INTERACTIONS OF ARBUSCULAR MYCORRHIZAL FUNGI AND SPORE-ASSOCIATED BACTERIA

By

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INTERACTIONS OF ARBUSCULAR MYCORRHIZAL FUNGI AND SPORE-ASSOCIATED BACTERIA

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ABSTRACT

Arbuscular mycorrhizal (AM) fungi are naturally occurring in roots of terrestrial plants. AM fungi are capable of benefiting the host plant through various mechanisms such as enhanced nutrient supply, alleviation of environmental stress and inhibition of plant fungal pathogens. AM fungal spore-associated bacteria have been previously isolated and shown to have plant growth-promoting (PGP) abilities by several authors. Some bacterial isolates are able to promote AM fungal colonisation of host plants and are known to be mycorrhizal helper bacteria (MHB). This study focused on the isolation of AM fungal spore-associated bacteria, characterization of the isolates according to plant growth promoting abilities and evaluation of their potential to enhance plant growth and mycorrhizal colonisation.

AM fungi were extracted from soils sampled from natural indigenous forest sources, raspberry (*Rubus idaeus* cv. Heritage) and strawberry (*Fragaria ananassa*) farms in South Africa and from a raspberry (*Rubus idaeus* cv. Autumn Bliss) plantation in Argentina. A total of 52 spore-associated bacteria were isolated from the external and internal surfaces of AM fungal spore morphotypes from the two countries. The bacterial isolates were evaluated for their PGP abilities such as phosphate solubilisation, indole-3-acetic acid production, ammonia production and inhibition of the fungal pathogens *Fusarium oxysporum* and *Phythophthora nicotianae* through mechanisms such as siderophore and/ or hydrolytic enzyme production. A total of 23 bacterial isolates from both South Africa and Argentina showing the most potential to be PGP, were identified molecularly as belonging to the genera *Acinetobacter, Alcaligenes, Bacillus, Microbacterium, Micrococcus, Serratia* and *Staphylococcus.*

The ability of ten selected bacterial isolates showing multiple PGP capacity were evaluated for their plant growth promotion and mycorrhizal colonisation enhancement ability on raspberry (*Rubus idaeus* cv. Meeker). Significant differences in increased shoot and root dry weights were shown by the treatments compared to the uninoculated control. The highest increase in shoot and root dry weights were shown by South African (*Bacillus mycoides*) and Argentinean (*Alcaligenes faecalis*) isolates. AM fungal colonisation was significantly enhanced by the South African (*Bacillus mycoides*) and Argentinean (*Micrococcus luteus*) isolates compared to the AM fungal singly inoculated control.

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LIST OF ABBREVIATIONS

<	Less than
>	Greater than
µ	Micron (10^{-6})
AM	Arbuscular Mycorrhizal
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
Bp	Base pairs
cm	centimetre
ddH_2O	distilled and deionized water
DNA	Deoxyribose Nucleic Acid
dNTP	Deoxyribose Nucleotide Triphosphate
ERH	extraradical hyphae
g	gram
hr(s)	hour(s)
i.e.	that is
IAA	indole acetic acid
IRH	intraradical hyphae
ITS	internal transcribed spacer
Kb	kilobase
l/L	litre
mg	milligram
mg/ml	milligram/millilitre

MHB	mycorrhizal helper bacteria
Min	minute(s)
N	nitrogen
nm	nanometer
NCBI	National Centre for Biotechnology Information
P	phosphorus
PCR	polymerase chain reaction
PGPR	plant growth promoting bacteria
PSB	phosphate solubilising bacteria
rDNA	ribosomal deoxynucleic acid
RNA	ribose nucleic acid
rpm	rotation per min
rRNA	ribosomal ribonucleic acid
Sec	seconds
sp.	species (singular)
spp.	species (plural)
Tm	melting temperature
V	volts
vol/vol	volume / volume
wt/vol	weight/ volume

CHAPTER 1

GENERAL INTRODUCTION

1 General Introduction[1]

1.1 Mycorrhizal fungi

Mycorrhiza, a term meaning "fungus root" (from the Greek words *mykes* = fungus and *rhiza* = root) describes a mutualistic symbiotic relationship between a soil fungus and plant roots (Smith and Read, 2008; Dighton, 2009). The mycorrhizal symbiosis is distinguished by the exchange of nutrients between the plant and fungus (Smith and Read, 2008). Mycorrhizal fungi improve the nutrient status of the host plant by providing soil derived inorganic nutrients (mainly phosphorus and nitrogen), other complexed compounds and water to the plant. This is achieved through the extensive network of their hyphae that forage for soil nutrients more effectively by increasing the absorptive surface area and exploiting smaller micro-niches than the coarser plant roots and root hairs alone could achieve (Finlay, 2004; Smith and Read, 2008). In return, the plants supply the mycorrhizal fungi with photosynthetically derived carbohydrates that support fungal growth and reproduction. The mycorrhizal fungi consume approximately 20% (5 billion tonnes of carbon per year) of the carbohydrate products synthesized from plants. They therefore contribute significantly to the global phosphate and carbon biogeochemical cycling, influencing primary production in terrestrial ecosystems (Parinske, 2008).

Mycorrhizal fungi associate with host plants at varying levels resulting in obligately mycorrhizal and facultatively mycorrhizal plants (Brundrett, 2004). Obligate mycorrhizal plants depend entirely upon the mycorrhizal fungi for nutrients such as phosphorous. As a result, the plant and fungus become more closely associated with each other. Facultative mycorrhizal plants can derive their own nutrients from the soil in high soil phosphorous conditions hence depending upon the mycorrhizal fungus only in poor soil conditions (Koide and Schreiner, 1992; Brundrett, 2004). Non-mycorrhizal plants are unable to support mycorrhizal colonisation and some belong to the families *Amaranthaceae, Brassicaceae, Caryophyllaceae* and *Proteacea* (Tester *et al.*, 1987; Giovannetti and Sbrana, 1998; Quilambo, 2003; Smith and Smith, 2011). AM fungi do however, sometimes colonise non-mycorrhizal plants to utilise carbon photoassimilates (Matsumura *et al.,* 2007).

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[1] Sections of this chapter have been published in a review article (Dames and Ridsdale, 2012. What we know about arbuscular mycorrhizal fungi and associated soil bacteria. *African Journal of Biotechnology*, **11**(73), pp. 13753-13760).

The non-mycorrhizal condition has been found to originate under extreme soil conditions such as highly disturbed or waterlogged soils in some plants (Smith and Smith, 2011). The inability to form mycorrhizal colonisation may be a result of accumulation of chemicals such alkaloids, cyanogenic glucosinolates and antifungal compounds found in the root cortical cells or root exudates that prevent hyphal branching (Giovannetti and Sbrana, 1998; Brundrett, 2002; Brundrett, 2004). Also enhanced concentrations of salicylic acid produced by plants have been found to inhibit mycorrhizal colonisation (Medina *et al.*, 2003; Quilambo, 2003). It is suggested that some of these plants function independently by changing the pH of the rhizosphere enhancing soil nutrient availability (Brundrett 2002; Brundrett, 2004; Smith and Read, 2008).

Seven different types of mycorrhizal associations have been classified into ectomycorrhizal and endomycorrhizal based upon their hyphal structures (Figure 1.1) (Brundrett, 2004; Smith and Read, 2008; Bonfante and Anca, 2009, Bonfante and Genre, 2010). Forest species such as trees and shrubs are colonised by ectomycorrhizal fungi. Their hyphae are extracellular comprising of the sheath or mantle wrapped around the outside of the root and extraradical hyphae that extend into the surrounding soil. Epidermal and sometimes the outer layer of cortical cells are surrounded and separated by the development of intercellular labyrinth-like hyphae termed ‘the Hartig net’ that enhances the contact area with root cells. These are characteristic for certain ectomycorrhizas, namely ectendomycorrhiza, arbutoid and monotropoid mycorrhizas (Bonfante and Anca, 2009, Dighton, 2009). Endomycorrhizas include arbuscular, ericoid and orchid mycorrhizas where the hyphae penetrate the root cortical cells forming an intracellular association with the host plant. In Ericoid mycorrhizas, the fungus develops within epidermal cells forming coils that are independent infection units. Orchid mycorrhizas develop highly coiled structures (peletons) within the cortical cells and upon death deposit cellulose and pectin within the host plant. Orchid mycorrhizas differ from arbuscular mycorrhizas by having septate hyphae that are limited to the plants epidermal cells (Bonfante and Anca, 2009, Dighton, 2009). The arbuscular mycorrhizal association is the most complex.

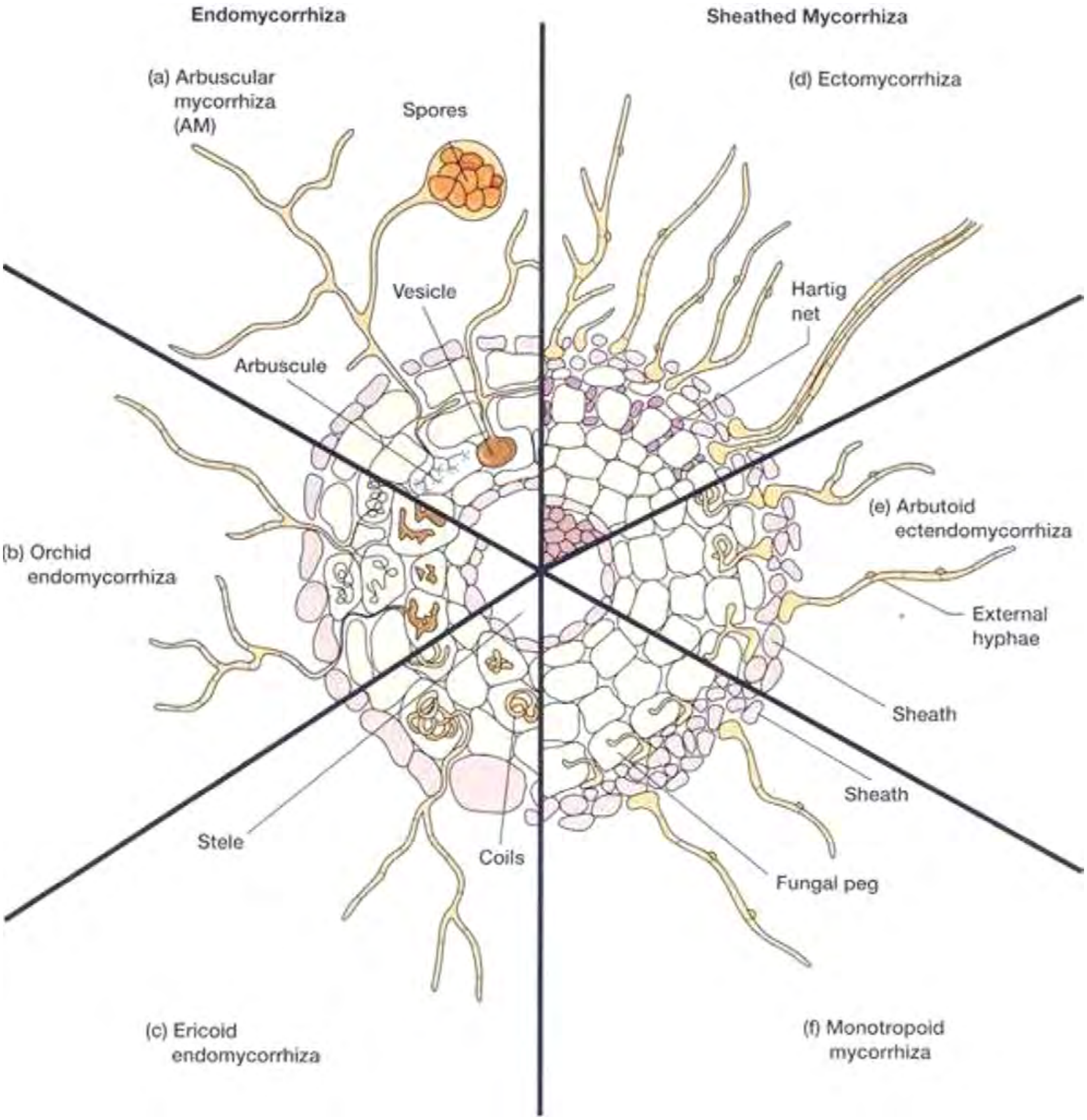


Figure 1.1 Root cross section illustrating different types of mycorrhizal relationships that exist within plants (Prescott *et al*., 2005).

1.2 Arbuscular Mycorrhizal fungi

1.2.1 Description

Arbuscular mycorrhizal (AM) fungi are obligate biotrophs, reproduce asexually, have large multinucleate spores with layered walls and non-septate hyphae (Smith and Read, 2008). The AM symbiosis is ancient and the fossil record from the Rhynie chert reveals AM structures present in early land plants approximately 400 million years ago. The high level of organisation and wide distribution of AM fungi in these plants suggest AM fungi were present in common ancestral terrestrial plants and probably were pivotal in the initial colonisation of land (Parinske, 2008; Bonfante and Anca, 2009; Bonfante and Genre, 2010). AM fungi belong to the monophyletic phylum Glomeromycota, comprising approximately 150 fungal species associating with the roots of approximately 80% of the world's terrestrial plant species. The AM fungi are comprised of three major components: the root which provides carbon to the fungus in the form of sugars, structures within the cortical cells of the plant root which provides the contact between the fungus and the plant cytoplasm, the extraradical hyphae which help take up the necessary nutrients such as nitrogen, phosphorous and water (Smith and Read, 2008).

The AM fungi are characterised by the finely branched tree-shaped hyphal structures termed arbuscules that are formed within root cortical cells of plants during root colonisation by the fungus. These arbuscules are thought to be the main site for nutrient exchange between fungus and plant (Smith and Read, 2008). Due to root colonisation by the AM fungi, root hair development by the plant is suppressed as the extraradical hyphae increase the absorptive area. Other important structures include intraradical hyphae (IRH), vesicles, extraradical hyphae (ERH) and extraradical auxillary cells. IRH allow the fungus to spread within short distances of the root cortical cells to form colonising units such as arbuscules and vesicles. Vesicles are storage compartments for carbon and are rich in lipids (Morton and Benny, 1990; Isaac, 1992; Dodd *et al.,* 2002) (Figure 1.2). Vesicles are found mainly in three genera of Glomeromycota: *Glomus*, *Acaulospora* and *Enthrophospora* (Isaac, 1992). Their formation and development depends upon various environmental conditions such as high or low phosphorous (P) levels (Smith and Read, 2008).

The ERH have been distinguished into three types, the "branching absorptive hyphae" which colonise the rhizosphere in search of nutrients, the "infective hyphae" which search along the surfaces of roots to establish new entry points into the plants and the "reproductive hyphae" which produce the fertile spores after colonisation of the roots (Nagahashi, 2000) (Figure 1.2). Extraradical auxillary vesicles are found mainly in Gigasporaceae and store lipids. They are also involved in nuclei and nutrient (phosphorous or carbon) partionining before sporulation (Morton and Benny, 1990; Isaac, 1992; Dodd *et al.,* 2002).

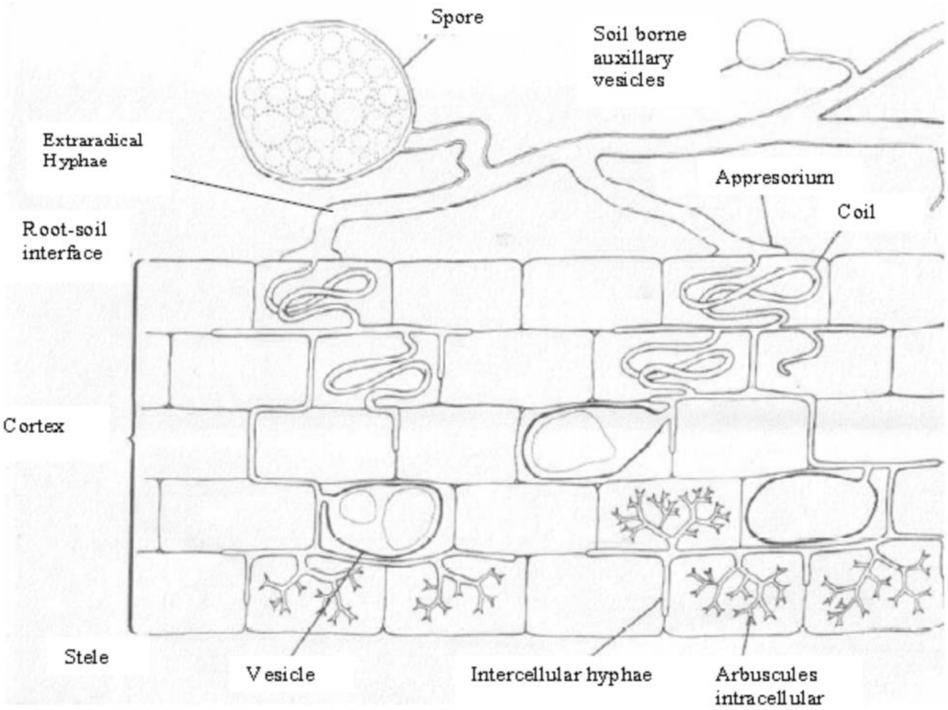


Figure 1.2 Diagrammatic representation of the structures characteristic of arbuscular mycorrhizal fungi in a cortical cell of a plant root (Modified from Isaac, 1992).

1.2.2 Reproduction and Life Cycle

The reproduction of AM fungi is thought to be solely asexual as there has been no evidence to suggest it reproduces sexually (Pawlowska and Taylor, 2004; Smith and Read, 2008). Under favourable conditions, the spores of the AM fungi germinate and undergo a sequence of events based on structural morphogenesis, which is still poorly understood (Barker, *et al.,* 1988). These events have been categorised into asymbiotic, presymbiotic and symbiotic stages (Giovannetti, 2000; Bago and Bécard, 2002).

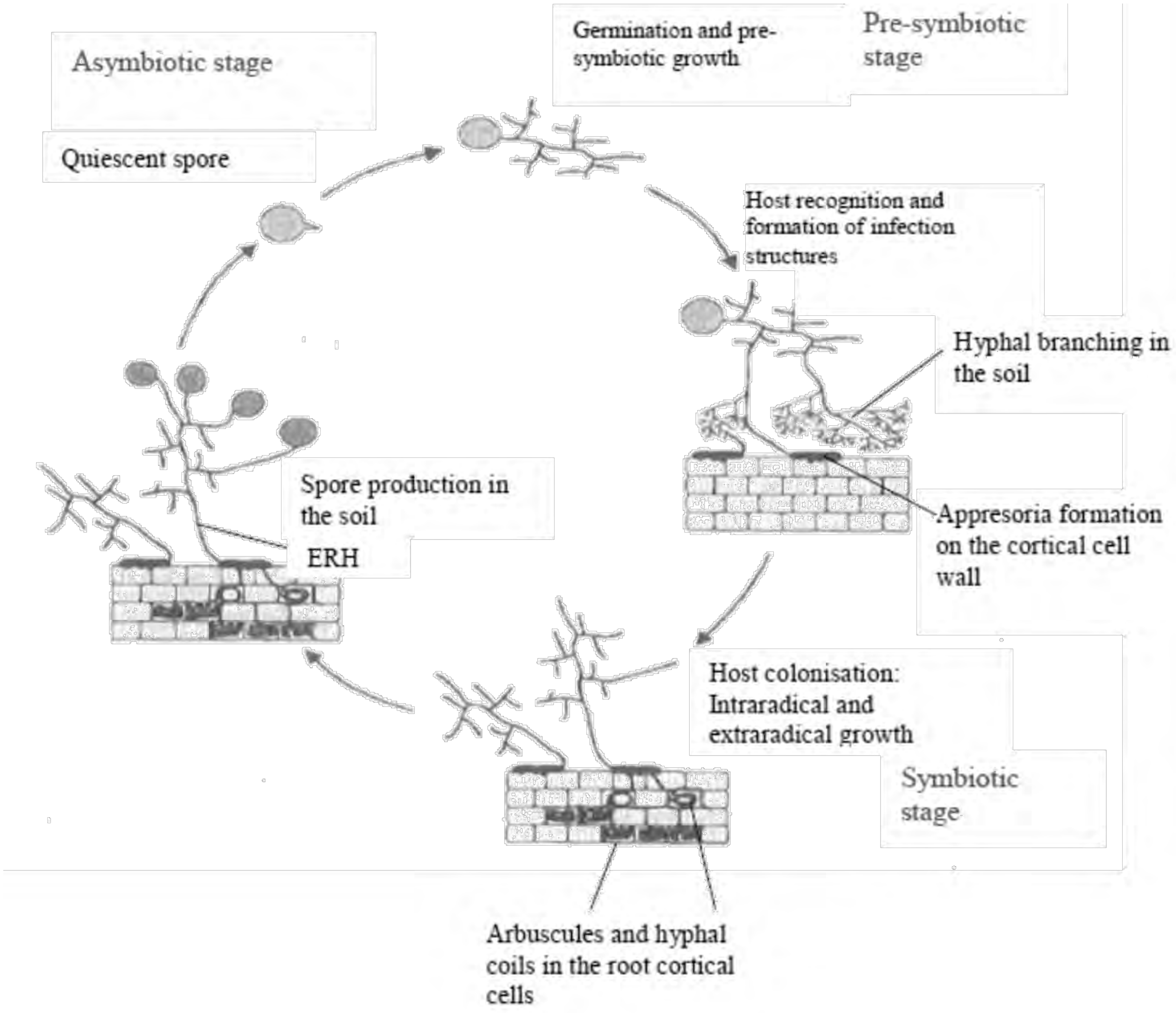


Figure 1.3 Diagrammatic representation of the life cycle of arbuscular mycorrhizal fungi showing the stages of colonization (Modified from Giovannetti, 2000).

Asymbiotic Phase

After a symbiotic association has occurred with a host plant, AM fungal spores are produced and released into the soil by the extraradical hyphae. This stage of the lifecycle can often be referred to as the resting stage (Bago and Bécard, 2002 ; Nagahashi, 2000). These dormant spores can remain alive in the soil for one or two years and the dormancy periods can differ greatly among species and genera (Giovannetti, 2000).

Various factors such as pH, temperature, moisture, CO_2 and organic nutrients are likely triggers that break the dormancy of the spore. This stage is host independent as the AM fungal spores contain energy reserves within stored lipids and carbohydrates used during initial spore germination to sustain the growth of the germ tube. When the presence of a host is not available, germination ceases before excess consumption of the energy supplies becomes depleted or the cytoplasm is retracted within the spore (Giovannetti and Sbrana, 1998; Bago and Bécard, 2002).

Presymbiotic Phase

Germinated spores grow toward the host root by producing hyphal branches. Structures called appressoria are formed which occur on the host root epidermal cell walls (Figure 1.3) (Nagahashi, 2000). An appressorium is used to describe hyphal tip enlargement that attaches to the root surface of the host. This stage is often considered as presymbiotic since no contact is required to occur between the fungus and host plant for the stimulation of hyphal branches to occur, instead branching is under the influence of root exudates such as organic acids, amino acids, phenolics and other compounds (Giovannetti and Sbrana, 1998). Strigolactones have recently been shown to stimulate AM fungal metabolism, branching and spore germination (Hause and Fester, 2005; Parinske, 2008; Bonfante and Genre, 2010). Plant hormones such as auxins have been thought to play a vital role in mycorrhizal colonisation of the host plant roots because the auxins are found in high concentrations during the formation of appressoria (Ludwig-Müller, 2000).

Symbiotic Phase

This stage refers to the penetration and development of IRH and subsequent formation of arbuscules in the cortex of roots. The ERH growth arises after arbuscule formation and is characterised by the release of spores into the soil. Bidirectional exchange of carbon and nutrients between the host plant and the AM fungi occurs only during the symbiotic phase (Nagahashi, 2000; Hause and Fester, 2005; Parinske, 2008; Bonfante and Genre, 2010). Root colonisation brings about the symbiotic interaction between the host plant and fungus.

In AM fungi there are two types of colonization strategies, which are based on the structures of the intraradical hyphae, and these are referred to as the *Arum*-type and *Paris*-type (Smith and Read, 2008). In the *Arum*-type, the intercellular hyphae run along a longitudinal channel between cortical cells in a linear form before entering the cortical cells to form arbuscules. These arbuscules are the main site for nutrient exchange. In the *Paris*-type colonisation, intracellular hyphae grow as coils within the cortical cells and in this type both the hyphal coils and arbuscules are thought to be involved in nutrient release but this has not been fully ascertained (Smith and Read, 2008). Although both of these colonisation strategies have similar percentage root colonisation, they differ in their sites of metabolic activity. AM fungal species are generally non-specific in their choice of host plants (Van Aarle *et al.*, 2005). Specificity, the ability of the fungus to colonize root cells; infectivity, the amount of colonization and effectivity and the plant's response to colonization are three major parameters that influence and determine root colonization (Sylvia *et al.*, 1998).

1.2.3 Benefits of AM fungi

AM fungi provide many benefits to their plant symbionts including enhanced nutrient uptake, increased tolerance to plant pathogens, drought resistance, tolerance to heavy metals and improved soil aggregation to name a few (Smith and Read, 2008).

Enhanced Nutrient Uptake

Plants require varying amounts of macro and micronutrients present in the soil. Soils generally contain high levels of total phosphorous, however only a small percentage is available for plant uptake. P is found in many soils as organic (phytic acid) and complex inorganic forms. Due to the low solubility and mobility of P, plants cannot readily use these forms. Plants therefore obtain P as orthophosphate anions (HPO_4^{2-} and $H_2PO_4^{1-}$) (Rodriguez and Fraga, 1999). The concentration of orthophosphates in the soil are however low (1 to 5 µM). AM fungi contribute to increased P uptake through solubilisation of phosphorous by the production of exudates by the fungal hyphae. The extraradical mycelium of AM fungi secretes phosphatases that aid in the uptake of phosphorous by hydrolysing and releasing phosphorous from organic phosphate complexes (Schachtman *et al.*, 1998).

The increased efficiency of P uptake by mycorrhizal fungi is achieved by the extraradical mycelium developing into the soil and allowing P to be absorbed by the mycorrhiza at distances upto several cm away from the root and transferred to the plant (Bucher, 2007). High mycorrhizal hyphal density provides a greater surface area for the absorption of orthophosphate by plants. The smaller size of hyphae in comparison to the roots and root hairs allows for better exploitation of soil pores and nutrient niches inaccessible to the plant roots.

This is beneficial when a depletion zone occurs around the plant root when freely available nutrients have been utilised and the mycorrhizal hyphae can extend past the ‘depletion zone’, providing nutrients to mycorrhizal plants, which would be inaccessible to non-mycorrhizal plants (Bonfante and Genre, 2010). In AM fungi, phosphate acquisition is achieved through membrane integral proteins, including PHT phosphate transporter and the P-type H^+ ATPase. Following the uptake of P, transport within the fungal structures is mainly in the form of polyphosphates. These polyphosphates may also be stored as nutrient reserves within polyphosphate granules in the cortical cells. Plants also have mycorrhizal specific phosphate transporters that receive Pi from the fungus and deliver it to the plant cells (Bonfante and Anca, 2009).

Host plants therefore have two mechanisms of P uptake, the ‘direct’ pathway via the plant-soil interface by the root hairs and the ‘mycorrhizal’ pathway via the fungal mycelium. Expression of plant epidermal P transporters is reduced in roots colonised by AM fungi and P uptake occurs via the fungal transporters and transferred to the plant. In some cases, AM colonisation results in a complete shutdown of the ‘direct’ pathway, resulting in all P uptake provided by the mycorrhizal pathway (Smith *et al.,* 2003).

Although phosphorous is the main nutrient required by plants, nitrogen is also of great importance for plant growth. Nitrogen is obtained by the extraradical hyphae of AM fungi in many different forms, for example organic nitrogen forms such as amino acids, peptides, ions (NO_3^-, NH_4^+). Li *et al.,* (1991) reported that different *Glomus* sp. can assimilate and metabolise both organic and inorganic sources of nitrogen by glutamate synthetase activity produced by the extraradical hyphae. The presence of a transporter for high-affinity uptake of NH_4^+ has been identified in the extraradical mycelium of *Glomus* sp. (Lopez-Pedrosa *et al.*, 2006).

AM fungal colonisation can also induce plant nitrate transporters and a fungal (*G.intraradices*) nitrate reductase may be involved in the transfer of nitrogen in a reduced form (Hause and Fetter, 2005). The concentrations of phosphorous and nitrogen in the soil can determine the rate of the uptake of other micro (Fe, Cu, Mn, Zn) and macronutrients (K, Ca) by mycorrhizal plants. Liu *et al.*, (2000) confirmed this in their study that determined the role of AM fungi in the uptake of Cu, Zn, Mn and Fe in maize, which showed the uptake of these nutrients was significantly influenced by soil phosphorous nutrition.

Drought Resistance and Water Relations

Drought stress is a major factor affecting agricultural industries in arid and semi-arid regions. Drought has a serious impact on nodule function, inhibits photosynthesis and disrupts oxygen control mechanisms in nodules of legumes. Drought stress inhibits cell expansion and division, resulting in reduced leaf area development. Nitrogen availability is also reduced resulting in decreased N uptake by the plants and lowered N assimilation rates (Quilambo, 2003, Boomsa and Vyn, 2008).

The AM fungal hyphae allows a greater access to water in the soil through mechanisms in the plant such as stomatal conductivity and regulations, higher transpiration rates, increased root hydraulic activity (enhanced stele tissue size), osmotic adjustments (which promotes turgor pressure maintenance) and maintenance of cellular water pressure, cell elasticity changes and increased photosynthetic activity (Davies *et al.*, 1993, Amerian and Stewart, 2001, Auge, 2001). These mechanisms may be a secondary consequence of enhanced plant P nutrition through mycorrhizal colonisation. Mycorrhizal fungi also promote drought tolerance by enhancing rooting depth and length by affecting the soil or plants cytokinin and indole-acetic acid concentrations (Smith *et al.,* 2010). Auge, (2001) discusses various experimental results conducted by researchers on various crop species and drought tolerance/ resistance mechanisms involved in his extensive review.

A study by Asrar and Elhindi (2011) on marigolds (*Tagetes erecta*) and an AM fungus (*Glomus contrictum*) revealed that in the mycorrhizal plants had an increased shoot dry weight, higher chlorophyll a and b concentrations in leaves and higher carotenoids in flowers compared to the non-mycorrhizal plants under severe drought stressed conditions.

The mycorrhizal colonisation improved drought resistance in marigold plants as a consequence of enhanced P uptake and water through the AM fungus which according to Koide (2000) increases stomatal conductance and transpiration rate. A similar study by Subramanian *et al* (2005) also showed an increase in the yield of tomato (*Lycopersicon esculentum*) fruits by 24.7% and higher ascorbic acid concentrations when grown inoculated with *Glomus intraradices* under severe drought stressed conditions.

Wu and Xia (2006) investigated the effect of *Glomus versiforme* on the drought tolerance of Tangerine (*Citrus tangerine*) and reported that the mycorrhizal plants had increases in leaf water potential (21%), transpiration rates (27%), photosynthetic rates (50%), stomatal conductance (29%), lowered leaf temperature (5%) and increased relative water content compared to the non-mycorrhizal plants under severe drought conditions. The authors also found increased levels in inorganic ions such as K^+, Ca^{2+} and Mg^{2+} in the leaves and roots of the tangerine plants. They concluded that organic solutes and inorganic ions are accumulated under water stress conditions to increase the osmotic adjustment of the plant. The AM fungal colonisation improved osmotic adjustment and photosynthetic parameters through accumulation of more carbohydrates, inorganic ions and water and increase plant biomass resulting in enhanced drought tolerance in tangerines.

Increased Tolerance to Plant Pathogens

Colonisation of plant roots by AM fungi is suggested to increase the plant's tolerance to pathogens by acting as biological control agents. Biological control preserves the quality of the environment by reducing the inputs of chemicals. The study of the AM symbiosis in plant protection against pathogens began in 1970's and a great deal of published information has been released on the subject, however little is known about the underlying mechanisms. Many reviews on the subject have focused on mechanisms of interaction such as enhanced nutritional status of host plant, competition, morphological changes, induced plant defence mechanisms and reduced infection sites (Hooker *et al.*, 1994; Azcon-Aguilar and Barea, 1996; Harrier and Watson, 2004; Akkopru and Demir, 2005; Wehner *et al.*, 2010).

Enhanced nutritional status of host plant

The ability of AM fungi to increase plant's tolerance to pathogens may be due to the increased nutrient uptake. The AM fungal interaction with soil root pathogens is brought about by the enhanced uptake of nitrogen, phosphorous and other nutrients. AM fungal symbiosis results in more vigorous plants so that the plant itself may be more resistant to or more tolerant of pathogens and their effects (Azcon-Aguilar and Barea, 1996). Some studies have indicated that phosphorous-induced changes in plant root exudation could potentially reduce spore germination of some fungal pathogen. While other studies have shown that phosphorous nutrition is not directly involved in reducing disease (Linderman, 2000).

Damage compensation

Arbuscular mycorrhizal fungi can increase the plants' tolerance to pathogens by compensating for the loss of root biomass or function caused by pathogens. This indirect mechanism contributes to biological control by conserving the root-system function both by the growth of fungal hyphae into the soil increasing the absorptive surface area of the roots and the maintenance of root cell activity through arbuscule formation (Azcon-Aguilar and Barea, 1996).

Competition for nutrients and infection sites

AM fungi have direct access to the plant photosynthetic products whereas pathogens, which are not obligate biotrophs can only access carbon from other organic sources. Therefore competition for nutrients between the AM fungi and pathogens such as *Fusarium* and *Phytophthora* seems unlikely or limited (Linderman, 2000). Fungal root pathogens and AM fungi, may both colonise the same host tissue (particularly in the case of AM fungi and *Phytophthora*). These two organisms may develop in different root cortical cells which indicate some likelihood of competition for space (Azcon-Aguilar and Barea, 1996). A study by Ozgonen and Erkilic, (2007) looked at growth enhancement by arbuscular mycorrhizal fungi in pepper and Phytophthora blight (*Phytophthora capsici* Leonian) control. The plant heights of the pepper (*Capsicum annuum* L.) plants was increased by the arbuscular mycorrhizal fungi (*Glomus fasciculatum* (GF), *Glomus etunicatum* (GE), *Gigaspora margarita* (GiM) and *Glomus mosseae* (GM) by 31.7, 28.3, 25 and 23.4% respectively compared to the control. Fresh weights of shoots were increased by GF (29%), GiM (26.6%) and GE (24.8%).

The fresh weight of roots in the control treatment was 4.9g, whereas in GiM, GF and GE treatments it was 8.1 (66.3%), 8.1 (66.3%) and 7.7 g (58.0%). In GM treatments it increased by 30.1%. Mycorrhizal plants showed a significantly higher dry weight of shoots and roots compared to the control. GiM treatment had the greatest effect on shoot and root dry weight, increasing them by 34.1 and 58.6% respectively. The AM fungi reduced the disease severity by *P.capsici* in pepper plants in field conditions. GM and GE reduced disease severity by 45.7 and 57.2% respectively, followed by GiM (30.7) and GF (14.4%). The amount of capsidiol accumulation in the pepper plants was measured with the control plants having 12.5 $\mu g.g^{-1}$ fresh weights, whereas among the AM fungi treatments, GM had the highest level of capsidiol (40.3$\mu g.g^{-1}$ fresh weight). Their results indicated that mycorrhizal inoculation stimulated growth of pepper plants due to enhanced nutrition. Also, the AM fungi, especially *G.mosseae* can be used against Phytophthora blight of pepper, due to capsidiol, a phytoalexin produced by the fungus which is an antimicrobial compound.

Improved Soil Aggregation

AM fungi improve soil structure through the secretion of glomalin, a “glue-like” proteinaceous, water-soluble and heat stable substance from their hyphae (Steinberg and Rillig, 2003). Glomalin aids in the soil aggregation process by binding soil particles together influencing soil porosity, which in turn promotes aeration and water movement required for good root growth, development and microbial activity. Glomalin is therefore a good indicator for soil aggregation and stability (Rillig, 2004).

Salinity Tolerance

Soil salinity is a major problem with saline soils occupying 7% of the world’s land surface. High levels of salinity (> 0.1% soil content) in soil is caused by soluble salts present in irrigated water and fertilisers, both used in agriculture (Evelin *et al.,* 2009). Soil salinity significantly effects the establishment, growth and development of plants, especially in arid and semi-arid environments. These effects lead to huge losses in productivity.

Direct effects of salt on the plant may be through (a) reduced osmotic potential of the soil (limiting the amount of available water to the plant) and reduced photosynthetic ability of the plant causing physiological drought; (b) toxicity of excessive Na^+ and Cl^- ions towards the cell causing disruption in enzyme structure, damage to cell organelles and plasma membrane, disruption of photosynthesis, respiration and protein synthesis; and (c) nutrient imbalance leading to ion deficiencies (Evelin *et al.,* 2009; Hajiboland *et al.,* 2010).

AM fungi have been known to occur naturally in saline environments. The most predominant species are *Glomus intraradices, Glomus versiform* and *Glomus etunicatum*. AM fungal spore density is high in saline soils since sporulation is stimulated under salt stress which means AM fungi may produce spores even at low-colonisation levels in severe saline conditions (Evelin *et al.,* 2009; Hajiboland *et al.,* 2010).

AM fungi enhance the ability of plants to cope with salt stress by improving uptake of nutrients other than P, improved ion balance, accumulation of osmoregulators, stimulation of protecting enzymes, higher water uptake. Evelin *et al.,* (2009) wrote an extensive review of the mechanisms involved in these processes.A study by Hajiboland *et al.,* (2010) looked at the role of *Glomus intraradices* on salt tolerance in two strains of tomato (*Solanum lycopersicum*) plants, cv. Behta (Salt-sensitive) and cv. Piazar (Salt-tolerant). The AM fungus stimulated growth greatest with Piazar than with Behta under saline conditions. The study revealed that the mycorrhisation alleviated salt-induced reduction of P, Ca, and K uptake. Growth improvement by *G.intraradices* was independent from plant P nutrition under high salinity. The authors also found the mycorrhisation improved net assimilation rates by elevating stomatal conductance and protecting photochemical processes. They concluded that AM fungi may protect plants against salt stress by alleviating salt-induced oxidative stress.

Porras-Soriano *et al.,* (2009) showed that *Glomus mosseae* was the most effective AM fungus (in a study with *G. intraradices* and *claroideum*) in increasing olive (*Olea europaea*) trees tolerance to salt. *G.mosseae* increased the biomass of the olive trees from 163% (shoot) and 295% (root) in non-saline conditions to 239% (shoot) and 468% (root) in saline conditions. *G.mosseae* also increased the number of shoots by 294% and the stem diameter by 61% in saline conditions. The authors also found an increase in K acquisition, which was enhanced under severe saline conditions by 6.4-fold with *G.mosseae.*

Potassium plays a major role in osmoregulation in plants. Therefore *G.mosseae* alleviated salt-induced adverse effects by increasing the acquisition of K which improves the plant's osmoregulation processes.

Toxic metals

Metal compounds are present in the atmosphere and originate from nature (terrestrial, marine, volcanic and biogenic) and anthropogenic (combustion, industrial) sources. Heavy metals in the soil constitute 10% of the sources whereas 90% enters the soil by dry and wet atmospheric deposition and agronomic practices (including fertiliser and sewage application). The toxicity of the metals is related to the concentrations in which they occur in the soil. Exposure to toxic levels of heavy metals reduces plant growth, causing leaf chlorosis and necrosis, turgor loss, decreased seed germination rate and plant death (Gamalero *et al.*, 2009). Heavy metals can affect some developmental stages of the AM fungi or eliminate establishment. The level and toxicity at which heavy metals affect both plants and mycorrhizal fungi is dependant and influenced by many factors such as soil pH, temperature, redox potential, cation exchange capacity and soil organic matter content (Gamalero *et al.*, 2009).

AM fungi help to alleviate plant stunting caused by heavy metals through binding to these metals in the extraradical hyphae in the root zone, or accumulated in the vacuoles, sequestered by siderophores, deposited into plant root apoplasm or into the soil, altering the plant cells ability to capture the metals (Upadhyaya *et al.,* 2010). The polyphosphates produced by AM fungi are proposed to be responsible for sequestering heavy metals (Haselwandter *et al.*, 1994). Glomalin produced by the AM fungi has been shown to sequester metals such as Cu, Cd and Zn. According to Gonzales-Chavez *et al.,* (2004) 1 g of glomalin could extract upto 4.3 mg Cu, 0.08 mg Cd and 1.12 mg of Pb from polluted soils. Therefore AM fungi can stabilise metals in the soil, reduce their availability and decrease their toxicity to other soil microorganisms and plants in the vicinity (Gamalero *et al.*, 2009).

A study by Audet and Chanest (2006) on *Nicotiana rustica* and *Glomus intraradices* revealed that the concentration of Zn was significantly lower in roots of mycorrihzal plants (difference of 18.6 mg total root mass, 1.06 mg in dry mass) than non mycorrhizal plants at the highest Zn concentrations in the soil. The fungus may have immobilised the Zn as a soil contaminant and prevented it from being taken up by the host plant, especially under increased toxic soil Zn concentrations.

Another study by Jianfeng *et al.,* (2009) on *Nicotiana tabacum* and different AM species such as *Glomus spp.* co-inoculated with *Acaulospora spp., A.mellea, G.versiforme* and *G.caledonium.* The authors observed a decrease in soil pH in the rhizosphere of mycorrhizal plants and a decrease in arsenic (As) concentrations compared to non-mycorrhizal plants. The lowered soil pH caused a decline in the As availability as a consequence of the AM fungi modifying the amount and composition of root exudates.

1.3 Soil microorganisms

1.3.1 Rhizosphere Micro-organisms

The soil is a complex environment comprising of a diverse range of microorganisms. Mycorrhizal fungi are critical soil microorganisms providing a direct link between plant roots and soil. AM fungal hyphae may directly interact with other soil microorganisms, providing a means of transport in the soil, substrates required for growth as well as a suitable niche environment. These interactions can affect root development and plant growth performance being either positive, neutral or negative (Johansson *et al.*, 2004).

Mycorrhizal formation can either directly or indirectly affect microbial communities through induced changes of root exudates, transport of carbon compounds or fungal exudation of stimulatory or inhibitory compounds (Gryndler, 2000). Mycorrhizal fungi interact with beneficial soil organisms such as Mycorrhizal Helper Bacteria (MHB), Phosphate-solubilising Bacteria (PSB) and Plant Growth Promoting Rhizobacteria (PGPR) to mention a few (Gryndler, 2000). These groupings refer to functionality of the bacterial species involved and several of these species may overlap between groups.

1.3.2 Mycorrhizal Helper Bacteria

Mycorrhizal Helper Bacteria are organisms which specifically promote the formation of the mycorrhizal symbiosis. Bowen and Theodore (1979) first showed that the presence of bacteria is directly involved in the mycorrhiza formation. They showed both promoting and inhibiting effects of the colonisation of *Pinus radiata* roots in a study with *Rhizopogon luteolus*. MHB are found in a various habitats from the fruiting bodies of ectomycorrhizal fungi, AM fungal spores, extraradical mycelium to other mycorrhizal structures (Gamalero *et al.,* 2003, Barea *et al.,* 2005). Examples of MHB strains include Gram-negative Proteobacteria (*Agrobacterium, Azospirillum,* and *Pseudomonas*), Gram-positive Firmicutes (*Bacillus, Brevibacillus* and *Paenibacillus)* and Gram-positive Actinomycetes (*Rhodococcus, Streptomyces,* and *Arthrobacter)* (Frey-Klett *et al.*, 2007, Tarkka and Frey-Klett, 2008).

Meyer and Linderman (1986) showed enhancement of AM fungal infection and growth of subterranean clover when co-inoculated with a rhizospheric *Pseudomonas putida* strain. A study by Ames (1989) found that seven out of twelve Actinomycetes from AM fungal spores stimulated AM establishment in onion seedlings. A different example of the helper effect is shown by *Rhizobia* which produces 1-aminocyclopropane-1-carboxylate (ACC) deaminase which modulates the ethylene levels in the plant, increasing the tolerance of the plant to environmental stress and stimulating nodulation (Ma *et al.*, 2002). This compound also produced by *Pseudomonas putida* UW4 promotes mycorrhization with the AM fungus *Gigaspora rosea* when inoculated into cucumber plants (Gamalero *et al.*, 2008).

Mechanisms of MHB effect on Mycorrhizal fungi

The extent of the mycorrhizal colonisation depends on various factors, abiotic and biotic environmental interactions, fungal physiology and susceptibility of the root to infection. MHB are able to promote mycorrhizal infection rate at various stages of the tripartite interaction (Tarkka and Frey-Klett, 2008). Five major hypotheses explaining the helper effect were reviewed in Garbaye, 1994, Frey-Klett *et al.*, 2007, Tarkka and Frey-Klett, 2008) (Figure 1.4).

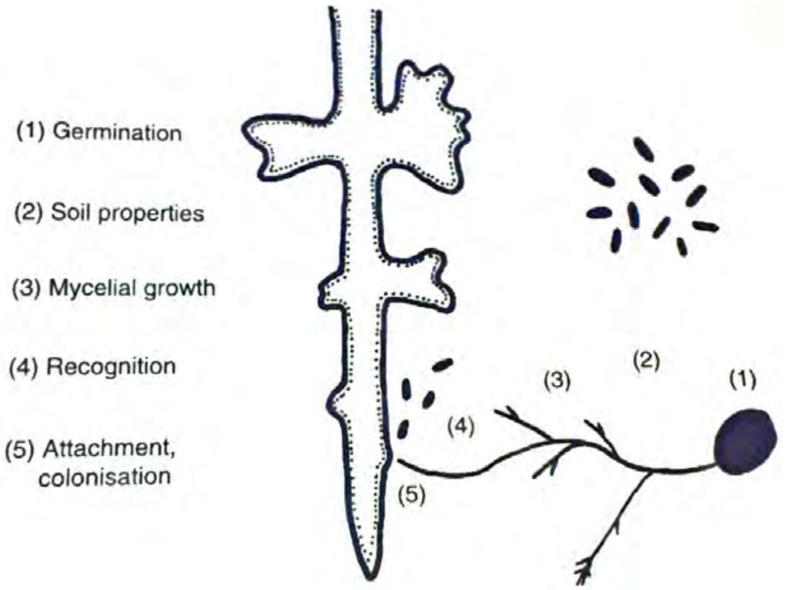


Figure 1.4. Five hypothesised mechanisms for the helper effect of MHB on mycorrhizas. Adapted from Garbaye, (1994) in Tarkka and Frey-Klett, (2008).

MHB effect on fungal propagule germination

Bacteria in the soil can trigger or accelerate the germination process of spores or any other dormant propagules (Figure 1.4-1). This is the first step in the formation of mycorrhizas. Mosse, 1962 found that some rhizosphere bacteria (for example *Pseudomonas* sp.) enhanced the germination of chlamydospores of the AM fungus *Glomus mosseae*. MHB exudates often stimulate fungal spore germination. Will and Sylvia (1990) showed that sea oats (*Unicola paniculata*) roots inoculated with *Klebsiella pneumoniae* increased spore germination and hyphal extension of *Glomus deserticola*.

MHB modification of the rhizospheric soil

The metabolic activity of the bacteria in the rhizosphere modifies the physiochemical properties of the soil in a way that facilitate mycorrhizal infection (Figure 1.4-2). Since the rhizospheric soil is the habitat for both the host plant and fungus, both can be affected by changes in pH or the complexing of ions by siderophore producing fluorescent pseudomonads (Frey-Klett *et al.*, 2007, Tarkka and Frey-Klett, 2008). Environmental factors such as drought and pollution stress have a strong influence on mycorrhizal symbiosis and on the extent of the mycorrhizal helper effect.

Vivas *et al.,* 2003a showed that a *Bacillus* sp. inoculation had a stronger positive effect on colonization intensity and arbuscule density in mycorrhizal roots of lettuce subjected to drought stress. Studies in the presence of *Brevibacillus brevi* using Cd- or Zn-contaminated substrates showed an increase in *Glomus mosseae* colonization and development of extraradical hyphae (Vivas *et al.*, 2003b). These effects were explained by an increased carbohydrate transport from host plant to the fungus. Bacterial inoculation reduced damage to *G.mosseae* hypahe but also increased the hyphal growth from 195% (no Cd) to 254% (with Cd) and from 125% (no Zn) to 232% (with Zn) (Vivaz *et al.,* 2005).

The potential role of siderophores is an important consideration as chelating ligands, especially hydroxamate siderophores produced by ectomycorrhizal fungi contributes to mineral uptake by the root (Garbaye, 1994). MHB also influence the concentrations of antagonistic substances produced by mycorrhizal fungi and of toxic substances produced by soil microbes. Riedlinger *et al.,* 2006 showed that antibiotic production by a *Streptomyces* sp. was suppressed by acidic substance production by *Amanita muscaria*.

Effect on fungal growth

Bacterial isolates may enhance fungal growth in the pre-symbiotic stage of its lifecycle (Figure 1.4-3). Duponnois (1992) compared bacterial isolates such as *Bacillus subtilis, Bacillus* sp., *Pseudomonas fluorescens* and *Pseudomonas* sp. which had the potential to be mycorrhization inhibitors or helpers towards *Laccaria laccata* and Douglas Fir seedlings and discovered a significant correlation between the various isolates in general to either reduce or promote the mycelia growth of the fungus and their effect on mycorrhiza formation.

This study strongly suggested that the MHB act by mainly by enhancing mycelial growth and colonization of the surface of the long roots and subsequently come into contact with the infection-receptive short roots. As a result, the larger volume of soil occupied by the mycelium increases the likelihood of the fungus encountering a root of the host plant. Garbaye and Bowen, 1989 investigated the symbiotic association between *Pinus radiata* and *Rhizopogon luteolus* and revealed the ability of some bacterial isolates from *R.luteolus* to limit or enhance mycorrhiza formation.

This was found to be mainly a result of their negative or positive effect on the growth of the fungus mycelium. Work by Bowen and Theodore, (1979) revealed an increase by up to 70% in the growth of some ectomycorrhizal fungi with one pseudomonad and a significant decrease by another pseudomonad. The study also showed that the one non-inhibitory pseudomonad could reduce the effect of the negative pseudomonad. This was shown by measuring the mycelial growth of ectomycorrhizal fungi along the roots of *Pinus radiata* in aseptic conditions and introducing individual bacterial strains. All of the experiments indicate the presence of some sort of trophic stimulative, perhaps involving nutritional relations, of the fungal growth by the bacterial isolates. This could explain how some MHBs are fungus-specific.

These interactions then led to two mechanisms within this hypothesis. The first mechanism discusses the involvement of the production of metabolites by the bacterium which is directly utilized by the fungus as nutrients or to enhance anabolism (growth factor-type effect). Experiments with MHB such as *Bacillus subtilis, Bacillus* sp*., Pseudomonas fluorescens* and *Pseudomonas* sp. associated with *Hebeloma crustilimforme, Paxillus involutus* and *Laccaria laccata* revealed that some organic acids (malic and citric acid) were excreted by the MHB. These represented a carbon source as sufficient as glucose for fungal growth.

The same authors also discovered in an experiment that volatile compounds were also involved in specificity. The second mechanism suggests the involvement of detoxification of the fungal environment by the bacterial isolates. Like most organisms, mycorrhizal fungi excrete metabolites into the soil that potentially inhibit hyphal growth. MHB could detoxify the fungal habitat by either using or breaking down the fungal metabolites in the soil (Garbaye, 1994).

Duponnois and Garbaye (1990) showed that the ectomycorrhizal fungi *Paxillus involutus* and *Hebeloma crustulimforme* produce toxic fungal metabolites such as dark polyphenols produced by the former and unidentified colourless substances in the case of the latter. The bacterial isolates such as *Bacillus subtilis, Bacillus* sp*., Pseudomonas fluorescens* and *Pseudomonas* sp. displayed an MHB effect toward these fungi and also detoxified the media in which the fungi were grown.

Effect on root-fungus recognition

The bacteria in the soil are involved with the plant root-mycorrhizal fungus recognition mechanisms which are the important first step in the symbiosis with the mycorrhizal fungi (Figure 1.4-4). The recognition process incorporates the reception of plant signals by the fungal hyphae, chemotrophic hyphal extension and growth to the potential site of infection, and changes in hyphal morphology (Frey-Klett *et al.*, 2007, Tarkka and Frey-Klett, 2008). Chemical elicitors and mediators such as phenolic and volatile compounds, enzymes, glycoprotein fibrils (permits surface attachment) and phytohormones produced either by the fungus or the plants involved in the mutual recognition was reviewed by Anderson, 1988.

The bacteria are then involved in this mechanism by breaking down or transforming the chemical substances or contributing to the production of some important compounds like auxins and enzymes which promote fungal-root recognition. Xie *et al.,* (1995) showed that MHB such as *Bradyrhizobium japonicum* stimulated AM colonization and direct hyphal growth towards the root by enhancing the production of stimulatory signals or by inducing changes in the host plants' flavonoid spectrum.

Bacteria can also modify the cell-wall properties or facilitate the establishment of the symbiosis by attaching to either the root or fungus and thereby provides a mechanical link between them (Anderson, 1988). Lateral root production can also be positively influenced by MHB is probably due to the production of auxins or auxin-related substances by the bacteria. The formation of new root tips may lead to the establishment of more mycorrhizas as the density of colonization sites per soil volume increases (Garbaye, 1994, Poole *et al.,* 2001).

Effect on root receptivity

Bacteria present in soil prior to mycorrhizal development may improve the roots' receptivity to the formation of mycorrhizae (Figure 1.4-5). A study performed with the Douglas Fir – *Laccaria lacata* symbiosis by Duponnois, 1992 showed that MHB which associated with the symbiosis produced Indole-3-Acetic Acid (IAA) and that exogenous IAA stimulated the initiation of short roots on Douglas Fir seedlings. The more short roots the plant can produce leads to an increase in the probability that the roots encounter mycorrhizal propagules in the same volume of soil.

Duponnois (1992) also hypothesized that the MHB were able to soften the cell walls of the middle lamella between the cortex cells by the production of specific cell wall-digesting enzymes which makes penetration by the fungus easier. In pure cultures of MHB such as *Bacillus subtilis, Bacillus* sp., *Pseudomonas fluorescens* and *Pseudomonas* sp. from the Douglas Fir – *L.lacata* experiment, cell wall-degrading enzyme activities (endoglucanase, cellobiose hydrolase, pectate lyase and xylanase) were detected in the system. Early work by Mosse, 1962 revealed that some microorganisms such as *Pseudomonas* sp which produce cell wall-degrading enzymes did promote the establishment of AM fungi on clover roots. The suppression of the plant defense response prior to fungal colonization could also potentially lead to enhanced mycorrhization (Garbaye, 1994, Frey-Klett *et al.*, 2007, Tarkka and Frey-Klett, 2008).

1.3.3 Plant-growth promoting Rhizobacteria

A number of bacteria in the rhizosphere are known to stimulate plant growth. This is achieved through direct or indirect interactions with the plant roots. These bacteria have been termed Plant-Growth Promoting Rhizobacteria (PGPR) (Bloemberg and Lugtenberg, 2001). Interactions between the fungi, bacteria and plants occur in the zone of soil surrounding the roots and hyphal network known as the “mycorrhizosphere”. PGPR mainly belong to the genera *Paenibacillus, Burkholderia, Pseudomonas* and *Bacillus sp* (Vessey, 2003; Martinez-Viveros *et al.*, 2010). The direct mechanisms of positive effect on plant growth is through the production of phytohormones and plant growth auxins such as indole acetic acid (IAA), nitrogen fixation and the solubilisation of phosphorous.

Indirect mechanisms include ability to decrease or prevent any deleterious effects of pathogenic microorganisms which can be by the production of antibiotics or siderophores by the bacteria (Singh and Kapoor, 1998).The beneficial effects of some PGPR may be due to their interactions with AM fungi. PGPR have a strong stimulatory effect on the growth of AM fungi. Increased mycelial growth from *G.mosseae* spores caused by an unidentified PGPR suggest that selected PGPR and AM fungi could be co-inoculated to optimize the formation and functioning of the AM fungi symbiosis (Gryndler, 2000).

Phosphate-Solubilising Bacteria

Phosphorous is an essential macronutrient required for growth and development by plants. Many soil bacteria are phosphate solubilising bacteria which are able to mobilize phosphate ions from soluble organic and inorganic phosphorous sources such as tricalcium phosphate, hydroxyapatite and rock phosphate (Gryndler, 2000; Vessey, 2003; Richardson *et al.*, 2009; Martinez-Viveros *et al.*, 2010). Through this process, the bacteria help improve the supply of phosphorous to the plants. It has been proposed that the phosphate solubilised by PSB could potentially be taken up more efficiently by the plant through a mycorrhizal-mediated channel between the plant roots and surrounding soil that allows better acquisition of phosphorous from the soil to the plants (Rodriguez and Fraga, 1999). Strains from the genera *Pseudomonas, Bacillus and Rhizobium* are among the most powerful phosphate solubilisers (Rodriguez and Fraga, 1999; Martinez-Viveros *et al.*, 2010; Suresh *et al.*, 2010). Singh and Kapoor, (1998) showed PSB such as *Bacillus circulans* together with the AM fungi increased plant yield and phosphorous uptake in wheat.

The major mechanism of mineral phosphate solubilisation occurs by the action of organic acids which are synthesized by the soil bacteria. The production of these organic acids results in acidification of the microbial cell and its environment. The production of these organic acids, particularly gluconic acid and 2-ketogluconic acid by phosphate solubilising bacteria has been well reported (Khan *et al.*, 2009). Other mechanisms have also been considered such as the production of chelating substances by the bacteria as well as the production of inorganic acids such as sulphidric, nitric and carbonic acid (Rodriguez and Fraga, 1999, Vessey, 2003; Richardson *et al.*, 2009; Martinez-Viveros *et al.*, 2010).

Organic phosphate sources are mineralised by the action of several phosphatase enzymes. Phosphatase activity involves the hydrolysis of phosphor-ester or phosphor-anhydride bonds, thereby catalysing the bound phosphorous into inorganic phosphorous (Rodriguez and Fraga, 1999; Richardson *et al.*, 2009; Martinez-Viveros *et al.*, 2010). Phytate (Myo-inositol hexakis-phosphate) constitutes upto 80% of organic phosphorous in the soil making it one of the most abundant sources of phosphorous for plants (Lim *et al.*, 2007). There are some PGPR which are able to produce phytase, an enzyme which degrades phytate to lower phosphate esters. Phytase producing PGPR have been shown to belong to the genera *Bacillus, Burkholderia, Pseudomonas, Serratia* and *Staphylococcus* (Hariprasad and Niranjana, 2009).

A study by Hariprasad and Niranjana, (2009) revealed an *Enterobacter* sp. of PGPR which was able to solubilise calcium phosphate and phytase to have the most significant increase in plant growth of tomato (*Lycopersicon esculentum* Mill.) plants. It was found that the bacterium increased shoot length by 1.55 fold; root length by 1.44 fold; fresh weight (g/ seedling) by 3.38 fold and dry weight (g/ seedling) by 2.68 fold compared to the control. In a broth assay determined that this *Enterobacter* sp. produced gluconic acid which indicated the solubilisation of CaP and decrease in pH was in relation to the production of the organic acid.

Fernandez *et al.*, (2007) investigated the influence of phosphate-solubilising ability of bacterial isolates on soybean (*Glycine max* L.) growth under greenhouse conditions. They found inoculation with *Bhurkolderia* sp. had 40% greater aerial heights (cm) than the uninoculated soil/seed control showing that phosphate-solubilisation by the bacteria had a significant effect on plant growth.

A study by Akhtar and Siddiqui, (2009) examined the effects of phosphate solubilising bacteria on the growth of chickpea (*Cicer arietinum* L.) under field conditions. They found that *Paenibacillus polymyxa*, *Pseudomonas putida*, *Pseudomonas alcaligenes* and *Pseudomonas aeruginosa*) significantly increased shoot dry weight, increased seed weight and yield over the uninoculated control with *P. polymyxa* having the greatest effect among the isolates.

Canbolat *et al.,* (2006) examined the effects of plant growth promoting bacteria on barley (*Hordeum vulgare*) seedling growth. The bacterial isolates (*Bacillus* RC01, *Bacillus* RC02, *Bacillus* RC03 and *Bacillus* M-13) were found to solubilise phosphate. Available phosphate in the soil was significantly increased by seed inoculation with *Bacillus* RC01 and *Bacillus* M-13.

Inoculations of barley with *Bacillus* RC01, *Bacillus* RC02, *Bacillus* RC03 and *Bacillus* M-13 increased root dry weight by 16.7, 12.5, 8.9 and 12.5% respectively compared to uninoculated control and shoot weight by 34.7, 34.7, 28.6 and 32.7% respectively. As a result of phosphate solubilisation and other activities, these bacterial isolates have potential plant growth promotion activity in barley.

Nitrogen fixation

Nitrogen is an essential plant nutrient. There are two types of nitrogen fixation carried out by PGPR: symbiotic and non-symbiotic. Non-symbiotic nitrogen fixation is carried out by free-living diazotrophs and can stimulate non-legume plants growth. These bacteria belong to the genera *Azoarcus, Azospirillum, Burkholderia, Gluconacetobacter* and *Pseudomonas* (Antoun and Prevost, 2005; Richardson *et al.*, 2009; Martinez-Viveros *et al.*, 2010).

Symbiotic nitrogen fixers include *Rhizobia,* develop symbiotic relationships with host legume plants and through atmospheric N2 fixation within nodules can provide upto 90% of the N requirements of the plant. Endophytic diazotrophs appear to have an advantage over root-surface organisms since they are capable of colonising the interior of roots and establish themselves within niches that are more conducive to effective N_2 fixation, transferring the fixed N to the host plants. In addition to their potential for supplying N through N_2 fixation to the host plants they may also promote plant growth through various other mechanisms such as phytohormone production (Richardson *et al.*, 2009).

Phytohormone production

Some PGPR can have an influence on plant growth by the production of phytohormones such as auxins, cytokinins and gibberellins. Auxins contribute to the endogenous pool of phytohormones produced by the plant (Martinez-Viveros *et al.*, 2010). The production of indole acetic acid has been shown to be widespread among PGPR (Xie *et al.*, 1996; Patten and Glick, 2002) and is predominantly synthesized by an alternate tryptophan-dependant pathway which is carried out through indole-pyruvic acid. The role of IAA produced by the PGPR in plant growth is however still undetermined. IAA in plants is the main auxin which controls many important and beneficial physiological processes including cell enlargement and division, tissue differentiation and responses to light and gravity (Patten and Glick, 2002; Spaepen *et al.*, 2007; Shahab *et al.*, 2009; Martinez-Viveros *et al.*, 2010). Therefore PGPR which produce IAA and interact with plants have the potential to interact with any of the fore-mentioned processes, resulting in a positive or negative effect. IAA produced by PGPR can promote root growth (Spaepen *et al.*, 2007), which can subsequently enhance mycorrhizal contact (Garbaye, 1994).

PGPR which produce IAA have been demonstrated as belonging to the following genera's of *Aeromonas, Bacillus, Burkholderia, Enterobacter, Pseudomonas* and *Rhizobium* (Martinez-Viveros *et al.*, 2010).Rapid establishment of roots can be achieved either by the elongation of primary roots or by the proliferation of lateral and adventitious roots. This establishment is advantageous for young seedlings as it increases their ability to anchor themselves in the soil. This increases their chances of survival by obtaining water and nutrient from the soil (Patten and Glick, 2002).

Dobbelaere *et al.*, (1999) showed in an experiment with *Azospirillum* that increased rooting was directly related to IAA synthesis. This increased rooting enhanced the plant mineral uptake and root exudation which in turn stimulated bacterial colonisation which further enhances the inoculation effect.

