P), electrical conductivity (EC), pH (H_2O), pH (KCl) and the percentage of Carbon (C) and Nitrogen (N).

2.2.3 Arbuscular mycorrhizal (AM) fungal spore enumeration

Ten soil subsamples from each of the intact and degraded thicket sites were used for enumeration of AM fungal spores. For each sample 100 g was placed in a 500 ml beaker filled with 200 ml water. The solution was agitated for 5 min and allowed to settle for 2 min. The supernatant was sieved through a nest of soil sieves having mesh sizes of 425 µm, 250 µm, 125 µm and 45 µm. The wash and decanting steps were repeated three times. The debris from the 425 µm sieve was carefully discarded as this mainly contained organic matter. The debris from the remaining sieves was washed into a 50 ml centrifuge tube with tap water and centrifuged at 3500 rpm for 5 min. After centrifugation the supernatant was discarded. The pellet was resuspended in a 60% sucrose solution and then centrifuged for a further 5 min (Heraeus Megafuge 1.OR). After sucrose centrifugation the supernatant was decanted into the 45 µm sieve and rinsed with water to remove the sucrose solution. A 9 cm labelled and gridded filter paper disc (Whatman # 1) was placed in a Buchner funnel attached to a water vacuum. The supernatant was decanted onto the filter paper, rinsed with water and filtered to remove moisture. The filter paper was transferred to the lid of a clean petri dish to quantify the AM fungal spores extracted. Spores were examined and counted using a dissecting microscope (Leica S4E L.2). Counts represented the number of spores per 100 g of soil (Smith and Dickson, 1997; Schenck, 1982).

2.3.4 Infectivity test

Twenty plastic pots (8 cm) were rinsed in a 3.5% sodium hypochloride bleach solution and allowed to dry. Soil from the intact and degraded site was used to fill 10 pots each. A standard host plant, *Sorghum bicolor* (L.) Moench, was selected because it is highly mycotrophic to test for mycorrhizal infectivity. Fungicide free seeds of *Sorghum bicolor* were obtained from Professor MacLaren, University of the Free State, Bloemfontein, South Africa. The seeds were surface sterilized for 10 min in a 3.5% sodium hypochloride solution followed by a water rinse. Two seeds were placed in each pot. Pots were placed in the Mycorrhizal Research tunnel. Temperatures in the tunnel ranged from 20°C to 35°C (min/ max temperatures) with natural light. Pots were automatically irrigated daily with filtered, UV treated water. Once germinated, seedlings were thinned to one per pot for a growth period of six weeks. After this period, the plants were removed from each pot, the roots were gently washed under running water to remove the soil in preparation for staining.

2.3.5 Assessing mycorrhizal root colonization

Roots from the seedlings were cut into sections of 1-3 cm after harvesting. The roots were cleared with a solution of 5% potassium hydroxide (KOH) for 30 min in a water bath at 90°C to remove the cytoplasm and coloured material from the plant cells. The KOH solution was discarded and roots were rinsed with distilled water. Roots were bleached in an alkaline hydrogen peroxide (H_2O_2) solution for 30 min. The bleaching solution was discarded and roots were rinsed with distilled water. Roots were covered with 0.1 M hydrochloric acid (HCl) and left overnight at room temperature to acidify roots to ensure the binding of the stain. The HCl solution was discarded. Roots were stained using a lactoglycerol solution which contained 0.05% Trypan Blue stain for 30 min in a water bath (90°C). After 30 min the stain solution was discarded and the roots were covered with lactoglycerol solution (without Trypan Blue) for 24 hr to remove unbound stain (Smith and Dickson, 1997).

Root sections were placed onto microscope slides in lactoglycerol and covered with cover slips. Roots were gently flattened by applying pressure to the coverslip. Individual fragments were examined using a compound light microscope (Leica CME) at 400 x magnification for the formation of intercellular hyphae which ramify along the root cortex and intracellular branched arbuscules as well as other structures. Colonization was scored based on a modified gridline intersect method (Smith and Dickson, 1997; Schenck, 1982). Percentage colonization was calculated using the following equation.

$$\text{Percentage colonization} = \frac{\text{No. of fields of views with mycorrhizal structures}}{\text{Total fields of view}} \times 100$$

(Smith and Dickson, 1997)

Images of colonized roots were captured using Olympus BX40 camera on analysis document software with camera attachment SC30.

2.4 STATISTIC ANALYSIS

Spore counts and percentage root colonization data was averaged per site. An in-dependent t-test was used to compare the number of spores and the mycorrhizal colonization percentage from the degraded and intact site. Comparison of mean effects was based on least significant difference $p < 0.05$ using STATISTICA 13.0.

2.5 RESULTS

All measured nutrients (expect P) were higher in soil from the intact site (Table 2.1). The pH(H_2O) approached neutral while pH(KCl) was more acidic. Available P was undetected in both soils while Ca, Na and C% levels were more than double in intact soil resulting in higher. Nitrogen was not detected in degraded soil EC (Table 2.1 and Table 2.2.).

Table 2.1: Soil nutrient analysis from the intact and degraded site

Nutrient status	Units	Intact site	Degraded site
Ca	mg/kg	2711	1173.5
Mg	mg/kg	334	208.5
K	mg/kg	375.5	246.5
Na	mg/kg	39.5	17.5
P	mg/kg	0	0
pH(H_2O)		7.06	6.83
pH(KCl)		6.35	5.95
EC	Ms/m	129	73
N	%	0.11	0
C	%	5.05	2.72

Table 2.2: Exchangeable cations in soil from the intact and degraded site

Exchangeable cations	Units	Intact site	Degraded site
Ca)	cmol(+)/kg	13.53	5.86
Mg (cmol(+)/kg)	cmol(+)/kg	2.75	1.72
K (cmol(+)/kg)	cmol(+)/kg	0.96	0.63
Na (cmol(+)/kg)	cmol(+)/kg	0.17	0.08
CEC (cmol(+)/kg)	cmol(+)/kg	16.59	9.23
S-value	cmol(+)/kg	17.41	8.28
Base saturation	%	104.94	89.66

Average AM spore counts for the two sites are presented in figure 2.4. Although generally low (less than 1 spore per gram), spore numbers from the intact site was significantly higher when compared to the degraded site.

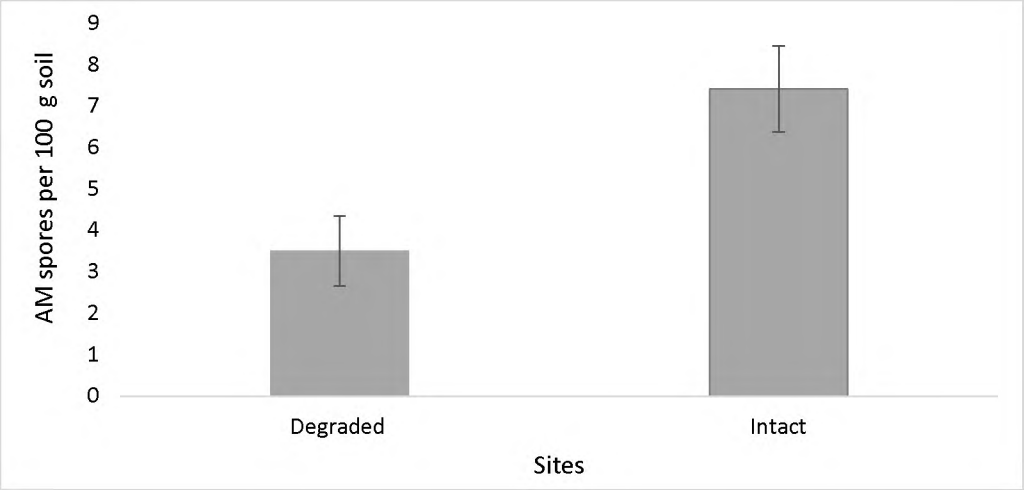

Figure 2.4: AM fungal spore count from the degraded and intact site. Bar represents treatment means ± standard error (t=-2.916, p=0.009)

Characteristic of AM fungal structures were observed in roots of *Sorghum* from soil collected from degraded and intact site (Figure 2.6). Mean colonization from degraded site was 43% and intact site 42.2%. No significant differences were recorded in colonisation (Figure 2.5).

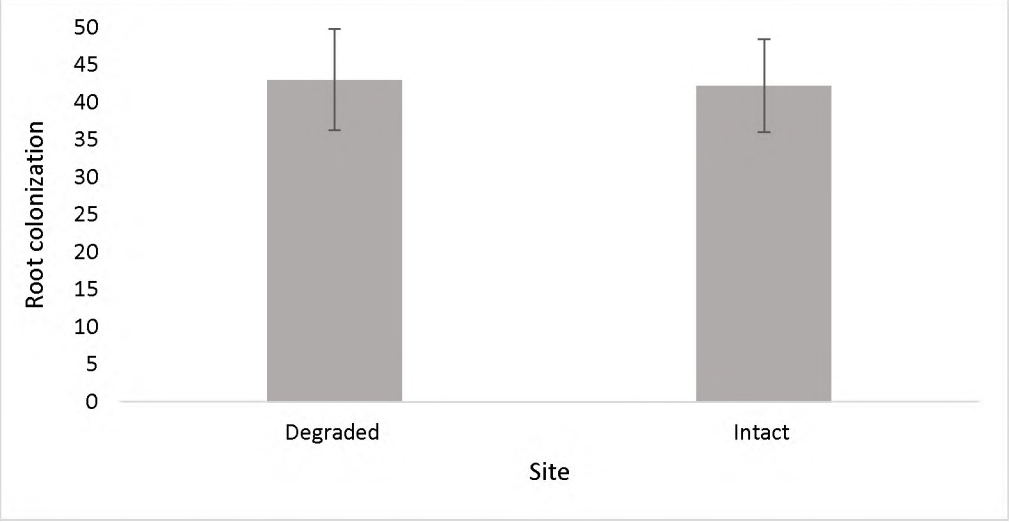

Figure 2.5: Percentage of mycorrhizal colonization in Sorghum roots grown in soil from the degraded and intact site. Bar represents treatment means± standard error (t=0.087, p=0.931).

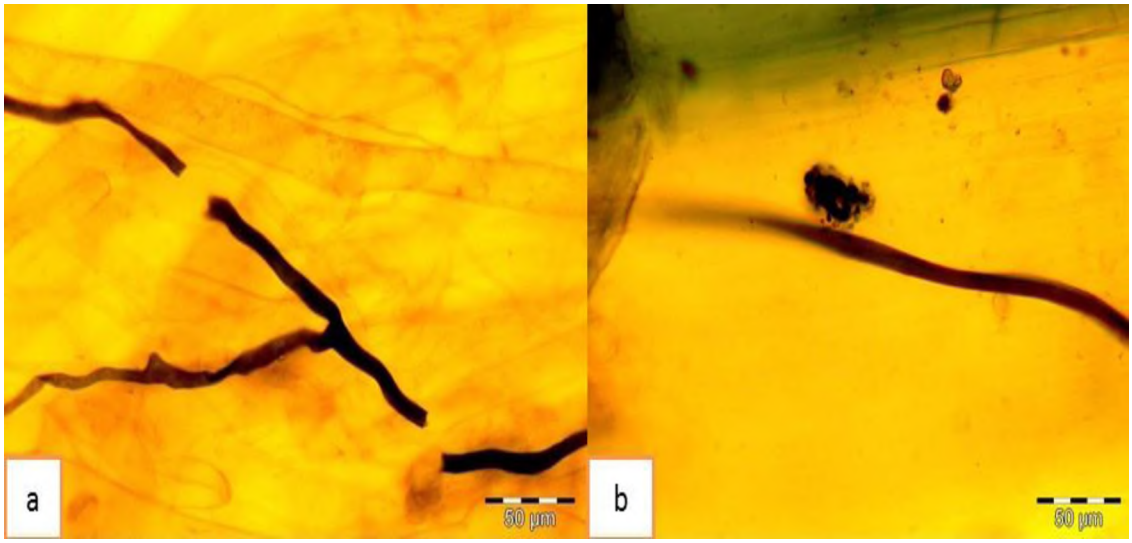

Figure 2.6: Micrographs of AM fungal structures (a) intercellular hyphae (b) intracellular arbuscules.

2.6 DISCUSSION

Cation exchange capacity **(**CEC) is a measure of a soil's capacity to retain exchangeable cations ions such as calcium (Ca), magnesium (Mg), sodium (Na) and potassium (K), which are generally referred to as base cations (CEC) (Kamau, 2000; Hazelton and Murphy, 2007). The CEC indicates to what extent the soil is buffering or protected from changes in pH which influence nutrient availability and soil structure (Hazelton and Murphy, 2007). CEC between 6-12 indicates low soil nutrients and water holding capacity (Kamau, 2000). According to Syibli *et al.* (2013) AM fungal activity is linked to increased CEC as the association aids access to nutrients including cations in the soil. The higher number of spores in the intact site was associated with a moderate increase in CEC of 16.59 cmol(+)/kg (Table 2.2), similar results were reported by Syibli *et al.* (2013) and Miller and Jackson, (1998).

Antoninka *et al.* (2011) reported a strong correlation between % organic C and AM fungal activity. AM fungi participate actively in the carbon cycling but also in soil carbon storage (Cardoso and Kuyper, 2006 and Syibli *et al.* 2013). Arbuscular mycorrhizal fungi produce a glycoprotein, glomalin, which is released into the soil and is a source of C storage (Driver *et al.* 2005). Cardoso and Kuyper, (2006) reported that increasing AM fungi will increase carbon flow into the soil. In this study, it was noted that the intact site had a higher % C content of 5.05 (Table 2.1) as well as

a significantly higher AM fungal spore numbers. Similar results were obtained by Syibli *et al.* (2013). This data suggest that increased C may have a close relationship with AM fungi.

Nitrogen is an essential constituent of proteins and enzymes required for biosynthesis such as chlorophyll responsible for plants' green foliage (Supply, 2004; Maathuis, 2009). Soil organic matter is one of the sources of nitrogen, that is, mineralized by the action of soil microorganisms, including AM fungi (Kamau, 2009). The total nitrogen content in soil from the intact site was low (0.11%) (Table 2.1) and was not detected in the degraded site. Although soil microbial activity was not the focus of this study the lower AM fungal spores number combined with the lower % C indicates poor microbial activity which would result in reduced N available in soil because of a lack of N-fixation (Kamau, 2009). These results support the findings of Satya *et al.* (2014) who also discovered lower number of AM fungal spores in soil with a low N concentration of 0.07%.

Soil P is an important macro nutrient for plant growth being required for the formation of membrane synthesis of ATP (supply energy) and nucleic acids (Maathuis, 2009). Phosphorous in the soil is found in the inorganic available form which is readily used by plants and AM fungi. This element quickly become complexed in the soil to form insoluble inorganic complexes such as phytates and organic matter which cycles P back into the soil through the action of microbial degradation. Arbuscular mycorrhizal fungal hyphae play an important role in absorbing available P from the soil, whilst also being able to hydrolyse P locked in organic matter and solubilize complexed inorganic P (Syibli *et al.* 2013). Mycorrhizal fungi are well known for their ability to access a variety of P sources and transport these to the host plant.

