Investigating the use of Arbuscular Mycorrhizas and Plant Growth Promoting Bacteria to improve the drought tolerance of maize (*Zea mays* L.).

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Abstract

Maize (*Zea mays* L.) is a direct staple food crop in Africa and remains an essential component of global food security, with maize crops accounting for over 60% of the total harvested area of annual food crops. Stress caused by drought and high soil salinity limits crop growth and productivity more than any other single environmental factor, with grain yield reductions up to 76% depending on the severity of the drought and the plant growth stage. Arbuscular mycorrhizal (AM) fungi and Plant Growth Promotion Rhizobacteria (PGPR) have previously been shown to improve tolerance of plants to drought stress through a number of chemical and physiological processes. The aim of this investigation was to determine whether mycorrhizal fungi and rhizobacteria adapted to drought and saline conditions and possessing plant growth promoting (PGP) traits were able to stimulate plant growth responses when applied to *Zea mays* seeds growing under greenhouse conditions

Bacterial isolates selected were tolerant to concentrations of NaCl up to 600 mM and maintained 50% growth at low water potentials (-1.44 MPa). They were positive for Indole Acetic Acid (IAA) production, phosphate solubilisation and secretion of siderophores. Bacterial isolates showing plant growth promoting potential were identified using 16S rDNA gene sequencing as *Achromobacter xylosoxidans* strains A8 and C54 and *Klebsiella oxytoca* strain M1. Mixed inoculum was prepared from indigenous communities of mycorrhizas in soils sampled from the Cerebos Salt Pan and the Kalahari Desert. Mycorrhizal diversity was investigated using 454-Pyrosequencing which revealed that the community composition was dominated by species in the *Ambispora*, *Glomus* and *Paraglomus* genera with a rare component represented by species in the *Redeckera*, *Archaeospora* and *Geosiphon* genera. Microscopic examination of plant roots at the end of the trial revealed the presence of diagnostic mycorrhizal structures within the root cells, confirming that colonization was successful.

Plant growth response to microbial inoculation was assessed by monitoring changes in plant photosynthetic capacity over the duration of a 7 week pot trial. A significant difference in photosynthetic and biomass data was observed between drought and well-watered groups but no mycorrhizal or bacterial treatment effect was evident within the groups, despite the high levels of colonization by mycorrhizas. These results suggest that the beneficial effects of mycorrhizal colonization may be primarily attributed to improved nutrient and mineral uptake in conditions where nutrients are limiting, resulting in improved growth. The

improved growth may then have secondary effects on the plant's ability to withstand drought. Having controlled for nutrient deficiency, it was not evident in this study that mycorrhizal fungi were able to stimulate a change in plant physiology and confer drought tolerance under the conditions imposed.

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Table of Contents

List of Figures

Each point represents an average of the drought and control groups (n = 32-56), vertical bars represent ± standard error. By measurement 4 (day 18) all of the plants were showing signs of severe water stress, indicated by leaves which were wilted, curled and turning brown. Effect of treatment on soil water content $F_{(1,875)} = 1213.6$, $p<0.0001$ and effect of time $F_{(11, 875)}=109.6$, $p<0.0001$.

Figure 3.12: Light micrograph of the diagnostic arbuscular mycorrhizal structures in root cells of *Zea mays* after inoculation with mixed mycorrhizal cultures. A-Intraradical Hyphae (IH), extraradical hyphae (EH) and arbuscules (AR). B- Germinated mycorrhizal spore (SP) with intra (IH) and extra-radical hyphae (EH). C- Soil borne auxilliary vesicles (AV), D- Intraradical vesicles (IV).

Figure 3.13: Colonization of *Zea mays* root cells by arbuscular mycorrhizal fungi at the end of the pot experiment. Each column represents a different treatment average (n = 9), vertical bars represent ± standard error. The treatment effect on colonization $F_{(15,128)} = 46.0$, $p<0.0001$. Significant differences at $P<0.05$ by Tukey's HSD Test.

Figure 3.14: Plant growth assessment parameters: **A**) Root biomass of drought stressed and control groups. Treatment effect on root growth $F_{(15, 128)}=3.6$, $p<0.0001$; **B**) Shoot biomass. Treatment effect on shoot growth $F_{(15, 128)}=14.4$, $p<0.0001$; **C**) Total plant biomass. Treatment effect on total plant biomass $F_{(15, 128)}=8.2.$, $p<0.0001$; **D**) Root-to-shoot ratio. Treatment effect on root: shoot ratio $F_{(15, 128)}=0.8$, $p=0,6$. Each point represents the average dry weight (g) of plants in the drought and control groups (n = 9), vertical bars represent ± standard error. Different letters are significantly different ($p<0.05$) by Tukey's Post-Hoc Test and an absence of letters indicates no significant differences.

Figure 3.15: Photosynthetic rate of pot-cultivated *Zea mays* during the drought /recovery pot experiment. Panel A and B represent the drought and watered control groups respectively and the legend applies to both graphs in each row. Panel C contains the combined data from each group. Each point represents a different treatment average (n = 3-7 for separate treatments and n = 32-56 for combined groups), vertical bars represent ± standard error. The effect on photosynthetic rate of the imposed treatments over time: $F_{(165,904)} = 4.4$, $p<0.0001$. Significant differences at $P < 0.05$ by Tukey's HSD Test.

Figure 3.16: Stomatal conductance of pot-cultivated *Zea mays* during the drought /recovery pot experiment. Panel A and B represent the drought and watered control groups respectively and the legend applies to both graphs in each row. Panel C contains the combined data from each group. Each point represents a different treatment average (n = 3-7 for separate treatments and n = 32-56 for combined groups), vertical bars represent ± standard error. The effect on stomatal conductance of the imposed treatments over time: $F_{(165,904)} = 3.7$, $p<0.0001$. Significant differences at $P < 0.05$ by Tukey's HSD Test.

Figure 3.17: Transpiration rate of pot-cultivated *Zea mays* during the drought /recovery pot experiment. Panel A and B represent the drought and watered control groups respectively and the legend applies to both graphs in each row. Panel C contains the combined data from each group. Each point represents a different treatment average (n = 3-7 for separate treatments and n = 32-56 for combined groups), vertical bars represent ± standard

error. The effect on transpiration rate of the imposed treatments over time: $F_{(165,903)} = 2.5$, $p<0.0001$. Significant differences at $P < 0.05$ by Tukey's HSD Test.

List of Tables

Chapter 1

General Introduction and Review of Literature

Impact of drought on agricultural sector

Rapid population growth and accelerating resource depletion presents a significant challenge to the agricultural sector to provide food for the coming generations (Ainsworth *et al*., 2008; Garrity *et al*., 2010). Population increases have placed enormous pressure on agriculture to match the growing global food demand which is estimated to rise by 60% in the next 40 years (Rosegrant & Cline, 2003).

South Africa is a country characterised by diversity, and the agricultural sector is no exception. A wide range of social, economic, political and environmental conditions collectively influence the distribution and development of agriculture across the country. Although commercial farming is well established, subsistence farming remains a large sector upon which millions of rural households are dependant. The dependence of these households upon agriculture highlights the vulnerability of many South Africans to the growing effects of Global Warming (Gbetibouo *et al*., 2010). Gbetibouo *et al*. (2010) quantitatively evaluated the relative vulnerability of the South African farming sector to climate change and assessed variability across the country and found that vulnerability to climate change is extrinsically linked to the level of social-economic development.

It is estimated that small-scale farmers represent 95% of agriculture in Africa and the majority of households in rural areas are involved in subsistence farming, producing food which is sold and consumed locally (Lamb, 2000). Agriculture is thus a core component of rural livelihoods and is essential to both food security as well as economic empowerment. Production in these regions is restricted by fragile ecosystems and the challenges smallholder farmers face are exacerbated by inherently low soil fertility, the highly variable rainfall which is characteristic of the region as well as limited financial resources with which to purchase inputs such as fertilizer and improved seed. Subsequently, water and nutrient use efficiency

in these areas is low (Groenewald, 2009; Mapfumo & Giller, 2001; Materechera, 2010; Mushayi *et al*., 1998; Smaling & Braun, 1996; Whitbread *et al*., 2010). The development of strategies to increase productivity remains a crucial food security issue (Devereaux & Maxwell, 2001; Schmidhuber & Tubiello, 2007).

Climate records have shown that South Africa is affected by highly variable inter-annual rainfall with prolonged periods of drought (O'Keeffe *et al*., 1992; Richard *et al*., 2001; Walker & Schulze, 2008) and global climate change is expected to significantly modify the crop production environment, exposing farmers to environmental conditions for which they are unprepared and ill equipped to handle (Gbetibouo *et al*., 2010). Increasing greenhouse gas concentrations, mean surface temperatures and evapotranspiration as well as re-distribution of rainfall patterns and soil degradation are just a few of the observed and predicted effects of climate change which have the potential to increase the occurrence of drought around the world. Precipitation events are set to become more intense, leading to increased flooding and climatic extremes such as heat waves and frost will also be more likely to occur (Ainsworth *et al*., 2008; IPCC, 2007; Walker & Schulze, 2008). Desertification as a result of drought and soil erosion will further increase the loss of fertile arable land and put pressure on the already strained resources of countries experiencing rapid population increase (Naveed *et al*., 2014; IPCC, 2007). Expansion of maize-growing areas onto marginal lands combined with climate change trends has the potential to create a higher number of drought-prone maize production environments, potentially reducing yields and further undermining food security (Boomsma & Vyn, 2008; IPCC, 2007; Walker & Schulze, 2008).

Drought and salinity are considered two of the major limiting factors affecting growth and productivity of crops worldwide (Marulanda *et al*., 2009; Naveed *et al*., 2014; Upadhyay *et al*., 2011) and it is estimated that by 2050 drought stress will have severely affected more than 50% of arable land (Vinocur & Altman, 2005). Water scarcity causes salts to remain un-dissolved where they accumulate in soil (Estrada *et al*., 2013; Evelin *et al*., 2009), causing ionic toxicity, nutrient imbalance, osmotic stress and secondary oxidative stress in plants (Ding *et al*., 2010; Parida & Das, 2005; Upadhyay *et al*., 2011). With increasing demand from industrial, municipal and agricultural sectors on diminishing water resources, the development of reduced-input agriculture has taken a higher priority (Woo *et al*., 2008).

Research has focused on strategies to develop crops with improved water use efficiencies and reduced drought-induced yield losses (Somerville & Briscoe, 2001; Woo *et al*., 2008; Zhang *et al*., 2004).

Maize as a staple crop

Globally, maize is considered the third most important food crop and is thus a staple source of energy and protein for millions of people worldwide (Naveed *et al*., 2014). Annual yield losses due to drought are estimated at 15% of potential yield (Edmeades, 2008; Naveed *et al*., 2014). In South Africa, maize is an important food crop and accounts for over 60% of the total harvested area of food crops with over 70 million tons being produced in 2013 alone (FAOSTAT, 2015) and feeding in the region of 200 million people (du Plessis, 2015). Smallholder farmers rely upon maize as a staple food crop, farmed throughout the country under diverse environmental conditions predominantly for subsistence and a small marketable surplus. However, production in rural areas is hampered by numerous challenges including land degradation, lack of tillage services, water scarcity and poor soil fertility (du Plessis, 2015; Mandiringana *et al*., 2005).

Physiological effects of drought

Drought stress affects the morphology and physiology of maize at both the cellular and whole-plant levels and was summarised by Boomsma and Vyn (2008) as follows: Cellular-level responses to drought stress include (a) abscisic acid (ABA) accumulation; (b decreased cell expansion and division; (c) osmotic adjustment (d) proline accumulation; (e) chlorophyll photo-oxidation; and (f) reduced enzyme activity. Whole-plant level responses to drought stress include (a) reduced leaf, silk, stem, root, and grain kernel expansion; (b) stomatal closure; (c) decreased photosynthesis and respiration; (d) reduced assimilate flux to growing organs; (e) accelerated leaf senescence; (f) delayed silk growth and greater ear and kernel abortion; (g) increased root to shoot ratio; and (h) stem reserve (i.e. photoassimilate) remobilization and subsequent lodging.

The ability to withstand drought stress is partially dictated by genetics but is also influenced by environmental conditioning such as repeated exposure to drought, heat or cold (Pinior *et al*., 2005). The response of plants to drought conditions has been evaluated by genetic, biochemical and physiological changes (Naveed *et al*., 2014). Parameters such as leaf gas exchange, photochemical efficiency of photosystem II (PSII), electron transport, relative water content (RWC) and chlorophyll content have been used as indicators of plant stress (Naveed *et al*., 2014; Woo *et al*., 2008).

Maize has a high water requirement and each plant is estimated to consume about 250 L of water to reach maturity (du Plessis, 2015) being particularly sensitive to water stress during the initial growth phase and during anthesis with overall yield severely reduced due to diminished photosynthetic capacity (Barnabas *et al*., 2008; Olawuyi *et al*., 2014). Moisture also plays a key role in the ability of maize plants to cope with heat induced by global warming. In a recent study by Lobell *et al*. (2011), a model based on historical maize trials in Africa was designed to investigate the effect on increased growing temperature under optimal rain-fed management and drought management. Their results found that yield in trials in optimal rain-fed sites with average temperatures above 25 °C decline rapidly, with more than 10% yield loss per °C of warming. They found that under drought conditions, even the trials with the coolest temperatures were damaged by a 1 °C increase in temperature, with the hottest sites (temperatures exceeding 30 °C) experiencing losses up to 40 %. It was thus estimated that 65% of present maize growing areas in Africa would experience yield losses for 1 °C of warming under optimal rain-fed management, with over 75 % of areas experiencing at least 20% yield loss under drought conditions (Lobell *et al*., 2011).

These results emphasize the key role of moisture in the ability of maize to withstand drought and the importance of developing strategies which enhance plant performance under conditions which will be exacerbated by climate change, such as drought, high temperatures and salinity (Estrada *et al*., 2013; Lobell *et al*., 2011; Vallejo *et al*., 2005) without relying upon additional inputs such as chemical fertilizers (Marulanda *et al*., 2009).

Plant response to drought stress occurs at morphological, anatomical and cellular levels through modifications which enable the plant to avoid the stress or increase tolerance (Bray, 1997; Marulanda *et al*., 2003). In addition to the natural responses to stress, plants also form associations with an assortment of soil microorganisms such as bacteria and mycorrhizal fungi which may help to moderate the stress symptoms. Current research has centred around developing practical strategies to isolate microorganisms such as arbuscular mycorrhizal (AM) fungi and plant growth promoting rhizobacteria (PGPR), apply them to crops in such a way that their beneficial activities are optimized and potentially confer tolerance to drought, salinity, metal toxicity and/or nutrient limitation (Augé, 2004; Gianinazzi *et al*., 2010; Godfray *et al*., 2010; Marulanda *et al*., 2000; Vivas *et al*., 2006).

Arbuscular Mycorrhizal Fungi

Arbuscular mycorrhizal fungi are a group of filamentous fungi grouped in a monophyletic clade, the Glomeromycota (Schüßler *et al*., 2001), within which four orders, the *Glomerales*, *Diversisporales*, *Archaeosporales* and *Paraglomerales* have been described (Krüger *et al*., 2009; Lumini *et al*., 2010). AM fungi are ubiquitous in soils around the world, forming a symbiotic relationship with a diverse range of around 250 000 plant species (Santos-González *et al*., 2007), comprising approximately 80% of all terrestrial plant species (Augé, 2004; Smith & Read, 2008).

Within the Glomeromycota, approximately 220 AM fungi have been described on the basis of morphological characteristics (Palenzuela *et al*., 2011; Santos-González *et al*., 2007). The majority of these fall within the *Glomerales* and *Diversisporales* (Schüßler *et al*., 2001) with nine species assigned to *Archaeosporales* and three to the *Paraglomaceae* (Goto *et al*., 2008; Renker *et al*., 2007; Walker, 2008). Recent molecular studies have however reported a species diversity and richness which strongly suggest that the number of species within the Glomeromycota is considerably underestimated (Hijri *et al*., 2006; Öpik *et al*., 2006; Santos-González *et al*., 2007), an assumption which is emphasized by the profuse number of environmental sequences deposited in databases (Palenzuela *et al*., 2011). Many of these

environmental sequences cannot however be assigned to a described fungal species with certainty and on the other hand, accurate and thorough morphological descriptions are often confounded by insufficient number of specimens as well as ambiguous morphological features (Oehl *et al*., 2009, 2010).

AM fungi form symbioses with plants in all natural ecosystems, irrespective of environmental conditions (Barea & Jeffries, 1995). Symbiosis can be defined as "the permanent association between two or more specifically distinct organisms, at least during part of the life cycle" (Krings *et al*., 2007). Singh *et al*. (2011) proposed that a successful plant-fungus symbiosis involves three core events: The penetration of the plant tissues by fungal hyphal filaments; Colonization of plant tissues by the infecting fungus; Expression of a fungal symbiotic lifestyle.

AM fungi produce a filamentous network of hyphae which disperse inside root cells and also extend outwards into the soil, effectively amplifying the extent of the plant root system several fold. In return, the plant provides the fungus with nutrition in the form of sugars, amino acids and vitamins (Harley & Smith, 1983). The AM symbiosis has therefore been defined as a "specialised system for nutrient uptake and transfer", more efficient than plant roots alone (Ruiz-Lozano, 2003; Varma & Hock, 1999). However, these symbiotic relationships have been shown to provide a diverse range of benefits above and beyond nutrient uptake (Augé, 2004; Ruiz-Lozano, 2003). The positive growth responses observed in mycorrhizal maize plants have mainly been attributed to mobilization and assimilation of poorly soluble minerals such as phosphorus (P), potassium, calcium, magnesium, sulphur, iron, zinc, copper and manganese (Boomsma & Vyn, 2008). They have also been shown to enhance uptake of nitrogen ions, particularly under drought conditions. Other effects which have been noted are protection against soil pathogens, synergistic interaction with beneficial microbes and improved soil water relations (Boomsma & Vyn, 2008; Miller & Jastrow, 2000).