Patten and Glick, (2002) showed that low concentrations of IAA stimulated the elongation of the primary root and demonstrated the direct influence of bacterial IAA in promotion of root elongation when associated with the host plant. However, high concentrations of IAA stimulate the formation of lateral and adventitious roots. IAA produced by PGPR can therefore have many beneficial influences on plant growth by altering root elongation. A study by Shahab *et al.,* (2009) revealed three bacterial isolates (two strains of *Bacillus thuringensis* and *Pseudomonas aeruginosa)* which were able to produce IAA had an effect on mung beans (*Vignia radiate)* in greenhouse experiments.

The *P.aeruginosa* isolate showed the most significant root and shoot elongation compared to the other two isolates and the control. It is therefore discussed that plant growth substances produced by these bacteria improve plant growth by directly having an effect on metabolic processes. Since they induce lateral root and root hair proliferation, increasing the nutrient absorbent surface areas leading to greater nutrient absorption rates and increases in shoot and root length of the plants (Shahab *et al.,* 2009).

Cytokinins are phytohormones which promote cell division and enlargement, tissue expansion and promote root hair development. Cytokinin production has been shown in various PGPR including *Azospirillum, Pseudomonas fluorescens* and *Paenibacillus polymyxa* (Madhaiyan *et al.*, 2010).

Gibberellins enhance the development of plant tissues, particularly stem tissue and promote root elongation and lateral root extension. Production of gibberellins has been reported in PGPR such as *Azospirillum, Bacillus punnilus, Bacillus licheniformis* and *Rhizobium* (Vessey, 2003; Martinez-Viveros *et al.*, 2010).

Ethylene is a phytohormone which effects plant growth by inhibiting root elongation. Plants produce ACC (1-aminocyclopropane-1-carboxylate) which is the precursor for ethylene. Some ACC is released into the soil and reabsorbed by the roots, which leads to diminished root growth. Some PGPR have the ability to synthesize ACC deaminase, an enzyme which cleaves ACC which thereby decreases ethylene production, promoting root lengthening. ACC deaminase activity has been reported in the genera *Achromobacter, Azospirillum, Bacillus* and *Pseudomonas* (Vessey, 2003; Martinez-Viveros *et al.*, 2010.

Madhaiyan *et al.*, (2010) studied the effect of co-inoculations of *Methylobacterium oryzae* with *Azospirillum brasilense* and *Burkholderia pyrrocinia* on the growth of tomato (*Lycopersicon esculentum* Mill.), red pepper (*Capsicum annum* L.) and rice (*Oryza sativa* L.). Studies showed that *Methylobacterium* through the production of phytohormones such as IAA and cytokinins improved plant growth. Other mechanisms such ACC deaminase and the production of siderophores have also been documented. *Azospirillum* is known as a nitrogen fixer and a producer of IAA whereas *Burkholderia* has been shown to solubilise phosphate and have ACC deaminase activity. Under greenhouse conditions, there was a significant increase in all plant growth paramters by the bacterial isolates compared to the non-inoculated control plants. In tomato, individual inoculation of *M.oryzae* or its co-inoculation with *A. brasilense* and *B. pyrrocinia* produced significant increases in root length compared to control or individual inoculations. In red peppers, inoculation with *M.oryzae* produced the greatest root and shoot lengths while a greater root and shoot growth was found with the dual inoculation of *M.oryzae* with *B. pyrrocinia*. In rice, no significant increases in root length were recorded. Cytokinins produced by *M.oryzae* may enhance stomatal opening and promote cell division in the presence of auxins resulting in an enahnced uptake of water and other nutrients from the soil. Thus cytokinin and IAA production by *M.oryzae* may have a positive effect on the growth of plants (Madhaiyan *et al.*, 2010).

Mena-Violante and Olalde-Portugal, (2007) investigated the effect of a PGPR (*Bacillus subtilis* BEB-13bs) on tomato (*Lycopersicon esculentum* Mill.) growth parameters under greenhouse conditions. *B.subtilis* was able to significantly increase the root dry weight and root length by 18-26% and 13-15% respectively in the two experiments. The inoculation with *B.subtilis* also increased the yield per plant (g), which was higher than the control by 21% and 25% in the two experiments. Fruits in the *B.subtilis* treatments were significantly heavier and longer by 18% than those in the control treatments. They attributed the effects of the *Bacillus* inoculation to the production of hormones, which are believed to change assimilate partitioning patterns in plants, altering growth in roots, fructification process and development of the fruit. They concluded that tomato root inoculaiton with a PGPR *Bacillus* strain enhances fruit quality under greenhouse conditions. They expressed however, field trials should be carried out to ensure those positive effects are maintained.

Effects of floral and foliar application of plant growth promoting rhizobacteria on the growth of sweet cherry was investigated by Esitken *et al.*, (2006). The bacterial isolates used to investigate the plant growth promoting effects on sweet cherry (*Prunus avium* L.) were *Pseudomonas* (BA-8) and *Bacillus* (OSU-142). BA-8 has been found to produce transzeatin and OSU-142 to fix nitrogen and produce IAA. The bacterial treatments BA-8, OSU-142 and BA-8 + OSU-142 affected yield per trunk cross-section area (TCSA), fruit weight and shoot length. The yield per TCSA was significantly increased in the bacterial treamtents except for OSU-142 compared with the control. Significant yield increase was obtained with *Pseudomonas* BA-8 (0.107 kg.cm^{-2}; 16.3%) and BA-8 + OSU-142 (0.112 kg.cm^{-2}; 21.7%) as compared with the control (0.0902 kg.cm^{-2}). Fruit weights was also significantly increased by the bacterial treatments with BA-8 (7.56g) and OSU-142 (7.65g) compared with the control (7.26g). In addition, the treatments significantly increased the shoot lengths by BA-8 (16.36cm; 11.3%), OSU-142 (16.43cm; 11.8%) and BA-8 + OSU-142 (19.05cm; 29.6%) compared with the control (14.70cm).

The growth enhancements of the bacteria on sweet cherry could be explained by the nitrogen fixation and IAA producing capacity (OSU-142) and trans-zeatin production (BA-8). They concluded that *Pseudomonas* BA-8 and *Bacillus* OSU-142 alone or in combination have a great potential to enhance sweet cherry growth parameters.

A study by Orhan *et al.*, (2006) looked at the effects of *Bacillus* OSU-142 (nitrogen fixing bacterium) and *Bacillus* M3 (nitrogen fixing and phosphate solubilising bacterium) on the growth parameters of raspberry. The results ahowed that M3 treatment stimulated plant growth and resulted in significant yield increase. Significant yield increase was obtained with M3 and OSU-142 + M3 by 33.9 and 74.9% respectively. M3 and OSU-142 + M3 treatments gave the highest cane length of rapsberry with increases of 13.6 and 15.0% respectively over the control. These two treatments also caused significant increases in the number of cluster per cane and the number of berries per cluster as compared with the control. In the study, the authors also found increases in N and P content of rapsberry leaves, and a decrease in pH of the soil, which correlates to the evidence of the bacterial isolates fixing nitrogen and solubilising phosphate due to the produciton of organic acids. Therefore these isolates, through their PGPR ability were able to increase the growth parameters of rapsberry and can be utilised for organic agricultural practices.

The plant growth promoting effects of *Bacillus* M3 (phosphate solubilisation), *Bacillus* OSU-142 (cytokinin and IAA production) and *Microbacterium* (N_2 fixing) were tested alone or in combination on apple (*Malus domestica* L. cv. Granny Smith) in terms of yield, growth and nutrient composition of leaves was investigated by Karlidag *et al.*, (2007). They found significant increases in yield was obtained with application of M3 + *Microbacterium* (12.71 kg/tree, 88%), OSU-142 + *Microbacterium* (10.96 kg/tree, 62.9%), OSU-142 (8.55 kg/tree, 26.5%) and M3 + *Microbacterium* (8.52 kg/tree, 26%) compared to the control (6.76 kg/tree). Average fruit weight also significantly increased by M3 + *Microbacterium* (193.7 g), M3 + OSU-142 (191.5 g), OSU-142 (186.9 g) and OSU-142 + *Microbacterium* (175.7 g) treatments compared with control (154.3 g). Results showed that the most significant shoot length increase was obtained with M3 + *Microbacterium* (73.25 cm) followed by M3 + OSU-142 (71.94 cm) treatments compared to controls (56.54 cm). Shoot diameter also significantly increased by M3 + OSU-142 (18.4%) and M3 + *Microbacterium* (15.9%) application compared to controls.

Effects of root colonization by plant growth promoting rhizobacteria (PGPR) on biomass and essential oils composition were determined in *Origanum majorana* L. by Banchio *et al.*, (2008). PGPR strains evaluated were *Pseudomonas fluorescens, Bacillus subtilis, Sinorhizobium meliloti,* and *Bradyrhizobium sp.* Inoculation with *P. fluorescens* or *Bradyrhizobium* sp. induced significant increases in number of leaf, shoot length, and number of nodes. Leaf number was 80% higher in *P. fluorescens* inoculated plants than in control, as reflected in a 3.2-fold increase in shoot fresh weight. Root dry weight in plants inoculated with *P. fluorescens* or with *Bradyrhizobium* was 6-fold higher than in control (non-inoculated) plants, and significantly higher than in plants inoculated with other strains. Plants inoculated with *P. fluorescens* or *Bradyrhizobium* showed significant increase in total essential oil yield 24- and 10-fold, respectively. In *P. fluorescens* inoculated plants; essential oil yield (% V/W) increased 0.14%, compared to 0.05% in non-inoculated plants.

Pathogen Inhibition

PGPR have been shown to mediate in the biological control of plant pathogens. The mechanisms involved can be direct via the production of antibiotics, siderophores, hydrogen cyanide, hydrolytic enzymes (chitinases, proteases and cellulases) or indirectly by competition with the pathogen for ecological niche such as infection and nutrient sites (Linderman, 2000; Bloemberg and Lugtenberg, 2001; Sharma and Johri, 2002).

PGPR have been to shown to produce various antibiotics effective against phytopathogens under laboratory conditions. These antibiotics include butyrolactones, zwittermycin A, kanosamine and 2.4-diacteylphloroglucinol (2,4-DAPG). 2,4-DAPG is one of the most efficient antibiotics and is produced by *Pseudomonas* strains. It has a wide spectrum including being an anti-fungal, antibacterial and anti-helmintic (Whipps, 2001; Martinez-Viveros *et al.*, 2010).

Some PGPR are capable of producing hydrogen cyanide (HCN), a volatile secondary metabolite which suppresses the development of other microbes. Many different bacterial genera produce HCN including *Alcaligenes, Aeromonas, Bacillus, Pseudomonas* and *Rhizobium* (Whipps, 2001; Martinez-Viveros *et al.*, 2010).

Iron is an essential element required for growth in all organisms and bioavailable iron in the soil is generally deficient. Therefore under iron-limiting environments, PGPR produce siderophores, low molecular weight compounds which competitively sequesters ferric iron. The bacterium which originally produces the siderophore, takes up the iron-siderophore complex through receptors specific to the complex on the outer cell membrane. Once inside the bacterium, the iron is released and available to support microbial growth (Siddiqui, 2005). The siderophores deprive the pathogenic fungi of the available iron in the soil as the PGPR siderophores have a greater affinity for the iron. Some PGPR can sequester iron from heterologous siderophores produced by other soil microorganisms (Suresh and Bagyaraj, 2002). Siderophore producing bacteria have been found to belong to *Bradyrhizobium, Pseudomonas, Rhizobium, Serratia,* and *Streptomyces* (Martinez-Viveros *et al.*, 2010).

The root surface and its surrounding rhizosphere are significant sources of carbon. Therefore along the surfaces of the roots there are a variety of nutrient rich niches which attracts a wide range of microorganisms, including phytopathogens. Competition for these nutrient niches is one of the major mechanisms by which PGPR protect the plant from pathogens. PGPR have the ability to reach the surfaces of the roots through active motility by their flagella and are guided by chemotrophic responses. They are also carried by the mycorrhizal hyphal network (Compant *et al.*, 2005).

1.3.4 Endocellular bacteria of AM fungi

Endocellular bacteria are reported to be found only in a few fungi including some Glomeromycota species (AM fungi). The cytoplasm of AM fungi contains many bacteria-like organisms (BLO's). Microscopy revealed these bacterial cells as Gram-negative and rod shaped, occur singly or in groups, often inside fungal vacuoles in both spores and hyphae and were described as being related to *Burkholderia.* They were placed in a new taxon related to *Burkholderia* but named '*Candidatus Glomeribacter gigasporarum*' because of their unculturability (Bianciotto and Bonfante, 2002; Bonfante and Anca, 2009).

Different mechanisms by which these endosymbionts can affect fungal performance including the release of substances which affect fungal gene expression, introduction of chemical compounds into the spores, attachment to fungal surface by producing lectins and degradation of fungal cell wall which may aid in germination or colonisation (Artursson *et al.*, 2006; Bonfante and Anca, 2009; Miransari, 2011).

The functional significance of AM fungal endobacteria is not clear as these bacteria are unculurable. The bacterial genes have been partially identified, some of which are involved in nutrient uptake, such as a putative phosphate transporter operon, *pst*; and a gene involved in colonisation events by bacterial cells, *vac.* A DNA region which contained putative nitrogenase coding genes (*nif* operon) has also been found (Bianciotto and Bonfante, 2002).

1.4 Interactions with other soil microorganisms

The use of AM fungi to enhance plant growth and yield of various crops has been researched in recent years due to their beneficial effects which have a large economic impact on agriculture and horticulture. The interactions between AM fungi and other rhizosphere micro-organisms therefore have the potential to increase plant growth and yield of plants more so than individual inoculations. Many studies have been carried out to analyse the interactions between AM fungi and rhizosphere micro-organisms.

1.4.1 AM fungal interactions with Mycorrhizal Helper Bacteria

There have been studies investigating the interactions between AM fungi and MHB on plant growth. These interactions may be beneficial to the host plant as the MHB helps increase AM fungal colonisation in the plant roots by mechanisms mentioned before. A study by Mamatha *et al.*, (2002) investigated the interactions between AM fungi and a mycorrhiza helper bacterium (*Bacillus coagulans*) in field established mulberry and papaya plants. Mulberry plants inoculated with *Glomus fasciculatum* showed a significant increase in plant height and number of leaves and in papaya plants inoculated with *Glomus mosseae* and *Glomus caledonium* a significant increase in plant height and and stem girth compared with the control given 100% recommended P. There was no significant difference in plant growth parameters between treatments with the AM fungi alone or the AM fungi with *Bacillus coagulans*.

Mycorrhizal colonisation however, was highest in plants inoculated with the AM fungi and *B.coagulans*. Increased AM fungal colonisation by the bacterium indicates that *B.coagulans* is a mycorrhizal helper bacterium. It has been suggested that MHB produce hydrolytic enzymes which cause the cortical cells to dilate, providing a larger intercellular surface area with which the AM fungi can penetrate and colonise more easily, increasing the percentage of AM fungi present in the plant, thereby providing more nutrients to the plant (Mamatha *et al.*, 2002).

1.4.2 AM fungal interactions with Plant-growth promoting Rhizobacteria

The improvement of plant growth and nutrition through the synergistic interaction between AM fungi and PGPR has been described in several studies. It has been mentioned that in P-deficient soils, phosphate-solubilising micro-organisms interact with AM fungi by releasing phosphate ions which are transferred by the AM fungi to the plant. There are different mechanisms by which PGPR and AM fungi interact together to improve plant growth (Artursson *et al.*, 2006).

A study by Khan and Zaidi, (2007) examined the co-inoculation of a nitrogen-fixing (*Azotobacter chroococcum*), a phosphate-solubilisng bacterium (*Bacillus* sp.8), and an AM fungus (*Glomus fasciculatum*) on the growth of wheat (*Triticum aestivum L.*). The dual inoculations of *A. chroococcum* and *Bacillus*; *A. chroococcum* and *G. fasciculatum*; and *Bacillus* and *G. fasciculatum* increased the dry matter significantly compared to the control. The co-inoculation of *Bacillus* and *G. fasciculatum* enhanced the dry matter accumulation in roots, shoots and whole plants by 1.7, 1.5 and 1.6-fold respectively compared with the control, and was superior to other single or dual inoculation treatments.

The triple inoculation of *A. chroococcum, Bacillus* and *G. fasciculatum* had a two-fold increase in total dry matter and doubled the grain yield of wheat relative to the control. The number of AM spores and percentage root colonisation were signifcantly greater in the triple inoculation (322.5 spores.g^{-1} soil and 90.2% respectively) compared with other treatments. The results were attributed to the ability of the phosphate-solubilising organisms to solubilse inorganic phosphate sources in the soil. *A. chroococcum* is a phyto-stimulator as well as nitrogen-fixer, therefore provides considerable amounts of plant growth promoting substances such as hormones. The AM fungi then provide these extra nutrient sources to the plant through their hyphal network (Khan and Zaidi, 2007).

A study by Bharadwaj *et al.*, (2008) investigated AM fungal spore-associated bacteria and their effects on potato (*Solanum tuberosum L.)* growth. They found an isolate, *Pseudomonas putida* biotype A, which solubilises phosphate and produces IAA had the most significant effect on potato growth by increasing the number of primary roots by 100%, the number of lateral roots by 65% and root length by 76%, shoot length by 73% and the number of leaves by 81% compared with the control. This isolate also increased the root colonisation by *G.mosseae.*

Pivato *et al*., (2008) performed a study which revealed a bacterial isolate *Pseduomonas fluorescens* which in combination with the AM fungi *G. mosseae* and *G. rosea*, increased shoot and root growth and promoted the mycorrhization of tomato (*Lycopersicon esculentum* Mill.) plants, however the combinations resulted in lower shoot and root growth than *P. fluorescens* isolate singly inocluated.

A study by Gamalero *et al.*, (2004) looked into the impact of two pseudomonads (*Pseudomonas fluorescens* and *Pseudomonas fluorescens* P190r) and an AM fungus (*G.mosseae)* on tomato (*Lycopersicon esculentum* Mill.) plant growth. They found that *Pseudomonas fluorescens* 92rk increased mycorrhizal colonisation by 41%, whereas *Pseudomonas fluorescens* P190r did not. *Pseudomonas fluorescens* 92rk and *Pseudomonas fluorescens* P190r significantly increased both the shoot and root fresh weights whereas *G.mosseae* only increased the shoot fresh weights. Co-inoculation of 92rk and BEG12 induced significant increases in shoot fresh weight. Co-inoculation of all three increased shoot and root fresh weights relative to all the other treatments.

Khan and Zaidi, (2006) investigated the inoculations of a phosphate-solubilisng bacterium (*Bacillus subtilis*), a nitrogen-fixing bacterium (*Bradyrhizobium sp.*) and an AM fungus (*G. fasciculatum*) on greengram (*Vignia radiata* L. wilczek). It was found, in general there was no significant increase in plant length (root and shoots) among the individual treatments. The dual inoculation of *G. fasciculatum* and *B. subtilis* significantly increased the root length at the flowering stage only. Among all the treatments, the tripartite inoculation of *Bradyrhizobium, G. fasciculatum* and *B. subtilis* enhanced the length of the root and shoots at flowering and harvest relative to the control. Single inoculations of *Bradyrhizobium* sp significantly increased the the total dry matter by 110 and 117% at flowering and harvest respectively.

In comparison, the inoculation of *Bradyrhizobium sp* and *G. fasciculatum* increased the total dry matter production by 117% at each of the stages compared to the control. Among all the treatments, the most significant inoculation combination was that of *Bradyrhizobium, G. fasciculatum* and *B. subtilis* which increased the dry matter production significantly by 200 and 183% at flowering and harvest stages respectively, compared to the control. They concluded the application of a phosphate-solubilising bacteria and AM fungus assists the plant root to utilise the sparingly available soluble P and making more P available to the crop.

The plant growth and yield of greengram plants was therefore increased. The study indicated a strong interaction exists between *Bradyrhizobium, G. fasciculatum* and *B. subtilis.* The fact that plant growth and nutrient uptake increased in the presence of AM fungi, it can be suggested that a strong synergistic relationship occurs between root colonisation, P uptake and growth promotion (Khan and Zaidi, 2006).

A study by Kohler *et al.*, (2007) studied the interactions between a PGPR (*B. subtilis*) and an AM fungus (*G. intraradices*) and their effects on lettuce plants (*Latuca sativa*). They found the dual inoculations of *B.subtilis* and *G. intraradices* had the highest effect (77%) on shoot biomass of the lettuce plants, They attributed the increase in plant growth by *B. subtilis* due to an enhanced P and K supply to the crop. It has been shown that *B. subtilis* is a phosphate- and potassium-solubilising rhizobacterium Kohler *et al.*, (2007), which may enhance mineral uptake by plants by solubilisng insoluble P and releasing K from silicate in the soil, which is then taken up by the extra-radical AM hypahe and transferred to the crop plant (Rodriguez and Fraga, 1999).

Mar Vazquez *et al.,* 2000 performed a study to investigate the influence of different microbial inoculants on maize (*Zea mays* L.). The bacterial isolate *Azospirillum brasilense* is known to produce IAA and was found to have a significant increase in shoot and root dry weight in the dual inoculation with *G. deserticola.* They confirmed the ability of growth promoting substances to stimulate plant susceptibility to mycorrhizal colonisation, spore germination, mycelial growth, which in turn increases the chance of contact between fungal hyphae and plant roots. The beneficial effects of *Azospirillum* on plant growth may not be related to only stimulation of colonisation by *G. deserticola* but an increase in the development of the extra-radical mycelium could also be involved. Their results indicate, as previoulsy reported, a functional compatibility between saprotrophic and symbiotic micro-organisms.

A study by Medina *et al*., (2003) looked at the interactions between AM fungi (*G. mosseae, G. intraradices* and *G. deserticola*) and PGPR (*Bacillus pumilus* and *Bacillus licheniformis*) on alfalfa plants (*Medicago sativa). B. pumilus* and *B. licheniformis* produce IAA and gibberellins respectively. Single bacterium treatments did not have an effect on plant growth parameters. Two of the *Glomus* spp. (*G. intraradices* and *G. deserticola*) caused increases in shoot weight. The most efficient treatment in plant growth (shoot and root dry weight, root length and surface area) was the dual (*G. deserticola* and *B. pumilus*) inoculation, which produced a 715% (shoot weight) and 190% (root length) increase over the uninoculated control. The direct effect of *B. pumilus* on root growth is a result of phytohormone production, since IAA production regulates adventitious root formation, increasing nutrient uptake, further enhanced by the AM fungal hyphal network.

Interactions between AM fungi and PGPR occurs naturally since they share common habitats such as the root surface (Barea *et al*., 2004). The interaction increases plant growth through mechanisms carried out by both the AM fungi (through increased nutrient uptake and an enhanced surface area for absorption) and PGPR (phosphate-solubilisation, nitrogen fixation and phytohormone production) (Antoun and Prevost, 2005; Miransari, 2011), which combined have a benefecial effect on plant growth in various crops as investigated by the studies mentioned.

1.5 Ecological Significance of AM fungal interactions

1.5.1 AM fungi in management of disturbed environments

Landscape Development

AM fungi are able to accelerate the natural processes of plant community development. AM fungi stimulate the growth of plant’s originally present in the area by increasing nutrient and water uptake. This leads to successional processes as revegetation occurs through the primary plants (Jeffries *et al.,* 2003; Johansson *et al*., 2004).

Horticulture and Agriculture

The use of AM fungi in agriculture could lead to decreased usage of chemicals, reducing pollution caused by these chemicals in soil water. Successful inoculation is usually achieved when AM fungi are introduced very early in the plant development process followed by the use of low amount of P fertilisers and selective use of pesticides. Colonisation by AM fungi will follow the development of the root of the seedlings/ cuttings, therefore the plants will be already mycorrhizal when transplanted in the field. Inoculation with AM fungi can reduce plant losses during the acclimatisation phase, stimulate plant development (including flowering) and increase productivity after transplant into the field (Jeffries *et al.,* 2003). Following field transplantation, the rhizosphere of a micro-propagated plant is usually colonised with a variety of other soil micro-organisms, some of them beneficial to the symbiosis.

Sustainable agricultural systems utilise natural processes to obtain acceptable levels of productivity and food quality while minimising adverse environmental impacts (Harrier and Watson, 2004). In low-input situations, the effective production requires the crop to have access to nutrients, water, and be relatively free of pests and diseases but also require minimal additional inputs such as chemicals and fertilisers. These can be influenced or be provided by AM fungi (Atkinson *et al.*, 2002). These low-input systems promote both mycorrhizal colonization of crops and the maintenance of AM fungi spores in the soil in comparison with the more intensive conventional or high-input systems. The low-input sustainable agriculture not only increase sporulation but also influences the composition of the AM fungi communities by increasing diversity and also changing the relative abundance of each species (Hamel, 1996).

AM fungi are important contributors to the maintenance of soil quality. Figure 1.4 shows the features of conventional agriculture soils which include high disturbance, high tillage and fertiliser input, high pesticide input compared to the sustainable agriculture soil which contains low disturbance, low tillage practices and fertiliser input and contains high biological input and also control of pathogens instead of chemicals (Johansson *et al.*, 2004).

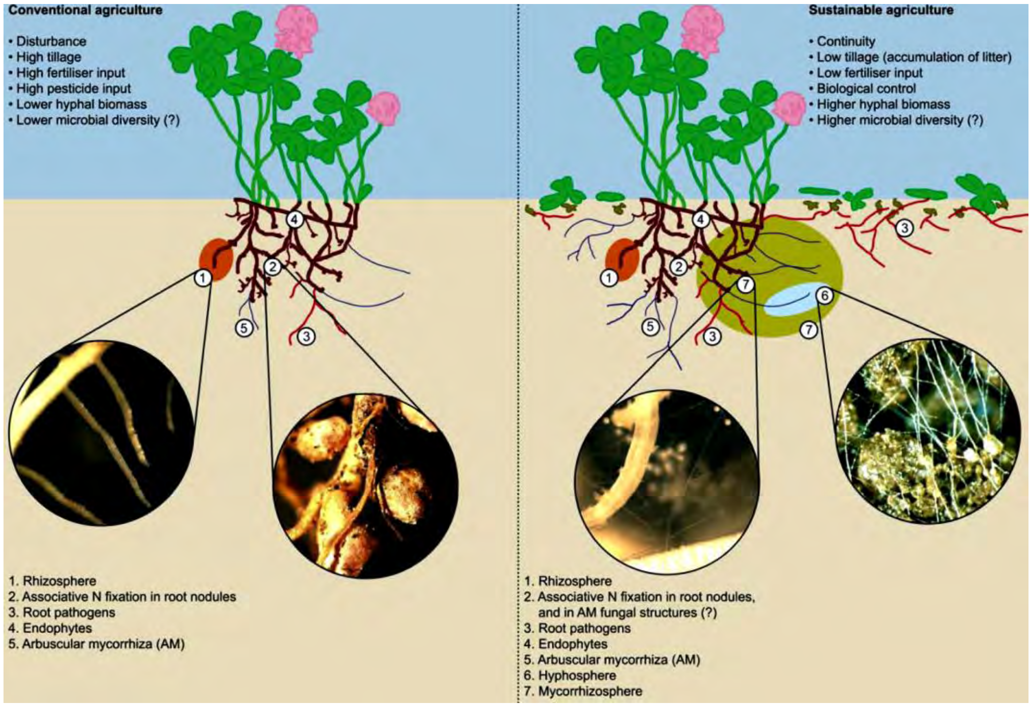


Figure 1.4: Schematic view of the rhizopshere and mycorrhizosphere with respect to features of conventionally managed agriculture soils versus sustainable agriculture soils (Johansson *et al.*, 2004).

Sustainable agricultural practices involves reduced soil tillage, increased crop diversity, maintenance of vegetation cover and reduced chemical inputs compare to the conventional counterparts. AM fungi have an influence on agriculture practices in both conventional and sustainable systems but are more predominant in sustainable systems (Jeffries *et al.*, 2003). These influences account for the application of AM fungi as a biological input for sustainable agriculture. The influences include crop nutrition, crop pests and diseases, the interactions with other soil micro-organisms, the crop water relations and soil structure (Gosling *et al.*, 2006).

Alleviating Desertification

Disturbance of natural plant communites is often accompanied by / or preceded by loss of physico-chemical and biological soil properties such as soil structure, plant nutrient availability, organic matter content and microbial activity (Jeffries *et al.*, 2003). It is becoming more crucial to recover not only the vegetation but also the soil properties.

Inoculation with symbiotic organisms such as AM fungi are becoming an alternative method to reduce the effects of desertification. The introduction of target indiginous plant species associated with a managed community of microbial symbionts to become a successful biotechnological tool to aid in the recovery of desertified ecosystems.

Bioremediationof polluted soils

Current remediation of pollutant from soil include physico-chemical extraction; this method is however extremely expensive and destroys all organisms existing in the substrate. Bioremediation is suggested as an alternative. Three important approaches have been proposed to clean up polluted soils (Jeffries *et al.*, 2003). Phytostabilisation- pollutants are immobilised by plant activity, resulting in attenuation of the wind and soil erosion and run off processes into the ground water or air; Phytoextraction- metal hyperaccumulation plants which can contain more than 1% of metals in harvestable tissues; and Phytodegradation- whole range of metabolic processes usually by plants and assisted by micro-organisms to degrade organic compounds such as hydrocarbons, pesticides and explosives etc (Jeffries *et al.*, 2003).

1.5.2 Influence of conventional farming practices on AM fungi

The management of crops utilises practices which can impact the AM fungi association both directly by damaging or killing AM fungi and indirectly by creating conditions which are unfavourable to AM fungi (Hamel, 1996). Agricultural practices in general have a negative effect on AM fungi association and agricultural soils are AM fungi impoverished especially in terms of the numbers of species. Different management strategies introduce different types of disturbances which influence the AM fungi. These disturbances include fertilisers, biocides, tillage and crop rotations (Gosling *et al.,* 2006).

Fertilisers

There is a strong influence of the host phosphorous status on AM association. Therefore the use of phosphate fertilisers has a significant impact on the relationship between the plant and fungus. Phosphate fertilisers in agriculture are usually used in excess of the crop requirements which results in a build up of total and easily available phosphorous in the soil (Gosling *et al.,* 2006).

This leads to the plants relying less on the AM association and results in a lower AM fungi colonization and propagule density, which also results in the soil containing increased phosphorous available. Fertilisation may also select for AM fungi species which are inferior in terms of providing a benefit to the host (Gosling *et al.,* 2006). The use of other readily soluble fertilisers such as nitrogen fertilisers also has a negative effect on AM fungi colonization and/or diversity. Organic sources of nutrients which include farmyard manure, compost and crop residues and also slow releasing fertilisers like rock phosphate do not appear to suppress the AM fungi and may also stimulate the colonization and development of AM fungi (Gosling *et al.,* 2006). However, the overuse of organic amendments, especially those which are high in phosphorous (such as chicken manure) may have a negative influence on the AM fungi. Liming acidic soil tends to increase the population density of the AM fungi and may also result in a change of species dominance among the AM fungi (Gosling *et al.,* 2006).

Biocides

The effect that biocide has on the AM fungi association is complex and not easily predictable. Fungicides have been shown to reduce to AM fungi colonization and spore production. Some fungicides have been shown to have deleterious effects on AM fungi and in others increase AM fungi colonization and nutrient uptake (at reduced application rates) (Hamel, 1996). Other types of biocides can have negative, neutral or positive effects on the AM fungi colonisation. At the recommended application dosage of these biocides results in reduced colonization and spore production but at half their application dosage they can increase colonization and spore production. Non-fungicidal biocides are known to alter root exudates type and their quantity which are important in plant growth promotion and also in AM colonization (Hamel, 1996).

Tillage

In natural ecosystems, seedlings are colonised by AM fungi through contact with the common mycorrhizal network (CMN). Soil tillage has been shown to cause severe disruption to the CMN which results in delayed or reduced root colonisation and also a reduction in the volume of soil which is exploited by the AM fungi (Gosling *et al.,* 2006). This leads to a reduced plant nutrient uptake which affects crop growth and yield. The exact effect of tillage on AM fungi depends on the type of soil. Deep inversion tillage is likely to bury AM fungal propagules below a depth which early seedling root growth cannot reach and delays the colonisation of the root by the AM fungi (Gosling *et al.,* 2006). Tillage has been shown to also cause a shift in community structure of the AM fungi and may favour species that colonize mainly from spores instead of other propagule sources such as root fragments and CMN. These species of AM fungi may not be the most beneficial fungi to the host plant since they use a large portion of their resources to producing spores instead of the uptake of nutrients (Gosling *et al.,* 2006). Reducing tillage has been shown to increase AM fungal colonization and nutrient uptake. In agro-ecosystems tillage increases nitrogen mineralisation, increased soil temperature, reduced weed numbers and improved soil physical properties all of which may impact on the association of the plant with AM fungi (Gosling *et al.,* 2006).

Crop rotations

Soils which are used in agricultural production have a low density of AM fungi compared to natural ecosystems and are often dominated by *Glomus* species. One reason for this is a low host diversity which mainly occurs in crop monoculture. Monoculture may select for AM fungi species which provide limited benefits to the host plant. These AM fungi grow and sporulate most rapidly and divert most of their resources to their own reproduction and growth (Hamel, 1996). In general, increasing crop diversity is beneficial to AM fungi whereas adding a non-mycorrhizal host crop to the fields can have a negative effect on MA fungi colonization, nutrient uptake and the yield of the crops (Johansson *et al*., 2004). Root and AM fungi hyphal fragments (important for early colonization) only survives for around six months in the soil and when non-mycorrhizal host crop plants occupy the soil, the propagule numbers decline and AM colonization of the subsequent crop will be delayed and reduced in quantity. Cropping with a non-mycorrhizal host plant can be more detrimental to a subsequent highly mycorrhizal dependant crop than either tillage of phosphate fertiliser (Gosling *et al.,* 2006; Johansson *et al*., 2004). Bare fallow periods have a similar effect to non-mycorrhizal host crops and reduced propagule numbers, colonization, nutrient uptake and in some cases the yield of subsequent mycorrhizal crops (Johansson *et al*., 2004). Cultivating the bare fallow can further reduce hyphal survival which can be detrimental. Area's which contain soils with low phosphate availability, an extended bare fallow results in a condition termed long fallow disorder in which the crops experience phosphate and zinc deficiency (Johansson *et al.,* 2004).

1.6 Motivation for study

The influence of AM fungi and associated soil micro-organisms such as mycorrhizal helper bacteria and plant-growth promoting rhizobacteria to enhance plant growth and mycorrhizal development has been extensively studied (reviewed in Dames and Ridsdale, 2012). Through these studies, the mechanisms underlying the benefits afforded by both AM fungi and associated bacteria can be determined. The use of these mechanisms can then be applied to trials to further understand the tripartite symbiosis which can then be enhanced and used in sustainable agriculture practices. Through this, one can hopefully enhance plant productivity and food quality and lessen the usage of chemical alternatives which are detrimental to the environment. This is a collaborative study between South Africa and Argentina, examining multi-trophic mycorrhizal complexes and raspberry is a focus host in both countries.

1.7 Hypothesis and Objectives

Hypothesis

Soil bacteria associated with arbuscular mycorrhizal fungi contribute to mycorrhizal establishment and improve plant growth.

In order to investigate this, the following objectives were addressed:

1. Isolation of AM fungi from various soil sources (South Africa and Argentina) and subsequent isolation of spore-associated bacterial isolates.

2. Characterisation of bacterial isolates according to selected PGPR characteristics.

3. Identification of selected spore-associated bacterial isolates.

4. Determine if spore-associated bacterial isolates contribute to mycorrhizal establishment and enhance plant growth.

CHAPTER 2

CHARACTERISATION OF AM FUNGAL SPORE-ASSOCIATED BACTERIA: SOUTH AFRICA

2.1 Introduction

Arbuscular mycorrhizal fungi associate with various terrestrial plant species, some of which are beneficial in the agriculture industry. Raspberry (*Rubus idaeus*) and strawberry (*Fragaria ananassa*) have both been shown to be host to various arbuscular mycorrhizal fungi in the genus *Glomus, Gigaspora and Scutellospora*. These AM fungi have been shown to promote and depress the growth and yield of both berry types (Taylor and Harrier, 2000; Taylor and Harrier, 2001; Stewart *et al.,* 2005; Douds, Jr *et al.,* 2008).

The use of microbial communities to alleviate the usage of chemicals and the industry looking to become more organic is increasing in order to produce high yield crops. These organisms include bacteria that are able to solubilise phosphate, fix nitrogen or inhibit pathogens, for example some *Bacillus* species are used to improve plant growth, and studies into tripartite symbiosis with AM fungi have been done (Vestberg *et al.,* 2004; Orhan *et al.,* 2006).

The berry industry is one of the smaller fruit markets in South Africa. Berries are high value crops due to the many health benefits they offer and hence the demand for berries is increasing (den Hartigh, 2011). There are approximately 40 berry growers across South Africa, of which a few produce 98% of the fresh berries. South Africa’s biggest competition for blueberries is Argentina and for raspberries are Spain, Portugal, Morroco and Mexico (den Hartigh, 2011).

In 2008 South Africa exported approximately 200 tonnes of berries, now, five years later the industry is exporting “1000t of both blueberries and raspberries, and 50t of blackberries per annum” (Erasmus, 2012). Approximately 80% of the berries exported by South Africa go to the United Kingdom and continental Europe markets and exports are expected to reach R12.9 billion this year (Erasmus 2012).

Strawberry production in South Africa has been occurring for the past 60 years. There is approximately 300 hectares of strawberries grown in South Africa, 80% of which are planted to locally bred cultivars. Yields before 1959 exceeded 6 t/ ha but now yields of 20 to 30 t/ha are obtained. “The annual value of the crop is estimated to be worth about 25 million Rands” (Human and Evans, 1989).

The aims of the experiment were to isolate bacteria from AM fungal spores from the soils associated with the roots of various natural indeginous forest sources, raspberry and strawberry samples in South Africa and characterise them according to plant-growth promoting abilities and then identify the isolates with the most beneficial characteristics.

2.2 Materials and Methods

2.2.1 Soil Samples

Ten replicate random soils samples were collected from the following sources: Natural indigenous forest soil samples, of which the spores extracted from the soils were combined from Forest Way, Stutterheim, Eastern Cape (32°34'S 27°25'E) and Mountain Drive, Grahamstown, Eastern Cape (33°18'S 26°32'E), in February. Raspberry (*Rubus idaeus* Heritage sp.) soil samples from Winterberg Berries, Nettle Grove Farm, Tarkastad, Eastern Cape, South Africa (32°01'S 26°16'E), in September. Strawberry (*Fragaria ananassa)* soil samples from de Kleine Maasstroom, Bedford, Eastern Cape (32°41'S 26°05'E), in October.

2.2.2 Estimation of Root and Soil Bacterial Populations

Root Samples

Root samples (10 x 1 cm) of the ten replicate samples from the raspberry and strawberry sources above were placed in sterile 0.2% saline in a 1:1 ratio and allowed to incubate at room temperature for an hour in the solution and then underwent serial dilution (five x 5 fold dilution). Aliquots (100 µl) from the dilutions 5^{-4} and 5^{-5} were spread onto Tryptone Soy Agar (TSA) (Biolab Catalog no: HG000C17) for total bacterial counts. Each dilution was spread onto two replicate plates. The number of colonies formed was counted after 24 hrs and 48 hrs incubation at 28°C (± 2°C). The Colony Forming Units per cm of root length (CFU.cm^{-1}) was calculated by Eqn 1 (Johnson and Case, 2007) and averaged per source.

Soil Samples

Soil samples (1 g) of the ten replicate samples from the raspberry and strawberry sources above were placed in sterile 0.2% saline in a 1:1 ratio and allowed to incubate at room temperature for an hour in the solution and then underwent serial dilution (five 5 fold dilution). Aliquots (100 µl) from the 5^{-4} and 5^{-5} dilutions were spread on Tryptone Soy Agar (TSA) (Biolab Catalog no: HG000C17) for total bacterial count. Each dilution was spread onto two replicate plates. The number of colonies formed was counted after 24 hrs and 48 hrs incubation at 28°C ($\pm 2^{o}$C). The Colony Forming Units per g of soil ($CFU.g^{-1}$) was calculated by Eqn 1 (Johnson and Case, 2007) and averaged per source.

Equation 1:

$$\text{CFU} = \text{number of colonies x dilution factor x } \frac{\text{Standard Volume}}{\text{Aliquot plated}}$$

2.2.3 AM Fungal Spore Extraction and Isolation

Spore Extraction

AM fungal spores were extracted from the various soil sources by the method described by Smith and Dickson, 1997. This involved weighing out 100g of soil in a beaker, then a soil solution was made with dH_2O which was stirred and allowed to settle. The supernatant was decanted through a nest of sieves (425 m, 250 m, 125 m, 45 m) and repeated three times. The sieves were washed and the debris from the 425 m discarded. The debris in the remainder of the sieves were washed into 50 ml centrifuge tubes and filled with sterile water (Smith and Dickson, 1997).

Spore Purification

The spore suspensions were centrifuged (1900 g for 5 min) on a Heraues Megafuge 1.0R and the supernatant discarded. The pellet was resuspended in 60% sucrose solution and centrifuged for a further 5 min. The supernatant (containing the spores) was filtererd through a Buchner funnel onto a filter paper disc (Whatman #1) and rinsed with distilled water. The filter paper was transferred to the lid of a clean petri dish (Smith and Dickson, 1997).

The spores were counted, microscopically examined using a Leica S4E dissecting microscope and morphologically separated according to only colour, spores of different sizes of similar colour were combined. Images were taken with a Fujifilm Finepix S2900 digital camera. The spores were only used to isolate potential mycorrhizal helper and plant growth promoting bacteria and not for identification or molecular purposes.

2.2.4 AM fungal colonisation

The colonisation of the plant roots was evaluated by using a method described by Koske and Gemma, (1989) and Smith and Dickson, (1997). This method involved using 1-3 cm long sections of the root and cleared with 5% KOH solution, which removes the cytoplasm and all coloured material from the plant cells. The roots in the KOH were heated in a water bath at just below boiling for 30 min. The KOH solution was discarded and the roots were rinsed with distilled water. The roots were bleached in an alkaline H_2O_2 solution for 10-30 min. When the roots turned white, the bleach was poured off and the roots rinsed with water. The roots underwent acidification to ensure the binding of trypan blue to the mycorrhizal structures by using 0.1M HCl solution. The HCl was removed and the roots covered with lactoglycerol solution containing 0.05% trypan blue and stained for 30 min at 80-90^{o}C in a waterbath. The stain was poured off and destained for 12-24 hrs.

Colonisation was characterised by the formation of intercellular hyphae and intracellular arbuscules. The percentage root colonisation was determined using a modified Line Intersect method (McGonigle *et al* ., 1990). Segments of the stained roots were placed on microscope slides and covered with a cover slip after placing a drop or two of the destain. The roots are squashed and examined with a Nixon YS100 compound microscope for their entire length, one field of view at a time using 40 x magnifications was recorded with or without mycorrhizal structures (arbuscules, vesicles or hyphae). The number of mycorrhizal structures present in a 100 fields of view was determined to be equal to the percentage colonisation by the AM fungi present. Images were taken using an Olypmus DP72 camera on an Olympus SZX16 microscope.

2.2.5 AM fungal Spore-associated Bacterial Isolation

External Surface of Spores

The spores of the AM fungi were placed in sterile 0.2% saline, vortexed (Labnet Int, Inc Vortex mixer) for 30 min and allowed to soak. A sample (200 µl) from the solution was serially diluted (five x 5 fold dilution) with sterile 0.2% saline solution and an aliquot (100 µl) from the 5^{-4} and 5^{-5} dilutions was spread onto TSA plates and incubated at 28^{o}C to prevent overgrowth of fast growing isolates. The number of CFU.ml^{-1} per spore solution was determined for each plate after 24 and 48 hours. Colonies were streaked discontinuously to obtain pure bacterial colonies which were cultured in Nutrient Broth (NB) (Biolab Catalog no: 1024537), at 150 rpm at 37^{o}C for 48 hrs. Glycerol stocks of the isolates were made by combining 0.5 ml of the NB cultures with 0.5 ml of 50% glycerol solution (Merck, Catalog no: 1040211) and stored at -80^{o}C for further anaylsis.

Internal Surface of Spores

AM fungal spores were surface sterilised by soaking in a 2% chloramine T trihydrate (Merck Catalog no: 1024260250) and Tween 20 solution for 30 min at constant vortexing (medium speed) (Labnet Int, Inc Vortex mixer), after which the solution was centrifuged (Hangzhou Allsheng Super mini centrifuge) at 10 000 g for 5 min, the supernatant was removed and 1 ml sterile dH_2O was added to wash the spores. The sample was vortexed for 5 mins continuously and then centrifuged at 10 000 g for 5 min, the supernatant was removed and replaced with new sterile H_2O. This wash process was repeated three times. A sample (100 µl) from the solution was spread onto TSA plates and incubated at 28^{o}C to determine the efficiency of the sterilisation step. There after 500 µl of each 10 mg.ml^{-1} of ampicillin sodium salt (Sigma Catalog no: A9518-5G), chlorampenicol (Sigma Catalog no: C0378-25G) and streptomycin sulphate *Streptomyces* sp. (Calbiochem Catalog no: 5711) solution was added and soaked for 45 min with constant vortexing. After which the spores are then washed three times with sterile dH_2O as mentioned above. A sample (100 µl) from the solution was spread onto TSA plates and incubated at 28^{o}C to determine the efficiency of the antibiotics. After the final wash, the water was replaced with 1 ml 0.2% sterile saline, centrifuged at 10 000 g for 5 min and the spores crushed using a sterile micropestle to release the internal bacteria into the saline solution.

This was serially diluted (five x 5 fold dilution) with distilled 0.2% saline solution and aliquot (100 µl) from the 5^{-4} and 5^{-5} dilutions was spread onto TSA plates and incubated at 28°C. The number of CFU.ml^{-1} per spore solution was determined for each plate after 24 hrs. Colonies were streaked discontinuously to obtain pure bacterial colonies which were cultured in NB, on a rotary shaker at 150 rpm at 37°C for 48 hrs. Glycerol stocks of the isolates were made by combining 0.5 ml of the NB cultures with 0.5 ml of 50% glycerol solution, stored at -80°C for further anaylsis.

2.2.6 Morphological Identification of Bacterial Isolates

A Gram stain was performed to ensure the purity and phenotypic characteristics of each bacterial isolate. Samples were heat fixed, stained with crystal violet, crystal violet was fixed with an iodine solution, excess stain was washed with 95% ethanol and counter-stained with safranine solution respectively for 60 sec each with a 5 sec wash interval with water (Madigan and Martinko, 2006). Samples were air dried and visualised under a compound microscope under immersion oil (Nixon YS100). The ratios of Gram-positive and Gram-negative bacteria, rods and cocci was determined for the spores.

2.2.7 Plant Growth Promoting Characterisation Tests

Preparation of Bacterial Isolates

The bacteria isolated from the spores were grown overnight at 37°C in 20 ml of NB in a 50 ml centrifuge tube, on a rotary shaker at 150 rpm. A sample (1 ml) of each isolate was collected and centrifuged at 10 000 g for 5 mins. The supernatant was removed and the cells resuspended in sterile 0.2% saline. Their concentrations were measured on a (Shimadzu UV mini – 1240 Vis Spectrophotometer) at an OD of 600 nm (Poole *et al.,* 2001). Concentrations were adjusted to an OD reading of 0.3 by dilution with sterile 0.2% saline (Sbrana *et al.,* 2002). These preparations were used in all subsequent testing.

Phosphate-Solubilisation

The potential of the bacterial isolates to produce organic acids to solubilise inorganic sources of phosphate such as calcium phosphate was tested using selective media. A modified phosphate medium-NBRIP (National Botanical Research Institute's Phosphate growth medium) with bromophenol blue was used (Mehta and Nautiyal, 2001; Chibuogwu and Nmesoma, 2011) (Appendix I A-1). Phosphate solubilisers produce clearing zones around the colonies in media containing $CaHPO_3$ (Rodriguez and Fraga, 1999). The prepared bacterial isolates (100 µl) were placed in wells in the media and incubated at 28^oC for five days. Five replicate wells for each bacteria isolated were perfomed. Controls were inoculated with sterile saline. Yellow halo formation indicates the release of organic acids (Mehta and Nautiyal, 2001). The halo's formed were measured across two diameters per replicate well and averaged.

Phytase Production

Phytate is an organic phosphate source commonly found in the soil. The production of phytase, an enzyme produced by PGPR to degrade phytate was tested by inoculating the prepared bacterial isolates (100 µl) into five replicate wells on phytase screening medium containing phytic acid (Appendix I A-2). Phytase activity was indicated by a clearing halo around the wells containing the bacterial isolates (Hariprasad and Niranjana, 2009). No halo measurements were taken, only positive or negative reactions were recorded.

Indole Acetic Acid Production

The bacterial isolates (1 ml) were grown in 10 ml of DEV Tryptophan Broth (Merck Catalog no: 1106940500) (Appendix I A-3) for 3 days at 37^oC. This media contains L-tryptophan which is a precursor for the formation of IAA. Cells were centrifuged (4500 rpm for 20 min) and the supernatant collected. To the supernatant (300 µl), 600 µl Kovacs Reagent (Merck Catalog no: 1092930100) was added. A colour change to red is indicative of IAA production (Cappuccino and Sherman, 2008). Three replicates for each bacteria isolate was performed. Un-inoculated broth media was used as a control.

Production of ammonia

The conversion of organic nitrogen sources to ammonia is an essential process providing nitrogen to plants. The ability of bacterial isolates to produce ammonia was investigated. Bacterial cultures (1 ml) were inoculated into 10 ml peptone water (Appendix I A-4) and incubated at 28^{o}C for 48 to 72 hrs. Control was un-inoculated peptone water. Nesslers reagent (Merck, catalogue number SAAR4425000KF) (0.5 ml) was added. Development of a brown to yellow colour was a positive test for ammonia production (Cappuccino and Sherman, 2008).

Fungal Pathogen Inhibition

This experiment was conducted to determine if any of the bacterial isolates had an inhibitory effect on selected soil-borne fungal pathogens. Bacterial isolates (100 µl) were inoculated into wells on Potato Dextrose agar (PDA) (Biolab Catalog no: HG00C100) placed at the four points along a perpendicular axis. The fungal isolates *Fusarium oxysporum f sp lycopersici* (Sacc.) W.C. Snyder and H.N. Hans (PPRI number 5457) and *Phythophthora nicotianae* Breda de Haan (PPRI number 10962) were obtained from the National Fungal Culture Collection, Pretoria. These were inoculated centrally onto each plate and incubated at 28^{o}C. The fungal growth (mm) was measured along the two perpendicular axes for approximately 10 days. Three replicates of each bacterial isolate was used as well as three control plates which contain sterile water. The percentage fungal inhibition was determined for each bacterial isolate against both pathogens (Prasanna Reddy *et al.,* 2010).

Siderophore Production

Siderophores are low molecular weight compounds capable of sequestering iron from a medium. Bacterial isolates were assayed for siderophore production on modified chrome azurole S agar (CAS) (Appendix I A-5). Isolates (1 ml) were inoculated into two replicate wells on the plates and incubated for 5 days at 30^{o}C. Controls were inoculated with sterile 0.2% saline. Development of yellow-orange halos around the isolates indicated siderophore production (Schwyn and Nielands, 1987; Alexander and Zuberer, 1991; Milagres *et al.,* 1998). No halo measurements were taken, only positive or negative reactions were recorded.

Protease Production

Protease is an important hydrolytic enzyme used to degrade protiens in the cell wall of fungi and plants. Protease activity (casein degradation) was determined using skimmed milk agar (Appendix I A-6). The prepared bacterial isolates (100 µl) were placed in wells in the media. Five replicate wells for each bacteria isolate was perfomed. Controls were inoculated with sterile 0.2% saline. Plates were incubated at 30^{o}C for 5 days. Development of halo zone around five replicate wells was considered positive for protease production (Chaiharn *et al.*, 2008). The halo's formed were measured across two diameters per replicate well and averaged.

Cellulase Production

Plant and fungi cell walls are composed of cellulose and the production of hydrolytic enzymes such as cellulase by bacteria enable the breakdown of cellulose. Cellulase activity was determined by using carboxymethylcellulose (CMC) agar (Appendix I A-7) (Kasana *et al.,* 2008). The prepared bacterial isolates (100 µl) were placed in wells in the media. Five replicate wells for each bacteria isolate was perfomed. Controls were inoculated with sterile 0.2% saline. Plates were incubated at 30^{o}C for 5 days. Grams iodine (5 ml) was added to the CMC plates and left for 5 min, as the iodine binds to undegraded cellulose. Development of halo zone around five replicate wells was considered positive for cell wall degrading enzymes (Chaiharn *et al.*, 2008). The halo's formed were measured across two diameters per replicate well and averaged.

Chitinase Production

Many fungal cell walls are comprised of chitin, and therefore the production of chitinases enable bacteria to breakdown fungal cell walls. Chitinase production is tested by plating on chitin agar (Appendix I A-8). The bacterial isolates were streaked onto the plate and incubated at 37^{o}C for 48 hrs. Halo formation around the isolate was positive for chitinase production.

Catalase production

Catalase production was tested by streaking a single line of the prepared bacterial isolates onto Nutrient Agar (NA) (Biolab Catalog no: HG0000C1) incubated at 37^{o}C for 18-24 hrs.

Control plates contained no bacterial isolates. Positive reaction for catalase was determined by instant bubble (O_2) formation upon the addition of 6% H_2O_2 solution (Chaiharn *et al.*, 2008).

2.2.8 Molecular Identification of Bacterial Isolates

DNA Extraction

Bacterial cultures were grown overnight in NB at 37^{o}C, 150 rpm. The cultures were centrifuged at 10 000 g for 5 min. The bacterial DNA of the selected isolates were extracted using the ZR Fungal/Bacterial DNA miniprep kit (Zymo Research Catalog No: D6005) according to manufacturer's instructions. This involves adding 750 µl of the lysis buffer to the bacterial cells. The mixture was transferred to a BashingBead^{TM} lysis tube and vortexed for 10 mins at maximum speed on a Labnet Int, Inc. vortex mixer. This process breaks down the bacterial cells, releasing the DNA into the lysate. The tubes were centrifuged 10 000 g for one min. Approximately 400 µl of the supernatant is then transferred to a Zymo-Spin^{TM} IV Spin filter in a collection tube and centrifuged at 7000 g for one min to filter the lysate and remove cell debris and other cellular components which was trapped in the filter. The DNA was in the collection tube, to which 1200 µl of Fungal/Bacterial DNA Binding Buffer is added. This helps bind the DNA to the column while other debris is washed away.

The filtrate solution (800 µl) is added to a Zymo-Spin^{TM} IIC Column in a collection tube and centrifuged for one min at 10 000 g. The flow through is discarded and the process repeated. DNA Pre-wash Buffer (200 µl) is added to the IIC column in a new collection tube and centrifuged at 10 000 g for one min. Fungal/Bacterial DNA Wash Buffer (500 µl) was added to the IIC column and centrifuged, thus washing any excess debris and compounds from the filter while retaining the DNA. The IIC column was transferred to a sterile 1.5 ml microcentrifuge tube and 100 µl of DNA Elution Buffer is added directly to the column and then centrifgued at 10 000 g for one min to elute the DNA. The DNA was visualised on a 0.8% Agarose gel, using tracking dye for visualisation and viewed with a Uviprochem UV Transilluminator. The concentration of the DNA was determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) to establish efficient quantity of DNA for PCR analysis.

PCR amplification

Amplification of the 16S rDNA bacterial gene is carried out with the bacterial primers Fd1 and rP2 (1500 bp product) (Weisburg *et al.*, 1991); and GM5F and R907 (500 bp product) (Muyzer *et al.,* 1994) (Table 2.1) using a 2720 Thermal Cycler (Applied Biosystems). A reaction volume of 50 µl with 5 µl template DNA was carried out. The reaction contained 25 µl KAPA *Taq* Readymix DNA polymerase (Kapa Catalog no: KK1024), 2 µl of primers. A control contained 10 µl of distilled water instead of DNA. The PCR was performed using the conditions listed in Table 2.2 and 2.3. PCR products were electrophoresed with a Lambda DNA/*Eco*RI + *Hind*III molecular marker (Promega Catalog no: HG1731) on an ethidium bromide stained agarose gel (0.8%) at 100V for 1 hr 15 mins. Gels were visualised using a Uviprochem UV transilluminator and a digital image recorded.

Table 2.1: PCR primers used to amplify the 16s rRNA structure in the bacterial isolates

Primer	Sequence	Tm (^oC)
Fd1	5’- AGAGTTTGATCCTGGCTCAG -3’	54
rP2	5’- ACGGCTACCTTGTTACGACTT -3’	56.1
GM5F	5’- CCTACGGGAGGCAGCAG - 3’	58.2
R907	5’CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCC GTCAATTCCTTTGAGTTT-3’	81.8

Table 2.2 PCR cycling conditions used for amplification of bacterial DNA from the isolates with the primers Fd1 and rP2.

Conditions	Temperature (^oC)	Time (seconds)	Cycles
Initial Denaturation	95	90	1
Denaturation	95	45	30
Annealing	50	45	
Extension	72	60	
Final Extension	72	120	1

Table 2.3 PCR cycling conditions used for amplification of bacterial DNA from the isolates with the primers Gm5F and R907.

Conditions	Temperature (^{o}C)	Time (seconds)	Cycles
Initial Denaturation	95	120	1
Denaturation	95	30	30
Annealing	53	30	
Extension	72	60	
Final Extension	72	120	1

Purification of DNA and Sequencing

The PCR product which was used for sequencing was purified using the Promega Wizard SV gel and PCR Clean Up Kit (Promega Catalog no: A9281) according to the manufacturer's instructions. To 30 µl of the PCR products was 90 µl of Membrane Binding Solution^{TM} was added. The mixture was added to a column and collection tube, incubated at room temperature for one min. The samples were centrifuged for 3 min at 12000 g. The flow through was discarded and 700 µl of Membrane Wash Solution^{TM} was added. The samples were centrifuged and flow through discarded. After which 500 µl of Membrane Wash Solution^{TM} was added and centrifuged at 12000 g for five min, the flow through was discarded and the samples centrifuged for one min. The column was transferred to a 1.5 ml microcentrifuge tube and 50 µl of nuclease-free water was added and the samples centrifuged.

The samples were sent to Inqaba Biotechnical Industries (Pty) Ltd, Pretoria where they underwent Sanger Sequencing which produces an ABI format chromatogram file, which is analysed by FinchTV, version 1.4.0, Geospiza, Inc. The sequence was cleaned up by replacing invalid base pairs using the software. Sequences were submitted and aligned for comparative analysis to the National Centre for Biotechnology Information (NCBI) online standard Basic Local Alignment Search Tool (BLAST) program (Wheeler *et al*., 2006).

The significant level of similarities with 16S rRNA in the Genbank database was determined by noting the percentage identity and the expectation value (E-value). The percentage identity is the percentage of nucleotides which are identical between two aligned sequences. The E-value shows the equivalent or similarity of a number of alignment score to the raw alignments score that are expected to occur in a database; the lower the E-value the more significant the score (Wheeler *et al*., 2006). The reliability of the percentage identity was related to the expectation value (E-value). A significant similarity value of > 95% and > 98% was accepted in this study as belonging to the same genus and species, respectively.

2.2.9 Statistical Analyses

To normalise data sets, data were log transformed if expressed in CFU. A t-test of independence between groups was used to compare CFU values, root colonisation % and spore counts. A repeated measure Analysis of Variance (ANOVA) with 2 way effects was used for the Fungal Inhibition *in vitro* study data set, that were obtained for a treatment over a period of time (Zar, 1999). This type of analysis was utilised under the principle that the dependent variable (i.e. treatment) was measured repeatedly (Statsoft, 2005; Lutgen *et al*., 2003). Significant difference between group means was determined using test of significance. The level of significance for ANOVA and Fischer LSD test was 5%. All analyses were carried out by means of StatSoft, Inc. (2009) STATISTICA (data analysis software system) Version 7. www.statsoft.com.

2.3 Results

2.3.1 Estimation of Root and Soil Bacterial Populations

The total culturable numbers of bacteria present in the rhizospheric (root) and bulk soil from raspberry and strawberry plantations was estimated. The log CFU.cm^{-1} was higher in the root samples (6.44 and 6.50) compared to the bulk soil samples (4.67 and 4.59) in both the raspberry (t-value = 8.79, df = 18, $p < 0.001$) and strawberry samples respectively (t-value = 4.93, df = 22, $p < 0.001$) (Figure 2.1) with no significant difference in the root (t-value = -0.135, df = 20, p = 0.89) and soil (t-value = 0.54, df= 20, p = 0.59) total culturable numbers of bacteria between the raspberry and strawberry samples (Figure 2.1).

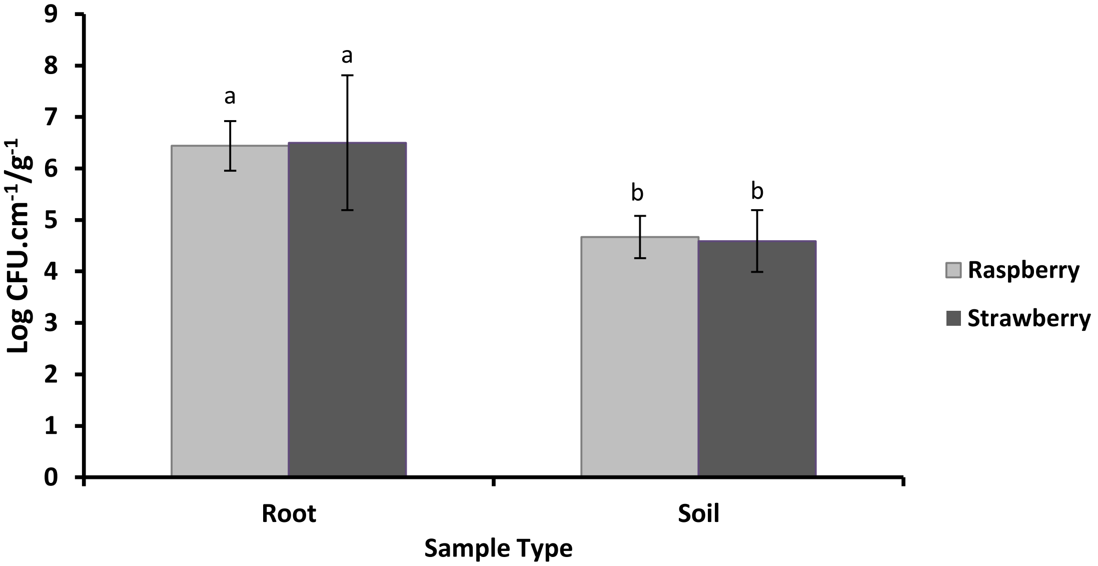


Figure 2.1 Total culturable bacterial counts of Raspberry (*Rubus idaeus* cv. Heritage) and Strawberry (*Fragaria ananassa*) root and bulk soil samples. Log CFU values are means of ten replicates, bars represent ± standard deviation. Column labels with the same letter represent no significant differences.

2.3.2 AM Fungal Colonisation and Spore Counts

The AM fungal root colonisation percentage was determined for both the raspberry and strawberry samples. The colonisation percentage was significantly higher in the strawberry samples than in the raspberry samples (t-value= -6.022, df= 20, $p < 0.001$) (Table 2.4).

The spores were extracted from the soil and placed on filter paper in petri dishes, they were microscopically counted and morphologically separated according to colour. The raspberry sample had a significantly higher spore count compared to the strawberry samples (t-value= 2.66, df=64, $p < 0.01$). The raspberry samples were collected in September, when they were still young plants whereas the strawberry samples were collected in October, and they were adult plants at harvest stage.