Increased Na concentration in the soil results in an increase in soil pH and impends soil nutrient uptake (Supply, 2004). In addition to this Na affects soil structure, water infiltration and reduces plant growth (Horneck *et al.* 2011). Aliasgharzadeh *et al.* 2001 stated that AM fungi sporulate is stimulated under salt stress producing more spores. This is probably because the thick spore walls may impart tolerance towards salinity. Mycorrhizal fungi do occur naturally in saline environments (Ho, 1987). Fulmaka, (2015) similarly recorded higher AM fungal spore numbers (112.7/ 100g) in soils collected from Helspoort, Eastern Cape (-33.169537 S and 26. 31622667 E). This site also had increased Na concentration in soil of 62.5 mg/kg when compared to another site (Hounslow, Eastern Cape (-33.19738082 S and 26.43825013 E) which also has high Na concentration in soil

(92.5 mg/kg) AM fungal spore numbers decreased to 77.2/100g soil. It is difficult from this study to correlate soil nutrient status to AM spore numbers because of limited nutrient analysis data.

Calcium plays important roles in maintaining plant membrane integrity, cell wall structures, as well as ion transport regulation and selectivity (Maathuis 2009; Evelin *et al.* 2012). It is the most abundant element in nature (Maathuis, 2009). Higher Ca concentration in AM fungal associated plants alleviates the toxic effects of NaCl by inducing a higher K:Na ratio leading to salt adaptation (Rabie and Almadini 2005; Evelin *et al.* 2009). When Ca is too high, soil pH tends towards alkalinity which can impede plant absorption of some nutrients (Supply, 2004) leading to plant growth reduction. In a study by Anderson *et al.* (1984) Ca was found to be the best predictor of spore abundance. High Ca concentrations enhances sporulation of AM fungi (Evelin *et al.* 2012). It can be concluded that the increase in AM spore abundance in the intact site may have some relationship with the higher Ca concentration.

According to Jha *et al.* (1992) and Trejo *et al.* (2015) degraded areas tend to have reduced vegetation cover as well as soil moisture because of increased evaporation which further reduces the survival of plants in the soil. Mycorrhizal activity in the soil is determined by the number of spores, hyphae fragments and colonize root fragments with adhering hyphae (Duponnoise *et al.* 2001). Arbuscular mycorrhizal fungi are dependent on the presence and successful colonization of host plants in order to complete their life cycle. The degraded site in this study was also characterised by sparse vegetation cover made up of weeds, grass and a limited number of woody trees and shrubs resulting in lower spore numbers observed.

Conversely the vegetation cover from the intact site was dense and more spores were recorded. Spore numbers were still low as compared to other reported studies such as 610.6 spores / 100 g from forest soil (Sharmah and Jha, 2014). Sarkar *et al.* (2014) also found 1380 AM fungal spores/ 100g soil from degraded forest and 500/ 100 g from Rubber plantation. Continuous vegetation cover means that AM fungi are able to grow and develop symbiotic relationships with roots over a long period of time therefore producing spores only to ensure long term survival during adverse climatic condition.

Soil pH is a measure of the soils acidity and alkalinity that gives an indication of the activity of the hydrogen ion (H^+) and hydroxyl ion in (OH^-) in a water solution (Horneck *et al.* 2011).

Solutions which contain equal concentrations of H^+ and OH^- ions are said to be neutral with a pH of 7.0. The pH of soil is determined in potassium chloride (KCl) or water (H_2O) (Van Schoor *et al.* 2000). Soil water is a solution of dissolved elements and pH(KCl) is a more suitable reflection of soil pH. According to Maun, (2009) and Sundar and Sabari, (2011) soil pH is an important factor which determines spore germination and AM fungal development. The pH(KCl) of intact site was 6.35 slightly acidic and degraded site 5.95 moderately acidic. Arbuscular mycorrhizal fungal spores are found in soil of varying pH (Clark, 1997) conditions which are favourable to spore germination.

Soils approaching neutrality are believed to support AM fungal colonization (Gashua *et al.* 2015). In this study, no significant effect was observed in mycorrhizal root colonization when comparing the intact and degraded site whose pH were comparable.

Mycorrhizal infectivity was not significantly different between the degraded and intact site. Propagules from degraded and intact site could colonize *S. bicolor*. Sorghum is highly mycotrophic (Eom *et al.* 2002; Hetrick and Bloom, 1986); and colonization can occur from spores (indicating at least one spore is viable) or from other propagules such as hyphal and colonized root fragments. Overall, these results revealed that the use of highly mycotrophic species could have encouraged root colonization. Although colonization percentage was below 50% given suitable host plants the soil from both sites had potential to initiate the mycorrhizal symbiosis.

2.7 CONCLUSION

In conclusion, some of the factors evaluated may have had an influence on AM spore number such as Na, Ca, CEC, N, C levels and vegetation cover reinforcing the significance of these factors on the association in degraded and intact site. Both sites had mycorrhizal potential but a continual decrease in AM propagules in the degraded site due to reduced vegetation cover would negatively affect the potential.

CHAPTER THREE: THE ROLE OF MICROBIAL INOCULANTS ON THE GROWTH OF *Mystroxylon aethiopicum, Scutia myrtina* AND *Aloe ferox* SEEDLINGS

3.1 INTRODUCTION

To ensure successful restoration programmes in degraded sites, it is necessary to apply the correct methods that improve soil quality and seedling germination and establishment (Caravaca *et al.* 2002). There is increasing evidence that soil microbial communities, particularly mycorrhizal fungi play a vital role in developing plant biomass (Duponnois *et al.* 2001; Pal and Pandey, 2014) and preventing soil erosion through production of a glycoprotein known as glomalin which binds soil particles. Compant *et al.* (2010) recognised the interaction between plants and various soil microorganisms as being important for plant development. Application of a multifunctional microbial inoculum and using appropriate combinations of beneficial microorganisms can be useful in promoting plant growth (Wang *et al.* 2007). Mycorrhizal fungi and plant growth promoting rhizobacteria (PGPR) are the most widely used inoculants for restoration purposes. These soil microorganisms, enhance nutrient absorption from degraded soil (Bharadwaj *et al.* 2012) and act as a tool to protect plants against biotic and abiotic stresses.

Soil microbial plant associations are complex (Richardson *et al.* 2009). Through mycorrhizal root colonization, roots form an extensive hyphal network in the soil thus enhancing nutrient uptake due to the increased absorptive capacity of the fungus. Plant growth promoting rhizobacteria also increase root growth through production of phytohormones. These microorganisms synergistically stimulate metabolic processes activating poorly available nutrient sources (Richardson *et al.* 2009) for example PGPR release phosphate ions from complexed inorganic nutrient sources enhancing mycorrhizal fungal absorption and transfer to the plant thus stimulating growth.

A positive correlation between AM fungal colonization and plant growth parameters was found by Avio *et al.* (2006). In addition to this, interaction with PGPR (Compant *et al.* 2010) also promotes plant health through the production of siderophores and other antimicrobial compounds that aid in protecting plants from pathogens. In this study the role of arbuscular mycorrhizal fungi and PGPR as important inoculants for improving growth of selected seedlings used in restoration of degraded thicket was investigated.

The aim of this experiment was to assess the ability of AM fungi and an *Enterobacter* sp. isolate in promoting plant growth of *Mystroxylon aethiopicum, Scutia myrtina* and *Aloe ferox* seedlings.

3.2 MATERIALS AND METHODS

3.2.1 Plant material

Three species were selected for this study *Mystroxylon aethiopicum, Scutia myrtina* and *Aloe ferox*. These plants were selected because they are appropriate for restoration and commonly found in thicket vegetation.

3.2.2 *Mystroxylon aethiopicum*

Mystroxylon aethiopicum (Thunb.) Loes belongs to the Celastraceae family and is commonly known as the spike-thorn/Kooboo berry. It is an attractive evergreen tree usually used as an ornamental plant (Figure 3.1). Local communities use the leaves to control helminthosis and black water in sheep (Gakunga *et al.* 2014). Ornamental plants play an important part of the economy and ecotourism in South Africa, for this purpose *M. aethiopicum* seedlings are produced in indigenous nurseries and are valuable in terms of restoration.

Figure 3.1: *Mystroxylon aethiopicum* plant (source M. J. Powell).

3.2.3 *Scutia myrtina*

Scutia myrtina (Burm.f.) Kurz from the family Rhamnaceae is commonly known as Cat thorn. It is an evergreen, broad-leaf species, with short, hooked thorns (Figure 3.2). It is a spinescent species that is common in mesic savanna areas on the eastern seaboard of southern Africa and are utilized as fodder for domestic livestock (Zharare and Scogings, 2011). Resource harvesting, livestock browsing and grazing of this vegetation has resulted in an uneven vegetation coverage (Pote *et al.* 2006).

Figure 3.2: *Scutia myrtina* plant (source M. J. Powell)

3.2.4 *Aloe ferox*

Aloe ferox Mill. L. belongs to the family Asphodelaceae and forms an important vegetation component of the South African landscape. They are the most familiar succulent plants species (Wintola *et al.* 2010) and occupy a wide range of habitats (Cousins and Witkowski, 2012). *Aloe ferox* is distributed through the Eastern Cape, Southern Cape, Southern parts of KwaZulu Natal and Lesotho. It is an arborescent single stemmed 2-3 m perennial shrub (Wintola *et al.* 2010) with a tall narrow stem that is enclosed in woody, dead leaves with a crown of succulent leaves at the very top (Figure 3.3). *Aloe ferox* has been harvested for its bitter sap for approximately 250 years

(Shackleton and Gambiza, 2007), local communities and traditional healers also use it for medicinal purposes. The sap extracted from these plants has been used to treat sexual transmitted disease (Kambizi *et al.* 2007).

In the Eastern Cape Province, South Africa *A. ferox* is an important vegetation type of the ATB (Hoare *et al.* 2006). They represent the "health" of the vegetation. When thicket vegetation becomes heavily browsed by livestock or elephants, aloes become exposed to further exploitation by herbivores (Cousins and Witkowski, 2012).

Figure 3.3: *Aloe ferox* plant (source Prof. J. Dames)

3.2.5 INOCULANTS

3.2.5.1 Bacteria

A bacterial culture (Isolate number RUMRL – SvA 51) isolated from a semi-arid environment in the Kalahari, Northern Cape, South Africa was obtained from the Mycorrhizal Research Laboratory, Rhodes University, South Africa. The pure isolate was tested for various PGPR characteristics, and was selected because tests for N fixation, P solubilisation, siderophore, IAA and biofilm production were positive. The bacterium was rod shaped, Gram negative and was molecularly identified as an *Enterobacter* species (S. van Aswegan personal communication)

Bacteria were inoculated into fresh sterile Nutrient Broth and incubated at 37°C for 24 hrs prior to use in pot trials.

3.2.5.2 Arbuscular mycorrhizal fungi

A mixed crude inoculum of AM fungi was obtained from Mycoroot, South Africa. The fungi were isolated in pot culture from two sites, Coega Salt Pan, Eastern Cape (33°46'41.47"S, 25°42'29.27"E), and Northern Cape, Haakdoorn Duin Farm, Kalahari (27°39'26.1"S, 21°03'94.6"E), South Africa. The AM fungal population in the inoculum was identified using next generation pyrosequencing after PCR amplification of the 18S (SSU) gene. The Salt Pan site was dominated by *Ambispora* (37%), *Glomus* (34%) and *Paraglomus* (22%) genera. The Kalahari site was dominated by *Paraglomus* (46%), *Glomus* (33%) and *Ambispora* (16%) genera. The *Archaeospora, Redeckera* and *Geosiphon* genera represented rare components of the AM fungal population at less than 1% at both sites (Moore, 2015). A 1:1 mix of each inoculum was used with a most probably number of propagules of 50 per gram (Mycoroot).

3.2.5.3 Seedling preparation

Mystroxylon aethiopicum and *S. myrtina* seeds were collected from trees occurring in the Thicket area surrounding Grahamstown, Eastern Cape, South Africa. The seed coat was removed and seeds were allowed to dry in the sun (24 hours). The seeds were sawn into a tray of vermiculite and watered and placed in Mycorrhizal Research tunnel until germination.

Seedlings of *A. ferox* were obtained from Weltevreden Succulent Nursery, Port Elizabeth, South Africa. Two days prior to planting seedlings were removed from trays, roots were pruned to 1 cm from the base and shoots were allowed to dry.

3.4 SEEDLING POT TRIAL

Pots (2L) sterilized with bleach and rinsed with water were filled with pasteurised soil from degraded thicket. Pasteurization was conducted at the Mycorrhizal tunnel, Rhodes University. The soil was steamed on two successive days for two hrs at 80°C.

A pot trial was conducted using four treatments (Table 3.1), three seedlings species with each treatment replicated ten times. Initial shoot height of *M. aethiopicum* and *S. myrtina* germinated

seedlings was recorded. After planting of seedlings pots were placed under the following conditions; temperatures max 35°C and min 20-25°C, irrigated daily with UV- treated water and subjected to natural lighting. *Aloe ferox* seedlings were planted in 0.5 L sterilized pots and watered only once a week due to their succulent nature.

Table 3.1: Pot trial experimental design

Treatments	Replicates	Inoculants
1. Control	10	Non-inoculated soil as the control
2. AM fungi	10	Five ml of AM fungal inoculum. Placed beneath the seedling.
3. Enterobacter sp.*	10	Five ml of *Enterobacter sp.* bacterial suspension. Applied to the soil surface
4. AM fungi + *Enterobacter* sp.*	10	Bacteria and AM fungi, applied as described above.

*Treatments 3 and 4 were inoculated a second time with bacterial suspension after four weeks.

3.5 PLANT GROWTH PARAMETERS

Shoot heights of the *M. aethiopicum* and *S. myrtina* seedlings were recorded every second week to determine growth rate. Final height was measured at harvest after five months. Seedling canopy was also determined by recording two diameter measure across the canopy. Cylinder formula was used to determine the canopy volume of each species (Yang *et al.* 1996; 2002).

Canopy volume = $\pi r^2 H$

Where, r = average radius of canopy (mm)

H = shoot height (mm)

The units for canopy volume after calculations were in mm^3. These values were converted to cm^3 by multiplying by 0.001.

All three seedlings were removed from the soil and separated into shoot and root components. Wet weight of both components was recorded and components were oven dried for 1 week at 60°C, after which dry weight was recorded. Fresh root subsamples were collected for analysis of percentage AM fungal colonization (Section 2.3.5) using a compound microscope (Leica CME). The wet weight of the subsample was recorded to correct the overall root biomass data after drying.