AM contribution to water relations

Of particular interest to this study are the contributions of AM symbiosis to plant water relations and tolerance to drought stress which result from a combination of physical, nutritional, physiological and cellular effects (Ruiz-Lozano, 2003). One of the primary mechanisms by which AM fungi enhance plant water relations is through the growth promotion provided by improved nutrient uptake (especially P). This results in increased plant size and consequently larger root systems which are then able to access more water from the soil (Augé, 2004). However, numerous studies have excluded the plant-size effect by controlling for nutrient uptake and reported than under conditions where nutrient levels are controlled and plants are of a similar size, AM symbiosis often allows plants to maintain higher water balances, stomatal conductance and transpiration as well as increased carbon fixation and osmoregulation during drought stress compared to non-AM controls (Augé, 2004, 2001; Zhu *et al*., 2012). The major mechanisms thought to be responsible for these effects are described below.

Uptake and transfer of water by fungal hyphae

Increased water uptake by plants may be due to a combination of synergistic mechanisms, including AM hyphal uptake, increased root uptake due to AM-induced changes in root morphology or root diameter (fineness), all of which enable the integrated root/hyphal system to access more of the soil water reserves (Augé, 2004). The advantage which mycorrhizas have over plants in terms of nutrient and water acquisition is due to the small diameter of their hyphae and the distance to which they extend outwards from the root zone where they are able to penetrate the spaces between soil particles to access poorly-soluble nutrients unavailable to the thicker and shorter plant roots (Aghari & Cavagnaro, 2012; Aghili *et al*., 2014; Jakobsen *et al*., 1992; Marulanda *et al*., 2003). Average diameters of hyphae are 2-20 µm compared to roots which are on average greater than 300 µm (Smith *et al*., 2010). As soil dries, it compacts and spaces between particles become much smaller and tighter and hold an increasing proportion of water. It has also been reported that AM fungi are able to adjust the diameter of their hyphae, enabling exploration of increasingly dry and compacted soils (Smith *et al*., 2010). AM plants have also been shown to maintain stomatal conductance even

when soil water potential is lowered osmotically, indicating that AM hyphae are able to access water of lowered activity more efficiently that non-AM plants. The superior ability of AM fungi to access bound water means they are able to access and transport water to plants even when water potentials are below that of the "permanent wilting point" of plants (Augé, 2004).

Osmotic adjustment

In order to maintain a favourable water potential gradient for water movement from soil to roots, plants must lower their internal water potential to match that of soils as they dry out. The primary plant mechanism by which this is achieved is known as osmotic adjustment or osmoregulation, the process of lowering water potential through the accumulation of compatible solutes in plant cells (Ruiz-Lozano, 2003). Osmoregulation allows the plant to maintain a favourable water potential gradient and facilitates cellular expansion and growth, stomatal opening and photosynthesis through the preservation of turgor pressure (Ruiz-Lozano, 2003). The solutes which are involved in osmotic adjustment are inorganic ions (e.g. K^+ and Cl^-), uncharged organic compounds (e.g. proline, glycine and betaine) and carbohydrates (e.g. sucrose, pinitol and mannitol) (Ruiz-Lozano, 2003). AM symbiosis has been reported to stimulate the increased accumulation of proline in cells (Azcón *et al*., 1996; Goioechea *et al*., 1998; Ruiz-Lozano *et al*., 1995), alter the amounts of free amino acids and sugars in roots (Augé *et al*., 1992) and increase overall levels of osmotic adjustment compared to non-AM control plants (Kubikova *et al*., 2001).

Enhancement of plant gaseous exchange

Attributed mainly to increased water uptake and use efficiency, AM plants are often reported to able to maintain higher gas exchange rates during soil drying compared to non-AM plants (Ruiz-Lozano, 2003). AM colonization has been reported to alter the critical point where stomatal behaviour is affected by leaf water potential with AM plant leaf water potentials dropping further than non-AM plants before stomata begin to close (Allen & Boosalis, 1983;

Augé, 2004). Stomatal conductance in AM plants has also been maintained for longer once soil water potential has declined (Augé, 2001). AM plants often show higher photosynthetic rates, which is consistent with the effects on stomatal conductance (Augé, 2001; Zhu *et al*., 2012). Rapid recovery from water stress and superior soil water extraction at low soil water potentials have been reported in AM plants (Hardie & Leyton, 1981; Safir *et al*., 1971).

Changes in soil water retention properties

AM fungi are also known to affect the physical structure of soils. The extensive network of thread-like hyphae which extend from the site of infection at the root surface outwards into the soil is thought to hold soil particles together via physical entanglement or enmeshment as well as through the aggregating activity of hyphal exudates and residues (Augé, 2004; Bedini *et al*., 2009; Miller & Jastrow, 1990, 2000). Among the compounds produced by AM fungi, glomalin is of particular interest (Bedini *et al*., 2009). Glomalin is an insoluble proteinaceous substance, comprised of glycoproteins and an assortment of minerals and ions, which is thought to improve stability of soil by preventing the disaggregation action of soil water (Bedini *et al*., 2009; Rillig, 2004; Wright & Upadhyaya, 1998; Wright *et al*., 2007). Soil aggregation has been positively correlated with plant drought resistance (Davies *et al*., 1992; Rillig, 2004) and Augé (2004) found that simply growing non-AM bean plants in mycorrhizal soil improved drought resistance. Augé *et al*. (2001) reportedly found that after 7 months of colonization by *Glomus intraradices*, the characteristic soil moisture curve of loam soil was altered in comparison to non-AM soil and that once the soil began to dry out, changes in soil matric potential per unit change in soil water content were smaller in the mycorrhizal soil than the non-mycorrhizal soil (Ruiz-Lozano, 2003). The dynamics of water movement and retention in soils is determined by the size of particles as well as the size and distribution of pores. The ability of AM fungi to increase aggregation and stability in soils has been shown to alter the water retention properties, thus directly influencing plant water uptake (Augé *et al*. 2001; Bedini *et al*., 2009)**.**

In addition to the above mechanisms, AM symbiosis has also been shown to alter the assimilative of ions (e.g. NO_3^-) (Bertrand *et al*., 1999) essential for plant growth, confer

protection against oxidative damage generated by drought (Ruiz-Lozano, 1996, 2001) and alter the activity of aquaporins, thus regulating bulk water flow (Krajinski *et al*., 2000).

Mycorrhizas in agriculture

A host plant may invest 4-20% of its total photosynthetic carbon into the mycorrhizal symbiosis through the arbuscules, sites of phosphorus, carbon, water and nutrient exchange (Lerat *et al*., 2003). The allocation of resources between plants and mycorrhizas are highly variable and context-dependant as the advantage gained by the symbiosis depends on the relative availability of resources required by either partner (Johnson *et al*., 1997; Hoeksema *et al*., 2010; Grman & Robinson, 2013). The prevalence of mycorrhizal symbioses in agriculture is expected to increase as population increase drives the expansion of agriculture onto barren lands with no additional resources to improve soil fertility, resulting in an overall reduction of crop productivity (Menge, 1983; Tilman *et al*., 2011). This reduction in fertility and plant growth may stimulate the crop plants to invest carbohydrate resources into the root system and its associated symbionts in order to improve access to nutrients. The limitation of crop productivity by inadequate supply of macro- and micronutrients may then be alleviated by AM fungi and PGPR (Bennet *et al*., 2013). These effects are not only important in low-fertility soils but also during drought conditions, where mobility of ions is limited by the reduced soil water potential (Boomsma & Vyn, 2008).

The use of arbuscular mycorrhizas in agriculture, particularly rural agriculture holds great promise as once the fungus has established within the rhizosphere, it forms a stable relationship with the host plants and propagules remain in soil after harvesting. This self-sustaining form of ‘biofertilisation’ suits the conditions under which most rural farmers operate (limited fertilizer input and rain-fed crops) and requires no specialized skill for its application and no need for frequent replenishment (Olawuyi *et al*., 2014).

The application of mycorrhizal fungi to improve crop growth and yield under field conditions has been reported with varying results (Augé, 2004; Boomsma & Vyn, 2008). Mycorrhizal

colonization of soil and plant roots is a complex multi-step process, in which the mycorrhizal association is influenced not only by the host plant and the AM fungi, but also by soil and other environmental conditions (Barea, 1991). The benefits obtained by mycorrhizal symbiosis are thought to be more pronounced when both partners in the symbiosis have evolved and been selected for under the same conditions (Johnson *et al*., 2010). Marulanda *et al*. (2006) reported that the plant growth promotion ability of an indigenous strain of *Glomus intraradices* was more effective for active colonization of roots and nodule development than a reference lab-cultured strain of *G. intraradices*

Colonization by diverse indigenous assemblages of mycorrhizal fungi occurs naturally in most field grown crops and thus the inoculation of test plants with a mixed assemblage instead of a single species is thought to provide a more realistic indication of field effect and relevance (Aghili *et al*., 2014). Colonization by members of a natural AM fungal assemblage are perhaps more likely to be effective than a single inoculant as different species will 'excel' at certain processes where others fail and thus a mixed assemblage provides a broad spectrum of activities that more than likely complement each other to provide a more balanced nutritional supplementation for plants (Aghili *et al*., 2014; Cooper & Tinker, 1978).

Plant Growth Promoting Rhizobacteria

Ecosystem functioning is largely driven by microbial populations as they are an integral part of the nutrient cycles of plant macro- and micro-nutrients (Barea *et al*., 2002; Marulanda *et al*., 2009). As habitats change and environmental stressors such as drought, heat and misuse of chemical fertilizers become more prevalent, so the interdependence of organisms in symbiotic relationships is expected to increase (Bertness & Callaway, 1994; Aghili *et al*., 2014). Organisms in symbiotic relationships often rely upon the partners to provide additional resources, e.g. nutrients and enzymes which are not required during growth under optimal conditions but rather confer an advantage or 'safety net' during periods of environmental stress and thus mutual benefits are pronounced under adverse conditions (Aghili *et al*., 2014).

Due to rhizodeposition of root exudates, which include root exudates and cells which are sloughed off during growth, the rhizosphere soil provides a rich source of nutrients and organic substances which support large and diverse communities of microorganisms (Sabir *et al*., 2013; Martens, 1991; Morgan *et al*., 2005). Root exudates condition the rhizosphere and have been shown to comprise predominantly of organic carbon in the form of simple molecules, mucilage and cells and tissues which are shed during root growth and elongation (Mechri *et al*., 2014; Rovira *et al*., 1983). The small molecules are a range of sugars, amino acids, organic acids and phenolics. It was estimated by Helal and Sauerbeck (1989) that approximately 19% of the total photosynthetic carbon of various plant species was released into the rhizosphere as organic material (Mechri *et al*., 2014).

Many of these microbes associated with the rhizosphere produce compounds or enzymes beneficial to the host plant and are broadly termed Plant Growth Promoting Rhizobacteria (Rodríguez & Fraga, 1999). A wide range of bacterial species such as *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Burkholderia*, *Bacillus* and *Serratia* amongst many others have been shown to promote plant growth under a range of different conditions (Chiarini, *et al*., 1998; Marulanda *et al*., 2009; Naveed *et al*., 2014; Rodríguez & Fraga, 1999).

PGPR either exist as intracellular, whereby colonization is inside the plant cells, or extracellular where they inhabit the spaces between cells, the root surface or the rhizosphere (Gray & Smith, 2005; Marulanda *et al*., 2010). Bacteria may be found living inside cortical root cells, living on the surface of the roots (rhizoplane) or in the soil around the roots (rhizosphere). The spatial relationships, in terms of root proximity, are crucial to the interaction between host and microorganisms and the extent of inter-dependence. A natural gradient exists within the soil extending outwards from the root and as the amount and solubility of root exudates and microbial metabolites varies, so the bacterial species and their relative contributions and dependencies vary in space and time (Gray & Smith, 2005; Marulanda *et al*., 2009). The relationships between host and microorganisms can vary from almost causal to tightly regulated and accommodated in specialized structures (Marulanda *et*

al., 2009).

These bacteria have been considered as potential biofertilisers and biocontrol agents of plant pathogens and the ease of large scale production has led to the formulation of commercial products. They are easily and routinely isolated and cultured from environmental samples and have been shown to improve the growth and vigour of numerous crops, both in laboratory and field trials (Adesemoye *et al*., 2008; Figueiredo *et al*., 2008; Glick *et al*., 2007; Rodríguez & Fraga, 1999; Shaharoona *et al*., 2008; Yang *et al*., 2008).

The idea that PGPR application may enable agriculturalists to reduce the costly irrigation and fertilizers which currently sustain their yields is an exciting one which has already produced promising results. An example of the practical utilization of these findings is a study performed by Shaharoona *et al*. (2008) where they were able to show under field conditions that the yield of wheat (*Triticum aestivum* L.) plants that were given 75% of the recommended amount of N-P-K fertilizer and inoculated with a PGPR strain was equivalent to that of un-inoculated plants given 100% of the recommended fertilizer application. Similarly, Hernández and Chailloux (2004) found that yields of tomato plant inoculated with two PGPR strains and given 50% fertilizer were greater than the un-inoculated control plants with 100% fertilization (Yang *et al*., 2008).

The mechanisms by which these bacteria promote plant growth can be classified as direct or indirect. Direct mechanisms include the production of phytohormones such as auxins, gibberellins and cytokinins, nitrogen fixation, production of antibiotics and iron-chelating siderophores as well as enzymes such as ACC-deaminase (Marulanda *et al*., 2010). Indirect mechanisms include the formation of symbiotic relationships with other beneficial microorganism such as mycorrhizal fungi and the suppression of pathogenic organisms through antibiotic production or competitive nutrient and mineral acquisition (Kloepper *et al*., 2004; Lugtenberg & Kamilova, 2009; Marulanda *et al*., 2010; Vessey, 2003; Yang *et al*., 2008).

Another phenomenon which has been reported is 'induced systemic resistance' which refers the induction of a range of internal host plant defences such as the production of toxins, tannins or acids within plant tissues (Bennett & Wallsgrove, 1994; van Loon *et al*., 2004). In addition, it has been documented by many researchers that inoculation of plants with PGPR improves tolerance to abiotic stresses such as drought and salinity (Kohler *et al*., 2009; Liddycoat *et al*., 2009; Marulanda *et al*., 2010) and this effect has been seen not only as increased biomass but also as an improvement in water relations (Kohler *et al*., 2009; Marulanda *et al*., 2010; Nadeem *et al*., 2007).

There is a vast array of mechanisms by which PGPR may improve the growth and stress tolerance of host plants, a small selection of which were investigated in this study and are described below.

Phytohormone production

Phytohormones produced by rhizosphere bacteria are considered key determinants of the interactions between plants and their associated microbes, as well as in the stimulation of plant growth and plant pathogenicity (Morris, 1986; Naveed *et al*., 2014; Sergeeva *et al*., 2002; Yamada, 1993).The release of these phytohormones is known to alter the root growth and architecture (Augé, 2004) and it has also been suggested that the stimulation of plant growth may result in increased nutrient release, thus directly benefiting the microbial population which utilize plant root exudates (Sergeeva *et al*., 2002; Smith & Read, 2008). Bacteria which colonize the rhizosphere of plants often produce a variety of physiologically active compounds such as hormones and auxins which are beneficial to the plant. Traits often characteristic of PGPR are the production of plant growth regulators such as auxin, gibberellin and ethylene, siderophores and antibiotics (Arshad & Frankenberger, 1991).

Indole acetic acid (IAA) is a product of L-tryptophan metabolism and is one of the most physiologically active auxins (Arshad & Frankenberger, 1991; Mohite, 2013). L-tryptophan is a physiological precursor for the biosynthesis of auxins and is a natural component of root

exudates, thus stimulating the production of auxins such as IAA by microbial assemblages in the rhizosphere (Martens & Frankenberger, 1994). IAA is the main plant hormone which controls many physiological processes including changes in root growth and development, cell enlargement and division, tissue differentiation and responses to light and gravity (Moore, 1989; Lüthen *et al*., 1999; Davies, 1995). These alterations may lead to an increase in total root surface area and consequently increase water and nutrient uptake, having beneficial effects on overall plant growth (Naveed *et al*., 2014; Somers *et al*., 2004). The amount of IAA produced by strains is considered a suitable marker for selecting bacteria effective at plant growth promotion, especially under drought stress (Marulanda *et al*., 2009).

Phosphorus solubilisation

Phosphorus is one of the major essential macronutrients required for biological growth and development of plants and microorganisms. Phosphorus is highly reactive with iron, aluminium and calcium in soils, and the rapid immobilization and precipitation of available phosphorus in soils can render up to 90% of applied phosphorus biochemically unavailable to plants (Dey, 1988; Gyaneshwar *et al*., 2002; Richardson, 1994; Yang *et al*., 2008). Although naturally abundant in soils, it is most often found in insoluble form as minerals such as apatite, hydroxyapatite and oxyapatite or associated with the surface of hydrated oxides of iron, aluminium and magnesium (Fernández & Novo, 1988; Rodríguez & Fraga, 1999). Microbial nutrient cycling plays a central role in the natural phosphorus cycle, as under the right conditions, microbes are able to solubilise bound phosphate and make it available for plants and other microbes (Rodríguez & Fraga, 1999).

The ability of several bacterial species to solubilise inorganic forms of phosphate has been documented and it has been found that substantial populations of phosphate solubilising bacteria exist in soil and plant rhizospheres (Rodríguez & Fraga, 1999). The release of phosphorus from insoluble phosphates by many microorganisms has been predominantly attributed to the production of organic acids which dissolve phosphate precipitated in the soil with aluminium, calcium and iron by the chelation of the cations that accompany the phosphate anion, thus releasing the phosphorus (Kucey, 1983).

A symbiotic relationship exists between these bacteria and the plant as evidenced by a considerably higher concentration of phosphate-solubilising bacteria in the rhizosphere soil compared to non-rhizosphere soil (Katznelson *et al*., 1962; Raghu & MacRae, 1966). The high levels of P required by plants for growth and development means that P availability is often a limiting step in plant nutrition (Goldstein, 1986) and thus the inoculation of P-solubilising microorganisms has the potential to increase plant growth (Rodríguez & Fraga, 1999). The inoculation of phosphate solubilising bacteria and the subsequent crop yield increase has been reported for a number of species such as *Pseudomonas*, *Bacillus*, *Rhizobium*, *Micrococcus*, *Flavobacterium*, *Burkholderia*, *Achromobacter*, *Erwinia*, and *Agrobacterium* (Rodríguez & Fraga, 1999; Vyas & Gulati, 2009).