Table 2.4 Average Arbuscular Mycorrhizal fungal root colonisation percentage (%) and Arbuscular Mycorrhizal fungal spore counts sampled from Raspberry (*Rubus idaeus* Heritage sp.) and Strawberry (*Fragaria ananassa*).

	Root Colonisation %	**Spore Count (100 g)**
Raspberry	4.9	10.3
Strawberry	45	3.9

Three different AM fungal spore morphotypes were found in the natural indigenous forest samples, raspberry and strawberry samples. The first was an orange-golden coloured spore, then a dark brown coloured spore and a clear-grey coloured spore; all with variations in size and colour intensity (Figure 2.2). The root stains of the raspberry (Figure 2.3) and strawberry (Figure 2.4) samples revealed AM fungal colonisation by the presence of arbuscules, hyphae, spores and vesicles, these images however do not represent the entire root samples investigated for colonisation, only a colonised segment of root. The presence of arbuscules was higher in the strawberry samples as the cortical cells were colonised.

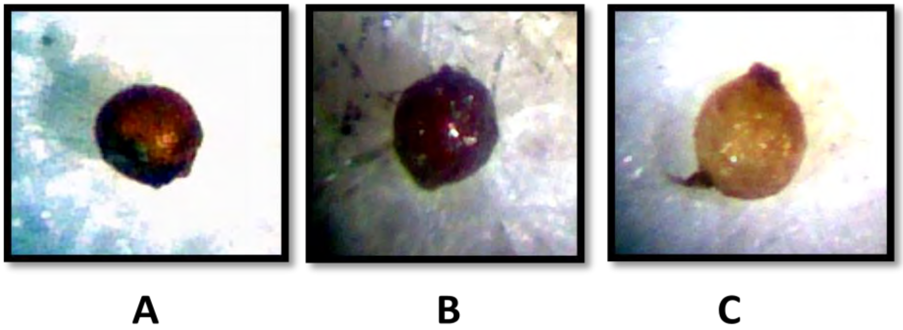


Figure 2.2 Arbuscular Mycorrhizal fungal spores morphotypes of Natural indigenous forest, Raspberry (*Rubus idaeus* Heritage sp.) and Strawberry (*Fragaria ananassa*) samples. A- Orange-gold coloured spore with dull surface, B- Dark brown coloured spore morphology will a glossy surface, C- Clear-grey coloured spore with glossy surface, attached is part of the subtending hyphae.

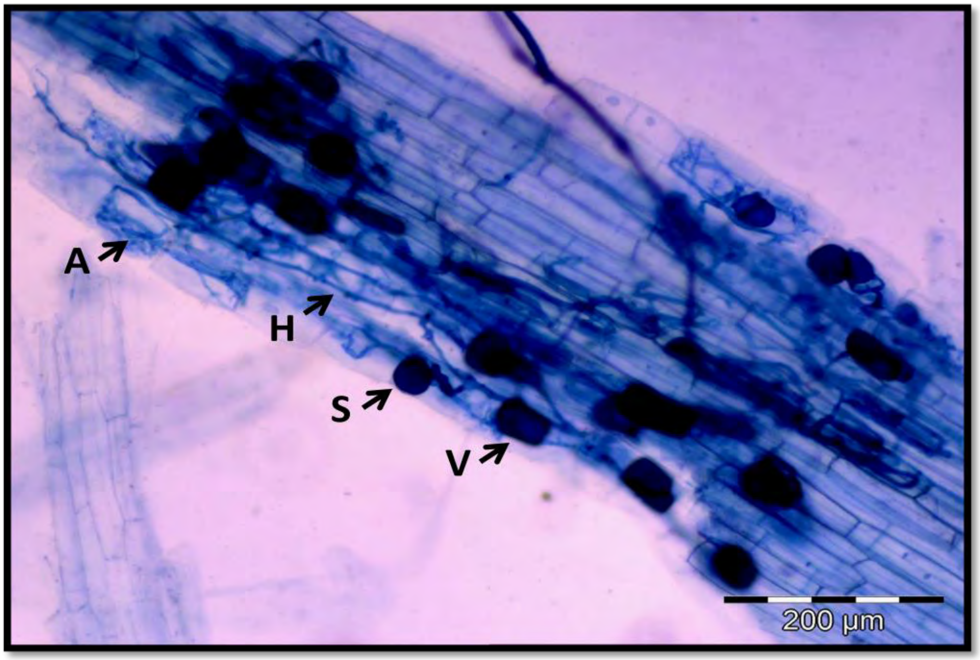


Figure 2.3 Arbuscular Mycorrhizal fungal spore colonisation of Raspberry (*Rubus idaeus* cv. Heritage) root samples. A- Arbuscule in cortical cell of root, H- intra-radical hyphae, S- spore, V- vesicle.

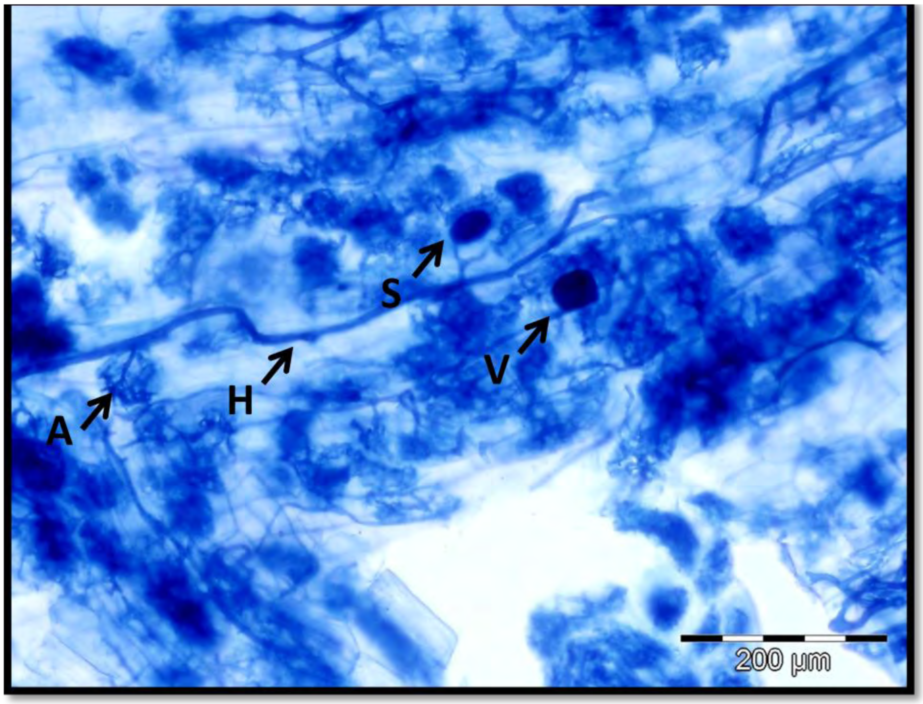


Figure 2.4 Arbuscular Mycorrhizal fungal spore colonisation of Strawberry (*Fragaria ananassa*) root samples. A- Arbuscule in cortical cell of root, H- intra-radical hyphae, S- spore, V- vesicle.

2.3.3 AM fungal Spore-associated Bacterial Isolation

Bacteria were isolated from the external and probable internal surfaces of the three main AM fungal spore morphotypes from natural indigenous forest, raspberry and strawberry samples. The control plates after the sterilisation processes showed no bacterial growth. The CFU.spore^{-1} was higher from the external surfaces of the spores when compared to internal surfaces in all three samples. There was no difference in the total bacterial numbers between the three samples (Table 2.5). A total of 27 bacteria were isolated from the surfaces of the AM fungal spores, of which 24 were Gram-positive while only three were Gram-negative. There was no difference in the number of Gram-positive bacteria between the three samples. Of the bacterial isolates, 23 were rods while 4 were cocci (Table 2.5).

Table 2.5 Bacterial cultures from the external and probable internal Arbuscular Mycorrhizal fungal surfaces sampled from Natural Indigenous Forest, Raspberry (*Rubus idaeus* cv. Heritage) and Strawberry (*Fragaria ananassa*).

SAMPLE	CFU.spore^{-1}	Total Number Isolated	Gram Positive	Gram Negative	Rods	Cocci	Rods:Cocci
NATURAL							
EXTERNAL	3.73E+04	6	5	1	6	0	6:0
INTERNAL	1.26E+03	2	2	0	0	2	0:2
TOTAL	4.98E+04	8	7	1	6	2	3:1
RASPBERRY							
EXTERNAL	2.55E+04	5	5	0	5	0	5:0
INTERNAL	3.24E+03	5	4	1	5	0	5:0
TOTAL	5.65E+04	10	9	1	10	0	10:0
STRAWBERRY							
EXTERNAL	1.87E+04	4	4	0	4	0	4:0
INTERNAL	9.86E+03	5	5	0	3	2	3:2
TOTAL	2.86E+04	9	9	0	7	2	7:2

2.3.4 Bacterial Isolate Coding

The bacteria were coded according to the isolation procedures, First letter with S represents South African Isolate, E and I represent isolation from the external and internal surfaces of the AM fungal spores respectively, N represents isolation from the combined Natural indigenous forest sources, R and S followed were isolated from the raspberry and strawberry sources. A, B and C represent the AM fungal spore morphotypes mentioned earlier (Figure 2.2) followed by the isolate number. Bacterial morphology represented by S, M, L represent cell size small, medium and large under immersion oil in relation to the field of view. "BM" represents cultures showing rhizoid colony morphology which are characteristic of *Bacillus mycoides* growth. ENDOS represents endospore formation by bacillus shaped bacteria, viewed under immersion oil.

2.3.5 Plant Growth Promoting Characterisation Tests

The bacteria isolated from the external and internal surfaces of AM fungal spores sampled from natural indeginous forest, raspberry (*Rubus idaeus* cv. Heritage) and strawberry (*Fragaria ananassa*) soils were subjected to plant-growth promoting characterisation tests to evaluate their ability to enhance plant growth and inhibit fungal pathogens by various mechanisms.

Phosphate Solubilisation and Phytase Production

Bacterial isolates were grown in media containing $CaHPO_3$ as the only phosphate source to test for phosphate solubilisation. The media also contained bromophenol blue which turns to yellow upon a decrease in pH indicative of organic acid production. Phytase production was evaluated using media containing phytic acid. Eight isolates (SEN2, SEN3, SEN4, SEN5, SEN9, SEN10, SIN1 and SIN2) from the natural indigenous forest (Table 2.6), nine isolates (SERA1, SERA2, SIRA1, SERB2, SERB3, SIRB1 and SIRB2, SIRC1 and SIRC2) from the raspberry samples (Table 2.7) and nine isolates (SESA1, SISA1, SISA2, SESB1, SESB2, SEB3, SISB1, SISB2 and SISC2) from the strawberry samples (Table 2.8) were shown to be phosphate solubilisers (Figure 2.5). The diameters of the halos produced were measured, and ranged from 13 to 25mm from the natural indeginous forest samples, 18-25mm from the raspberry samples and 12-28mm from the strawberry samples (Table 2.7, 2.8, 2.9). None of the bacterial isolates produced phytase (Table Table 2.7, 2.8, 2.9).

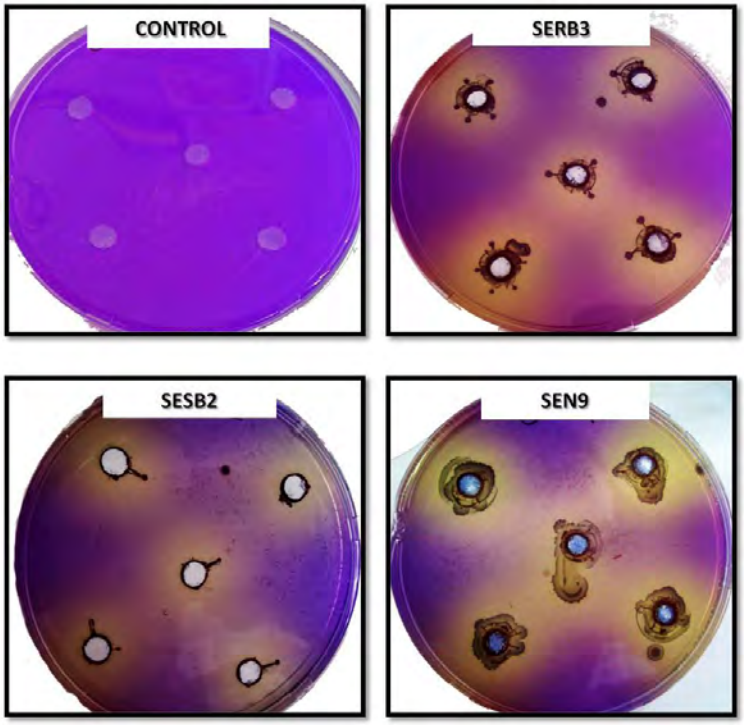


Figure 2.5 Phosphate solubilisation of $CaHPO_3$ by selected bacterial isolates from the surfaces of Arbuscular Mycorrhizal fungal spores from raspberry (*Rubus idaeus* Heritage sp.)- SERB3, strawberry (*Fragaria ananassa*) - SESB2 and natural indigenous forest sources- SEN9. Control- Inoculated with 0.2% sterile saline.

Indole Acetic Acid Production

Bacteria isolates were grown in DEV Tryptophan Broth to which Kovacs Reagent was added to the samples. All the isolates were negative for indole acetic acid production (Table 2.7, 2.8, 2.9) as all produced a yellow colour whereas a red colour is indicative of a positive reaction.

Production of Ammonia

Development of brown to yellow colour is positive test for ammonia production (Figure 2.6). One isolate (SEN9) from the natural indeginous forest samples (Table 2.7), three isolates (SIRA1, SIRB2 and SIRC2) from the raspberry samples (Table 2.8) and two isolates (SESB1 and SISA1) from the strawberry samples (Table 2.9) produced ammonia.

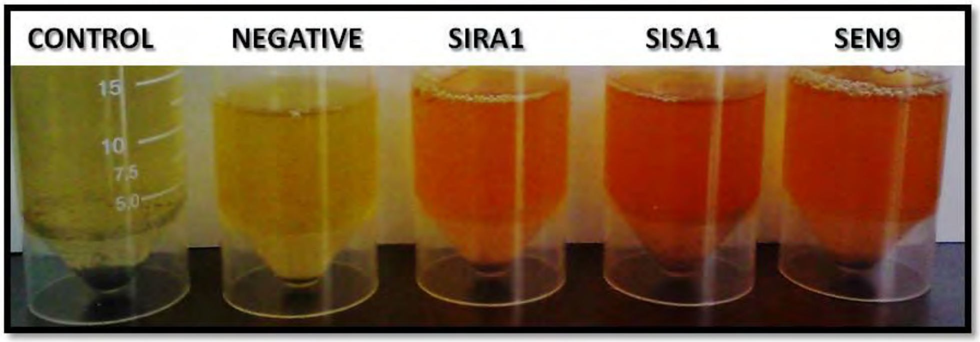


Figure 2.6 Production of ammonia by the selected bacterial isolates from the surfaces of Arbuscular Mycorrhizal fungal spores from raspberry (*Rubus idaeus* Heritage sp.) - SIRA1, strawberry (*Fragaria ananassa*) - SISA1 and natural indigenous forest sources - SEN9. Control - un-inoculated broth.

Fungal Pathogen Inhibition

This experiment was conducted to screen the bacterial isolates in their *in vitro* antagonstic effect on the hyphal growth of *Fusarium oxysporum* and *Phythophthora nicotineae*. Bacterial isolates were inoculated into wells on PDA placed at the four points along a perpendicular axis. The fungal growth (mm) was measured along the two perpendicular axes for 10 days.

Dual culture with Fusarium oxysporum

Dual culture studies of the bacteria against *F. oxysporum* revealed significant antagonistic ability by all the bacterial isolates ($F_{(20,618)}$= 4.4110, $p < 0.001$). The inhibition of growth was measured as a reduction in the hyphal growth compared to the control, however a reduction in the mycelium growth was observed, with the control showing more vigorous mycelium growth (Figure 2.7). Among the isolates, SIN1, SERA2 and SISC2 were the most effective in inhibition of *F. oxysporum* (Figure 2.8 A-C), which was confirmed by Fischer's LSD test and the percentage inhibition of the growth by the isolates ranged from 6-23% (Table 2.5).

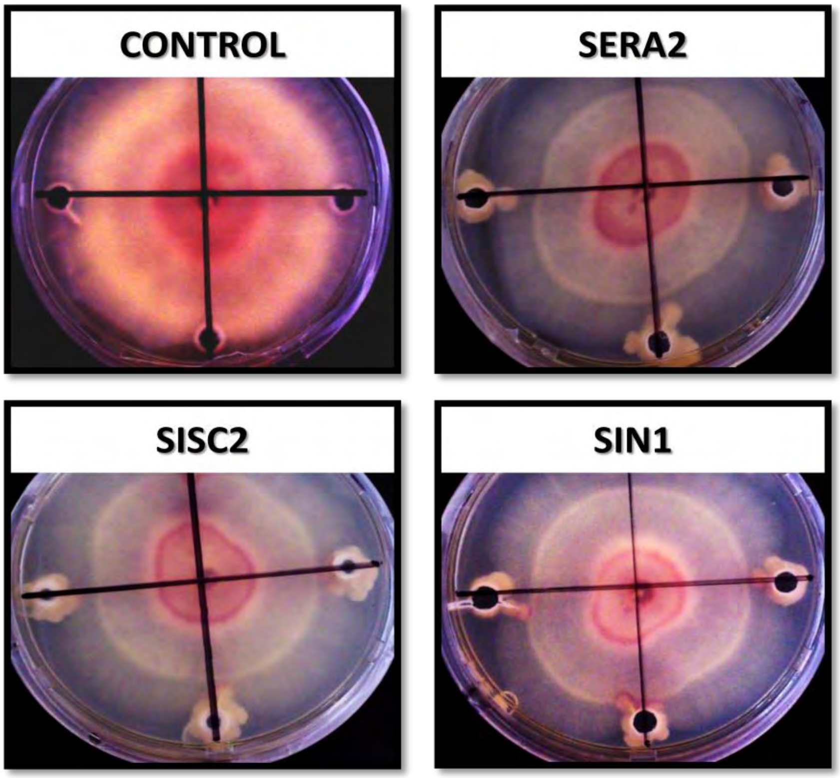
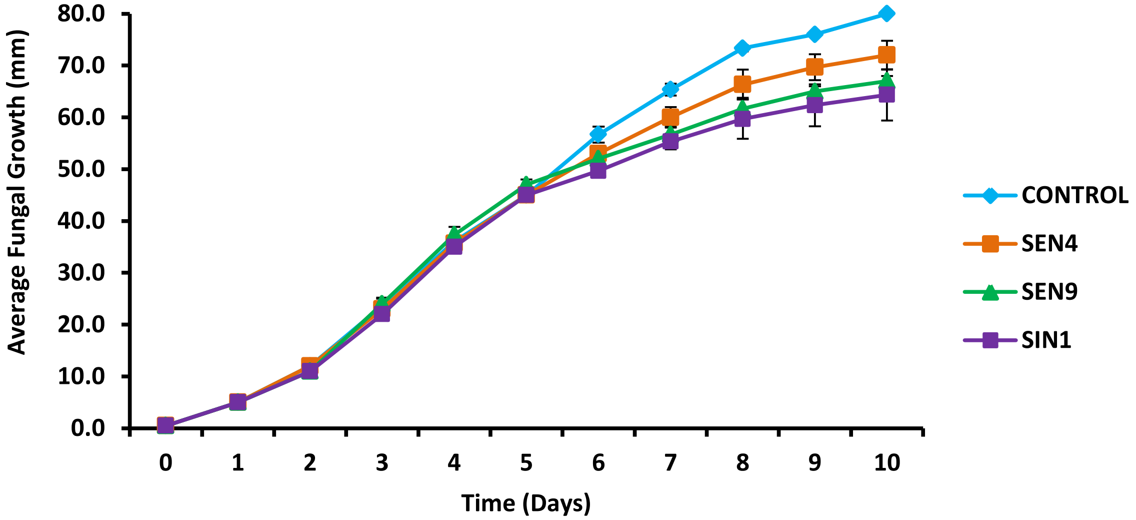
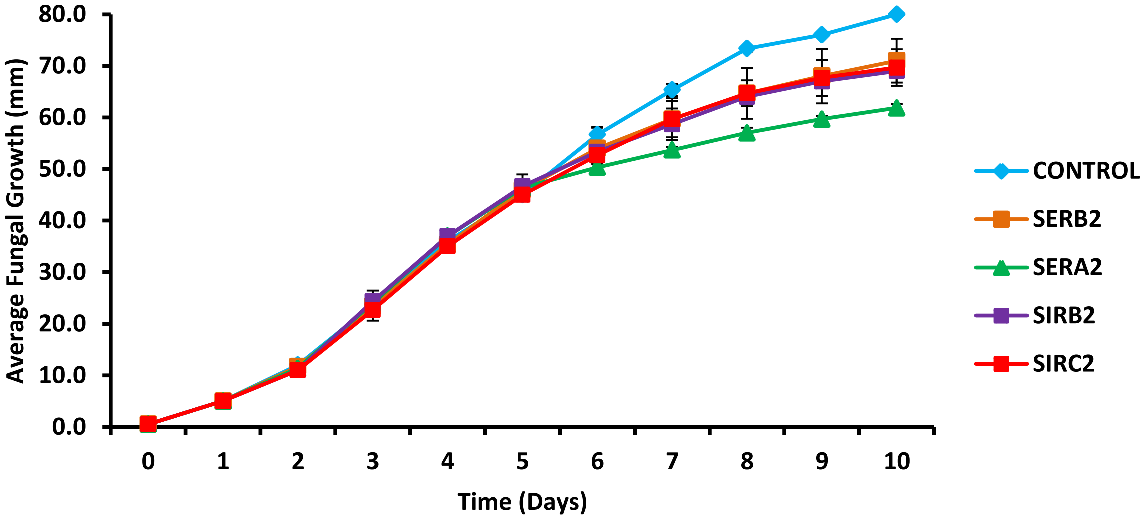


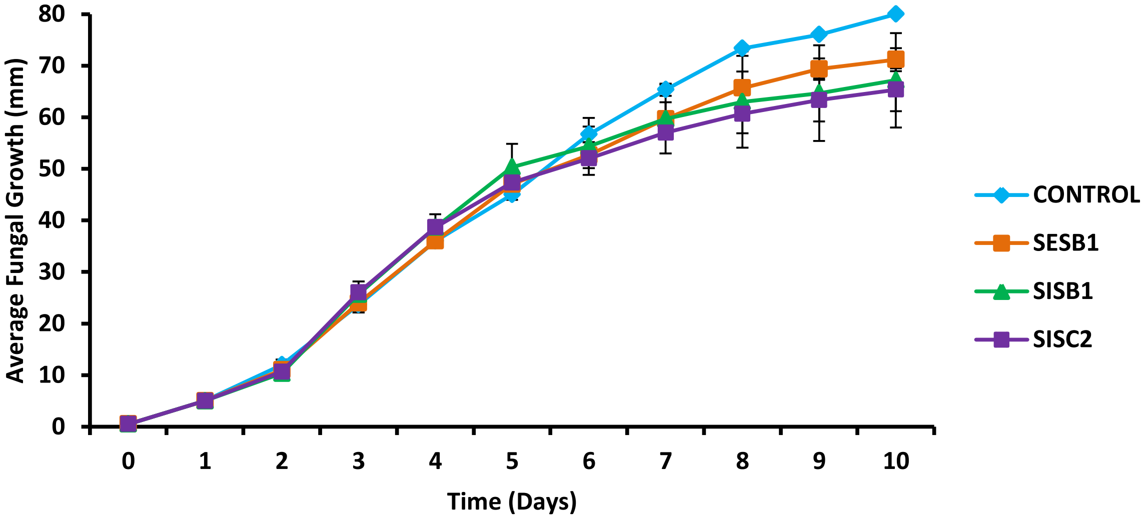
Figure 2.7 Fungal hyphal growth inhibition of *Fusarium oxysporum* by selected bacterial isolates from the surfaces of Arbuscular Mycorrhizal fungal spores from raspberry (*Rubus idaeus* Heritage sp.)- SERA2, strawberry (*Fragaria ananassa*) - SISC2 and natural indigenous forest sources- SIN1. Control- Inoculated with 0.2% sterile saline.



A



B



C

Figure 2.8 A-C Dual cultures of bacterial isolates and *F.oxysporum f sp lycopersici* showing bacterial effect on fungal growth (Mean ± SD) growth rate ($F_{(20,618)} = 4.110$; $p < 0.001$). Inhibition by selected bacterial isolates from the surfaces of AM fungal spores from **A-** natural indigenous forest sources, **B-** raspberry (*Rubus idaeus* Heritage sp.) and **C-** strawberry (*Fragaria ananassa*). Control- Inoculated with 0.2% sterile saline.

Dual culture with Phythophthora nicotianae

Dual culture studies of the bacteria against *P. nicotianae* revealed significant antagonistic ability by all the bacterial isolates ($F_{(20,618)} = 16.713$, $p < 0.001$). The inhibition of growth was measured as a reduction in the hyphal growth compared to the control (Figure 2.9). Among the isolates, SERB2, SISB1 and SEN9 were the most effective in inhibition of *P. nicotianae* (Figure 2.10 A-C), which was confirmed by Fischer's LSD test and the percentage inhibition of the growth by the isolates ranged from 33-49% (Table 2.5).

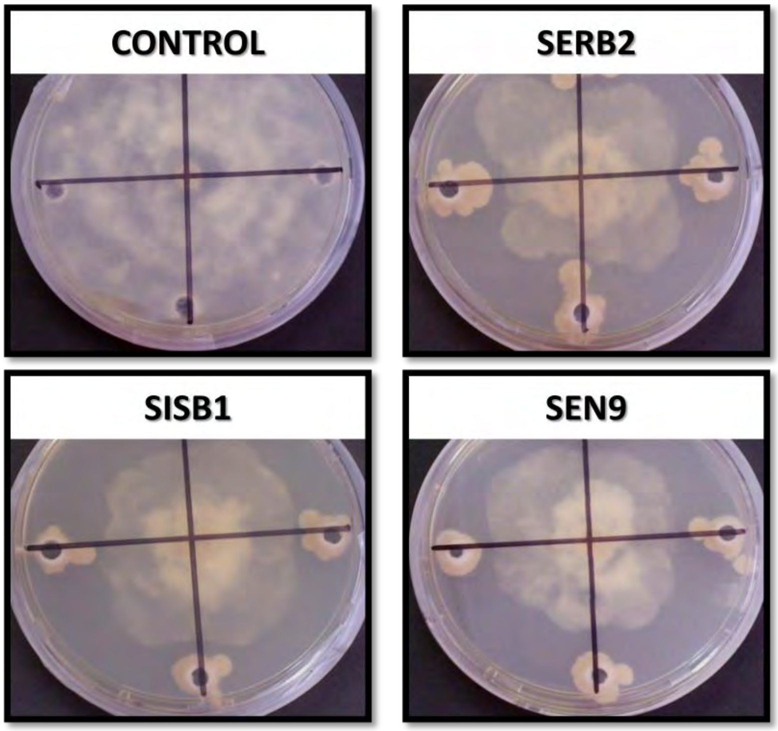
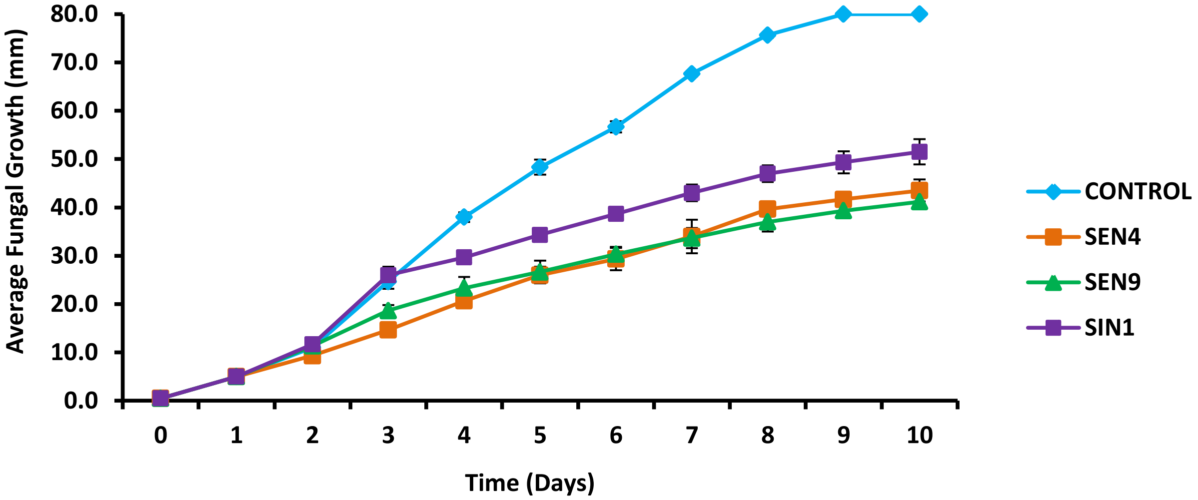
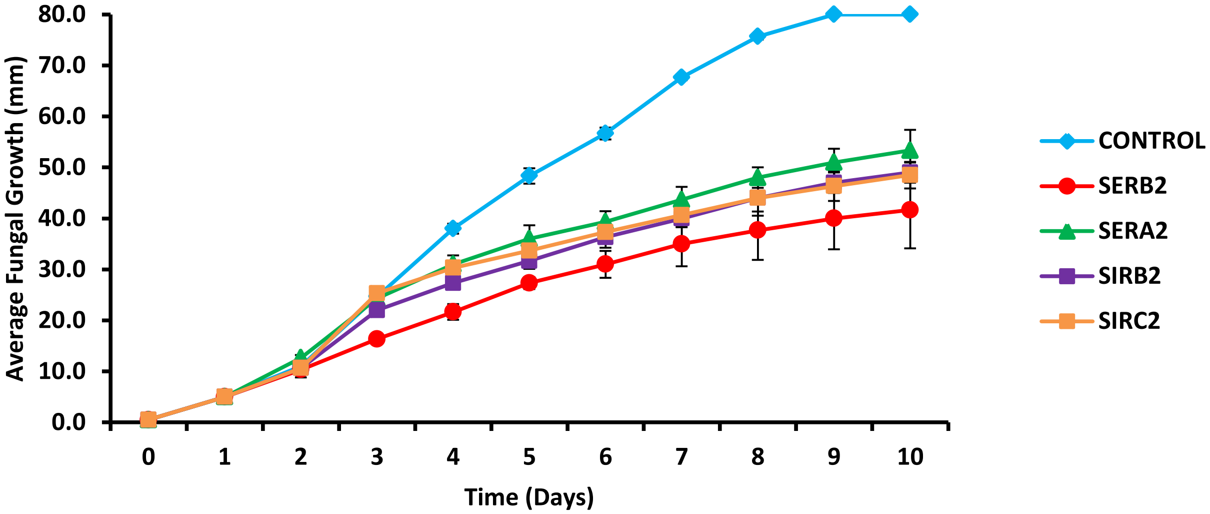


Figure 2.9 Fungal hyphal growth inhibition of *Phythophthora nicotianae* by selected bacterial isolates from the surfaces of Arbuscular Mycorrhizal fungal spores from raspberry (*Rubus idaeus* Heritage sp.)- SERB2, strawberry (*Fragaria ananassa*) - SISB2 and natural indigenous forest sources- SEN9. Control- Inoculated with 0.2% sterile saline.


Average Fungal Growth (mm)
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0
1
2
3
4
5
6
7
8
9
10
Time (Days)
CONTROL
SEN4
SEN9
SIN1

A


Average Fungal Growth (mm)
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0
1
2
3
4
5
6
7
8
9
10
Time (Days)
CONTROL
SERB2
SERA2
SIRB2
SIRC2

B

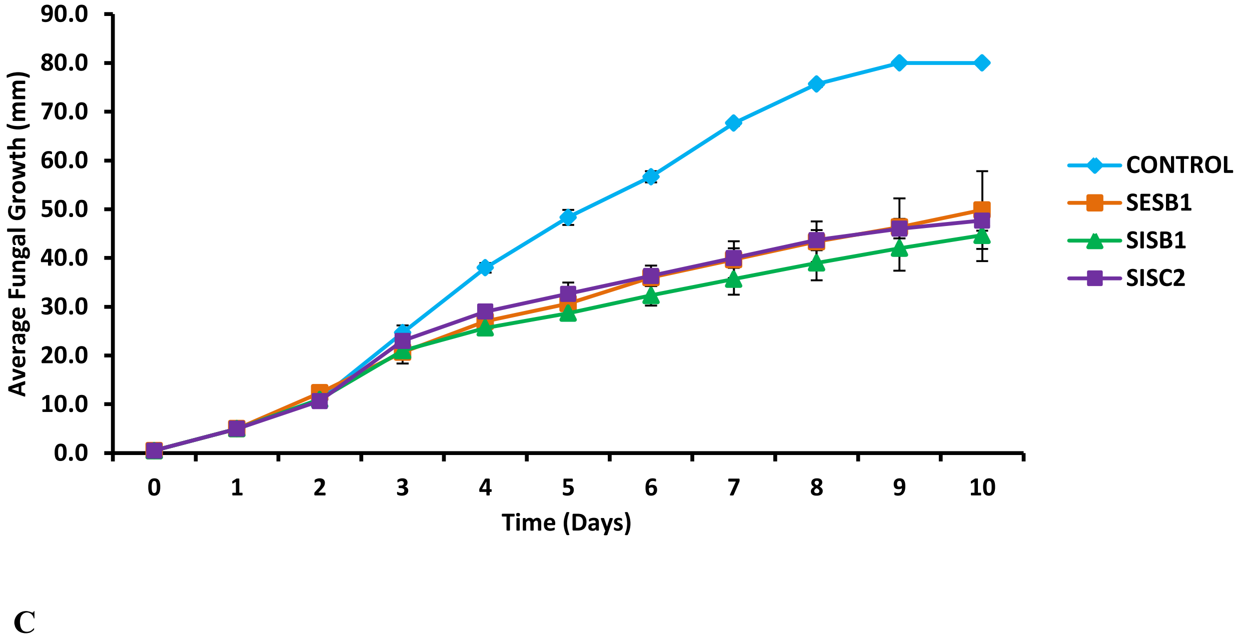


Figure 2.10 A-C Dual cultures of bacterial isolates and *Phythophthora nicotianae* showing bacterial effect on fungal growth (Mean ± SD) growth rate ($F_{(20,618)}$ = 16.713; $p < 0.001$). Inhibition by selected bacterial isolates from the surfaces of AM fungal spores from **A**- natural indigenous forest sources, **B**- raspberry (*Rubus idaeus* Heritage sp.) and **C**- strawberry (*Fragaria ananassa*). Control- Inoculated with 0.2% sterile saline.

Table 2.6 Fungal hyphal growth inhibition (%) of dual cultures of bacterial isolates against *Fusarium oxysporum* and *Phythophthora nicotianae*

Isolate	*F.oxysporum*	*P.nicotianae*
SEN4	6	46
SEN9	16	49
SIN1	19	36
SERB2	11	48
SERA2	23	33
SIRB2	14	39
SIRC2	13	44
SISC2	19	41
SESB1	11	38
SISB1	16	44

* Bold indicates highest % inhibition shown against the pathogens

Siderophore Production

Development of yellow-orange halos around the isolates indicated siderophore production (Figure 2.11). Four isolates (SEN3, SEN4, SEN9 and SIN1) from the natural indeginous forest samples (Table 2.7), four isolates (SERA2, SERB2, SIRB2 and SIRC2) from the raspberry samples (Table 2.8) and two isolates (SISB1 and SISC2) from the strawberry samples (Table 2.9) produced siderophores.

Figure 2.11 Siderophore production by selected bacterial isolates from the surfaces of Arbuscular Mycorrhizal fungal spores from raspberry (*Rubus idaeus* Heritage sp.)- SERA2, strawberry (*Fragaria ananassa*) - SISB1 and natural indigenous forest sources- SEN4. Control- Inoculated with 0.2% sterile

Protease Production

Protease activity by the bacterial isolates was determined using skimmed milk agar. Development of halo zone around five replicate wells was considered positive for protease production (Figure 2.12). One isolate (SISB1) from the strawberry samples produced protease.

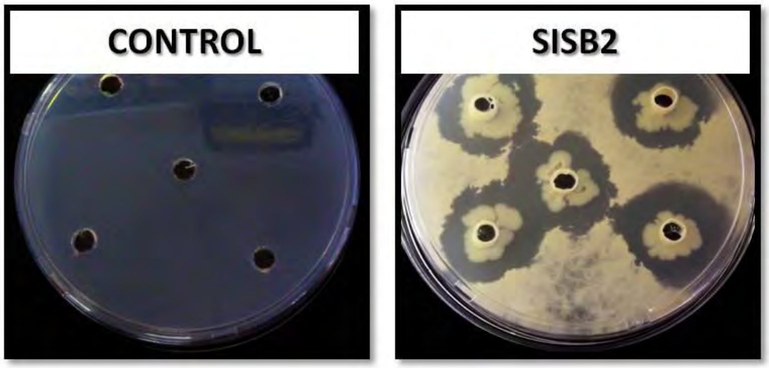


Figure 2.12 Protease production by selected bacterial isolates from the surfaces of Arbuscular Mycorrhizal fungal spores from strawberry (*Fragaria ananassa*) - SISB2. Control- Inoculated with

Cellulase Production

Cellulase activity was determined by using carboxymethylcellulose (CMC) agar. Grams Iodine was added to the CMC plates. Development of halo zone around five replicate wells was considered positive for cellulase production (Figure 2.13). Four isolates (SEN3, SEN4, SEN10 and SIN2) from the natural indeginous forest samples, three isolates (SERB2, SERB3 and SIRB1) from the raspberry samples and three isolates (SESB1 and SESB2) from the strawberry samples produced cellulase (Table 2.7, 2.8, 2.9).

Figure 2.13 Cellulase production by selected bacterial isolates from the surfaces of Arbuscular Mycorrhizal fungal spores from strawberry (*Fragaria ananassa*)- SESB2 and natural indigenous forest sources- SEN10. Control- Inoculated with 0.2% sterile saline.

Chitinase Production

Chitinase was tested by plating on chitin agar. None of the isolates produced clearing zones (Table 2.7, 2.8, 2.9).

Catalase Production

Catalase was tested by streaking diluted bacterial isolates on Nutrient agar. Positive reaction for catalase was determined by instant bubble (O_2) formation by the addition of 6% H_2O_2 solution. Four isolates (SEN2, SEN3, SEN4, SEN9) from the natural indeginous forest samples, six isolates (SERA1, SERB3, SIRA1, SIRB2, SIRC1 and SIRC2) from the raspberry samples and eight isolates (SESA1, SESB1, SESB2, SESB3, SISA2, SISB1, SISB2 and SISC2) from the strawberry samples produced catalase (Table 2.7, 2.8, 2.9).

Table 2.7 PGPR characterisation results of the bacterial populations of the external and internal AM fungal surfaces sampled from Natural indigenous forest. P-solub- phosphate solubilisation, IAA- Indole Acetic Acid, NH_3- Ammonia, CMC- cellulase, SMA- Protease, Phyt- phytase, Sidph- Siderophore, Catalase and Chitin production.

Isolate	Gram	Morphology	P-solub	IAA	NH_3	CMC	SMA	Phyt	Sidph	Catalase	Chitin
SEN2	+	Rods (L)	+ 15mm[1]	-	-	-	-	-	-	+	-
SEN3	+	Rods (S)	+13mm	-	-	+ 26mm	-	-	+	+	-
***SEN4**	-	Rods (S)	+ 22mm	-	-	+ 27mm	-	-	+	+	-
SEN5	-	Rods (S)	+ 22mm	-	-	-	-	-	-	-	-
***SEN9**	+	Rods (L)	+ 24mm	-	+	-	-	-	+	+	-
SEN10	+	Rods (S)	+ 18mm	-	-	+ 28.5mm	-	-	-	-	-
***SIN1**	+	Cocci	+ 25mm	-	-	-	-	-	+	-	-
SIN2	+	Cocci	+ 24mm	-	-	+ 23mm	-	-	-	-	-

* Bacterial isolates selected for molecular analysis
1 Average diameter of zone of clearance
L Large bacterial cell size (100x magnification)
S Small bacterial cell size (1000x magnification with immersion oil)
BM *Bacillus mycoides* – colony morphology

Table 2.8 PGPR characterisation results of the bacterial populations of the external and internal AM fungal surfaces sampled from Raspberry (*Rubus idaeus* cv. Heritage). P-solub- phosphate solubilisation, IAA- Indole Acetic Acid, NH_3- Ammonia, CMC- cellulase, SMA- Protease, Phytase, Sidph- Siderophore, Catalase and Chitin production.

Isolate	**Gram**	**Morphology**	**P-solub**	**IAA**	**NH_3**	**CMC**	**SMA**	**Phytase**	**Sidph**	**Catalase**	**Chitin**
SERA1	+	Rods (L) “BM”	+ 23mm[1]	-	-	-	-	-	-	+	-
***SERA2**	+	Rods (M) ENDOS	+ 25mm	-	-	-	-	-	+	-	-
***SERB2**	+	Rods (L) “BM”	+ 25mm	-	-	+ 35mm	-	-	+	-	-
SERB3	+	Rods (L)	+ 20mm	-	-	+ 24mm	-	-	-	+	-
SERB4	+	Rods (L) “BM”	-	-	-	-	-	-	-	-	-
SIRA1	-	Rods (M)	+ 20mm	-	+	-	-	-	-	+	-
SIRB1	+	Rods (L)	+ 22mm	-	-	+ 17mm	-	-	-	-	-
***SIRB2**	+	Rods (L)	+ 23mm	-	+	-	-	-	+	+	-
SIRC1	+	Rods (S)	+ 18mm	-	-	-	-	-	-	+	-
***SIRC2**	+	Rods (S)	+ 22mm	-	+	-	-	-	+	+	-

*	Bacterial isolates selected for molecular analysis
2	Average diameter of zone of clearance
L	Large bacterial cell size (100x magnification)
M	Medium bacterial cell size (400x magnification)
S	Small bacterial cell size (1000x magnification with immersion oil)
BM	*Bacillus mycoides* – colony morphology
ENDOS	Endospore presence

Table 2.9 PGPR characterisation results of the bacterial populations of the external and internal AM fungal surfaces sampled from Strawberry (*Fragaria ananassa*). P-solub- phosphate solubilisation, IAA- Indole Acetic Acid, NH_3- Ammonia, CMC- cellulase, SMA- Protease, Phytase, Sidph- Siderophore, Catalase and Chitin production.

Isolate	Gram	Morphology	P-solub	IAA	NH_3	CMC	SMA	Phytase	Sidph	Catalase	Chitin
SESA1	+	Rods (L) “BM”	+ 26mm^1	-	-	-	-	-	-	+	-
***SESB1**	+	Rods (L) “BM”	+ 20mm	-	+	+ 35mm	-	-	-	+	-
SESB2	+	Rods (L) “BM”	+ 22mm	-	-	+ 13.5mm	-	-	-	+	-
SESB3	+	Rods (L)	+ 26mm	-	-	-	-	-	-	+	-
SISA1	+	Cocci	+ 21mm	-	+	-	-	-	-	-	-
SISA2	+	Cocci	+ 22mm	-	-	-	-	-	-	+	-
***SISB1**	+	Rods (L) “BM”	+ 12mm	-	-	-	+ 7mm	-	+	+	-
***SISB2**	+	Rods (L)	+ 22mm	-	-	+ 32mm	-	-	-	+	-
***SISC2**	+	Rods (L)“BM”	+ 28mm	-	-	-	-	-	+	+	-

*	Bacterial isolates selected for molecular analysis
1	Average diameter of zone of clearance
L	Large bacterial cell size (100x magnification)
S	Small bacterial cell size (1000x magnification with immersion oil)
BM	*Bacillus mycoides* – colony morphology

2.3.6 Molecular Identification of Bacterial Isolates

Bacterial isolates were selected for molecular identification based on the best results of the plant-growth promoting characterisation tests. These isolates are represented in Tables 2.6, 2.7 and 2.8 by the asterisks. The DNA was extracted and underwent PCR amplification which was successful and efficient judging by the bands obtained (Figure 2.14). Amplification of the 16s rRNA of the bacterial isolates resulted in bands approximately 1500bp for the primers FD1 and rP2 and approximately 500 bp for the primers Gm5F and R907 in size, which is visualised using agarose gel electrophoresis (Figure 2.14). The sequences of the isolates were obtained (Appendix II) and identified using BLAST on the National Centre for Biotechnology Information (NCBI) website. Percentage Identity values greater than 95% were chosen as the cut off for significant identification at the genus level and 98% at species level (Table 2.10). Control was negative (not shown in selected gel).

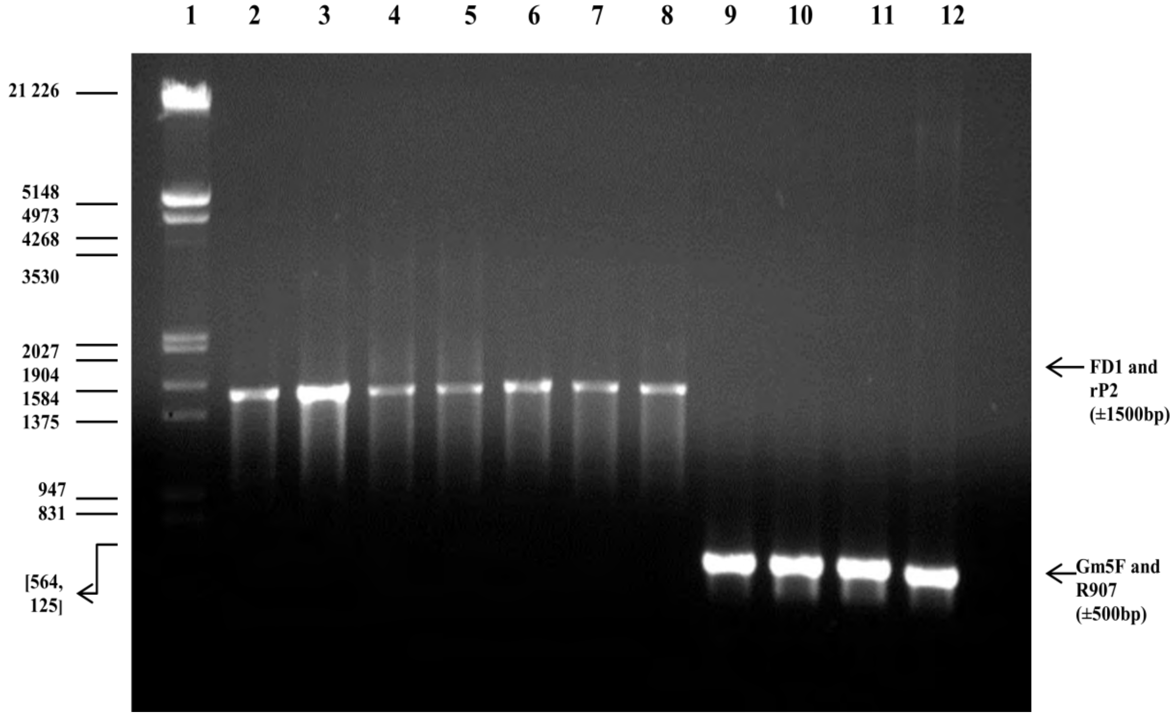


Figure 2.14 Ethidium bromide stained agarose gel (0.8%) showing PCR amplification by the primers FD1 and rP2, and primers Gm5F and R907 of the selected bacterial isolates from natural indigenous forest, raspberry and strawberry samples. Lane 1- Lambda DNA/*Eco*RI + *Hind*III molecular marker, Lane 2- isolate SERB2, Lane 3- isolate SESB1, Lane 4- isolate SISB1, Lane 5- isolate SISB2, Lane 6- isolate SEN9, Lane 7- isolate SISC2, Lane 8- isolate SIN1, Lane 9- isolate SEN4, Lane 10- isolate SERA2, Lane 11- isolate SIRB2, Lane 12- isolate SIRC2.

Table 2.10 Identified bacterial isolates obtained from the analysis of partial 16s rRNA using the National Centre for Biotechnology Information (NCBI) website.

Bacterial Isolate	Most Significant Alignment (NCBI)	% Identity	e-values	NCBI Accession Number
SEN4	*Acinetobacter ursingii*	99	0.0	AJ275039.1
SEN9	*Bacillus thuringiensis*	98	0.0	CP003687.1
SIN1	Unidentified	-	-	-
SERB2	*Bacillus mycoides*	98	0.0	AB681413.1
SERA2	*Bacillus cereus*	99	0.0	HQ333012.1
SIRB2	*Bacillus thuringiensis*	99	0.0	HQ873480.1
SIRC2	*Microbacterium nematophilum*	99	0.0	AF19539.1
SESB1	*Bacillus mycoides*	98	0.0	AB679984.1
SISB1	Unidentified	-	-	-
SISB2	*Bacillus sp.*	98	0.0	HM567041.1
SISC2	*Bacillus mycoides*	98	0.0	AB592538.1

From the sequences identified, it can be seen that the bacterial isolates from the natural indigenous forest were from the genus *Acinetobacter* and *Bacillus,* with one isolate unidentifiable. The raspberry sample contained bacterial isolates from the genus *Bacillus* and *Microbacterium.* The bacterial isolates from the strawberry samples were identified in the genus *Bacillus,* with one isolate unidentified. Three of the isolates were identified as *Bacillus mycoides* (Table 2.10).

2.4 Discussion

Estimation of Root and Soil Bacterial Populations

The total number of culturable bacteria present in the root and soil of raspberry and strawberry samples were determined. Colony forming units (CFU) gives an indication of the number of bacteria present whether beneficial or deleterious (Madigan and Martinko, 2006). The number of bacteria present in the rhizospheric soil was significantly higher than the bulk soil in both the raspberry and strawberry samples, however there was no significant differences in the numbers between the raspberry and strawberry samples (Figure 2.1). This indicates the system for increased bacterial numbers in the rhizospheric soil is consistent regardless of site. The increase in the rhizospheric bacterial numbers could be due to a number of reasons, the first is that in the root elongation process, the root passes through the bulk soil and as a result bacteria may be become attached to surface of the root and are transported by the root as it progresses through the soil (Burges, 1967).

The second reason is that the surface area of the rhizospheric soil is vastly different to the surface area of bulk soil; hence the likelihood of culturing accurate numbers from the rhizospheric soil is more efficient than the bulk soil since volume of bulk soil sampled may not fully represent the true total volume of bulk soil bacterial populations (Madigan and Martinko, 2006). Thirdly the location of sampling near the root surface limits the bulk soil to only the few top centimetres of the soil and doesn't represent the full bulk soil of the sample. This top soil is influenced by water, pH, organic matter such as leaf litter, and cations such as Ca, Mg, K, and Na (Garbeva *et al.,* 2004). These influence bacterial communities and numbers. Cations stabilize cell membranes and promote bacterial growth (Chiarini *et al.,* 1998). The pH of the soil also influences the availability of these cations. Therefore the distribution of microbes throughout the soil is not even as the microbes tend to cluster around plant roots, which is known as the "rhizosphere effect" (Heritage *et al.,* 1999). To eliminate the effect of these biotic and abiotic factors on the CFU values, one should sample deeper past the root to achieve true bulk soil populations and soil nutrient analyses should be performed on the soil sampled from the different sites to compare the numbers to these factors, which was not carried out in this study.

Fourthly, the increase in rhizospheric numbers could be due to production of exudates by plant roots and possibly by the hyphae of AM fungi associated with roots. These exudates contain carbohydrates, hormones and other compounds which promote bacterial growth (Bianciotto and Bonfante, 2002). The presence of other bacteria associated in the rhizospheric soil could provide nutrients otherwise unavailable in the soil to the rhizospheric bacterial communities. These microbes could be phosphate-solubilising, nitrogen fixing bacteria or belong to bacteria that produce siderophores in iron limiting conditions that alter the pH of the soil (Gray and Williams, 1971). In addition, although not the scope of this study, the type of soil, irrigation, land use history and management practices (for example, input of fertilizers and herbicides) may create unique soil environments that may change the microbial community populations (Johansson *et al.*, 2004). Both the raspberry and strawberry fields are intensively cultivated.

Only a tiny fraction of the soil microbes can be cultivated upon artificial culture media and appropriate conditions may not exist for the vast majority of microbes. Many microbes live in complex communities which cannot be replicated once removed from the environment onto culture media, thus resulting in an underestimation of the microbial activity in the soil (Heritage *et al.,* 1999). An estimate is further influenced by the fact that some bacteria are present as dormant spores in the soil which may germinate when in contact with the rich media. In addition, the numbers may not represent the unculturable bacteria which cannot grow on artificial media. These bacteria can be investigated by other techniques such as polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) by exploiting the 16s rRNA structure which is conserved within members of a species, whereas different species show divergent 16s rRNA structures which can lead to taxonomic studies and give a more accurate representation of the soil microbial communities (Heritage *et al.,* 1999, Madigan and Martinko, 2006).

AM Fungal Colonisation and Spore Counts

AM fungi are a natural occurrence in most soils and the advantage is that they are non-host specific. The presence of AM fungi in the soil are usually determined through the rate of colonisation by structures such as spores, hyphae and other structures but spore density in a given soil is usually the major determinant (Smith and Dickson, 1997).

The roots of the raspberry and strawberry samples were separated from the soil and the spores from the soil of the natural indigenous forest, raspberry and strawberry samples were extracted. The spores were counted per 100 g of soil and morphologically separated according to colour. The percentage root colonisation in the raspberry samples was significantly lower than the in the strawberry samples (Table 2.4). This difference could be due to the difference in the growth stages of the raspberry and strawberry plants.

The raspberry plants were already established and have young immature roots with less surface area to colonise whereas the strawberry samples were at harvest stage and had mature roots with a larger surface area to colonise. The raspberry samples were undergoing re-growth from the winter dormancy. The spores produced by and possibly released by previous AM fungal colonisation of the raspberry plant in the soil had become dormant (Bago and Bécard, 2002; Nagahashi, 2000). These dormant spores can remain alive in the soil for one or two years and the dormancy periods can differ greatly among species and genera (Giovannetti, 2000). Hence when the raspberry were sown, the spores were released from dormancy by the presence of new host roots, changes in pH, exudates secreted by the roots or by other signal molecules (Giovannetti and Sbrana, 1998; Bago and Bécard, 2002). The spores would then germinate and begin the AM fungal lifecycle with the raspberry plants. In the case of the strawberry plants which were at harvest stage, the AM fungi have probably completed their lifecycle as the roots showed a high number of arbuscules, spores and hyphae within the root cortex (Figure 2.4). The spore counts also represent the differences between the two samples. The raspberry soils had a higher spore count (10.3 / 100 g) compared to the strawberry samples (3.9 / 100 g).

The spores present in the raspberry soil were most likely the dormant remnants of previous colonisation whereas in the strawberry soils, any spores initially in the soil had germinated and colonised the strawberry roots. Since the strawberry plants showed full AM fungal colonisation, the AM fungi were in the process of completing their lifecycle which is by the production of spores, which is visible in the root stains. Sporulation by AM fungi may be season-dependant. Therefore a species of AM fungi though not active at one seasonal period may become effective depending on environmental conditions that aid colonisation and the subsequent release of spores into the soil (Kabir *et al*., 1997; Anderson *et al*., 1983).

Hence with the strawberry samples, although colonised by AM fungi, may not yet be releasing spores into the soil due to environmental conditions. The samples of the strawberry were collected in October, which is spring in South Africa, and may therefore only sporulate towards November and December when summer arrives with the increase in temperature and moisture. It is known that moisture affects spore germination phases including hydration, activation, germ tube emergence and hyphal growth processes therefore increased rainfall during the summer period may very well trigger sporulation (Sinegani *et al.*, 2005). In contrast, a study by Lovelock *et al.,* (2003) in Costa Rica showed that more spores were present in the dry season than the wet and that in particular *Glomus* and *Entrophospora* had higher spore numbers in the dry season compared to the wet however the reverse was found for *Acaulospora sp.* They also mentioned how predation of the spores is reduced in the drier periods. Therefore the low spore counts in this study could be due to predation which occurred in the previous wet season and the AM fungi had not yet released new viable spores due to lack of moisture not yet received. It is important to note that season has two effects on AM fungi, one being the seasonal patterns in weather and the other seasonal applied management practices (fertilisation, manuring and tillage), both of which could affect sporulation and colonisation by the AM fungi.

There are limitations to the method of spore sieving, such as distinguishing between dead and viable spores, loss of spores smaller than 45 µm, or spores adhering to particles which may be discarded (Schenck, 1982). Spore production also varies with respect to AM fungal species composition, dormancy and variability (Smith and Read, 2008). Therefore although reliable, may give an underestimation of total AM fungal spore population. Therefore the differences in percentage colonisation and spore counts may attribute to the stage in the AM fungal life cycle, circumstances of the host plant and soil properties, land use history, time or seasonal and agricultural practices.

The range in colour of spores is described by Morton (1988). Colour varies according to how the light interacts with the specimen. The three spore colour morphotypes isolated from the natural indigenous forest, raspberry and strawberry samples (Figure 2.2) could represent a number of AM fungal species since colour morphotypes ranging from white (hyaline) to red-black is present in the genera *Glomus* and *Scutellospora*, white (hyaline) to dark orange- brown in the genera *Acaulospora* and *Entrophospora,* and ranging from white to yellow in *Gigaspora.*

Therefore the AM fungal diversity is quite low, even from the natural indigenous soil, which is considered undisturbed from agricultural practices compared to the raspberry and strawberry samples which may affect AM fungal diversity. Since the spores of the colour types differed in size as well it becomes more difficult to make comparisons between species since they have a minimum and maximum size range, from 10 µm to 800 µm, and a mean size is difficult to obtain. Identification of the AM fungi was not the scope of this study.

AM Fungal Spore-associated Bacterial Isolation

Bacteria were extracted from the external and internal surfaces of the AM fungal spores from the natural indigenous forest, raspberry and strawberry samples. The CFU.spore^{-1} was determined for both spore surface types (Table 2.5), and the external surfaces amongst all three samples had a higher count than the internal surface of the spores. There is a greater presence of bacteria in the soil surrounding the spore as it is produced by the extraradical hyphae into the hyphosphere compared to the amount of bacteria present in the hyphae and plant root. In addition, bacteria have been known to attach to the external surface of the spores either through the production of biofilms or through appendages such as pili, fimbriae and flagella (Bianciotto and Bonfante, 2002). The bacteria present on the internal surfaces are found only in the cytoplasm of spores and hyphae.

The bacteria isolated from the surfaces of the spores were of both Gram-positive and Gram-negative, and of rod and cocci morphology (Table 2.5), which is consistent with the presence of these types of bacteria in the rhizosphere (Bharadwaj *et al.*, 2008). The presence of Gram-positive isolates was higher than the Gram-negative, particularly on the external surfaces compared to the internal surfaces of the spores. The rod morphology of the bacteria was also higher than the number of cocci isolates, been greatest on the external surfaces of the spores (Table 2.5). This could be due to some Gram-positive bacteria being able to produce resistant resting structures such as spores and are able to be cultured when inoculated on artificial media. The sterilisation of the external surface of the spores was a success as no bacteria was found on the control plates after the sterilisation step. Therefore the use of chloramine T and antibiotics such as ampicillin, chloramphenicol and streptomycin were efficient in inhibiting the presence of bacteria on the external surface of the spores.

The internal isolates can then be determined to be internal, except for the Gram-positive rods, which may be from the external surface and produce endospores which survive the sterilisation process since the supernatant may contain residual antibiotics inhibiting the spore germination and once cultured with the supernatant from the internal surface of the spores are able to germinate on the solid media

A study by Xavier and Germida, (2003) isolated bacteria from the external and internal surfaces of *Glomus clarum* spores and found a variety of genera present, with Gram-positive and Gram-negative isolates such as *Alcaligenes, Bacillus spp., Burkholderia, Flavobacterium* and *Pseudomonas spp.* The external surface showed an almost equal ratio of Gram-positive to Gram-negative isolates, with the Gram-negative having a higher count (23:28) However the internal surfaces had approximately 80% Gram-positive isolates of the genera *Bacillus spp.,* and found no Gram-negative isolates (Xavier and Germida, 2003).

Other studies have also revealed a variety of genera isolated from the external and internal spores of AM fungi such as *Glomus intraradices* and *Glomus mosseae*, including Gram-positive and Gram-negative bacteria such as *Pseudomonas spp., Corynebacterium, Pseudomonas putida, Paenibacillus, Bacillus spp., Stenotrophomonas spp.* (Mayo *et al.,* 1986; Walley and Germida, 1997; Budi *et al.,* 1999; Bharadwaj *et al.*, 2008).

Plant Growth Promoting Characterisation

A number of bacteria in the rhizosphere are known to stimulate plant growth. This is achieved through direct or indirect interactions with the plant roots. These bacteria have been termed Plant-Growth Promoting Rhizobacteria (PGPR) (Bloemberg and Lugtenberg, 2001). The direct mechanisms are through the production of phytohormones and plant growth auxins such as indole acetic acid (IAA), nitrogen fixation and the solubilisation of phosphorous. Indirect mechanisms include ability to decrease or prevent any deleterious effects of pathogenic microorganisms which can be by the production of antibiotics or siderophores by the bacteria (Singh and Kapoor, 1998).

Phosphate Solubilisation

Phosphorous is an essential macronutrient required for growth and development by plants. Many soil bacteria are phosphate solubilising bacteria which are able to mobilize phosphate ions from soluble organic and inorganic phosphate sources such as tricalcium phosphate, hydroxyapatite and rock phosphate (Gryndler, 2000; Vessey, 2003; Richardson *et al.*, 2009; Martinez-Viveros *et al.*, 2010). Through this process, the bacteria help improve the supply of phosphorous to the plants. The bacteria isolated from the natural indigenous forest, raspberry and strawberry samples were evaluated for their phosphate solubilising ability on media containing $CaHPO_{3.}$ The media also contained bromophenol blue which turns to yellow upon a decrease in pH. Of the 27 isolates screened, eight isolates from the natural indigenous forest, nine isolates from the raspberry and nine isolates from the strawberry samples produced yellow halos on the media (Figure 2.5). The decrease in pH causing the production of the yellow halos is due to the production of organic acids (Harisprasad and Niranjana, 2009; Khan *et al.,* 2009).

Organic acids such citrate, lactate and succinate are known to directly dissolve mineral phosphate sources as a result of anion exchange or the organic acids can chelate to Fe and Al ions associated with phosphorous. The insoluble form of P is then converted to soluble monobasic H_2PO_4 and dibasic HPO_4^{2-} ions (Harisprasad and Niranjana, 2009; Khan *et al.,* 2009). Other mechanisms have also been considered such as the production of chelating substances by the bacteria as well as the production of inorganic acids such as sulphidric, nitric and carbonic acid (Rodriguez and Fraga, 1999, Vessey, 2003; Richardson *et al.*, 2009; Martinez-Viveros *et al.*, 2010).

Organic phosphate sources are mineralised by the action of several phosphatase enzymes. Phosphatase activity involves the hydrolysis of phosphor-ester or phosphor-anhydride bonds, thereby catalysing the bound phosphorous into inorganic phosphorous (Rodriguez and Fraga, 1999; Richardson *et al.*, 2009; Martinez-Viveros *et al.*, 2010). Studies into these alternative mechanisms have revealed the use of techniques such as thin-layer chromatography to determine the organic acids involved and to quantify the production of these compounds and various others involved (Harisprasad and Niranjana, 2009; Khan *et al.*, 2009).

These isolates can therefore be considered as PGPR as phosphate solubilisation has been shown to enhance plant growth by releasing phosphorous from complexed inorganic sources (Artursson *et al.*, 2006). This process could be further facilitated by the use of AM fungi to translocate the phosphorous source to the plants more efficiently through their extra radical hyphae.