In this study the term microbial dependency (MiD) was used to evaluate the effect of *Enterobacter* sp. alone and in combination with AM fungi on seedling growth. Microbial Dependency (MiD) was adapted from the formular of Plenchette *et al.* (1983) as shown below.

$$MiD = \frac{\text{Total plant biomass of inoculated plants-Total plant biomass of uninoculated plants}}{\text{Total plant biomass of inoculated plants}} * 100$$

Habte and Manjunath, (1991) used the following scale to categorise dependency:

1. Highly dependent plants (>50%)
2. Moderately dependent plants (25-50%)
3. Marginally dependent plants (<25 %)
4. Independent plants (<0%)

3.6 STATISTIC ANALYSIS

The data was tested for normality using the Kolmogorov- Smirnov test. If the data was normally distributed, parametric tests were used. Percentage colonization was arcsin-transformed, and the other parameters were log-transformed to compensate for heterogeneity of variance, before analysis of variance. The effect of arbuscular mycorrhizal fungi, *Enterobacter* sp. and the combination of AM fungi and *Enterobacter* sp. inoculation on measured variables was tested by a one-way analysis of variance, and comparisons among means were made with the Tukey's significant difference (HSD) test, calculated at $P<0.05$. Before analyses, root biomass and the final shoot height were log transformed. Seedlings growth over time was analysed using repeated measures analysis of variance. The relationship between the root biomass and the final shoot height was tested using a multiple-regression analysis. Statistical procedures were carried out with the software package STATISTICA 13.0.

3.7 RESULTS

Figure 3.4 and 3.5 indicate seedling growth and conclusion after pot trial

Mystroxylon aethiopicum

Figure 3.4: *Mystroxylon aethiopicum* seedlings

Figure 3.5: Growth development of *Mystroxylon aethiopicum* seedlings, A- control, B- *Enterobacter* sp., C- AM fungi and D- AM fungi plus *Enterobacter* sp.

The effect of the treatment on seedling growth was assessed by recording shoot height every two weeks. The AM fungal treatment of *M. aethiopicum* seedlings was significantly higher than

inoculation with *Enterobacter* sp. and the combination of *Enterobacter* sp. and AM fungi and the control (Figure 3.6). Inoculation effects become more apparent after 14 weeks.

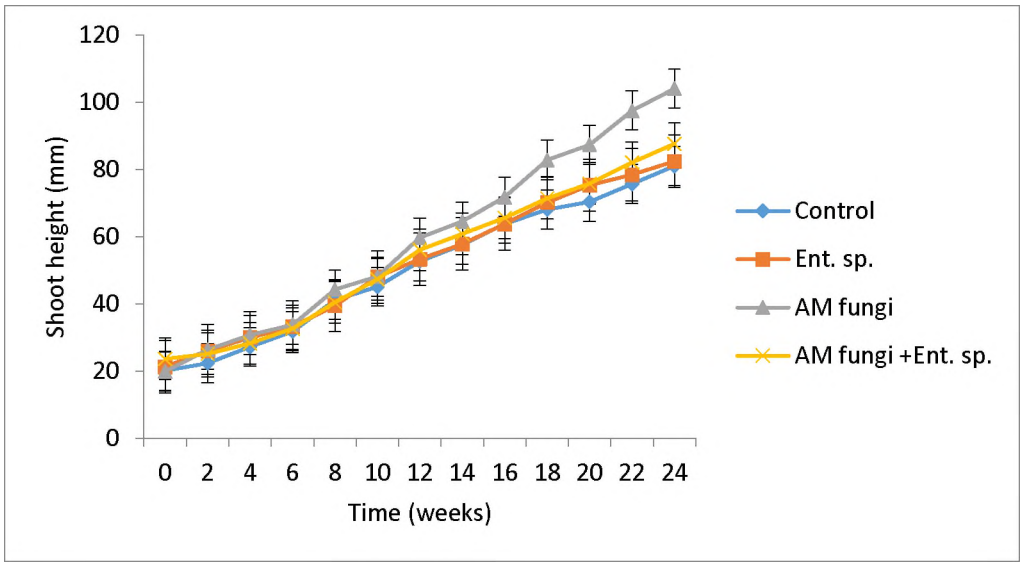

Figure 3.6: Growth of *Mystroxylon aethiopicum* seedlings in response to microbial inoculants. Points represents treatment means ± standard error. $F_{(3,36)}$=9.491; p =0.001.

Shoot biomass of *M. aethiopicum* seedlings were recorded at harvest. AM fungal inoculation promoted shoot biomass in *M. aethiopicum* seedlings when compared to all other treatments (Figure 3.7). Combined inoculation reduced the beneficial growth effect obverse to the uninoculated control levels.

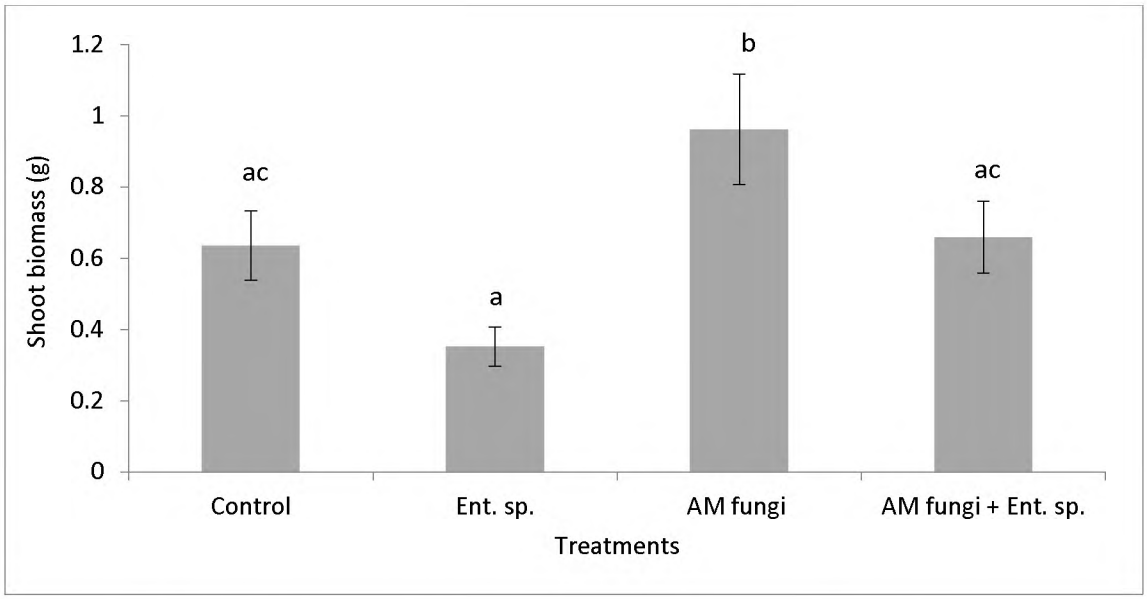

Figure 3.7: Shoot biomass of *Mystroxylon aethiopicum* seedlings in response to microbial inoculation effect. Bar represents treatment means ± standard error. Different letters indicate significant differences. $F_{(3,36)}$=5.321; p =0.004.

No significant difference was observed in *M. aethiopicum* seedlings root biomass in response to microbial inoculants (Figure 3.8).

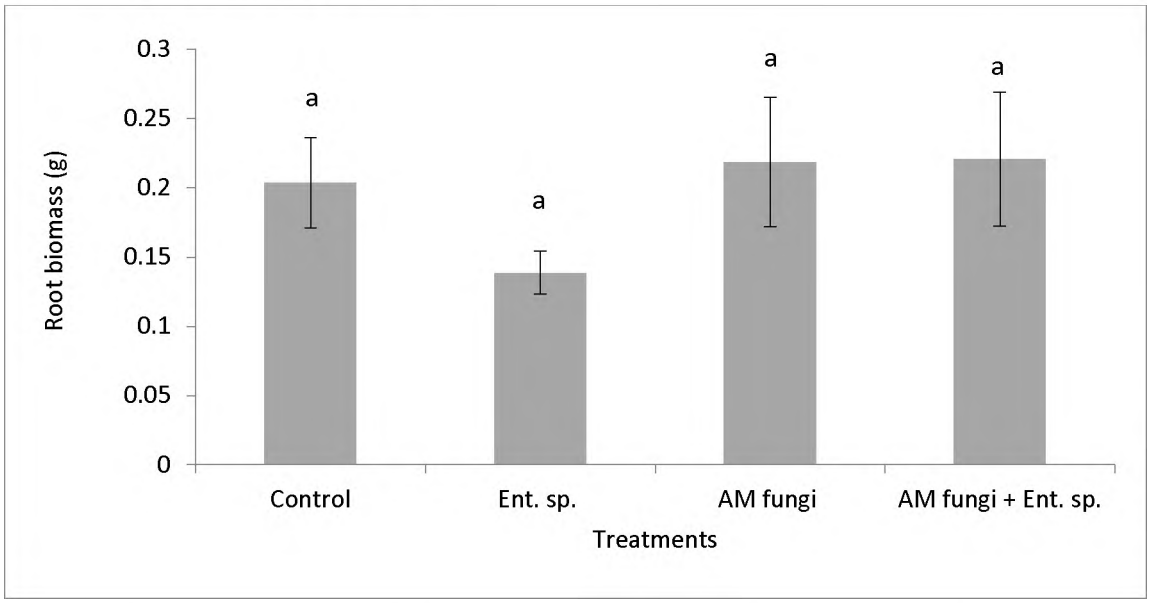

Figure 3.8: Root biomass of *Mystroxylon aethiopicum* seedlings shown no significant response to microbial inoculants. Bars represent means ± standard errors.

AM fungal treatment significantly increased canopy volume when compared to other treatments (Figure 3.9).

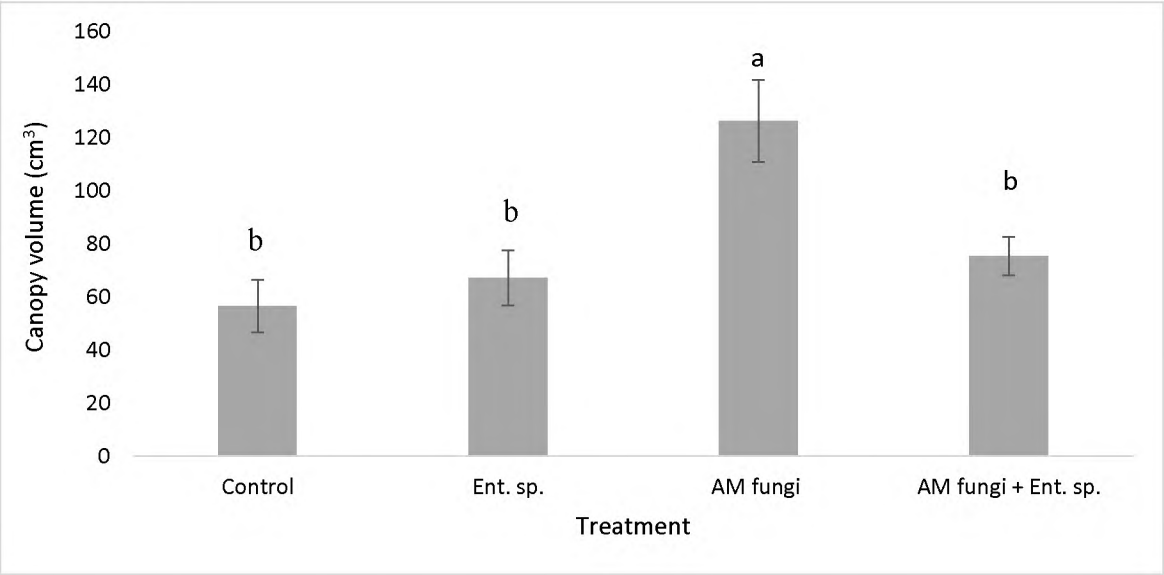

Figure 3.9: Canopy volume of *Mystroxylon aethiopicum* seedlings in response to microbial inoculants. Bar represents treatment means ± standard error. Different letters indicate significant difference. $F_{(3,36)}=7.699$; $p = 0.001$.

A positive relationship was found between shoot height and dry root biomass in *M. aethiopicum* seedlings inoculated with AM fungi inoculant ($p = 0.001$) (Figure 3.10). No other significant relationships were found.

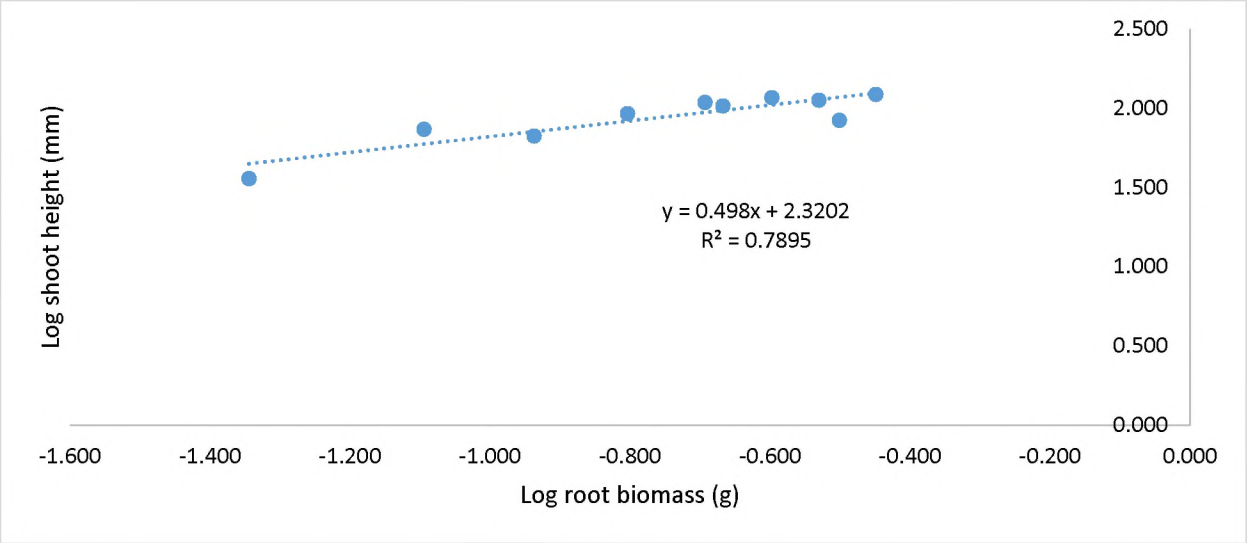

Figure 3. 10: A positive relationship between log root biomass and log shoot height of *Mystroxylon eathiopicum* seedlings inoculated with AM fungi plus *Enterobacter* sp.

Characteristic AM fungal structures were observed in roots of *M. aethiopicum* seedlings confirming that *M. aethiopicum* seedlings associate with AM fungi (Figure 3.11).