The phosphate solubilising ability of bacteria is highly variable as bacteria utilize various forms of phosphate through the production of a range of organic acids (Vyas & Gulati, 2009). Their ability is also affected by environmental conditions such as temperature, soil moisture content and physiochemical variables such as pH and the availability of other micronutrients. The efficiency of these organisms is also dependant on the soil type and specific plant cultivar (Rodríguez & Fraga, 1999). The variability exhibited by these bacteria has led to research into using mixed inoculum versus single species inoculum. Researchers have found that often mixed inocula produce superior results and this is possibly attributed to the provision of a more balanced nutrition to plants, increasingly so when the bacteria simultaneously produce other growth promoting substances such as IAA (Alagawadi & Gaur, 1992; Belimov *et al*., 1995; Kundu & Gaur, 1984; Monib *et al*., 1984; Rodríguez & Fraga, 1999).

Siderophore production

Under iron-limited conditions, bacteria may produce iron-chelating siderophores which are low molecular weight (<10 000 D), ferric iron specific ligands in order to access insoluble iron (Neilands, 1995; Sayyed *et al*., 2005). Siderophores differ greatly in structure, although

most siderophores are either hydroxamates or catechols and most are soluble in water (Neilands, 1995). Iron is almost always an essential element for growth and cell functioning and hence siderophores are produced in order to scavenge available iron and make it available for the cell to access and utilize (Raven, 1988; Sayyed *et al*., 2010).

Siderophore production is not only beneficial to the bacteria as increased uptake of iron, but it also serves as a protective competitive mechanism which prevent pathogens from accessing the iron essential for nutrition, proliferation and colonization of plants (Ahmad *et al*., 2008; Lemanceau & Albouvette, 1993; Sayyed & Chincholkar, 2009). Chemical pesticides are detrimental both to the environment and to indigenous populations of beneficial microorganisms and inevitably result in broad-scale pesticide resistance in pathogens. Siderophore-mediated iron scavenging has thus been indicated as a potential bio-control approach (Sayyed & Chincholkar, 2009). Biocontrol strategies such as these are of interest as they are considered safer, do not build up in the environment or induce pesticide resistance and due to self- replication, do not require repeated application (Sayyed & Chincholkar, 2009).

Iron is an abundant transition metal and plays a key role in the biogeochemistry of soils, particularly in the fixation of phosphorus in agricultural soils (Barrow, 2002; Borch *et al*., 2009; Shang *et al*., 1992; Zhang *et al*., 2014). Much of the phosphorus applied as fertilizer is immediately bound up and fixed by iron oxides and hydroxides, rendering it unavailable to plants. Bacteria which are able to reduce Fe(III), resulting in the desorption of phosphate, have been shown to work synergistically with other soil microorganisms like mycorrhizas to improve colonization and phosphate uptake by plants (Zhang *et al*. 2014).

Zhang *et al*. (2014) showed under greenhouse conditions that co-inoculation of *Medicago sativa* (Alfalfa) under high and low inorganic phosphorus levels with an iron reducing bacteria strain of *Klebsiella pneumoniae* and *Glomus mossea*e increased the shoot P content as well as increased colonization and hyphal length of *G. mosseae*. Compared to individual inoculation, dual inoculation increased the available P content in soil at both low and high levels of P application. Inoculation with *G. mosseae* alone significantly increased the shoot P

content at low levels of inorganic phosphorus but had no effect at high rates. Their results suggest that iron reducing bacteria may work synergistically with mycorrhizas on the mobilization of Fe(III)-fixed phosphorus.

Osmotic adjustment compounds

One of the major mechanisms which enable bacteria to withstand drought stress is the accumulation of small compatible solutes called osmolytes which serve as osmoprotectants. These solutes include amino acids such as glutamate, glutamine, proline and alanine; quaternary amines such as glycinebetaine and sugars such as sucrose, trehalose and polyglucosyl (Csonka, 1989; Potts, 1994; Sandhya *et al*., 2010). Accumulation of these solutes in the cytoplasm lowers the internal water potential, thereby enabling the cell to maintain turgor and prevent cell damage (Sandhya *et al*., 2010). Osmolytes have also been shown to act as free-radical scavengers and chemical chaperones by stabilizing membranes and proteins (Diamant *et al*., 2001). Certain bacterial species also produce exo-polysaccharides which reduce desiccation and improve drought tolerance (Potts, 1994).

Upadhyay *et al*. (2011) isolated bacteria from wheat crops grown in saline and non-saline soils. The isolates were then screened for tolerance to salt up to 60g L^{-1}. Isolates were then screened for the ability to produce exo-polysaccharides. They were able to show that isolates which were able to produce exo-polysaccharides under saline conditions were able to improve the growth of wheat plants under saline conditions. The primary explanation for this was attributed to the ability of exo-polysaccharides to bind to cations, including Na^+, thereby reducing the content of Na^+ for plant uptake. Secondary plant growth promotion could then potentially occur through increased uptake of nutrients and minerals, antibiotic activity and stimulation of other biochemical and enzymatic pathways (Barriuso *et al*., 2008; Han & Lee, 2005; Upadhyay *et al*., 2011).

Enzymatic assistance

PGPR activity can be important in mitigating the damage caused to plant tissues by drought and heat stress (Kohler *et al*., 2008). One of the mechanisms which combat stress is the antioxidant defence system which includes antioxidant enzymes and low-molecular antioxidants (Foyer & Noctor, 2005; Mittler, 2002). Oxidative stress compounds such as H_2O_2 damage leaf tissue and catalase produced by PGPR may help to neutralize the acid before it causes harm (Kohler *et al*., 2008). Increased levels of superoxide dismutases have been found in plants under stress conditions and are expressed as a defence mechanism, allowing the plant to escape cellular damage and maintain growth under adverse conditions (Wang *et al*., 2009).

Mycorrhizal Helper Bacteria

The variability in the root exudates and the resulting rhizosphere carbohydrate profile are considered to be largely responsible for the composition and diversity of the microbial communities which these plants harbour (Mechri *et al*., 2014). In the same way that root exudates from plants stimulate and encourage the colonization of the rhizosphere and plant tissues by beneficial microorganisms, so the exudates from mycorrhizal hyphae are likely to facilitate a relationship between the fungus and what are commonly termed mycorrhiza helper bacteria (MHB). The extent of mycelia exploration is far beyond that of plant roots and results in the deposition of significant amounts of organic carbon and hence influences the activity and richness of the microbial communities within the rhizo- and hyphosphere (Smith *et al*., 2010).

AM fungi and the microbial community within the plant rhizosphere are intrinsically linked and interact with each other at the physical, metabolic and functional levels (Finlay, 2008; Franco-Correa *et al*., 2010; Johansson *et al*., 2004). AM fungi exudates have been found to comprise of different sugars (Hooker *et al*., 2007), proline and isocitrate (Lioussanne *et al*., 2008) and citric acid (Tawaraya *et al*., 2006) and as microbial species differ in their ability to utilize and compete for different carbon sources, different AM species will exist in symbiosis

with different assemblages of bacteria (Mechri *et al*., 2014).

Functional relationships between mycorrhizas and specific bacterial species have been documented and have been observed to promote AM symbiosis, root colonization and mycelial growth (Frey-Klett *et al*., 2007; Vivas *et al*., 2006), thus leading to the term 'helper bacteria'. The activity of MHB has been described in many reports and involves a diverse range of bacterial genera including *Agrobacterium, Azospirillum, Azotobacter, Burkholderia, Bradyrhizobium, Enterobacter, Pseudomonas, Klebsiella, Rhizobium, Bacillus, Brevibacillus, Paenibacillus,* Actinomycetes, *Rhodococcus, Streptomyces* and *Arthrobacter* (Frey-Klett *et al., 2007).*

In a study by Marulanda *et al*. (2009), it was reported that the growth promotion ability of two bacterial isolates was only observed when they were applied to plants already colonized with AM fungi, suggesting that any substances produced were actually stimulating mycorrhizal functioning rather than directly promoting plant growth. The main effect that MHB have on the AM symbiosis is the significant improvement of root colonization (Frey-Klett *et al*., 2007) however, increased fungal metabolism and higher photosynthetic and water use efficiency has also been observed in plants co-inoculated with MHB and AM fungi (Vivas *et al*., 2003).

For example, Vivas *et al*. (2003) found that the inoculation of a *Bacillus* sp. was able to influence the development and activity of two *Glomus* species colonizing Lettuce (*Lactuca sativa* L. cv Romana). The *Bacillus* sp. was reported to promote plant growth when inoculated separately from the mycorrhizal fungi, however in combination with *Glomus* species, the effects were shifted to stimulation of mycelial development rather than plant growth promotion. These results infer perhaps that the bacteria obtain greater benefits and resources from interactions with mycorrhizal fungi than they do from plant roots and exudates. The ability of this bacterium to influence the mycelial growth indicates that it has a direct effect on the metabolic status of the fungus.

The relationships between MHB and AM fungi have also been shown to be highly specific. Pivato *et al*. (2009) tested 6 strains of rhizospheric bacteria for the ability to influence saprophytic growth and colonization of *Glomus mosseae* BEG12 and *Gigaspora rosea* BEG9 on *Medicago truncatula* roots. Their results found that plant growth promotion by was greatest when *P. fluorescens* was in combination with *G. mosseae* compared to *G. rosea*. The apparent fungal-specificity was then further tested by using a different host plant species in order to determine whether the effect was host specific as well. Results showed that *P. fluorescens* in combination with *G. mosseae* on *Lycopersicon esculentum* was also growth promoting whereas *P. fluorescens* in combination with *G. rosea* had no significant or even negative effects on plant growth, showing definitively that the bacterial growth promotion was fungal and not host-specific.

Similarly, in an unpublished study, Marulanda *et al*. (2009) found that inoculation of B. *megaterium* onto plants colonized by *Glomus intraradices* increased the amount of photosynthetic pigments in leaves, where an increase in both total chlorophyll and carotenoids was observed. However, co-inoculation of *B. megaterium* with *G. mosseae* had no effect on plant gas exchange irrespective of whether the plants were under drought stress or not. These results highlight the specificity of the interactions between bacterial and mycorrhizal species and the importance of elucidating the compatibility of the relationship prior to the *in vivo* application and testing of the microorganisms (Marulanda *et al*., 2006, 2009).

Motivation

The observed and predicted changes brought about by global climate change and land use practises mean that the already-vulnerable agricultural ecosystems are going to be placed under further pressure in the near future. Population growth is placing enormous pressure on agriculture to produce more food for people, however dwindling natural resources and increasing financial difficulty means that this challenge is unlikely to be met by farmers under the current regime. The use of microorganisms such as AM fungi and PGPR may provide an economical and environmentally sustainable solution to help alleviate the main biotic and abiotic stresses which undermine crop yields. If locally-sourced microorganisms can be applied in such a way that crop growth may be improved under osmotic stress, this could impact in meaningful ways upon the livelihoods of subsistence farmers in South Africa.

Hypothesis

Drought and saline adapted mycorrhizal fungi and PGPR improve the drought tolerance of *Zea mays*.

Objectives

1. Isolation of AM fungi and bacteria adapted to osmotic stress.
2. Assessment of bacterial Plant Growth Promoting (PGP) characteristics.
3. Isolation and molecular identification of select bacterial and mycorrhizal isolates.
4. *In vitro* pot trial to assess the effect of select mycorrhizas and rhizobacteria in sustaining plant growth under stress conditions.

Chapter 2

Methods

Rhizobacterial isolation and characterisation

Isolation of bacteria adapted to salt-induced osmotic stress

In order to isolate bacteria already adapted to drought and/or high salinity, the root-sheaths of *Stipgrostis cilata* var *capensis* (Tall Bushman's grass), collected from Haakdoorn Duin Farm, Kalahari, Northern Cape (27°39'26.1"S, 21°03'94.6"E) were ground up and homogenized in 0.8% sterile saline. Similarly, 1g of soil collected from the rhizosphere of selected plants growing in the Cerebos Salt Pans in the Eastern Cape (33°47'49.5"S, 25°40'16.8"E) was homogenized in 100 ml sterile saline. Serial dilutions up to 10^{-8} were prepared and plated onto Nutrient Agar (Merck, catalogue no. HG000C1.500) supplemented with NaCl (0, 100, 200, 400 and 600 mM) and incubated at 28 °C for 24 hrs. As prolific growth was observed even at 600 mM NaCl, the most abundant colonies (based on morphology) were sub-cultured onto 600 mM NaCl plates in order to obtain pure colonies. Any contaminated cultures were discarded and the 50 most prolific cultures were selected for further testing.

Evaluation of Plant Growth Promoting characteristics

Indole Acetic Acid (IAA) production

A dual approach was applied to Indole Acetic Acid (IAA) production assays. Literature has shown that Kovac's and Salkowski's reagents react to different forms and concentrations of indolic compounds (Glickmann & Dessaux, 1995; Lombard & Dowell, 1983; Sergeeva *et al*., 2002). In addition, the use of Kovac's reagent does not allow for quantification of the amount of IAA produced whereas Salkowski's reagent develops a colour range which can be measured spectrophotometrically (Gordon & Weber, 1951).

Assay using Kovac's reagent was performed as follows: Isolates were inoculated into 10 ml DEV Tryptophan Broth (Merck, catalogue no. 1106940500) and incubated at 36°C for 48 hrs. 5 drops of Kovac's reagent (Merck, catalogue no. 3745000) was then carefully dispensed into each culture. The formation of a cherry-red ring on top of the culture indicated a positive reaction for the production of IAA (Kerkar *et al*., 2012).

Assay using Salkowski's reagent was performed as follows: Isolates were inoculated into 10 ml DEV Tryptophan Broth and incubated at 36 °C for 72 hrs. The bacterial cell concentrations were measured at a wavelength of 660 nm and adjusted to an OD reading of 0.5 using 0.1% sterile saline. An aliquot of 1.5 ml from each culture was dispensed into a sterile microcentrifuge tube and centrifuged using a desktop centrifuge (Super Mini Centrifuge, Hangzhou Allsheng Instruments) for 1 min at 13000 rpm. A 1 ml sample of supernatant was then mixed with 2 ml Salkowski's reagent (Appendix A) and incubated at room temperature for 25 min. The appearance of a pink-red colouration in the supernatant is indicative of the presence of IAA. The colour change was then measured spectrophotometrically (UV mini-120 UV-VIS spectrophotometer) at 530 nm and quantified using a standard curve prepared from known concentrations of IAA (Appendix A) (Internet 1, 2014).

Siderophore production

Bacterial isolates were assayed for siderophore production using modified Chrome Azurol S (CAS) Agar (Milagres *et al*., 1999) (Appendix C for ingredients). Due to poor results with direct inoculation onto CAS medium, an 'overlay' method was tested whereby a culture was grown on standard growth media and CAS agar poured over the culture and incubated. Isolates were streaked onto Nutrient Agar and incubated at 28 °C for 72 hrs in order to obtain prolific growth. CAS agar was then prepared, allowed to cool until just before setting point and then very gently poured over the cultures on NA plates. The plates were then sealed with Parafilm (Bemis Company, Inc.) and incubated at 28 °C for up to 5 days. After incubation the development of a yellow colour change surrounding the bacteria growth was considered positive for siderophore production.

Phosphate solubilisation

Pikovskaya's phosphate solubilisation agar was prepared according to protocol (Appendix B). A 'drop' method was used for inoculation. Isolates were inoculated into Luria broth and incubated for 24 hrs. The bacterial cell concentrations were measured at a wavelength of 660 nm and adjusted to an OD reading of 0.5 using 0.1% sterile saline. A 1.5 ml sample of culture was then pelleted by centrifugation and re-suspended in sterile saline. From each culture 200 µl was carefully dispensed into the centre of the agar plate and allowed to stand until the liquid had been absorbed. Petri dishes were then sealed with Parafilm and incubated at 28 °C for 5 days. A clear halo was observed around isolates which were positive for phosphate solubilisation (Gupta *et al.*, 1994).

Ammonia production

Bacterial isolates were assayed for the production of ammonia. Isolates were inoculated into 10 ml Peptone water (Merck, catalogue no. HG000BX5.250) and incubated at 36 °C for 48 hrs. 0.5 ml HCl (32%) was added to each culture. Development of a yellow-brown colouration in the media was a positive reaction for the presence of ammonia. The darker the colour change, the more ammonia present in the media (Cappuccino and Sherman, 2011).

Catalase production

Isolates were streaked onto Nutrient Agar and incubated at 36 °C for 48 hrs. Hydrogen peroxide (30% H_2O_2) was then carefully dropped onto cultures using a Pasteur pipette. A vigorous bubbling at the site of contact was indicative of the production of catalase by the isolates. The more vigorous the bubbling, the more enzyme produced (Taylor & Achanzar, 1972).

PEG 8000 drought tolerance

Poly (ethylene glycol) (PEG) is a high molecular-weight carbohydrate which is hydrophilic and binds to water molecules, thereby reducing the water potential of a solution (Michel & Kaufmann, 1973). In order to test the ability of the isolates to tolerate drought, bacterial isolates were grown in liquid media supplemented with increasing amounts of PEG 8000 (Sigma-Aldrich, catalogue no. P5413). This method was adapted from Halverson & Firestone 1997. Halverson & Firestone (1997) used a basal medium (-0.15MPa) and lowered it by adding set amounts of PEG 8000 to achieve a range of water potentials (-0.125, -0.25, -0.375, -0.5, -1.0, -1.5 MPa). The water potential of standard solutions of Luria Broth and Nutrient Broth were determined by Dew Point Thermocouple Pyschrometry (HR-33T Dew point Microvoltameter, Wescor Inc.) to be -1.06 MPa and -0.80 MPa, respectively (Appendix E). As the isolates had been routinely cultured in Luria Broth, it was used as the control growth media which was then supplemented with PEG 8000 to achieve a range of water potentials (100, 150 and 262 g/L to achieve water potentials of -1.22, -1.44 and -2.0 MPa, respectively).

The wilting point of plants is estimated by the water content at -1.5 MPa (Tolk, 2013) thus the bacterial isolates were unlikely to be exposed to water potentials below this point. PEG 8000 was added to the Luria Broth (Merck, catalogue no. C147) and then autoclaved at 121°C for 15 min. Isolates were inoculated into fresh Luria Broth (LB) and incubated at 36 °C for 24 hrs. The bacterial cell concentrations were measured spectrophotometrically and adjusted to an OD_{660nm} reading of 0.5 using 0.1% sterile saline. An aliquot of 50 µl of this culture was used to inoculate 40 ml sterile LB or LB + PEG 8000. Cultures were incubated at 36 °C for 24 hrs and the optical density measured spectrophotometrically using a Bio-Tek Instruments Power Wave X at an absorbance of 660 nm using un-inoculated growth medium as a blank.