Phytase production

Phytate (Myo-inositol hexakis-phosphate) constitutes upto 80% of organic phosphorous in the soil making it one of the most abundant sources of phosphorous for plants (Lim *et al.*, 2007). Plants are known to produce phytase; however these are in low activity in the roots and other plant organs, suggesting that plant roots may not possess the ability to acquire phosphorous directly from the soil phytate sources. There are some PGPR which are able to produce phytase, an enzyme which degrades phytate to lower phosphate esters. Phytase producing PGPR have been shown to belong to the genera *Bacillus, Burkholderia, Pseudomonas, Serratia* and *Staphylococcus* (Hariprasad and Niranjana, 2009).

The 27 isolates were screened for the production of phytase using phytic acid as the major phosphate source. None of the isolates appeared to produce phytase as there was an absence of halos on the media (Table 2.7, 2.8, 2.9). Factors in the test should be evaluated and improved such as the length and temperature of incubation, which may influence secondary metabolite production of the enzyme. Perhaps to further eliminate the absence of phytase production, a colourmetric assay to quantify any phytase production by culturing the isolates in a liquid medium and measuring the production spectrophotometrically at 700 nm could be performed (Hariprasad and Niranjana, 2009).

Indole Acetic Acid Production

Some PGPR can have an influence on plant growth by the production of phytohormones such as auxins. Auxins contribute to the endogenous pool of phytohormones produced by the plant (Martinez-Viveros *et al.*, 2010). The production of indole acetic acid has been shown to be widespread among PGPR (Xie *et al.*, 1996; Patten and Glick, 2002) and is predominantly synthesized by an alternate tryptophan-dependant pathway which is carried out through indole-pyruvic acid.

IAA in plants is the main auxin which controls many important and beneficial physiological processes including cell enlargement and division, tissue differentiation and responses to light and gravity (Patten and Glick, 2002; Spaepen *et al.*, 2007; Shahab *et al.*, 2009; Martinez-Viveros *et al.*, 2010). Therefore PGPR which produce IAA and interact with plants have the potential to interact with any of the fore-mentioned processes, resulting in a positive or negative effect. IAA produced by PGPR can promote root growth (Spaepen *et al.*, 2007), which can subsequently enhance mycorrhizal contact (Garbaye, 1994).

None of the 27 isolates screened produced IAA (Table 2.7, 2.8, 2.9), which is usually indicated by a colour change to red upon the addition of Kovak's reagent to the supernatant after being cultured in DEV tryptone media. This result is questionable since many genera such as *Aeromonas, Bacillus, Burkholderia, Enterobacter, Pseudomonas* and *Rhizobium* have been shown to produce IAA (Martinez-Viveros *et al.*, 2010). An alternative method to further quantify if IAA was produced or not could be to use Salkowski's reagent which has been shown to be effective in quantifying IAA production (Joseph *et al.,* 2007; Chaiharn *et al.*, 2008; Suresh *et al.*, 2010). Perhaps these isolates, although not showing IAA production may be capable of other hormone production such as cytokinins and giberrellins, both important in root elongation promotion, which was not examined in this study (Martinez-Viveros *et al.*, 2010).

Ammonia production

Nitrogen is an essential plant nutrient. There are two types of nitrogen fixation carried out by PGPR: symbiotic and non-symbiotic. Non-symbiotic nitrogen fixation is carried out by free-living diazotrophs and can stimulate non-legume plants growth. These bacteria belong to the genera *Azoarcus, Azospirillum, Burkholderia, Gluconacetobacter* and *Pseudomonas* (Antoun and Prevost, 2005; Richardson *et al.*, 2009; Martinez-Viveros *et al.*, 2010). The conversion of organic nitrogen to ammonia by bacteria in the soil through a process termed ammonification may provide nitrogen to the soil which can be taken up by the plant roots (Madigan and Martinko, 2006).

Of the 27 isolates screened for ammonia production in peptone water, one isolate from the natural indigenous forest, three isolates from the raspberry and two isolates from the strawberry samples produced ammonia, indicated by the colour change of media to orange upon addition of Nessler's reagent (Figure 2.6). These isolates can therefore be possibly considered as PGPR as the production of ammonia through the conversion of organic nitrogen has been shown to enhance plant growth by improving the structure of the plant roots (Artursson *et al.*, 2006). Perhaps to evaluate the isolates ability to provide nitrogen from other forms, such as by atmospheric nitrogen could be determined through the use of nitrogen-free media (Cappucino and Sherman, 2008).

Siderophore Production

Iron is an essential element required for growth in all organisms and bioavailable iron in the soil is generally deficient. Therefore under iron-limiting environments, PGPR produce siderophores, low molecular weight compounds which competitively sequesters ferric iron. The selected bacterial isolated were screened for their siderophore producing ability on CAS media.

Four isolates from the natural indigenous forest, four isolates from the raspberry and two isolates from the strawberry samples produced siderophores, indicated by the orange halos around inoculated wells (Figure 2.11). These isolates could therefore be considered to be potential PGPR as siderophores have been shown to be important in plant growth promotion.

The bacterium which originally produces the siderophore, takes up the iron-siderophore complex through receptors specific to the complex on the outer cell membrane. Once inside the bacterium, the iron is released and available to support microbial growth (Siddiqui, 2005). The siderophores deprive the pathogenic fungi of the available iron in the soil as the PGPR siderophores have a greater affinity for the iron. Some PGPR can sequester iron from heterologous siderophores produced by other soil microorganisms (Suresh and Bagyaraj, 2002). Siderophore producing bacteria have been found to belong to *Bradyrhizobium, Pseudomonas, Rhizobium, Serratia,* and *Streptomyces* (Martinez-Viveros *et al.*, 2010). Different types of siderophores have been shown to be produced by bacteria, such as catecholates (by *E.coli* and *Bacillus spp.*); hydroxamates (by *Streptomyces spp.* and *Burkholderia spp.*) and carboxylates (Siddiqui, 2005).

Cell wall degrading enzyme production

Biocontrol of fungal pathogens has been shown to be mediated by the production of extracellular enzymes such as chitinase, cellulase and protease that are able to hydrolyse cell wall components consisting of chitin, β- 1, 3- glucanase and proteins (Siddiqui, 2005). Protease activity was evaluated as casein degradation of skim milk. One isolate (SISB1) from the strawberry samples produced protease, as indicated by the formation of a halo on the skim milk agar (Figure 2.12). Cellulase activity was determined by using carboxymethylcellulose (CMC) agar. Four isolates from the natural indeginous forest samples, three isolates from the raspberry samples and three isolates from the strawberry samples produced cellulase as indicated by the halos produced (Figure 2.13). Chitinase was tested by plating on chitin agar. None of the isolates produced clearing zones therefore it was assumed none produced chitinase (Table 2.7, 2.8, 2.9). Catalase was tested by streaking diluted bacterial isolates on Nutrient agar. Positive reaction for catalase was determined by instant bubble (O_2) formation by the addition of 6% H_2O_2 solution. Four isolates from the natural indeginous forest samples, six isolates from the raspberry samples and eight isolates from the strawberry samples produced catalase (Table 2.7, 2.8, 2.9). These isolates could be considered as potential PGPR as through the production of these enzymes could potentially inhibit fungal pathogens and thereby promote plant growth through indirect mechanisms.

Fungal Pathogen Inhibition

PGPR have been shown to mediate in the biological control of plant pathogens. The mechanisms involved can be direct via the production of antibiotics, siderophores, hydrogen cyanide, hydrolytic enzymes (chitinases, proteases and cellulases) or indirectly by competition with the pathogen for ecological niche such as infection and nutrient sites (Linderman, 2000; Bloemberg and Lugtenberg, 2001; Sharma and Johri, 2002).

The selected bacterial isolates were grown in dual culture with *Fusarium oxysporum* and *Phythopthora nicotianae* to establish the ability of the isolates to inhibit the growth of these fungal pathogens. There was significant antagonistic ability resulting in decreased growth of *F.oxypsorum* by the selected bacterial isolates ($p < 0.001$) (Figure 2.7).

Among the isolates, SIN1, SERA1 and SISC2 showed to be most effective in the inhibition of *F.oxypsorum* (Figure 2.8). The range of percentage inhibition was from 6 - 23 % (Table 2.6). The dual culture studies with *P. nicotianae* revealed significant reduction in hyphal growth by the selected bacterial isolates ($p < 0.001$) (Figure 2.9). Isolates SERB2, SISB1 and SEN9 were the most effective isolates in the inhibition of *P. nicotianae* (Figure 2.10). The range of percentage inhibition was from 33 – 49 % (Table 2.6). Therefore all isolates could be considered as PGPR as they inhibit fungal pathogens which could indirectly promote future plant growth of host plants.

It can be seen that all the bacterial isolates were most effective in inhibiting the hyphal growth of *P. nicotianae* compared to *F.oxypsorum* (Table 2.6). There are many mechanisms involved in the inhibition of fungal pathogens such as competition for nutrients, production of siderophores, antibiotics hydrogen cyanide, antifungal compounds and hydrolytic enzymes. The bacterial isolates used in the dual culture studies were evaluated for the production of siderophores and hydrolytic enzymes as previously discussed. The isolates which showed the most inhibition of *F.oxypsorum* were shown to only produce siderophores; therefore their mechanism of action was competing for iron in the media, which is known to be present in potatoes. Siderophore production by a *Pseudomonas sp.* has been shown to control *Fusarium sp* (Siddiqui, 2005).

The inhibition of *F.oxypsorum* began to occur after day 6, indicating secondary metabolite production by the isolates, possibly siderophores (Figure 2.8). *F.oxypsorum* belongs to the fungal division Ascomycota. Their cell walls are mostly comprised of chitin and β- glucans and have septate hyphae which provide the fungus with stability and rigidity (Madigan and Martinko, 2006). All of the isolates showed no production of chitinase and therefore would not be able to degrade the hyphae of *F.oxypsorum* which could account for the lower percentage inhibition compared to *P. nicotianae.* The bacterial isolates had higher percentage inhibition of *P. nicotianae* compared to *F.oxypsorum.* The inhibition of *P. nicotianae* began after day 3 of inoculation indicating the production of primary metabolites (Figure 2.10).

The isolates with the most inhibition were shown to produce protease, cellulase and siderophores. *P. nicotianae* belongs to the fungal division Oomycota. Their cell walls are composed mainly of cellulose rather than chitin. In addition, their hyphae are aseptate, and are therefore easier to degrade (Madigan and Martinko, 2006). Therefore the mechanisms of action by these isolates were to degrade the cellulose and protein components of the fungal cell wall and compete for iron. There may be however other mechanisms by which the isolates may have inhibited the two fungal pathogens which were not identified in this study but should be considered in future studies such as the production of hydrogen cyanide, antibiotics and antifungal compounds (Akhtar and Siddiqui, 2010).

In summary the plant growth promoting characterisation tests revealed a number of bacterial isolates (27) were able to solubilise phosphate (96%), produce ammonia (22%), and produce cellulase (37%), protease (3%), sideropohore (37%) and catalase (66%). Many of these isolates were able to show multiple characterisation ability. These isolates represent a range of bacteria, including Gram-positive and negative, rods and cocci which show potential to be PGPR, although their efficiency would need to be further tested. A number of authors have investigated potential rhizobacteria as possible PGPR from various host sources, also finding a variety of bacterial isolates showing PGPR abilities similar to this study. Most of the isolates were from the genera *Bacillus, Azotobacter, Pseudomonas, Serratia, Streptomyces, Rhizobium* (Joseph *et al.*, 2007; Ahmad *et al.*, 2008; Chaiharn *et al.*, 2008; Hariprasad and Niranjana, 2009; Yasmin *et al.*, 2009; Franco-Correa *et al.*, 2010; Martinez-Viveros *et al.*, 2010; Suresh *et al.*, 2010).

Molecular Identification of Bacterial Isolates

Isolate SEN4 was identified as *Acinetobacter ursingii* by 16s rRNA anaylsis. *Acinetobacter* is a genus of Gram-negative bacteria belonging to the Gammproteobacteria, often displaying coccobacillary morphology (Madigan and Martinko, 2006). The isolate in this study showed PGPR characteristics such as phosphate-solubilisation, cellulase and siderophore production but was negative for IAA and ammonia production. Studies by other authors researching various strains of *Acinetobacter sp.* have concluded that *Acinetobacter sp.* to be a PGPR.

This was revealed through characterisation tests such as the ones performed in this study (Huddedar *et al.,* 2002; Indiragandhi *et al*., 2008; Kang *et al*., 2012). Most of the studies found different species of *Acinetobacter* to solubilise inorganic phosphate sources such as $CaHPO_3$ and to produce siderophores. However differences in IAA production has been reported, with some species and/ or strains capable of producing IAA while others not. The same findings exist for nitrogen fixation. One study by Gopalakrishnan *et al*., (2010) revealed *Acinetobacter tandoii*, which is closely related to the isolate in this study, was positive for phosphate-solubilisation and siderophore production and negative for IAA production and nitrogen reduction, which correlates with the results in this study. A study by Prashant *et al*., (2009) found a different species of *Acinetobacter* to be antagonistic towards *Fusarium oxysporum* through the production of catechol type siderophores. The isolate in this study had an inhibitory effect on *Fusarium oxysporum*, and therefore correlates with the results by Prashant *et al.,* (2009). Therefore isolate SEN4 can be said to be a species of *Acinetobacter* with possible multiple PGPR characteristics; which should be further investigated for efficiency.

Through 16s rRNA analysis, isolates SEN9 and SIRB2 were identified as *Bacillus thuringiensis. Bacillus thuringiensis* is a Gram-positive soil dwelling, rod shaped bacterium capable of producing endospores (Madigan and Martinko, 2006). These isolates showed phosphate solubilisation, ammonia production and siderophore producing capabilities but were indole negative in this study. Although widely known as a successive biocontrol agent, other studies on *Bacillus thuringiensis* have revealed PGPR characteristics such as phosphate solubilisation, phytase production, IAA production, nitrogen fixation and siderophore production (Raddadi *et al*., 2008 and Vassilev *et al*., 2007). Therefore the isolates in this study correspond to the results shown by those authors and can be concluded to be *Bacillus thuringiensis,* although the IAA results don’t correspond, which could be due to error in the media used in this study or this isolate could be a different strain to the one in those studies. Therefore these two isolates have capabilities to be PGPR through the characteristics they possess.

Isolates SERB2, SESB1 and SISB1 were identified as *Bacillus mycoides*. *Bacillus mycoides* are known to be Gram-positive rods capable of producing endospores. They are easily identifiabel through their characteristic rhizoid growth pattern upon incubation on suitable agar medium after 48 hrs (Paul *et al.*, 1995). Isolate SISC2 also displayed this characteristic growth form and had similar PGPR characterisation results, however was unidentifiable as the BLAST results showed the isolate to be of *Serratia sp.*, however this is inaccurate since the Gram-stain revealed large Gram-positive rods, whereas *Serratia sp.* are small Gram-negative rods. The isolates also showed rhizoid growth on solid media which is characteristic of *B. mycoides* (Paul *et al.*, 1995). The *Serratia* was thought to be an introduced contaminant. Different species of *B. mycoides* have been shown to accelerate early AM fungi formation of *Glomus etunicatum, Glomus mosseae* and *Glomus intraradices* and hence suggested to be potential MHB (von Alten *et al.*, 1993). Other studies have focused mainly on the biocontrol potential of *B. mycoides.* Antagonstic ability by *B. mycoides* has been shown against *Beauveria bassiana, Botrytis cinereae, Pythium mamillatum* and *Fusarium oxysporum* (Paul *et al.*, 1995; Hammad and EI-Mohandes, 1999; Guetsky *et al.*, 2002; Toledo *et al.*, 2011). The isolates in this study have shown antagonistic ability against *Fusarium oxysporum* and *Phythophthora nicotianae.* Few studies have been revealed on the plant growth promoting abilities of *B. mycoides*. In this study however, the isolates showed multiple PGPR characteristics such as phosphate solubilisation, cellulase, protease and siderophore production. Therefore further investigation into their potential PGPR abilities should be performed.

Isolate SERA2 was identified as *Bacillus cereus. B.cereus* belongs to a Gram-positve group of bacteria of rod morphology and capable of producing endospores (Idris *et al.,* 2009). The isolate in this study showed only phosphate solubilisation and siderophore production capabilities. The isolate also had the highest antagonistic ability against *Fusarium oxysporum.* Other studies have shown various PGPR characteristics for different strains of *B.cereus,* all with differing results, possibly due to different strains used. For instance a study by Kumar *et al.,* (2011) revealed four *B.cereus* isolates, all were negative for siderophore production and positve for IAA production, however only one isolate was positive for phosphate solubilisation. The study by Idris *et al.,* (2009) showed seven isolates of *B.cereus*, with only one isolate having produced siderophore and all isolates except one solubilised phosphate and produced IAA.

Therefore different strains of *B.cereus* are capable of different multiple PGPR characteristics. Therefore isolate SERA2 is considered to be *B.cereus* and a PGPR since it is able to solubilise phosphate and supply phosphorous to the plant. In addition through the produciton of organic acids, which results in a lowering of the soil pH makes other soil ions such iron become more readily available for uptake through the bacterial siderophores or directly by the plant roots (Idris *et al.,* 2009).

Isolate SIRC2 was indentified as *Microbacterium nematophilum. Microbacterium* belong to aerobic Gram-positive bacilli. The isolate in this study was shown to be able to solubilise phosphate, produce ammonia and siderophores. Other studies on *Microbacterium spp.* have revealed similar PGPR characteristics. A study by Karlidag *et al.,* (2007) showed a species of *Microbacterium* was able to fix nitrogen, wereas studies by Tsavkelova *et al.*, (2007) and Choudhary *et al.*, (2011) revealed the ability to produce IAA. Phosphate solubilisation ability was shown by a strain of *Microbacterium* in a study by Malboobi, *et al*., (2009). Research by John *et al.*, (2001) showed a strain of *Microbacterium flavescens* was able to produce siderophores. Therefore the characteristation results of the isolate in this study correspond to the abilities presented by these other authors. It is interesting to note that the *nematophilum* species of *Microbacterium* has been shown to be isolated from the rectum of nematodes, in particular *Caenorhabditis elegans* (Hodgkin *et al.,* 2000). *M. nematophilum* has been shown to have an ability to be pathogenic towards *C. elegans.* Since it is located in the rectum of the nematode, it is likely to be excreted into the surrounding soil by the nematode and may come into contact with AM fungal structures such as spores and hyphae. In this study it showed antagonstic ability towards the fungal pathogens *F.oxysporum* and *P.nicotianae* and therefore could be used for its biocontrol ability against antagonistic fungi and nematodes in agriculture. Therefore it can be said that SIRC2 is indeed a species of *Microbacterium* with multiple plant growth promoting characteristics and therefore has potential to be a PGPR.

Isolate SISB2 was identified by 16s rRNA analysis as a *Bacillus sp. Bacillus sp.* are known to be Gram-positive rods, some able to produce endospores (Madigan and Martinko, 2006). Several studies investigating potential PGPR charactersitics of various *Bacillus spp.* Joseph *et al.,* (2007) showed *Bacillus spp.* able to produce IAA, ammonia and few showed siderophore production.

A study by Orhan *et al.,* (2006) and Karlidag *et al.,* (2007) revealed *Bacillus spp.* with phosphate solubilising, nitrogen fixing and IAA producing capabilities. Calvo *et al.,* (2010) characterised *Bacillus* isolates and found 81% produced IAA and 58% solubilised phosphate. A study by Hariprasad and Niranjana (2009) investigated five different species consisting of *Bacillus subtilis, Bacillus amyloliquefaciens* and *Bacillus megaterium.* Characterisation tests revealed all solubilised phosphate, produced IAA, produced phytase. None produced siderophores and only one isolate of *B.megaterium* produced cellulase.

Two isolates (one *B. subtilis* and one *Bacillus megaterium*) showed antagonistic ability towards *Fusarium oxysporum.* The isolate in this study showed PGPR characteristics such as phosphate solubilisation and cellulase production, but did not produce IAA or siderophores. These results correspond to those found by the authors mentioned and therefore the isolate SISB2 can potentially be a PGPR.

2.5 Conclusion and Summary

Bacterial isolates were extracted from the external and internal surfaces of AM fungal spores from various samples such as natural indigenous forest, raspberry and strawberry samples. They were characterised according to plant growth promoting abilities such as phosphate solubilisation, IAA production, ammonia production, siderophore and hydrolytic enzyme production. These characteristics are important for promoting plant growth through providing nutrients and other resources necessary for growth and establishment and for controlling plant pathogens which may inhibit plant growth. A total of 27 bacterial isolates were characterised, some with multiple abilities, of those 11 were identified by 16s rRNA molecular analysis. The identitities correlated with other studies published by numerous authors, all reporting similar PGPR characterisation results to the different genera of bacteria isolated in this study. These isolates can then be said to be plant growth promoting rhizobacteria. A similar study to this one was performed in Argentina but due to differences in methods, soil characteristics, environmental factors and plant stages, the two studies could not be compared. The methods and results of the study carried out in Argentina is presented in the next chapter.

CHAPTER 3

CHARACTERISATION OF AM FUNGAL SPORE-ASSOCIATED BACTERIA: ARGENTINA

3.1 Introduction

Arbuscular mycorrhizal fungi associate with approximately 80% of terrestrial plant species, some of which are beneficial in the agriculture industry. Raspberry (*Rubus idaeus*) is known to be host to various arbuscular mycorrhizal fungi in the genus *Glomus, Gigaspora and Scutellospora*. It has been shown that the AM fungi both promote and depress the growth and yield of raspberry (Taylor and Harrier, 2000). The industry is looking to increase more organic production in order to produce high yield crops (Made-in Argentina, 2010).

Total value of Argentine organic production is estimated at US$20 million (1999), of which 85 percent is exported and the remaining 15 percent sold in the domestic market (Made-in Argentina, 2010). The use of microbial communities to alleviate the usage of chemicals has been looked into as an alternative management strategy. These organisms include a variety of bacteria that are able to solubilise phosphate, fix nitrogen or inhibit pathogens, for example some *Bacillus* species are used to improve plant growth (Orhan *et al.,* 2006), and studies into tripartite symbiosis with AM fungi have been conducted.

Argentina produces and export raspberries, both fresh and frozen. Fresh raspberry production is approximately 580 tons/ year whereas frozen raspberries are 110 tons/year. Majority of the fresh raspberries are exported to Germany, France, Italy and the UK, where they go for U.S $ 4/ pound. The main areas of raspberry production in Argentina include Comarca Andina el Paralelo 42°, Upper Black River Valley, Neuquen and Buenos Aires (Made-in Argentina, 2010).

The Andean region contributes approximately 45% of the natural production, including El Bolson, El Hoyo, Lago Epuyen and Puelo. They yield approximately 4.5 to 15 tons/ ha. The frozen raspberries are exported for around U.S $ 2100/ ton, with 25% going to the US, 15% to Germany and 9% to Canada. Approximately 90% of the sales occur between February and May (Made-in Argentina, 2010).

The aims of the experiment were to isolate bacteria from AM fungal spores from the soils associated with the roots of raspberry samples in Argentina and characterise them according to plant-growth promoting abilities and then identify the isolates with the most beneficial characteristics.

3.2 Materials and Methods

3.2.1 Soil Samples

Ten replicate random soils samples (approximately 300 g) were collected from Raspberry (*Rubus idaeus* cv. Autumn Bliss) soil from Chacra El Monje, El hoyo, Rio Negro, Argentina (41^{o} 58'S, 71^{o} 32'W) (Figure 3.1). The raspberry samples were at full growth and harvest stage, ranging from 1.6 m in height.

Figure 3.1 A- Fruit of raspberry (*Rubus idaeus* cv. Autumn Bliss) and **B-** the raspberry plantation in Rio Negro, Argentina.

3.2.2 Estimation of Root and Soil Bacterial Populations

Root Samples

Root samples (10 x 1 cm) of the ten replicate samples from the three sources above were placed in sterile 0.2% saline in a 1:1 ratio and allowed to mix for an hour in the solution and serially diluted (five x 5 fold dilution). Aliquots (100 µl) from the dilutions 5^{-4} and 5^{-5} were spread onto Tryptone Soy Agar (TSA) (Britania, Catalog no: B0210206-436) for total bacterial counts. Each dilution was spread onto two replicate plates. The number of colonies formed was counted after 24 hrs and 48 hrs incubation at 28^{o}C (± 2^{o}C). The Colony Forming Units per cm of root length ($CFU.cm^{-1}$) was calculated by Eqn 1 (Johnson and Case, 2007) and averaged per source.

Soil Samples

Soil samples (1 g) of the ten replicate samples from the four sources above were placed in sterile 0.2% saline in a 1:1 ratio and allowed to mix for an hour in the solution and serially diluted (six x 5 fold dilution). Aliquots (100 µl) from the 5^{-5} and 5^{-6} dilutions were spread onto Tryptone Soy Agar (TSA) for total bacterial count. Each dilution was spread onto two replicate plates. The number of colonies formed was counted after 24 hrs and 48 hrs incubation at 28^{o}C ($\pm 2^{o}$C). The Colony Forming Units per g of soil (CFU.g^{-1}) was calculated by Eqn 1 (Johnson and Case, 2007) and averaged per source.

Equation 1:

$$\text{CFU} = \text{number of colonies x dilution factor x } \frac{\text{Standard Volume}}{\text{Aliquot plated}}$$

3.2.3 AM Fungal Spore Extraction and Isolation

Spore Extraction

AM fungal spores were extracted from the various soil sources by the method described by Smith and Dickson, 1997. This involves weighing out 200 g of soil in a beaker, making a soil solution with sterile tap water which was stirred and allowed to settle. The supernatant was decanted through a nest of sieves (500 µm, 250 µm, 53 µm, 35 µm) which was pre-washed with sterile tap water and repeated three times. The sieves were washed and the debris from the 500 µm discarded. The debris in the remainder of the sieves were washed into 50 ml centrifuge tubes and filled with water (Smith and Dickson, 1997).

Spore Purification

The spore suspensions were centrifuged (1900 g for 5 min) and the supernatant discarded. The pellet was resuspended in 60% sucrose solution (made in sterile 1 L beakers) and centrifuged (Sigma Sigmo Centrifuge) for a further 5 min. The supernatant was filtererd through a Buchner funnel onto a filter paper disc (Schleicher and Schüll 589[1] Shwarzband) and rinsed with sterile distilled water. The filter paper was transferred to the lid of a sterile petri dish.

The spores were counted, microscopically examined (Smith and Dickson, 1997) with an Olympus SZX9 dissecting microscope, morphologically separated according to only colour morphology spores of different sizes of similar colour were combined. Images were taken with an Olympus Evolt E-410 digital camera. The spores were only used to isolate potential mycorrhizal helper and plant growth promoting bacteria and not for identification or molecular purposes.

3.2.4 AM fungal colonisation

AM fungal colonisation was assessed as described in section 2.2.4. The roots were examined with an Olympus BX40 compound microscope Pictures of the colonization were taken using an Olympus Evolt E-140 digital camera.

3.2.5 AM fungal Spore-associated Bacterial Isolation

External Surface of Spores

The spores of the AM fungi were placed in sterile 0.2% saline/Tween 80 solution, vortexed for one hour and allowed to soak. A sample (200 µl) from the solution was serially diluted (five x 5 fold dilution) with sterile 0.2% saline solution and an aliquot (100 µl) from the 5^{-4} and 5^{-5} dilutions was spread onto TSA plates and incubated at 28°C. The number of CFU.ml^{-1} per spore solution was determined for each plate after 24 hours. Colonies were streaked discontinuously to obtain pure bacterial colonies which were cultured in 400 µl Nutrient Broth (NB) (Biolab Catalog no: 1024537) in Cryotubes containing sterile glass beads at 30°C for 48 hrs. Glycerol stocks of the isolates were made by combining 0.1 ml of 60% glycerol solution to the Cryotubes, vortexed to homogenise the glycerol and cultures. The isolates were frozen in steps: 4°C for 2 hrs, shaken, -20°C for 2 hrs and then stored at -80°C for further anaylsis.

Internal Surface of Spores

AM fungal spores were surface sterilised by soaking in a 2% chloramine T (Squibb Industria Farmaceútica, S.A, Catalog no: 731810 E.F.P) and Tween 20 solution for one hour at constant vortexing (medium speed), after which the solution was centrifuged at 10 000 g for 5 mins, the supernatant removed and 1 ml sterile H_2O was added to wash the spores.

The sample was vortexed for 5 mins continuously and centrifuged at 10 000 g for 5 mins, the supernatant was removed and replaced with new sterile H_2O and repeated three times. A sample (100 µl) from the solution was spread onto TSA plates and incubated at 28^{o}C to determine the efficiency of the sterilisation step. Thereafter 500 µl of each 10 mg.ml^{-1} of ampicillin (Amp, Sigma®), chloramphenicol (Clo, Anedra®) and streptomycin sulphate (Str, Richet®) solution was added and soaked for 1 hr 30 mins with constant vortexing. After which the spores are washed three times with sterile H_2O. A sample (100 µl) from the solution was spread onto TSA plates and incubated at 28^{o}C to determine the efficiency of the antibiotics. After the final wash, the water was replaced with 1 ml 0.2% sterile saline soaked for 1 hr 30 mins, centrifuged at 10 000 g for 5mins and the spores crushed using a sterile micropestle to release the internal bacteria into the saline solution, which was serially diluted (three x 5 fold dilution) with distilled 0.2% saline solution and an aliquot (100µl) from the 5^{-1}, 5^{-2} and 5^{-3} dilutions was spread onto TSA plates and incubated at 28^{o}C. The number of CFU.ml^{-1} per spore solution was determined for each plate after 24 hours. Colonies were streaked discontinuously to obtain pure bacterial colonies which were cultured in 400 µl (NB) in Cryotubes containing sterile glass beads at 37^{o}C for 48 hrs. Glycerol stocks of the isolates were made by combining 0.1 ml of 60% glycerol solution to the Cryotubes, vortexed to homogenise the glycerol and cultures. The isolates were frozen in steps: 4^{o}C for 2 hrs, shaken, -20^{o}C for 2 hrs and then stored at -80^{o}C for further anaylsis.

3.2.6 Morphological Identification of Bacterial Isolates

The bacterial isolates were morphologically identified by Gram staining as described in section 2.2.6. Samples were air dried and visualised under a compound microscope under oil immersion (Olympus BX40).

3.2.7 Transportation of Isolates to South Africa

The prepared isolates were streaked onto slant nutrient agar in 1.5 ml microcentrifuge tubes, sealed with parafilm and incubated at 30^{o}C for 48 hrs. The samples were transported to the Mycorrhizal Research Laboratory, Department of Biochemistry, Microbiology and Biotechnology, Rhodes University, South Africa (Permit number: P0049730).

The isolates were transferred to NB, incubated at 37°C, on a rotary shaker at 150 rpm for 48 hrs. Glycerol stocks of the isolates were made by combining 0.5 ml of the NB cultures with 0.5 ml of 50% glycerol solution and stored at -80°C for further anaylsis.

3.2.8 Plant-Growth Promoting Characterisation Tests

The bacterial isolates were prepared for and underwent Plant Growth Promoting characterisation tests as described in section 2.2.7.

3.2.9 Molecular Identification of Bacterial Isolates

Molecular identification of the bacterial isolates was carried out by DNA extraction and PCR protocols as described in section 2.2.8.

3.2.10 Statistical Analyses

Statistical analysis on the results was carried out using the same software and procedures as outlined in section 2.2.9.

3.3 Results

3.3.1 Estimation of Root and Soil Bacterial Populations

Ten replicate soil samples were taken from raspberry and the bacterial community cultured from the root surfaces and bulk soil. The bacteria were grown on TSA and the CFU.cm^{-1} root and CFU.g^{-1} soil was determined. There was a significant difference (t-value = -7.36, df = 47, $p < 0.001$) in the total number of culturable bacteria between the root and soil samples, the numbers being slightly higher in the soil samples (Figure 3.2).

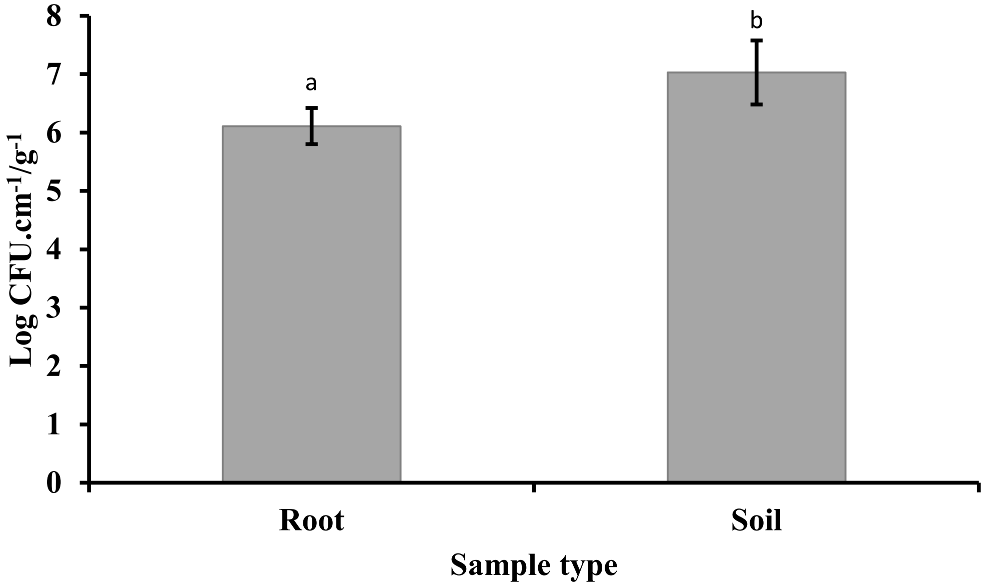


Figure 3.2 Total culturable bacterial counts of Raspberry (*Rubus idaeus* cv. Autumn Bliss) root and soil samples. Log CFU values are means of ten replicates, bars represent ± standard deviation. Column labels with same letter represent no significant differences.

3.3.2 AM Fungal Colonisation and Spore Counts

The roots isolated from the ten samples were stained with trypan blue and the percentage root colonisation of the raspberry plants was determined. The AM fungal spores were extracted from the soil and counted per 100 g. The root colonisation of the raspberry plants was higher than the number of spores present in the bulk spore of the raspberry plants (Table 3.1).

Table 3.1 Average Arbuscular Mycorrhizal fungal root colonisation percentage (%) and AM fungal spore counts sampled from Raspberry (*Rubus idaeus* cv. Autumn Bliss).

	Root Colonisation %	**Spore Count (100 g)**
Raspberry	75	8.25

Three different spore morphotypes were found in the bulk soil of the raspberry samples and were separated according to differences in colour. The first was an orange-golden coloured spore, then a dark brown to black coloured spore and then a cream, clear-grey coloured spore, all with variations in size and intensity of colour (Figure 3.3). The raspberry samples were colonised by the AM fungi through structures including arbuscules (which had a low presence), spores, vesicles and intra-radical hyphae (Figure 3.4).

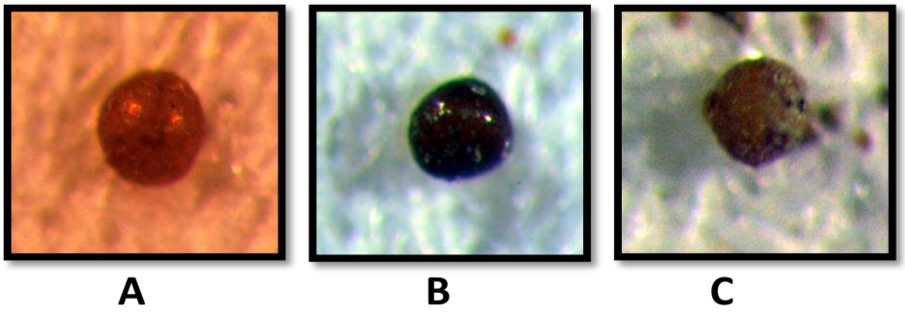


Figure 3.3 Different Arbuscular Mycorrhizal fungal spores of Raspberry (*Rubus idaeus* cv. Autumn Bliss) samples. A- Orange-gold coloured spore with dull surface, B- Dark brown coloured spore morphology with a glossy surface, C- Clear-grey coloured spore with glossy surface, attached is part of the subtending hyphae.

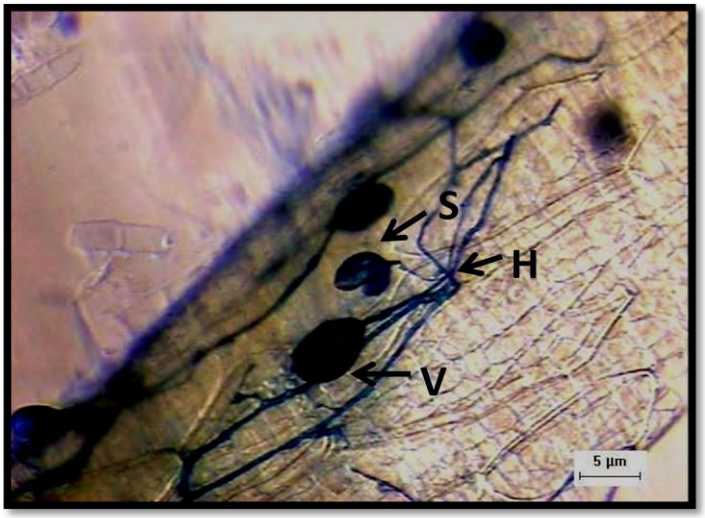


Figure 3.4 Arbuscular Mycorrhizal fungal spore colonisation of Raspberry (*Rubus idaeus* cv. Autumn Bliss) root cortical cells. H- intra-radical hyphae, S- spore, V- vesicle.

3.3.3 Bacterial Isolate Coding

The bacteria were coded according to the isolation procedures, First letter with A represents Argentinean Isolate, E and I represent isolation from the external and internal surfaces of the AM fungal spores respectively, R were isolated from the raspberry sources. A, B and C represent the AM fungal spore morphotypes mentioned earlier (Figure 3.3) followed by the isolate number. Bacterial morphology represented by S, M, L represent cell size small, medium and large under immersion oil in relation to the field of view. "BM" represents cultures showing rhizoid colony morphology which are characteristic of *Bacillus mycoides* growth. ENDOS represents endospore formation by bacillus shaped bacteria, viewed under immersion oil.

3.3.4 AM fungal Spore-associated Bacterial Isolation

Bacteria were isolated from the external and internal surfaces of the AM fungal spores and there was no bacterial growth on the control plates after the sterilisation steps. The total numbers of culturable bacteria per spore (CFU.spore^{-1}) was determined by culturing on TSA and counting the colonies after 24 hrs. The numbers were significantly higher in the external surface of the spores compared to the internal (Table 3.2). A total of 25 bacteria were isolated from the AM fungal spores, a gram stain was performed on these samples, with all 25 being Gram-positive and none Gram-negative. Of these Gram-positive bacteria, 13 were rods of various sizes and 12 isolates were cocci whereas the gram negative showed only rod morphology (Table 3.2).

Table 3.2 Bacterial cultures of the external and internal Arbuscular Mycorrhizal fungal surfaces sampled from Raspberry (*Rubus idaeus* cv. Autumn Bliss) samples.

Spore surface	CFU.spore^{-1}	Total Number Isolated	Gram Positive	Gram Negative	Rods	Cocci	Rods:Cocci
EXTERNAL	230	9	9	0	7	2	7:2
INTERNAL	26	16	16	0	6	10	3:5
TOTAL	256	25	25	0	13	12	10:7

3.3.5 Plant Growth Promoting Characterisation Tests

Bacteria isolated from the external and internal surfaces of AM fungal spores sampled from the Raspberry (*Rubus idaeus* cv. Autumn Bliss) soils were subjected to plant-growth promoting characterisation tests to evaluate their ability to enhance plant growth and inhibit fungal pathogens by various mechanisms.

Phosphate-Solubilisation

The selected bacterial isolates were grown in media containing $CaHPO_3$ as the only phosphate source to test for phosphate solubilisation. The media also contained bromophenol blue which turns to yellow indicaticive of organic acid poroduction (Figure 3.5). Seventeen bacterial isolates were shown to be phosphate solubilisers. The diameter of the halos' produced were measured for the five replicate wells and averaged which ranged from 17-28mm (Table 3.4, 3.5).

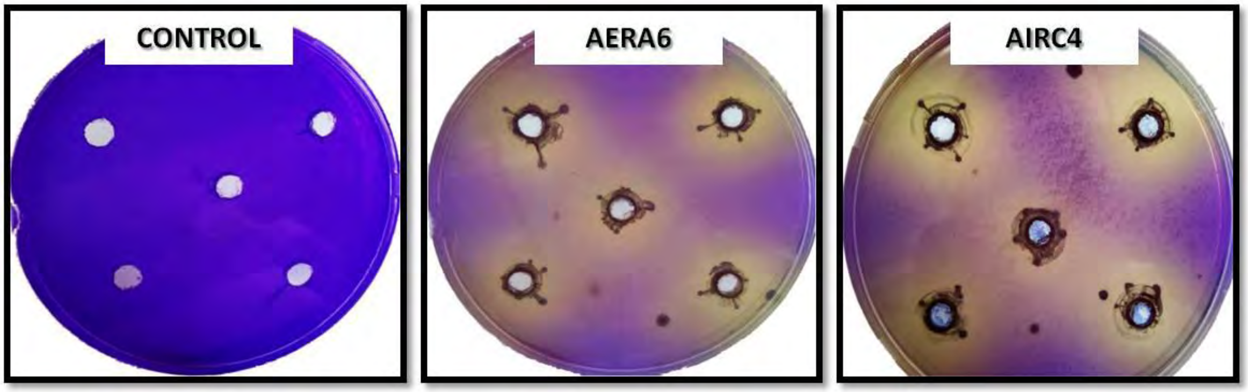


Figure 3.5 Phosphate solubilisation of $CaHPO_3$ by selected bacterial isolates from the external (AERA6) and internal (AIRC4) surfaces of Arbuscular Mycorrhizal fungal spores from raspberry (*Rubus idaeus* cv. Autumn Bliss). Control- Inoculated with 0.2% sterile saline.

Phytase production

Phytase production was evaluated usng media containing phytic acid. Isolates were incubated for 5 days. None of the bacterial isolates produced phytase (Table 3.4, 3.5).

Indole Acetic Acid Production

Bacteria isolated were grown in DEV Tryptophan Broth and then Kovacs Reagent was added to the samples. All the bacterial isolates were negative for indole acetic acid production (Table 3.4, 3.5) as all produced a yellow colour whereas a red colour is indicative of a positive reaction.

Production of Ammonia

Bacterial isolates were tested for ammonia production in peptone water and Nesslers reagent was added. Development of brown to yellow colour is positive test for ammonia production (Figure 3.3). Two bacterial isolates (AIRC2 and AIRC9) produced ammonia (Table 3.4, 3.5).

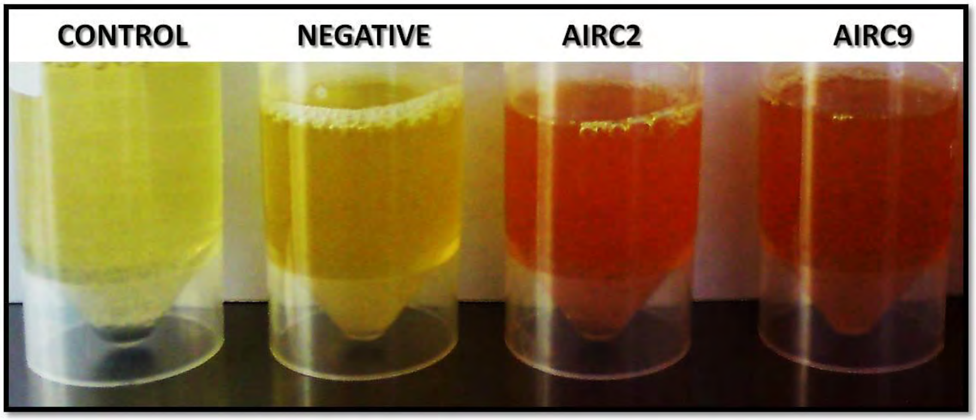


Figure 3.6 Production of ammonia by the bacterial isolates from the internal (AIRC2 and AIRC9) surface of Arbuscular Mycorrhizal fungal spores from raspberry (*Rubus idaeus* cv. Autumn Bliss). Control- un-inoculated broth.

Fungal Pathogen Inhibition

This experiment was conducted to determine if any of the bacterial isolates have an inhibitory effect on *Fusarium oxysporum* and *Phythopthora nicotianae*. Bacterial isolates were inoculated into wells on PDA placed at the four points along a perpendicular axis. The fungal growth (mm) was measured along the two perpendicular axes for approximately 20 days.

Dual culture with Fusarium oxysporum

Antagonistic ability of the bacteria against *F. oxysporum* revealed significant inhibition by all the bacterial isolates in the dual culture studies ($F_{(20,618)} = 10.572$, $p < 0.001$). Reduction in the hyphal growth compared to the control was indicative of inhibition (Figure 3.7). Among the isolates, AERA4.1 and AIRC4 were shown to be the most effective in inhibition of *F. oxysporum* (Figure 3.8 A-B), which was confirmed by Fischer's LSD test and the percentage inhibition of the growth by the isolates ranged from 6-25% (Table 3.3).

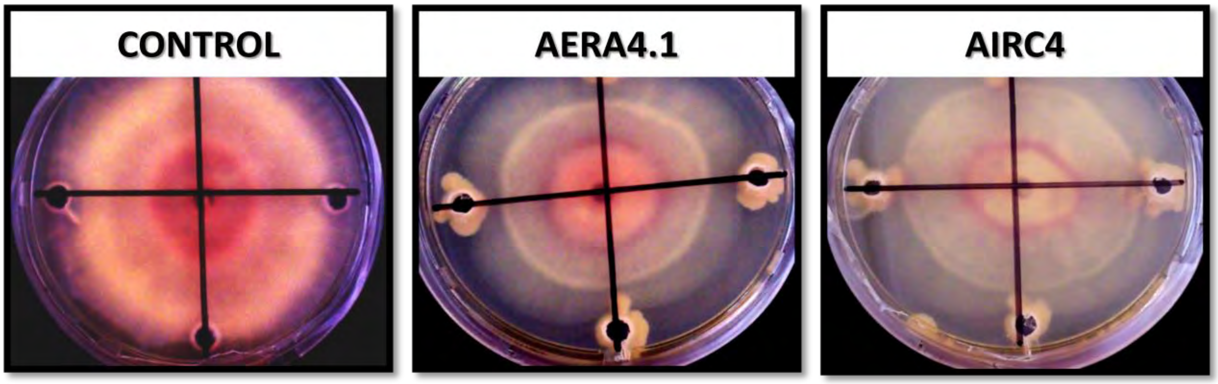


Figure 3.7 Fungal hyphal growth inhibition of *Fusarium oxysporum* by selected bacterial isolates from the surfaces of Arbuscular Mycorrhizal fungal spores from raspberry (*Rubus idaeus* cv. Autumn Bliss)- AERA4.1 and AIRC4. Control- Inoculated with 0.2% sterile saline.

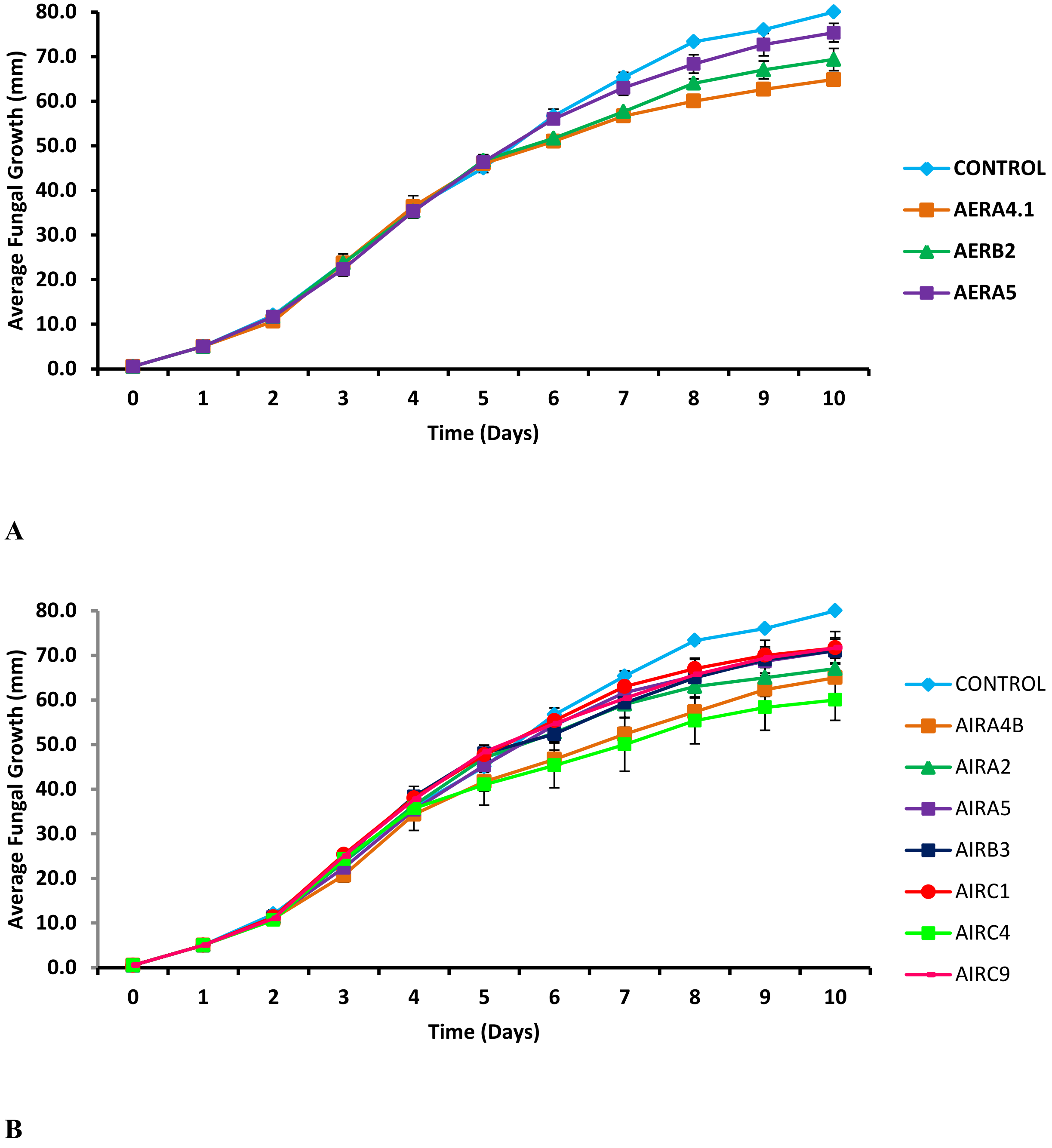


Figure 3.8 A and B Dual cultures of bacterial isolates from raspberry (*Rubus idaeus* cv. Autumn Bliss) and *F.oxysporum f sp lycopersici* showing bacterial effect on fungal growth (Mean ± SD) growth rate ($F_{(20,618)}$ = 10.572; $p < 0.001$). **A-** Bacteria isolated from external surface of spores; **B-** Bacteria isolated from internal surface of spores. Control- Inoculated with 0.2% sterile saline.

Dual culture with Phythophthora nicotianae

Antagonistic ability of the bacteria against *P. nicotianae* revealed significant inhibition by all the bacterial isolates in the dual culture studies ($F_{(20,618)} = 17.720$, $p < 0.001$). Reduction in the hyphal growth compared to the control was indicative of inhibition (Figure 3.9). Among the isolates, AERB2 and AIRA4B were shown to be the most effective in inhibition of *P. nicotianae* (Figure 3.10 A-B), which was confirmed by Fischer's LSD test and the percentage inhibition of the growth by the isolates ranged from 36 - 55% (Table 3.3).

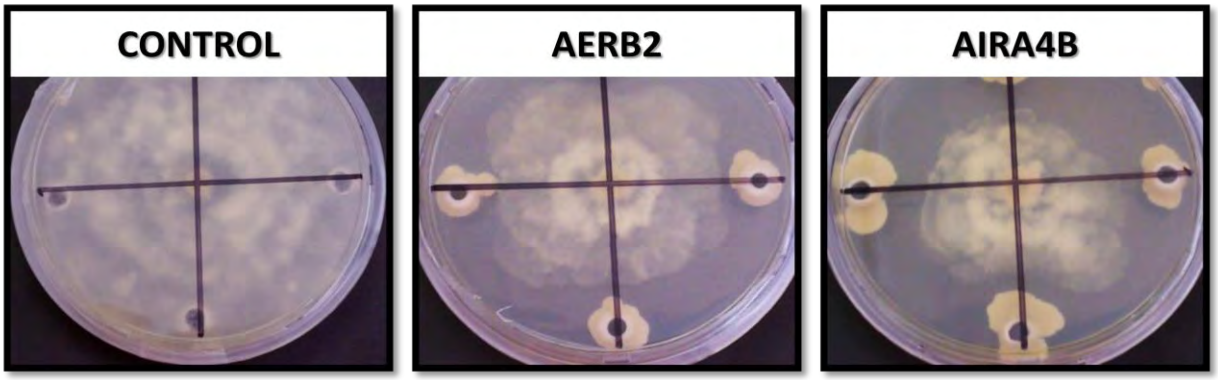


Figure 3.9 Fungal hyphal growth inhibition of *Phythophthora nicotianae* by selected bacterial isolates from the surfaces of Arbuscular Mycorrhizal fungal spores from raspberry (*Rubus idaeus* cv. Autumn Bliss) - AERB2 and AIRA4B. Control- Inoculated with 0.2% sterile saline.

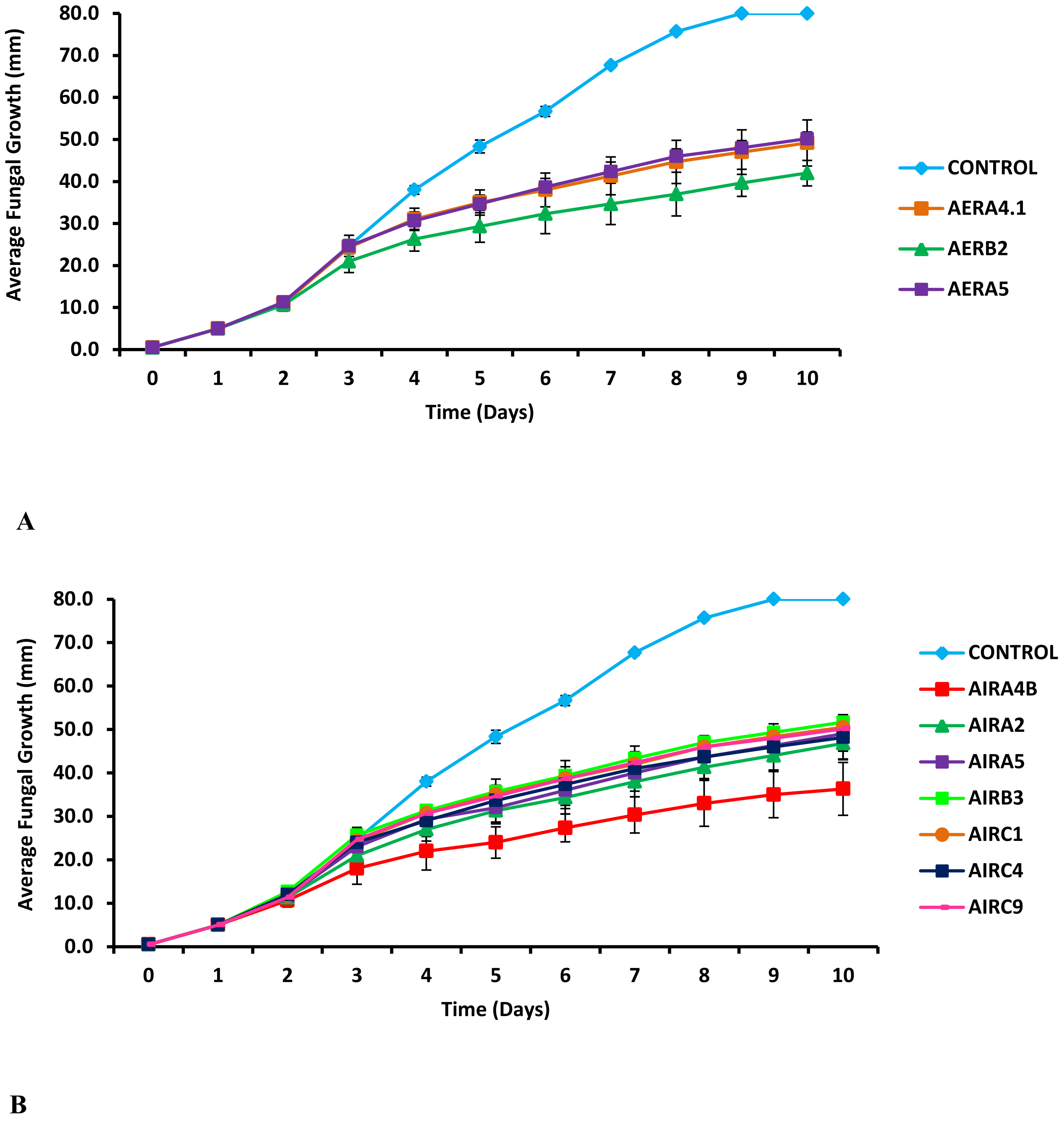


Figure 3.10 A and B Dual cultures of bacterial isolates from raspberry (*Rubus idaeus* cv. Autumn Bliss) and *Phythophthora nicotianae* showing bacterial effect on fungal growth (Mean ± SD) growth rate ($F_{(20,618)} = 17.720$; $p < 0.001$). **A-** Bacteria isolated from external surface of spores; **B-** Bacteria isolated from internal surface of spores. Control- Inoculated with 0.2% sterile saline.

Table 3.3 Fungal hyphal growth inhibition (%) of dual cultures of bacterial isolates against *Fusarium oxysporum* and *Phythophthora nicotianae*

Isolates	*F. oxysporum*	*P. nicotianae*
AERA4.1	19	39
AERB2	13	48
AERA5	6	42
AIRA4B	19	**55**
AIRA2	16	42
AIRA5	11	39
AIRB3	15	36
AIRC1	10	37
AIRC4	**25**	40
AIRC9	10	48

- * Bold indicates highest % inhibition shown against the pathogens

Siderophore production

Development of yellow-orange halos around the isolates indicated siderophore production (Figure 3.11). Two bacterial isolates (AERA4.1 and AERA5) from the external surface and nine bacterial isolates (AIRA2, AIRA4A, AIRA4B, AIRA4C, AIRA5, AIRB2, AIRB3, AIRC1 and AIRC9) from the internal surface of AM fungal spores were shown to produce siderophores (Table 3.4, 3.5). The halo's produced were less than 5mm in diameter.

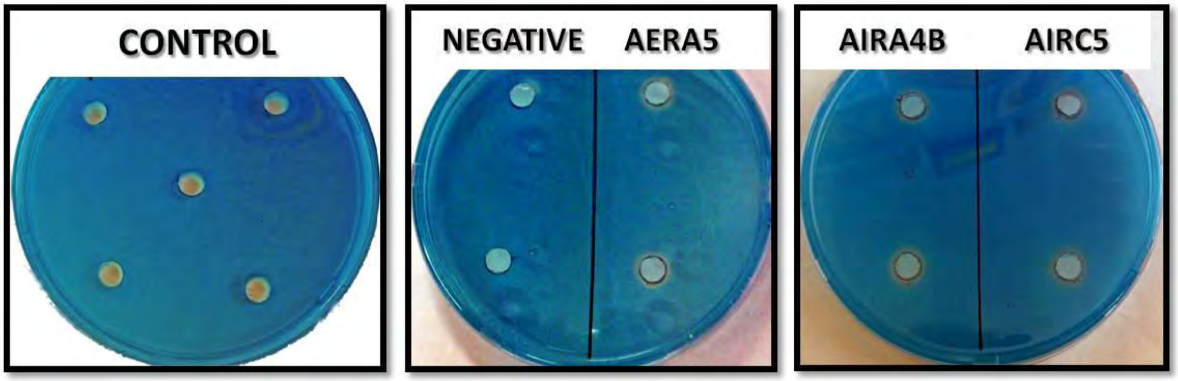


Figure 3.11 Siderophore production by the selected bacterial isolates from the external (AERA5) and internal (AIRA4B and AIRC5) surfaces of Arbuscular Mycorrhizal fungal spores from raspberry (*Rubus idaeus* cv. Autumn Bliss). Control- Inoculated with 0.2% sterile saline.

Protease Production

Protease activity (casein degradation) by the bacterial isolates was determined using skimmed milk agar. Development of halo zone around five replicate wells was considered positive for protease production (Figure 3.12). Two isolates (AERB2 and AERA4.1) from the external surface of AM fungal spores produced protease. The halo's produced were measured for the five replicate wells and averaged, which were 13mm and 22mm (Table 3.4, 3.5).

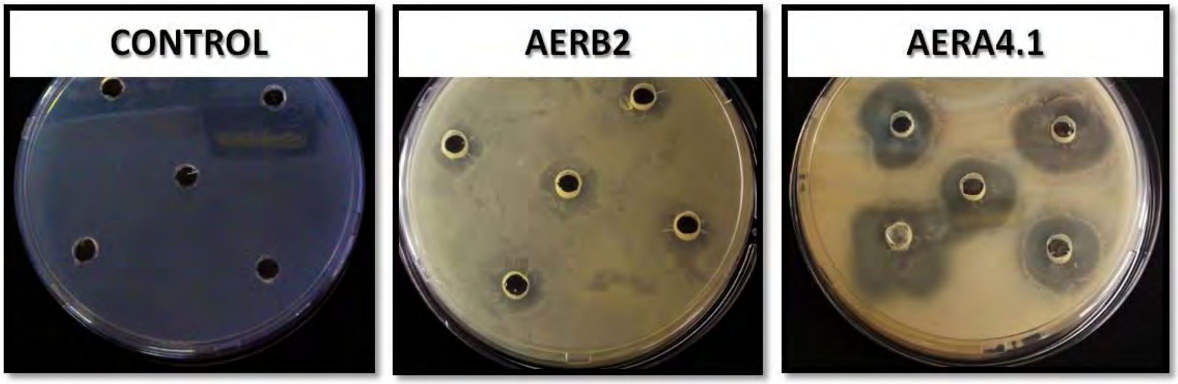


Figure 3.12 Protease production by the selected bacterial isolates from the external (AERB2 and AERA4.1) surfaces of Arbuscular Mycorrhizal fungal spores from raspberry (*Rubus idaeus* cv. Autumn Bliss). Control- Inoculated with 0.2% sterile saline.

Cellulase Production

Cellulase activity was determined by using carboxymethylcellulose (CMC) agar. Grams Iodine was added to the CMC plates. Development of halo zone around five replicate wells was considered positive for cellulase production (Figure 3.13). One isolate (AERA1) from the external surface and ten isolates (AIRA2, AIRA4A, AIRA4B, AIRA4C, AIRA5, AIRB2, AIRB3, AIRC4, AIRC5, and AIRC7) from the internal surface of AM fungal spores produced cellulase (Table 3.4, 3.5). The halo's produced were measured along their diameter for the five replicate wells and averaged, the range was 21 to 33 mm.