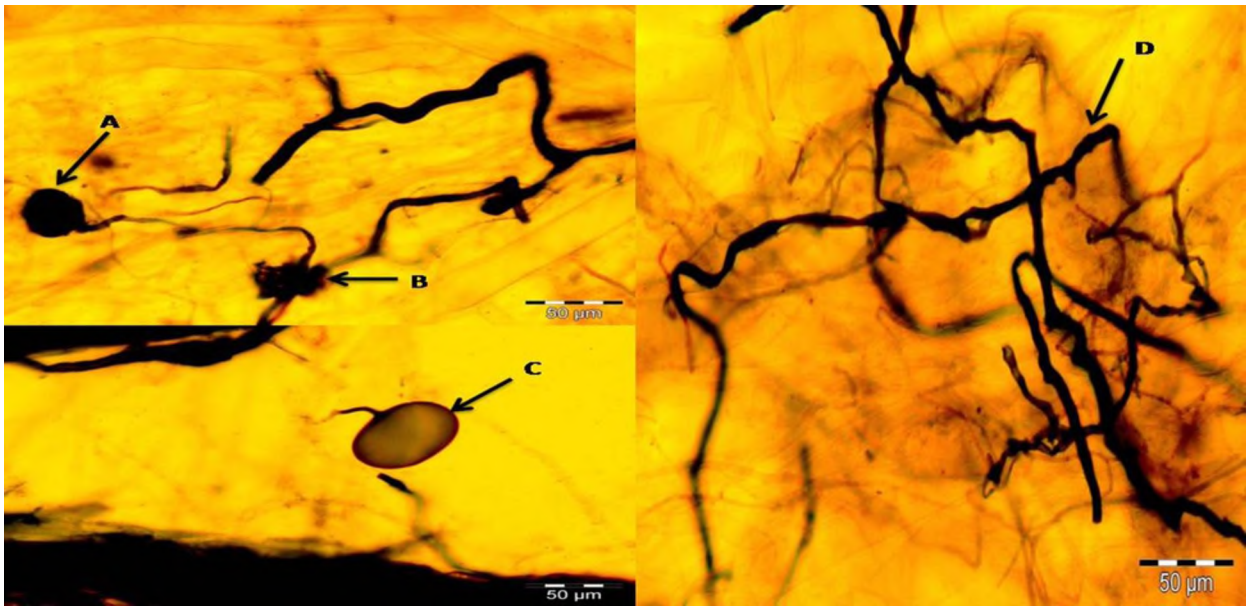

Figure 3.11: Arbuscular mycorrhizal fungal root colonization of *Mystroxylon aethiopicum* A-vesicles, B-arbuscules, C-spores and D-intercellular hyphae.3.

Seedlings inoculated with AM fungal had significantly more colonization when compared to other treatments as expected. Colonization was distinguished by higher percentage of intraradical hyphae, arbuscules and vesicles. Colonization was significantly reduced in combined inoculation of AM fungi and *Enterobacter* sp. (Table 3.2).

Table 3.2: Arbuscular mycorrhiza fungal root colonization of *M. aethiopicum* seedlings

	Intraradical structures (% contribution to total colonization)			**Extraradical structure**		
Treatment	**Hyphae**	**Arbuscules**	**Vesicles**	**Hyphae**	**Spores**	**Total colonization**
Control	3.11±7.03^{c}	0.00^{d}	0.00^{b}	6.02±4.02^{a}	0.00	3.4±0.67^{b}
Ent. sp.	44.24±10.05^{c}	3.01±7.17^{d}	6.02±4.02^{b}	6.02 ±8.15^{a}	0.00	5±1.28b
AM fungi	91.44 ±8.81^{b}	37.88 ±9.44^{c}	27.09±9.40^{a}	31.86±9.78^{b}	3.01±3.01	17.8±3.19^{a}
AM fungi+ Ent. sp.	30.61±10.66^{c}	19.82±3.01^{d}	6.02±4.02^{b}	13.8 ±4.02	3.01±3.01	7±2.238^{b}
$F_{value\ (3,36)}$	9.42	6.70	4.63	3.06	1	9.80
P <0.05	0.001	0.001	0.007	0.04	0.4	0.001

Values are means ± standard errors. Different letters indicate significant difference

The microbial dependency of *M. aethiopicum* varied with inoculation. Seedlings were moderately dependent on AM fungi but this was reduced with combined inoculation (Figure 3.12).

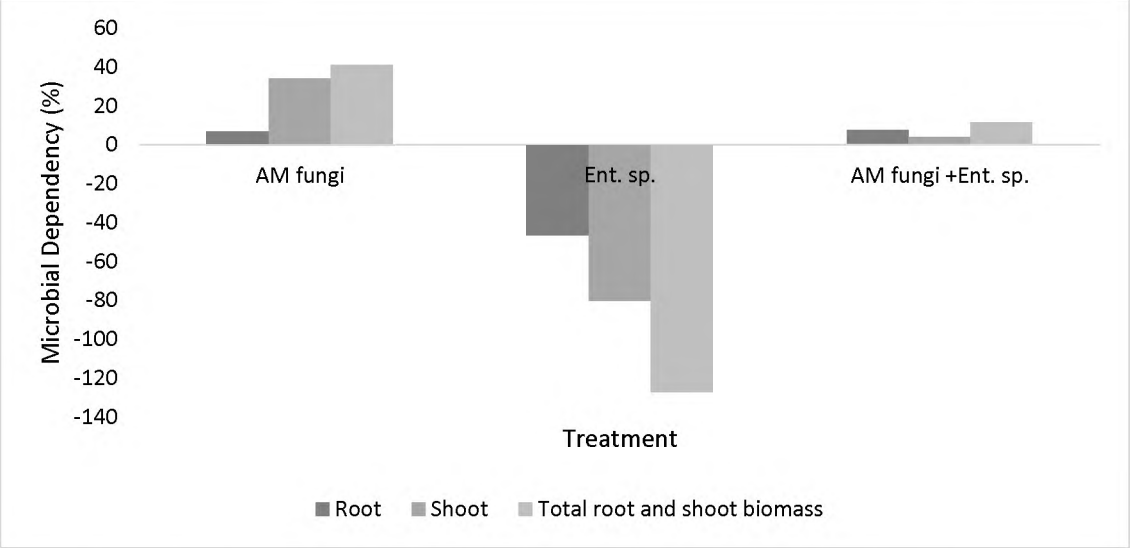

Figure 3.12: Microbial dependency of *Mystroxylon aethiopicum* seedlings in response to microbial inoculants.

Scutia myrtina

Figure 3.13 and 3.14 indicate seedling growth and conclusion after pot trial

Figure 3.13: *Scutia myrtina* seedlings

Figure 3.14: The growth development of *Scutia myrtina* seedlings A-control, B-*Enterobacter* sp., C-AM fungi, and D-AM fungi plus E*nterobacter* sp.

The AM fungal plus *Enterobacter* sp. treatment significantly improved growth of *S. myrtina* seedlings becoming apparent after 14 weeks. Significant increase was also noted for AM fungal and *Enterobacter* sp. treatments alone when compared with the control (Figure 3.15).

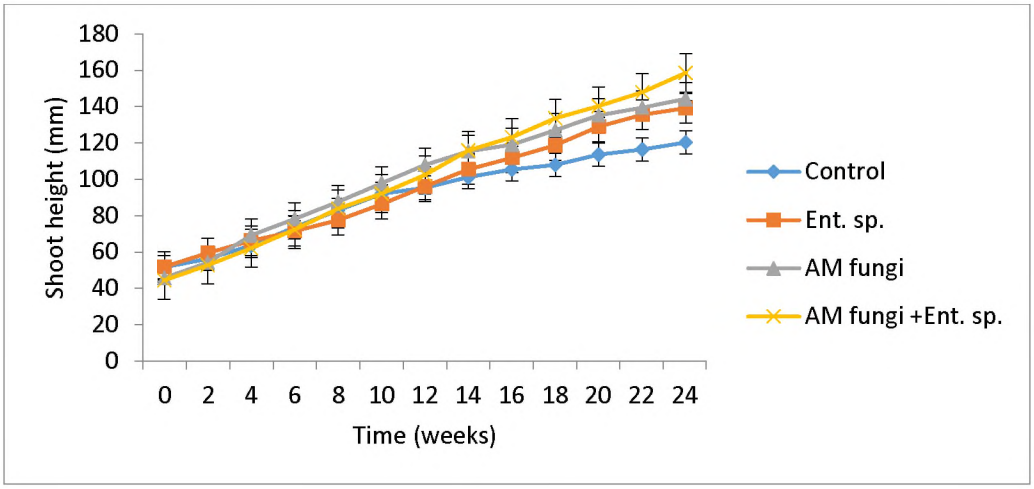

Figure 3.15: Growth of *Scutia myrtina* seedlings in response to microbial inoculants. Points represents treatment means ± standard error. $F_{(3,36)}$= 8.381; p =0.001

Shoot and root biomass of *S. myrtina* seedlings was not significantly affected by inoculation (Figure 3.16 and 3.17).

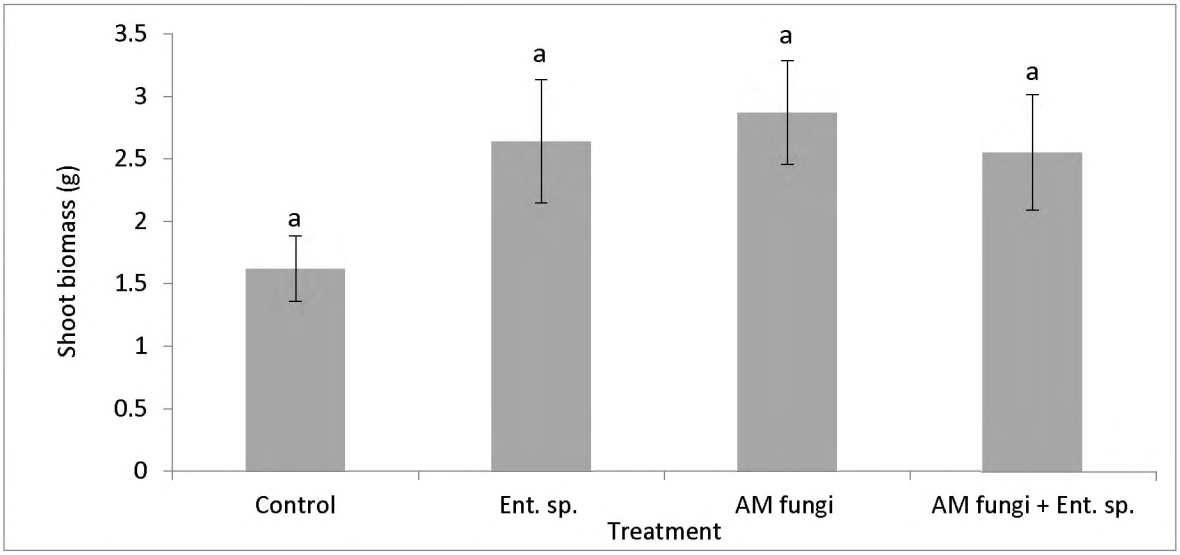

Figure 3.16: Shoot biomass of *Scutia myrtina* seedlings showed no significant response to microbial inoculants. Bar represents treatment means ± standard error.

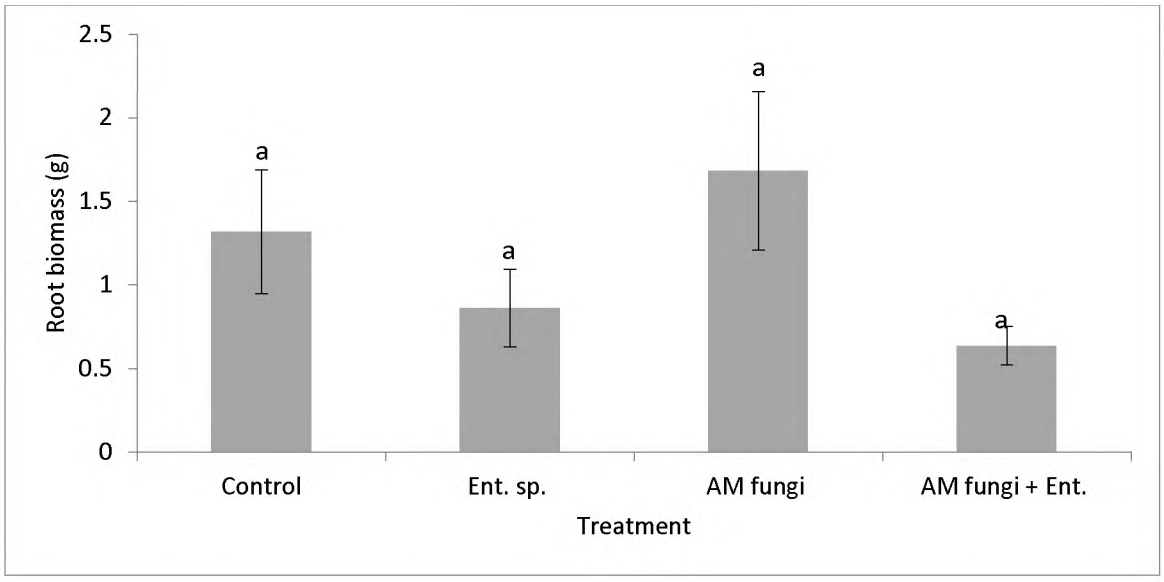

Figure 3.17: Root biomass of *Scutia myrtina* seedlings showed no significant response to microbial inoculants. Bars represent treatment means ± standard error.

The canopy volume of *S. myrtina* seedlings was not affected by the inoculants (Figure 3.19).

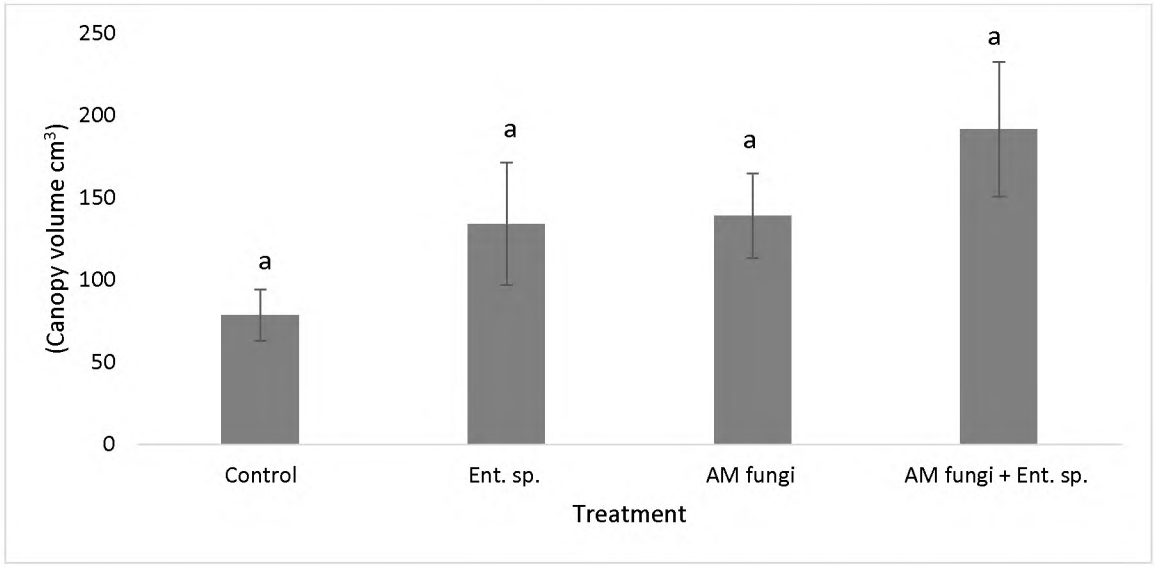

Figure 3.18: Canopy volume of *Scutia myrtina* seedlings showed no significant response to microbial inoculants. Bar represents treatment means ± standard error.