Gram staining

Gram staining was performed upon cultures of each isolate in order to check that the cultures were pure and to allow preliminary identification based on Gram reaction, cell size and morphology. Following standard procedure, a sterile metal inoculating loop was dipped into 24hr liquid culture and a smear placed in the centre of a clean glass slide. The slide was then passed above a flame to heat fix the culture to the glass. The smear was covered with Crystal violet stain for 60 s, staining the cells dark purple. The smear was then covered with Iodine solution for 30s, fixing the crystal violet stain to the cell walls of Gram-positive bacteria. The smear was then rinsed with distilled water and de-stained using 100% ethanol. The smear was then counter-stained with Safranin solution for 30 s, rinsed with distilled water and allowed to dry (Madigan *et al*., 2010). The slides were then examined under 1000x magnification and immersion oil using a Nikon YS100 compound microscope.

Molecular identification of selected bacterial isolates

DNA extraction

Isolates were incubated overnight in 600 mM Nutrient Broth at 28 ℃. A 1.5 ml sample of culture was dispensed into a microcentrifuge tube and centrifuged at 13000 g for 30 s. The pelleted cells were then re-suspended in 200 µl supernatant. Genomic DNA was extracted using the ZR Fungal/Bacterial DNA Mini-Prep^{TM} Kit (catalogue No. D6005) according to the manufacturer's instructions. The only deviation from the protocol was that extracted DNA was eluted into 100 µl nuclease-free water instead of elution buffer.

A 200 µl aliquot of concentrated bacterial culture was added to 750 µl Lysis solution in a ZR BashingBead Lysis Tube. The tubes were fitted into a Vortex Mixer 230V EU (Labnet International) and vortexed for 5 min at maximum speed. The tubes were then centrifuged using a desktop centrifuge (Super Mini Centrifuge, Hangzhou Allsheng Instruments) for 1 min at 10 000 rpm. A 400 µl aliquot of supernatant was then transferred into a Zymo-Spin IV Spin Filter and centrifuged at 7,000 rpm for 1 min, after which 1200 µl of Binding Buffer was added to the filtrate in the collection tube. A 800 µl aliquot of the mixture was then transferred to a Zymo–Spin IIC Column and centrifuged at 10,000 rpm for 1 min. The filtrate

was discarded and this step repeated with the remaining mixture. 200 µl DNA Pre-Wash Buffer was then added to the Zymo- Spin IIC Column and centrifuged at 10,000 rpm for 1 minute in a clean collection tube. A 500 µl aliquot of DNA Wash Buffer was then added to the Zymo-Spin IIC Column and centrifuged at 10,000 rpm for 1 min. The column was then centrifuged once again with the lid open in order to allow for the evaporation of any excess alcohol remaining in the filter. The Zymo- Spin Column was transferred into a clean microcentrifuge tube before the addition of 100 µl of nuclease-free water directly to the column matrix and centrifugation at 10,000 rpm for 30 sec. Extracted DNA was visualized after electrophoresis as described below.

Agarose Gel Electrophoresis (AGE)

The standard protocol used for electrophoresis and visualization of genomic DNA and PCR products was as follows: A 0.8% agarose gel was prepared using Tris-Acetate-EDTA (TAE) buffer and stained with 0.5 µg/ml ethidium bromide. A 5 µl aliquot of template DNA mixed with 5µl 1X Blue/Orange Loading Dye (Promega, catalogue no. G1881), loaded onto the agarose gel and electrophoresed for 45 min at 100V. The size of the DNA was estimated using the Lambda/Pst DNA molecular marker (Promega, catalogue no G210A) for bacterial DNA. The gels were visualized using a UV Fluorescence Uvitec Gel Doc System.

Amplification of target DNA sequence by Polymerase Chain Reaction (PCR)

The target 16S rDNA gene region required for identification was amplified by PCR using the universal primers rP2 and Fd1 (Table 2.1) (Weisburg *et al.*, 1991).

Table 2.1: Nucleotide sequence of 16S rDNA primers used in this study.

Primer	Sequence
rP2	5'ACGGCTACCTTGTTACGACTT 3'
Fd1	5'AGAGTTTGATCCTGGCTCAG 3'

The 50 µl reaction mixture was prepared as follows: 25 µl Kapa HiFi Hotstart ReadyMix (KAPA Biosystems, catalogue no. KK2501), 2µl fd1, 2µl rP2, 2-5 µl template DNA and made up to 50 µl with nuclease-free water. Amplification was performed using a 2720 Thermal Cycler (Applied Biosystems) with the following cycling parameters (Table 2.2):

Table 2.2: Thermal cycling parameters used in the amplification of 16S rDNA.

Parameters	Temperature (°C)	Time (seconds)	Cycles
Initial Denaturation	95	300	1
Denaturation	98	20	
Annealing	54.5	15	30
Extension	72	30	
Final Extension	72	300	1

Purification of PCR amplification product

PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, catalogue no. A9281) following the manufacturer's instructions. An equal volume of Membrane Binding solution was added to the PCR reaction. The mixture was transferred to an SV Minicolumn fitted into a Collection Tube. The column was incubated at room temperature for 1 min before centrifuging at 16000 g for 1 min. The flow-through was discarded and the column re-inserted into the Collection Tube. A 700 µl aliquot of Membrane Wash solution was added to the SV Minicolumn before centrifuging at 13000 g for 1 min. The flow-through was discarded and the column re-inserted into the Collection Tube. The above step was repeated with 500 µl Membrane Wash solution and centrifuged at 16000 g for 5 min. The Collection Tube was then emptied and the column centrifuged with the lid open to allow evaporation of any residual ethanol. The SV Minicolumn was then transferred to a clean 1.5 ml microcentrifuge tube and 50 µl nuclease-free water added to the column. The column was incubated at room temperature for 1 min and then centrifuged at 13000 g for 1 min. The minicolumn was discarded and the eluted DNA stored at -4 °C.

Gel purification of PCR product

In the event that multiple bands were observed after visualization of PCR products, gel purification using the Wizard SV Gel and PCR Clean-Up System (Promega, catalogue no. A9281) was employed in order to eliminate the undesired product from the sample. A 20 µl aliquot of PCR product was mixed with 5 µl 6X Blue/Orange Loading Dye (Promega, catalogue no. G1881) and loaded onto a 0.8% agarose gel and electrophoresed for 45 min at 100V as described above. The gel was then placed onto a portable UV light (Vilber Lourmat 365 nm) and a clean blade used to excise the band of the desired size. The excised slice of gel was then placed into a clean 1.5 ml microcentrifuge tube and covered with approximately 300 µl Membrane Binding Solution. The mixture was transferred to an SV Minicolumn fitted into a Collection Tube and the manufacturer's instructions followed as described previously.

Sequencing

Purified PCR products were sent to Inqaba Biotechnology, Pretoria, South Africa for sequencing. Big Dye V3.1 was used to sequence the sample by Sanger sequencing. The sequences were read using Finch TV software (Geospiza Inc., version 1.4.0) and the nucleotide sequences submitted to the National Centre for Biotechnology Information (NCBI) website and identified using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1990). Similarity matches of 97-98% are considered sufficient for identification down to genus level and ≥99% is considered identification at species level (Drancourt *et al*., 2000).

Mycorrhizal isolation and identification

Mycorrhizal spore extractions

Arbuscular mycorrhizal spores were extracted from soil collected from 6 sites from the Cerebos Salt Pans (from here on referred to Salt Pan samples) and from 6 sites from Haakdoorn Duin (Kalahari samples) using a wet sieving, decanting and sucrose centrifugation technique (Smith & Dickson, 1997). A sample of each soil (100 g) was added

to 500 ml water and vigorously agitated. The soil was allowed to settle for 30 sec and the water poured off over a set of sieves of varying mesh sizes: 425 µm, 250 µm, 125 µm, 45 µm. The washing process was repeated three times per sample. The material trapped by the sieves was then carefully washed off with distilled water into conical centrifuge tubes. Taking care to balance the tubes, the tubes were then centrifuged at 1900 rpm for min using a Megafuge 1.0 R. Supernatant was poured off and the remnants re-suspended in 60% sterile sucrose. Tubes were then centrifuged at 1900 rpm for 5 min. Whatman filter paper which had grid lines drawn upon it was then placed into a Buchner funnel and the supernatant poured over it and drained until dry. The filter paper was then carefully transferred to a clean petri dish and allowed to dry before examination. The spores trapped by the filter paper were examined using a Leica S 4E dissecting microscope to allow counting of the spores.

Trap cultures

In order to enrich the mycorrhizal cultures for molecular identification and field trial inoculum, mixed trap cultures were prepared from the Salt Pan and Kalahari samples, individually. Composite soil samples (100 g) were prepared by combining equal amounts of the 6 Kalahari samples and 6 Salt Pan samples. The composite soils were then mixed into sterilized sand as a growth medium and planted with surface-sterilized grass (*Cynodon dactylon*) seeds. The pots were placed in a greenhouse (min, 19 ^{o}C; max. 30 ^{o}C, natural lighting and automatic watering) and the plants grown for 6 months.

Mycorrhizal DNA extraction

In order to concentrate mycorrhizal spores for efficient and comprehensive DNA extraction, spore extractions were performed on 50 g trap culture samples. The extraction was performed using only the 425 um sieve to remove large debris and then the 45 um sieve was used to retain all possible spores. The fine debris and spores were carefully transferred into a sterile dish and two DNA extractions performed from each sample. DNA was extracted using the ZR Soil Microbe DNA Kit (Zymo Research, catalogue D6001). The extracted DNA was then

combined and visualized using Agarose Gel Electrophoresis (AGE) as described previously. The size of the DNA was estimated using the 1000 bp DNA molecular marker for fungal DNA.

Amplification of 18S rDNA for pyrosequencing

454-Pyrosequencing was employed to investigate the mycorrhizal community composition within each of the sample sites from which they were collected. The AM fungal specific primers NS31 and AML2 (Lee & Young, 2008; Krüger *et al*., 2009) were used to amplify the 18S rDNA for sequencing. The primers were tagged with short unique sequence tags called multiplex identifiers (MIDs) which enable identification of reads from different samples after sequencing (Table 2.3).

Table 2.3: Nucleotide sequences of the 18S rDNA primers used in this study. The region in blue represents the adaptor sequence, followed by the MID tag in red and the site-specific primer in black.

Primer	Sequence
NS31	5' - TTGGAGGGCAAGTCTGGTGCC - 3'
NS31-MID1	5' - CGTATCGCCTCCCTCGCGCCATCAGACGAGTGCGTTTGGAGGGCAAGTCTGGTGCC - 3'
NS31-MID2	5' - CGTATCGCCTCCCTCGCGCCATCAGACGCTCGACATTGGAGGGCAAGTCTGGTGCC - 3'
AML2	5' - GAACCCAAACACTTTGGTTTCC – 3'
AML2-MID1	5' - CTATGCGCCTTGCCAGCCCGCTCAGACGAGTGCGTGAACCCAAACACTTTGGTTTCC – 3'
AML2-MID2	5' - CTATGCGCCTTGCCAGCCCGCTCAGACGCTCGACAGAACCCAAACACTTTGGTTTCC – 3'

A nested-PCR approach was employed in order to achieve amplification. The untagged primer set was used for initial amplification of the DNA and the resulting product was visualized using AGE as described previously. DNA gel extraction and purification was performed on bands of the correct size and then MID-tagged primer sets were subsequently used to achieve a uniquely tagged amplification product for 454-pyrosequencing. The 50 µl reaction mixture was prepared as follows: 25 µl Kapa HiFi Hotstart ReadyMix, 2µl NS31,

2µl AML2, 2-5 µl template DNA and made up to 50 µl with nuclease-free water. Amplification was performed using a 2720 Thermal Cycler (Applied Biosystems) with the following cycling parameters (Table 2.4).

Table 2.4: Thermal cycling parameters used in the amplification of 18S rDNA.

Parameters	Temperature (°C)	Time (seconds)	Cycles
Initial Denaturation	98	300	1
Denaturation	98	30	
Annealing	49.7	45	5
Extension	72	60	
Denaturation	98	30	
Annealing	65	45	20
Extension	72	60	
Final Extension	72	300	1

PCR products were electrophoresed and visualized as described previously. PCR product was then purified using the Wizard SV Gel and PCR Clean-Up System (Promega, catalogue no. A9281) and subjected to emulsion PCR before sequencing using the GS FLX Titanium Sequencer (454 Life Sciences, Roche) at the Rhodes University Pyrosequencing Facility. The resulting sequence reads were curated using MOTHUR (Schloss *et al*., 2009) according to the protocol supplied by the sequencing facility (Appendix G). Sequences were assigned to Operational Taxonomic Units (OTU's) using a 97% species identity threshold.

Due to the lack of a comprehensive mycorrhizal sequence database for the SSU 18S region, a database for manual blasting was created using a stand-alone blast programme downloaded from the NCBI website. A reference database was created using the Virtual Taxa (VT) sequences supplied by the Maarjam website (Öpik *et al*., 2010). Using the stand-alone blast programme, the curated pyrosequencing dataset was blasted against the VT's supplied by Maarjam and the dominant OTU's identified down to Genus level.

Greenhouse plant trial

Growing conditions

Plastic pots (7L) were sterilized in 1% sodium hypochlorite for 15 min, fitted with a sterile plastic mesh bottom to prevent soil leakage and then filled to approximately 4 cm below pot rim with river sand which had been collected from un-cultivated riverine habitat in Grahamstown, Eastern Cape. The river sand was sieved and pasteurised. The physiochemical composition of the river sand was analysed by North-West University Eco-Analytica, Potchefstroom, South Africa and is presented in Table 2.5 below.

Table 2.5: Physiochemical variables of experimental soil used in pot trial.

Parameter	Nutrient Status (mg/kg)
Calcium	95.5
Magnesium	37.5
Potassium	13.0
Sodium	15.0
Phosphate	16.5
CEC	9.75
pH (H_2O)	6.44
pH (KCl)	5.75
EC (mS/m)	71

Pots were then transported to a to a naturally lit, clear polyethylene tunnel greenhouse tunnel. The average day / night temperatures in the tunnel were 27.7 °C ± 0.17 s.e / 16.8 °C ± 0.8 and and the average day / night relative humidity was 52.0% ± 0.48 s.e / 82.4% ± 0.20. Once in the tunnel, all pots were watered to field capacity (approximately 24.41%) (Appendix F) in order to rinse off any trace amounts of bleach as well as to standardize the soil moisture content before planting.

Maize (*Zea mays*) seeds were obtained from Pannar seeds (variety BG 3292). The seeds had been pre-treated with pesticide and herbicide which had a red colouring. In order to minimize the potential harmful effect of these chemicals on bacterial and mycorrhizal establishment the chemicals were removed. Seeds were placed in a large beaker of warm water (approximately 40 °C) amended with 25 g Bicarbonate soda and 50 ml of a common household dishwashing liquid. The seeds were then vigorously agitated until the chemical treatment began to wash off. The process was repeated until very little red-colour could be seen on the seed surface.

The seeds were then rinsed 5 times in distilled water and left to soak overnight at room temperature in distilled water.

A germination test was conducted to ensure that the washing process had not damaged the seed. Seeds were surface sterilized in 3.2% sodium hypochlorite for 15 min and rinsed 3 times in sterile distilled water before planting. Four maize seeds were planted approximately 5 cm apart in the centre of each pot. Seeds were covered with 500 g (approximately 1.5 cm) of river sand and the pots watered lightly. Pots were watered daily to maintain constant soil water content close to field capacity. Treatments were arranged randomly but grouped into alternating sets of 8 control and drought treatments. The river sand analysis (Table 2.5) revealed that the soil was relatively low in nutrients so in order to supplement the nutrient composition of the growth medium to optimize maize growth, pots were watered once per week with 50 ml of a modified nutrient solution (Smith *et al.*, 1983) (Appendix D). The solution was modified to be lower in phosphorus so as to stimulate the phosphate solubilising activity of the applied microorganisms.

Bacterial inoculum

The three bacterial isolates showing the highest potential for PGP activity were selected for application in a greenhouse pot trial. Bacterial isolates were inoculated into 4 L of LB supplemented with 300 mM NaCl and incubated overnight at 28 °C. Cell concentrations were measured spectrophotometrically at 660 nm and adjusted to an OD reading of 0.5 using sterile LB. 50 ml of each culture was aseptically dispensed into sterile conical centrifuge tubes and the cells pelleted by spinning at 3000 rpm for 2 min. Cells were then re-suspended in 0.8% sterile saline. Each pot was inoculated twice at one week intervals with 25 ml bacterial suspension poured directly into the centre of the pot covering the area of seeds planted.

Mycorrhizal inoculum

Trap cultures containing indigenous mycorrhizal propagules was mixed with sterile growth medium and placed into 7 L pots. Surface sterilized *Sorghum bicolor* seeds were planted as host plants and grown for 6 months in a greenhouse with controlled humidity and automatic watering. Soil and root material (1.2 kg) from each starter culture was removed from the pots.

Root material was cut up into 1 cm pieces and mixed back into the sample. The two soil samples were combined and used as mycorrhizal inoculum in the plant trial. In pots assigned to a mycorrhizal treatment, 30 g of mycorrhizal inoculum was placed in the centre of each pot before planting. A spore extraction was performed on a sample of the inoculum and the spore count was estimated to be 150 spores / 30 g inoculum. The spores counted included large, darker spores as well as very small, pale spores which indicate that the started cultures had successfully allowed the proliferation and reproduction of the mycorrhizal communities within.

Experimental design

The treatments imposed during the trial were as follows (Table 2.6):

Table 2.6: Plant pot trial experimental design

Treatment	Replicates per Group	
	Drought	**Control (Watered)**
Myc + B1	9	9
Myc + B2	9	9
Myc + B3	9	9
B1	9	9
B2	9	9
B3	9	9
Myc	9	9
Control (Uninoculated)	9	9

Maize plants were thinned to one plant per pot after 3 weeks of growth and the drought trial was initiated at 6 weeks. Pots which were marked for drought had saucers placed beneath them to prevent any water being absorbed from below the pots whereas pots which were kept at field capacity were allowed to drain freely. Drought was initially imposed by severely restricted watering (50 ml/day) and then pots were re-watered on day 19 and maintained at the field capacity for 7 days. A more gradual drought (250 ml every 2nd day) was then imposed for the rest of the trial period. The control group was watered with 400 ml water every second day.