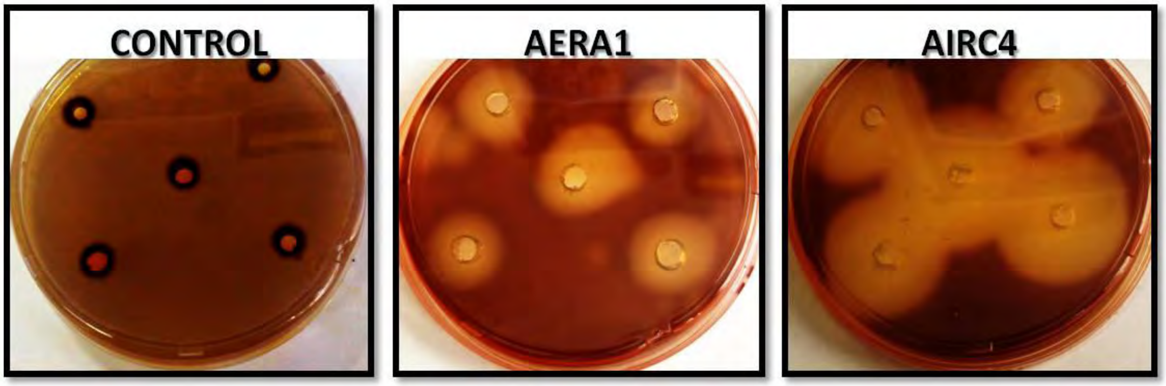


Figure 3.13 Cellulase production by the selected bacterial isolates from the external (AERA1) and internal (AIRC4) surfaces of Arbuscular Mycorrhizal fungal spores from raspberry (*Rubus idaeus* cv. Autumn Bliss). Control- Inoculated with 0.2% sterile saline.

Chitinase Production

Chitinase was tested by plating on chitin agar. There was no halo formation amongst all the isolates (Table 3.4, 3.5).

Catalase Production

Catalase was tested by streaking diluted bacterial isolates on Nutrient agar. Positive reaction for catalase was determined by instant bubble (O_2) formation by the addition of 6% H_2O_2 solution. Two isolates (AERA1 and AERA5) from the external surface and twelve isolates (AERC3, AIRA4A, AIRA4B, AIRA4C, AIRB2, AIRB3, AIRC1, AIRC2, AIRC4, AIRC5, AIRC7 AIRC8) from the internal surface of AM fungal spores produced catalase (Table 3.4, 3.5).

Table 3.4 PGPR characterisation results of the bacterial populations of the external AM fungal surfaces sampled from Raspberry (*Rubus idaeus* cv. Autumn Bliss). P-solub- phosphate solubilisation, IAA- Indole Acetic Acid, NH_3- Ammonia, CMC- cellulase, SMA- Protease, PhyM- phytase, SidPh- Siderophore, Catalase and Chitin production.

Isolate	Gram	Morphology	P-solub	IAA	NH_3	CMC	SMA	Phytase	Sidph	Catalase	Chitin
***AERA1**	-	Rods (S)	+ 20mm[1]	-	-	+ 33mm	-	-	-	+	-
AERA2	+	Rods (L)	+ 17mm	-	-	-	-	-	-	-	-
***AERA4.1**	+	Rods (M) ENDOS	-	-	-	-	+ 22mm	-	+	-	-
***AERA5**	+	Rods (M) ENDOS	+ 27mm	-	-	-	-	-	+	+	-
AERA6	+	Cocci	+ 22mm	-	-	-	-	-	-	-	-
AERB1	+	Rods (S)	+ 22mm	-	-	-	-	-	-	-	-
***AERB2**	-	Cocco-bacilli	+ 19mm	-	-	-	+ 13mm	-	-	-	-
AERC2	+	Rods (S)	-	-	-	-	-	-	-	+	-
AERC3	+	Rods	-	-	-	-	-	-	-	+	-

* Bacterial isolates selected for molecular identification

1 Average diameter of zone of clearance

L Large bacterial cell size (100x magnification)

M Medium bacterial cell size (400x magnification)

S Small bacterial cell size (1000x magnification with immersion oil)

ENDOS Endospore presence

Table 3.8 PGPR characterisation results of the bacterial populations of the internal AM fungal surfaces sampled from Raspberry (*Rubus idaeus* cv. Autumn Bliss). P-solub - phosphate solubilisation, IAA- Indole Acetic Acid, NH_3- Ammonia, CMC- cellulase, SMA- Protease, Phytase, Sidph- Siderophore, Catalase and Chitin production.

Isolate	Gram	Morphology	P-solub	IAA	NH_3	CMC	SMA	Phytase	Sidph	Catalase	Chitin
***AIRA2**	+	Rods (M)	+ 21mm[1]	-	-	+ 20mm	-	-	+	-	-
AIRA3	+	Rods (L)	+ 17mm	-	-	-	-	-	-	-	-
***AIRA4B**	+	Cocci	+ 24mm	-	-	+ 26mm	-	-	+	+	-
***AIRA5**	+	Rods (M)	+ 28mm	-	-	+ 20mm	-	-	+	-	-
***AIRB3**	+	Cocci	+ 26mm	-	-	+ 21mm	-	-	+	+	-
***AIRC1**	+	Rods (L)"BM"	+ 20mm	-	-	-	-	-	+	+	-
AIRC2	+	Rods (S)	+ 25mm	-	+	-	-	-	-	+	-
***AIRC4**	+	Cocci	+ 21mm	-	-	+28mm	-	-	-	+	-
***AIRC5**	+	Cocci	+ 20mm	-	-	+ 30mm	-	-	-	+	-
AIRC8	+	Rods (L)"BM"	+ 21mm	-	-	-	-	-	-	+	-
***AIRC9**	+	Cocci	+ 28mm	-	+	-	-	-	+	-	-

* Bacterial isolates selected for molecular identification
1 Average diameter of zone of clearance
L Large bacterial cell size (100x magnification)
M Medium bacterial cell size (400x magnification)
S Small bacterial cell size (1000x magnification with immersion oil)
BM *Bacillus mycoides* colony morphology on solid media
ENDOS Endospore presence

3.3.6 Molecular Identification of Bacterial Isolates

Based on the best results of the plant-growth promoting characterisation tests, bacterial isolates were selected for molecular identification. These isolates are represented in Tables 3.6 and 3.7 by the asterisks. The DNA was extracted and underwent PCR amplification which was successful and efficient judging by the bands obtained (Figure 3.14). The 16s rRNA of the bacterial isolates was amplified and resulted in bands of approximately 1500bp for the primers FD1 and rp2 and approximately 500bp for the primers Gm5F and R907 in size, visualised using agarose gel electrophoresis (Figure 3.14).

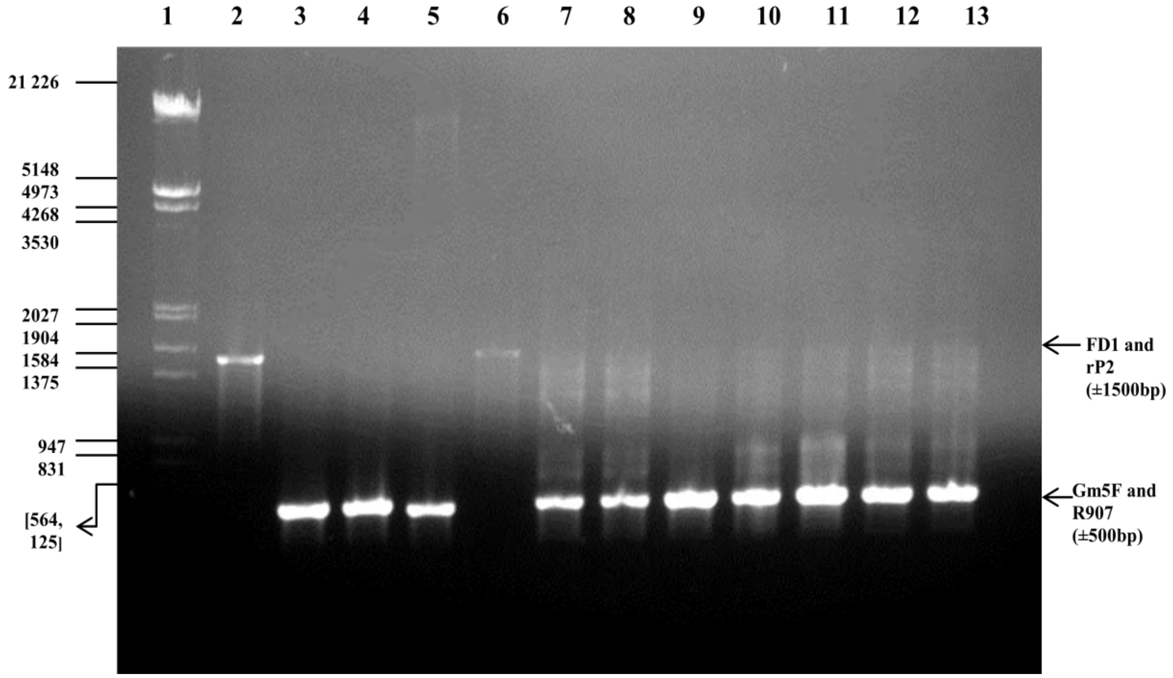


Figure 3.14 Ethidium bromide stained agarose gel (0.8%) showing PCR amplification by the primers FD1 and rP2, and primers Gm5F and R907 of the selected bacterial isolates from raspberry samples. Lane 1- Lambda DNA/*Eco*RI + *Hind*III molecular marker, Lane 2- isolate AERA1, Lane 3- isolate AERB2, Lane 4- isolate AIRA4B, Lane 5- isolate AIRC5, Lane 6- isolate AERA4.1, Lane 7- isolate AIRA2, Lane 8- isolate AIRA5, Lane 9- isolate AIRB3, Lane 10- isolate AIRC1, Lane 11- isolate AERA5, Lane 12- isolate AIRC9, Lane 13- isolate AIRC4.

The sequences of the isolates were obtained (Appendix II) and identified using BLAST on the National Centre for Biotechnology Information (NCBI) website. Percentage Identity values greater than 95% were chosen as the cut off for significant identification at the genus level and 98% at species level (Table 3.9).

Table 3.6 Identified bacterial isolates obtained from the analysis of partial 16s rRNA using the National Centre for Biotechnology Information (NCBI) website.

Bacterial Isolate	Most Significant Alignment (NCBI)	% Identity	e-values	NCBI Accession Number
AERA1	*Alcaligenes faecalis*	99	0.0	KC172062.1
AERA4.1	*Bacillus pumilus*	98	0.0	JX847610.1
AERB2	*Serratia marcescens*	99	0.0	GU294130.1
AERA5	*Bacillus pumilus*	99	0.0	KC113516.1
AIRA4B	*Micrococcus luteus*	99	0.0	FJ229461.1
AIRC5	*Staphylococcus sp.*	100	0.0	KC121048.1
AIRA2	*Bacillus sp.*	97	0.0	JX402437.1
AIRA5	Unidentified	-	-	-
AIRB3	Unidentified	-	-	-
AIRC1	Unidentified	-	-	-
AIRC9	*Staphylococcus pasteuri*	99	0.0	JX994109.1
AIRC4	*Bacillus sp.*	96	0.0	JF723202.1

The bacterial isolates from the raspberry samples were identified as belonging to the genera *Alcaligenes*, *Bacillus*, *Serratia*, *Micrococcus* and *Staphylococcus* with three isolates unidentified (Table 3.9).

3.4 Discussion

Estimation of Root and Soil Bacterial Populations

Soil samples were taken from raspberry plants and the bacterial community cultured from the root surfaces and bulk soil to determine the colony forming units. Colony forming units (CFU) is an indication of the number of bacteria present (Madigan and Martinko, 2006). The $CFU.cm^{-1}$ root and $CFU.g^{-1}$ soil was determined. There was a significant difference ($p < 0.0001$) in the total number of culturable bacteria between the root and soil samples, the numbers being slightly higher in the soil samples (Figure 3.2). Microbes generally gather around plant roots. This is known as the "rhizosphere effect". The dispersal of microbes throughout the soil is therefore not uniform (Heritage *et al.,* 1999). However in the study, the bacterial numbers were higher in the bulk soil. A study by Baudoin *et al.,* (2003) examined the bacterial community of bulk and rhizospheric soils from maize (*Zea mays*). They found the bacterial counts in the bulk soil to be slightly lower than the rhizospheric soil.

Soil is a complex environment which is influenced by factors such as pH, organic matter content (such as leaf litter or added compost), and cations (Ca, Mg, K, and Na) (Garbeva *et al.,* 2004). The nature of soil, irrigation, land use history and management practices (for example, input of fertilizers and herbicides) may also create unique soil environments. Nutrients such as phosphorous and nitrogen is introduced by these practices and input systems. These nutrients are important for biochemical processes in the cell and hence bacterial growth (Madigan and Martinko, 2006). Cell membranes are stabilised and bacterial growth is promoted by cations. The pH of the soil influences the availability of these cations (Chiarini *et al.,* 1998). Microbial community populations in the bulk soil may be changed by these factors (Johansson *et al.*, 2004). Soil analysis, although not performed in this study should be considered to determine the effect of environmental parameters on soil bacterial communities.

Although lower than the bulk soil, bacterial communities are still present in the rhizospheric soil. The rhizospheric soil numbers could be due to secretion of exudates by plant roots and by AM fungal hyphae associated with roots. These exudates contain carbohydrates, hormones and other compounds which promote bacterial growth (Bianciotto and Bonfante, 2002).

Baudoin *et al.,* (2003) showed the addition of artificial root exudates (such as sugars, amino acids and organic acids) increased the CFU.g^{-1} of both the bulk and rhizospheric soil, with similar counts of bacteria. Some compounds released by the plant root could also be deleterious to the rhizospheric microbial community. Some microbes associated in the rhizospheric soil could provide nutrients otherwise unavailable in the soil to the rhizospheric bacterial communities. These microbes could be phosphate-solubilising, nitrogen fixing bacteria or belong to bacteria that produce siderophores in iron limiting conditions that alter the pH of the soil (Gray and Williams, 1971). However some microbes in the soil are able to compete with other soil microbes or produce inhibitory compounds such as antibiotics and siderophores which could affect the natural rhizospheric communities (Campbell, 1979). In addition, no investigation into the dominant bacterial species present in the soil was performed in this study and the results only indicate easily culturable isolates.

Soil and rhizospheric microbes can be cultured on artificial media. However a small proportion of microbes may be unculturable due to the absence of suitable conditions. An underestimation of the microbial activity in the soil may occur. Microbes tend to live in complex communities and once removed from the environment onto culture media, these conditions may not be able to be replicated (Heritage *et al.,* 1999). In addition accurate estimates are further affected since some bacteria in the soil are present as dormant spores. When in contact with the suitable culture media these spores may germinate. Techniques utilising the 16s rRNA structure such as polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) may aid in determining unculturable microbial communities. Taxonomic studies can be performed since members within a species have conserved 16s rRNA structures. Different species show divergent 16s rRNA structures. A more accurate representation of the soil microbial communities can then be determined (Heritage *et al.,* 1999, Madigan and Martinko, 2006).

AM Fungal Colonisation and Spore Counts

AM fungi are non host specific and found naturally occurring in most soils. The presence of AM fungi in the soil can be ascertained through the rate of colonisation by AM fungal structures such as spores, hyphae and other fragments. This is usually mainly determined by spore density in a given soil (Smith and Dickson, 1997).

The percentage colonisation of the raspberry roots was high however the spore density in the soil was lower, this could be due to AM fungi lifecycle, environmental factors or seasonality. The colonisation percentage of the host root by AM fungi may relate to the lifecycle of the AM fungi. Since the raspberry plants were at fruiting stage implies the AM fungi has adequately penetrated the extensive root system and completed the colonisation of the roots, since the lifecycle is initiated with spore germination upon the recognition of a host root and rapidly establishes in the root cortical cells.

There are many limitations to spore sieving which may give an underestimation of total AM fungal spore population in the soil. Distinguishing between dead and viable spores, spores smaller than 45 µm may be lost, or spores adhering to particles which may be discarded are a few examples (Schenck, 1982). AM fungal species composition, dormancy and variability may also determine spore production (Smith and Read, 2008). The distribution of AM fungi in the soil is related to soil pH, phosphorous levels, salinity, soil disturbances and hydrologic conditions of the soil (Escudero and Mendoza, 2005). An increase in soil pH, P or salinity may result in a decreased AM root colonisation percentage or spore density.

Spore counts in the soil and root colonisation percentage are not necessarily correlated with each other. The rate of extension by mycorrhiza formation is not always related to spore density (Ingham and Wilson, 1999; Mendoza *et al.,* 2002). The pattern observed in spore density does not reflect the activity of the AM fungi within the roots but instead their capacity to sporulate along a hydrologic gradient (Escudero and Mendoza, 2005). Sporulation by AM fungi may be dependent upon time of year. Although not functioning at one seasonal period, a species of AM fungi may become operational depending on environmental conditions. These conditions may assist colonisation and release of spores into the soil (Kabir *et al*., 1997; Anderson *et al*., 1983). Spore germination phases such as hydration, activation, germ tube emergence and hyphal growth processes may be affected by moisture. Sporulation could be triggered by increased rainfall after the winter rainfall or during the summer period (Sinegani *et al.,* 2005). Lugo and Cabello (2002) studied the effect of seasonality on various AM fungal sporulation and spore density in a temperate climate of mountain grassland in Cordoba Argentina.

Their results revealed that spore density of *Acaulospora* and *Scuttellospora* was highest in spring and of *Glomus* in autumn, when the weather becomes cooler and drier and decreased in the wet summer periods, which they found was coincidental with the lack of flowering and fruiting in the autumn season. In contrast, root colonisation percentage of the grasses was found to be highest in summer period, followed by autumn (Lugo *et al.,* 2003). The samples in this study were collected in late autumn in El hoyo, Argentina, which is located further south and receives more rainfall during the autumn and winter period compared to the study by Lugo and Cabello (2002). Therefore the release of spores may be dormant until increased winter rains have surpassed, resulting in the decreased spore density in this time period. The results from this study correlate with those from the study by Escudero and Mendoza, (2005) on seasonality on AM fungi from the Buenos Aires province. Their results revealed a decrease in AM fungal (*Glomus* and *Acaulospora*) spore density from the wetter autumn period (April to July) with increases in the drier summer (October to January). Therefore soil analyses should be performed and a second sampling should occur in summer to take in account these soil properties and seasonal affects on spore density, which was however limited by time constraints of the project.

Three spore colour morphotypes isolated from raspberry samples which could represent a number of AM fungal species. Spore colour changes according to how the light interacts with the specimen Morton (1988). AM fungal spore colour morphotypes range from white (hyaline) to red-black in *Glomus* and *Scutellospora*, white (hyaline) to dark orange- brown in *Acaulospora* and *Entrophospora,* and ranging from white to yellow in *Gigaspora.* Schalamuk *et al.,* (2006) isolated various AM fungi belonging to the genera *Acaulospora, Entrophospora, Gigaspora, Glomus* and *Scuttellospora* from the soils associated with spring wheat in the Pampa region (central) of Argentina. This shows a wide variety of AM fungal diversity is present in the soils of Argentina, which could relate to the AM fungi collected from the raspberry soils in this study, although identification of the AM fungi was not the scope of this study, and colour morphology only gives a tentative identification of the AM fungal diversity in a given soil, and other characteristics would have to be taken into account for accurate identification of AM fungi from their spores.

AM Fungal spore-associated Bacterial Isolation

AM fungal spores were collected from the raspberry samples and bacteria were extracted from the external and internal surfaces of the spores and the number of culturable bacteria was determined for both spore surface types. Since no bacteria were found on the control plates after the sterilisation step, the use of chloramine T and antibiotics such as ampicillin, chloramphenicol and streptomycin were efficient in inhibiting the presence of bacteria on the external surface of the spores. The internal isolates can then be confirmed to be internal, except for the Gram-positive rods, which may be from the external surface and produce endospores which survive the sterilisation process and are able to germinate on the artificial media

The external surfaces had a higher count than the internal surface of the spores. Since spores are produced and released by the extraradical hyphae in the soil, they come into contact with rhizospheric microbial communities. Through the production of biofilms or through appendages such as pili, fimbriae and flagella, bacteria are able to attach to the external surface of the spores (Bianciotto and Bonfante, 2002). Endophytic bacteria are found only in the cytoplasm of spores and hyphae and are taken up through the cytoplasm of the root. Both Gram-positive and Gram-negative bacteria were isolated from the surfaces of the spores. The quantity of Gram-positive isolates were higher and were of rod and cocci morphology, whereas Gram-negative were consisted only of rods. This is consistent with the presence of these types of bacteria in the rhizosphere (Bharadwaj *et al.*, 2008).

Some Gram-positive bacteria are able to produce resistant resting structures such as spores and are able to be cultured when inoculated on artificial media. For example bacteria isolated from the external and internal surfaces of *Glomus clarum* spores revealed a variety of genera present, including *Alcaligenes, Bacillus spp., Burkholderia, Flavobacterium* and *Pseudomonas spp.* They also found that the internal surfaces had approximately 80% Gram-positive and no Gram-negative isolates (Xavier and Germida, 2003). Genera including *Pseudomonas spp., Corynebacterium, Pseudomonas putida, Paenibacillus, Bacillus spp., Stenotrophomonas spp.* were isolated from the spores of AM fungi such as *Glomus intraradices* and *Glomus mosseae*, (Mayo *et al.,* 1986; Walley and Germida, 1997; Budi *et al.,* 1999; Bharadwaj *et al.*, 2008).

Plant Growth Promoting Characterisation

Rhizospheric bacteria have been termed Plant-Growth Promoting Rhizobacteria (PGPR) are known to stimulate plant growth (Bloemberg and Lugtenberg, 2001). Direct mechanisms of growth promotion include the production of phytohormones and plant growth auxins such as indole acetic acid (IAA), nitrogen fixation and the solubilisation of phosphorous. Indirect mechanisms involve the ability to decrease or prevent any deleterious effects of pathogenic microorganisms. This can be achieved through the production of antibiotics, siderophores or other anti-microbial compounds by the bacteria (Singh and Kapoor, 1998).

Phosphate Solubilisation

A key macronutrient required for growth and development of plants is phosphorous. Within the soil many bacteria are phosphate solubilising bacteria which are able to mobilize phosphate ions from organic and inorganic phosphorous sources such as tricalcium phosphate, hydroxyapatite and rock phosphate (Gryndler, 2000; Vessey, 2003; Richardson *et al.*, 2009; Martinez-Viveros *et al.*, 2010). The bacteria isolated from the raspberry samples were evaluated for their phosphate solubilising ability on media containing $CaHPO_3$. The media contained bromophenol blue which turns to yellow upon a decrease in pH. Seventeen bacterial isolates were shown to be phosphate solubilisers. The halos' produced were measured which ranged from 17-28 mm indicating varying levels of phosphate solubilisation.

A decrease in pH is due to the production of organic acids (citrate, lactate and succinate etc) which are known to directly dissolve mineral phosphate sources through anion exchange (Harisprasad and Niranjana, 2009; Khan *et al.,* 2009). The Fe and Al ions associated with phosphorous can be chelated by the organic acids. The insoluble form of P is then converted to soluble monobasic H_2PO_4 and dibasic HPO_4^{2-} ions (Harisprasad and Niranjana, 2009; Khan *et al.,* 2009). Alternative techniques such as thin-layer chromatography can be utilised to determine the organic acids involved and to quantify the production of these compounds (Harisprasad and Niranjana, 2009; Khan *et al.,* 2009).

Phytase production

Upto 80% of organic phosphorous in the soil constitutes phytate (Myo-inositol hexakis-phosphate); making it one of the most abundant sources of phosphorous for plants (Lim *et al.*, 2007). Some PGPR have the ability to produce phytase which is able to degrade phytate to lower phosphate esters. The plant roots may not possess the ability to acquire phosphorous directly from the soil phytate sources.

Phytase producing PGPR have been shown to belong to the Genera *Bacillus, Burkholderia, Pseudomonas, Serratia* and *Staphylococcus* (Hariprasad and Niranjana, 2009). None of the isolates appeared to produce phytase as there was an absence of halos on the media. Perhaps to improve the study, one could increase the length of incubation time and temperature since phytase is a secondary metabolite. To further quantify any phytase production that was unable to occur in solid media, one could culture the isolates in a liquid medium and measuring the production spectrophotometrically at 700 nm (Hariprasad and Niranjana, 2009).

Indole Acetic Acid Production

Production of phytohormones such as auxins can have an influence on plant growth. Many PGPR are capable of producing indole acetic acid. It is primarily produced using an alternate tryptophan-dependant pathway which is carried out through indole-pyruvic acid (Xie *et al.*, 1996; Patten and Glick, 2002). Many important and beneficial physiological processes including cell enlargement and division, tissue differentiation and responses to light and gravity are controlled by IAA (Patten and Glick, 2002; Spaepen *et al.*, 2007; Shahab *et al.*, 2009; Martinez-Viveros *et al.*, 2010). PGPR can therefore promote root growth through the production of IAA (Spaepen *et al.*, 2007).

None of the bacterial isolates screened produced IAA, which upon addition of Kovak's reagent to the supernatant should change to red upon IAA production (Cappucino and Sherman, 2008). This was surprising as many bacteria from the genera such as *Aeromonas, Bacillus, Burkholderia, Enterobacter, Pseudomonas* and *Rhizobium* have been shown to produce IAA (Martinez-Viveros *et al.*, 2010).

The use of Salkowski's reagent has been shown to be effective in quantifying IAA production in other studies (Joseph *et al.,* 2007; Chaiharn *et al.*, 2008; Suresh *et al.*, 2010). Therefore an aletranative method to quanitfy IAA production by the isolates in this study would be to use Salkowski's reagent. The isolates in this study could be capable of other hormone produciton that was not accounted for in this study such as cytokinins and giberrellins. These hormones are known to be important in plant growth through the promotion of root processes such as elongation of primary root tip, and extrapolation of adventitous roots (Martinez-Viveros *et al.*, 2010).

Ammonia production

Another key plant nutrient required for growth is nitrogen. Symbiotic and non-symbiotic are two types of nitrogen fixation carried out by PGPR. Free-living diazotrophs are capable of non-symbiotic nitrogen fixation and stimulate non-legume plant growth. Genera such as *Azoarcus, Azospirillum, Burkholderia, Gluconacetobacter* and *Pseudomonas* have been shown to fix nitrogen non-symbiotically (Antoun and Prevost, 2005; Richardson *et al.*, 2009; Martinez-Viveros *et al.*, 2010). Ammonification by bacteria in the soil is important in the conversion of organic nitrogen to ammonia (Madigan and Martinko, 2006), which would enhance the soil nitrogen content which could be taken up by the plant roots.

Only two isolates were capable of ammonia production. The production of ammonia by converting organic nitrogen has been shown to improve the structure of plant roots and hence enhancing plant growth (Artursson *et al.*, 2006). Other sources of nitrogen utilisation that convert to ammonia should also be considered, such as atmospheric nitrogen fixation which can be performed using nitrogen free media. These isolates can therefore be possibly considered as PGPR.

Siderophore Production

An important element necessary for growth in all organisms is iron which is generally deficient in the soil in an utilisable form. Under iron-limiting environments PGPR are capable of producing siderophores. Siderophores are low molecular weight compounds which competitively sequesters ferric iron.

Through receptors specific to the complex on the outer cell membrane the iron-siderophore complex is taken up by the bacterium which originally produces the siderophore. The iron is released once inside the bacterium, and available to support microbial growth (Siddiqui, 2005). Genera such as *Bradyrhizobium, Pseudomonas, Rhizobium, Serratia,* and *Streptomyces* have been found to produce siderophores (Martinez-Viveros *et al.*, 2010). Bacteria are known to produce different types of siderophores. These types include catecholates (for example by *E.coli* and *Bacillus spp.*); hydroxamates (by *Streptomyces spp.* and *Burkholderia spp.*) and carboxylates (Siddiqui, 2005). Two bacterial isolates from the external surface and nine bacterial isolates from the internal surface of AM fungal spores were shown to produce siderophores.

Since the availability of iron in the soil is improved through the reduction of the soil pH due to the release of organic acids by bacteria to solubilise phosphate and iron can be provided by the bacteria through the use of siderophores has been shown to enhance plant growth, these isolates could therefore be considered to be potential PGPR.

Cell wall degrading enzyme production

The production of extracellular enzymes such as chitinase, cellulase and protease are able to hydrolyse cell wall components consisting of chitin, β- 1, 3- glucanase and proteins; have been shown to mediate in the biocontrol of fungal pathogens (Siddiqui, 2005). Two isolates from the external surface of AM fungal spores produced protease. The halo’s produced was measured and averaged, which were 13 mm and 22 mm, indicating a difference in protease production. One isolate from the external surface and ten isolates from the internal surface of AM fungal spores produced cellulase. The halo’s produced ranged was 21 to 33 mm, indicating a difference in the ability to produce cellulases.

None of the isolates produced clearing zones on chitin agar therefore it was assumed none produced chitinase. Two isolates from the external surface and twelve isolates from the internal surface of AM fungal spores produced catalase. The production of these enzymes are known to inhibit fungal pathogens and thereby promote plant growth. These isolates could then be considered as potential PGPR.

Fungal Pathogen Inhibition

Biological control of plant pathogens has been shown to be facilitated by PGPR. This achieved through the production of antibiotics, siderophores, hydrogen cyanide and hydrolytic enzymes (chitinases, proteases and cellulases). Indirect inhibition may occur through competition with the pathogen for ecological niches such as infection and nutrient sites (Linderman, 2000; Bloemberg and Lugtenberg, 2001; Sharma and Johri, 2002).

There was significant antagonistic ability resulting in decreased growth of *F.oxypsorum* by the selected bacterial isolates. Among the isolates, AERA4.1 and AIRC4 showed to be most effective in the inhibition of *F.oxypsorum.* The range of percentage inhibition was from 6-25%. Significant reduction in hyphal growth by the selected bacterial isolates was revealed in dual culture studies with *P. nicotianae*. Isolates AERB2 and AIRA4B were the most effective isolates in the inhibition of *P. nicotianae.* The range of percentage inhibition was from 36 - 55%. All the bacterial isolates were most effective in inhibiting the hyphal growth of *P. nicotianae* compared to *F.oxypsorum* (Table 3.6). Many mechanisms are known to be involved in the inhibition of fungal pathogens such as competition for nutrients, production of siderophores, antibiotics hydrogen cyanide, antifungal compounds and hydrolytic enzymes. The bacterial isolates used in the dual culture studies were evaluated for the production of siderophores and hydrolytic enzymes as previously discussed. Isolate AERA4.1 was shown to be able to produce protease, and therefore could degrade the proteins present in the fungi cell wall, accounting for its ability to inhibit *F.oxypsorum.* Isolate AIRC4, showed the greatest percentage inhibition of 25%, and however was shown to not be able to produce siderophores, only cellulases. The isolates with the most inhibition against *P. nicotianae* were shown to produce protease, cellulase and siderophores. Therefore the mechanisms of action by these isolates were to degrade the cellulose and protein components of the fungal cell wall and compete for iron.

The inhibition of *P. nicotianae* began after day 3 of inoculation indicating the production of primary metabolites. The inhibition of *F.oxypsorum* began to occur after day 6, indicating secondary metabolite production by the isolates. *F.oxypsorum* belongs to the fungal division Ascomycota. Their cell walls are mostly comprised of chitin and β- glucans and have septate hyphae which provide the fungus with stability and rigidity (Madigan and Martinko, 2006).

All of the isolates showed no production of chitinase and therefore would not be able to degrade the hyphae of *F.oxypsorum* which could account for the lower percentage inhibition compared to *P. nicotianae. P. nicotianae* belongs to the fungal division Oomycota. Their cell walls are composed mainly of cellulose rather than chitin. In addition, their hyphae are aseptate, and are therefore easier to degrade (Madigan and Martinko, 2006). Other mechanisms by which the isolates may have inhibited the two fungal pathogens may be involved which were not identified in this study but should be considered in future studies such as the production of hydrogen cyanide, antibiotics and antifungal compounds (Akhtar and Siddiqui, 2010).

Perhaps combinations of bacterial isolates may improve the inhibition of the fungal pathogens, especially looking at those with different abilities and enzyme productions, can aid in the inhibition process. All isolates used in the inhibition study could be considered as PGPR as they inhibit fungal pathogens which could indirectly promote future plant growth of host plants.

In summary, the plant growth promoting characterisation tests revealed a number of bacterial isolates were able to solubilise phosphate (68%), produce ammonia (8%), and produce cellulase (28%), protease (8%), sideropohore (32%) and catalase (44%). Multiple characterisation ability was shown by many of these isolates. A range of bacteria are represented by these isolates, including Gram-positive and negative, rods and cocci which show potential to be PGPR, although their efficiency would need to be further tested. The research in this study is consistent with a number of authors which have investigated from various host sources potential rhizobacteria as possible PGPR. Isolates from the genera *Bacillus, Azotobacter, Pseudomonas, Serratia, Streptomyces, Rhizobium* were found to have PGPR abilities through characterisation (Joseph *et al.*, 2007; Ahmad *et al.*, 2008; Chaiharn *et al.*, 2008; Hariprasad and Niranjana, 2009; Yasmin *et al.*, 2009; Franco-Correa *et al.*, 2010; Martinez-Viveros *et al.*, 2010; Suresh *et al.*, 2010).

Molecular Identification of Bacterial Isolates

Isolate AERA1 was identified by 16s rRNA analysis to be *Alcaligenes faecalis.* Members of *Alcaligenes* are Gram-negative rods, which corresponds to the morphology of isolate AERA1. This isolate showed PGPR characteristics such as phosphate solubilisation and cellulase production. This isolate showed negative results for IAA, ammonia and siderophore production. Studies by authors on the PGPR characteristics of *Alcaligenes spp.* have found species to produce IAA, ammonia and siderophore, however these species were negative for phosphate solubilisation and cellulase (Sayyed *et al.,* 2010; Kumar *et al.,* 2011; Manjunath *et al.,* 2011). Therefore the results by the isolate differ from those species of *Alcaligenes,* this could be due to differences in media used to evaluate PGPR ability or different strains are capable of different activities. It has however been shown that species of *Alcaligenes* are potential PGPR (Rodriguez and Fraga, 1999; Orhan *et al.,* 2006; Karlidag *et al.,* 2007). Therefore it can be said that isolate AERA1 is a *Alcaligenes sp.* and has potential to be a plant growth promoting rhizobacteria.

Isolates AERA4.1 and AERA5 were identified as *Bacillus pumilus. Bacillus pumilus* are known Gram-positive rods capable of producing endospores. The morphology of both isolates indicated Gram-positive rods with endospore formation. These isolates, although both *B.pumilus* have shown different PGPR characteristics in this study. Isolate AERA4.1 showed protease and siderophore production but did not solubilise phosphate, produce IAA, ammonia or catalase, whereas isolate AERA5 solubilised phosphate, produced siderophores and catalase but also did not produce IAA or ammonia. Therefore these isolates are possibly different strains of *B.pumilus* capabale of different PGPR abilities. Studies by other authors have also shown differences in PGPR abilities among different strains of *B.pumilus.* For example a study by Kumar *et al.,* (2011) investigated PGPR abilities of four different strains of *B.pumilus*. Two isolates were capable of solubilising phosphate, one isolate produced siderophores and all produced IAA. Whereas a study by Hernandes *et al.,* 2009 revealed nitrogen fixing abilities of a strain of *B.pumilus.* Therefore different strains of *B.pumilus* are capable of different PGPR characteristics. Isolates AERA4.1 and AERA5 although similar, are capable of different PGPR abilities which should be further investigated for their efficacy as possible PGPR.

Isolate AERB2 was identified as *Serratia marscecens*, which is a Gram-negative, rod shaped bacterium. The isolate in this study showed an ability to solubilise phosphate and produce siderophores and protease. Studies by other authors have presented a wide range of PGPR abilities, some varying with species or strains of *Serratia.* A study by Hariprasad and Niranjana (2009) revealed a species of *Serratia* was able to solubilise phosphate, produce phytase but was negative for IAA, siderphore, chitinase and glucanase ability. Idris *et al.,* (2009) found *Serratia* able to produce siderophores but was negative for phosphate solubilsation and IAA, however Yasmin *et al.,* (2009) showed IAA production, Phosphate solubilisation, nitrogen reduction and siderophore production by an isolate of *Serratia.* Hayat *et al.,* (2012) showed isolates of *Serratia sp.* to produce ammonia, IAA and solubilise phosphate. Results by El-Azeem *et al.,* (2007) showed differences in phosphate solubilisation and siderophore production by *Serratia sp.* Therefore different species and/ or strains of *Serratia* were capable of different PGPR abilities, some which correspond and differ to the results found in this study. This could be due to differences or inaccuracies in characterisation media used or may be different strains to the one in this study. *Serratia sp.* have however been known to be PGPR and therefore this isolate has potential to be PGPR, however its efficiency as a PGPR should be further tested.

Isolate AIRA4B was identified as *Micrococcus luteus.* This bacterium is a Gram-positive coccus commonly found in the soil environment. This isolate showed PGPR characteristics such phosphate solubilisation, cellulase and siderophore production but was negative for IAA, ammonia and phytase ability. Studies by other authors revealed differences in PGPR abilities between species and strains of *Micrococcus.* Ali *et al.,* (2010) showed a species of *Micrococcus* had the ability to produce IAA and siderophores but was negative for phosphate solubilisation. This differs from the results of Rodriguez and Fraga (1999) who have shown *Micrococcus* to be a genera able to solubilise phosphate, which was also shown in a study by Chibuogwu and Nmesoma (2011).

The study by El-Azeem *et al.,* (2007) studied ten different isolates of *Micrococcus* from three different species. All the isolates were able to produce IAA and siderophores, however only four isolates were able to solubilise phosphate. Therefore most *Micrococcus spp.* are able to produce IAA and siderophores and some may posses the ability to solubilise phopsphate. The PGPR characterstics of the isolate in this study corresponds to results shown by the authors mentioned. Therefore isolate AIRA4B can be considered to be PGPR with multiple abilities which should be investigated further.

Isolate AIRC5 was identified as a *Staphylococcus sp.* and isolate AIRC9 was identified as *Staphylococcus pasteuri. Staphylococci* belong to a genus of Gram-positive cocci bacteria. Isolate AIRC5 showed PGPR characterisation abilities such phosphate solubilisation, cellulase and catalase production but was negative for IAA, ammonia and siderophore production. Isolate AIRC9 showed an ability to solubilise phosphate, produce ammonia and siderophores and was negative for IAA and catalase. Therefore it can be said from the characterisation tests that these two isolates are indeed different species or strains of *Staphylococcus.* Species of *Staphylococcus* are characterised by the production of catalase, however only two species, *Staphylococcus saccharolyticus* and *Staphylococcus aureus* subsp. *anaerobius*, are not able to produce catalase (Dezfulian *et al.,* 2010). A study has shown another catalase negative strain of *Staphylococcus* different to the other two, which was identified as *S. aureus* subsp. a*ureus* (Dezfulian *et al.,* 2010). PGPR characterisation tests by other authors have revealed similar results. A study by Ali *et al.,* (2010) showed characterisation ability such as IAA and siderophore production but was negative for phosphate solubilisation. Other studies on *Staphylococcus spp.* have revealed positive results for IAA and siderophore production and an ability to solubilise phosphate (Barriuso *et al.,* 2005; Kumar *et al.,* 2011; Hayat *et al.,* 2012). Therefore these two isolates have an ability to be PGPR since it is known that acquisition of phosphate, production of indole acetic acid and sequestering iron are able to enhance plant growth.

Analysis of 16s rRNA structures identified Isolate AIRA2 and AIRC4 as a *Bacillus sp.* *Bacillus sp.* are a genus comprised of Gram-positive, rod shaped bacteria, capable of endospore production (Madigan and Martinko, 2006). Potential PGPR charactersitics of various *Bacillus spp.* have been investigated by many studies. A *Bacillus spp.* able to produce IAA, ammonia and showed few siderophore production was revealed by Joseph *et al.,* (2007). Orhan *et al.,* (2006) and Karlidag *et al.,* (2007) showed different *Bacillus spp.* able to solubilise phosphate, fix nitrogen and produce IAA. *Bacillus* isolates were characterised by Calvo *et al.,* (2010) and revealed 81% produced IAA and 58% solubilised phosphate. Five different species consisting of *Bacillus subtilis, Bacillus amyloliquefaciens* and *Bacillus megaterium* were investigated for PGPR abilities by Hariprasad and Niranjana (2009). Characterisation tests revealed phosphate solubilisation, IAA and phytase production by all the isolates. None of the isolates produced siderophores and cellulase was produced by only one isolate of *B.megaterium*. Antagonistic ability towards *Fusarium oxysporum* was shown by an isolate of *B. subtilis* and *Bacillus megaterium.* Isolate AIRA2 in this study showed PGPR characteristics such as phosphate solubilisation, cellulase and siderophore production, but did not produce IAA. Isolate AIRC4 solubilised phosphate, prodcued cellulase but was negative for IAA and siderophore production. These results correspond to those found by the authors mentioned and therefore isolates AIRA2 and AIRC4 can potentially be PGPR, however further investigations into their efficiency should be carried out.

Isolates AIRA5, AIRB3 and AIRC1 were unidentified by 16s rRNA analysis. The sequencing results revealed the three isolates to be *Serratia spp.* however this was thought to be incorrect since *Serratia sp.* are Gram negative rods. AIRA5 and AIRC1 were identified morphologically as medium to large Gram-positive rods, with isolate AIRC1 forming rhizoid colony morphology on agar characteristic of *Bacillus mycoides* growth (Paul *et al.*, 1995). Isolate AIRB3 was determined morphologically to be Gram positive cocci. Therefore these isolates are suspected to be of *Bacillus or Staphylococcus* genera which does not support the identification of *Serratia sp.*

3.5 Conclusion and Summary

Bacterial isolates were extracted from the external and internal surfaces of AM fungal spores from raspberry samples. They were characterised according to plant growth promoting abilities such as phosphate solubilisation, IAA production, ammonia production, siderophore and hydrolytic enzyme production. These characteristics are important for promoting plant growth through providing nutrients and other resources necessary for growth and establishment and for controlling plant pathogens which may inhibit plant growth. A total of 25 bacterial isolates were characterised, some with multiple abilities, of those 12 were identified by 16s rRNA molecular analysis. The identitities correlated with other studies published by numerous authors, all reporting similar PGPR characterisation results to the different genera of bacteria isolated in this study. These isolates can then be said to be plant growth promoting rhizobacteria.

CHAPTER 4

PLANT GROWTH PROMOTION AND AM FUNGAL COLONISATION ENHANCEMENT OF RASPBERRY (*RUBUS IDAEUS* MEEKER sp.)

4.1 Introduction

Raspberries comprise a variety of some 500 species in the genus *Rubus*, members of the *Rosaceae* family. Raspberries are closely related to strawberries in the subfamily *Rosoideae.* Raspberries are often termed under “Bramble” fruits, which are aggregate fruits which are formed by the aggregation of several smaller fruits called drupelets (Graham and Woodhead, 2009). *Rubus* species are prostrate to erect, generally with fine thorns on the shrubs. They produce renewal shoots from the ground called canes. They are perennials only because each bush consists of biennial canes. Two major types of raspberries exist, flurocane and primocane. Flurocane varieties fruit on the second years of growth, for example the Meeker cultivar, whereas primocane varieties fruit on the first years growth such as the Heritage and Autumn Bliss cultivars (Graham and Woodhead, 2009).

Rubus species are an important horticultural source of income and labour. In most countries fruit from *Rubus* species is produced for the fresh market. Rapsberries are gaining popularity because of their increasing all year round availability, been marketed as fresh dessert fruits, and processed from frozen berries into conserves, purees and juices (Graham and Woodhead, 2009). Raspberries are a major source of antioxidants and compounds which have been shown to be used against cardiovascular and epithelial cancers and other diseases. As a result of the increasing health benefits of raspberry the consumption and production is also increasing (Graham and Woodhead, 2009).

Agricultural practices that avoid the use of chemicals and fertilizers which could be detrimental to the human health are been replaced with more organic or biological processes. These organic systems rely on biofertilisation, crop rotations, animal manure, legumes and biological pest control to maintain soil productivity (Orhan *et al.,* 2006). However an important problem in organic systems is a reduction in the yield of crops (Lind *et al.,* 2003). Therefore the use of the microorganisms in the biofertilisation process is used to improve plant growth by supplying the plant with nutrients and may aid in sustaining environmental health and soil productivity. Bramble fruits are relatively easy to produce using organic systems provided an adequate supply of nutrients is available due to the application of biofertiliser into the soil (Kuepper *et al.,* 2003, Orhan *et al.,* 2006).

Micropropagation is a current method of producing large quantities of uniform high-value horticultural crops such as fruits, vegetables, plantation and spices in a short period of time. Microplants are initially grown *in vitro* under aseptic conditions, devoid of microorganisms (Taylor and Harrier, 2001; Vestberg *et al.,* 2004). In nature, beneficial rhizosphere microorganisms are essential for plant growth and development, especially in microbe-rich and nutrient-poor environments (Cordier *et al.,* 2000). Hence, by creating a beneficial rhizopshere in microplants at an early stage could better protect the plants against biotic and abiotic stresses that occur in the greenhouse or field. The beneficial rhizosphere could be created with various microorganisms such as arbuscular mycorrhizal fungi, plant growth-promoting rhizobacteria and biocontrol fungi and bacteria (Taylor and Harrier, 2001; Vestberg *et al.,* 2004).

The aim of the current experiment is to evaluate the ability of AM fungi and AM fungal spore-associated bacteria isolated from natural indigenous forest, raspberry and strawberry sources in South Africa and from raspberry in Argentina, inoculated singly or in combination to enhance AM fungal colonisation and plant growth parameters of Raspberry (*Rubus idaeus* cv. Meeker).

4.2 Materials and Methods

4.2.1 Bacterial Isolates

Five South African (SEN4, SERB2, SESB1, SISB1 and SISB2) and five Argentinean (AERA1, AERA4.1, AERB2, AIRA4B and AIRC5) bacterial isolates were selected upon their plant growth promoting abilities according to the characterisation tests (Table 4.1). Isolates were grown in NB, on a rotary shaker at 120 rpm at 37^{o}C for 72 hrs. A sample (1 ml) of each isolate was collected and centrifuged at 10 000 rpm for 5 mins. The supernatant was removed and the cells resuspended in NB. Their concentrations were measured at an OD of 600 nm and adjusted to an OD reading of 1.5 by dilution with sterile NB.

Table 4.1 Summary of PGPR characteristics and identity of isolates inoculated in the study.

Isolate	Coding*	P-solub	NH_3	Sidph	Cellulase	Identity (NCBI)
SEN4	1	+	-	+	+	*Acinetobacter ursingii*
SERB2	2	+	-	+	+	*Bacillus mycoides*
SESB1	3	+	+	-	+	*Bacillus mycoides*
SISB1	4	+	-	+	-	*Unidentified*
SISB2	5	+	-	-	+	*Bacillus sp.*
AERA1	6	+	-	-	+	*Alcaligenes faecalis*
AERA4.1	7	-	-	+	-	*B.pumilus*
AERB2	8	+	-	-	-	*Serratia marscecens*
AIRA4B	9	+	-	+	+	*Micrococcus luteus*
AIRC5	10	+	-	-	+	*Staphylococcus sp.*

* Coding used in trial, represented in Appendix IV

4.2.2 Pot Trial Set Up

Raspberry (*Rubus idaeus* Meeker sp.) was used as the host plant to evaluate plant growth promotion and mycorrhizal colonisation enhancement. The Raspberry seedlings were recieved from (Microprop CC). The AM fungal incolulum used was from Mycoroot (Pty) Ltd. Mycoroot products contain a combination of naturally occurring Southern African arbuscular mycorrhizal isolates which include *Glomus clarum*, *Gigaspora gigantea*, *Glomus mosseae*, *Glomus etunicatum* and *Paraglomus occulum* (molecular determination). The experiment involved four treatments; a control without the bacteria or AM fungal inoculum (Control); treatment with just AM fungal inoculum (AM); treatments with only the selected bacteria (selected bacterial isolate) and treatments with both the bacteria and AM fungal inoculum (AM with selected bacterial isolates). Each treatment was replicated five times (110 pots). The raspberry seedlings were planted into 250ml pots set up with stones (bleached) at the bottom and a mix of pasteurised sand, autoclaved compost and vermiculite in a ratio of 10:5:1. AM fungal inoculum (5ml) was added to the pots before the raspberry seedlings were planted. The bacterial isolates in Nutrient Broth (5ml) were added to the surface of the soil around the raspberry seedlings. To the control and AM fungal pots, 5 ml of sterile uninoculated Nutrient Broth was added.

The experiments were undertaken in a Mycorrhizal Tunnel where it was watered daily with UV sterilised water and received a low phosphorous Long Ashton solution weekly (Smith *et al.*, 1983). The initial plant height of the raspberry seedlings and any secondary shoots was measured upon planting. Plants were grown for 12 weeks.

4.2.3 Harvest and Analysis

Shoot height, Shoot weight, Root weight

At harvest the plants were removed and the roots separated from the shoots. Roots were fresh weighed and a subsample of the fresh root material was removed for AM fungal colonisation assessment. Shoots and roots were dried at 65^{o}C in an oven for three days and weighed. The root dry weights were corrected for the subsample removed after moisture determinations.

AM fungi colonisation

*C*olonisation of the plant roots was evaluated by using a method described by Koske and Gemma, (1989) and Smith and Dickson, (1997). Sections of roots were cleared with 5% KOH solution at 70^{o}C, 10 minutes. The roots were bleached in an alkaline H_2O_2 solution for 15 mins until the roots turned white. The roots underwent acidification using 0.1M HCl solution and stained with lactoglycerol solution containing 0.05% tryptan blue, stained for 30 mins at 80-90^{o}C in a waterbath. The roots were destained for 12-24 hrs. Colonisation is characterised by the formation of intercellular hyphae and intracellular arbuscules. The percentage root colonisation was determined microscopically using a modified gridline intersect method (McGonigle *et al.*, 1990).

4.2.4 Statistics

Shoot and root dry weights and percentage AM fungal colonisation was analysed by grouping all the data, including South African and Argintinean bacterial isolates for comparison between the countries using a one-way ANOVA. Significant difference between group means was determined using test of significance. The level of significance for ANOVA and Fischer LSD test was 5%. All analyses were carried out by means of StatSoft, Inc. (2009) STATISTICA (data analysis software system) Version 7. www.statsoft.com.

4.3 Results

4.3.1 Shoot weight

Shoot wet and dry weights were measured of the raspberry (*Rubus idaeus* cv. Meeker) inoculated singly with AM fungi or bacteria or dual inoculated. There was a significant difference in the dry shoot weight amongst treatments ($F_{21,88} = 2.212$, $p < 0.005$). Treatments SISB1, AERA1, AM+SESB1 and AM+SISB1 were significantly different to the uninoculated control. The most significant increase in shoot weight was with isolate AERA1 singly inoculated, confirmed by Fischer's LSD ($p < 0.002$) however there was no significant difference between these inoculated treatments (Figure 4.1). Treatments SISB1, AERA1 and AM+SESB1 were significantly different to the AM fungal treatment singly inoculated. Treatment SERB2 had a significantly decreased shoot dry weight in comparison with SISB1, AERA1, AM+SESB1 and AM+SISB1 whereas treatment AM+SERB2 was lower than all the treatments but had a significantly decreased shoot weight in comparison with SISB1, AERA1, AM+SEN4, AM+SESB1, AM+SISB1, AM+SISB2, AM+AERA2 and AM+AIRA4B. Treatment SISB2 was only significantly lower than AERA1. Treatment AERA4.1 had a significantly lower shoot dry weight than treatments SESB1, SISB1, AERA1, AM+SEN4, AM+SESB1, AM+SISB1, AM+SERB2, AM+SISB2, AM+AERA1, AM+AERB2 and AM+AIRA4B. There were no other significant differences between the increased dry weight treatments (Table 4.2, Appendix IV). There was a difference in shoot height as seen in Figure 4.2, where some of the treatments such as the controls had reduced and withered shoots compared to others hence measuring shoot height was difficult in some treatments and a comparison could not be adequately performed.

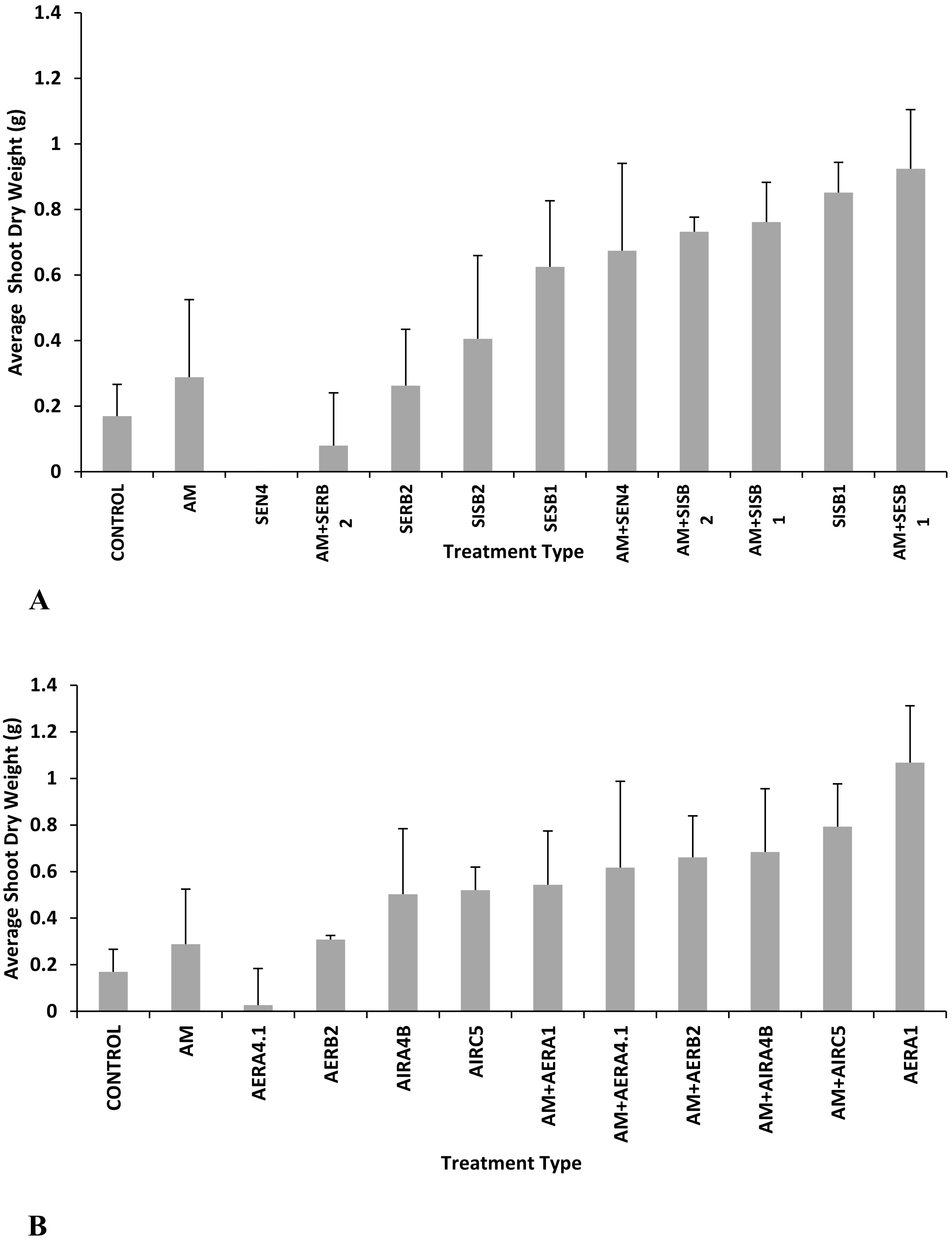


Figure 4.1 A and B. Average shoot dry weight of raspberry (*Rubus idaeus* cv. Meeker) (Mean ± SE), inoculated with AM fungi, selected bacterial isolates and dual inoculation. **A** – inoculated with South African bacterial isolates and **B-** inoculated with Argentinean bacterial isolates, ($F_{21,88} = 2.212$, $p < 0.005$).

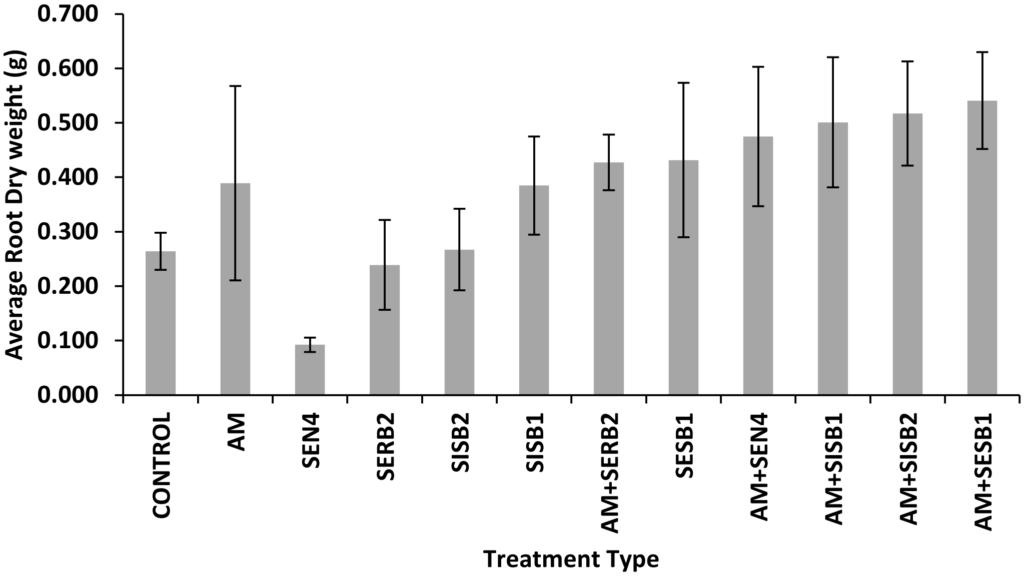
Table 4.2 Significant differences in shoot dry weight by the treatments as compared by Fischers LSD.

Treatment	Control	AM fungi	SEN4	SERB2	SESB1	SISB1	SISB2	AERA1	AERA4.1	AERB2	AIRA4B	AM+SEN4	AM+SERB2	AM+SESB1	AM+SISB1	AM+SISB2	AM+AERA1	AM+AERA4.1	AM+AERB2	AM+AIRA4B	AIRC5	AM+AIRC5
Control						*		**						**	*							
AM fungi						*		**						*								
SEN4					*	**		***				*		*	**	*	*		*	*		
SERB2						*		**						**	*							
SESB1			*																			
SISB1	*	*	**	*					**				**									
SISB2								*														
AERA1	**	**	***	**			*		***	*	**		***					**				
AERA4.1					*	**		***				*		**	*	*	*		*	*		
AERB2								*														
AIRA4B								**														
AM+SEN4			*						*				*									
AM+SERB2						**		***				*		**	*	*			*	*		
AM+SESB1	**	*	**	**					**				**					*				
AM+SISB1	*		**	*					**				*									
AM+SISB2			*						**				*									
AM+AERA1			*						*													
AM+AERA4.1								**						*								
AM+AERB2			*						*				*									
AM+AIRA4B			*						*				*									
AIRC5																						
AM+AIRC5																						

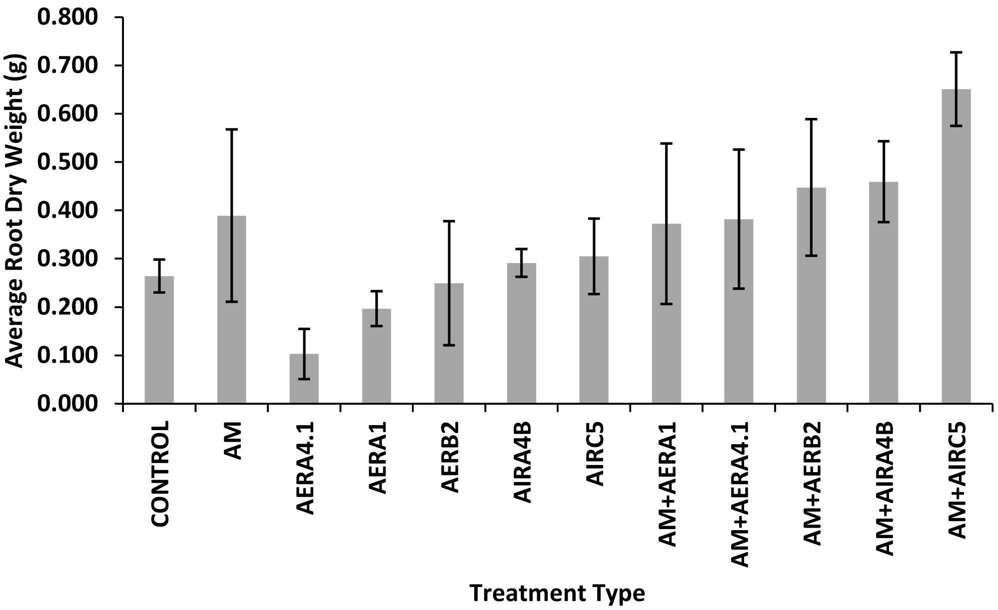
Significant differences indicated by: * ($p < 0.05$), ** ($p < 0.001$), *** ($p < 0.0001$)

4.3.2 Root weight

Root wet and dry weights were measured from the raspberry plants. There was a significant difference among treatments on root dry weight ($F_{21, 88} = 1.897$, $p = 0.021$). The most significant increase in root dry weight compared to the control was isolate AERA1 singly inoculated, confirmed by Fisher's LSD ($p < 0.009$) (Figure 4.2). Isolate SEN4 had a significantly reduced root dry weight than all treatments except SERB2, SISB2, AIRA4B, AIRC5, AM+AERA1, AM+AERA4.1 and AM+AIRC5. Isolate AERA1 was significantly higher than treatments SEN4, SERB2, SISB2, AERA4.1, AIRA4B, AIRC5 and AM+AIRC5. Isolate AERA4.1 had a significantly reduced root dry weight compared to SESB1, AERA1, AERB2, all the dual inoculations with AM fungi and South African isolates. There were no other significant differences amongst treatments (Table 4.3, Appendix IV).