A positive relationship was found in *S. myrtina* seedlings between shoot height and dry root biomass in AM fungal plus *Enterobacter* sp. inoculation ($p = 0.046$) and in AM fungal inoculation ($p = 0.022$) (Figure 3.19 and 3.20).

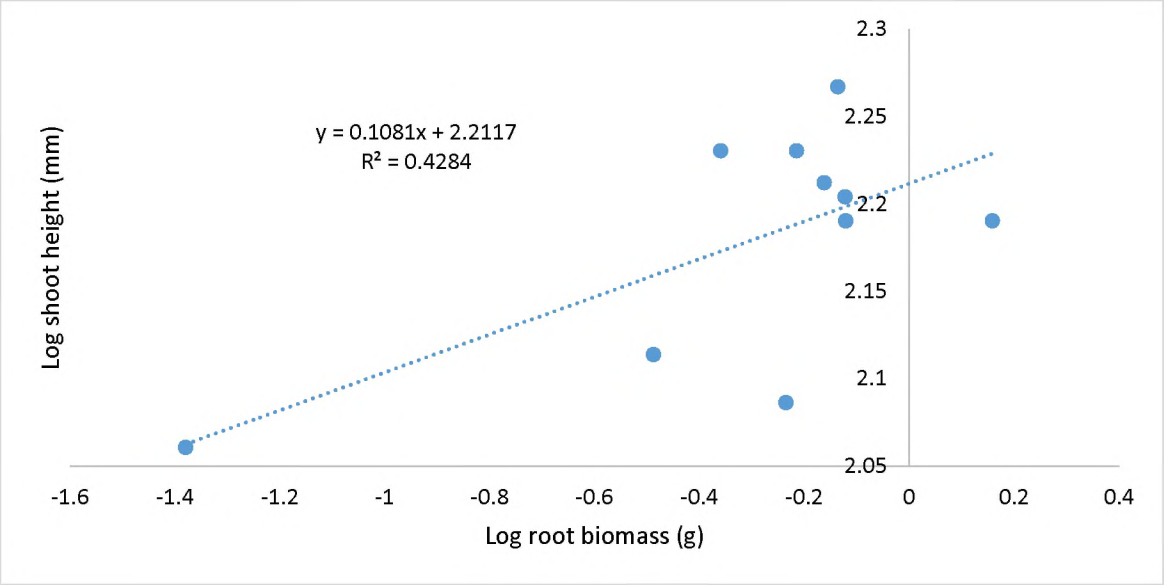

Figure 3.19: A positive relationship between log root biomass and log shoot height of *Scutia myrtina* seedlings inoculated with AM fungi plus *Enterobacter* sp.

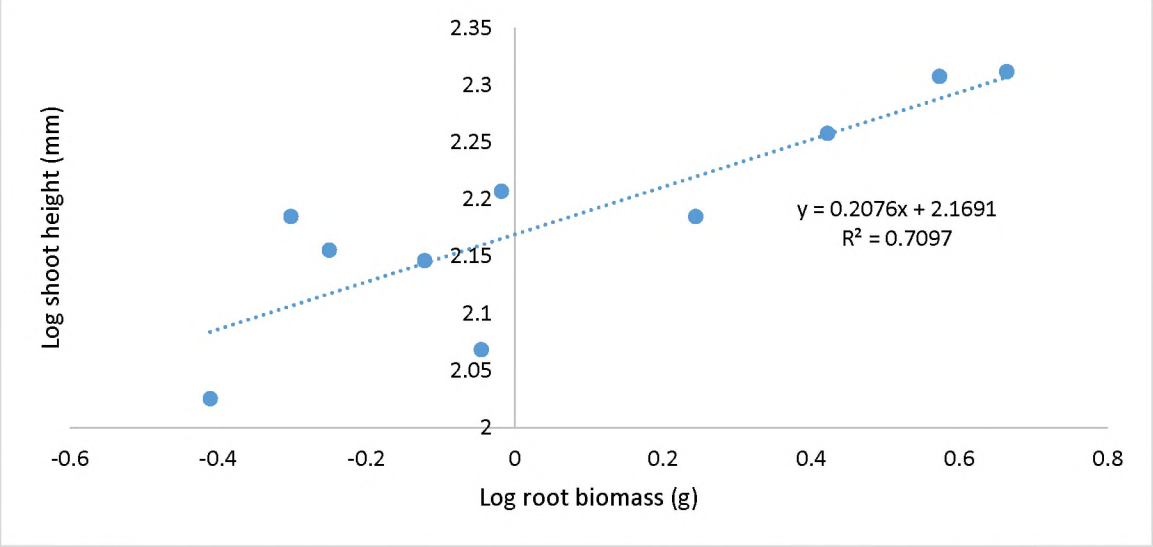

Figure 3.20: A positive relationship between root biomass and shoot height of *Scutia myrtina* seedlings inoculated with AM fungi

The microbial dependency varied with inoculation. Seedlings were highly dependent on AM fungal inoculation. This was negatively affected with *Enterobacter* sp. inoculation for root and total biomass (Figure 3.21).

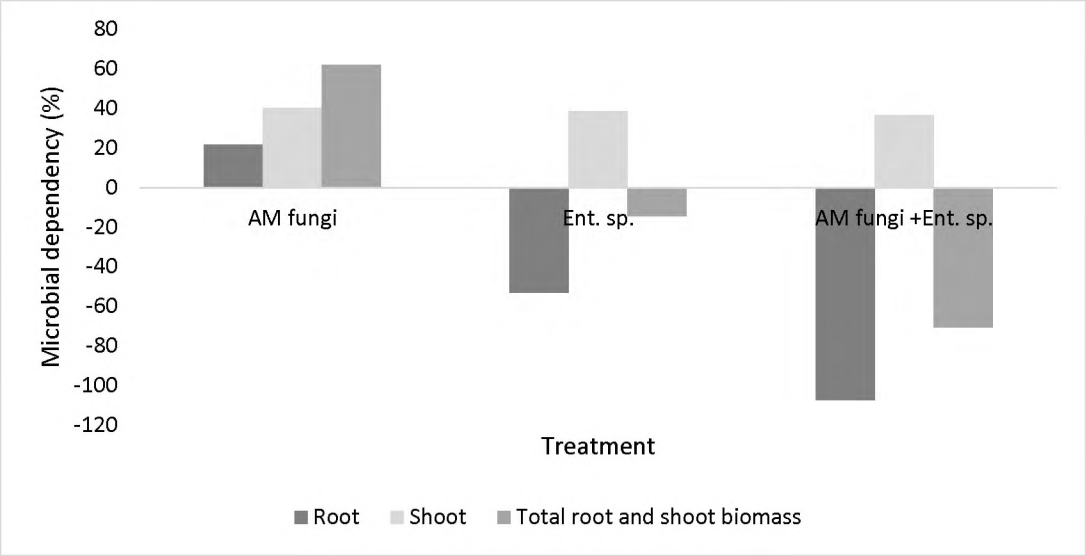

Figure 3.21: Microbial dependency of *Scutia myrtina* seedlings in response to microbial inoculation.

Mycorrhizal colonization could not be assessed in *S. myrtina* seedlings due to dense root pigmentation.

Aloe ferox

Figure 3.22 indicate seedling growth

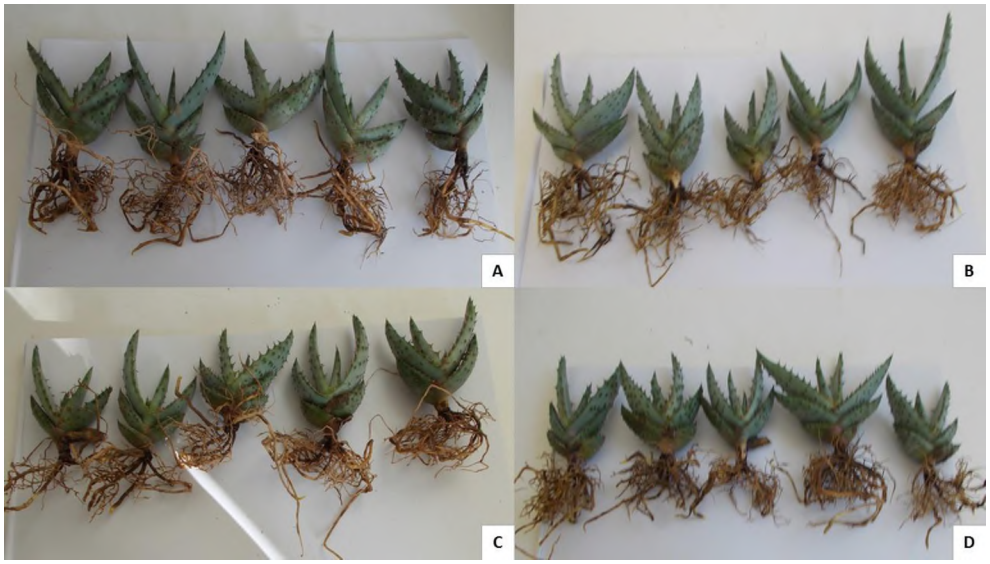

Figure 3.22: The growth development of *Aloe ferox* seedlings A- control, B- *Enterobacter* sp., C- AM fungi and D- AM fungi plus *Enterobacter* sp. combination

The effect of AM fungi, *Enterobacter* sp. alone and in combination did not significantly increase biomass of *Aloe ferox* seedlings (Figure 3.23).

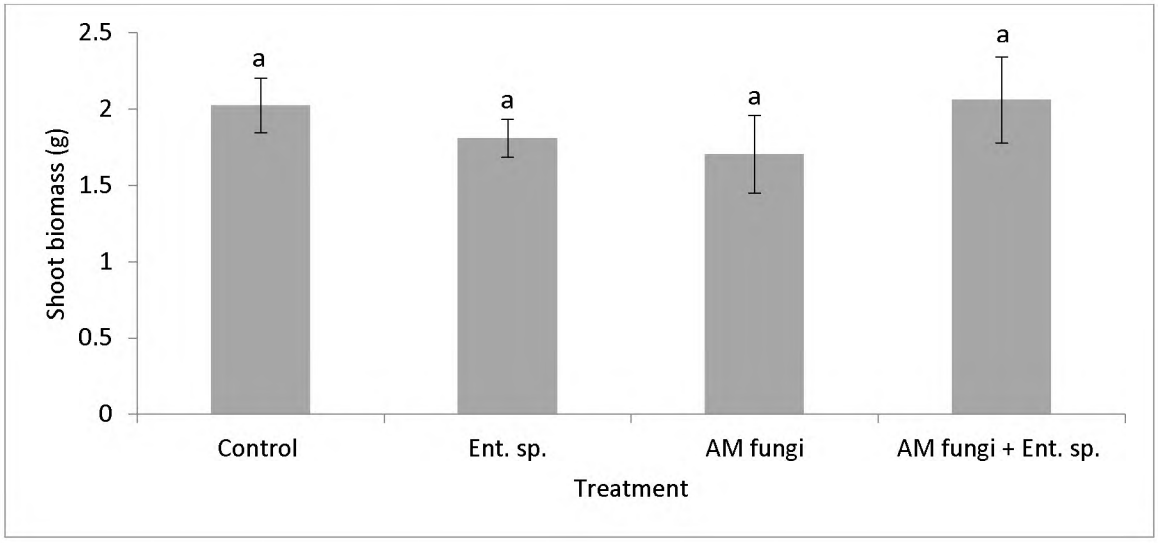

Figure 3.23: Shoot biomass of *Aloe ferox* seedlings showed no significant response to microbial inoculants. Bars represent treatment means ± standard error.

No significant difference was recorded between treatments and root biomass of *A. ferox* seedlings (Figure 3.24).

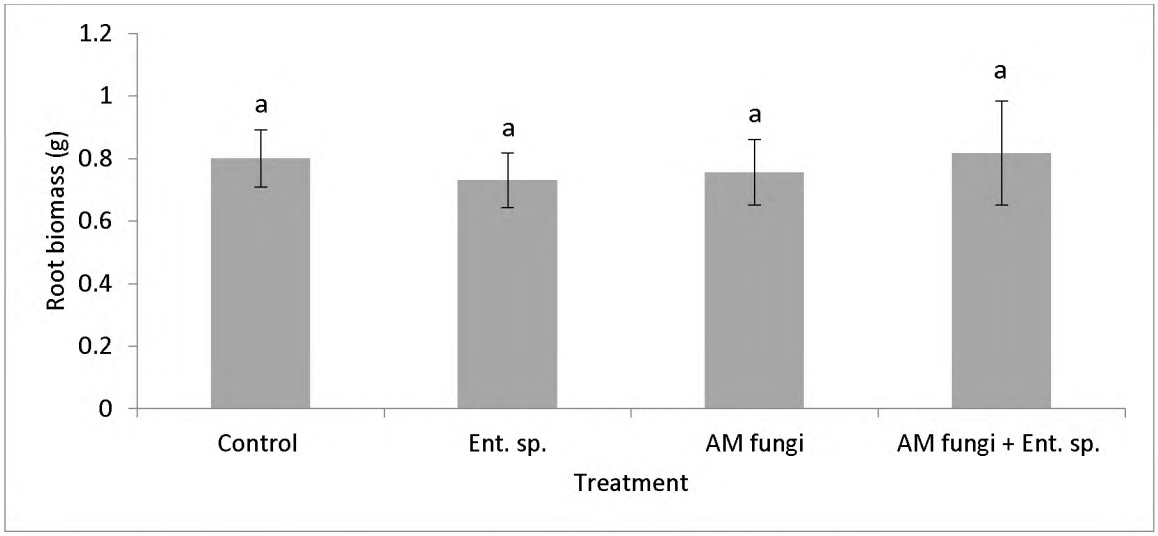

Figure 3.24: Root biomass of *Aloe ferox* seedlings showed no significant response to microbial inoculants. Bar represents treatment means ± standard error. Effect of inoculating *Aloe ferox* seedlings with selected inoculants on microbial dependency.

Biomass of *A. ferox* seedlings in the *Enterobacter* sp. and AM fungal treatment were independent of inoculation. Whereas combined inoculation of seedlings was marginally dependent on the inoculants (Figure 3.25).