Determination of soil water contents

The soil moisture content of the pots was monitored on alternating days using a soil moisture probe (ThetaProbe, type ML2x, Delta-T Devices) which had been calibrated to the specific growth medium according to the manufacturer's instructions (Appendix F).

Physiological measurements

Measurements of photosynthetic rate, stomatal conductance and transpiration rate were carried out between 9 am and 4 pm over a period of 7 weeks in a climate controlled, naturally lit, clear polyethylene tunnel using a LiCor LI-6400XT Portable Photosynthesis System (Li-Cor Inc., Lincoln, NE, USA). Measurements were taken on an attached, fully expanded leaf (3^{rd}-youngest) approximately 6 cm from the leaf tip. Leaves were clamped into the 2 cm^2 chamber of the flourometer for 2-4 min until the stomatal conductance values displayed by the instrument had stabilized. A photosynthetic photon flux density of 1500 μmol $m^{-2}s^{-1}$ was supplied by the red-blue LED internal light source and air temperature was set at 30 °C. Five replicate measurements were taken per leaf and 3-7 plants were measured per treatment. Measurements taken over 2 consecutive days were combined into single measurement sets and a total of 12 sets of measurements were taken in the duration of the trial.

Plant Growth Parameters

The trial plants were harvested the day after the last physiological measurement had been taken using the LiCor. Plants were dissected into roots and shoots. The shoots were placed into a paper bag and weighed on site. The roots were carefully removed from the pots and the soil gently washed off, after which they were placed into a paper bag and weighed. A 2 g subsample of the root material was retained for AM fungal colonisation assessment. The roots and shoots were placed in a drying oven at 60°C for 72 hrs after which they were weighed again and the dry weights recorded. The root dry weight was corrected for the removed subsample.

Mycorrhizal colonisation

A subsample of root material was retained from each plant in order to assess the colonisation

of plant roots by arbuscular mycorrhizal fungi. Colonisation was assessed using the method described by Smith and Dickson, 1997. Roots were cleared by soaking in a 5% KOH solution overnight. This solution was then carefully poured off and the roots washed with distilled water. The roots were then bleached by immersion in an alkaline H_2O_2 solution for 30 min. In order to ensure adequate binding of Trypan Blue stain, the roots were acidified by immersion in 0.1M HCl for 2-3 hours. The HCl was then poured off and the roots covered with a lactoglycerol solution containing 5% Trypan Blue. The roots were stained overnight at room temperature and then de-stained for 24hrs using a lactoglycerol solution without Trypan Blue. Roots were then mounted onto glass slides in 2 cm sections and viewed under a compound microscope at 100x magnification. Colonisation was characterized by the observation of intra- and extra-cellular structures such as arbuscules, vesicles, hyphae and spores. Fifty fields-of-view were examined per slide and percentage colonisation was estimated as follows:

Percentage colonisation = colonised root material in field-of-view/50 x 100

Data analyses

Data was collected from all of the replicates with n=3-9 depending on the experiment. All statistical analyses were performed using STATISTICA 12. General Linear Model ANOVA was used to compare treatments and Tukey's Post-hoc test was performed on any significant variables measured (StatSoft Inc, 2013).

Chapter 3

Results

Isolation and characterisation of bacterial isolates

Isolation of drought and saline adapted bacteria

Bacterial isolates were sub-cultured and maintained on 600 mM NaCl Nutrient agar and many of them appeared to require salt for growth as growth was strongly diminished when cultured on standard Nutrient Agar. The isolated colonies exhibited a variety of colours and morphologies ranging from white and creamy to glossy pink. The selection of morphologically different colonies from cultures inoculated from rhizosphere soil and rhizosheath material resulted in 50 isolates.

Indole Acetic Acid (IAA) production

One of the main characteristics of Plant Growth Promoting bacteria is the production of Indole Acetic Acid. For this reason, production of IAA was used for initial screening of isolates. The assay for IAA production employed both Kovac's Reagent (Kerkar *et al*., 2012) as well as Salkowski's Reagent (Gordon & Weber, 1951). The 9 isolates which produced the highest concentrations of IAA were selected for further testing. Salkowski's reagent produced a pink-colouration in the presence of IAA Fig 3.1(A) and Kovac's reagent produced a red ring on the surface of the culture Fig. 3.1(B).

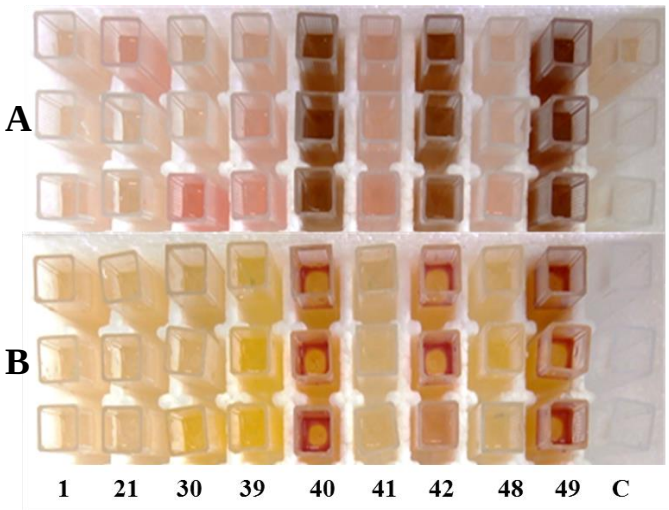

Figure 3.1: Assays for the production of IAA (A) using Salkowski's reagent a positive reaction was indicated by a pink-red colouration in the medium, (B) using Kovac's reagent a positive reaction was indicated by a red ring on top of the medium. Each column contains a different isolate and each cuvette holds a replicate sample.

Isolates 1, 21, 30, 40, 41, 42, 48 and 49 all tested positive for varying amounts of IAA using Salkowski's reagent (Fig 3.1A). Only isolates 40, 42 and 49 tested positive for IAA when assayed with Kovac's reagent (Fig 3.1B).

Table 3.1 details the estimated concentration of IAA produced by the 9 isolates which produced the most IAA. Concentration of IAA in the media was calculated using a standard curve prepared from known quantities of IAA (Appendix A).

Table 3.1: Concentration of IAA produced by isolates after testing with Salkowski's reagent.

Isolate number	OD_{530}	IAA (µg/ml)
1	0,1651	3,067
30	0,1847	3,873
21	0,2042	4,673
39	0,2906	8,230
48	0,3169	9,313
41	0,3812	11,959
40	0,4689	15,568
42	0,8106	29,630
49	0,8901	32,900

As seen in Figure 3.1, the Kovac's assay for IAA failed to detect isolates producing relatively

high levels of IAA as only isolates 40, 42 and 49 tested positive. Table 3.1 illustrates the range of concentrations detected by the Salkowski's assay.

PEG 8000 drought tolerance

PEG simulates physiological drought in the field by binding to water molecules, thereby making them physiologically inaccessible to organisms growing in liquid culture (Michel & Kaufmann, 1973). In this way, PEG can be used to lower the water potential of a liquid solution and simulate the progression of drought in a pot trial. Isolates were grown in Luria Broth with a range of lowered water potentials which exceed the water potential which causes permanent wilting in plants (Tolk, 2013). Figure 3.2 below illustrates the growth as measured by cell density (OD_{660}) in relation to optimal growth as determined by cell density after 24 hrs growth in standard Luria Broth.

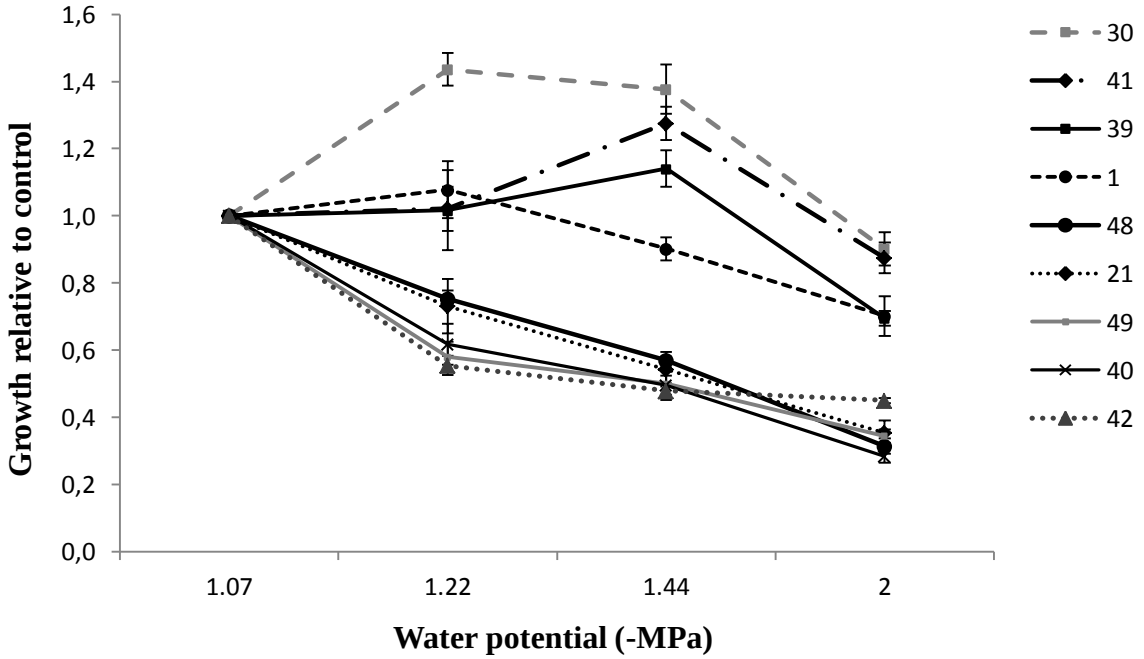

Figure 3.2: Growth of isolates in solutions of varying water potentials (-1.22, -1.44, -2.0 MPa) relative to growth in the control medium (Luria Broth, -1.07 MPa). Solutions comprise of Luria Broth supplemented with 100, 150 and 262 g/L PEG 8000 respectively. Each point represents the average growth relative to the LB control of individual isolates as measured by Optical Density (OD) at 660 nm. Vertical bars are standard errors (n=3).

The growth trends observed in Figure 3.2 indicate that some isolates thrive at a lower water potential whereas others exhibited an immediate decline in growth. The isolates exhibited a marked decline at -2 MPa but it is interesting to note that isolates 30 and 41 maintained 90% of their 'normal' growth at a water potential which is far beyond the wilting point of most plants.

Siderophore production

Under nutrient limiting conditions such as those imposed during a drought, bacteria rely upon mechanisms such as the production of iron-chelating siderophores in order to assimilate essential micronutrients. Siderophores are low-molecular weight, Fe(III)-specific ligands which access insoluble iron in the surrounding media, allowing bacteria to maintain cell functioning (Schwyn & Neilands, 1987). The overlay method was used to assay isolates for the production of siderophores.

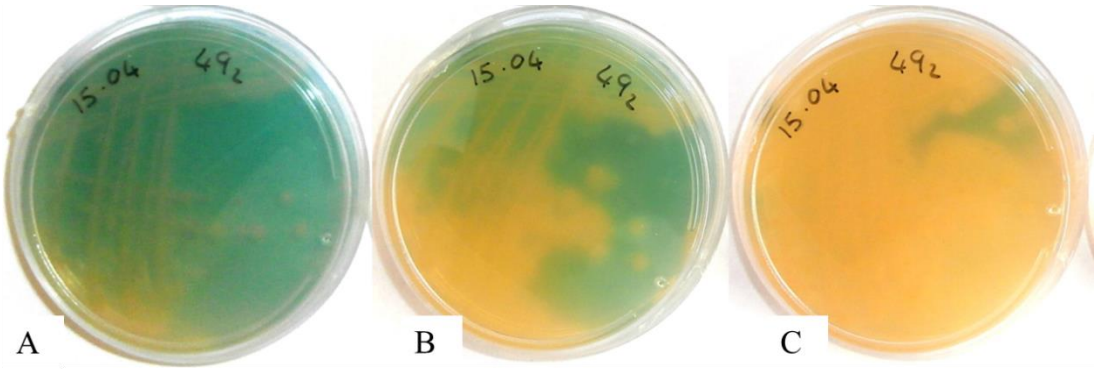

Figure 3.3: Assay for the production of siderophores on CAS agar. Figure 3.3(A-C) illustrates the colour change from blue to yellow after (24, 72 and 120 hrs) which indicates the production of siderophores by the bacteria.

The overlay method produced a clear indication of the excretion of siderophores into media (Fig 3.3). All of the isolates tested positive for the production of siderophores but to varying degrees (Table 3.2). Some isolates appear to produce less-soluble forms where the agar directly in contact with the colony shows the colour change but it does not spread outwards into the media. This is in comparison to the widespread colour change observed in Figure 3.3 (C).

Phosphate solubilisation

Phosphorus is one of the major limiting nutrients required for plant growth and is often bound in insoluble forms in the soils (Nautiyal, 1999). Pikovskaya's phosphate solubilisation agar was used to assay the 9 selected isolates for the ability to solubilise phosphate. Figure 3.4(A) illustrates a negative reaction for phosphate solubilisation whereas the development of a zone of clearance around the culture (Fig. 3.4B) indicates the ability of the isolate to solubilise phosphate in the media. The width of the zone of clearance is directly proportional to the amount of enzyme produced (Nautiyal, 1999).

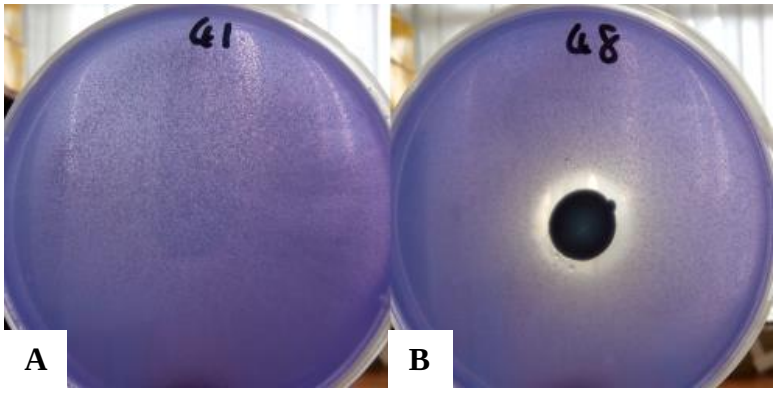

Figure 3.4: Assay for phosphate solubilisation using Pikovskaya's agar supplemented with 0.01% Bromophenol Blue. (A) negative response (B) clear zone of clearance indicated a positive response.

Of the 9 isolates tested for phosphate solubilisation, 7 tested positive to varying degrees. Some isolates produced a larger, more diffuse halo which was not completely clear whereas others, such as isolate 48 pictured above produced a small, completely clear halo (Fig 3.4B).

Production of ammonia from peptones

Isolates were tested for the ability to breakdown proteins using proteases, forming smaller molecules such as ammonia as a by-product. Isolates were cultured in Peptone Broth for 48 hrs and assayed for the presence of ammonia by the addition of 0.5 ml Hydrochloric acid (HCl). The development of a yellow-brown colouration in the media indicates the presence of

ammonia in the media. Figure 3.5 illustrates the range of colour changes observed from various isolates.

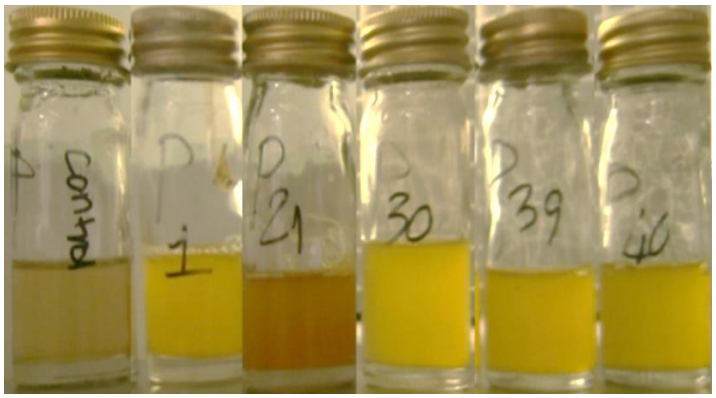

Figure 3.5: Assay for the production of proteases. Isolates displaying a yellow-brown colour development are considered positive for presence of ammonia, the product of protein hydrolysis.

All of the isolates tested positive for the production of ammonia with isolate 21 exhibiting a stronger ability to breakdown protein (Table 3.2). The amount of ammonia produced is relative to the ability of the bacteria to break down proteins in the environment, thereby making them more easily accessible for assimilation and hydrolysis into amino acids.

Catalase production

The production of the catalase enzyme is a cellular defence mechanism against the harmful oxidative effects of hydrogen peroxide (H_2O_2), a by-product of oxygen metabolism. The catalase enzyme facilitates the breakdown of hydrogen peroxide into water and oxygen, resulting in the rapid formation of bubbles (Taylor & Achanzar, 1972). Vigorous bubbling at the site of contact between H_2O_2 and the bacteria is a positive reaction for the production of catalase and 8 of the 9 isolates tested positive for varying levels of catalase production (Table 3.2).

Summary of Plant Growth Promoting characteristics

The results from the assays which characterise isolates as potentially plant growth promoting (PGP) are summarised below.

Table 3.2: Summary of the selected isolates PGP characteristics.