A

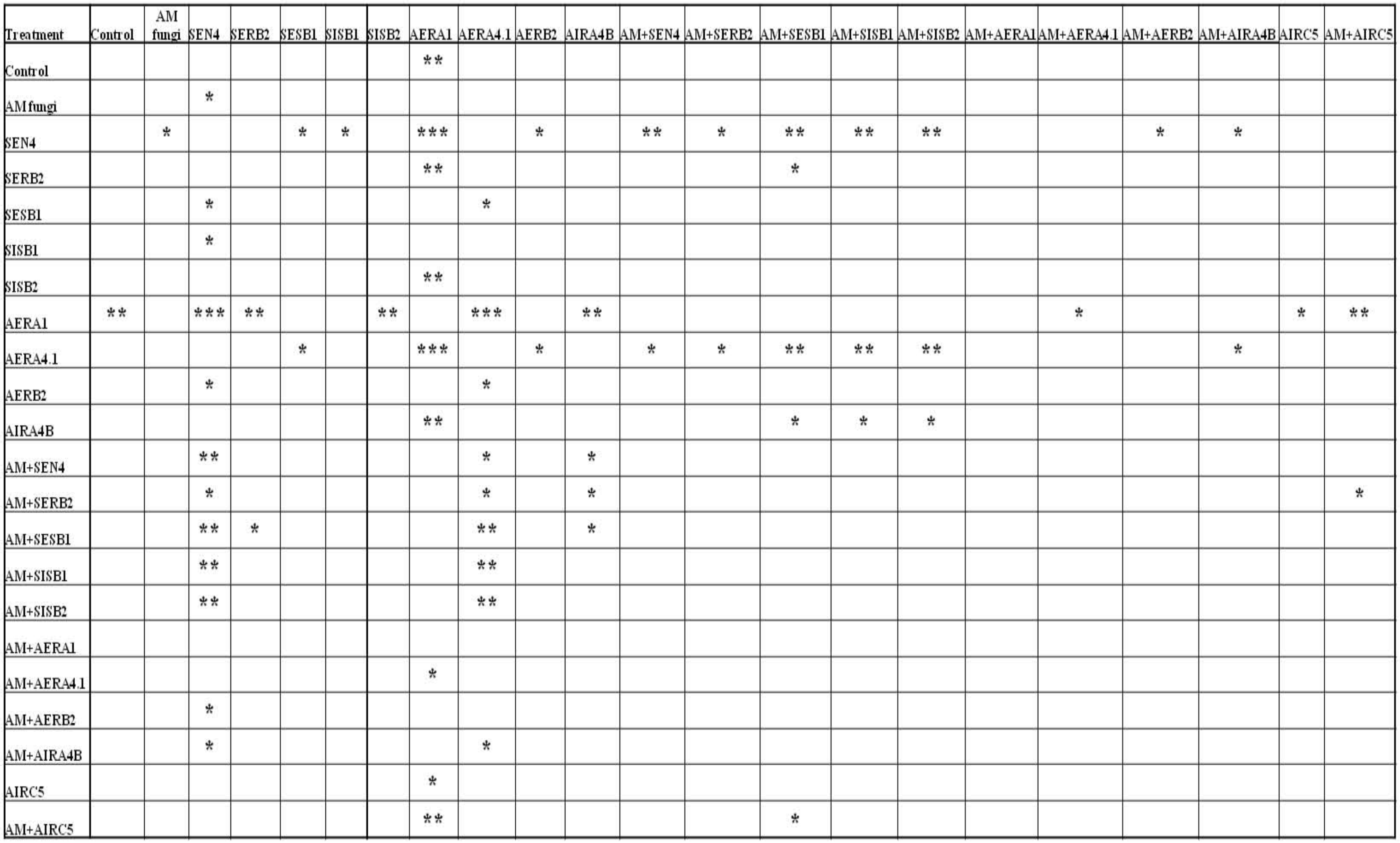


B

Figure 4.2 A and B. Average root dry weight of raspberry (*Rubus idaeus* cv. Meeker) (Mean ± SE), inoculated with Arbuscular Mycorrhizal fungi, selected bacterial isolates and dual inoculation. **A** – inoculated with South African bacterial isolates and **B-** inoculated with Argentinean bacterial isolates, ($F_{21, 88}$

Figure 4.3 A and B. **A-** Final wet shoot and root of raspberry (*Rubus idaeus* cv. Meeker) of treatment AERA1 at 12 weeks. **B-** Enhanced image of root section showing the new root formation.

Table 4.3 Significant differences in root dry weight by the treatments as compared by Fischers LSD (p < 0.05).



Treatment	Control	AM fungi	SEN4	SERB2	SESB1	SISB1	SISB2	AERA1	AERA4.1	AERB2	AIRA4B	AM+SEN4	AM+SERB2	AM+SESB1	AM+SISB1	AM+SISB2	AM+AERA1	AM+AERA4.1	AM+AERB2	AM+AIRA4B	AIRC5	AM+AIRC5
Control								**														
AM fungi			*																			
SEN4		*			*	*		***		*		**	*	**	**	**			*	*		
SERB2								**						*								
SESB1			*						*													
SISB1			*																			
SISB2								**														
AERA1	**		***	**			**		***		**							*			*	**
AERA4.1					*			***		*		*	*	**	**	**				*		
AERB2			*						*													
AIRA4B								**						*	*	*						
AM+SEN4			**						*		*											
AM+SERB2			*						*		*											*
AM+SESB1			**	*					**		*											
AM+SISB1			**						**													
AM+SISB2			**						**													
AM+AERA1																						
AM+AERA4.1								*														
AM+AERB2			*																			
AM+AIRA4B			*						*													
AIRC5								*														
AM+AIRC5								**						*								

Significant differences indicated by:* (p <0.05), ** (p< 0.001), *** (p<0.0001).

4.3.3 AM Fungal Colonisation Percentage

Roots of the raspberry plants were stained with trypan blue and the roots examined for AM fungal colonisation. The percentage colonisation was determined and there was no colonisation present in the uninoculated control and single inoculations of bacterial isolates. There was a significant difference among treatments ($F_{21, 88} = 19.703$, $p < 0.001$). The treatments significantly different (increased AM fungal colonisation %) to the AM fungal inoculation was AM+SEN4, AM+SERB2, AM+AERA4.1, AM+AIRA4B and AM+AIRC5. The most significant increase in AM fungal colonisation percentage was the dual inoculation with SERB2, confirmed by Fischer's LSD compared to the AM fungi singly inoculated ($p < 0.001$) (Figure 4.4). Treatment AM+SEN4 had a significantly higher AM fungal colonisation percentage than AM+SESB1 and AM+SISB2 whereas AM+SERB2 had a significantly higher AM fungal colonisation percentage compared to all treatments except AM+SEN4, AM+AIRA4B and AM+AIRC5 (Table 4.4). The raspberry roots showed colonisation structures by AM fungi such as arbuscules, spores, intra-radical hyphae and vesicles. Interesting to note, isolate SERB2 had the lowest shoot dry weight, but a higher root dry weight, almost double the dry weight compared to the shoot and also had the highest percentage root colonisation of AM fungi, therefore even though a low root dry weight, most of the root was colonised.

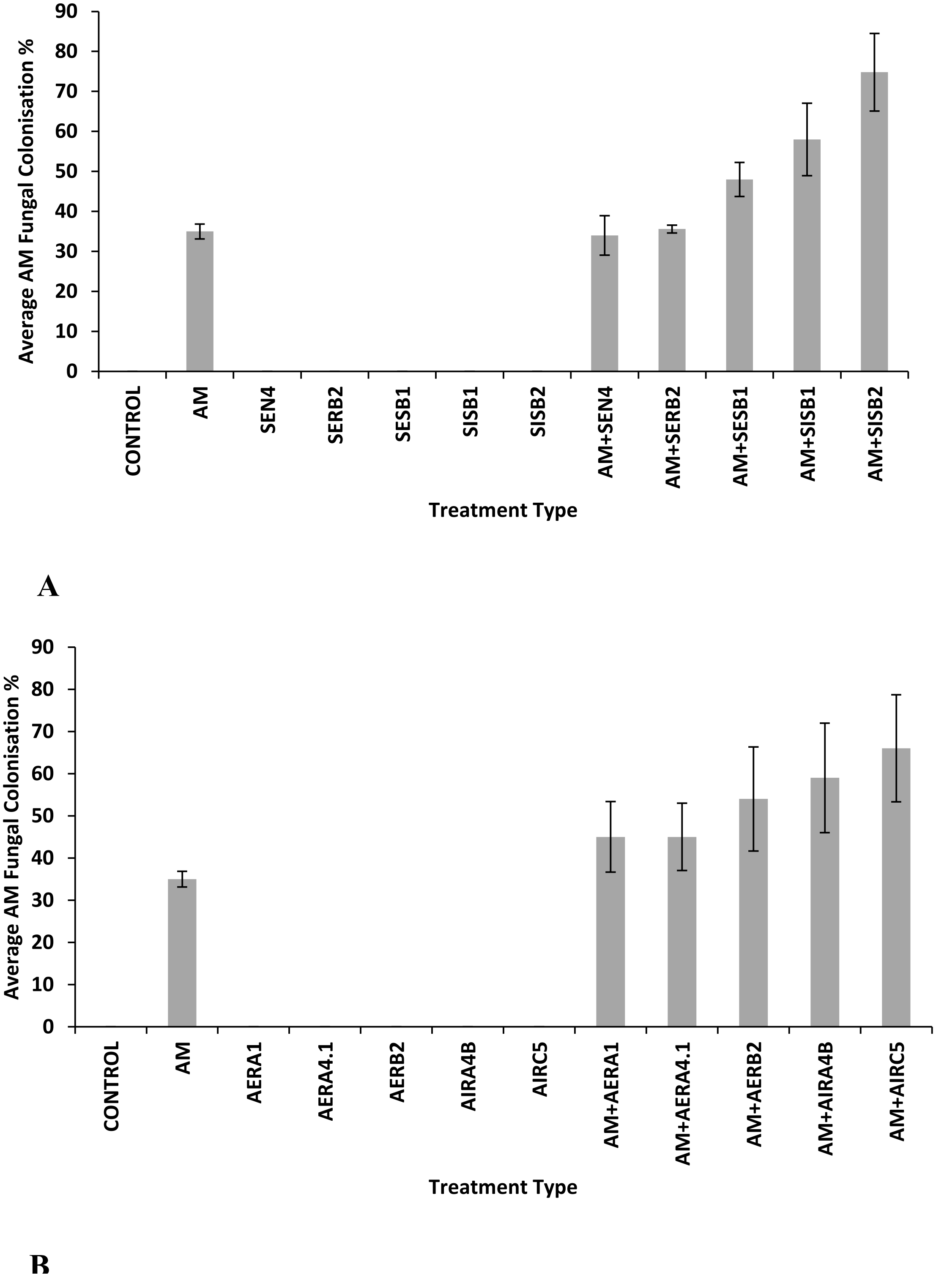


Figure 4.4 A and B. Average AM fungal colonisation percentage of raspberry (*Rubus idaeus* cv. Meeker) roots (Mean ± SE), inoculated with Arbuscular Mycorrhizal fungi, selected bacterial isolates and dual inoculation. **A** – inoculated with South African bacterial isolates and **B-** inoculated with Argentinean bacterial isolates, ($F_{21, 88} = 19.703$, p <0.00).

Table 4.4 Significant differences in % root colonisation by the treatments as compared by Fischers LSD

Treatment	Control	AM fungi	SEN4	SERB2	SESB1	SISB1	SISB2	AERA1	AERA4.1	AERB2	AIRA4B	AM+SEN4	AM+SERB2	AM+SESB1	AM+SISB1	AM+SISB2	AM+AERA1	AM+AERA4.1	AM+AERB2	AM+AIRA4B	AIRC5	AM+AIRC5
Control		***										***	***	***	***	***	***	***	***	***		***
AM fungi	***		***	***	***	***	***	***	***	***	***	***	***					*		***	***	**
SEN4		***										***	***	***	***	***	***	***	***	***		***
SERB2		***										***	***	***	***	***	***	***	***	***		***
SESB1		***										***	***	***	***	***	***	***	***	***		***
SISB1		***										***	***	***	***	***	***	***	***	***		***
SISB2		***										***	***	***	***	***	***	***	***	***		***
AERA1		***										***	***	***	***	***	***	***	***	***		***
AERA4.1		***										***	***	***	***	***	***	***	***	***		***
AERB2		***										***	***	***	***	***	***	***	***	***		***
AIRA4B		***										***	***	***	***	***	***	***	***	***		***
AM+SEN4	***	***	***	***	***	***	***	***	***	***	***			*		***					***	
AM+SERB2	***	***	***	***	***	***	***	***	***	***	***			***	**	***	***	*	***		***	
AM+SESB1	***		***	***	***	***	***	***	***	***	***	*	***					*		***	***	**
AM+SISB1	***		***	***	***	***	***	***	***	***	***		**							*	***	
AM+SISB2	***		***	***	***	***	***	***	***	***	***	**	***					*		***	***	**
AM+AERA1	***		***	***	***	***	***	***	***	***	***		***							*	***	
AM+AERA4.1	***	*	***	***	***	***	***	***	***	***	***		*	*		*					***	
AM+AERB2	***		***	***	***	***	***	***	***	***	***		***							*	***	
AM+AIRA4B	***	***	***	***	***	***	***	***	***	***	***			***	***	***	*		*		***	
AIRC5		***										***	***	***	***	***	***	***	***	***		***
AM+AIRC5	***	**	***	***	***	***	***	***	***	***	***			**		**					***	

Significant differences indicated by:* (p <0.05), ** (p< 0.001), *** (p<0.0001).

4.4 Discussion

Through their natural ability to provide nutrients otherwise inaccessible to plants, PGPR play an important role in maintaining the soil environment. Nitrogen and phosphorous are key macronutrients required by the plants and which are supplied by the PGPR. The inoculation effects of PGPR such as phosphate solubilisers, nitrogen fixers and siderophore producers, as well as AM fungi have been investigated in this study for their potential to promote plant growth and improve mycorrhizal colonisation. The synergistic effect of phosphate solubilisers, nitrogen fixers and AM fungi has been studied extensively for their plant growth promoting abilities with various crop hosts such as sweet cherry, raspberry, apple, wheat, wheat and oat, chickpea, tomato and sorghum (Esitken *et al.,* 2006; Orhan *et al.,* 2006*;* Karlidag *et al.,* 2007; Khan and Zaidi, 2007; Yao *et al.,* 2008; Akhtar and Siddiqui, 2009; Hariprasad and Niranjana, 2009; Idris *et al.,* 2009).

The shoot dry weight of raspberry (*R.idaeus* cv.Meeker) was significantly increased compared to uninoculated control. The highest increase in weight was by single inoculations with isolates SISB1, AERA1 and dual inoculations with AM fungi and isolates SESB1 and SISB1. Isolate SISB1 was shown to solubilise phosphate and produced siderophores, isolate AERA1, which showed the highest increase in shoot dry weight from the Argentinean samples only solubilised phosphate, and the highest increase in shoot weight from the South African samples was SESB1 which showed phosphate solubilisation and ammonia production. The enhancement of shoot dry weight by these bacteria could be explained with the phosphate solubilising, production of ammonia and iron provided by the bacteria through their siderophore producing capacity.

Phosphate solubilising bacteria (PSB) have been shown to solubilise and mineralise phosphorous from inorganic and organic sources of total soil P, thereby increasing availability of the P to the plants, which may promote plant growth (Rodriguez and Fraga, 1999; Khan *et al.*, 2009). The phosphate solubilising ability has been attributed to the ability of the bacteria to lower the pH of the surrounding soil environment by releasing either organic acids or protons. Organic acids such as gluconic acid, citrate and lactate for example have been known to be secreted by PSB into the rhizosphere.

These secreted organic acids can dissolve mineral phosphates through anion exchange or by chelating Fe and Al ions associated with phosphorous. The insoluble form of P is solubilised and converted to soluble monobasic and dibasic ions (H_2PO_4 and HPO_4^{2-}). This leads to increased availability of P to plants and hence uptake of P, which can promote the growth of the plant (Rodriguez and Fraga, 1999; Khan *et al.*, 2009; Martinez-Viveros *et al.*, 2010; Suresh *et al.*, 2010). A study by Hariprasad and Niranjana (2009) showed an isolate of *Enterobacter sp.* had the highest significant increase in dry weight of tomato by 168%, which was attributed to its phosphate solubilising activity as it increased the phosphate accumulation in the plant. Akhtar and Siddiqui (2009) showed phosphate solubilising organisms such as *Pseudomonas putida*, *Pseudomonas alcaligenes* and *Pseudomonas aeruginosa* significantly increased shoot dry weight of chickpea compared to the controls by 13 %, 8.8% and 9.96% respectively.

Nitrogen is an essential plant nutrient. There are two types of nitrogen fixation carried out by PGPR: symbiotic and non-symbiotic. Non-symbiotic nitrogen fixation is carried out by free-living diazotrophs and can stimulate non-legume plants growth. Endophytic diazotrophs appear to have an advantage over root-surface organisms since they are capable of colonising the interior of roots and establish themselves within niches that are more conducive to effective N_2 fixation, transferring the fixed N to the host plants. The production of ammonia through the conversion of molecular nitrogen by nitrogenase has been shown to enhance plant growth by improving the structure of the plant roots (Artursson *et al.*, 2006). A study by Khan and Zaidi (2007) showed an isolate of *Azotobacter*, a nitrogen fixing bacterium significantly improved the shoot dry weight of wheat by 52% through its ability to fix nitrogen and supply it to the plant which was evident in the increased N concentrations of the plant. A study on the effects of PGPR on raspberry by Orhan *et al.,* (2006) showed an isolate of *Microbacterium* capable of nitrogen fixation significantly increased the yield by 33.9%. Mycorrhizal formation may be affected by nitrogen fixation via the formation of ammonia. Arbuscular mycorrhizal fungi may be associated with nitrogen fixation through spore-associated bacteria. A study by Cruz and Ishii (2012) revealed an isolate of *Bacillus thuringiensis* isolated from the internal surface of *Gigaspora margarita* was able to produce nitrogenase through an acetylene reduction assay and therefore is capable of fixing atmospheric nitrogen.

Bacterial siderophore production has been shown to enhance iron nutrition in the soil which can be utilised by plants for growth. Iron in the soil is deficient in neutral or alkaline pH due to low solubility (Sharma and Johri, 2003). Solubilisation of phosphate by the bacteria decreases the pH of the soil, which increases the availability of Fe for uptake.

Iron is essential for cellular growth and metabolism and therefore its availability for plant growth is essential. A study by Idris *et al.,* 2009 revealed isolates of *Serratia marscecens* and *Bacillus cereus* significantly improved plant growth of sorghum by increasing shoot dry weights and showed the isolate capable of producing siderophores. They concluded the isolates may have improved plant growth by the action of siderophores.

At harvest, there was a difference in shoot heights observed, with some of the shoots showing necrosis, yellowing of the leaves or complete withering of the shoots (Figure 4.2). This could be due to the raspberry plantlets arising from bare cane rooted tissue cultured plants and showed some poor root development when transplanting. Micropropagation present disadvantages in the technique. Micropropagation has been widely used in the production many agricultural crops. It is used as it provides a source of pathogen free material and a large number of plantlets in a short period of time. The hardening and transfer stage of micropropagation involves transferring of the in vitro plants to the soil after acclimatisation (Jha and Ghosh, 2005).

Significant loss of plants after transplant, rapid development of shoot and leaf chlorosis and difficulty in shoots efficiently elongating are major problems encountered with micropropagated plants. These problems can be overcome if acclimatisation is carried out carefully in order for the micropropagated plants to survive transfer to the soil (Jha and Ghosh, 2005). The plants must be gradually hardened to withstand exposure to the stress of lower humidity (20-60%), higher light intensity and exposure to pathogens. Plants should be maintained under high humidity for 2- 3 weeks just after transfer to a greenhouse. Light intensity should be gradually increased during the hardening stage (Jha and Ghosh, 2005). Stomatal morphology of micropropagated plants prior to hardening have revealed inconsistencies in stomatal functioning, with the stomata either remaining open or closing too slowly, hence plants must be protected until the stomata respond more appropriately (Jha and Ghosh, 2005).

A study by Wu *et al.,* (2009) on micropropagated *Rubus* plants revealed the addition of compounds such as 6-benzyladenine, indole-3-butyrc acid and activated charcoal and reduced light intensity improved performance of the plantlets, which originally had an explants survival of 35%.

Taylor and Harrier (2000) studied micropropagated raspberry, they acclimatised the plantlets first with elevated humidity of (80 ± 2%) before transfer to pots. The plants were grown with 18 hr day length, at a temperature of 16-22 ^{o}C and natural light supplemented with 400 W high pressure sodium lamps. The plants were watered daily and received Long Ashton nutrient solution weekly after six weeks. Future use of micropropagated plants for the study of PGPR effects should first be hardened efficiently with high humidity and low light intensity, perhaps supplemented with compounds or AM fungi, which have been shown to improve survival of plants during the weaning stage before inoculation with the bacterial isolates to reduce loss of plants to chlorosis as was observed in this study.

The root dry weight of the raspberry plants were significantly increased by treatments with the highest increase by AERA1 compared to the control. This isolate as mentioned before showed an ability to only solubilise phosphate. Although the isolate showed no IAA production in this study, the results may be inconclusive as the isolate showed significant increase in root formation (Figure 4.). Some PGPR can have an influence on plant growth by the production of phytohormones such as auxins, cytokinins and gibberellins. IAA in plants is the main auxin which controls many important and beneficial physiological processes including cell enlargement and division, tissue differentiation and responses to light and gravity (Patten and Glick, 2002; Spaepen *et al.*, 2007; Shahab *et al.*, 2009; Martinez-Viveros *et al.*, 2010). IAA produced by PGPR can promote root growth (Spaepen *et al.*, 2007). Rapid establishment of roots can be achieved either by the elongation of primary roots or by the proliferation of lateral and adventitious roots. This establishment is advantageous for young seedlings as it increases their ability to anchor themselves in the soil. This increases their chances of survival by obtaining water and nutrient from the soil (Patten and Glick, 2002).

Dobbelaere *et al.*, (1999) showed in an experiment with *Azospirillum* that increased rooting was directly related to IAA synthesis. This increased rooting enhanced the plant mineral uptake and root exudation which in turn stimulated bacterial colonisation which further enhances the inoculation effect.

Patten and Glick, (2002) showed that low concentrations of IAA stimulated the elongation of the primary root and demonstrated the direct influence of bacterial IAA in promotion of root elongation when associated with the host plant. However, high concentrations of IAA stimulate the formation of lateral and adventitious roots. IAA produced by PGPR can therefore have many beneficial influences on plant growth by altering root elongation. A study by Shahab *et al.,* (2009) revealed two strains of *Bacillus thuringensis* and *Pseudomonas aeruginosa* which were able to produce IAA had an effect on mung beans (*Vignia radiate)* in greenhouse experiments. The *P.aeruginosa* isolate showed the most significant root and shoot elongation compared to the other two isolates and the control. It is therefore discussed that plant growth substances produced by these bacteria improve plant growth by directly having an effect on metabolic processes. Since they induce lateral root and root hair proliferation, increasing the nutrient absorbent surface areas leading to greater nutrient absorption rates and increases in shoot and root length of the plants (Shahab *et al.,* 2009).

If the isolate did not produce IAA, then there may be other auxins produced by the isolate that promoted root growth which were not tested in this study such as cytokinins and gibberellins. Cytokinins are phytohormones which promote cell division and enlargement, tissue expansion and promote root hair development (Madhaiyan *et al.*, 2010). Gibberellins enhance the development of plant tissues, particularly stem tissue and promote root elongation and lateral root extension. Production of gibberellins has been reported in PGPR (Vessey, 2003; Martinez-Viveros *et al.*, 2010).

SEN4, SERB2 and AERA4.1 had the lowest shoot and root dry weight increase compared to the other isolates. This probably indicates that the ability of these bacterial isolates to display PGPR characteristics may not necessarily mean that the bacteria are PGPR (Vessey, 2003). Isolate SERB2 was shown to solubilise phosphate but was negative for siderophore production and isolate AERA4.1 showed no phosphate solubilisation ability but produced siderophores.

Both of these isolates showed cellulase production. Idris *et al.,* (2009) showed two isolates of *S. marscecens* were capable of solubilising phosphate, producing IAA and siderophores, yet only one was able to significantly promote the growth of sorghum while the other did not.

The strain which did not promote plant growth was shown to be effective in the suppression of fungal pathogens. Isolate SERB2 was shown to effective in inhibition of *Phythophthora nicotianae* in the characterisation process (Section 2.3.5) and isolate AERA4.1 showed inhibition of *Fusarium oxysporum* and *Phythophthora nicotianae* (Section 3.3.5). Although they may not be PGPR through direct mechanisms of biofertilisation, they may possess an ability to be antagonistic to fungal pathogens, which indirectly may promote plant growth. Further investigations into this ability should be studied *in vivo* with a host plant, to determine if the isolates are capable of reducing the effects of the fungal isolates and hence promote plant growth. There was no significant difference amongst the other treatments, although there was evidence in increased shoot dry weights. The variations in success of the microbial inoculations in this study could be due to the differences in their functionality, survivability and colonisation efficiency of the microbial strains (Khan and Zaidi, 2007). The plants also release beneficial or detrimental compounds such as signal compounds or exudates such as amino acids, carbohydrates that influence the bacterial isolates in the soil which could affect their colonisation ability and survivability (Khan and Zaidi, 2007).

Isolate SESB1 only improved shoot dry weight significantly when in co-inoculation with AM fungi. Dual inoculations with the bacterial isolates and AM fungi except treatments AM+ SERB2, AM+ SISB1 and AM+ AERA1 increased the shoot dry weight, although not significant, compared to the single inoculations. AM fungi also increased root dry weights of dual inoculated treatments AM+ AERA1, AM+ AERB2 and AM+ AIRC5. Isolate SESB1, a strain of *B. mycoides* was shown to solubilise phosphate and produce ammonia. Therefore the supply and uptake of P and N provided by this isolate was enhanced by the AM fungus. It has been mentioned that in P-deficient soils, phosphate-solubilising micro-organisms interact with AM fungi by releasing phosphate ions which are transferred by the AM fungi to the plant. There are different mechanisms by which PGPR and AM fungi interact together to improve plant growth (Artursson *et al.*, 2006).

Through their extraradical hyphae, AM fungi are able to supply these nutrients to plant which enhances plant growth (Rodriguez and Fraga, 1999). Gamalero *et al.*, (2004) studied the impact of *Pseudomonas fluorescens* and *Pseudomonas fluorescens* P190r and *G.mosseae* on tomato (*Lycopersicon esculentum* Mill.) plant growth.

Significant increases in both the shoot and root fresh weights were shown by *Pseudomonas fluorescens* 92rk and *Pseudomonas fluorescens* P190r. Co-inoculation of 92rk and BEG12 induced significant increases in shoot fresh weight. Co-inoculation of all three increased shoot and root fresh weights relative to all the other treatments. Kohler *et al.*, (2007) studied the interactions between *B. subtilis* and *G. intraradices* and their effects on lettuce plants (*Latuca sativa*). Dual inoculations of *B.subtilis* and *G. intraradices* had the highest effect (77%) on shoot biomass of the lettuce plants. The increase in plant growth by *B. subtilis* was due to an enhanced P supply to the crop. *B. subtilis* is a phosphate-solubilising rhizobacterium, which may enhance mineral uptake by plants by solubilisng insoluble P in the soil. The P is then taken up by the extra-radical AM hyphae and transferred to the crop plant (Rodriguez and Fraga, 1999). Mar Vazquez *et al.,* 2000 investigated the influence of different microbial inoculants on maize (*Zea mays* L.). The bacterial isolate *Azospirillum brasilense* was found to have a significant increase in shoot and root dry weight in the dual inoculation with *G. deserticola.* A study by Medina *et al.*, (2003) studied the interactions between *G. mosseae, G. intraradices* and *G. deserticola* and *Bacillus pumilus* and *Bacillus licheniformis* on alfalfa plants (*Medicago sativa).* Single bacterium treatments did not have an effect on plant growth parameters.

The most efficient treatment in plant growth (shoot and root dry weight, root length and surface area) was the dual (*G. deserticola* and *B. pumilus*) inoculation, which produced a 715% (shoot weight) and 190% (root length) increase over the uninoculated control. Interactions between AM fungi and PGPR occurs naturally since they share common habitats such as the root surface (Barea *et al.*, 2004). The interaction increases plant growth through mechanisms carried out by both the AM fungi (through increased nutrient uptake and an enhanced surface area for absorption) and PGPR (phosphate-solubilisation, nitrogen fixation and phytohormone production) (Antoun and Prevost, 2005; Miransari, 2011), which combined have a benefecial effect on plant growth in various crops as investigated by the studies mentioned.

It is interesting to note that some treatments had higher root dry weights compared to shoot dry weights, in particular treatment with AM fungi and isolate SERB2 had almost double the root dry weight, and this isolate also showed the highest percentage root colonisation by the AM fungus. Therefore when in combination with AM fungi, SERB2 and the AM fungi put more energy into root production compared to the shoot, which is evident since most of the root surface was colonised by AM fungi. So the nutrients acquired by the bacteria and AM fungi was tranlocated to root development by the plant. Also there was large deviations in the standard error in treatments, this could be due to variability in the plants response to the environment and treatment. These deviations could be reduced by increasing the number of replicates, perhaps from five to ten which would eliminate outlying results. In addition, root and shoot samples should be harvested over time to determine the effects of environment and ensure accurate colonisation of the roots by both the AM fungi and bacterial isolates

The AM fungal colonisation percentage of the raspberry roots had a significant increase compared to the control and AM fungal inoculated treatments. The treatments which significantly increased AM fungal colonisation % compared to the AM fungal inoculation was AM+ SEN4, AM+ SERB2, AM+ AERA4.1, AM+ AIRA4B and AM+ AIRC5. The most significant increase in AM fungal colonisation percentage was the dual inoculation with SERB2. These isolates could then be considered to be potential mycorrhizal helper bacteria. Isolates SERB2 and AERA4.1, both identified as *Bacillus spp.* showed little increase in shoot or root dry weights in this study, however were shown to be potential biocontrol isolates of *Fusarium oxysporum* and *Phythophthora nicotianae* (Sections 2.3.5 and 3.3.5). It has been said that although MHB may have the potential to be PGPR and PGPR may have the potential to be MHB, the two are not mutually exclusive (Garbaye, 1994). The extent of the mycorrhizal colonisation depends on various factors, abiotic and biotic environmental interactions, fungal physiology and susceptibility of the root to infection. MHB are able to promote mycorrhizal infection rate at various stages of the tripartite interaction (Tarkka and Frey-Klett, 2008).

It has been suggested that MHB promote mycorrhizal colonisation through five mechanisms: Bacteria in the soil can trigger or accelerate the germination process of spores or any other dormant propagules; the metabolic activity of the bacteria in the rhizosphere modifies the physiochemical properties of the soil in a way that facilitate mycorrhizal infection; enhancing mycelial growth and colonization of the surface of the long roots and subsequently come into contact with the infection-receptive short roots. The bacteria in the soil are involved with the plant root-mycorrhizal fungus recognition mechanisms and bacteria present in soil prior to mycorrhizal development may improve the roots' receptivity to the formation of mycorrhizae (Garbaye, 1994, Frey-Klett *et al.*, 2007, Tarkka and Frey-Klett, 2008).

Isolate SERB2 was identified as a strain of *Bacillus mycoides.* A study by von Alten *et al.,* (1993) showed significant increase in AM fungal colonisation percentage of a variety of host plants by strains of *B. mycoides*, showing non host specificity by the AM fungi. Xie *et al.,* (1995) showed that MHB such as *Bradyrhizobium japonicum* stimulated AM colonization and direct hyphal growth towards the root by enhancing the production of stimulatory signals or by inducing changes in the host plants' flavonoid spectrum. Increased AM fungal (*Glomus fasciculatum, Glomus mosseae,* and *Glomus caledonium*) colonisation by *B.coagulans* indicates the bacterium that is a mycorrhizal helper bacterium. It has been suggested that MHB produce hydrolytic enzymes which cause the cortical cells to dilate, providing a larger intercellular surface area with which the AM fungi can penetrate and colonise more easily, increasing the percentage of AM fungi present in the plant, thereby providing more nutrients to the plant (Mamatha *et al.*, 2002).

A study by Gamalero *et al.*, (2004) found that *Pseudomonas fluorescens* 92rk increased *G.mosseae* mycorrhizal colonisation by 41% on tomato (*Lycopersicon esculentum* Mill.). AM fungal (*G.mosseae*) colonisation was increased by 7-fold by a *Pseudomonas* isolate and 6-7 fold by *Stenotrophomonas* and *Arthrobacter* isolates in potatoes (*Solanum tuberosim)* (Bharadwaj *et al.,* 2008). A study by Budi *et al.,* (1999) showed a species of *Paenibacillus* was able to promote *G.mosseae* colonisation of Sorghum.

Pivato *et al.,* (2008) showed a *Pseudomonas* isolate promoted mycorrhization by *G.mosseae* in barrel medic and tomato plants. *Pseudomonas monteilli* significantly improved mycorrhizal colonisation of *G.intraradices* by 79% in Acacia plants (Duponnois and Plendette, 2003). Another study by Toro *et al.,* (1997) showed increased colonisation of *G.intraradices* by *Bacillus subtilis.*

4.5 Conclusion and Summary

Bacterial isolates from South African and Argentinean origin were evaluated for their plant growth promoting and AM fungal colonisation enhancement abilities of raspberry (*Rubus idaeus* cv. Meeker). Significant increases in shoot and root dry weight was found in the treatments, with an isolate from both South Africa and Argentina having the highest increase in biomass. Variations among the other isolates were found, some with lower increases, which however showed potential to promote growth through indirect mechanisms such as fungal pathogen inhibition. The root colonisation percentage by the AM fungi was significantly increased by the bacterial isolates, with one isolate of *Bacillus mycoides* having the highest percentage. The isolates in this study can then either be considered to be plant growth promoting rhizobacteria or mycorrhizal helper bacteria, capable of promoting plant growth and colonisation of raspberry.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

5.1 General Discussion

5.1.1 Estimation of Root and Soil Bacterial Populations

Bacteria are not uniformly distributed in the soil and are generally known to surround the roots of plants. The numbers and diversity of bacteria isolated from the rhizospheric soil is therefore generally higher compared to the bulk soil (Heritage *et al.,* 1999). This is due to the production of beneficial compounds such as amino acids and carbohydrates and other exudates secreted by plant roots which promote and maintain bacterial growth (Bianciotto and Bonfante, 2002). Different environmental conditions such as pH, organic matter such as leaf litter, and cations such as Ca, Mg, K, and Na may influence the soil environment (Garbeva *et al.,* 2004). The type of soil, irrigation, land use history and management practices (for example, input of fertilizers and herbicides) may create unique environments that may change the soil microbial community populations (Johansson *et al.*, 2004). The presence of other microbes associated in the rhizospheric soil could provide otherwise unavailable nutrients in the soil to the rhizospheric bacterial communities. These microbes could be mycorrhizal fungi or bacteria capable of solubilising phosphate, providing nitrogen or producing. Some microbes may produce antagonistic substances which may inhibit growth of certain bacteria. These factors can all influence the soil properties and bacterial communities (Gray and Williams, 1971).

The total number of culturable bacteria determined from the rhizospheric soil and bulk soil may vary depending upon the effect these biotic and abiotic factors impose upon the soil and root environments, which was shown in this study. CFU may not be a reliable estimation of the total culturable bacteria in the soil as it only provides a temporary glimpse in time of the bacteria present in the soil at a given time under certain conditions. The CFU was greater in the rhizospheric soil of the South African samples whereas the CFU was greater in the bulk soil of the Argentinean samples. This can be accounted for by the differences between the two countries, such as soil practices utilised by the farmers, seasonal, environmental factors and host plant species may all influence the bacterial communities present at the time of sampling. However these numbers only represent bacterial communities which are able to be cultured on artificial media and does not take into account the bacteria which are unculturable due to the specific environments they occupy not been replicable on artificial media.

These communities would therefore need to be studied through techniques such as PCR and DGGE which utilise the 16s rRNA structures to determine different species composition of the rhizospheric and bulk soil (Heritage *et al.,* 1999, Madigan and Martinko, 2006). To give an accurate estimation of bacterial communities present in the rhizospheric and bulk soil, one would need to incorporate both culturable and unculturable estimation techniques, and perform soil nutrient and environmental analysis over time to determine the factors influencing the bacterial communities in the soil.

5.1.2. AM Fungal Colonisation and Spore Counts

AM fungi occur naturally in the soil of approximately 80% of terrestrial plant species (Smith and Read, 2008). Their presence and quantity is usually determined through techniques such as spore density counts, percentage colonisation studies which evaluate the presence of propagules such as arbuscules, vesicles, hyphae and spores in the cortical cells of plant roots colonised by the AM fungi (Smith and Dickson, 1997). In order for successful colonisation of a host plant the AM fungal spores present in the soil are released from dormancy due to environmental conditions such as pH, temperature, moisture, CO_2 and organic nutrients. The spores germinate and hyphal branches produced recognise the plant root surface by the secretion of exudates such as organic acids, amino acids, carbohydrate monomers, phenolics, or volatiles by the plant root. This recognition of a host plant root triggers hyphal elongation, penetration and subsequent colonisation of the plant root which results in the production and release of spores (Jones *et al.*, 2004; Smith and Read, 2008). The percentage colonisation of a certain host plant depends on the specificity of the AM fungal species and also on environmental conditions such as climate. AM fungal spore production and release is seasonly dependant (Kabir *et al.*, 1997; Anderson *et al.*, 1983). The roots were stained with trypan blue and the percentage colonisation determined by the presence of structures such as spores, vesicles, and arbuscules. The size of the spores present in the cortical cells of the roots was similar in size to the vesicles, however factors such as the spherical morphology of the spores and presence of subtending hyphae made the spore distinguishable from the vesicles as vesicles are more flat and rectangular in shape terminating from the intraradical hyphae with the presence of lipid droplets internally (Smith and Read, 2008).

The AM fungal colonisation percentage and spore density of the South African and Argentinean samples were different when comparing isolation from raspberry samples. This was due to the different growth stages of the host plant, which in turn affects AM fungal colonisation, as different growth stages of plants are dependent upon different conditions and compounds for efficient growth which affect AM fungi.

In addition, microbial communities present in the soil during AM fungal colonisation affect AM fungi either positively or negatively. For example some bacteria are termed mycorrhizal helper bacteria which secrete compounds such as auxins which promote either the plant root growth or compounds which aid in the recognition of the AM fungi by the root. Other microbes might secrete antagonistic substances in the soil which inhibit AM fungal germination. Season affect the colonisation percentage of the AM fungi and spore production since moisture affects spore germination phases including hydration, activation, germ tube emergence and hyphal growth processes therefore increased rainfall during the summer period trigger sporulation (Sinegani *et al.,* 2005).

Spore sieving although reliable, gives an underestimation of total AM fungal spore population. Factors such as distinguishing between dead and viable spores, loss of spores smaller than 45 µm, or spores adhering to particles which may be discarded all affect spore density counts (Schenck, 1982). Spore production also varies with respect to AM fungal species composition, dormancy and variability (Smith and Read, 2008). Both countries showed similar AM fungal spore colour morphotypes which was from clear or hyaline to dark brown. These are known to represent a wide range of AM fungal species such as *Glomus, Scutellospora*, *Acaulospora, Entrophospora* and *Gigaspora.* However identification of AM fungi cannot be accurately determined based only on colour morphology of the spores.

5.1.3 AM Fungal Spore-associated Bacterial Isolation

AM fungal spores when produced and released from the extraradical hyphae into the soil come into contact with bacteria present in the root surfaces and rhizospheric bacteria. These bacteria are able to attach to the surfaces of the spores either by producing substances like carbohydrates or biofilms or through structures such as pili, fimbriae and flagella (Bianciotto and Bonfante, 2002).

Bacteria isolated from the surfaces of spores have been shown to include a variety of Gram positive and negative, rod and cocci isolates, which is consistent with the presence of these types of bacteria in the rhizosphere (Bharadwaj *et al.*, 2008). The South African and Argentinean samples both contained Gram positive and negative isolates, with the presence of Gram-positive bacteria been higher. This is probably due to the fact that most Gram-positive bacteria are capable of producing endospores, which are resistant structures and can withstand harsh environmental or culturing conditions (Madigan and Martinko, 2006).

The viability of the spores may also determine the presence and type of bacteria isolated. If spores are dead, the bacteria could be decomposers utilising the organic source of nutrients, whereas if the spores are viable, the bacteria could utilise the spores for nutrients, transport, and in turn may be utilised by the AM fungi for benefits the bacteria may possess such as enzymes which my aid the AM fungi (Artursson *et al.*, 2006). In this study, care was given to isolate only viable spores, which is identifiable through their translucent appearance and presence of lipid droplets which can be seen under a dissecting microscope, to eliminate the possibility of saprophytic bacteria.

The bacteria isolated from the surfaces of AM fungal spores in this study do not represent the bacterial communities which are unculturable on artificial media. The cytoplasm of AM fungi contains many endocellular bacteria. Some of these bacterial cells have been revealed though microscopy to be Gram-negative and rod shaped. They tend to occur singly or in groups, often located inside fungal vacuoles in both spores and hyphae.

The bacteria were described as being related to *Burkholderia* and are known of their unculturability (Bianciotto and Bonfante, 2002; Bonfante and Anca, 2009). However studies have shown their importance through molecular identification by DGGE or through gene analysis to determine the presence of beneficial genes such as phosphate transporter genes important for nutrient uptake and transfer of phosphorous (Bianciotto and Bonfante, 2002).

5.1.4 Plant Growth Promoting Characterisation

Bacterial isolates from both the South African and Argentinean samples showed an ability to solubilise phosphate, produce ammonia, siderophores and hydrolytic enzymes such as cellulase and protease. Some isolates showed antagonistic ability towards *Fusarium oxysporum* and *Phythophthora nicotianae.* Through these abilities, the bacteria are able to promote plant growth by supplying enhanced nutrients otherwise in a form inaccessible to plants such as P, N and iron; and reduce the effects of pathogenic fungi.

5.1.5 Molecular Identification of Bacterial Isolates

The bacterial isolates which showed the greatest plant growth promoting abilities were selected and identified by 16s rRNA. Molecular identification of the bacterial isolates was performed using two different sets of primers. This was necessary as the first set of primers (Fd1 and rP2) was unable to amplify the 16s rRNA of some of the isolates and a second set of universal bacterial primers (Gm5F and R907) (Muyzer *et al.,* 1994) was used which showed success in the amplification of the 16s rRNA, utilising the same reagents and similar cycling conditions. This error could occur as different bacteria have different specificity towards primers depending on the region being amplified by the primer. For instance Weisburg *et al.,* (1991) showed that a different primer to rP2, rD1 was preferred for amplification since it is closer to the 3' end of the sequence.

Many studies on PGPR have revealed bacteria from various genera having the ability to demonstrate plant growth promoting abilities such as the ones in this study (Rodriguez and Fraga, 1999; Siddiqui, 2005; Orhan *et al.*, 2006; Karlidag *et al.*, 2007; Hariprasad and Niranjana, 2009; Madhaiyan *et al.*, 2010; Martinez-Viveros *et al.*, 2010). This study identified bacteria predominantly belonging to the Genera *Acinetobacter, Alcaligenes, Bacillus, Microbacterium,* *Micrococcus, Serratia* and *Staphylococcus*. These isolates showed PGPR abilities such as phosphate solubilisation, ammonia production, siderophore production and hydrolytic enzymes (cellulase and protease).

5.1.6 Plant Growth Promotion and AM fungal colonisation enhancement of raspberry

PGPR play an important role in maintaining the soil environment through their natural ability to provide nutrients otherwise inaccessible to plants. PGPR are able to supply the key macronutrients required by the plants such as nitrogen and phosphorous (Singh and Kapoor, 1998; Bloemberg and Lugtenberg, 2001). The inoculation effects of PGPR such as phosphate solubilisers, nitrogen fixers and siderophore producers, as well as AM fungi have been investigated in this study for their potential to promote plant growth and improve mycorrhizal colonisation.

Bacterial isolates from both South Africa and Argentina were able to enhance the dry shoot weight of raspberry probably as a result of their ability to solubilise phosphate, convert organic nitrogen sources and produce siderophores. Phosphate solubilising bacteria (PSB) are able to solubilise inorganic phosphorous sources of total soil P, thereby increasing availability of the P to the plants, which promote plant growth. Through this action, the soil pH is reduced due to the production of organic acids. This reduction in pH makes other ions like iron become more available for utilisation (Rodriguez and Fraga, 1999). Bacteria capable of producing siderophores are then able to sequester the iron and make it more accessible to plants, thereby promoting their growth since iron is essential for cellular growth and metabolism (Suresh and Bagyaraj, 2002).

The root dry weight of the raspberry plants were significantly increased by treatments with the highest increase by an Argentinean bacterial isolate (AERA1). IAA in plants is the main auxin which controls many important and beneficial physiological processes (Patten and Glick, 2002; Spaepen *et al*., 2007; Shahab *et al*., 2009; Martinez-Viveros *et al*., 2010). IAA produced by PGPR can promote root growth (Spaepen *et al*., 2007). Other auxins produced by AERA1 could have promoted root growth which was not tested in this study such as cytokinins and gibberellins. Cytokinins are phytohormones which promote cell division and enlargement, tissue expansion and promote root hair development (Madhaiyan *et al*., 2010).

Gibberellins enhance the development of plant tissues, particularly stem tissue and promote root elongation and lateral root extension. Production of gibberellins has been reported in PGPR (Vessey, 2003; Martinez-Viveros *et al.*, 2010).

The AM fungal colonisation percentage of the raspberry roots was significantly increased compared to the AM fungal control. Both South African and Argentinean bacterial isolates increased AM fungal colonisation. These isolates could then be considered to be potential mycorrhizal helper bacteria. The extent of the mycorrhizal colonisation depends on various factors, abiotic and biotic environmental interactions, fungal physiology and susceptibility of the root to infection (Garbaye, 1994). MHB are able to promote mycorrhizal infection rate at various stages of the tripartite interaction (Tarkka and Frey-Klett, 2008). Several different mechanisms have been suggested by Garbaye (1994) to which MHB can promote mycorrhizal establishment and colonisation.

Garcia-Garrido *et al.,* (1992) showed that spore extracts from *Glomus* mosseae contain cellulolytic enzymes which are involved in the establishment of the fungus by degrading the host cell wall thus allowing penetration by the fungus. It was difficult for them to confirm the production of cellulases by the AM fungi at the time due to the unculturability of AM fungi axenically outside a host plant. They therefore attributed the cellulase activities detected in the colonised roots of lettuce and onion to the fungus since the activity present inside spores and extraradical hyphae showed the same electrostatic mobility to the activity inside the mycorrhizal root extracts. However, recently Martin (2013) showed research on the genome diversity of mycorrhizal fungi on the functionality of the symbiosis and discovered that the genera *Glomus* had a lack of cellulase production. It is interesting to note that in this study, a number of bacteria isolated from in particular the internal surfaces of AM fungal spores from both South Africa and Argentina were capable of producing cellulases. Therefore the cellulases produced by these endobacteria could be utilised by the AM fungi to penetrate the cell wall of the host cell and aid in establishment. The production of cellulases by endobacteria could have accounted for the cellulase production detected in the spore extracts discovered by Garcia-Garrido *et al.,* (1992) and not by the AM fungi.

In this study, it was shown that the internal bacteria were capable of enhancing the AM fungal colonisation of raspberry roots, in particular the Argentinean internal isolate (AIRA4B – *Micrococcus luteus*) had the highest colonisation percentage by the internal isolates and showed a cellulase production of 26 mm in solid media (Section 3.3.5) and showed a similar colonisation percentage to isolate SERB2 (*Bacillus mycoides*), a South African isolate from the external surface of AM fungal spores which showed the highest overall colonisation percentage and showed a cellulase production of 35 mm. Therefore these PGPR both external and in particular internal play a vital role in the AM fungal establishment and symbiosis and should perhaps be examined in more depth.

5.2 Summary and Conclusion

This study successfully isolated bacteria from the external and internal surfaces of AM fungal spores from various samples such as natural indigenous forest, raspberry and strawberry from South Africa and raspberry from Argentina. The bacterial isolates were successfully characterised according to plant growth promoting abilities such as phosphate solubilisation, ammonia production, siderophore and hydrolytic enzyme (cellulase and protease) production. The isolates also showed an ability to inhibit fungal pathogens such as *Fusarium oxysporum* and *Phythophthora nicotianae*. These characteristics are important for promoting plant growth through providing nutrients and other resources necessary for growth and establishment and for controlling plant pathogens which inhibit plant growth. A total of 27 South African and 25 Argentinean bacterial isolates were characterised, some with multiple abilities. Through 16s rRNA molecular analysis 18 of the isolates were identified. The identitities correlated with other studies published by numerous authors, all reporting similar PGPR characterisation results to the different genera of bacteria isolated in this study such as *Acinetobacter, Alcaligenes, Bacillus, Microbacterium, Micrococcus, Serratia* and *Staphylococcus*. These isolates can then be said to be plant growth promoting rhizobacteria.

Bacterial isolates from South African and Argentinean origin were evaluated for their plant growth promoting and AM fungal colonisation enhancement abilities of raspberry (*Rubus idaeus* cv. Meeker). Significant increases in shoot and root dry weight was found in the treatments, with an isolate from both South Africa and Argentina having the highest increase in biomass. Variations among the other isolates were found, some with less of an effect, these isolates showed capable of other PGPR abilities such as fungal pathogen inhibition. The root colonisation percentage by the AM fungi was significantly increased by the bacterial isolates, with one isolate of *Bacillus mycoides* having the highest percentage. The isolates in this study can then either be considered to be plant growth promoting rhizobacteria or mycorrhizal helper bacteria, capable of promoting plant growth and colonisation of raspberry.

This study therefore met its objectives in isolating AM fungi from various soil sources from two different countries, extracting AM fungal spore-associated bacteria, characterisation of the bacterial isolates according to plant growth promoting abilities, identification of the bacterial isolates and studying *in vivo* their ability to promote plant growth and enhance mycorrhizal colonisation of raspberry.

5.3 Recommendations and Future Work

This study successfully isolated plant-growth promoting rhizobacteria from the surfaces of AM fungal spores and was shown to enhance shoot and root dry weights of raspberry and enhanced AM fungal colonisation. However, some difficulties were encountered in the study with regards to isolation, characterisation, and pot trial analysis. In the isolation and analysis of root and soil culturable bacterial communities should include soil nutrient analysis such as phosphorous and nitrogen; and environmental analysis such as temperature, pH, salinity, moisture. This would give a better indication as to the effect these factors would have on the bacterial communities. The extraction of AM fungal spores and root colonisation should be performed at different times of the year to evaluate the effect of seasonality on AM fungi and their lifecycle. In addition the effect of biotic and abiotic factors on native AM fungal species could be determined through soil nutrient and environmental analysis; and the effect of associated bacteria.

The characterisation of bacterial isolates for plant growth promoting abilities could be enhanced. For instance the use of Salkowski's Reagent instead of Kovak's for the determination of IAA has been shown to be successful by many authors and perhaps both should be tried to determine the efficiency of each. To quantify IAA production, once red colour develops, the absorbance can be measured at 535nm and IAA produced calculated using a standard curve produced with known concentrations of pure IAA (Suresh *et al.*, 2010).

This is important since IAA is essential for root elongation processes; therefore if IAA production is accurately determined by the bacterial isolates, they could be potentially applied for further plant growth promoting studies, both in future pot and field trials.

The phytase production test could be enhanced by perhaps increasing the incubation temperature and length, or utilising liquid media to determine production instead of solid media, and pre-incubating the isolates in a solution containing calcium phytate and then measuring the production spectrophotometrically at 700 nm could be performed (Hariprasad and Niranjana, 2009). If phytase production was accurately determined, then this would give an indication of the use of organic phosphorous sources by the isolates. This could be included in future studies in cases where isolates are unable to solubilise inorganic phosphate sources, therefore could potentially still be able to supply phosphorous to the plant through the application of organic P sources. Chitinase production was also inconclusive and could be improved by making a powder form out of chitin flakes, or by using a liquid media, since growth occurred on plates with yeast extract but did not grow without the yeast extract but neither produced any clearing halos which would have indicated chitinase production. Chitinase production is an important enzyme utilised in the inhibition of fungal pathogens, such as those belonging to the Ascomyocta family; whose cell walls are primarily composed of chitin. These isolates which if shown to produce chitinase could then be potentially applied in industry as biocontrol agents against pathogens such as *Fusarium spp.* for example.

The pot trial analysis although successful in showing growth enhancement of raspberry by the PGPR and mycorrhizal colonisation enhancement by MHB encountered problems with some of the replicate plants experiencing leaf chlorosis and reduced shoot elongation as a result of utilising micropropagated plantlets. In future studies, the micropropagated plantlets should be correctly hardened before transplant to the pots for the trials.

This can be achieved by incubating the plantlets at high humidity for two to three weeks and slowly exposing them to increased light intensity (Jha and Ghosh, 2005). Those that survive the hardening process can then be utilised in the pot trials as this process should eliminate the leaf chlorosis process which was shown to be successful in increasing the survival percentage of explants in the *Rubus* genus (Wu *et al.,* 2009).

In addition, the variability shown by the isolates in the plant growth parameters should be further investigated. This could be determined by estimating the CFU of the root, rhizospheric and bulk soil after inoculation of the plants and at harvest. This could aid in establishing the efficiency in survival and colonisation ability of the isolates utilised. The growing media could be improved from the compost/vermiculite mix used in this study. The pH of the growing mix should also be measured to determine if the soil was too acidic or basic to sustain the growth of the isolates or to determine if the isolates produced compounds such as organic acids which lead to the solubilisation of phosphate and promote the availability of other essential nutrients such as iron, which could then be taken up by the bacteria and utilised by the plant for growth.

The bacterial isolates which showed the most plant growth promotion and have been identified could be utilised to study mycorrhizal niches which harbour the bacterial isolates. This can be carried out by first transforming the bacterial isolates with a plasmid containing the gene which codes for the green fluorescent protein. Once the bacterial cells are competent, their suspensions can be used to inoculate a suitable host plants' seeds, and together grown in seedling trays. After 3-4 weeks, the plants should be removed and the roots cut into small sections, and placed on microscope slides and the bacterial colonisation of the mycorrhizal niches observed under confocal microscopy (Hassen and Labushachagne, 2010). Unculturable bacterial isolates associated with AM fungal spores could be identified by performing the DNA extraction and PCR amplification procedures as for the culturable bacteria in this study. The PCR product would then undergo pyrosequencing (Qiagen, 2010) and the sequences identified by BLAST and Genbank.

All these improvements would give more of an indication as to the mechanisms occurring at the soil, root, bacterial and mycorrhizal interfaces in order to improve plant growth of various crop plants, which have benefits in the agricultural industry.

REFERENCES

Ahmad, F., Ahmad, I., and Khan, M. (2008). Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiological Research,* **163** , 173-181.

Akhtar, M., and Siddiqui, Z. (2009). Effects of phosphate solubilizing microorganisms and *Rhizobium* sp. on the growth, nodulation, yield and root-rot disease complex of chickpea under field condition. *African Journal of Biotechnology,* **8** (15) , 3489-3496.

Akhtar, M., and Siddiqui, Z. (2010). Role of Plant Growth Promoting Rhizobacteria in Biocontrol of Plant Diseases and Sustainable Agriculture. In Maheshwari, D; *Plant Growth and Health Promoting Bacteria* (pp. 157-194). Berlin, Heidelberg: Springer-Verlag.

Akkopru, A., and Demir, S. (2005). Biological Control of Fusarium Wilt in Tomato Caused by *Fusarium oxysporum f. sp. lycopersici* by AMF *Glomus intraradices* and some Rhizobacteria. *Journal of Phytopathology,* **153** , 544-550.

Alexander, D., and Zuberer, D. (1991). Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bateria. *Biology and Fertility of Soils,* **12** , 39-45.

Ali, B., Sabri, A., and Hasnain, S. (2010). Rhizobacterial potential to alter auxin content and growth of *Vigna radiata* (L.). *World Journal of Microbiology and Biotechnology* **26** , 1379–1384.

Amerian, M.R and Stewart, W.S. (2001). Effect of 2 species of arbuscular mycorrhizal fungi on growth assimilation and leaf water relations in maize (*Zea mays*). *Aspects of Applied Biology.* **63** , 1-6.

Ames, B. (1989). Mycorrhiza development in onion in response to chitin-decomposing actinomycetes. *New Phytologist,* **112** , 423-427.

Anderson, A. (1988). Mycorrhize- Host Specificity and recognition. *Bulletin of the American Phytopathological Society* **78** , 375-378.

Anderson, R., Liberta, A., Dickman, L., and Katz, A. (1983). Spatial variation in vesicular arbuscular mycorrhiza spore density. *Bulletin of the Torrey Botanical Club.* **10** , 519-525.

Antoun, H., and Prevost, D. (2005). Ecology of Plant Growth Promoting Rhizobacteria. In Z. Siddiqui, *PGPR: Biocontrol and Biofertilization* (pp. 1-38). Netherlands: Springer.

Artursson, V., Finlay, R., and Jansson, J. (2006). Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. *Environmental Microbiology* **8** (1) , 1-10.

Asrar, A.-W. A., and Elhindi, K. (2011). Alleviation of drought stress of marigold (*Tagetes erecta*) plants by using arbuscular mycorrhizal fungi. *Saudi Journal of Biological Sciences* **18** , 93-98.

Atkinson, D., Baddeley, J., Goicoechea, N., Green, J., Sanchez-Diaz, M., and Watson, C. (2002). Arbuscular Mycorrhizal Fungi on low input agriculture. In S. Gianinazzi, H. Shuepp, J. Barea, and K. Haseleandter, *Mycorrhizal Technology in Agriculture* (pp. 211-233). Switzerland: Birkhauser Verlag.

Audet, P., and Charest, C. (2006). Effects of AM colonization on "wild tobacco" plants grown in zinc contaminated soil. *Mycorrhiza,* **16** , 277-283.

Augé, R. (2001). Water relations, drought and vesicular arbuscular mycorrhizal symbiosis. *Mycorrhiza* **11** , 3-42.

Azcon-Aguilar, C., and Barea, J. (1996). Arbuscular mycorrhizas and biological control of soil-borne plant pathogens - an overview of the mechanisms involved. *Mycorrhiza,* **6** , 457-464.

Bago, B., and Bécard, G. (2002). Bases of biotrophy of arbuscular mycorrhizal fungi. In S. Gianinazzi, H. Scüepp, J. Barea, and K. Haselwandter, *Mycorrhizal Technology in Agriculture: From Genes to Bioproducts* (pp. 33-48). Basel: Birkhäuser Verlag.

Banchio, E., Bogino, P., Zygadlo, J., and Giordano, W. (2008). Plant growth promoting rhizobacteria improve growth and essential oil yield in Origanum majorana L. *Biochemical Systematics and Ecology,* **36** , 766-771.

Barea, J., Azcon, R., and Azcon-Aguilar, C. (2004). Mycorrhizal Fungi and Plant Growth Promoting Rhizobacteria. In A. Varma, L. Abbott, D. Werner, and R. Hampp, *Plant Surface Microbiology* (pp. 351-371). Heidelberg, Berlin: Springer-Verlag.

Barea, J., Pozo, M., Azcon, R., and Azcon-Aguilar, C. (2005). Microbial co-operation in rhizosphere. *Journal of Experimental Botany,* **56** , 1761-1778.

Barker, J., Tagu, D., and Delp, G. (1998). Regulation of root and fungal morphogenesis in mycorrhizal symbioses. *Plant Physiology* **116** , 1201-1207.

Barriuso, J., Pereyra, M., Lucas Garcıa, J., Megıas, M., Gutierrez Man˜ero, F., and Ramos, B. (2005). Screening for Putative PGPR to Improve Establishment of the Symbiosis *Lactarius deliciosus-Pinus* sp. *Microbial Ecology,* **50** , 82-89.

Baudoin, E., Benizri, E., and Guckert, A. (2003). Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere. *Soil Biology and Biochemistry* **35** , 1183–1192.

Bharadwaj, D., Lundquist, P.-O., and Alstrom, S. (2008). Arbuscular mycorhhizal fungal spore-associated bacteria affect mycorrhizal colonization, plant growth and potato pathogens. *Soil Biology and Biochemistry* **40** , 2494-2501.

Bianciotto, V., and Bonfante, P. (2002). Arbuscular mycorrhizal fungi: a specialised niche for rhizospheric and endocellular bacteria. *Antonie van Leeuwenhoek,* **81** , 365-371.

Bloemberg, G., and Lugtenberg, B. (2001). Molecular basis of plant growth promotion and biocontrol by rhizobacteria. *Current opinions in plant biology,* **4 ,** 343-350.

Bonfante, P., and Anca, I.-A. (2009). Plants, Mycorrhizal fungi, and Bacteria: A network of Interactions. *Annual Review of Microbiology,* **63** , 363-383.

Bonfante, P., and Genre, A. (2010). Mechanisms underlying beneficial plant-fungus interactions in mycorrhizal symbiosis. *Nature Communications,* **1** *(48)* , 1-11.

Boomsma, C., and Vyn, T. (2008). Maize drought tolerance: Potential improvements through arbuscular mycorrhizal symbiosis? *Field Crops Research* **108** , 14-31.

Bowen, G., and Theodorou, C. (1979). Interactions between bacteria and ectomycorrhizal fungi. *Soil Biology and Biochemistry* **11** , 119-126.

Brundrett, M. (2002). Co-evolution of roots and mycorrhizas of land plants. *New Phytologist,* **154** , 275-304.

Brundrett, M. (2004). Diversity and classification of mycorrhizal associations. *Biological Review,* **79** , 473-495.

Bucher, M. (2007). Functional biology of plant phosphate uptake at root and mycorrhiza interfaces. *New Phytologist,* **173** , 11–26.

Budi, S., Van Tuinen, D., Martinotti, G., and Gianinazzi, S. (1999). Isolation from the Sorghum bicolor Mycorrhizosphere of a bacterium compatible with Arbuscular Mycorrhiza Development and Antagonistic towards Soilborne Fungal Pathogens. *Applied and Environmental Microbiology,* **65** (11) , 5148-5150.

Burges, A. (1967). *Micro-organisms in the soil.* London: Hutchinson and Co Ltd. .

Calvo, P., Ormeno, O. E., Martinez-Romero, E., and Zuniga, D. (2010). Characterisation of *Bacillus* isolates of potato rhizosphere from Andean soils of Peru and their potential PGPR characteristics. *Brazilian Journal of Microbiology,* **41** , 899-906.

Campbell, R. B. (1979). Microbiology of Soil, Air and Water. In L. Hawker, and A. Linton, *Micro-organsims: Form, Function and Environment* (pp. 243-257). London: Edward Arnold Ltd.

Canbolat, M., Bilen, S., Cakmakci, R, Sahin, F., and Aydin, A. (2006). Effect of plant growth-promoting bacteria and soil compaction on barley seedling growth, nutrient uptake, soil properties and rhizosphere microflora. *Biology and Fertility of Soils,* **42** , 350-357.

Cappuccino, J., and Sherman, N. (2008). *Microbiology:A Laboratory Manual, 9th Edition.* San Francicsco: Benjamin Cummings Publishing Company.

Chaiharn, M., Chunhaleuchanon, S., Kozo, A., and Lumyong, S. (2008). Screening of Rhizobacteria for their plant growth promoting activities. *KMITL Science and Technology Journal,* **8**(1) , 18-23.

Chiarini, L., Bevivino, A., Dalmastri, C., Nacamulli, C., and Tabacchioni, S. (1998). Influence of plant development, cultivar and soil type on microbial colonization of maize roots. *Applied Soil Ecology.* **8** , 11-18.

Chibuogwu, O., and Nmesoma, E. (2011). Batch Culture Studies of Phosphate Solubilisation by Micrococcus sp. PSB 7 isolated from Rhizospheric Soil. *American-Eurasian Journal of Agricultural and Environmental Science,* **10**(4) , 667-674.

Choudhary, D., Sharma, K., and Gaur, R. (2011). Biotechnological perspectives of microbes in agro-ecosystems. *Biotechnology Letters* **33** , 1905-1910.

Compant, S., Duffy, B., Nowak, J., Clement, C., and Barka, E. (2005). Use of Plant Growth-Promoting Bacteria for Biocontrol of Plant Diseases: Principles, Mechanisms of Action and Future Prospects. *Applied and Enivronmental Microbiology* **71**(9) , 4951-4959 .

Cordier, C., Lemoine, M., Lemanceau, P., Gianinazzi-Pearson, V., and Gianinazzi, S. (2000). The beneficial rhizosphere: a necessary strategy for microplant production. *Acta Horticulturae.* **530** , 259–268.

Cruz, A., and Ishii, T. (2012). Arbuscular mycorrhizal fungal spores host bacteria that affect nutrient biodynamics and biocontrol of soil borne plant pathogens. *Biology Open,* **1**, 52-57.

Dames, J.F., and Ridsdale, C.J. (2012). What we know about arbuscular mycorhizal fungi and associated soil bacteria. *African Journal of Biotechnology* **11** (73) , 13753-13760.

Davies, F., Potter, J., and Linderman, R. (1993). Drought resistance of mycorrhizal pepper plants independent of leaf P concentration- response in gas exchange and water relations. *Physologia Plantarum.* **87** , 45-53.

den Hartigh, W. (2011). *SA's burgeoning berry industry*. Retrieved October 5, 2012, from Media Club South Africa: http://www.mediaclubsouthafrica.com

Dezfulian, A., Salehian, M., Amini, V., Dabiri, H., Azimirad, M., Aslani, M., Zali, MR and Fazel, I (2010). Catalase-negative Staphylococcus aureus isolated from a diabetic foot ulcer. *Iranian Journal of Microbiology,* **2** (3) , 165-167.