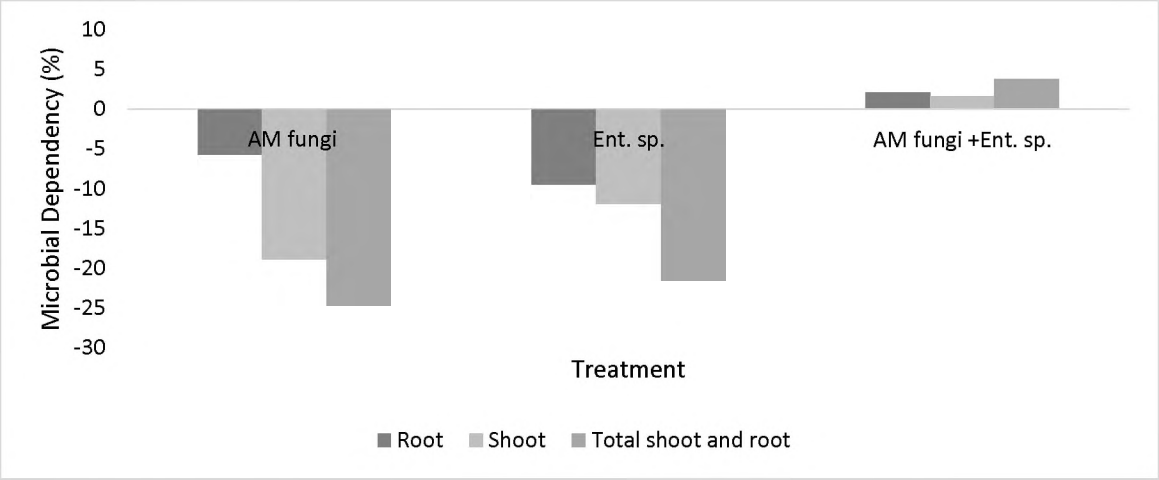

Figure 3.25: Microbial dependency of *Aloe ferox* seedlings in response to microbial inoculation.

Characteristic AM fungal structures were observed in roots of *Aloe ferox* seedlings confirming association with AM fungi (Figure 3.26 and Table 3.3).

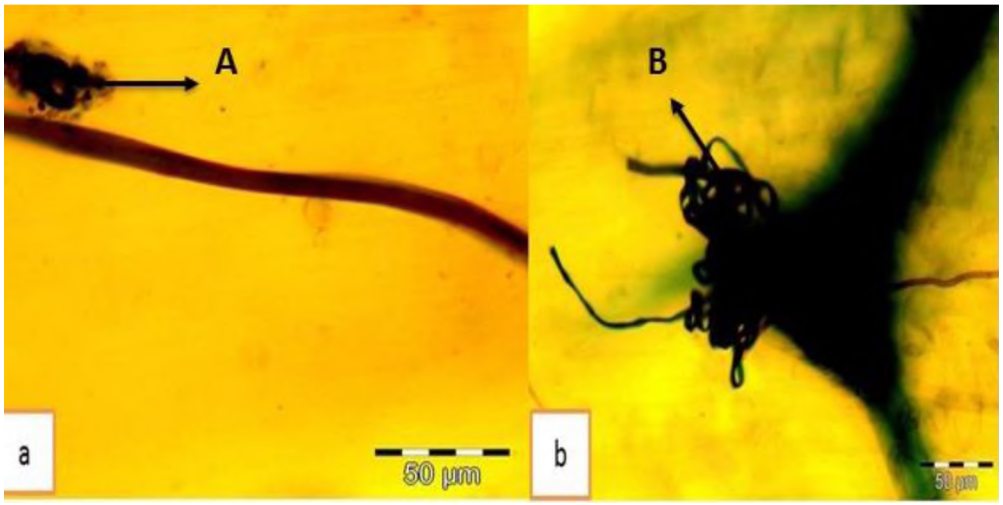

Figure 3.26: Features of AM fungi root colonization taken from *Aloe ferox* root A-arbuscules, B-intercellular hyphae.

Seedlings inoculated with AM fungi alone and in combination with *Enterobacter* sp. had significantly more colonization when compared to the bacterial and control treatments. Colonization was characterised by higher percentage of intraradical hyphae, arbuscules and vesicles (Table 3.3).

Table 3.3: AM fungal root colonization of *Aloe ferox* seedlings

	Intraradical structures (% contribution to total colonization)			**Extraradical structure**		
Treatment	**Hyphae**	**Arbuscules**	**Vesicles**	**Hyphae**	**Spores**	**Total colonization**
Control	67.25 ± 10.64^{c}	9.03 ± 4.60^{b}	0.00^{b}	6.02 ± 4.02^{a}	3.01 ± 3.01	6.40 ± 0.98^{b}
Ent. sp.	72.76 ± 9.59^{b}	19.82 ± 9.17^{b}	9.03 ± 6.43^{b}	15.05 ± 6.74^{a}	6.02 ± 4.02	9.8 ± 2.26^{b}
AM fungi	97 ± 13.86^{a}	93.62 ± 11.95^{a}	37.09 ± 11.38^{a}	37.88 ± 11.38^{b}	9.03 ± 6.43	41.6 ± 2.84^{a}
AM fungi+ Ent. sp.	95 ± 12.79^{b}	72.48 ± 9.67^{c}	26.81 ± 11.35^{a}	34.87 ± 10.17^{b}	9.03 ± 9.04	30.6 ± 2.29^{a}
$F_{value\ (3,36)}$	5.51	19.5	3.77	3.23	0.22	60.13
$P < 0.05$	0.003	0	0.019	0.033	0.878	0

Values are means ± standard errors. Different letters indicate significant difference.

3.8 DISCUSSION

3.8.1 Growth evaluation of selected seedlings

The synergistic effect of PGPR and AM fungal inoculants in promoting plant productivity are receiving increased attention (Khan and Zaid, 2007). In this study the effect of single and combined inoculation of AM fungi and *Enterobacter* sp. in promoting plant development were investigated in *M. aethiopicum*, *S. myrtina* and *A. ferox* seedlings used for the restoration of degraded thicket. Arbuscular mycorrhizal fungal inoculation stimulates the production of phytohormones e.g. cytokinins (Ramakrishnan and Selvakumar, 2012; Kavitha and Nelson, 2013). Cytokinins promote cell division and expansion of shoots playing a major role in shoot morphology (Kavitha and Nelson, 2013). Even though cytokinins content was not tested in this study, this indirectly may have contributed to increased shoot height and biomass and canopy volume of AM fungal inoculated *M. aethiopicum* seedlings. The promotion of seedling growth was clearly evident over a period of time in relation to shoot height for both *M. aethiopicum* and *S. myrtina* seedlings. After

14 weeks, the AM inoculated seedlings grew taller. Establishment of AM fungi can take approximately 4 weeks with measurable difference being observed after 8-12 weeks (J. Dames personal communication).

The combined inoculation of AM fungi and *Enterobacter* sp. had no significant or synergistic effect on *M. aethiopicum* seedlings. Medina *et al.* (2003) reported similar results on the shoot biomass of *Medicago sativa* plants inoculated with *Bacillus* spp. and AM fungi. However, Fulmaka, (2015) found better shoot biomass when *P. afra* inoculated seedlings inoculated with AM fungi in combination with *Bacillus megaterium* and with *Bacillus thuringiensis*. Combination of inoculants may have a negative or poor impact on plant growth resulting from a negative interaction between the inoculants (Zhang *et al.* 2010). The lack of synergistic effects from the inoculum suggests the presence of competition for root exudates by both agents (Kohler *et al.* 2007) leading to a poor plant development. Roots exudates provide PGPR with C and nutrient sources while these exudates are also known to stimulate AM spore germination and hyphal growth initiating the symbiotic interaction (Bais *et al.* 2006). Utilization of root exudates by PGPR could reduce their concentration in the rhizosphere to levels below those required for AM fungal signalling.

The combined inoculation of AM fungi and *Enterobacter* sp. did not significantly affect shoot height of *M. aethiopicum* seedlings. However, Fulmaka, (2015) found no significant difference in shoot height of *P. afra* seedlings inoculated when inoculated with AM fungi and in combination with single inoculants of selected bacterial strains (M2 *Bacillus megaterium*, HB2 *Bacillus thuringiensis*, HP5 *Bacillus subtilis* and HP6 *Bacillus* sp.) but, it promoted shoot height of *S. myrtina* seedlings. This growth promotion was observed with single inoculants highlighting the influence of AM fungal inoculation and synergistic effect of these inoculants on the plant growth. Barea *et al.* (2005) stated that inoculum combinations could promote plant development, but requires selection of compatibility microbes that interact in the rhizosphere (Kohler *et al.* 2007). The microbes selected for this study indicate compatibility with *S. myrtina* as a host plant. Although AM fungi are regarded as non-specific associating with over 80% of plant species interaction between AM fungi, other soil microbes and host plants may influence the symbiotic associations.

The shoot biomass of *M. aethiopicum* seedlings was significantly increased by the AM fungal inoculation. Increase in above ground biomass is a commonly cited benefit of the AM fungal symbiosis for a variety of host plants. Youpensuk *et al.* (2005) reported increase in shoot biomass of *Macaranga denticulata* when inoculated with *Acaulospora* spp. (an AM fungus) increases in shoot biomass are a direct result of water and nutrient uptake promoted by the AM fungi in exchange for the C required for growth (Zhang *et al.* 2010).

Mystroxylon aethiopicum seedlings inoculated with *Enterobacter* sp. had a lower shoot biomass and height than other treatments. Fulmaka, (2015) found similar results on the shoot biomass of *P. afra* seedlings inoculated with M1 *Enterobacter aerogenes*, M2 *Bacillus megaterium*, HB2 *Bacillus thuringiensis*, HP5 *Bacillus subtilis* and HP6 *Bacillus* sp. inoculants. Ahmad *et al.* (2005) stated that the effect of rhizobacteria on plant growth is highly dependent on microorganism strains. Even though some bacterial isolates display PGPR characteristics, it is possible their ability to promote plant growth development in soil is hampered (Vessey, 2003). This could be due to the inability of the bacterial strain to maintain its growth in nutrient and C limiting soils (Nie *et al.* 2015).

No significant differences in the root dry biomass was recorded for any of the host plants regardless of inoculants. Fulmaka, (2015) found similar results in roots of *P. afra* seedlings when inoculated with single inoculant of M1 *Enterobacter aerogenes*, M2 *Bacillus megaterium*, HB2 *Bacillus thuringiensis*, HP5 *Bacillus subtilis*, HP6 *Bacillus* sp. and in AM fungi treatment from soil collected from the Thicket in the Eastern Cape. The *Enterobacter* sp. selected was shown to produce IAA which promotes root growth but did not have an influence on this study. Although some roots were lost while removing the soil at the end of the trial, the loss was deemed insignificant across all the treatments and would have little impact on results.

Selected inoculants had no significant increase on the shoot biomass of *S. myrtina* and *A. ferox* seedlings. This was also observed for canopy volume of *S. myrtina* seedlings. Similar results were found by Badenhorst, (2014) when *A. ferox* was inoculated with bacterial strain of *Bacillus toyonensis, Bacillus thuringienesis, Bacillus subtilis subsp.* and in combination with AM fungal inoculant in the Eastern Cape. Kohler *et al.* 2007 stated that although interactions between bacterial species and AM fungi have been reported the mechanisms are not completely understood. It has also been shown that when plants and microbial inoculant inhabit the rhizosphere they may not

result in any beneficial effect on plant development (Valdenegro *et al.* 2001). Perhaps an indication of the regulatory soil environment and the need for complex microbial populations rather than single inoculants.

The positive relationship between shoot height and root biomass in *S. myrtina* seedlings when inoculated with both *Enterobacter* sp. and AM fungi was shown and could have indicated a synergistic effect of these inoculants. Kohler *et al.* (2007) reported on the significant role of the bacteria, *B. subtilis,* and *Glomus intraradices* (an AM fungus) in the solubilization of inorganic phosphate and mineralization of organic phosphates. Phosphate is immobile in soil and diffusion is limited often resulting in a depletion zone around roots. AM fungal mycelium being able to reach these sources of P, facilitate their transport and transfer to the host plant (Barea *et al.* 2005; Arthursson *et al.* 2006).

When the supply of P increases in the soil AM fungi absorb P through hyphae and greater shoot growth is observed (Marschner *et al.* 1996). Therefore, it can be concluded that this could have directly contributed to the increase in shoot height rather than root biomass of *M. aethiopicum* and *S. myrtina* seedlings.

3.8.2 Mycorrhizal colonization

Successful AM fungal colonization was observed in roots of *M. aethiopicum* and *A. ferox*. Confirming the mycorrhizal interaction, colonization has been generally regarded to be linked to enhanced uptake of nutrients and growth (Clark and Zeto, 2000) of the seedlings.

Some PGPR are known to enhance AM fungal growth, exhibiting helper characteristics which support spore germination and mycelia extension (Xavier and Germida, 2003; Dames and Risdale, 2012) enabling root colonization of the host plant (Garbaye, 1994). Toro *et al.* (1997) observed that when *Enterobacter* sp. was inoculated with AM fungi in *Allium cepa* plants it increased root colonization. Use of combined inoculants on *A. ferox* seedlings did not significantly alter colonization percentage but negatively affected *M. aethiopicum* colonization. The *Enterobacter* sp. therefore did not show mycorrhizal helper characteristic in this study.

Roots in the control and *Enterobacter* sp. treatments showed low levels of colonization. For the duration of pot trial seedlings were irrigated through overhead sprinkler which may have splashed

inoculum from AM fungal inoculated pots. Wandeto, (2014) stated that contamination could also occur during experiment when spacing between pots is limited or soil is not highly sterilized. Pasteurisation reduces microbial load but does not eliminate it entirely and could potential carry over some AM fungal propagules. Seeds and vermiculite used for germination were not initially sterilized and Aloe roots were pruned but not totally severed. This may have contributed to some contamination as well due to carry over of AM inoculum.

Wang and Qiu, (2006), found that species from Rhamnaceae family can be colonized by AM fungi. However, AM fungal colonization was not confirmed in *S. myrtina* seedlings, due to highly pigmented root epidermal cells which did not respond to clearing. Although colonization was not confirmed increase in shoot growth and canopy volume was observed in seedlings inoculated with AM fungi in combination with *Enterobacter* sp. indicating a symbiotic interaction.

Arbuscules were observed in *M. aethiopicum* seedlings inoculated with AM fungi. Similar results were observed by Birhane *et al.* (2013) in *Acacia etbaica* seedlings when inoculated with AM fungi. Arbuscules are specialized haustorial organs capable of tremendous metabolic activity. They are formed within roots cortical cells (Brundrett *et al.* 1996) and are responsible for nutrient exchange between the fungus and host plant especially P and C (Smith and Read, 2008; Lewandowski, 2012). The high level of AM fungal colonization observed was closely related to the observed improvement in *M. aethiopicum* seedlings growth. Although colonization and nutrients exchange structures such as arbuscules were recorded in *A. ferox* seedlings this was not accompanied by an increase in biomass.

Vesicles were also recorded in both *M. aethiopicum* and *A. ferox* inoculated seedlings. Vesicles are lipids storage structures for the storage of excess C providing an energy reserve for the fungus (Smith and Read, 2008; Gopal *et al.* 2012).

External hyphae were observed in *M. aethiopicum* and *A. ferox* seedlings inoculated with AM fungi. AM fungi develops an extensive network of external hyphae of about >10 µm which ramifies into the soil to explore nutrients beyond the limited zone of the root system. It is responsible for the uptake and transport of nutrient ions (Wandeto, 2014; Srivastava, 2012).