Isolate number	IAA Salkowski (µg/ml)	IAA Kovacs	Growth (%) at low water potential -1.44 mPa	Siderophore production	Phosphate	Catalase	Ammonia
1	3.07	-	90.2	++	+	+	-
21	4.7	-	54.3	+	+	+	++
30	3.9	-	137.7	+++	++	+	+
39	8.2	-	114.0	++	-	+	-
40	15.6	+	49.5	+	+	+	+
41	12.0	-	127.5	+	-	-	++
42	29.6	+	47.8	+	+	+	++
48	9.3	-	56.9	+++	+++	+	+
49	32.9	+	49.9	+++	++	++	+

Table 3.2 above illustrates that single isolates or species may possess many potentially advantageous characteristics however the distribution of beneficial traits across many different species emphasizes the need for diversity and commensalism within microbial communities. Isolates 42 and 49 had by far the highest IAA activity and were positive for all PGP characteristics tested. Isolate 48 had a lowered IAA production but very high phosphate solubilisation and siderophore excretion activity. All three isolates were moderately tolerant (~50% relative growth) to PEG induced drought conditions. Isolates 42, 48 and 49 were selected for testing in the plant pot trial and will subsequently be referred to as isolates B1, B2 and B3 respectively.

Gram staining

The selected isolates were further characterised by their cell shape and Gram stain reaction. All three isolates were small, short Gram-negative rods. They did not appear to be chain

forming and no endospores were observed. Isolate B2 appeared much smaller than isolates B1 and B3 and also appeared to be slime-forming due to the clumping pattern observed under the microscope.

Molecular identification of bacterial isolates

DNA extraction

Genomic DNA was extracted and electrophoresed in order to determine the quality and quantity of DNA for PCR.

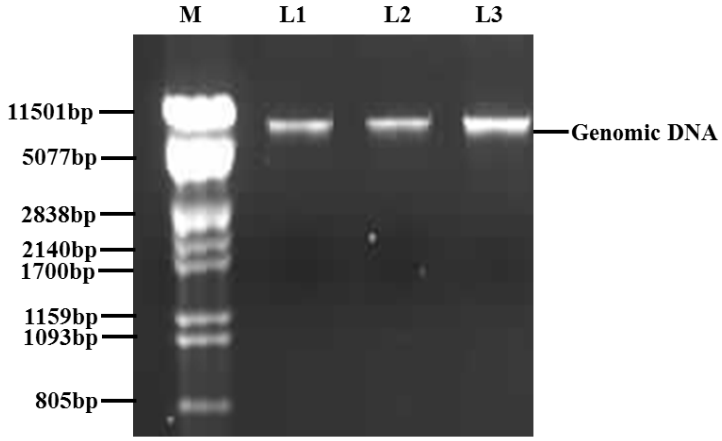

Figure 3.6: Genomic DNA of bacterial isolates. Lane 1 (M) contains molecular weight marker, Lanes 1-3 contains genomic DNA extracted from isolates B1, B2 and B3.

Amplification and purification of target DNA sequence by Polymerase Chain Reaction (PCR)

The 16S rDNA was amplified using PCR and the product visualized after gel electrophoresis in order to determine the quality, quantity and size of the amplified product for sequencing. The visualization of the gel confirmed that the gene had amplified correctly, however the PCR product appeared as if there may have been some non-specific binding and shearing of the DNA. For this reason, the PCR product was purified using gel-extraction in order to ensure that the product was high quality, free of contamination and ready for sequencing.

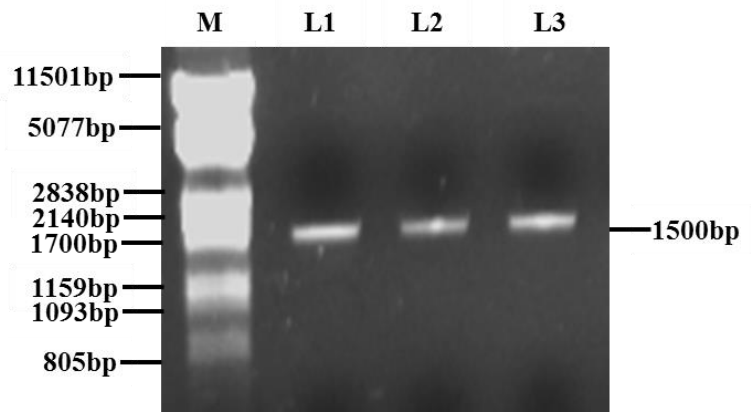

Figure 3.7: PCR product of bacterial isolates. Lane 1 (M) contains Lambda/*Pst* molecular weight marker. Lanes 1-3 contain the gel-purified PCR products of isolates B1, B2 and B3.

Sequencing and identification

Sequences returned after Sanger sequencing were read using Finch TV software and the nucleotide sequences submitted to the National Centre for Biotechnology Information (NCBI) website for identification using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.* 1997) (Table 3.3).

Table 3.3: Nucleotide sequence alignments and identities of selected isolates.

Isolate	Species	Query coverage (%)	E-value	Identity (%)	Accession
B1	***Achromobacter xylosoxidans* C54**	100%	0.0	98	CP009448.1
B2	***Klebsiella oxytoca strain* M1**	100%	0.0	98	CP008841.1
B3	***Achromobacter xylosoxidans* A8**	100%	0.0	97	NC_014640.1

Molecular identification of mycorrhizal isolates

Mycorrhizal spore extractions

Arbuscular mycorrhizal spores were extracted from individual soil samples using the wet sieving and decanting method (Smith & Dickson, 1997). Spores were examined under a

microscope, being sure to count only whole, viable-looking spores.

Table 3.4: Number of spores counted within each fraction of soil samples from the two sample sites.

Sample	45 µm	125 µm	Total/100g soil
Kalahari	1282	170	1452
Salt Pan	2691	530	3221

An abundance of spores were observed under the microscope with a wide range of morphologies. Spores ranged in size and colours ranged from pale-yellow to ocre, reddish-brown and brown-black. Spores appeared singly or in grape-like clusters and some spores were irregularly shaped and ornamented. Many spores had subtending hyphae still attached. The vast range of morphologies indicates a variety of species present within the communities sampled.

Soil analysis

The physiochemical composition of soils was analysed in order to further comprehend the environments from which the mycorrhizas and bacteria were isolated. The soil analysis provides insight into the nutrients and minerals which are available to the plants and the microorganism communities.

Table 3.5: Physiochemical composition of soils from sample sites.

Parameter	Salt Pan	Kalahari
Organic C (%)	1.10	1.29
Phosphate (mg/L)	38	20
Potassium (mg/L)	278	1299
Calcium (mg/L)	3939	4528
Magnesium (mg/L)	778	293
Zinc (mg/L)	7.6	1.1
pH (KCl)	7.72	7.50
Exchange acidity (cmol/L)	0.08	0.09
Total cations (cmol/L)	26.85	28.41

The soil analysis revealed substantial differences in the nutrient content of the soils. The Salt Pan soils were notably higher in Magnesium (Mg) and Zinc (Zn) and were slightly higher in Phosphorus whereas the Kalahari soil was much higher in Potassium.

Mycorrhizal DNA extraction

Genomic DNA was extracted and visualized after gel electrophoresis in order to determine the quality and quantity of DNA for PCR.

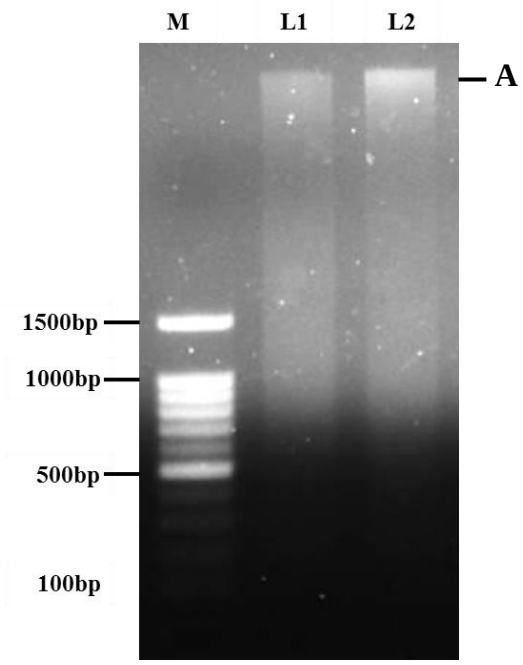

Figure 3.8: Genomic DNA extracted from soil samples. Lane 1 (M) contains molecular weight marker, Lanes 1 and 2 contain the DNA obtained from the Salt Pan and Kalahari samples respectively. The bands in L1 and L2 at position A represent supercoiled genomic DNA.

The presence of bands at the top of the agarose gel confirmed that DNA had been successfully extracted from the soil samples although it is not specific to mycorrhizas. PCR using AM fungal specific primers will selectively amplify mycorrhizal DNA from the mixed samples.

Amplification of 18S rDNA for pyrosequencing

The 18S rDNA was amplified using PCR and the product visualized after gel electrophoresis and purification in order to assess the quality, quantity and size of the amplified product for sequencing.

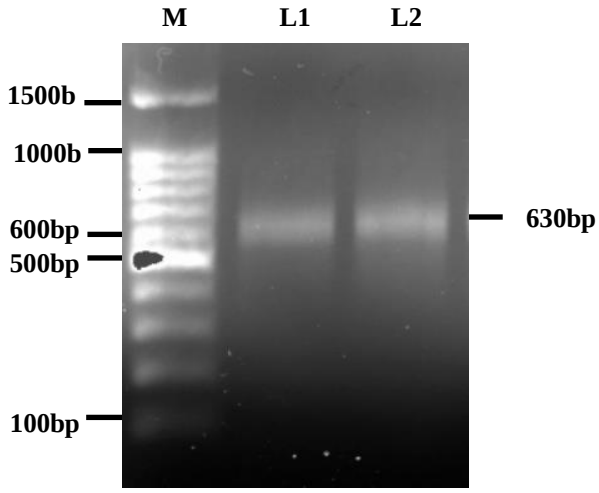

Figure 3.9: PCR product after gel purification. Lane 1 (M) contains molecular weight marker, Lanes 1 and 2 contain the amplified AMF 18S rDNA product of approximately 630 bp obtained from the Salt Pan and Kalahari samples respectively.

When compared to the molecular weight marker bands in Lanes 1 and 2 appear to be larger than the anticipated 570 bp. This may be due to the lengthy adaptor sequences which are fused to the primers in order to facilitate pyrosequencing. The region which was amplified was approximately 570 bp so the bands on the gel may be around 630 bp due to the 57 bp primers. The bands may also have migrated further had the gel been electrophoresed for a longer time period. The visualized product was sent for sequencing at the Rhodes University Pyrosequencing Facility.

Pyrosequencing analysis

Nucleotide sequences returned after 454-Pyrosequencing were curated using Mothur and a brief summary of the curation steps is presented in Table 3.6 below. The sequences which remained after curation were separated into Operational Taxonomic Units (OTU) using a species identity threshold of 97%.

Table 3.6: Summary of the pyrosequencing data curation steps using Mothur (Schloss *et al.*, 2009).

Curation step		Number of reads	
		Salt Pan (MID1)	Kalahari (MID2)
Summary at start		10512	12013
Trim all sequences <500bp		8071	9138
Merge files	Total:	17 209	
	Unique:	9478	
Align sequences	Total:	13520	
	Unique:	7497	
Precluster and trim	Total:	13520	
	Unique:	3387	
Remove chimeras (Uchime)	Total:	11293	
	Unique:	2187	
Final number of sequences		5256	6037
Number of OTU's		347	323

The representative sequences from each OTU were then identified down to Genus level using the VT sequences obtained from the Maarjam website and the stand-alone BLAST programme obtained from the NCBI website.

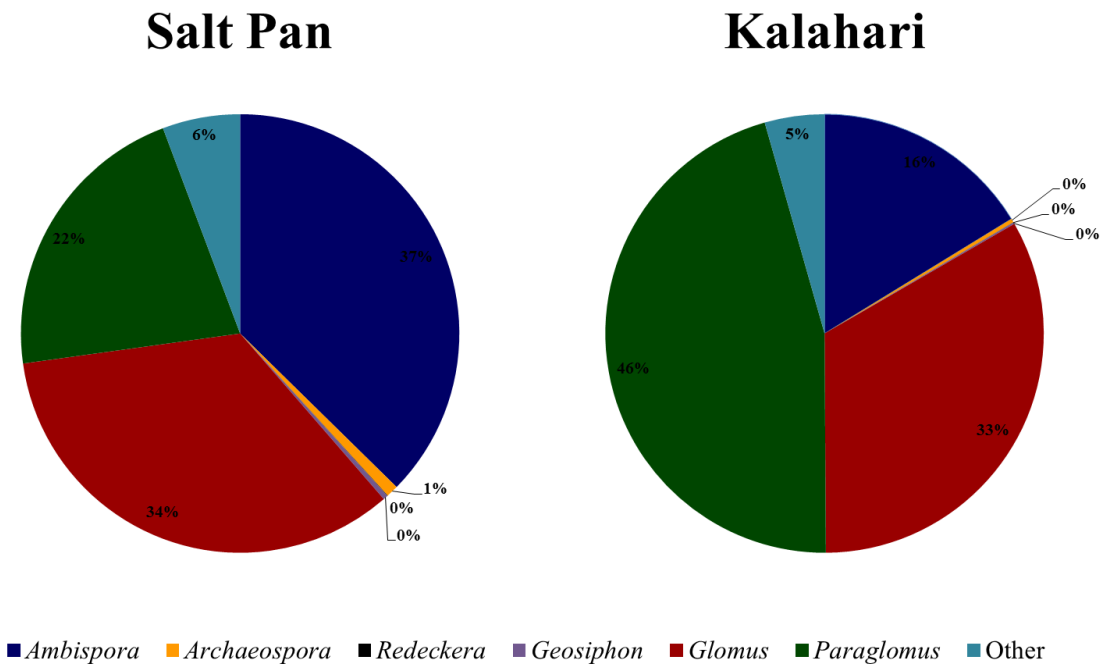

Figure 3.10: Proportion of the 18S fungal OTUs assigned to different fungal genera (according to the Maarjam VT sequence data) within each sample. Percentage labels were included for clarification where sections could not be discerned. OTU's which contained only 1 or 2 sequences were assigned to "Other".

A total of 11 293 sequences were assigned to 593 OTU's with 439 OTU's containing singletons. The Salt Pan sample contained a total of 5256 sequences assigned to 398 OTU's and the Kalahari sample contained a total of 6037 sequences assigned to 324 OTU's. There were a total of 10 601 sequences which were common to both samples and they were assigned to 77 OTU's. The sequence matches obtained from the stand-alone BLAST created using the Maarjam VT sequences ranged from 79% -99% which meant sequences could only be confidently assigned to Genus level.

The Salt Pan site was dominated by species in the *Ambispora* (37%) and *Glomus* (34%) genera and to a lesser extent by *Paraglomus* (22%). The Kalahari site was dominated by the *Paraglomus* (46%) and *Glomus* (33%) genera with the *Ambispora* representing only 16%. The *Archaeospora*, *Redeckera* and *Geosiphon* genera represent the rare components at less than 1% of the total species composition at both sites.

Evaluation of plant growth parameters

Soil moisture content

Gravimetric and probe-measured soil moisture values were correlated in order to develop a substrate-specific calculation of soil moisture content (Appendix F). The decline in soil water potential during imposed droughts was monitored using a ThetaProbe and plotted over the plant growth period.

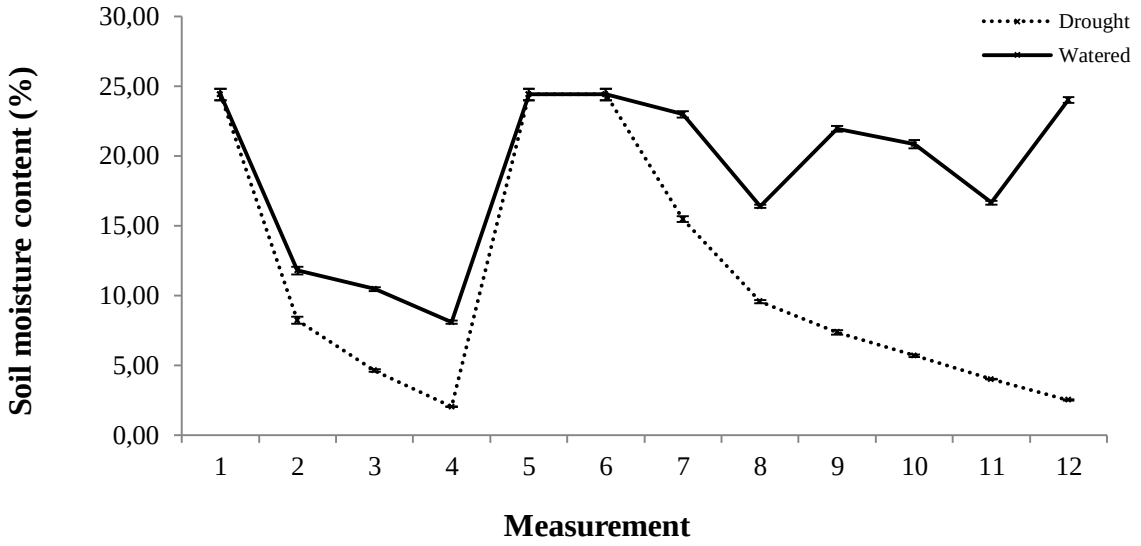

Figure 3.11: Soil moisture content of pot-cultivated *Zea mays* during the drought /recovery pot experiment. Each point represents an average of the drought and control groups (n = 32-56), vertical bars represent ± standard error. By measurement 4 (day 18) all of the plants were showing signs of severe water stress, indicated by leaves which were wilted, curled and turning brown. Effect of treatment on soil water content $F_{(1,875)=}1213.6$, $p<0.0001$ and effect of time $F_{(11, 875)}=109.6$, $p<0.0001$.

Drought imposition resulted in a significant difference in soil moisture content of the drought and watered groups (Figure 3.11) and the effects of drought on plant physiology were immediately evident (Figure 3.15-3.17). The first implemented drought resulted in a rapid decline in soil moisture (~ 3.2% per day for the first 5 days, after which 0.7% per day for 9 days) which caused plants to wilt and curl their leaves. At this point the plant photosynthetic capacity was significantly reduced (Figure 3.15). The recovery was implemented after measurement 4 and plants were maintained at field capacity until their photosynthetic rate had returned to the pre-drought range. The second drought was then imposed more gradually at approximately 1.9% per day for the first 8 days, after which 0.5% per day for 15 days.

Mycorrhizal colonisation

Colonization of maize roots was assessed by examining the roots under magnification and identifying mycorrhizal structures such as hyphae, spores and vesicles.