Dighton, J. (2009). Mycorrhizae. *Mutualism and Commensalism*, 153-162.

Dobbelaere, S., Croonenborghs, A., Thys, A., Vande Broek, A., and Vanderleyden, J. (1999). Phytostimulatory effect of *Azospirillum brasilense* wild type and mutant strains altered in IAA production on wheat. *Plant and Soil,* **212** , 155-164.

Dodd, J. C., Dougall, T. A., Clapp, J. P., and Jeffries, P. (2002). The role of arbuscular mycorrhizal fungi in plant community establishment at Samphire Hoe, Kent, UK. *Biodiversity Conservation.* **11** , 39–58.

Douds, J. D., Nagahashi, G., Shenk, J., and Demchak, K. (2008). Inoculation of Strawberries with AM Fungi Produced On-Farm Increased Yield. *Biological Agriculture and Horticulture* **26** , 209-219.

Duponnois, R. (1992). Les bacteries auxiliaries de la mycorrhization du Douglas (*Pseudotsuga menziesii* (Mirb.) Franco) par *Laccaria laccata* souche S238. These de l'Universite de Nancy I, France.

Duponnois, R., and Garbaye, J. (1990). Some mechanisms involved in growth stimulation of ectomycorrhizal fungi by bacteria. *Canadian Journal of of Botany,* **68** , 2148-2152.

Duponnois, R., and Plenchette, C. (2003). A mycorrhiza helper bacterium enhances ectomycorrhizal and endomycorrhizal symbiosis of Australian Acacia species. *Mycorrhiza* **13** , 85–91.

El-Azeem, A. S., Mehana, T., and Shabayek, A. (2007). Some plant growth promoting traits of rhizobacteria isolated from Suez Canal region, Egypt. *African Crop Science Conference Proceedings* **8** , 1517-1525.

Erasmus, D. (2012). *Berries to the Force*. Retrieved October 5, 2012, from Farmer's Weekly: http://www.farmersweekly.co.za/article.aspx?id=23439andh=Berries-to-the-fore

Escudero, V., and Mendoza, R. (2005). Seasonal variation of arbuscular mycorrhizal fungi in temperate grasslands along a wide hydrologic gradient. *Mycorrhiza* **15** , 291–299.

Esitken, A., Pirlak, L., Turan, M., and Sahin, F. (2006). Effects of floral and foliar application of plant growth promoting rhizobacteria on the yield, growth and nutrition of sweet cherry . *Scientia Horticulturae,* **110** , 324-327.

Evelin, H., Kapoor, R., and Giri, B. (2009). Arbuscular mycorrhizal fungi in alleviation of salt stress: a review. *Annals of Botany* **104** , 1263–1280.

Fernandez, L., Zalba, P., Gomez, M., and Sagardoy, M. (2007). Phosphate-solubilization activity of bacterial strains in soil and their effects on soybean growth under greenhouse conditions. *Biological Fertilised Soils,* **43** , 805-8009.

Finlay, R. (2004). Mycorrhizal Fungi and thier multifunctional roles. *Mycologist* **18**(2) , 91-96.

Franco-Correa, M., Quintana, A., Duque, C., Suarez, C, Rodriguez, M., and Barea, J-M. (2010). Evaluation of actinomycete strains for key traits related with plant growth promotion and mycorrhiza helping activities. *Applied Soil Ecology,* **45** , 209-217.

Frey-Klett, P., Garbaye, J., and Tarkka, M. (2007). The mycorrhiza helper bacteria revisited. *New Phytologist,* **176** , 22-36.

Gamalero, E., Berta, G. M., Glick, B., and Lingua, G. (2008). Synergistic interactions between the ACC deaminase-producing bacterium *Pseudomonas putida* UW4 and the AM fungus *Glomus rosea* positively affect cucumber growth. *FEMS Microbiological Ecology,* **64** , 459-467.

Gamalero, E., Fracchia, L. C., Garbaye, J., and Frey-Klett, P. V. (2003). Characterisation of functional traits of two fluorescent pseudomonads isolated from basidiomes of ectomycorrhizal fungi. *Soil Biology and Biochemistry,* **35** , 55-65.

Gamalero, E., Lingua, G., Berta, G., and Glick, B. (2009). Beneficial role of plant growth promoting bacteria and arbuscular mycorrhizal fungi on plant responses to heavy metal stress. *Canadian Journal of Microbiology,* **55 ,** 501-514.

Gamalero, E., Trotta, A., Massa, N., Copetta, A., Giovanna Martinotti, M., and Berta, G. (2004). Impact of two fluorescent pseudomonads and an arbuscular mycorrhizal fungus on tomato plant growth, root architecture and P acquisition. *Mycorrhiza,* **14** , 185-192.

Garbaye, J. (1994). Helper Bacteria: A new dimension to the mycorrhizal symbiosis. *New Phytologist,* **128** , 197-210.

Garbaye, J., and Bowen, G. (1989). Stimulation of ectomycorrhizal infection of Pinus radiata by some microorganisms associated with the mantle of ectomycorrhizas. *New Phytologist* **112** , 383-388.

Garbeva, P., van Veen, J., and van Elsas, J. (2004). Microbial diversity in soil: Selection microbial populations by plant and soil type and implications for disease suppressiveness. *Annual Review of Phytopathology.* **42** , 243-270.

Garcia-Garrido, J., Garcia-Romera, I., and Ocampo, J. (1992). Cellulase production by the vesicular-arbuscular mycorrhizal fungus Glomus mosseae (Nicol. and Gerd.) Gerd. and Trappe. *New Phytologist,* **121** (2) , 221-226.

Giovannetti, M. (2000). Spore Germination and Pre-Symbiotic Mycelial Growth. In Y. Kapulnik, and D. Douds Jr, *Arbuscular Mycorrhizas: Physiology and Function* (pp. 47-68). Netherlands: Kluwer Academic publishers.

Giovannetti, M., and Sbrana, C. (1998). Meeting a non-host: the behaviour of AM fungi. *Mycorrhiza* **8 ,** 123-130.

Gonzalez-Chavez, M., Carrillo-Gonzalez, R., Wright, S., and Nichols, K. (2004). The role of glomalin, a protein produced by arbuscular mycorrhizal fungi, in sequestering potentially toxic elements. *Environmental Pollution,* **130** , 317–323.

Gopalakrishnan, S., Humayun, P., Kiran, B., Kannan, I., Vidya, M., Deepthi, K., and Rupela, O (2010). Evaluation of bacteria isolated from rice rhizosphere for biological control of charcoal rot of sorghum caused by *Macrophomina phaseolina* (Tassi) Goid. *World Journal of Microbiology and Biotechnology online*.

Gordon, S., and Weber, R. (1951). Colorimetric Estimation of indole acetic acid. *Plant Physiology* **26** (1) , 192-195.

Gosling, P., Hodge, A., Goodlass, G., and Bending, G. (2006). Arbuscular mycorrhizal fungi and organic farming. *Agriculture, Ecosystems and Environment* **113** , 17-35.

Graham, J., and Woodhead, M. (2009). Raspberries and Blackberries: The Genomics of Rubus. In K. Folta, and S. Gardiner, *Genetics and Genomics of Rosaceae, Plant Genetics and Genomics: Crops and Models 6.* LLC: Springer Science+Business Media.

Gray, T., and Williams, S. (1971). *Soil Micro-organisms.* Edinburgh: Oliver and Boyd.

Gryndler, M. (2000). Interactions of arbuscular mycorrhizal fungi with other soil organisms. In Y. Kapulnik, and D. Douds Jr, *Arbuscular Mycorrhizas: Physiology and Function* (pp. 239-262). Dordrecht, The Netherlands: Kluwer Academic Publishers.

Guetsky, R., Shtienberg, D., Lad, Y., Fischer, E., and Dinoor, A. (2002). Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. *Phytopathology,* **92** , 976-985.

Hajiboland, R., Aliasgharzadeh, N., Laiegh, S., and Poschenrieder, C. (2010). Colonisation with arbuscular mycorrhizal fungi improves salinity tolerance of tomato (*Solanum lycopersicum* L.) plants. *Plant and Soil,* **331** , 313-327.

Hamel, C. (1996). Prospects and problems pertaining to the management of arbuscular mycorrhizae in agriculture. *Agriculture, Ecosystems and Environment* **60** , 197-210.

Hammad, A., and EI-Mohandes, M. (1999). Controlling fusarium wilt disease of cucumber plants via antagonistic microorganisms in free and immobilized states. *Microbiological Research* **154** , 113-117.

Hariprasad, P., and Niranjana, S. (2009). Isolation and characterisation of phosphate-solubilising rhizobacteria to improve plant health of tomato. *Plant and Soil* **316** , 13-24.

Harrier, L., and Watson, C. (2004). The potential role of arbuscular mycorrhizal (AM) fungi in the bioprotection of plants against soil-borne pathogens in organic and/ or other sustainable farming systems. *Pest Management Science,* **60** , 149-157.

Haselwandter, K., Leyval, C., and Sanders, F. (1994). Impact of arbuscular mycorrhizal fungi on plant uptake of heavy metals and radionuclides from soil,. In S. Gianinazzi, and H. Schuepp, *Impact of Arbuscular Mycorrhizas on Sustainable Agriculture and Natural Ecosystems* (pp. 179-189). Basel Switzerland: BirkhaÈuser Verlag.

Hassen, A., and Labushachagne, N. (2010). Root colonisation and growth enhancement in wheat and tomato by rhizobacteria isolated from the rhizoplane of grasses. *World Journal of Microbiology and Biotechnology,* **26** , 1837-1846.

Hause, B., and Fester, T. (2005). Molecular and cell biology of arbuscular mycorrhizal symbiosis. *Planta,* **221** , 184-196.

Hayat, R., Sheirdil, R., Iftikhar-ul-Hassan, M., and Ahmed, I. (2012). Characterization and identification of compost bacteria based on 16S rRNA gene sequencing. *Annals of Microbiology* , 1-8.

Heritage, J., Evans, E., and Killington, R. (1999). *Microbiology in action.* United Kingdom: Cambridge University Press.

Hernandez, J.-P., de-Bashana, L., Rodriguez, D., Rodriguez, Y., and Bashan, Y. (2009). Growth promotion of the freshwater microalga *Chlorella vulgaris* by the nitrogen-fixing, plant growth-promoting bacterium *Bacillus pumilus* from arid zone soils. *European Journal of Soil Biology,* ***45***, 88-93.

Hodgkin, J., Kuwabara, P., and Corneliussen, B. (2000). A novel bacterial pathogen, *Microbacterium nematophilum,* induces morphological change in the nematode *C. elegans*. *Current Biology,* **10** (24), 1615-1618.

Hooker, J., Jaizme-Vega, M., and Atkinson, D. (1994). Biocontrol of plant pathogens using arbuscular mycorrhizal fungi. In S. Gianinazzi, and H. Schuepp, *Impact of Arbuscular Mycorrhizas on Sustainable agriculture and Natural ecosystems* (pp. 191-200). Switzerland: Birkhauser Verlag.

Huddedar, S., Shete, A., Tilekar, J., Gore, S., Dhavale, D., and Chopade, B. (2002). Isolation, characterization and plasmid pUPI126 mediated indole 3 acetic acid (IAA) productions in *Acinetobacter* strains from rhizosphere of wheat. *Applied Biochemistry and Biotechnology,* **102** , 21-29.

Human, J., and Evans, E. (1989). Strawberry production in South Africa. *Acta Horticulturae (ISHS)* **265**, 757.

Idris, A., Labusschagne, N., and Korsten, L. (2009). Efficacy of rhizobacteria for growth promotion in sorghum under greenhouse conditions and selected modes of action studies. *Journal of Agricultural Science,* **147** , 17-30.

Indiragandhi, P. A., Madhaiyan, M., and Sa, T. (2008). Characterization of plant growth-promoting traits of bacteria isolated from larval guts of Diamondback moth *Plutella xylostella* (Lepidoptera: Plutellidae). *Current Microbiology,* **56** , 327-333.

Ingham E, and Wilson. M. (1999). The mycorrhizal colonization of six wetland species at sites differing in land use history. *Mycorrhiza,* **9** , 233–235.

Isaac, S. (1992). *Fungal-plant interactions.* London: Chapman and Hall.

Jeffries, P., Gianinazzi, S., Perotto, S., Turnau, K., and Barea, J. (2003). The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. *Biology and Fertility of Soils,* **37**, 1-16.

Jha, T., and Ghosh, B. (2005). *Plant Tissue Culture: Basic and Applied.* India: Universities Press.

Jianfeng, H., Xiangui, L., Rui, Y., Qian, J., and Yufang, S. (2009). Effects of arbuscular mycorrhizal fungi inoculation on arsenic accumulation by tobacco (*Nicotiana tabacum* L.). *Journal of Environmental Sciences,* **21**, 1214-1220.

Johansson, J., Paul, L., and Finlay, R. (2004). Micorbial interactions in the rhizosphere and their siginficance for sustainable agriculture. *FEMS Microbiology Ecology,* **48**, 1-13.

John, S., Ruggiero, C., Hersman, L., Tung, C.-S., and Neu, M. (2001). Siderphore mediated plutonium accumulation by *Microbacterium flavescens* (JG-9). *Environmental Science and Technology,* **35** (14), 2942-2948.

Johnson, R., and Case, C. (2007). *Laboratory experiments in microbiology.* San Fransisco: Benjamin and Cummings Publishing Co.

Joseph, B., Ranjan Patra, R., and Lawrence, R. (2007). Characterization of plant growth promoting rhizobacteria associated with chickpea (*Cicer arietinum* L.). *International Journal of Plant Production,* **1** (2), 141-152.

Kabir, Z., Halloran, I., Fyles, J., and Hamel, C. (1997). Seasonal changes of arbuscular mycorrhizal fungi as affected by tillage practices and fertilization: Hyphal density and mycorrhizal root colonisation. *Plant and Soil,* **192**, 285-293.

Kang, S.-M., Khan, A., Hamayun, M., Shinwari, Z., Kim, Y.-H., Joo, G.-J., and Lee, I-J. (2012). *Acintobacter calcoaceticus* ameliorated plant growth an influenced gibberellins and functional biochemicals. *Pakistan Journal of Botany,* **44** (1), 365-372.

Karlidag, H., Esitken, A., Turan, M., and Sahin, F. (2007). Effects of root inoculation of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrient element contents of leaves of apples. *Scientia Horticulturae,* **114**, 16-20.

Kasana, R., Salwan, R., Dhar, H., Dutt, S., and Gulati, A. (2008). A Rapid and Easy Method for the Detection of Microbial Cellulases on Agar Plates Using Gram's Iodine. *Current Microbiology,* **57,** 503–507.

Khan, A., Jilani, G., Akhtar, M., Naqvi, S., and Rasheed, M. (2009). Phosphorous solubilizng bacteria: Occurrence, Mechanisms, and their role in crop production. *Journal of Agriculture and Biological Science,* **1**(1), 48-58.

Khan, M., and Zaidi, A. (2006). Influence of composite inoculations of phosphate solubilising organisms and an arbuscular mycorrhizal fungus on yield, grain protein, and phosphorous and nitrogen uptake by greengram. *Archives of Agronomy and Soil Science,* **52**(5), 579-590.

Khan, M., and Zaidi, A. (2007). Synergistic effects of the inoculation with Plant-Growth-Promoting Rhizobacteria and an Arbuscular Mycorrhizal Fungus on the performance of Wheat. *Turkish Journal of Agriculture and Forestry,* **31**, 355-362.

Kohler, J., Caravaca, F., Carrasco, L., and Roldan, A. (2007). Interactions between a plant growth-promoting rhizobacterium, an AM fungus and a phosphate-solubilising fungus in the rhizosphere of *Latuca sativa*. *Applied Soil Ecology,* **35**, 480-487.

Koide, R. (2000). Mycorrhizal symbiosis and plant reproduction. In Y. D. Kapulnik, *Arbuscular Mycorrhizas: Physiology and Function* (pp. 19-46). Dordrecht: Kluwer.

Koide, R., and Schreiner, R. (1994). Regulation of the vesicular-arbuscular mycorrhizal symbiosis. *Annual Review of Plant Physiology and Plant Molecular Biology,* **43**, 557-581.

Koske. R.E., and Gemma J N. (1989). A modified procedure for staining roots to detect VA mycorrhizas. *Mycological Research,* **92**, 486–505.

Kuepper, G., Born, H., and Bachmann, J. (2003). *Organic culture of bramble fruits* . Retrieved from Horticulture production guides: http://www.attra.ncat.org

Kumar, K., Amaresan, N., Bhagat, S., Madhuri, K., and Srivastava, R. (2011). Isolation and characterization of rhizobacteria associated with coastal agricultural ecosystem of rhizosphere soils of cultivated vegetable crops. *World Journal of Microbiology and Biotechnology,* **27**, 1625-1632.

Li, X., George, E., and Marschner, H. (1991). Phosphorus depletion and pH decrease at the root-soil and hyphae-soil interfaces of VA mycorrhizal white clover fertilized with ammonium. *New Phytologist* **119**, 397-404.

Lim, B., Yeung, P., Cheng, C., and Hill, J. (2007). Distribution and diversity of phytate-mineralizing bacteria. *ISME Journal,* **1**, 321-330.

Lind, K., Lafer, G., Schloffer, K., Innerhoffer, G., and Meister, H. (2003). *Organic Fruit Growing* . Wallingford, UK: CABI Publishing.

Linderman, R. (2000). Effects of Mycorrhizas on Plant Tolerance to Diseases. In Y. Kapulnik, and D. Douds Jr, *Arbuscular Mycorrhizas: Physiology and Functionq* (pp. 345-365). Dordrecht, The Netherlands: Kluwer Academic Publishers .

Liu, A. H. (2000). Acquisition of Cu, Zn, Mn and Fe by mycorrhizal maize (*Zea mays* L.) grown in soil at different P and micronutrient levels. *Mycorrhiza* **9**, 331-336.

López-Pedrosa, A., González-Guerrero, M., Valderas, A., Azcón-Aguilar, C., and Ferrol, N. (2006). GintAMT1 encodes a functional high-aYnity ammonium transporter that is expressed in the extraradical mycelium of *Glomus intraradices. Fungal Genetics and Biology,* **43**, 102-110.

Lovelock, C., Andersen, K., and Morton, J. (2003). Arbuscular Mycorrhizal Communities in Tropical Forests Are Affected by Host Tree Species and Environment. *Oecologia,* **135** (2), 268-279.

Ludwig-Müller, J. (2000). Hormonal balance in plants during colonization by mycorrhizal fungi. In Y. Kapulnik, and D. Douds Jr, *Arbuscular Mycorrhizas: Physiology and Function* (pp. 263-285). Netherlands: Kluwer Publishers.

Lugo, M., and Cabello, M. (2002). Native arbuscular mycorrhizal fungi (AMF) from mountain grassland (Cordoba, Argentina) I. Seasonal variation of fungal spore diversity. *Mycologia,* **94** (4), 579-586.

Lugo, M., González-Maza, M., and Cabello, M. (2003). Arbuscular Mycorrhizal Fungi in a Mountain Grassland II: Seasonal Variation of Colonization Studied, along with Its Relation to Grazing and Metabolic Host Type. *Mycologia,* **95** (3), 407-415.

Lutgen, E., Muir-Clairmont, D., Graham, J., and Rillig, M. (2003). Seasonality of arbuscular mycorrhizal hyphae and glomalin in Western Montana grassland. *Plant and Soil,* **257**, 71-83.

Ma, W., Penrose, D., and Glick, B. (2002). Strategies used by *Rhizobia* to lower plant ethylene levels and increase nodulation. *Canadian Journal of Microbiology,* **48**, 947-954.

Made-in-Argentina. BIBLIOGRAPHY (2010). Retrieved October 5, 2012 from Made-in-Argentina: http://www.made-in-argentina.com/eng/food/fruits/berries/related%20topics/argentina%20raspbe rry.htm

Madhaiyan, M., Poonguzhali, Kang, B.-G., Lee, Y.-J., Chung, J.-B., and Sa, T.-M. (2010). Effect of co-inoculation of methylotrophic *Methylobacterium oryzae* with *Azospirillum brasilense* and *Bhurkolderia pyrrocinia* on the growth and nutrient uptake of tomato, red pepper and rice. *Plant and Soil,* **328**, 71-82.

Madigan, M., and Martinko, J. (2006). *Brock Biology of Microorganisms, eleventh edition.* New Jersey: Pearson Prentice Hall.

Malboobi, M., Owlia, P., Behbahani, M., Sarokhani, E., Moradi, S., Yakhchali, B., Deljou, A; and Heravi, K.M (2009). Solubilization of organic and inorganic phosphates by three highly efficient soil bacterial isolates. *World Journal of Microbiology and Biotechnology,* **25**, 1471-1477.

Mamatha, G., Bagyaraj, D., and Jagnath, S. (2002). Inoculation of field-established mulberry and papya with arbuscular mycorrhizal fungi and a mycorrhiza helper bacterium. *Mycorrhiza,* **12**, 313-316.

Manjunath, M., Prasanna, R., Sharma, P., Nain, L., and Singh, R. (2011). Developing PGPR consortia using novel genera *Providencia* and *Alcaligenes* along with cyanobacteria for wheat. *Archives of Agronomy and Soil Science,* **57** (8), 873-887.

Mar Vazquez, M., Cesar, S. A., and Barea, J. (2000). Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (*Azospirillum, Pseudomonas, Trichoderma*) and their effects on microbial population and enzyme activities in the rhizosphere of maize plants. *Applied Soil Ecology,* **15**, 261-272.

Martin, F. (2013). Exploring the genome diversity of mycorrhizal fungi to understand the evolution and functioning of symbiosis. *7th International Conference on Mycorrhiza, "Mycorrhiza for All: An Under-Earth Revolution"*, (Key note lecture: Developmental, Functional and Environmental Genomics of Mycorrhiza). New Delhi, India.

Martinez-Viveros, O., Jorquera, M., Crowley, D., Gajardo, G., and Mora, M. (2010). Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. *Journal of Soil Science and Plant Nutrition,* **10** (3), 293-319.

Matsumura, A., Horii, S., and Ishii, T. (2007). Effects of arbuscular mycorrhizal fungi and intercropping with bahiagrass on growth and anti-oxidative enzyme activity of radish. *The Japanese Society for Horticultural Science,* **76** (3), 224-229.

Mayo, K. D. (1986). Stimulation of germination of spores of *Glomus versiforme* by spore-associated bacteria. *Mycologia,* **78**, 426-431.

McGonigle, T. M. (1990). A new which gives an objective measure of colonisation of roots by vesicular-arbuscular mycorrhizal fungi. *New Phytologist.* **115**, 495-501.

Medina, M., Gagnon, H., Piche, Y., Ocampo, J., Garrido, J., and Vierheilig, H. (2003). Root colonisation by arbuscular mycorrhizal fungi is affected by the salicilic acid content of the plant . *Plant Science,* **164** (6), 993-998.

Mehta, S., and Nautiyal, C. (2001). An efficient Method for Qualitative Screeningof Phosphate-Solubilizing Bacteria. *Current Microbiology,* **43** , 51-56.

Mena-Violante, H., and Olalde-Portugal, V. (2007). Alteration of tomato fruit quality by root inoculation with plant growth-promoting rhizobacteria (PGPR): *Bacillus subtilis* BEB-13bs. *Scientia Horticulturae,* **113**, 103-106.

Mendoza, R Goldmann, V., Rivas, J., Escudero, V, Pagani, E., Collantes, M and Marbán, L. (2002). Poblaciones de hongos micorrízicos arbusculares en relación con propiedades del suelo y planta hospedante en pastizales de Tierra del Fuego. *Ecologia Austral,* **12**, 9-20.

Meyer, J., and Linderman, R. (1986). Response of subterranean clover to dual inoculation with vesicular arbuscular mycorrhizal fungi and a plant growth promoting bacterium. *Soil Biology and Biochemistry,* **18** 185-190.

Milagres, A., Machuca, A., and Napoleao, D. (1999). Detection of siderophore production from several fungi and bacteria by a modification of chrome azurol S (CAS) agar plate assay. *Journal of Microbiological Methods,* **37**, 1-6.

Miransari, M. (2011). Interactions between arbuscular mycorrhizal fungi and soil bacteria. *Applied Microbiology Biotechnology* **89**, 917-930.

Morton, J. (1988). Taxonomy of VA mycorrhizal fungi: classification, nomenclature, and identification. *Mycotaxon,* **32**, 267-324.

Morton, J., and Benny, G. (1990). Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): A new order, Glomales, two new sub-orders, Glomineae and Gigasporineae and two new families, Acaulosporaceae and Gigasporaceae, with emendation of Glomaceae. *Mycotaxon,* **37**, 471-491.

Mosse, B. (1962). The establishment of vesicular-arbuscular mycorrhiza under aseptic conditions. *Journal of General Microbiology,* **27**, 509-520.

Muyzer, G., Teske, A., Wirsen, C., and Jannasch, H. (1995). Phylogenetic relationships of thiomicrospira species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Archives of Microbiology,* **164**, 165-172.

Nagahashi, G. (2000). In vitro and in situ techniques to examine the role of root exudates during AM fungus-host interactions. In Y. K. Douds, *Arbuscular Mycorrhizas: Physiology and Function* (pp. 287-305). Netherlands: Kluwer Academic Publishers,.

Orhan, E., Esitken, A., Ercisli, S., Turan, M., and Sahin, F. (2006). Effects of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrient contents inorganically growing raspberry. *Scientia Horticulturae,* **111**, 38-43.

Ozgonen, H., and Erkilic, A. (2007). Growth enhancement and Phytophthora blight (*Phytophthora capsici* Leonian) control by arbuscular mycorrhizal fungal inoculation in pepper. *Crop protection,* **26**, 1682-1688.

Parani, K., and Saha, B. (2009). Studies on interaction of *Serratia marscecens* strain (SR1) with fungal pathogens. *American-Eurasian Journal of Agriculture and Environmental Science,* **5** (2), 215-218.

Parinske, M. (2008). Arbuscular mycorrhiza: the mother of plant root endosymbioses. *Nature Reviews Microbiology,* **6**, 763-775.

Patten, C., and Glick, R. (2002). Role of *Pseudomonas putida* Indoleacetic acid in Development of the Host Plant Root System. *Applied and Environmental Microbiology* **68** (8), 3795-3801.

Paul, B., Charles, R., and Bhatnagar, T. (1995). Biological control of *Pythium mamillatum* causing damping-off of cucumber seedlings by a soil bacterium, *Bacillus mycoides*. *Microbiological Research,* **150**, 71-75.

Pawlowska, T., and Taylor, J. (2004). Organization of genetic variation in individuals of arbuscular mycorrhizal fungi. *Nature.* **427**, 733-737.

Pivato, B., Offre, P., Marchelli, S., Barbonaglia, B., Mougel, C., Lemanceau, P., and Berta, G. (2008). Bacterial effects on arbuscular mycorrhizal fungi and mycorrhiza development as influenced by the bacteria, fungi, and host plant. *Mycorrhiza,* **19**, 81-90.

Poole, E., G.D, B., Whipps, J., and Read, D. (2001). Bacteria associated with *Pinus sylvestris-Lactarius rufus* ectomycorrhizas and their effects on mycorrhiza formation in vitro. *New Phytologist,* **151** (3), 743-751.

Porras-Soriano, A., Soriano-Martın, M., Porras-Piedra, A., and Azcon, R. (2009). Arbuscular mycorrhizal fungi increased growth, nutrient uptake and tolerance to salinity in olive trees under nursery conditions. *Journal of Plant Physiology* **166**, 1350—1359.

Prasanna Reddy, B., Rani, J., Reddy, M., and Krishna Kumar, K. (2010). Isolation of Siderophore-producing strains of Rhizobacterial fluorescent Pseudomonads and their biocontrol against rice fungal pathogens. *International Journal of Applied Biology and Pharmaceutical Technology,* **I** (1), 133-137.

Prashant, S., Makarand, R., Bhushan, C., and Sudhir, C. (2009). Siderophoregenic *Acinetobacter calcoaceticus* isolated from wheat rhizosphere with strong PGPR activity. *Malaysian Journal of Microbiology,* **5** (1), 6-12.

Prescott, L., Harley, J., and Klein, D. (2005). *Microbiology.* New-York: McGraw-Hil.

Qiagen. (2010). *Pyrosequencing- the synergy of sequencing and quantification.* Retrieved March 21, 2011, from Qiagen: www.qiagen.com

Quilambo, O. (2003). The vesicular-arbuscular mycorrhizal symbiosis. *African Journal of Biotechnology,* **2** (12), 539-546.

Raddadi, N., Cherief, A., Boudabous, A., and Daffonchio, D. (2008). Screening of plant growth promoting traits of *Bacillus thuringiensis*. *Annals of Microbiology,* **58** (1), 47-52.

Richardson, A., Barea, J., McNeill, A., and Prigent-Combaret, C. (2009). Acquisition of phosphorous and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant and Soil,* **321**, 305-339.

Riedlinger, J., Schrey, S., Tarkka, M., Hampp, R., Kapur, M., and Fiedler, H. (2006). Auxofuran, a novel substance stimulating growth of fly agaric, produced by the mycorrhizal helper bacterium *Streptomyces* AcH 505. *Applied Environmental Microbiology,* **72**, 3550-3557.

Rillig, M. (2004). Arbuscular mycorrhizae, glomalin and soil aggregation. *Canadian Journal of Science,* **84**, 355-363.

Rodriguez, H., and Fraga, R. (1999). Phosphate Solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances,* **17**, 319-339.

Sayyed, R., Gangurde, N., Patel, P., Joshi, S., and Chincholkar, S. (2010). Siderophore production by *Alcaligenes faecalis* and its applicaiton for growth promotion in *Arachis hypogaea*. *Indian Journal of Biotechnology,* **9**, 302-307.

Sbrana, C., Agnolucci, M., Bedini, S. L., Toffanin, A., Giovannetti, M., and Nuti, M. (2002). Diversity of culturable bacterial populations associated to *Tuber borchii* ectomycorrhizas and their activity on *T.borchii* mycelial growth. *FEMS Microbiology Letters,* **211**, 195-201.

Schachtman, D. R. (1998). Phosphorus uptake by plants: From soil to cell. *Plant Physiology,* **116**, 447-453.

Schalamuk, S., Velazquez, S., Chidichimo, H., and Cabello, M. (2006). Fungal Spore Diversity of Arbuscular Mycorrhizal Fungi Associated with Spring Wheat: Effects of Tillage. *Mycologia,* **98** (1), 16-22.

Schenck, N. (1982). *Methods and principles of mycorrhizal research.* St. Pauls Minnesota: American Phytopathological Society.

Schwyn, B., and Nielands, J. (1987). Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry,* **160**, 46-56.

Shahab, S., Ahmed, N., and Khan, N. (2009). Indole acetic acid production and enhanced plant growth promotion by indegenous PSBs. *African Journal of Agricultural Research* **4** (11), 1312-1316.

Sharma, A., and Johri, B. (2002). Arbuscular-Mycorrhiza and Plant Disease. In A. Sharma, and B. Johri, *Arbuscular Mycorrhizae-Interaction in plants, rhizosphere and soil* (pp. 69-96). New Hampshire: Science Publishers.

Siddiqui, Z. (2005). PGPR: Prospective biocontrol agents of plant pathogens. In Z. Siddiqui, *PGPR: biocontrol and biofertilisation* (pp. 111-142). Netherlands: Springer.

Sinegani, A., Mahboobi, A., and Nazarizadeh, F. (2005). The effect of agricultural practices on the spatial variability of arbuscular mycorrhiza spores. *Turkish Journal of Biology,* **29**, 149-153.

Singh, S., and Kapoor, K. (1998). Effects of inoculation of phosphate-solubilizing microorganisms and an arbuscular mycorrhizal fungus on mungbean grown under natural soil conditions. *Mycorrhiza,* **7**, 249-253.

Smith, G., Johnston, C., and Cornforth, I. (1983). Comparison of nutrient solutions for growth of plants in sand culture. *New Phytologist,* **94** (4), 537-548.

Smith, S., and Dickson, S. (1997). *VA mycorrhizas: Basic research techniques.* Glen Osmond: Cooperative Research Centre for Soil and Land Management.

Smith, S., and Read, D. (2008). *Mycorrhizal Symbiosis.* UK: Academic Press.

Smith, S., and Smith, F. (2011). Roles of Arbuscular Mycorrhizas in Plant Nutrition and Growth: New Paradigms from Cellular to Ecosystem Scales. *Annual Review of Plant Biology,* **62**, 227-250.

Smith, S., Facelli, E., Pope, S., and Smith, F. (2010). Plant performance in stressful environments: interpreting new and established knowledge of the roles of arbuscular mycorrhizas. *Plant and Soil,* **326**, 3-20.

Smith, S., Smith, F., and Jakobsen, I. (2003). Mycorrhizal Fugus can dominate phosphate supply to plants irrespective of growth responses. *Plant Physiology,* **133**, 16-20.

Spaepen, S., Vanderleyden, J., and Remans, R. (2007). Indole-3-acetic acid in microial and microganism-plant signaling. *FEMS Microbiological Review* **31**, 425-448.

StatSoft, Inc. STATISTICA (data analysis software system) Version 7. (2009). Retrieved from www.statsoft.com.

Steinberg, P., and Rillig, M. (2003). Differential decomposition of arbuscular mycorrhiza fungal hyphae and glomalin. *Soil Biology and Biochemistry* **35**, 191-194.

Stewart, L., Hamel, C., Hogue, R., and Moutoglis, P. (2005). Response of strawberry to inoculation with arbuscular mycorrhizal fungi under very high soil phosphorus conditions. *Mycorrhiza* **15**, 612–619.

Subramanian, K., Santhanakrishnan, P., and Balasubramanian, P. (2006). Responses of field grown tomato plants to arbuscular mycorrhizal fungal colonization under varying intensities of drought stress. *Scientia Horticulturae* **107**, 245–253.

Suresh, A., Pallavi, P., Srinivas, P., Praveen Kumar, V., Jevan Chandra, S., and Ram Reddy, S. (2010). Plant growth promoting activities of fluorescent pseudomonads associated with some crop plants. *African Journal of Microbiology Research,* **4** (14), 1491-1494.

Suresh, C., and Bagyaraj, D. (2002). Mycorrhiza-microbe interaction: Effect on rhizosphere. In A. Sharma, and B. Johri, *Arbuscular Mycorrhizae: Interactions in Plants, Rhizosphere and Soils* (pp. 7-28). Hampshire: Science Publishers Inc.

Sylvia, D., Fuhrmann, J., Hartel, P., and Zuberer, D. (1998). *Principles and Applications of Soil Microbiology.* New Jersey: Prentice Publishers.

Tarkka, M., and Frey-Klett, P. (2008). Mycorrhiza Helper Bacteria. In A. Varma, *Mycorrhiza* (pp. 113-132). Berlin: Heidelberg: Springer-Verlag.

Taylor, J., and Harrier, L. (2001). A comparison of development and mineral nutrition of micropropagated *Fragaria × ananassa* cv. Elvira (strawberry) when colonised by nine species of arbuscular mycorrhizal fungi. *Applied Soil Ecology,* **18** , 205–215.

Taylor, J., and Harrier, L. (2000). A comparison of nine species of arbuscular mycorrhizal fungi on the development and nutrition of micropropagated *Rubus idaeus* L. cv. Glen Prosen (red raspberry). *Plant and Soil,* **225**, 53–61.

Tester, M., Smith, S., and Smith, F. (1987). The phenomenon of "non-mycorrhizal plants". *Canadian Journal of Botany,* **65**, 419-431.

Toledo, A., Alippi, A., and de Remes Lenicov, A. (2011). Growth inhibition of *Beauveria bassiana* by bacteria isolated from the cuticular surface of the corn leafhopper, *Dalbulus maidis* and the planthopper, *Delphacodes kuscheli*, two important vectors of maize pathogens. *Journal of Insect Science* **11** (29), 1-13.

Toro, M., Azcon, R., and Barea, J. (1997). Improvement of Arbuscular Mycorrhiza Development by Inoculation of Soil with Phosphate-Solubilizing Rhizobacteria To Improve Rock Phosphate Bioavailability (32P) and Nutrient Cycling. *Applied and Environmental Microbiology,* **63** (11), 4408–4412.

Tsavkelova, E., Cherdyntseva, T., Klimova, S., Shestakov, A., Botina, S., and Netrusov, A. (2007). Orchid-associated bacteria produce indole-3-acetic acid, promote seed germination, and increase their microbial yield in response to exogenous auxin. *Archives of Microbiology,* **188**, 655-664.

Upadhyaya, H., Panda, S., Bhattacharjee, M., and Dutta, S. (2010). Role of Arbuscular mycorrhiza in heavy metal tolerance in plants: Prospects for Phytoremediation. *Journal of Phytology,* **2** (7), 16-27.

Van Aarle, I. C., and Dickson, S. (2005). Metabolic activity of Glomus intraradices in Arum- and Paris-type arbuscular mycorrhizal colonization. *New Phytologist,* **166** (2), 611-618.

Vassilev, N., Nikolaeva, I., and Vassileva, M. (2007). Indole-3-Acetic Acid Production by Gel-Entrapped *Bacillus thuringiensis* in the Presence of Rock Phosphate Ore. *Chem. Eng. Comm.,* **194**, 441-445.

Vessey, J. (2003). Plant growth promoting rhizobacteria as biofertilizers. *Plant and Soil,* **255**, 571-586.

Vestberg, M., Kukkonen, S., Saari, K., Parikka, P., Huttunen, J., Tainio, L., Devos, N; Weekers, F; Keversd, C; Thonart, P; Lemoine, M-C; Cordier, C; Alabouvette, C and Gianinazzi, S. (2004). Microbial inoculation for improving the growth and health of micropropagated strawberry. *Applied Soil Ecology,* **27**, 243–258.

Vivas, A., Barea, J.M and Azcon, R. (2005). Brevibacillus brevis isolated from cadmium - or zinc- contaminated soils improves in vitro spore germination and growth of *Glomus mosseae* under high Cd or Zn concentrations. *Microbial Ecology,* **49**, 416-424.

Vivas, A., Marulanda, A., Ruiz-Lozano, J., Barea, J., and Azcon, R. (2003 a). Influence of a *Bacillus* sp. on physiological activities of two arbuscular mycorrhizal fungi and on plant responses to PEG-induced drought stress. *Mycorrhiza,* **13**, 249-256.

Vivas, A., Voros, I. B., Campos, E., and Barea, J. A. (2003 b). Symbiotic efficiency of autochthonous arbuscular mycorrhizal fungus (*Glomus mosseae*) and *Brevibacillus* sp. isolated from cadium polluted soil under increasing cadmium levels. *Environmental Pollution,* **126**, 179-189.

von Alten, H., Lindemann, A., and Schonbeck, F. (1993). Stimulation of vesicular-arbuscular mycorrhiza by fungicides or rhizosphere bacteria. *Mycorrhiza,* **2**, 167-173.

Walley, F. G. (1997). Response of spring wheat (*Triticum aestivum*) to interactions between *Pseudomonas* species and *Glomus clarum* NT4. *Biology and Fertility of Soils,* **24**, 365–371.

Wehner, J., Antunes, P., Powell, J., Mazukatow, and Rillig, M. (2010). Plant pathogen protection by arbuscular mycorrhizas: A role for fungal diversity. *Pedobiologia,* **53**, 197-201.

Weisburg, W., Barns, S., Pelletier, D., and Lane, D. (1991). 16s Ribosomal DNA Amplification for Phylogenetic Study. *Journal of Bacteriology,* **173** (2), 697-703.

Wheeler, D., Barrett, T., Benson, D., Bryant, S., Canese, K., Chetvernin, V., et al. (2006). *Database resources of the national center for biotechnology information*. Retrieved from Nucleic Acids Research: www.ncbi,nlm.nih.gov

Whipps, J. (2001). Micorbial interactions and biocontrol in the rhizosphere. *Journal of Experimental Biology,* **52**, 487-511.

Will, M., and Sylvia, D. (1990). Interaction of rhizosphere bacteria, fertilizer and vesicular arbuscular mycorrhizal fungi with sea oats. *Applied Environmental Microbiology,* **56**, 2073-2079.

Wu, J.-H., Miller, S., Hall, H., and Mooney, P. (2009). Factors affecting the efficiency of micropropagation from lateral buds and shoot tips of *Rubus. Plant Cell Tissue Organ Culture,* **99**, 17-25.

Wu, Q.-S., and Xia, R.-X. (2006). Arbuscular mycorrhizal fungi influence growth, osmotic adjustment and photosynthesis of citrus under well-watered and water stress conditions. *Journal of Plant Physiology,* **163**, 417-425.

Xavier, L., and Germida, J. (2003). Bacteria associated with *Glomus clarum* spores influence mycorrhizal activity. *Soil Biology and Biochemistry,* **35**, 471-478.

Xie, H., Pasternak, J., and Glick, B. (1996). Isolation and characterization of mutants of the plant growth-promoting rhizobacterium *Pseudomonas putida* GR12-2 that overproduce indoleacetic acid. *Current Microbiology,* **32**, 67-71.

Yao, T., Yasmin, S., and Hafeez, F. (2008). Potential role of rhizobacteria isolated from Northwestern China for enhancing wheat and oat yield. *Journal of Agricultural Science,* **146**, 49-56.

Yasmin, F., Othman, R., Sijam, K., and Saad, M. (2009). Characterization of beneficial properties of plant growth-promoting rhizobacteria isolated from sweet potato rhizosphere. *African Journal of Microbiology Research,* **3** (11), 815-821.

Zar, J. (1999). *Biostatistical Analysis.* New Jersey: Prentice Hall.

APPENDICES

APPENDICES

Appendix I: Media and Reagents

Appendix II: Nucleotide Sequences of Bacterial Isolates

Appendix III: Culture Morphology of Bacterial Isolates Identified

Appendix IV: Fischer’s LSD of Significance- Pot Trial Results

Appendix V: Literature Published

APPENDIX I: MEDIA AND REAGENTS

A Selective Media

A-1 National Botanical Research Institute's Phosphate growth medium (NBRIP) (Mehta and Nautiyal, 2001).

Component	Gram / Litre
Glucose	10.0
$Ca_3(PO_4)_2$	5.0
$MgCl_2$. $6H_2O$	5.0
$MgSO_4.7H_2O$	0.25
KCl	0.2
$(NH_4)_2SO_4$	0.1
Agar	15.0
Bromophenol Blue	0.025

Autoclave at 121°C for 15 mins and allow to cool before dispensing into Petri dishes.

A-2 Phytase Media (Hariprasad and Niranjana, 2009)

Component	Gram / Litre
Phytic Acid	5.0
Glucose	10.0
$(NH_4)_2SO_4$	0.3
$MgSO_4$	0.5
$CaCl_2$	0.1
$MnSO_4$	0.01
$FeSO_4$	0.01
Agar	17.0

Autoclave at 121°C for 15 min and allow to cool before dispensing into Petri dishes.

A-3 Indole acetic acid media (Cappucino and Sherman, 2008)

16 g DEV Tryptophan Broth in 1 L, Autoclave at 121°C for 15 min and allow to cool before dispensing into Petri dishes.

A-4 Ammonia Production Media (Cappucino and Sherman, 2008; Chaiharn *et al.*, 2008)

Component	Gram / Litre
Peptone	1.0
NaCl	5.0

Autoclave at 121°C for 15 min and allow to cool before dispensing into Petri dishes. pH 7.2.

A-5 Siderophore Producing media (Milagres *et al.*, 1999)

Step 1: mix 60.5 mg of Chrome Azurol S (CAS) in 50 ml ddH_20

Step 2: make 10 ml Fe^{3+} solution (1 mM $FeCl_3.6H_20$, 10 mM HCl)
1 mM $FeCl_3.6H_20$: 0.003 g $FeCl_3.6H_20$ in 10 ml dd H_20
10 mM HCl: 10 µl of 32% HCl

Step 3: Under stirring, slowly add reagents from step 1 and 2 to 72.9 HDTMA dissolved in 40 ml ddH_20
Autoclave at 121°C for 15 min and allow to cool

Step 4: Mix 750 ml ddH_20, 15 g agar, 30.24 g PIPES, and slowly add approximately 12 ml 50% NaOH, pH to 6.8, and prepare an additional 160 ml ddH_20 separately to bring total volume to 1 L. Autoclave at 121 °C for 15 min and allow to cool

Final step: Add mixture of reagents from step 3 to step 4 along glass slide and dispense into Petri dishes

A-6 Skim Milk Agar

Component	Gram / Litre
Yeast Extract powder	1.0
Skim Milk Powder	1.0
Glucose Monohydrate	1.0
Casein enzymic hydrolysate	5.0
Agar	15.0

Autoclave at 121°C for 15 min and allow to cool before dispensing into Petri dishes.

A-7 Carboxymethylcellulose media (Kasana *et al.*, 2008)

Component	Gram / Litre
$NaNO_3$	2.0
K_2HPO_4	1.0
$MgSO_4$	0.5
KCl	0.5
CMC Na Salt	2.0
Peptone	0.2
Agar	17.0

Autoclave at 121°C for 15 min and allow to cool before dispensing into Petri dishes.

A-8 Chitin Media (Parani and Saha, 2009)

Component	Gram / Litre
Chitin from shrimp shell	0.2
Yeast extract	5.0
$MgSO_4$	0.5
Sodium nitrate	2
KCl	0.5
$FeSO_4$	Pinch
K_2HPO_4	1
Agar	20

pH 6.0; Autoclave at 121°C for 15 min and allow to cool before dispensing into Petri dishes.

B Root clearing and staining solutions (Smith and Dickson, 1997).

50% ethanol

1000 ml ethanol

1000 ml distilled water

5% KOH

100 g KOH

2 L distilled water

Alkaline Peroxide H2O2

3 ml NH_4OH(Ammonia)

30 ml 10% H_2O_2

567 ml distilled water

0.1M HCl (32% MW36.46)

22.79 ml HCl

2 L Distilled water

Lactoglycerol trypan blue stain

Lactic acid: Glycerol: Water (13:12:16)

520 ml lactic acid

480 ml Glycerol

640 ml distilled water

0.82 g Trypan blue

Lactoglycerol Destain

Lactic acid: Glycerol: Water (13:12:16)

520 ml lactic acid

480 ml Glycerol

640 ml distilled water

Appendix II: Nucleotide Sequences of Bacterial Isolates

> *Acinetobacter ursingii* (SEN4)

TGGGGCACGAGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGGTRCTG
GTATTAATACTACCAGGTACTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGG
GTGCGAGCGTTAATCGGATTTACTGGGCGTAAAGCGTGCGTAGGCGGCTAATTGAGTCGGATGTGAAATCCCCGAGCTTAACT
TGGGAATTGCATTCGATACTGGTTAGCTAGAGTGTGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGA
TCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAACACTGACGCTGAGGTACGAAAGCATGGGGAGCAAACAGGA
TTAGATACCCTGGTAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGGTCTCTTTGAGGGATTAGTGGCGCAGCTAACGCGAT
AAGTAGACCGCCTGGGGAGTACGGTCGCAAGACTAAAACTCAAAGGAATTGACGGCGGGCGGGGGCGGCGGGACGGGCGC
GGGGCSGGGGCGGGCGAA

> *Bacillus thuringiensis* (SEN9)

TCATGCAAGTCGTaTCGAGGTAtCCGATaaCTTGcTCTCAaGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGcC
CATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAAcATTTTGAACtGCATGGWTCGAAATTGAAAGGCGGC
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aAGTGACAGGTGGTTGCATGGtTGCGTCAgcTTCGTgGTCGGgAGATGTGGGTTTAActCCCCgCACCAcCCcCCACcCCT

> *Bacillus mycoides* (SERB2)

GGGSCKWCWWTAWAMATGCAAGTCGWRACRARTGTGAKYMRRARCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGT
AASACGTGGGTAACCTACCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATATTTTGAACTGCATAGT
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CAAACCCGAAGAAGKTGGGGAAWTKRAMCGAYCATAT

> *Bacillus thuringiensis* (SIRB2)

GMGAAGTCTGACGGAGCACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCT
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CGCGGGGCGCGGGCGGGCGA

> *Microbacterium nematophilum* (SIRC2)

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MGA

> *Bacillus mycoides* (SESB1)

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GAACGATCCTGAT

> *Bacillus cereus* (SERA2)

GCGTAGTCTGACGGAGCACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTA
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GGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCG
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CGGGGCCGGGCGGGGCGA

> *Bacillus sp.* (SISB2)

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AGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGC
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GTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGC
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AGAGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC
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GAAWKKAACSTYCAAAC

> *Bacillus mycoides* (SISC2)

GGGGWGCGCTCTMATGCAGTCGAGCgAtGGATtaRgAGCTTGCTCTTATGAAGTTAGCGGCGGACGGgTGAGTAACACGTGG
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GAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCG
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GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGT
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GgGGGGGAATGTGAAMST

> *Alcaligenes faecalis* (AERA1)

ATATMCATGCAGTCGAACGGCAGCGCGAGARAGCTTGCTCTCTTGGCGGCGAGTGGCGGACSGGTGAGTAATATATCGGAAC
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CATTAGTTGCTACGCAGAGCACTCTAATGAGACTGCCAGTGACAATCGAGGATGGTGGGATGACGTCAGTCCTCATGGCCCTT
TATKGGGATG

> *Bacillus pumilus* (AERA4.1)

GGCGCTAATMATGCAGTCGAGCGGASAGAAGGGTGCTTGCTCCCKGATGTTAGCGGCGGACGGGTGAKTAACACGTGGGTA
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CGTCAAATTCATCAKGCCCCCATATG

> *Serratia marcescens* (AERB2)

GGCGCAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGTGGT
GARCTTAATACGYTCATCAATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGG
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GCGCGGGGCSSGGGGCSGGGCGA

> *Bacillus pumilus* (AERA5)

GGcGAGTCTGACGGAGCACGCCGCGTGAGTGATGAAGGTtTTCGGATCGTAAAgCTCTGTTgTTAGGGAAGAACAAgTGcGagAGTAACTGctCgCacCTTGACGGTACCTAAcCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTgTCCGGAATTATTGGGCGTAAAGgGCtCGcAGGCGGTTTcTTAAGTCTGATGTGAAAGCCCcCGGCTCAACCGgGGAGGGTCATTGGAAACTGGgAAACTTGAGTGCAGAAGAGGAgAGTGGAATTCCAcGTGTAGCGGTGAAATGCGtAGAGATgTGGAGGAACACCAGTGGCGAAGGCGACTcTCTGGTCTGTAACTGACGCTGAtGaGCGAAAGCGTGGGGAgCgAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGtCGCAAGacTGAAACTCAAAGGAATTGACGGCGGGCGGGGGCGGCGGGACGGGCGCGGGGCggGGGcgGGGCAaA

> *Micrococcus luteus* (AIRA4B)

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> *Staphylococcus sp.* (AIRC5)

GGSGGAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATYMGGGAAGAACAAATGTGTAAGTAACTATGCACGTCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGCGGGCGGGGGCGGCGGGACGGGCGCGGGGCGCGGCGGGCGA

> *Staphylococcus pasteuri* (AIRC9)

GGGCGAAGCCTGACGGAGCACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATCAGGGAAGAACAAATGTGTAAGTAACTGTGCACATCTTGACGGTACCTGATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCRCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGCGGGCGGGGGCGGCGGGACGGGCGCGGGGGGGGGCSSGGGSRAA

> *Bacillus sp.* (AIRA2)

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GGTTTAAGTCCCGCAACGAGCGCAACCCTGATCTTTAGTTGCCAGCATTCARGTGGGGCACTCKAAAGGGTGACTGCGTGACG
ACCGGGAGAAGGGTGGGGGAATKGAACSGTCA

> *Bacillus sp.* (AIRC4)

TGaGaAGTCTgACAtgCAGCAcCgCCGCGTGagTGATGAAGGccTTCGGAtcTGTAAAGCaCTtTTGgcAGaGAAGAACAAGTGcg
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CgCCTGGaGAGTACgGTCgCAAgACTGAAACTCAAAgGAATTGACgGCGGGCGGGGGCGGCGggACGGGCGCGGGCGcCCGCg
GGGCGATT

Appendix III: Culture Morphology of Bacterial Isolates Identified

A- South African Isolates


SESB1


SERB2


SEN9


SERA2


SEN4


SISB2

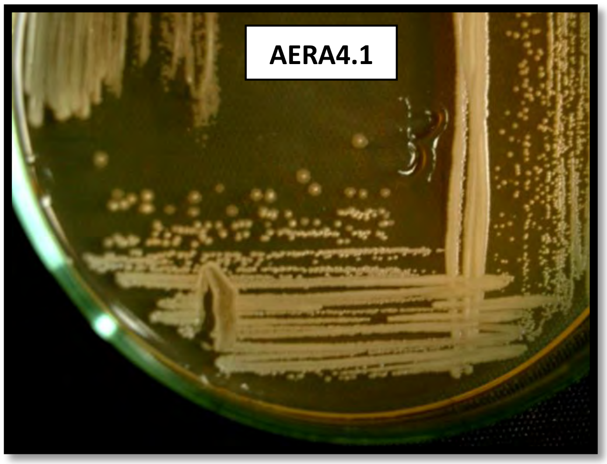

SIRC2


SIRB2


SISC2

B- Argentinean Isolates


AIRA4B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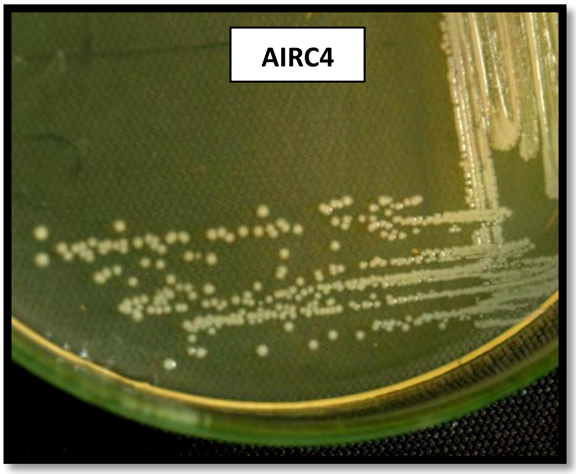
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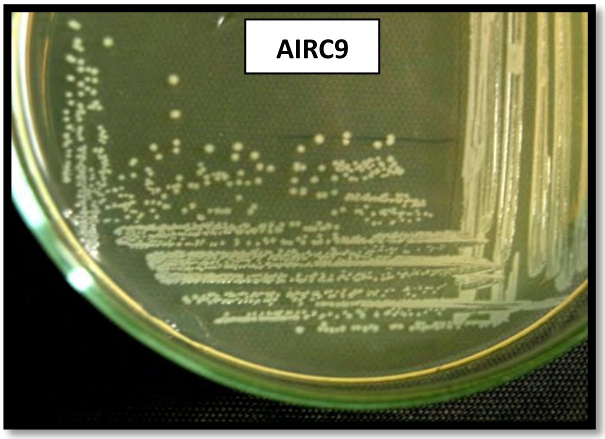
AERA1


AIRA5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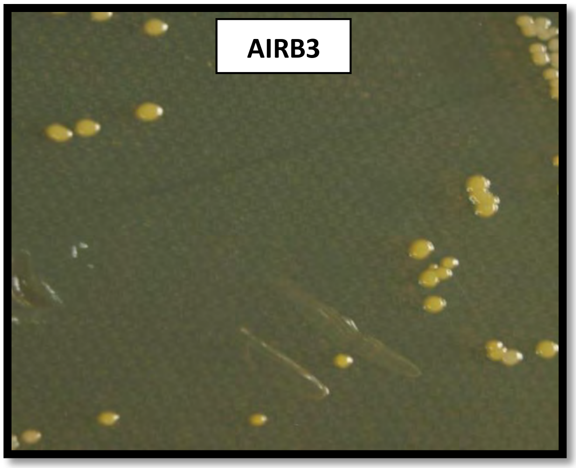
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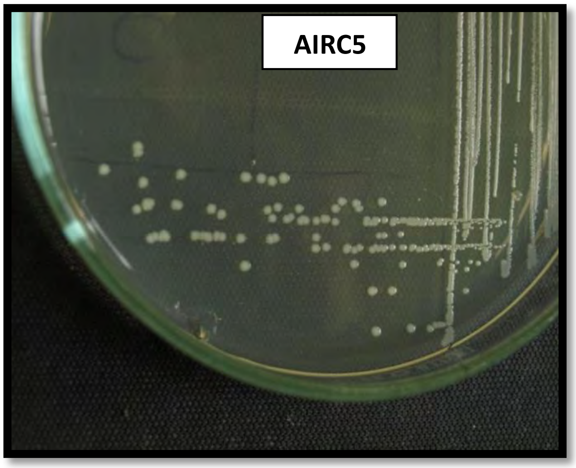
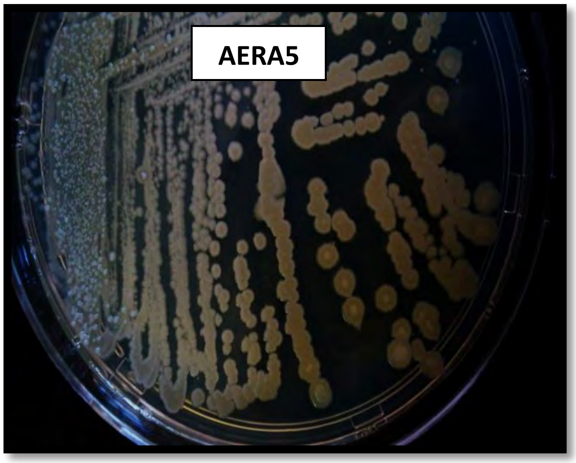
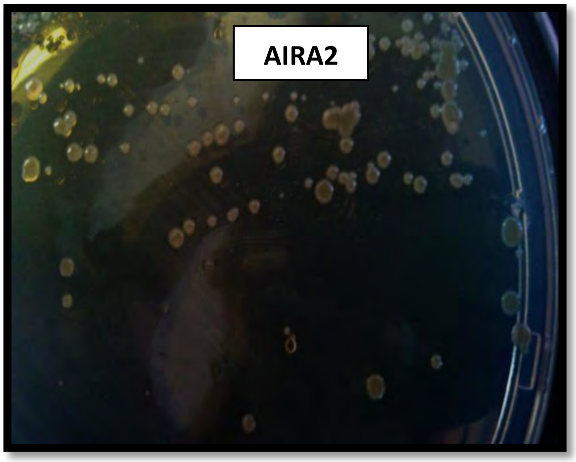

AIRC5


AERA5


AIRA2


AERB2

Appendix IV: Fischer's LSD of Significance- Pot Trial Results

Shoot Dry Weight:

LSD test; variable shoot weight (Spreadsheet1)
Probabilities for Post Hoc Tests
Error: Between MS = .20079, df = 88.000

Cell No.	treatment	{1} .16960	{2} .28300	{3} 0.0000	{4} .17980	{5} .62460	{6} .85120	{7} .40520	{8} 1.0672	{9} .02680	{10} .50240	{11} .36120	{12} .65740	{13} .06900	{14} .92380	{15} .76180	{16} .73220	{17} .61660	{18} .28820	{19} .69080	{20} .68440	{21} .54320	{22} .52080
1	0		0.690026	0.551084	0.971371	0.111970	0.018265	0.408041	0.002116	0.615609	0.243444	0.500771	0.088725	0.723461	0.009254	0.039540	0.050241	0.118325	0.676613	0.069277	0.072700	0.190837	0.218558
2	1	0.690026		0.320738	0.716623	0.231300	0.048046	0.667387	0.006894	0.368458	0.440911	0.783248	0.189898	0.452201	0.026219	0.094671	0.116549	0.242322	0.985402	0.153716	0.160202	0.361063	0.403695
3	21	0.551084	0.320738		0.527446	0.030140	0.003475	0.156326	0.000299	0.924876	0.079733	0.205839	0.022674	0.808209	0.001587	0.008595	0.011427	0.032258	0.311977	0.016801	0.017808	0.058521	0.069487
4	22	0.971371	0.716623	0.527446		0.120122	0.020021	0.428561	0.002363	0.590653	0.258083	0.523786	0.095488	0.696771	0.010210	0.042982	0.054461	0.126841	0.703018	0.074798	0.078446	0.203116	0.232114
5	23	0.111970	0.231300	0.030140	0.120122		0.426115	0.440911	0.121941	0.037754	0.667387	0.355216	0.908126	0.053106	0.293978	0.629507	0.705105	0.977544	0.238422	0.815847	0.833370	0.774617	0.715049
6	24	0.018265	0.048046	0.003475	0.020021	0.426115		0.119139	0.448000	0.004591	0.221695	0.087319	0.495879	0.007032	0.798416	0.753166	0.675585	0.410026	0.050082	0.572850	0.557664	0.280098	0.246834
7	25	0.408041	0.667387	0.156326	0.428561	0.440911	0.119139		0.021770	0.185255	0.732436	0.876975	0.375948	0.238699	0.070651	0.211620	0.251694	0.457696	0.680729	0.316338	0.327243	0.627513	0.684339
8	26	0.002116	0.006894	0.000299	0.002363	0.121941	0.448000	0.021770		0.000414	0.049369	0.014609	0.151731	0.000681	0.614128	0.284151	0.240367	0.115431	0.007259	0.187566	0.180247	0.067822	0.057081
9	27	0.615609	0.368458	0.924876	0.590653	0.037754	0.004591	0.185255	0.000414		0.096862	0.241203	0.028632	0.881970	0.002130	0.011127	0.014691	0.040328	0.358863	0.021387	0.022634	0.071831	0.084810
10	28	0.243444	0.440911	0.079733	0.258083	0.667387	0.221695	0.732436	0.049369	0.096862		0.619566	0.585816	0.129785	0.140609	0.362534	0.419635	0.687956	0.451780	0.507932	0.522417	0.885858	0.948381
11	29	0.500771	0.783248	0.205839	0.523786	0.355216	0.087319	0.876975	0.014609	0.241203	0.619566		0.298815	0.305348	0.050241	0.161027	0.193913	0.369948	0.797330	0.247971	0.257205	0.522417	0.574762
12	31	0.088725	0.189898	0.022674	0.095488	0.908126	0.495879	0.375948	0.151731	0.028632	0.585816	0.298815		0.040794	0.349790	0.713475	0.792448	0.885858	0.196064	0.906453	0.924317	0.687956	0.631004
13	32	0.723461	0.452201	0.808209	0.696771	0.053106	0.007032	0.238699	0.000681	0.881970	0.129785	0.305348	0.040794		0.003346	0.016496	0.021540	0.056549	0.441326	0.030867	0.032587	0.097833	0.114479
14	33	0.009254	0.026219	0.001587	0.010210	0.293978	0.798416	0.070651	0.614128	0.002130	0.140609	0.050241	0.349790	0.003346		0.569035	0.500771	0.281341	0.027426	0.413214	0.400552	0.182738	0.158561
15	34	0.039540	0.094671	0.008595	0.042982	0.629507	0.753166	0.211620	0.284151	0.011127	0.362534	0.161027	0.713475	0.016496	0.569035		0.917054	0.609694	0.098252	0.802765	0.785410	0.442573	0.397423
16	35	0.050241	0.116549	0.011427	0.054461	0.705105	0.675585	0.251694	0.240367	0.014691	0.419635	0.193913	0.792448	0.021540	0.500771	0.917054		0.684339	0.120781	0.884191	0.866448	0.506585	0.457696
17	36	0.118325	0.242322	0.032258	0.126841	0.977544	0.410026	0.457696	0.115431	0.040328	0.687956	0.369948	0.885858	0.056549	0.281341	0.609694	0.684339		0.249684	0.794074	0.811480	0.796244	0.736143
18	37	0.676613	0.985402	0.311977	0.703018	0.238422	0.050082	0.680729	0.007259	0.358863	0.451780	0.797330	0.196064	0.441326	0.027426	0.098252	0.120781	0.249684		0.158970	0.165625	0.370695	0.414014
19	38	0.069277	0.153716	0.016801	0.074798	0.815847	0.572850	0.316338	0.187566	0.021387	0.507932	0.247971	0.906453	0.030867	0.413214	0.802765	0.884191	0.794074	0.158970		0.982034	0.603806	0.550147
20	39	0.072700	0.160202	0.017808	0.078446	0.833370	0.557664	0.327243	0.180247	0.022634	0.522417	0.257205	0.924317	0.032587	0.400552	0.785410	0.866448	0.811480	0.165625	0.982034		0.619566	0.565232
21	210	0.190837	0.361063	0.058521	0.203116	0.774617	0.280098	0.627513	0.067822	0.071831	0.885858	0.522417	0.687956	0.097833	0.182738	0.442573	0.506585	0.796244	0.370695	0.603806	0.619566		0.937181
22	310	0.218558	0.403695	0.069487	0.232114	0.715049	0.246834	0.684339	0.057081	0.084810	0.948381	0.574762	0.631004	0.114479	0.158561	0.397423	0.457696	0.736143	0.414014	0.550147	0.565232	0.937181	