3.8.3 Microbial dependence of selected seedlings

Giri *et al.* (2007) defined "mycorrhizal dependency (MD) as the degree to which plant species are dependent on mycorrhizal association to produce its maximum growth at a given level of soil fertility". This indicator was adapted to include both microbial inoculants and renamed Microbial Dependency (MiD) *M. aethiopicum* and *A. ferox* seedlings inoculated with *Enterobacter* sp. were independent meaning that plant growth was not directly associated with the bacterium. *M. aethiopicum* seedlings were dependent on AM fungi suggesting that plant growth was directly influenced by this symbiosis (van der Heijden *et al.* 1998). To ensure *M. aethiopicum* seedling establishment when transplanted into field soil AM fungal inoculation is recommended in order to ensure access to nutrients and water.

Shoot biomass of *S. myrtina* seedlings was marginally dependent on *Enterobacter* sp. which showed various plant growth promoting characteristics. Indicating that the interacting could indirectly promote survival under field conditions (Schachtman *et al.* 1998).

M. aethiopicum and *S. myrtina* seedling were moderately to highly dependent on AM fungi when combined with *Enterobacter* sp. this dependency was reduced in *M. aethiopicum* seedlings as indicated by the reduced colonization of roots. Shoot biomass dependency was consistent regardless of inoculants indicating that inoculation would be beneficial for sustaining shoot development. The marginal dependency of *A. ferox* to combined inoculation could indicate that activities of *Enterobacter* sp. could be required for AM establishment and subsequently influence biomass in the longer term (Barea *et al.* 2005 and Artursson *et al.* 2006).

3.9 CONCLUSION

It can be concluded that at the seedling stage *M. aethiopicum* benefits from AM fungal inoculation promoting shoot biomass and canopy volume while *S. myrtina* benefits in terms of shoot growth. Although these benefits were not evident for *A. ferox* at the seedling stage indications are that combined inoculation is necessary for adequate acquisition nutrients and water.

CHAPTER FOUR: THE ROLE OF MICROBIAL PLANTS INTERACTIONS ON SOIL PARAMETERS

4.1 INTRODUCTION

Plant growth promoting rhizobacteria and AM fungi improve the availability and utilization of soil nutrients by host plants through their synergistic interaction (Marschner and Dell, 1994; Dames and Ridsdale, 2012). Wu *et al.* (2005) reported that microorganisms increased soil nutrient uptake of N, P, Zn, Ni and improved soil properties. According to Han and Lee, (2005) several species of *Pseudomonas* can convert insoluble rock phosphate to soluble forms making it available for plant growth. Plant growth promoting rhizobacteria can also, exert a beneficial effect on plant growth by fixing nitrogen from the atmosphere, solubilizing phosphate and improving soil fertility (Ramakrishnan and Bhuvaneswari, 2014). Extracellular polysaccharides produced by bacterial species contribute in improving soil structure and protect roots against harmful substances (Ullrich, 2009).

In P-deficient soils, phosphate-solubilising micro-organisms release phosphate ions in the soil and AM fungi transfer them to the plant (Artursson *et al.* 2006). The AM hyphal network can also grow extensively in soil, extend across nutrient depletion zones, and absorb P from up to several centimetres away from the root surface (Smith and Read, 2008). Arbuscular mycorrhizal fungi also produce and release glomalin into the soil environment improving soil structure and water holding capacity (Wright and Upadhyaya, 1998).

The aim of this study was to assess the role of AM fungi, *Enterobacter* sp. and plant interaction in improving soil water holding capacity and nutrient availability.

4.2 MATERIALS AND METHODS

4.2.1 Nutrient analysis

Approximately 500 g of soil from each treatment was sampled after harvesting of the pot trials and sent to Eco-Analytica Laboratories, Potchefstroom, South Africa, for nutrient analysis. The nutrient analysis included quantification of Calcium (Ca), Magnesium (Mg), Potassium (K), Sodium (Na) and Phosphorus (P), electrical conductivity (EC), pH(H_2O), pH(KCl) and the

percentage of Carbon (C) and Nitrogen (N). Results were compared to initial nutrient analysis conducted on the soil from degraded thicket (Section 2.5, Table 2.1 and 2.2)

4.2.2 Soil water holding capacity

To assess water holding capacity, retention of water in a known quantity of soil was monitored. Initial soil sample was collected from degraded site before restoration. Soil from each treatment after restoration was also collected, combined, dried, sieved and weighed. Four filter paper disc (15 cm diameter Whatman #1) were placed in a funnel on top of a 1000 ml standard measuring cylinder. A 100 g sample of soil from each treatment was placed in the funnel and 100 ml of water was gently poured onto the soil surface. The soil was allowed to drain for 2 hours and the amount of water in the measuring cylinder was recorded. This value was used to calculate the percentage water holding capacity of the initial soil sample as well as the soil collected after restoration from the pot trial.

$$\text{SWH capacity (\%)} = \frac{Vol_{Total} - Vol_{collected}}{Vol_{Total}} * 100$$

(Okalebo *et al.* 1993)

The analysis was conducted using 3 replicates

4.3 ANALYSES

Statistical analysis was not performed on soil nutrients analysis data as only composite samples from the treatment pots were chemical analysed. The average soil nutrient concentration interacting with *M. aethiopicum*, *S. myrtina* and *A. ferox* seedlings was calculated. Soil nutrients values after restoration were compared to the value obtained before restoration. SWH capacity was analysed using one-way analysis of variance, and comparisons among means were made with the Tukey's significant difference (HSD) test, calculated at $P<0.05$. Statistical procedures were carried out with the software package STATISTICA 13.0 for Windows.

4.4 RESULTS

All measured nutrients before propagation (expect P) were higher in soil from the degraded site. (Section 2.5, Table 2.1).

The average total amount of soil nutrients interactive with *M. aethiopicum, S. myrtina* and *A. ferox* seedlings after propagation revealed that pH(H_2O) and pH(KCl) were acidic (Table 4.2). The C content was moderate and the N was not detected in the soil. The available P however had increased the concentration of K, Mg was moderate; while Na increased and Ca was reduced (Table 4.1 and 4.2) as compared to the initial soil used in the pot trial.

Table 4.1: The concentration of soil nutrients in each treatment interaction after propagation.

Treatment	Ca (mg/kg)	Mg (mg/kg)	K (mg/kg)	Na (mg/kg)	P (mg/kg)	%C	% N
S. myrtina Cont	924.5	193.5	143	119.5	16.7	1.46	0
S. myrtina Ent. sp.	917.5	189.5	139.5	154.5	17.1	1.46	0
S. myrtina AM fungi	925	199	148.5	123.5	16	1.46	0
S. myrtina AM fungi+ Ent. sp.	982.5	203.5	158.5	262.0	19.5	1.46	0
M. aethioicum Cont.	937	203	165.5	111	15.1	1.46	0
M. aethioicum Ent. sp.	938.5	209.5	173.5	114.5	18.2	1.38	0
M. aethioicum AM fungi	934.5	189	206.5	169.5	16.1	1.5	0
M. aethioicum AM fungi+ Ent. sp.	914	194	190.5	115.5	16.1	1.14	0
A. ferox Cont.	797	202.5	115	139.5	16.8	1.34	0
Aloe Ent. sp.	910	207.5	136	153.5	18	1.54	0
A. ferox AM fungi+ Ent. sp.	840	214	136	153.5	18	1.61	0
A. ferox AM fungi+ Ent. sp.	850	211.5	150	204	21.5	1.54	0
Average	905.88	201.38	155.22	151.71	17.43	1.44	0

Table 4.2: The concentration of exchangeable cations in each treatment after propagation

Treatment	**Ca** (cmol(+)/kg)	**Mg** (cmol(+)/kg)	**K** (cmol(+)/kg)	**Na** (cmol(+)/kg)	**pH**(H2O)	**pH**(KCl)
S. myrtina cont.	4.61	1.59	0.37	0.52	6.96	6.33
S. myrtina Ent. sp.	4.58	1.56	0.36	0.67	6.96	6.33
S. myrtina AM fungi	4.62	1.64	0.38	0.54	6.82	5.87
S. myrtina AM fungi +Ent. sp.	4.9	1.67	0.41	1.14	6.33	5.7
M. aethioicum Cont.	4.68	1.67	0.42	0.48	6.7	5.72
M. aethioicum Ent. sp.	4.68	1.72	0.44	0.5	6.75	5.82
M. aethioicum AM fungi	4.66	1.56	0.53	0.74	6.34	5.55
M. aethioicum AM fungi +Ent. sp.	4.56	1.6	0.49	0.5	6.73	5.71
A. ferox Cont.	3.98	1.67	0.29	0.61	5.95	5.23
A. ferox Ent. sp.	4.54	1.71	0.4	0.94	6.01	5.33
A. ferox AM fungi	4.19	1.76	0.35	0.67	5.83	5.21
A. ferox AM fungi +Ent. sp.	4.24	1.74	0.38	0.89	5.87	5.15
Average	4.52	1.66	0.4	0.68	6.44	5.66

No significant difference in SWH capacity was recorded between treatments for either of the seedlings (Figure 4.1, 4.2 and 4.3).

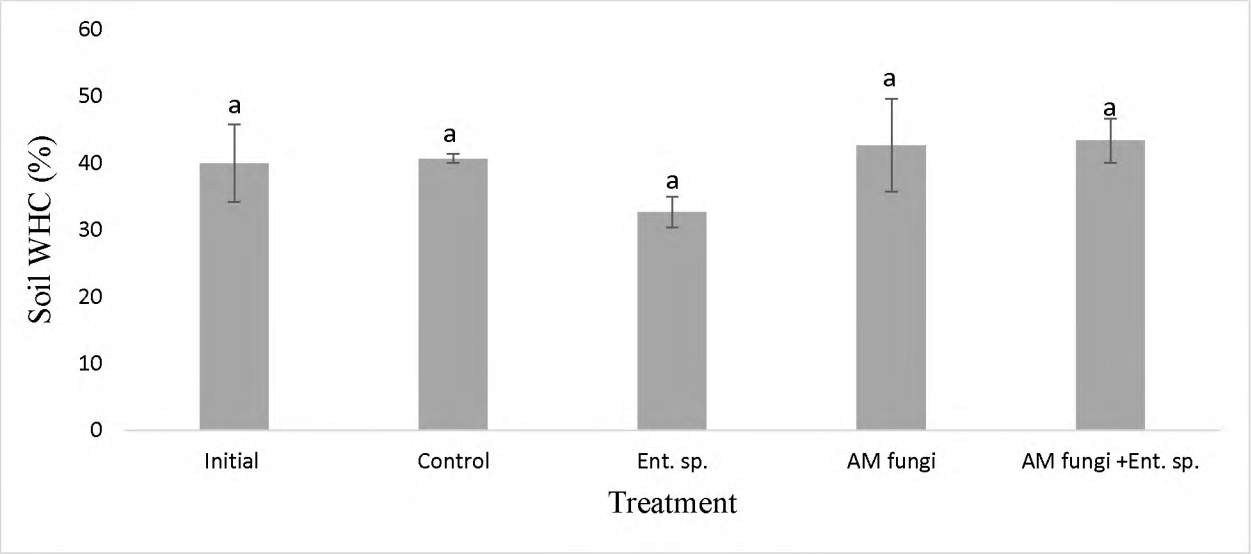

Figure 4.1: Soil water holding SWH (%) capacity of soil with *Mystroxylon aethiopicum* seedlings showed no significant differences. Bar represents ± standard error.

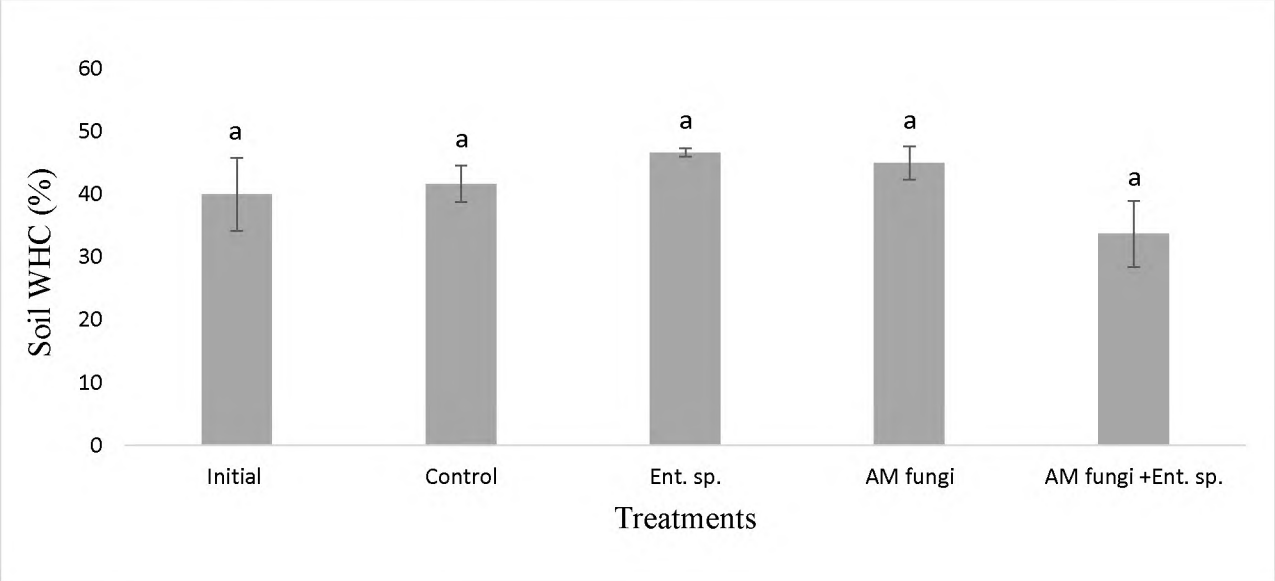

Figure 4.2: Soil water holding SWH (%) capacity of soil with *Scutia myrtina* seedlings showed no significant differences. Bar represents ± standard error.

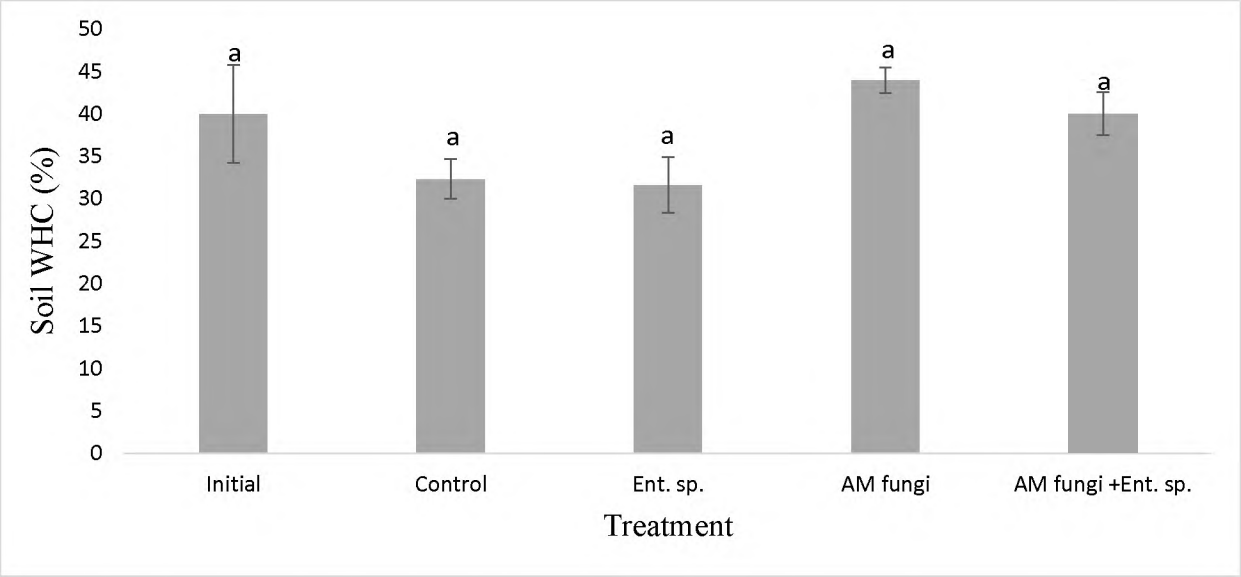

Figure 4.3: Soil water holding SWH (%) capacity of soil with *Aloe ferox* seedlings showed no significant differences. Bar represents ± standard error.