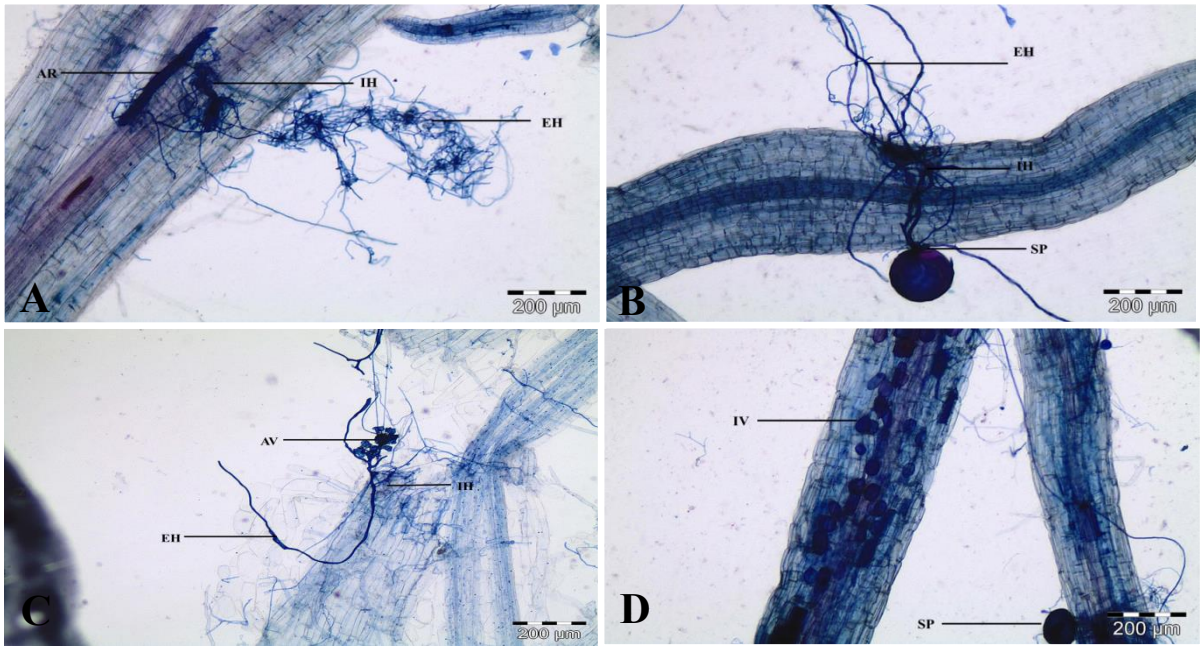

Figure 3.12: Light micrograph of the diagnostic arbuscular mycorrhizal structures in root cells of *Zea mays* after inoculation with mixed mycorrhizal cultures. A-Intraradical Hyphae (IH), extraradical hyphae (EH) and arbuscules (AR). B- Germinated mycorrhizal spore (SP) with intra (IH) and extra-radical hyphae (EH). C- Soil borne auxilliary vesicles (AV), D- Intraradical vesicles (IV).

In order to assess the extent of root colonization by mycorrhizas, roots were examined microscopically and the frequency of colonized roots assessed as a percentage of total root length examined.

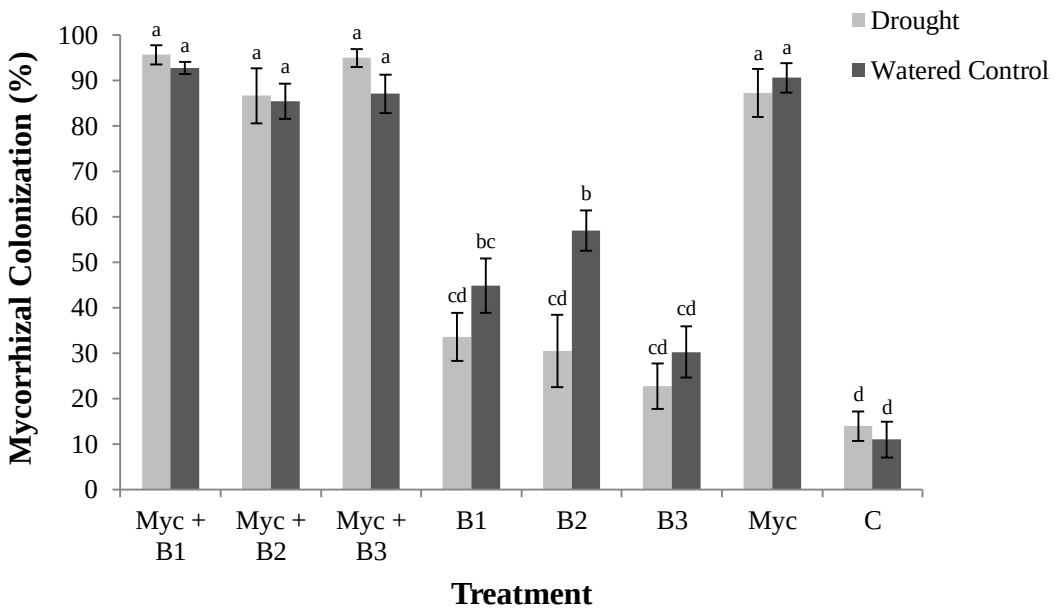

Figure 3.13: Colonization of *Zea mays* root cells by arbuscular mycorrhizal fungi at the end of the pot experiment. Each column represents a different treatment average (n = 9), vertical bars represent ± standard error. The treatment effect on colonization $F_{(15,128)} = 46.0$, $p<0.0001$. Significant differences at $P<0.05$ by Tukey's HSD Test.

The colonization by mycorrhizal fungi was evident in all treatments as characterized by the presence of spores, hyphae, vesicles and arbuscules (Figure 3.12). Percentage colonization was significantly higher in the treatments which were inoculated with mycorrhizal cultures. The colonization in un-inoculated treatments ranged between 11% and 57% and may possibly have resulted from cross-contamination due wind motion or splashing during watering (Duryea and Dougherty, 1991). Mycorrhizal helper bacterial effect was noted in the increase in colonisation in bacterial treatments. Despite the colonization observed in the un-inoculated controls, the significant difference in colonization levels is expected to have been large enough to stimulate a response in the plant physiology between treatments.

Biomass production

A mixed assemblage of mycorrhizal fungi and three bacterial isolates showing promise as PGPR were inoculated, either alone or in combination, onto maize seeds. Drought and watered control groups were grown for ~7 weeks and plants were subsequently harvested and separated into above- and below-ground biomass. Plant material was dried for 72 hrs at 60 °C and the dry weight data is presented below.

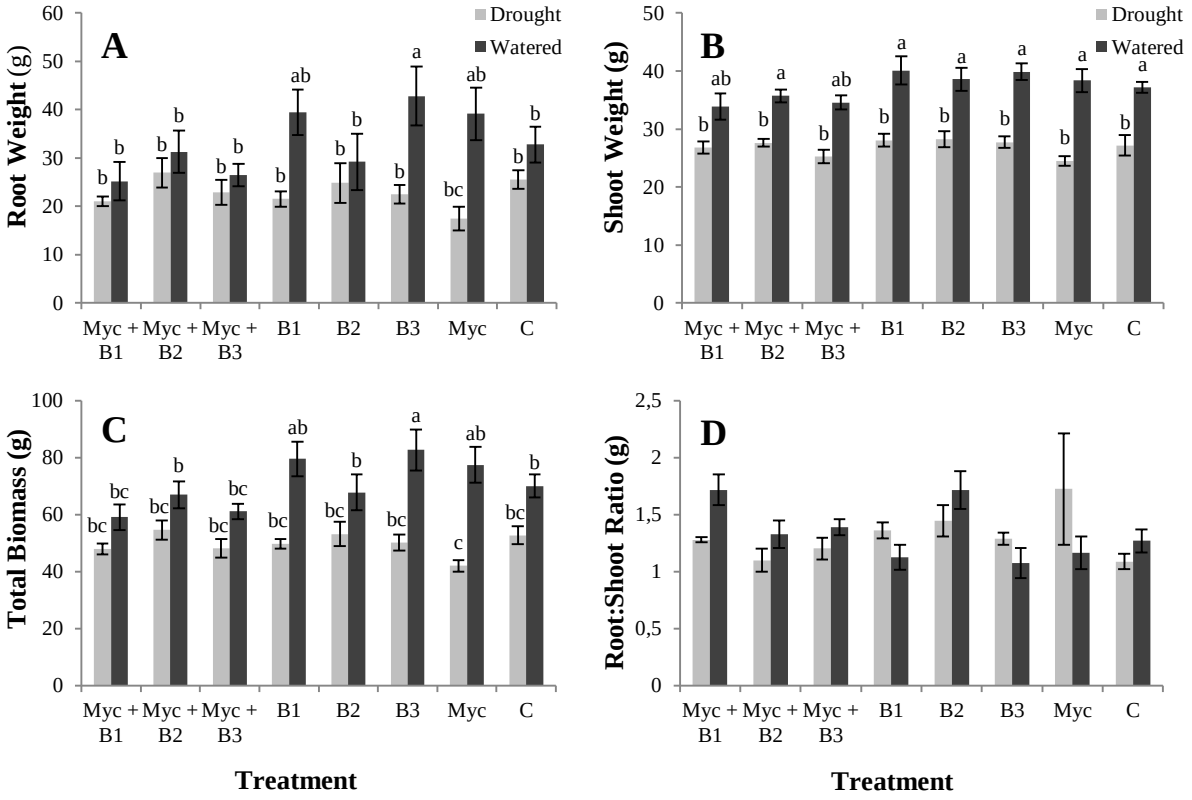

Figure 3.14: Plant growth assessment parameters: **A**) Root biomass of drought stressed and control groups. Treatment effect on root growth $F_{(15,\ 128)}$=3.6, p<0.0001; **B**) Shoot biomass. Treatment effect on shoot growth $F_{(15,\ 128)}$=14.4, p<0.0001; **C**) Total plant biomass. Treatment effect on total plant biomass $F_{(15,\ 128)}$=8.2., p<0.0001; **D**) Root-to-shoot ratio. Treatment effect on root: shoot ratio $F_{(15,\ 128)}$=0.8, p=0,6. Each point represents the average dry weight (g) of plants in the drought (D) and control groups (n = 9), vertical bars represent ± standard error. Different letters are significantly different (p<0.05) by Tukey's Post-Hoc Test and an absence of letters indicates no significant differences.

Photosynthetic capacity of Maize plants-Leaf Gas Exchange Measurements

Measurements of photosynthetic rate, stomatal conductance and transpiration rate were taken in order to evaluate the effect of microorganism treatments.

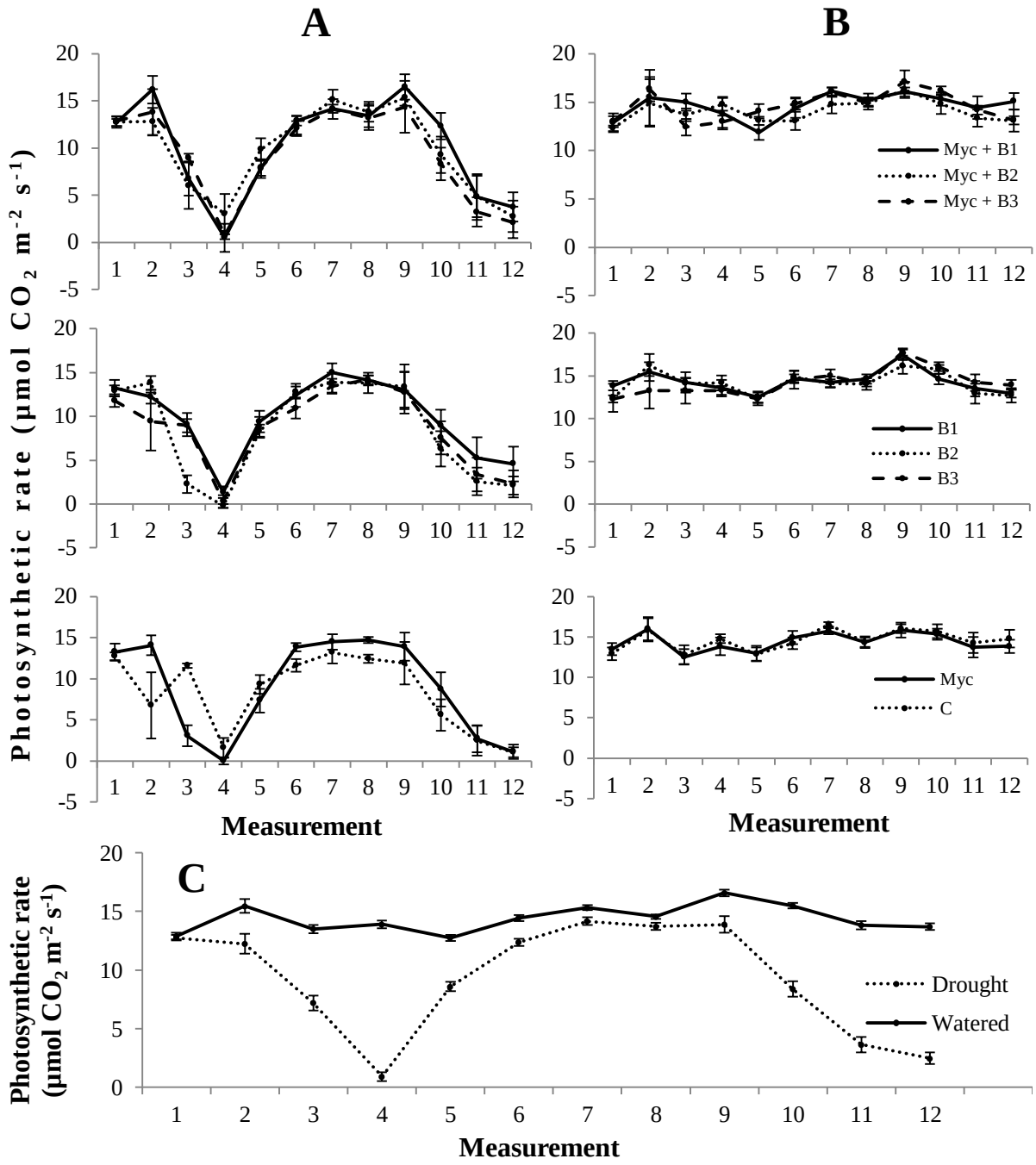

Figure 3.15: Photosynthetic rate of pot-cultivated *Zea mays* during the drought /recovery pot experiment. Panel A and B represent the drought and watered control groups respectively and the legend applies to both graphs in each row. Panel C contains the combined data from each group. Each point represents a different treatment average (n = 3-7 for separate treatments and n = 32-56 for combined groups), vertical bars represent ± standard error. The effect on photosynthetic rate of the imposed treatments over time: $F_{(165,904)} = 4.4$, $p<0.0001$. Significant differences at $P < 0.05$ by Tukey's HSD Test.

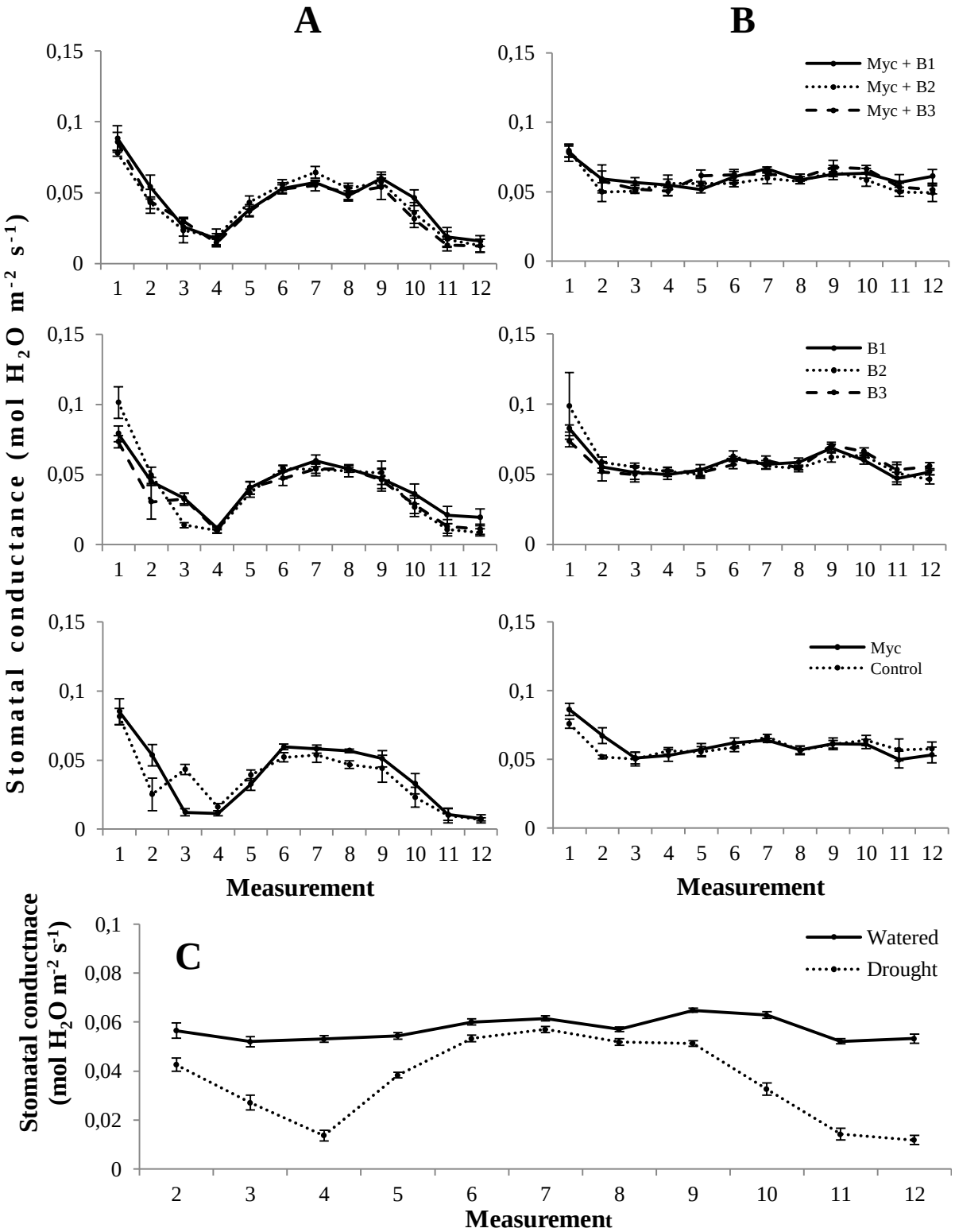

Figure 3.16: Stomatal conductance of pot-cultivated *Zea mays* during the drought /recovery pot experiment. Panel A and B represent the drought and watered control groups respectively and the legend applies to both graphs in each row. Panel C contains the combined data from each group. Each point represents a different treatment average (n = 3-7 for separate treatments and n = 32-56 for combined groups), vertical bars represent ± standard error. The effect on stomatal conductance of the imposed treatments over time: $F_{(165,904)} = 3.7$, $p<0.0001$. Significant differences at $P < 0.05$ by Tukey's HSD Test.