Treatments:
0: un inoculated control
1: treatment with just AM fungal inoculum
2: treatments with only the selected bacteria (with representive bacterial coding – Section 4.2.1)
3: treatments with both the bacteria and AM fungal inoculum (3; with representive bacterial coding- Section 4.2.1)

Root Dry Weight:

LSD test; variable shoot weight (Spreadsheet1)
Probabilities for Post Hoc Tests
Error: Between MS = .05282, df = 88.000

Cell No.	treatment	{1} .26400	{2} .38901	{3} .09240	{4} .23900	{5} .43160	{6} .38480	{7} .26720	{8} .65100	{9} .10280	{10} .44740	{11} .19660	{12} .47479	{13} .42707	{14} .54075	{15} .50075	{16} .51693	{17} .37220	{18} .30486	{19} .38184	{20} .45920	{21} .29120	{22} .24958
1	0		0.392134	0.240973	0.863842	0.252030	0.408197	0.982486	0.009224	0.270461	0.210390	0.644022	0.150577	0.264995	0.060189	0.106947	0.085348	0.458649	0.779302	0.419729	0.182760	0.851995	0.921210
2	1	0.392134		0.044297	0.304912	0.770196	0.976978	0.404317	0.074908	0.052104	0.688866	0.189043	0.556607	0.794070	0.299378	0.444108	0.381227	0.908204	0.564138	0.960798	0.630361	0.502798	0.340097
3	21	0.240973	0.044297		0.315962	0.021900	0.047325	0.232380	0.000229	0.943125	0.016597	0.475368	0.010060	0.023676	0.002725	0.006118	0.004438	0.057476	0.147409	0.049559	0.013418	0.174905	0.282504
4	22	0.863842	0.304912	0.315962		0.188603	0.318595	0.846621	0.005694	0.351328	0.155204	0.771209	0.108356	0.199121	0.040821	0.075174	0.059126	0.361995	0.651609	0.328458	0.133386	0.720373	0.942135
5	23	0.252030	0.770196	0.021900	0.188603		0.748246	0.261131	0.134788	0.026162	0.913691	0.109526	0.767073	0.975183	0.454712	0.635455	0.558685	0.683784	0.385626	0.732931	0.849836	0.336749	0.213815
6	24	0.408197	0.976978	0.047325	0.318595	0.748246		0.420679	0.070436	0.055579	0.667770	0.198802	0.537460	0.771916	0.286263	0.427205	0.365836	0.931110	0.583742	0.983809	0.610040	0.521300	0.354792
7	25	0.982486	0.404317	0.232380	0.846621	0.261131	0.420679		0.009798	0.261131	0.218386	0.628391	0.156798	0.274421	0.063155	0.111701	0.089309	0.472004	0.796183	0.432419	0.189967	0.869237	0.903803
8	26	0.009224	0.074908	0.000229	0.005694	0.134788	0.070436	0.009798		0.000294	0.164829	0.002401	0.228664	0.127011	0.450203	0.304132	0.358877	0.058351	0.019409	0.067426	0.190431	0.015230	0.007002
9	27	0.270461	0.052104	0.943125	0.351328	0.026162	0.055579	0.261131	0.000294		0.019939	0.520413	0.012202	0.028238	0.003379	0.007486	0.005460	0.067187	0.168015	0.058139	0.016187	0.198330	0.315367
10	28	0.210390	0.688866	0.016597	0.155204	0.913691	0.667770	0.218386	0.164829	0.019939		0.087969	0.850976	0.889063	0.522406	0.714485	0.633597	0.606207	0.329471	0.653092	0.935476	0.285500	0.177022
11	29	0.644022	0.189043	0.475368	0.771209	0.109526	0.198802	0.628391	0.002401	0.520413	0.087969		0.058895	0.116443	0.020096	0.039282	0.030155	0.230273	0.458396	0.205886	0.074247	0.516870	0.716370
12	31	0.150577	0.556607	0.010060	0.108356	0.767073	0.537460	0.156798	0.228664	0.012202	0.850976	0.058895		0.743449	0.651101	0.858668	0.772567	0.482189	0.245544	0.524200	0.914845	0.209923	0.124893
13	32	0.264995	0.794070	0.023676	0.199121	0.975183	0.771916	0.274421	0.127011	0.028238	0.889063	0.116443	0.743449		0.436252	0.613483	0.538020	0.706740	0.402783	0.756449	0.825540	0.352508	0.225346
14	33	0.060189	0.299378	0.002725	0.040821	0.454712	0.286263	0.063155	0.450203	0.003379	0.522406	0.020096	0.651101	0.436252		0.783814	0.870206	0.249362	0.108204	0.277281	0.576208	0.089535	0.048243
15	34	0.106947	0.444108	0.006118	0.075174	0.635455	0.427205	0.111701	0.304132	0.007486	0.714485	0.039282	0.858668	0.613483	0.783814		0.911618	0.378904	0.181236	0.415550	0.775680	0.152965	0.087511
16	35	0.085348	0.381227	0.004438	0.059126	0.558685	0.365836	0.089309	0.358877	0.005460	0.633597	0.030155	0.772567	0.538020	0.870206	0.911618		0.322130	0.148139	0.355252	0.692220	0.124032	0.069253
17	36	0.458649	0.908204	0.057476	0.361995	0.683784	0.931110	0.472004	0.058351	0.067187	0.606207	0.230273	0.482189	0.706740	0.249362	0.378904	0.322130		0.644322	0.947254	0.551015	0.578788	0.401212
18	37	0.779302	0.564138	0.147409	0.651609	0.385626	0.583742	0.796183	0.019409	0.168015	0.329471	0.458396	0.245544	0.402783	0.108204	0.181236	0.148139	0.644322		0.597720	0.291230	0.925350	0.704652
19	38	0.419729	0.960798	0.049559	0.328458	0.732931	0.983809	0.432419	0.067426	0.058139	0.653092	0.205886	0.524200	0.756449	0.277281	0.415550	0.355252	0.947254	0.597720		0.595929	0.534523	0.365368
20	39	0.182760	0.630361	0.013418	0.133386	0.849836	0.610040	0.189967	0.190431	0.016187	0.935476	0.074247	0.914845	0.825540	0.576208	0.775680	0.692220	0.551015	0.291230	0.595929		0.250904	0.152828
21	210	0.851995	0.502798	0.174905	0.720373	0.336749	0.521300	0.869237	0.015230	0.198330	0.285500	0.516870	0.209923	0.352508	0.089535	0.152965	0.124032	0.578788	0.925350	0.534523	0.250904		0.775309
22	310	0.921210	0.340097	0.282504	0.942135	0.213815	0.354792	0.903803	0.007002	0.315367	0.177022	0.716370	0.124893	0.225346	0.048243	0.087511	0.069253	0.401212	0.704652	0.365368	0.152828	0.775309	

Treatments:
0: un inoculated control
1: treatment with just AM fungal inoculum
2: treatments with only the selected bacteria (with representive bacterial coding – Section 4.2.1)
3: treatments with both the bacteria and AM fungal inoculum (3; with representive bacterial coding- Section 4.2.1)

AM Fungal Root Colonisation Percentage:

LSD test; variable shoot weight (Spreadsheet1)
Probabilities for Post Hoc Tests
Error: Between MS = 190.25, df = 88.000

Cell No.	treatment	{1} 0.0000	{2} 35.000	{3} 0.0000	{4} 0.0000	{5} 0.0000	{6} 0.0000	{7} 0.0000	{8} 0.0000	{9} 0.0000	{10} 0.0000	{11} 0.0000	{12} 58.000	{13} 74.800	{14} 35.600	{15} 48.000	{16} 34.000	{17} 45.000	{18} 54.000	{19} 45.000	{20} 66.000	{21} 0.0000	{22} 59.000
1	0		0.000126	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	0.000000	0.000000	0.000098	0.000000	0.000189	0.000002	0.000000	0.000002	0.000000	1.000000	0.000000
2	1	0.000126		0.000126	0.000126	0.000126	0.000126	0.000126	0.000126	0.000126	0.000126	0.000126	0.009900	0.000016	0.945321	0.139741	0.908998	0.254768	0.032079	0.254768	0.000614	0.000126	0.007209
3	21	1.000000	0.000126		1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	0.000000	0.000000	0.000098	0.000000	0.000189	0.000002	0.000000	0.000002	0.000000	1.000000	0.000000
4	22	1.000000	0.000126	1.000000		1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	0.000000	0.000000	0.000098	0.000000	0.000189	0.000002	0.000000	0.000002	0.000000	1.000000	0.000000
5	23	1.000000	0.000126	1.000000	1.000000		1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	0.000000	0.000000	0.000098	0.000000	0.000189	0.000002	0.000000	0.000002	0.000000	1.000000	0.000000
6	24	1.000000	0.000126	1.000000	1.000000	1.000000		1.000000	1.000000	1.000000	1.000000	1.000000	0.000000	0.000000	0.000098	0.000000	0.000189	0.000002	0.000000	0.000002	0.000000	1.000000	0.000000
7	25	1.000000	0.000126	1.000000	1.000000	1.000000	1.000000		1.000000	1.000000	1.000000	1.000000	0.000000	0.000000	0.000098	0.000000	0.000189	0.000002	0.000000	0.000002	0.000000	1.000000	0.000000
8	26	1.000000	0.000126	1.000000	1.000000	1.000000	1.000000	1.000000		1.000000	1.000000	1.000000	0.000000	0.000000	0.000098	0.000000	0.000189	0.000002	0.000000	0.000002	0.000000	1.000000	0.000000
9	27	1.000000	0.000126	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000		1.000000	1.000000	0.000000	0.000000	0.000098	0.000000	0.000189	0.000002	0.000000	0.000002	0.000000	1.000000	0.000000
10	28	1.000000	0.000126	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000		1.000000	0.000000	0.000000	0.000098	0.000000	0.000189	0.000002	0.000000	0.000002	0.000000	1.000000	0.000000
11	29	1.000000	0.000126	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000		0.000000	0.000000	0.000098	0.000000	0.000189	0.000002	0.000000	0.000002	0.000000	1.000000	0.000000
12	31	0.000000	0.009900	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000		0.057355	0.011923	0.254768	0.007209	0.139741	0.647702	0.139741	0.361618	0.000000	0.908998
13	32	0.000000	0.000016	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.057355		0.000021	0.002828	0.000010	0.000963	0.019257	0.000963	0.315853	0.000000	0.073523
14	33	0.000098	0.945321	0.000098	0.000098	0.000098	0.000098	0.000098	0.000098	0.000098	0.000098	0.000098	0.011923	0.000021		0.158722	0.854897	0.284182	0.037765	0.284182	0.000770	0.000098	0.008730
15	34	0.000000	0.139741	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.254768	0.002828	0.158722		0.112109	0.731744	0.493391	0.731744	0.042022	0.000000	0.210657
16	35	0.000189	0.908998	0.000189	0.000189	0.000189	0.000189	0.000189	0.000189	0.000189	0.000189	0.000189	0.007209	0.000010	0.854897	0.112109		0.210657	0.024253	0.210657	0.000418	0.000189	0.005202
17	36	0.000002	0.254768	0.000002	0.000002	0.000002	0.000002	0.000002	0.000002	0.000002	0.000002	0.000002	0.139741	0.000963	0.284182	0.731744	0.210657		0.305043	1.000000	0.018161	0.000002	0.112109
18	37	0.000000	0.032079	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.647702	0.019257	0.037765	0.493391	0.024253	0.305043		0.305043	0.172440	0.000000	0.567997
19	38	0.000002	0.254768	0.000002	0.000002	0.000002	0.000002	0.000002	0.000002	0.000002	0.000002	0.000002	0.139741	0.000963	0.284182	0.731744	0.210657	1.000000	0.305043		0.018161	0.000002	0.112109
20	39	0.000000	0.000614	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.361618	0.315853	0.000770	0.042022	0.000418	0.018161	0.172440	0.018161		0.000000	0.424468
21	210	1.000000	0.000126	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	0.000000	0.000000	0.000098	0.000000	0.000189	0.000002	0.000000	0.000002	0.000000		0.000000
22	310	0.000000	0.007209	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.908998	0.073523	0.008730	0.210657	0.005202	0.112109	0.567997	0.112109	0.424468	0.000000	

Treatments:
0: un inoculated control
1: treatment with just AM fungal inoculum
2: treatments with only the selected bacteria (with representive bacterial coding – Section 4.2.1)
3: treatments with both the bacteria and AM fungal inoculum (3; with representive bacterial coding- Section 4.2.1)

Appendix V: Published Literature

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Review

What we know about arbuscular mycorhizal fungi and associated soil bacteria

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Mycorrhizal fungi are common soil microorganisms and are well known for their symbiotic association with the roots of host plants. The soil is a complex environment harbouring a wide diversity of microorganisms. The interaction between soil bacteria and arbuscular mycorrhizal fungi has been shown in several studies to be both beneficial in terms of mycorrhizal establishment and as well as plant growth promotion. This has resulted in groups of bacteria being functionally termed Mycorrhizal Helper Bacteria, Phosphate Solubilising Bacteria and Plant Growth Promoting Rhizobacteria. Several of these groups overlap and in such a complex environment, it is likely that the combinations of microorganisms interacting with arbuscular mycorrhizal fungi enhance the benefits that are attributed to the relationship. Many different microorganisms inhabit the soil. This review will focus on the bacterial interactions and their potential use in agricultural biotechnology.

Key words: Mycorrhizal fungi, bacteria, soil, microorganisms, growth

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi are obligate biotrophs and associate with the roots of approximately 80% of all plant species. The AM fungi were named because of the finely branched hyphal structures 'arbuscules' that occur within root cortical cells. These are responsible for the exchange of carbon which is required by the AM fungi for energy, and nutrients from the soil needed by the plants (Smith and Read, 2008). The AM associations provide many benefits to their host and the soil environment which includes enhanced nutrient uptake, increased tolerance to drought and root pathogens, and improved soil aggregation (Bago and Bécard, 2002; Finlay, 2004; Smith and Read, 2008).

Nutrients present in the soil are required by plants in varying amounts. The uptake of inorganic nutrients from the root zone creates a depletion zone limiting nutrient uptake by non-mycorrhizal plants but gives mycorrhizal plants a greater advantage as the extraradical hyphal network can increase the surface area available for uptake as well as enhance mobilization (Sylvia and Zuberer, 2001; Smith and Read, 2008; Li et al., 1991).

Macronutrients like nitrogen and phosphorous may be in forms or niches inaccessible to plant roots. Phosphorus is immobile occurring mainly in organic or complex inorganic forms. The AM fungi aid in uptake by secreting phosphatase enzymes into the soil environment which hydrolyses and releases phosphorus facilitating its absorption by extraradical hyphae and translocation to the host plant (Schachtman et al., 1998). Amino acids, peptides, ions (NO_3^-, NH_4^+) and recalcitrant organic compounds are all forms of nitrogen found in the soil. The extraradical hyphae of different *Glomus* sp can assimilate and metabolise both organic and inorganic sources of nitrogen by glutamate synthetase activity (Li et al., 1991).

Pathogenic microorganisms are a major threat affecting plant health and ecosystem stability (Azcon-Aguilar and Barea, 1996). AM fungal colonization of plant roots increases the plants' tolerance to pathogens acting as biological control agents. Reviews on the subject have focused on mechanisms of interaction such as enhanced nutrition, competition, morphological changes, induced plant defence mechanisms and reduced infection sites (Hooker et al., 1994; Azcon-Aguilar and Barea, 1996).

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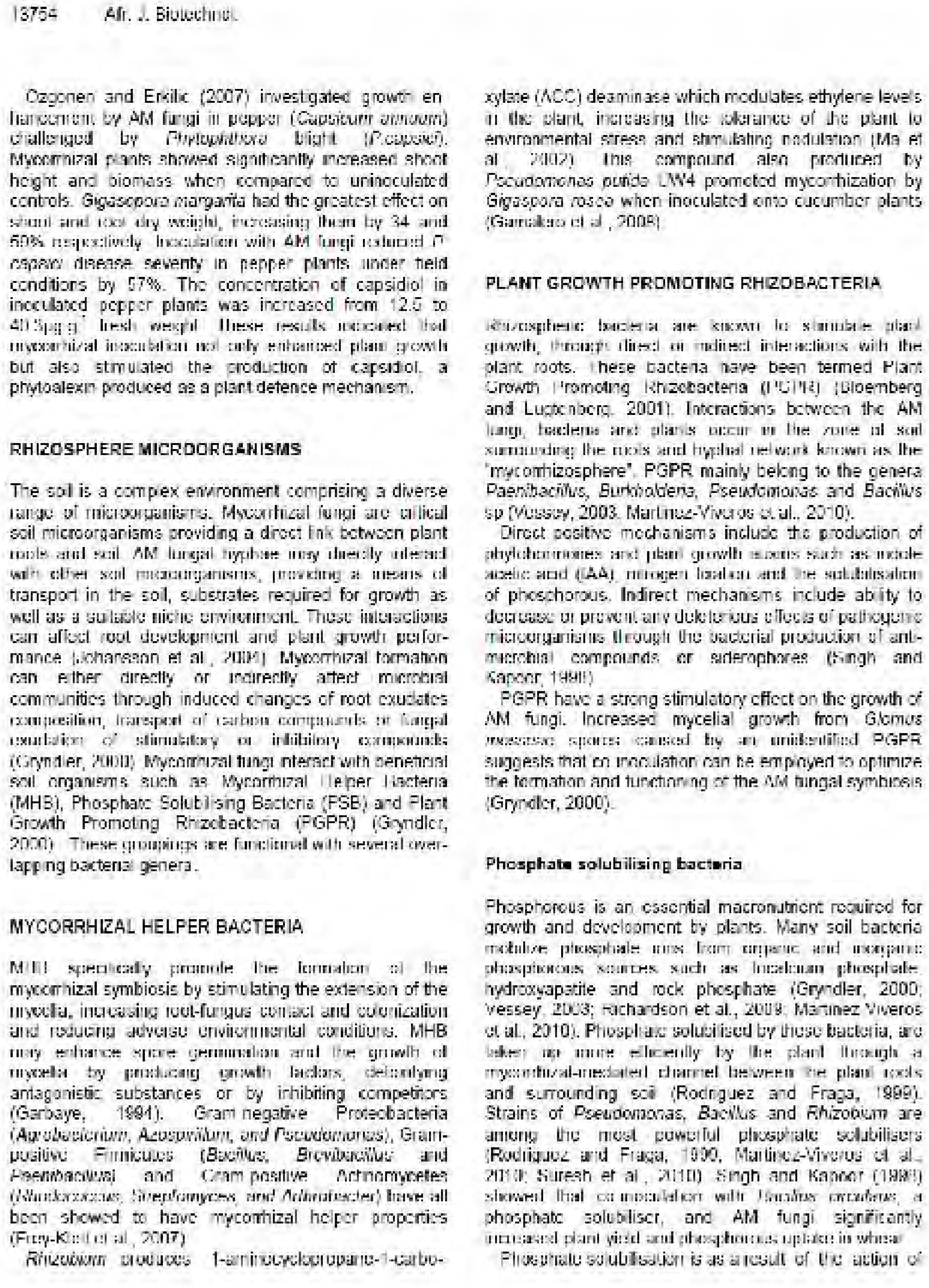


Ozgonen and Erkilic (2007) investigated growth enhancement by AM fungi in pepper (*Capsicum annuum*) challenged by *Phytophthora* blight (*P.capsici*). Mycorrhizal plants showed significantly increased shoot height and biomass when compared to uninoculated controls. *Gigaspora margarita* had the greatest effect on shoot and root dry weight, increasing them by 34 and 60% respectively. Inoculation with AM fungi reduced *P. capsici* disease severity in pepper plants under field conditions by 57%. The concentration of capsidiol in inoculated pepper plants was increased from 12.5 to $40.5 \mu g\ g^{-1}$ fresh weight. These results indicated that mycorrhizal inoculation not only enhanced plant growth but also stimulated the production of capsidiol, a phytoalexin produced as a plant defence mechanism.

RHIZOSPHERE MICROORGANISMS

The soil is a complex environment comprising a diverse range of microorganisms. Mycorrhizal fungi are critical soil microorganisms providing a direct link between plant roots and soil. AM fungal hyphae may directly interact with other soil microorganisms, providing a means of transport in the soil, substrates required for growth as well as a suitable niche environment. These interactions can affect root development and plant growth performance (Johansson et al., 2004). Mycorrhizal formation can either directly or indirectly affect microbial communities through induced changes of root exudates composition, transport of carbon compounds or fungal exudation of stimulatory or inhibitory compounds (Gryndler, 2000). Mycorrhizal fungi interact with beneficial soil organisms such as Mycorrhizal Helper Bacteria (MHB), Phosphate Solubilising Bacteria (PSB) and Plant Growth Promoting Rhizobacteria (PGPR) (Gryndler, 2000). These groupings are functional with several overlapping bacterial genera.

MYCORRHIZAL HELPER BACTERIA

MHB specifically promote the formation of the mycorrhizal symbiosis by stimulating the extension of the mycelia, increasing root-fungus contact and colonization and reducing adverse environmental conditions. MHB may enhance spore germination and the growth of mycelia by producing growth factors, detoxifying antagonistic substances or by inhibiting competitors (Garbaye, 1994). Gram negative Proteobacteria (*Agrobacterium, Azospirillum, and Pseudomonas*), Gram-positive Firmicutes (*Bacillus, Brevibacillus* and *Paenibacillus*) and Gram positive Actinomycetes (*Rhodococcus, Streptomyces, and Arthrobacter*) have all been showed to have mycorrhizal helper properties (Frey-Klett et al., 2007).

Rhizobium produces 1-aminocyclopropane-1-carboxylate (ACC) deaminase which modulates ethylene levels in the plant, increasing the tolerance of the plant to environmental stress and stimulating nodulation (Ma et al., 2002). This compound also produced by *Pseudomonas putida* UW4 promoted mycorrhization by *Gigaspora rosea* when inoculated onto cucumber plants (Gamalero et al., 2008).

PLANT GROWTH PROMOTING RHIZOBACTERIA

Rhizospheric bacteria are known to stimulate plant growth, through direct or indirect interactions with the plant roots. These bacteria have been termed Plant Growth Promoting Rhizobacteria (PGPR) (Bloemberg and Lugtenberg, 2001). Interactions between the AM fungi, bacteria and plants occur in the zone of soil surrounding the roots and hyphal network known as the "mycorrhizosphere". PGPR mainly belong to the genera *Paenibacillus, Burkholderia, Pseudomonas* and *Bacillus* sp (Vessey, 2003; Martinez-Viveros et al., 2010).

Direct positive mechanisms include the production of phytohormones and plant growth auxins such as indole acetic acid (IAA), nitrogen fixation and the solubilisation of phosphorous. Indirect mechanisms include ability to decrease or prevent any deleterious effects of pathogenic microorganisms through the bacterial production of antimicrobial compounds or siderophores (Singh and Kapoor, 1998).

PGPR have a strong stimulatory effect on the growth of AM fungi. Increased mycelial growth from *Glomus mosseae* spores caused by an unidentified PGPR suggests that co inoculation can be employed to optimize the formation and functioning of the AM fungal symbiosis (Gryndler, 2000).

Phosphate solubilising bacteria

Phosphorous is an essential macronutrient required for growth and development by plants. Many soil bacteria mobilize phosphate ions from organic and inorganic phosphorous sources such as tricalcium phosphate, hydroxyapatite and rock phosphate (Gryndler, 2000; Vessey, 2003; Richardson et al., 2009; Martinez Viveros et al., 2010). Phosphate solubilised by these bacteria, are taken up more efficiently by the plant through a mycorrhizal-mediated channel between the plant roots and surrounding soil (Rodriguez and Fraga, 1999). Strains of *Pseudomonas, Bacillus* and *Rhizobium* are among the most powerful phosphate solubilisers (Rodriguez and Fraga, 1999; Martinez-Viveros et al., 2010; Suresh et al., 2010). Singh and Kapoor (1998) showed that co-inoculation with *Bacillus circulans*, a phosphate solubiliser, and AM fungi significantly increased plant yield and phosphorous uptake in wheat.

Phosphate solubilisation is as a result of the action of bacterial producing organic acids, particularly gluconic acid and 2-ketogluconic acid (Khan et al., 2009). Production of chelating substances or inorganic acids such as sulphidric, nitric and carbonic acid may also contribute to the process (Rodriguez and Fraga, 1999; Vessey, 2003; Richardson et al., 2009; Martinez Viveros et al., 2010).

Organic phosphate sources are mineralised by the action of several phosphatase enzymes. Phosphatase activity involves the hydrolysis of phosphor ester or phosphor anhydride bonds, thereby catalysing the bound phosphorous into inorganic phosphorous (Rodriguez and Fraga, 1999; Richardson et al., 2009; Martinez Viveros et al., 2010). Phytate (Myo-inositol hexakis-phosphate) constitutes 80% complexed inorganic phosphorous in the soil making it one of the most abundant sources of phosphorous for plants (Lim et al., 2007). Several PGPR (*Bacillus, Burkholderia, Pseudomonas, Serratia* and *Staphylococcus*) produce the enzyme phytase, which degrades phytate to lower phosphate esters (Hariprasad and Niranjana, 2009).

Hariprasad and Niranjana (2009) showed that an *Enterobacter* sp. which was able to solubilise calcium phosphate through the production of gluconic acid significantly increased growth of tomato (*Lycopersicon esculentum*). Fernandez et al. (2007) investigated the influence of phosphate solubilising ability of bacterial isolates on soybean (*Glycine max*) growth under greenhouse conditions. Inoculation with *Burkolderia* significantly increased plant height by 40%.

Another study by Akhtar and Siddiqui (2009) examined the effects of phosphate solubilising bacteria on the growth of chickpea (*Cicer arietinum*) under field conditions. Results indicated that *Paenibacillus polymyxa*, *Pseudomonas putida*, *Pseudomonas alcaligenes* and *Pseudomonas aeruginosa* significantly increased shoot dry weight, seed weight and yield with *P. polymyxa* having the greatest effect. Canbolat et al. (2006) examined the effects of PGPR on barley (*Hordeum vulgare*) seedling growth. Four *Bacillus* isolates all solubilised phosphate. Available phosphate in the soil was significantly increased by seed inoculation with two of these isolates. The isolates also increased root and shoot weights.

Nitrogen fixation

Nitrogen is an essential plant nutrient. Symbiotic and non-symbiotic nitrogen fixation is a function of some PGPR. Non symbiotic nitrogen fixation is carried out by free living diazotrophs and can stimulate non legume plant growth. These bacteria belong to the genera *Azoarcus, Azospirillum, Burkholderia, Gluconacetobacter* and *Pseudomonas* (Antoun and Prevost, 2005; Richardson et al., 2009; Martinez Viveros et al., 2010). Symbiotic nitrogen fixers including *Rhizobium*, develop symbiotic relationships with legume plants and convert atmospheric N_2 to an inorganic form within nodules, providing 90% of the nitrogen requirements of the plant. Endophytic diazotrophs appear to have an advantage over root surface organisms since they are capable of colonising the interior of roots and establish themselves within niches that are more conducive to effective N_2 fixation, transferring the fixed nitrogen to the host plants. In addition to their potential for supplying nitrogen through N_2 fixation to the host plants they may also promote plant growth through various other mechanisms such as phytohormone production (Richardson et al., 2009).

Phytohormone production

PGPR can have an influence on plant growth through the production of phytohormones such as auxins, cytokinins and gibberellins. Auxins contribute to the endogenous pool of phytohormones produced by the plant (Martinez-Viveros et al., 2010). The production of indole acetic acid (IAA) has been shown to be widespread among PGPR (Xie et al., 1996; Patten and Glick, 2002) and is predominantly synthesized by an alternate tryptophan dependant pathway which is carried out through indole-pyruvic acid. The role of IAA produced by the PGPR in plant growth is still undetermined. IAA in plants is the main auxin which controls many important physiological processes including cell enlargement and division, tissue differentiation and responses to light and gravity (Patten and Glick, 2002; Spaepen et al., 2007; Shahab et al., 2009; Martinez-Viveros et al., 2010). Therefore, PGPR which produce IAA have the potential to interact with any of the fore mentioned processes. IAA produced by PGPR can promote root growth (Spaepen et al., 2007), subsequently increasing mycorrhizal contact (Garbaye, 1994). PGPR which produce IAA belong to the following genera: *Aeromonas, Bacillus, Burkholderia, Enterobacter, Pseudomonas* and *Rhizobium* (Martinez Viveros et al., 2010).

Rapid establishment of roots can be achieved either by the elongation of primary roots or by the proliferation of lateral and adventitious roots. This is advantageous for young seedlings as it increases their stability in the soil and survival through rapid access to water and nutrients (Patten and Glick, 2002). Dobbelaere et al. (1999) showed that increased rooting was directly related to IAA synthesis by *Azospirillum*. Increased plant mineral uptake and root exudation in turn stimulated bacterial colonisation further enhancing the inoculation effect. Patten and Glick (2002) showed that low concentrations of bacterial produced IAA stimulated primary root elongation. High IAA concentrations stimulated the formation of lateral and adventitious roots. IAA produced by PGPR can therefore have many beneficial influences on plant growth by altering root development. Shahab et al. (2009) investigated two IAA producing strains of *Bacillus* *fluorogenes* and *Pseudomonas aeruginosa* inoculated onto mung beans (*Vignia radiate*) in greenhouse experiments. The *P. aeruginosa* isolate showed the most significant root and shoot effects, suggesting a direct effect on metabolic processes. Development of lateral roots and increased root hair proliferation increased the surface area available for nutrient uptake and increased shoot and root growth (Shahab et al., 2009).

Cytokinins are phytohormones which promote cell division and enlargement, tissue expansion and root hair development. Cytokinin production has been shown in various PGPR including *Azospirillum*, *Pseudomonas fluorescens* and *Paenibacillus polymyxa* (Madhaiyan et al., 2010). Gibberellins enhance the development of plant tissues, particularly stem tissue and promote root elongation and lateral root extension. Production of gibberellins has been reported in *Azospirillum*, *Bacillus pumilus*, *Bacillus licheniformis* and *Rhizobium* (Vessey, 2003; Martinez-Viveros et al., 2010).

Ethylene effects plant growth by inhibiting root elongation. Plants produce 1-aminocyclopropane-1-carboxylate (ACC) which is the precursor for ethylene. Some ACC is released into the soil and reabsorbed by the roots, which leads to diminished root growth. Some PGPR have the ability to synthesize ACC deaminase, an enzyme which cleaves ACC which thereby decreases ethylene production, promoting root lengthening. ACC deaminase activity has been reported in the genera *Achromobacter*, *Azospirillum*, *Bacillus* and *Pseudomonas* (Vessey, 2003; Martinez-Viveros et al., 2010.

Madhaiyan et al. (2010) studied the effect of co-inoculations of *Methylobacterium oryzae* with *Azospirillum brasilense* and *Burkholderia pyrrocinia* on the growth of tomato (*Lycopersicon esculentum*), red pepper (*Capsicum annum*) and rice (*Oryza sativa*). Results showed that *M. oryzae* through the production of phytohormones such as IAA and cytokinins improved plant growth. Other mechanisms such ACC deaminase and the production of siderophores have also been documented. *Azospirillum* is a known nitrogen fixer and a producer of IAA whereas *Burkholderia* has been shown to solubilise phosphate and have ACC deaminase activity. Under greenhouse conditions, there was a significant increase in all plant growth parameters by the bacterial isolates compared to the non-inoculated control plants. In tomato, individual inoculation of *M.oryzae* or its co-inoculation with *A. brasilense* / *B. pyrrocinia* produced significant increases in root length compared to control or individual inoculations. In red peppers, inoculation with *M.oryzae* produced the greatest root and shoot lengths while a greater root and shoot growth was found with the dual inoculation of *M.oryzae* with *B. pyrrocinia*. In rice, no significant increases in root length were recorded. Cytokinins produced by *M.oryzae* may enhance stomatal opening and promote cell division in the presence of auxins resulting in an enhanced uptake of water and other nutrients from the soil. Thus cytokinin and IAA production by *M.oryzae* may have a positive effect on plant growth (Madhaiyan et al., 2010).

Mena-Violante and Olalde-Portugal (2007) showed in a greenhouse trial that inoculation of tomato with *Bacillus subtilis* significantly increased root dry weight and root length by 18 to 26% and 13 to 15%, respectively in two experiments. Yield per plant was increased by 21 to 25% and fruit weight and length was significantly greater. These significant effects were attributed to the production of hormones, which are believed to change assimilate partitioning patterns in plants, altering growth in roots, fructification process and development of fruit.

Effects of floral and foliar inoculation of *Pseudomonas* and *Bacillus* rhizobacteria on the growth of sweet cherry (*Prunus avium* L.) was investigated by Esitken et al. (2006). *Pseudomonas* produces transzeatin and *Bacillus* has the ability to fix nitrogen and produce IAA. The bacterial treatments alone and in combination significantly affected to varying degrees yield per trunk cross-section area, fruit weight and shoot length. This indicates that these PGPR are not restricted to the soil environment, application to above plant parts has great potential for commercial applications.

A similar study by Orhan et al. (2006) examined the effects of two *Bacillus* isolates, both of which had nitrogen fixing properties and one with phosphate solubilising capabilities on the growth of raspberry. A significant 75% increase in yield was achieved with co-inoculation of the two isolates. The authors also reported increased N and P content of rapsberry leaves, and a decrease in pH of the soil, due to the production of organic acids. Similar significant yield increases have also been reported in apple (*Malus domestica* L.) cv. Granny Smith when co-inoculated with *Bacillus* and *Microbacterium* (Karlidag et al., 2007).

Banchio et al. (2008) demonstrated improved plant growth and essential oils composition in *Origanum majorana* L. by the PGPR strains *Pseudomonas fluorescens*, *Bacillus subtilis*, *Sinorhizobium meliloti*, and *Bradyrhizobium* sp. Inoculation with *P. fluorescens* or *Bradyrhizobium* sp. induced significant increases in number of leaves, shoot length, and number of nodes. Leaf number was 60% higher in *P. fluorescens* inoculated plants resulting in a 3.2 fold increase in shoot fresh weight. Plants inoculated with *P. fluorescens* or *Bradyrhizobium* showed significant increase in total essential oil yield, 24 - and 10-fold, respectively.

Pathogen Inhibition

PGPR have been shown to mediate in the biological control of plant pathogens. The mechanisms involved can be direct via the production of antibiotics, siderophores, hydrogen cyanide, hydrolytic enzymes (chitinases, proteases and cellulases) or indirectly by competition with the pathogen for ecological niches such as infection and nutrient sites (Linderman, 2000; Bloemberg and Lugtenberg, 2001; Sharma and Johri, 2002).

PGPR have been shown to produce various antibiotics effective against phytopathogens under laboratory conditions. These antibiotics include butyrolactones, zwittermycin A, kanosamine and 2,4-diacetylphloroglucinol (2, 4-DAPG). 2, 4-DAPG is one of the most efficient antibiotics and is produced by *Pseudomonas* strains. It has a wide spectrum including being an antifungal, antibacterial and antihelmintic (Whipps, 2001; Martinez-Viveros et al., 2010). Some PGPR such as *Alcaligenes, Aeromonas, Bacillus, Pseudomonas* and *Rhizobium* are capable of producing hydrogen cyanide (HCN), a volatile secondary metabolite which suppresses the development of other microbes (Whipps, 2001; Martinez-Viveros et al., 2010).

Iron is an essential element required for growth in all organisms and bioavailable iron in the soil is deficient. Under iron-limiting environments, PGPR produce siderophores, low molecular weight compounds which competitively sequesters ferric iron. The bacterium, takes up the iron-siderophore complex through specific receptors specific on the outer cell membrane. Once inside the bacterium, the iron is released and available to support microbial growth (Siddiqui, 2005). The siderophores deprive pathogenic fungi of the available iron in the soil as the PGPR siderophores have a greater affinity for the iron. Some PGPR can sequester iron from heterologous siderophores produced by other soil microorganisms. Siderophore producing bacteria have been found to belong to *Bradyrhizobium, Pseudomonas, Rhizobium, Serratia* and *Streptomyces* (Martinez-Viveros et al., 2010).

The root surface and its surrounding rhizosphere are significant sources of carbon. Therefore, along the surfaces of the roots there are a variety of nutrient rich niches which attracts a wide range of microorganisms, including phytopathogens. Competition for these nutrient niches is one of the major mechanisms by which PGPR protect the plant from pathogens. PGPR have the ability to reach the surfaces of the roots through active motility by their flagella and are guided by chemotrophic responses. They are also carried by mycorrhizal hyphal network (Compant et al., 2005).

ENDOSYMBIOTIC BACTERIA OF AM FUNGI

Endocellular bacteria are reported to be found only in a few fungi including AM fungi. The cytoplasm of AM fungi contains many bacteria-like organisms. Microscopy revealed these bacterial cells as Gram negative and rod shaped, occurring singly or in groups, often inside fungal vacuoles in both spores and hyphae and have been described as being related to *Burkholderia* and were placed in a new unculturable taxon named *Candidatus Glomeribacter Gigasporarum*"(Bianciotto and Bonfante, 2002; Bonfante and Anca, 2009).

The functional significance of these endosymbionts is unclear but they are thought to affect AM fungal performance through the release of substances which affect fungal gene expression, introduction of chemical compounds into the spores stimulating germination, increasing fungal attachment by producing lectins and degradation of fungal cell walls (Artursson et al., 2006; Bonfante and Anca, 2009; Miransari, 2011).

Endosymbiotic bacterial genes have been partially identified, some of which are involved in nutrient uptake, such as a putative phosphate transporter operon, *pst*, a gene involved in colonisation events by bacterial cells, *vac* and nitrogenase coding genes, *nif* (Bianciotto and Bonfante, 2002).

INTERACTIONS BETWEEN AM FUNGI AND RHIZOSPHERE MICRO-ORGANISMS

The use of AM fungi to enhance plant growth and yield of various crops is gaining importance. The interactions between AM fungi and other rhizosphere micro-organisms therefore have the potential to synergistically enhance crop production. Several studies have been carried out to analyse the interactions between AM fungi and rhizosphere micro-organisms.

Interactions between AM fungi and MHB

Studies investigating the interactions between AM fungi and MHB on plant growth have shown that MHB increase AM fungal colonisation in the plant roots. Mamatha et al. (2002) investigated the interactions between AM fungi and *Bacillus coagulans* in field established mulberry and papaya plants. Mulberry plants inoculated with *Glomus fasciculatum* showed a significant increase in plant height and number of leaves and in papaya plants inoculated with *Glomus mosseae* and *Glomus caledonium* a significant increase in plant height and and stem girth compared with the control given 100% recommended P. There was no significant difference in plant growth parameters between treatments with the AM fungi alone or the AM fungi with *Bacillus coagulans*. Mycorrhizal colonisation however, was highest in plants inoculated with the AM fungi and *B.coagulans*, a possible mechanism here may be that MHB produce hydrolytic enzymes which cause the cortical cells to dilate, providing a larger intercellular surface area with which the AM fungi can penetrate and colonise more easily, increasing the percentage root colonisation, thereby providing more nutrients to the plant (Mamatha et al. 2002).

Interactions between AM fungi and PGPR

The improvement of plant growth and nutrition through the synergistic interaction between AM fungi and PGPR has been described in several studies. In P-deficient soils, phosphate solubilising micro organisms release phosphate ions which are transferred by the AM fungi to the plant (Artursson et al., 2006).

Khan and Zaidi (2007) examined the co inoculation of a nitrogen-fixing (*Azotobacter chroococcum*), a phosphate-solubilising bacterium (*Bacillus* sp.), and *Glomus fasciculatum* on the growth of wheat (*Triticum aestivum* L.). The dual inoculations of *A. chroococcum* and *Bacillus*, *A. chroococcum* and *G. fasciculatum*, and *Bacillus* and *G. fasciculatum* increased dry matter significantly. The co-inoculation of *Bacillus* and *G. fasciculatum* enhanced dry matter accumulation in roots, shoots and whole plants by 1.7, 1.5 and 1.6-fold, respectively and was superior to other single or dual inoculation treatments. The triple inoculation of *A. chroococcum*, *Bacillus* and *G. fasciculatum* increased total dry matter and doubled the grain yield of wheat. Increased number of AM spores and percentage root colonisation was also recorded. The results were attributed to solubilisation of inorganic phosphate by *Bacillus*. *A. chroococcum* is a phyto-stimulator as well as nitrogen fixer, providing plant growth promoting substances such as hormones (Khan and Zaidi, 2007).

Bharadwaj et al. (2008) investigated the effects of the AM fungal spore-associated *Pseudomonas putida* biotype A on potato (*Solanum tuberosum* L.) growth. The bacteria was shown to solubilises phosphate and produce IAA and significantly increased the number of primary roots by 100%, the number of lateral roots by 95% and root length by 79%, shoot length by 73% and the number of leaves by 81%. This isolate also increased the root colonisation by *G. mosseae*. Gamalero et al. (2004) investigated the impact of two pseudomonads (*Pseudomonas fluorescens* 92rk and *Pseudomonas fluorescens* P190r) and *G. mosseae* on tomato (*Lycopersicon esculentum* Mill.) plant growth. *Pseudomonas fluorescens* 92rk increased mycorrhizal colonisation by 41%. *Pseudomonas fluorescens* 92rk and *Pseudomonas fluorescens* P190r significantly increased both the shoot and root fresh weights whereas *G. mosseae* alone increased the shoot fresh weights. Co-inoculation of 92rk and *G. mosseae* induced significant increases in shoot fresh weight. Co-inoculation of all three microorganism increased shoot and root fresh weights relative to all other treatments.

Khan and Zaidi (2006) investigated interactions between *Bacillus subtilis*, *Bradyrhizobium* sp. and *Glomus fasciculatum* on greengram (*Vignia radiata* (L.) Wilczek). Dual inoculation of *G. fasciculatum* and *B. subtilis* significantly increased the root length at the flowering stage. The most significant inoculation combination was that of *Bradyrhizobium*, *G. fasciculatum* and *B. subtilis* which increased the dry matter production significantly by 200 and 103% at flowering and harvest stages respectively. The more efficient use of P through the interaction with *Bacillus* enhances nitrogen fixation which is highly dependent on P. Plant growth and yield of greengram plants was therefore increased.

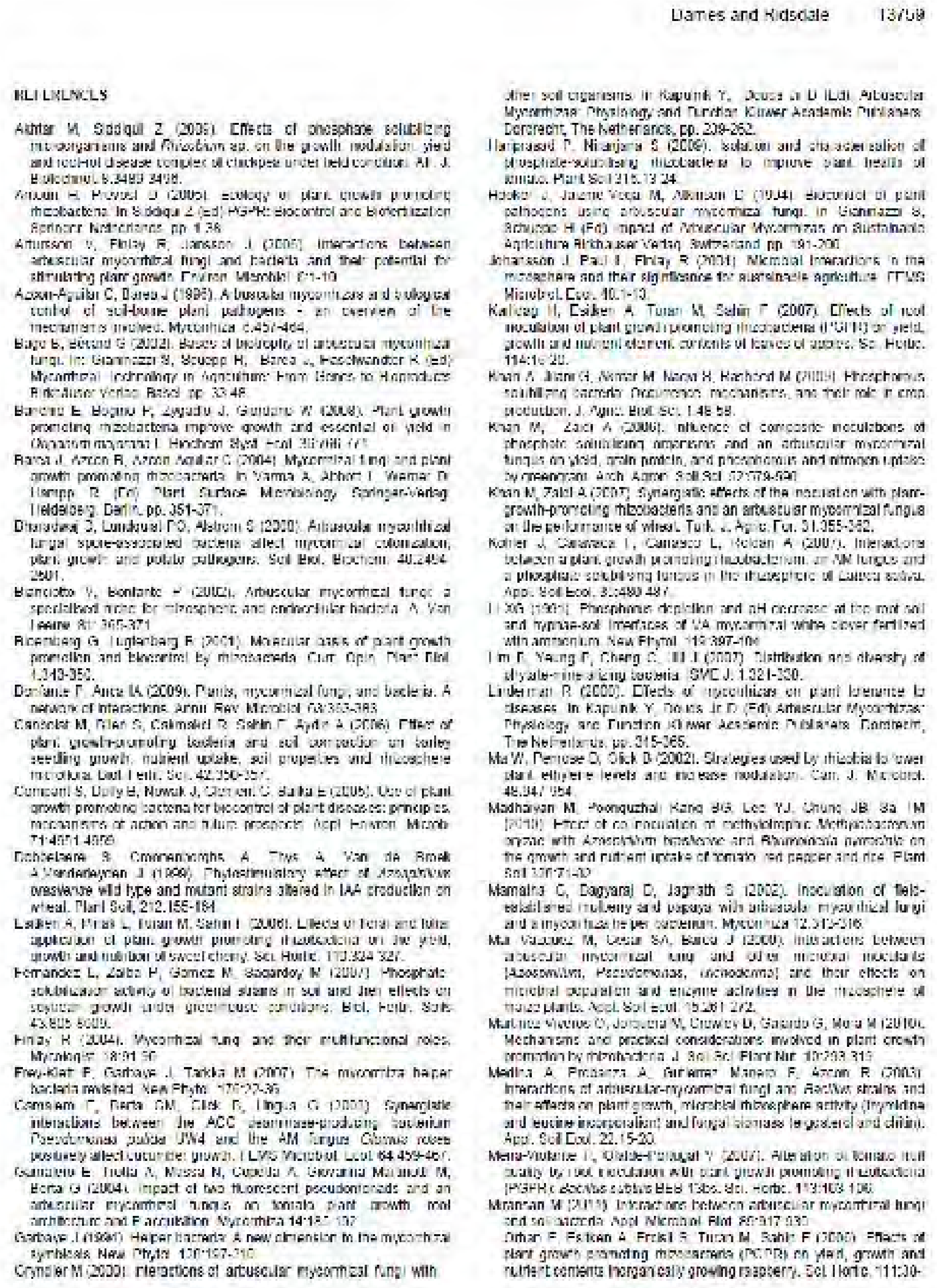
Kohler et al. (2007) studied the interactions between a *B. subtilis* and *Glomus intraradices* and their effects on lettuce (*Latuca sativa*). Dual applications had the highest effect (77%) on shoot biomass and this was attributed to an enhanced P and K nutrition. *B. subtilis* has been shown to be a phosphate- and potassium-solubilising rhizobacterium releasing these nutrients from silicates in the soil which are than translocated by extra radical AM hyphae and transferred to the crop plant (Kohler et al., 2007; Rodriguez and Fraga, 1999).

Mar Vazquez et al. (2000) inoculated maize (*Zea mays* L.) with *Azospirillum brasilense*, known to produce IAA and was found to have a significant increase in shoot and root dry weight in the dual inoculation with *Glomus deserticola*. They confirmed the ability of growth promoting substances to stimulate plant susceptibility to mycorrhizal colonisation, enhance spore germination and mycelial growth, which in turn increased the chance of contact between fungal hyphae and plant roots, indicating a functional compatibility between saprotrophic and symbiotic micro organisms.

Medina et al. (2003) similarly investigated interactions between *G. mosseae*, *G. intraradices* and *G. deserticola* and *Bacillus pumilus* and *Bacillus licheniformis* on alfalfa plants (*Medicago sativa*). *B. pumilus* and *B. licheniformis* produce IAA and gibberellins respectively. Single bacterium treatments did not have an effect on plant growth parameters. *G. intraradices* and *G. deserticola* increased shoot weight. *G. deserticola* and *B. pumilus* was the most efficient treatment resulting in 715% increase in shoot weight and 180% increase in root length.

CONCLUSION

Interactions between AM fungi, PGPR and P solubising bacteria occur naturally since they share common habitats such as the root surface (Barea et al., 2004). It is well known from the many studies cited that these interactions increase plant growth through several mechanisms carried out by both the AM fungi and the bacteria (Antoun and Prevost, 2005; Miransari, 2011). In combination this has a beneficial effect on plant growth and health. The challenge in agrobiotechnology is harnessing these interactions either through promotion of bacterial growth in the soil or through the development and application of inoculants. These inoculants can be applied separately or formulations of effective multiagents combined with AM fungi should be further investigated. Harnessing the soil microbial communities is required in order to substantially enhance crop production with reduced chemical inputs and environmental damage.



REFERENCES

Akhtar M, Siddiqui Z (2009). Effects of phosphate solubilizing microorganisms and *Rhizobium* sp. on the growth, nodulation, yield and root-rot disease complex of chickpea under field condition. Afr. J. Biotechnol. 8:3489-3496.

Antoun H, Prévost D (2005). Ecology of plant growth promoting rhizobacteria. In Siddiqui Z (ed) PGPR: Biocontrol and Biofertilization Springer, Netherlands, pp. 1-38.

Artursson V, Finlay R, Jansson J (2006). Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. Environ. Microbiol. 8:1-10.

Azcón-Aguilar C, Barea J (1996). Arbuscular mycorrhizas and biological control of soil-borne plant pathogens – an overview of the mechanisms involved. Mycorrhiza 6:457-464.

Bago B, Bécard G (2002). Bases of biotrophy of arbuscular mycorrhizal fungi. In: Gianinazzi S, Schüepp H, Barea J, Haselwandter K (ed) Mycorrhizal Technology in Agriculture: From Genes to Bioproducts Birkhäuser Verlag, Basel, pp. 33-48.

Banchio E, Bogino P, Zygadlo J, Giordano W (2008). Plant growth promoting rhizobacteria improve growth and essential oil yield in *Origanum majorana* L. Biochem. Syst. Ecol. 36:766-771.

Barea J, Azcón R, Azcón-Aguilar C (2004). Mycorrhizal fungi and plant growth promoting rhizobacteria. In Varma A, Abbott L, Werner D, Hampp R (Ed) Plant Surface Microbiology. Springer-Verlag, Heidelberg, Berlin, pp. 351-371.

Bharadwaj D, Lundquist PO, Alström S (2008). Arbuscular mycorrhizal fungal spore-associated bacteria affect mycorrhizal colonization, plant growth and potato pathogens. Soil Biol. Biochem. 40:2494-2501.

Bianciotto V, Bonfante P (2002). Arbuscular mycorrhizal fungi: a specialized niche for rhizospheric and endocellular bacteria. A. Van Leeuw. 81: 365-371.

Bloemberg G, Lugtenberg B (2001). Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr. Opin. Plant Biol. 4:343-350.

Bonfante P, Anca IA (2009). Plants, mycorrhizal fungi, and bacteria: A network of interactions. Annu. Rev. Microbiol. 63:363-383.

Canbolat M, Bilen S, Çakmakçi R, Sahin F, Aydin A (2006). Effect of plant growth-promoting bacteria and soil compaction on barley seedling growth, nutrient uptake, soil properties and rhizosphere microflora. Biol. Fertil. Soils 42:350-357.

Compant S, Duffy B, Nowak J, Clément C, Barka E (2005). Use of plant growth promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action and future prospects. Appl. Environ. Microb. 71:4951-4959.

Dobbelaere S, Croonenborghs A, Thys A, Vande Broek A, Vanderleyden J (1999). Phytostimulatory effect of *Azospirillum brasilense* wild type and mutant strains altered in IAA production on wheat. Plant Soil, 212:155-164.

Esitken A, Pirlak L, Turan M, Sahin F (2006). Effects of floral and foliar application of plant growth promoting rhizobacteria on the yield, growth and nutrition of sweet cherry. Sci. Hortic. 110:324-327.

Fernández L, Zalba P, Gómez M, Sagardoy M (2007). Phosphate-solubilization activity of bacterial strains in soil and their effects on soybean growth under greenhouse conditions. Biol. Fertil. Soils 43:805-809.

Finlay R (2004). Mycorrhizal fungi and their multifunctional roles. Mycologist 18:91-96.

Frey-Klett P, Garbaye J, Tarkka M (2007). The mycorrhiza helper bacteria revisited. New Phytol. 176:22-36.

Gamalero E, Berta GM, Glick B, Lingua G (2008). Synergistic interactions between the ACC deaminase-producing bacterium *Pseudomonas putida* UW4 and the AM fungus *Glomus mosseae* positively affect cucumber growth. FEMS Microbiol. Ecol 64:459-467.

Gamalero E, Trotta A, Massa N, Copetta A, Giovanna Martinotti M, Berta G (2004). Impact of two fluorescent pseudomonads and an arbuscular mycorrhizal fungus on tomato plant growth, root architecture and P acquisition. Mycorrhiza 14:185-192.

Garbaye J (1994). Helper bacteria: A new dimension to the mycorrhizal symbiosis. New Phytol. 128:197-210.

Gryndler M (2000). Interactions of arbuscular mycorrhizal fungi with other soil organisms. In Kapulnik Y, Douds Jr D (Ed). Arbuscular Mycorrhizas: Physiology and Function Kluwer Academic Publishers. Dordrecht, The Netherlands, pp. 239-262.

Hariprasad P, Niranjana S (2009). Isolation and characterization of phosphate-solubilizing rhizobacteria to improve plant health of tomato. Plant Soil 316:13-24.

Hooker J, Jaizme-Vega M, Atkinson D (1994). Biocontrol of plant pathogens using arbuscular mycorrhizal fungi. In Gianinazzi S, Schüepp H (Ed) Impact of Arbuscular Mycorrhizas on Sustainable Agriculture Birkhäuser Verlag, Switzerland, pp. 191-200.

Johansson J, Paul L, Finlay R (2004). Microbial interactions in the mycorrhizosphere and their significance for sustainable agriculture. FEMS Microbiol. Ecol. 48:1-13.

Karlidag H, Esitken A, Turan M, Sahin F (2007). Effects of root inoculation of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrient element contents of leaves of apple. Sci. Hortic. 114:16-20.

Khan A, Jilani G, Akhtar M, Naqvi S, Rasheed M (2009). Phosphorus solubilizing bacteria: Occurrence, mechanisms, and their role in crop production. J. Agric. Biol. Sci. 1:48-58.

Khan M, Zaidi A (2006). Influence of composite inoculations of phosphate solubilizing organisms and an arbuscular mycorrhizal fungus on yield, grain protein, and phosphorous and nitrogen uptake by greengram. Arch. Agron. Soil Sci. 52:579-590.

Khan M, Zaidi A (2007). Synergistic effects of the inoculation with plant-growth-promoting rhizobacteria and an arbuscular mycorrhizal fungus on the performance of wheat. Turk. J. Agric. For. 31:355-362.

Kohler J, Caravaca F, Carrasco L, Roldán A (2007). Interactions between a plant growth promoting rhizobacterium, an AM fungus and a phosphate solubilising fungus in the rhizosphere of *Lactuca sativa*. Appl. Soil Ecol. 35:480-487.

Li XG (1991). Phosphorus depletion and pH decrease at the root-soil and hyphae-soil interfaces of VA mycorrhizal white clover fertilized with ammonium. New Phytol. 119:397-404.

Lim B, Yeung P, Cheng C, Hill J (2007). Distribution and diversity of phytate-mineralizing bacteria. ISME J. 1:321-330.

Linderman R (2000). Effects of mycorrhizas on plant tolerance to diseases. In Kapulnik Y, Douds Jr D (Ed) Arbuscular Mycorrhizas: Physiology and Function Kluwer Academic Publishers. Dordrecht, The Netherlands, pp. 345-365.

Ma W, Penrose D, Glick B (2002). Strategies used by rhizobia to lower plant ethylene levels and increase nodulation. Can. J. Microbiol. 48:947-954.

Madhaiyan M, Poonguzhali S, Kang BG, Lee YJ, Chung JB, Sa TM (2010). Effect of co-inoculation of methylotrophic *Methylobacterium oryzae* with *Azospirillum brasilense* and *Burkholderia pyrrocinia* on the growth and nutrient uptake of tomato, red pepper and rice. Plant Soil 328:71-82.

Mamatha G, Bagyaraj D, Jaganath S (2002). Inoculation of field-established mulberry and papaya with arbuscular mycorrhizal fungi and a mycorrhiza helper bacterium. Mycorrhiza 12:313-316.

Mar Vázquez M, César SA, Barea J (2000). Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (*Azospirillum*, *Pseudomonas*, *Trichoderma*) and their effects on microbial population and enzyme activities in the rhizosphere of maize plants. Appl. Soil Ecol. 15:261-272.

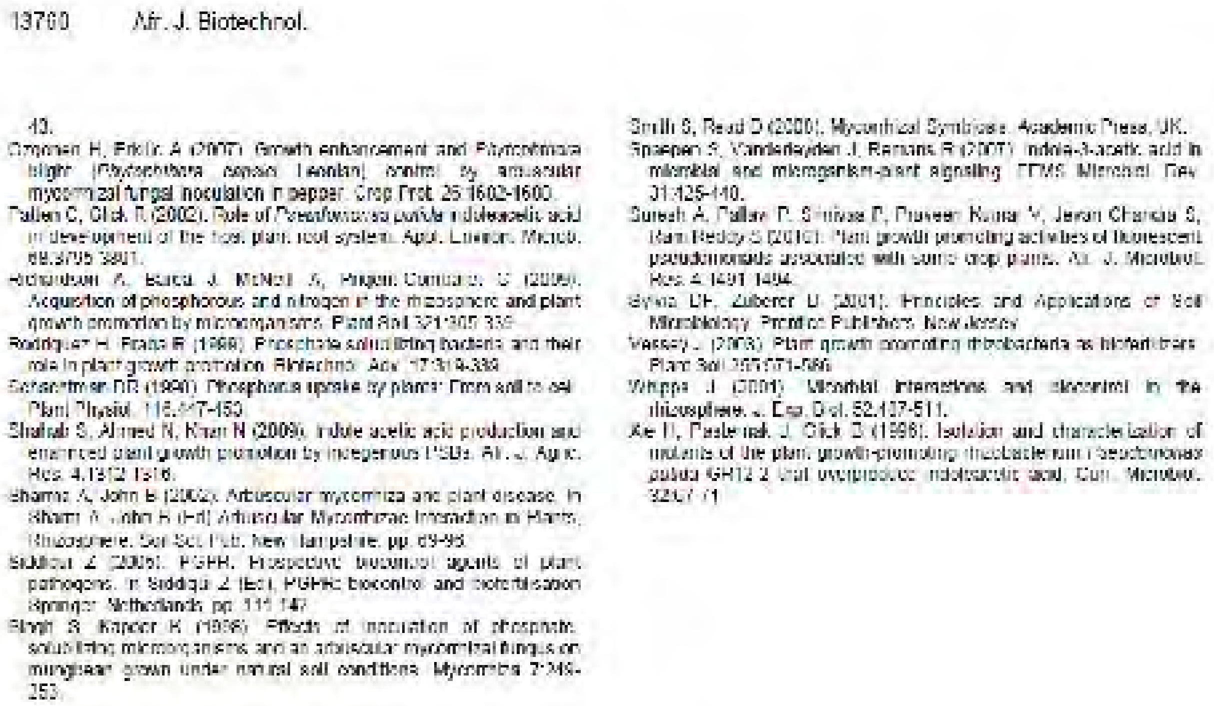
Martínez-Viveros O, Jorquera M, Crowley D, Gajardo G, Mora M (2010). Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. J. Soil Sci. Plant Nutr. 10:293-319.

Medina A, Probanza A, Gutierrez Mañero F, Azcón R (2003). Interactions of arbuscular-mycorrhizal fungi and *Bacillus* strains and their effects on plant growth, microbial rhizosphere activity (thymidine and leucine incorporation) and fungal biomass (ergosterol and chitin). Appl. Soil Ecol. 22:15-28.

Mena-Violante H, Olalde-Portugal V (2007). Alteration of tomato fruit quality by root inoculation with plant growth promoting rhizobacteria (PGPR): *Bacillus subtilis* BEB-13bs. Sci. Hortic. 113:103-106.

Miransari M (2011). Interactions between arbuscular mycorrhizal fungi and soil bacteria. Appl. Microbiol. Biot. 89:917-930.

Orhan E, Esitken A, Ercisli S, Turan M, Sahin F (2006). Effects of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrient contents in organically growing raspberry. Sci. Hortic. 111:38-



13760 Afr. J. Biotechnol.

43.
Ozgonen H, Erkilic A (2007). Growth enhancement and *Phytophthora* blight (*Phytophthora capsici* Leonian) control by arbuscular mycorrhizal fungal inoculation in pepper. Crop Prot. 26:1682-1688.
Patten C, Glick B (2002). Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. Appl. Environ. Microb. 68:3795-3801.
Richardson A, Barea J, McNeil A, Prigent-Combaret C (2009). Acquisition of phosphorous and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant Soil 321:305-339.
Rodriguez H, Fraga R (1999). Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol. Adv. 17:319-339.
Schachtman DP (1998). Phosphorus uptake by plants: From soil to cell. Plant Physiol. 116:447-453.
Shahab S, Ahmed N, Khan N (2009). Indole acetic acid production and enhanced plant growth promotion by indigenous PSBs. Afr. J. Agric. Res. 4:1312-1316.
Sharma A, Johri B (2002). Arbuscular mycorrhiza and plant disease. In Sharma A, Johri B (Ed) Arbuscular Mycorrhizae Interaction in Plants, Rhizosphere. Sci Sci Pub. New Hampshire. pp. 69-96.
Siddiqui Z (2005). PGPR: Prospective biocontrol agents of plant pathogens. In Siddiqui Z (Ed), PGPR: biocontrol and biofertilisation Springer Netherlands. pp. 111-142.
Singh S, Kapoor K (1998). Effects of inoculation of phosphate-solubilizing microorganisms and an arbuscular mycorrhizal fungus on mungbean grown under natural soil conditions. Mycorrhiza 7:249-253.
Smith S, Read D (2008). Mycorrhizal Symbiosis. Academic Press, UK.
Spaepen S, Vanderleyden J, Remans R (2007). Indole-3-acetic acid in microbial and microorganism-plant signaling. FEMS Microbiol. Rev. 31:425-448.
Suresh A, Pallavi P, Srinivas P, Praveen Kumar V, Jeevan Chandra S, Ram Reddy S (2010). Plant growth promoting activities of fluorescent pseudomonads associated with some crop plants. Afr. J. Microbiol. Res. 4:1491-1494.
Sylvia DH, Zuberer D (2001). Principles and Applications of Soil Microbiology. Prentice Publishers. New Jersey.
Vessey J (2003). Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255:571-586.
Whipps J (2001). Microbial interactions and biocontrol in the rhizosphere. J. Exp. Bot. 52:487-511.
Xie H, Pasternak J, Glick B (1996). Isolation and characterization of mutants of the plant growth-promoting rhizobacterium *Pseudomonas putida* GR12-2 that overproduce indoleacetic acid. Curr. Microbiol. 32:67-71.