4.5 DISCUSSION

4.5.1 Soil nutrients

Calcium is an essential nutrient required for plant growth and development (White and Broadly, 2003) and contributes to various structural roles such as cell wall and membrane formation (White and Broadly, 2003). The initial soil used in the trial had a higher Ca content than after the seedlings were harvested indicating that Ca was utilised to sustain seedling growth and development.

Berthrong *et al.* (2009) noted that afforestation accelerated the loss of Ca from the mineral soil under conifers, native and exotic tree species. Loss of Ca from the thicket biome soil maybe closely linked to the increase in Na in the soil. Increase in Na impairs drainage and increase compaction (Rahim *et al.* 2011).

Magnesium plays an important role in plant physiological processes acting as a co-factor for >300 enzymes (Senbayram *et al.* 2016). Magnesium concentration was found to be moderate in the soil between 1-3 cmol(+)/kg (Horneck *et al.* 2011) before and after propagation under *M. aethiopicum, S. myrtina* and *A. ferox* seedlings. Salt stress inhibits the uptake of essential mineral elements such as Mg due to the antagonistic relationship between the elements (Hashem *et al.* 2016).

Potassium is essential for plant growth (Zain and Ismail, 2016) and was found to be moderate (0.3-0.7 cmol(+)/kg) (Horneck *et al.* 2011) in the soil before and after propagation in the seedlings. Increased Na also results in decreased soil K possibly due to removal of Ca from the cell membranes altering its integrity and affecting K uptake (Cramer *et al.* 1985).

Sodium concentration was lower in the soil before propagation (Table 2.2), Na is not essential for growth and is found in low concentration in soil, but Ca, Mg and K are the most essential elements needed by plants (Whitehead, 2000). The high Na and reduced Ca may have been a result of irrigation which causes leaching (Provin and Pitt, 2001). Pasteurization of soil can also release elements bound on soil particles (Wandeto, 2014). The seedlings were irrigated with filtered, UV treated municipality water with no additional fertilization, all nutrients were obtained from the soil.

The concentration of Na in the soil after propagation was higher (0.7-2 cmol(+)/kg) (Horneck *et al.* 2011) in *S. myrtina* seedlings inoculated with a combination of AM fungi and *Enterobacter* sp. inoculants (Table 4.2). Similar results were found by Kohler *et al.* (2009, 2010) in areas that were affected by high concentration of Na recognising that PGPR and AM fungi contributes to plant salt avoidance by restricting the uptake of Na from the soil. This could result in accumulation of higher concentrations of Na in the soil.

Arbuscular mycorrhizal fungi reduce the uptake of Na, which reduces its negative toxic effect on plants thereby promoting the growth of host plants (Tian *et al.* 2004; Elhindi *et al.* 2016). Elhindi *et al.* (2016) showed that mycorrhizal plants prevent Na transfer to the shoots. The increase in Na did not affect the growth rate and development of the *M. aethiopicum*, *S. myrtina* and *A. ferox* seedlings. Emphasising the important role played by AM fungi alone and in combination with *Enterobacter* sp. under these conditions. Almethyeb *et al.* (2013), supported the conclusion that AM fungi with *Enterobacter radicincitans* inoculants applied to faba bean (*Vicia faba*) had a positive influence on salt stress.

The control seedlings showed that they can be adapted to tolerate saline soil. No literature could be found on the tolerance of these seedlings. But it is possible that these Thicket plants have some adaptive qualities.

Phosphate was totally absent in the baseline soil (Table 2.2), but increased after propagation (Table 4.2). Although the concentration of P in the soil may be adequate it is often unavailable for plant

uptake (Schachtman *et al.* 1998). Soil microbes such as AM fungi and PGPR enhance the availability P for plant development (Khan and Zaid, 2007; Miransari, 2011). In P-deficient soils, inoculation using phosphate-solubilizing rhizobacteria increases the available P content (Kumar and Narula 1999) by releasing phosphate ions into the rhizosphere through chelation, exchange reaction and organic acid production (Arthursson *et al.* 2006; Ponmurugan and Gopi, 2006).

The concentration of soil P in *S. myrtina* and *A. ferox* seedlings after propagation was adequate18-25 mg/kg (Horneck *et al.* 2011) in soil inoculated with AM fungi in combination with *Enterobacter* sp. inoculants (Table 3.3). These results are supported by Kohler *et al.* (2007) who noted that the combination of *B. subtilis* and the AM fungus, *Glomus intraradices*, played an important role in solubilisation of inorganic phosphate and mineralization of organic phosphates. Khan and Zaid, (2007) also detected an increase in P when *Bacillus* sp. and *Glomus fasiculatum* were inoculated in the soil. Overall, the inoculation effects of the AM fungi and *Enterobacter* sp. combination used in this study suggests a synergism in increasing P content in the soil through their mineralisation and solubilisation abilities which release bound and complex P into the soil environment. The *Enterobacter* isolate chosen for this study was shown to solubilize P *in vitro* (S. van Aswegen personal communication).

Carbon plays a role in improving water use efficiency through its effect on soil structure and associated soil physical properties (Chan, 2010). After propagation, a decrease in C concentration between the various treatments and control was observed in *M. aethiopicum* and *S. myrtina* seedlings (Table 4.1). Moss had grown on the surface of the soil in the pots (Figure 3.4 and 3.13) which can act as a C sink and assist in moisture retention (Woodin *et al.* 2009). In addition, microbial activity requires C for energy. Carbon was high in the soil before inoculation (Table 2.1), microbes utilize various organic sources of C as a source of energy (Gougoulias *et al.* 2014). Arbuscular mycorrhizal fungi participate in carbon cycling but also serve as carbon storage (Syibli *et al.* 2013).

Arbuscular mycorrhizal fungi also play an important role in regulating carbon fluxes between the biosphere and the atmosphere. Hyphae can live and turn over rapidly for five to six days, this suggests that C can respired back to the atmosphere quickly by AM fungi hyphae (Zhu and Miller, 2003). Arbuscular mycorrhizal fungi also produce glomalin which can store soil carbon (Driver *et*

al. 2005). Glomalin, could influence soil carbon storage indirectly by stabilizing soil (Duchicela *et al.* 2012).

Soil pH was not altered after propagation remaining slightly acidic (Table 3.4). Rosen and Bierman, (2008) noted that soil pH less than 7.0 indicates nutrients can be still be absorbed by plants for growth and development. Changes in the soil pH are limited as the soil buffering capacity helps maintain pH.

Nitrogen was deficient in the soil before and after propagation but plants did not show any of N deficiency (yellowing). A characteristic of the *Enterobacter* sp. used in this study was that it had the ability to fix N. Bacteria with N fixing abilities convert atmospheric N into ammonia which can be utilised by plants. This result in an improved N status for plants and consequently promotes plant growth and development (Azcón-Aguilar and Barea, 2015).

4.5.2 Soil water holding capacity for selected seedlings

Plant growth promoting rhizobacteria and AM fungi are known to promote soil structure. However, the SWH capacity of the treatments was not significantly altered. In this study the glomalin concentration of the soil was not analysed although the presence of AM fungus has been correlated to glomalin. The selected *Enterobacter* isolate was positive for biofilm production indicating ability to produce polysaccharides that also contribute to soil structure. In the long term these compounds could influence soil structure particularly once seedlings are transplanted into the field.

4.6 CONCLUSION

Soil water holding capacity was not altered by any microbial host plant interaction. Soil nutrients particularly Ca, Na, P, and C were however altered indicating the important role of these interactions in the soil ecosystem.

CHAPTER FIVE: CONCLUSION AND RECOMMENDATIONS

5.1. Soil mycorrhizal and nutrient status

Mycorrhizal spores were higher in the intact site as compared to the degraded site. The increase in spore numbers from the intact site represent a positivity diversity response by the AM fungi (Burrows and Pfleger 2002). In addition to this, higher nutrient status in the intact site with higher spore numbers implies greater nutritional benefits to the plant species growing in the soil which will promote plant development. Improved soil health and increased vegetation cover plays a critical role in determining the abundance of AM fungal populations (Johnson *et al.* 2005). These fungi also have a binding action on the soil through the secretion of glomalin that contributes to soil stability and water retention (Duchicela *et al.* 2012). The degraded site had low nutrient availability coupled with the lower number of spores in the soil indicating a reduced ability of AM fungi to form a symbiotic association thus negatively affecting potential benefits (Johnson *et al.* 2005). In general spore numbers were low being less than one spore per gram of soil. However, the presence of active growing roots in the soil which maintains mycorrhizal activity can result in less sporulation (Johnson *et al.* 2005) as spore are produced as a survival mechanism to withstand adverse growing conditions (Smith and Read, 2008).

The mycorrhizal potential of the soils from the sites was not significantly different despite the difference in spore numbers. The host plant used in this bioassay was *Sorghum* which is highly mycotrophic. However, to improve success in soil restoration, these results suggest that attention should be given to mycotrophic host species which could enhance the mycorrhizal infectivity of the soil. Further degradation of the soil would result in loss of mycorrhizal potential. This would require further investigation into the mycorrhizal associations of other thicket species and their mycorrhizal dependency.

5.2 Microbial inoculation of thicket seedlings

Seedling height, biomass, and canopy volume of *M. aethiopicum* seedlings increased as a result of inoculation with AM fungi. However, *S. myrtina* seedlings only increased growth when inoculated with AM fungi in combination with *Enterobacter* sp. The use of single or combined inoculum maybe a strategy to improve plant growth and development. It is recommended that seedlings should be inoculated under nursery conditions before transplanting out in the field.

In this study, there was only one isolate of PGPG tested, the *Enterobacter* sp. Plant growth promoting rhizobacteria isolates differ in their ability in promoting plant development and contribution to nutrient availability (Rodríguez and Fraga, 1999). It is recommended that further investigations consider the response of thicket plants associated with different PGPR isolates. The selection of bacterial isolates and their combination with mycorrhizal fungi also requires more attention. Development of a multifunctional microbial inoculum could strengthen ecological taxa of soil microorganisms and enhance successful restoration once seedlings are transplanted into the field (Wandeto, 2014).

Increase in canopy volume of *M. aethiopicum* seedlings inoculated with AM fungi can be extrapolated over time to result in higher canopy cover when planted out into the field. This would reduce soil erosion, improve soil structural properties such as aggregation and stability and provide cover for seed dispersal by animals and birds, thereby improving the vegetation recovery in degraded thicket sites (Mills *at al.* 2007). Increased canopy volume also indicates improved photosynthetic potential of the plant species (Balota *et al.* 2011; Ferrazzano and Williamson, 2013). The mycorrhizal association provides a for the bidirectional flow of nutrients between the fungi and their host plant. Fungal growth is sustained through the allocation of photosynthetically derived carbon from the host. In order to compensate for this transfer of carbon the host plant's photosynthetic rate increases from 5-30% (Smith and Read, 2008). Photosynthesis and chlorophyll rate was not measured in this study but can be included in further investigation. The use of AM fungal inoculation to promote canopy volume and photosynthesis of seedlings is also recommended.

Aloe ferox seedlings were not dependent on selected inoculants for plant development, this study revealed that these seedlings would grow and survive in degraded thicket soil without the application of inoculants. This indicates that *A. ferox* is a good plant for rehabilitation purpose in degraded soils. Aloes however, are mycorrhizal and would recruit fungi and become colonised from soil over time (Badenhorst, 2014).

Colonization of *M. aethiopicum* and *A. ferox* seedlings was confirmed. The response of seedlings to AM colonization suggested better utilization of soil nutrients. Colonization in *S. myrtina*

seedlings was not confirmed, it is recommended that modification of the staining protocols needs to be investigated for these seedlings. Plants selected for restoration of degraded soil requires the analysis of their mycorrhizal association. Inoculation are most easily achieved in the nursery during plant production before transplanting to the field (Wandeto, 2014). The inoculum used in this study was supplied by Mycoroot and inoculation is conservatively calculated as being less than R0.10 c per seedling. Given that re-inoculation is not required at transplant nor at later stages of growth suggests that the long-term benefits out weight the initial inoculation cost (J. Dames personal communication)

The problem of low soil fertility should be addressed in degraded sites. The introduction of microbial inoculants should be focussed on the best combination that will enhance the availability and uptake of nutrients. Iinoculation with mycorrhizal fungi would stimulate the efficient use of nutrient resources and reduce pollution runoff from inorganic fertilizers (Kamau, 2009). Microbial inoculation had a positive effect on Na avoidance indicating that mycorrhizal intervention can be used to develop strategies for re-establishing plant growth in saline soils. Ashrafi *et al.* (2014) reported that under these conditions mycorrhizal plants are able to increase their chlorophyll content ensuring efficient photosynthesis.

Microbial inoculants can be supported in the nursery by the application of compost. Compost application is beneficial to plant growth due to its chemical and physical properties (Harso, 2016) and serves as a source of nutrients and C to PGPR. This results in reduced utilization of mineral inorganic fertilizers. Generally, under nursery conditions the growth medium is pasteurized to eliminate weeds and reduce potential pathogens. The seedling experiments were conducted in pasteurized degraded soil. The process of pasteurization can also release bound nutrients. No additional nutrients were provided during the experiment but nutrients in the long term can be depleted in pots. This study relied solely on nutrients available in the degraded soil. Compost which is unpasteurised is also a good source of soil microbial that can contribute to plant growth promotion (Harso, 2016).

Although no effect on SWH capacity was recorded the long-term benefits of mycorrhizal association is production of glomalin which aids in improved soil structure. Glomalin production can be assessed in future studies.

5.3 Microbial potential for improved seedling growth

The objective of this study was to assess the potential of microorganisms for the improved biomass production of selected woody and succulent seedlings. This study revealed that interaction between microbial inoculants and plants can be synergistic or even antagonistic. *Mystroxylon aethiopicum* and *S. myrtina* responded to microbial inoculation at this seedling stage. The lack of response in *A. ferox* seedlings does not mean that aloes do not require these microbial associations, but rather that they are microbially independent at the seedling growth stage. The use of AM fungal inoculants combined with selected PGPR is recommended for the long-term establishment of thicket plants when planted in the field and inoculation the propagation stage is strongly recommended.

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