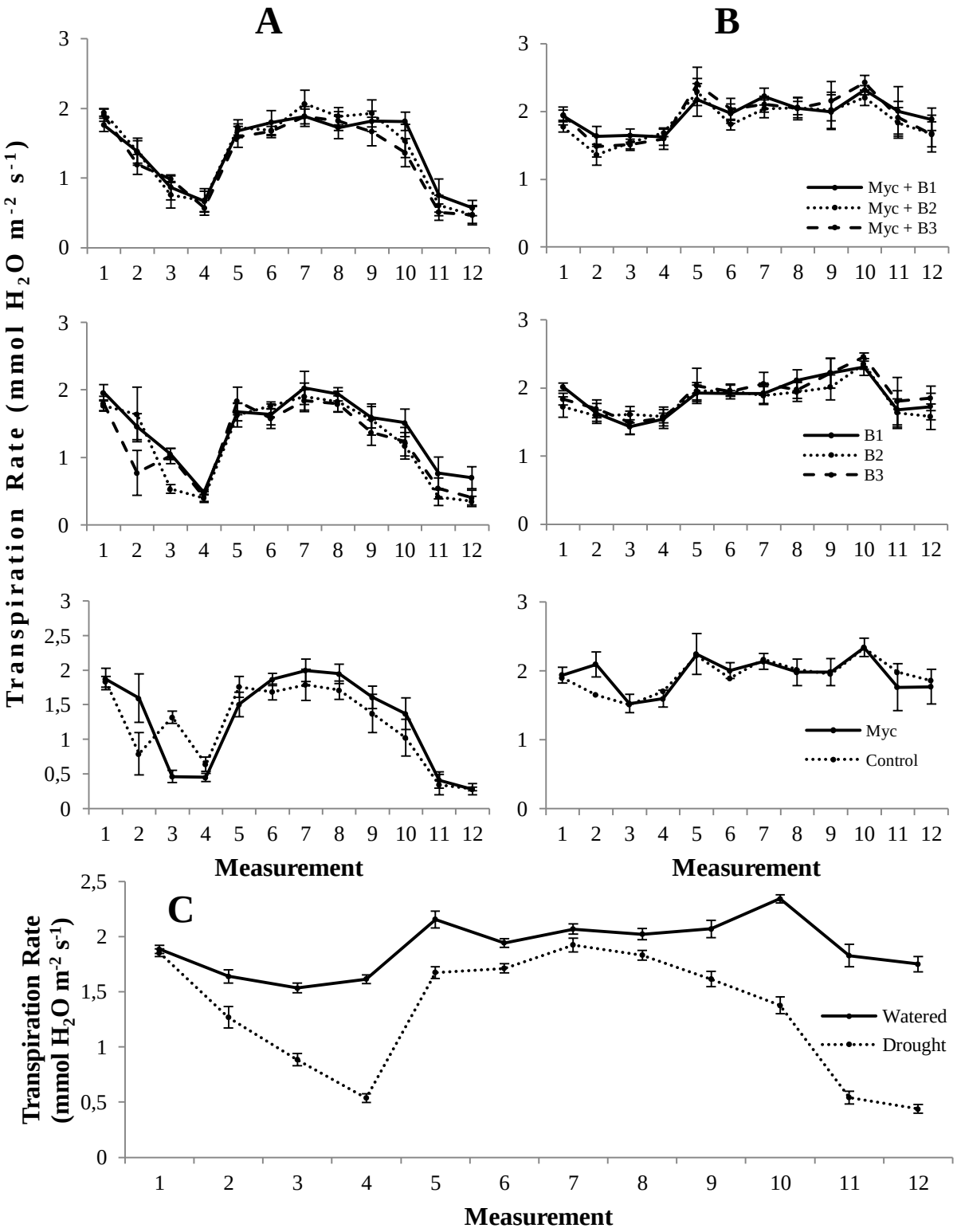

Figure 3.17: Transpiration rate of pot-cultivated *Zea mays* during the drought /recovery pot experiment. Panel A and B represent the drought and watered control groups respectively and the legend applies to both graphs in each row. Panel C contains the combined data from each group. Each point represents a different treatment average (n = 3-7 for separate treatments and n = 32-56 for combined groups), vertical bars represent ± standard error. The effect on transpiration rate of the imposed treatments over time: $F_{(165,903)} = 2.5$, $p<0.0001$. Significant differences at $P < 0.05$ by Tukey's HSD Test.

As can be seen from Figure 3.15, there was no significant difference in the photosynthetic rate of the plants inoculated with different mycorrhizal and bacterial treatments. This trend was observed both under drought (panel A) and well-watered conditions (panel B). The imposed drought resulted in a significant reduction in photosynthetic capacity of plants within all treatments (panel C). These same trends were mirrored by the stomatal conductance (Figure 3.16) and leaf transpiration (Figure 3.17) data.

Chapter 4

Discussion

Isolation of bacteria adapted to osmotic stress conditions

In the first part of this study the aim was so isolate rhizosphere bacteria which had been naturally adapted to tolerate drought stress and high salinity. It has been shown in literature that bacteria living in association with plants under conditions of drought stress or high salinity have co-evolved mechanisms to withstand these adverse conditions whilst simultaneously maintaining symbiotic relationships with host plants (Marulanda *et al*., 2009; Sleator & Hill, 2001). Bacteria used in this study were therefore isolated from rhizosphere soils in the Kalahari desert and the Cerebos Salt Pans. Isolations were performed across a gradient of increasing salinity (0-600 mM NaCl) in order to impose osmotic stress on the bacteria. It was found that there were prolific communities of bacteria thriving even at the highest osmolarity and the selection of morphologically different colonies from cultures inoculated from rhizosphere soil and rhizosheath material resulted in 50 isolates.

Detection of Indole Acetic Acid production

One of the most commonly cited criteria for effective plant growth promoting bacteria is the ability to produce the auxin Indole Acetic Acid (IAA) (Morris, 1986; Naveed *et al*., 2014; Sergeeva *et al*., 2002; Yamada, 1993). Having isolated salt-tolerant bacteria, these isolates were then tested for the ability to produce IAA from L-tryptophan. L-tryptophan is a common component of plant root exudates and is thus assumed to facilitate the symbiosis between PGPR and the plant host, where the bacteria are provided with an energy source and the plant benefits from the stimulating effect of increased IAA (Martens & Frankenberger, 1994). IAA is an auxin which is known to stimulate root tip elongation, alter root architecture and subsequently promote the growth of plants (Augé, 2004). The increased growth of plants then has a secondary feedback effect through increased production of root exudates, thus further benefiting microbial populations and forming the basis of the symbiosis (Sergeeva *et al*.,

2002; Smith & Read, 2008).

The initial approach used in this study to detect IAA production was the Kovac's test for Indole. Initial results yielded 11 positive reactions out of the 50 isolates tested. Literature has however stated that the Kovac's test is not conclusive for the detection of IAA (Lombard & Dowell, 1983) and thus the Salkowski's reagent was used in subsequent tests to confirm the results. Testing with Salkowski's reagent yielded many more positive results and furthermore enabled quantification of IAA production. It was interesting to note that the two reagents yielded very different results. When comparing the results (Figure 3.1; Table 3.2), it appears that the Kovac's reagent was only able to detect IAA yields in the range of approximately 20 µg/ml whereas Salkowski's reagent detected IAA concentrations as low as 4 µg/ml. The highest average yield obtained was 32.9 µg/ml, produced by *Achromobacter xylosoxidans* A8, followed by 29.6 µg/ml and 15.5 µg/ml by *Klebsiella oxytoca* strain M1 and an unidentified isolate respectively.

The discrepancy observed between results obtained using Kovac's and Salkowski's reagents is consistent with literature which has reported a difference in sensitivity amongst the various reagents. Lombard and Dowell (1983) compared the sensitivity of Ehrlich reagent, Kovac's reagent and p-dimethylaminocinnamaldehyde (DCMA) in detecting indole, 3-indoleacetic acid, 3-indolebutyric acid, 3-indolepropionic acid, 3-indolepyruvic acid, and 3-indolemethyl. The DMCA reagent was reported to be 100 times more sensitive than Kovac's reagent and 10 times more sensitive than Ehrlich reagent in detecting indole.

Similarly, Glickmann and Dessaux (1995) tested the specificity of Salkowski's reagent for a range of indolic compounds. There are multiple recipes for preparation of Salkowski's reagent and their study tested 3 formulations against the various indolic compounds. The formulations were all comprised of $FeCl_3$ and H_2SO_4 in different ratios and different amounts of reagent were added to culture supernatants in the assays. It was observed that the formulations were sensitive to different concentration ranges and that some reached a colour saturation point where the reagent was unable to discriminate between IAA concentrations any higher than 20 µg/ml. Irrespective of formulation, it was observed that Salkowski's

reagent is specific for IAA, Indole-3-acetamide (IAM) and Indole-3-pyruvic acid (IPyA) rather than for IAA alone. A difference in sensitivity was also observed when Salkowski's reagent was prepared with perchloric acid instead of sulfuric acid.

Sergeeva *et al*. (2002) compared the sensitivity of Salkowski's reagent, GC-MS and ELISA for detection of IAA and the results obtained showed that the Salkowski's assay was the least sensitive of the three techniques applied. It was observed that some of the Salkowski-negative strains responded positively using ELISA and GC-MS but never the reverse. These findings illustrate the wide range of variance within results obtained through the various methods used by researches to screen for IAA production. It is therefore important to decide how crucial accurate identification of this trait is in the overall experimental design. If the production of IAA is the foundation of a study then methods such as GC-MS and HPLC should be employed and if colorimetric assays are to be used for preliminary screening, it would be prudent to use at least two different reagents in order to enhance the detection of the indolic compounds (Glickmann & Dessaux, 1995; Lombard & Dowell, 1983; Sergeeva *et al*., 2002)

Tolerance to osmotic stress *in vitro*

IAA production was used as the primary screening criterion after salt tolerance as it is considered a highly active plant growth stimulator and a suitable selection criterion for PGPR (Arshad & Frankenberger, 1991; Marulanda *et al*., 2009; Mohite, 2013). The IAA-positive isolates were then tested for the ability to grow under osmotic stress induced by PEG 8000. PEG 8000 is often used to simulate water deficit stress *in vitro* as it is a non-permeating solute which is not absorbed or metabolized by bacterial cells (Busse & Bottomley, 1989; McAneney *et al*., 1982).

Tolerance to osmotic stress caused by solutes such as NaCl is achieved through selective uptake of external solutes in order to regain water potential equilibrium (Busse & Bottomley, 1989) and prevent water movement out of the bacterial cells. PEG has been used in bacterial studies due to the fact that it is a non-permeating solute which has the ability to lower the

water potential of the growth medium without permeating the cell walls, thus inducing the internal osmoregulation of bacteria, a trait considered desirable in PGPR (Busse & Bottomley, 1989; McAneney *et al*., 1982). For example, Marulanda *et al*. (2009) found that the ability to tolerate high levels of NaCl (600mM) correlated with the higher levels of proline accumulation in bacterial cells which would possibly account for their osmotic tolerance.

The permanent wilting point of plants is generally accepted to occur at a soil water potential of -1.5 MPa (Tolk, 2013). In the pot trial conditions under which the bacterial isolates would be assessed for plant growth promotion, plants would be drought stressed until the point of wilting and leaves began to turn brown. Bearing this in mind, bacteria were cultured in a range of lowered water potentials, reaching as far down as -1.44 MPa in order to simulate the drought which was to be imposed upon the plants and under which they would be expected to survive.

The bacterial isolates generally showed two growth trends (Figure 3.2), where one group of isolates' growth declined immediately from the onset of osmotic stress, whilst the others showed increased growth as water potential declined up until the point of -1.0 MPa after which they showed a marked decline.

These trends are consistent with the results reported by Busse and Bottomley (1989) who tested the ability of a number of bacterial isolates to grow under PEG-induced osmotic stress in YEM broth and observed that their bacterial isolates formed two distinct groups based on their growth responses to PEG-induced drought with the differences being greatest at –1.0 MPa. Although no isolates were able to grow at -1.5 MPa, Busse and Bottomley were able to confirm that the cultures were unharmed by exposure to physiological drought (by growing them on YEM agar plates) but that the two groups showed different morphological changes (change in cell shape) induced by drought stress. In addition, it was observed that the addition of calcium to the YEM medium dramatically increased the growth rate of the poorly-performing group, indicating that the growth inhibition observed was due to nutrient deficiency rather than inability to cope with the lowered water potential. The addition of

calcium also eliminated the morphological changes previously observed. These results indicate that the composition of the substrate and availability of minerals such as calcium may play a key role in the ability of inoculants to maintain growth under drought stress**.**

The bacterial isolates which showed the highest IAA production were unfortunately not the most drought tolerant isolates. However the observations of Busse and Bottomley (1989) suggests that perhaps the observed growth responses may have been due to the depletion of a specific nutrient or mineral required for osmoregulation-a factor which may have been elucidated with further testing. Nonetheless, the isolates maintained up to 40% growth under the highest osmotic stress and were therefore considered to be relatively drought tolerant.

Detection of siderophore production

Iron is essential element for growth of plants and microorganisms, however it is often found in insoluble form which is unavailable for direct assimilation by plants and microbes (Compant *et al*., 2005). The ability to competitively access bound iron is thus advantageous to PGPR and directly beneficial to host plants (Zhang *et al*. 2014). Siderophores are small (<10 000 D), mostly soluble, ferric iron-specific ligands which are produced by some bacteria under iron-limiting conditions (Neilands, 1995; Sayyed *et al*., 2005).

Isolates were tested for the ability to produce siderophores using CAS agar (Milagres *et al*., 1999). The initial use of the well method produced inconclusive results which were attributed to the toxicity of the detergent HDTMA (Alexander & Zuberer, 1991; Neilands, 1995). The method was thus modified where CAS agar was instead applied as an overlay onto well-grown (up to 72 hrs) pure cultures of each isolate (Figure 3.3). This method produced conclusive results where bacteria were observed to produce high levels of siderophores, as visualized by the large areas of colour change caused by the chelation of iron, reducing the chrome-azurol complex and changing the colour of the dye from blue to yellow (Neilands, 1995).

The reasons why this method allowed bacteria to produce such large amounts of siderophores are unknown however it may be possible that the CAS-agar does not contain sufficient nutrients to enable or sustain bacterial growth. It may also be that the production of siderophores requires a specific nutrient or mineral which was absent from the CAS agar but present in the agar used to establish the cultures. For example, Sayyed *et al*. (2005) and Deelip *et al*. (1998) found that organic acids and amino acids positively affected siderophore production whereas the presence of sugars and citric acid suppressed siderophore production**.**

Sayyed *et al*. (2005) tested two known siderophore-producing *Pseudomonas* species for the production of siderophores under a range of media and conditions. They reported that siderophores are not produced in nutrient broth, probably due to the high levels of iron as the stress of iron deficiency is known to induce siderophore production (Sayyed *et al*., 2005). This supports the conclusion that the colour-change observed in our results was due to the chelation of iron from the dye complex in the CAS agar overlay and not from any other interactions. The CAS-agar overlay plates were incubated for 5 days as siderophore production appeared to increase over time (data not shown). It is possible then that this increase in production could be due to the increasing iron limitation as it was depleted from the nutrient agar below as cultures had been growing for 8 days by the conclusion of the experiment. It was also observed that the extent of colour change from the point of contact with bacterial cultures was larger in some isolates compared to others. Siderophores are a group of molecules with many different compositions and conformations and thus varying solubility (Neilands, 1995). The differences observed in the colour change may be due to the production of varying types of siderophores by the different isolates.

Detection of phosphate solubilisation

Phosphate is an essential nutrient for plant growth and is often a limiting nutrient (Rodríguez & Fraga, 1999) due to the fact that although it is abundant in soils, it is usually in an insoluble form which is biochemically unavailable to plants (Dey, 1988; Gyaneshwar *et al*., 2002;

Richardson, 1994; Yang *et al*., 2008). The ability to solubilise bound phosphate is considered a key trait in effective PGPR due to the direct effects it has on plant growth. Phosphate solubilisation is has been reported in many bacterial genera (Rodríguez & Fraga, 1999) and thus isolates were tested for the ability to solubilise phosphate using Pikovskaya's agar.

The majority of isolates tested (7/9) were able to solubilise phosphate, as evidenced by the development of 'zones of clearance' around the bacterial colonies (Figure 3.4). The extent of solubilisation varied amongst the isolates (Table 3.2) but it was observed that the zones of clearance continued to develop over time (plates were stored indefinitely at 4 °C) indicating that the process is not immediate and may be dependent upon the accumulation of enzymes in the media, or possibly that enzyme production is increased as the available phosphorus becomes more limiting. It has been noted that Pikovskaya's agar does provide conclusive results for all bacterial species (Nautiyal, 1999**)**, so it is possible that the negative results obtained may have been false however the isolates which produced the highest levels of IAA were all positive for phosphate solubilisation so the results were not further confirmed.

Detection of ammonia production

Organic nitrogen in soils is often unavailable to plants and organisms as it is bound up in proteins and other complex compounds (Shmaefsky, 1991). Bound up nitrogen is made available the form of organic molecules through microbial processes involved in the decomposition of plant and animal tissues. During decomposition, proteins are broken down in polypeptides (peptones) and then into amino acids, after which the amino groups in the amino acids are removed through deamination (Internet 2, 2014). Production of ammonia from organic compounds is called ammonification, a process which yields ammonia and ammonium ions. In addition to ammonia, other compounds such as nucleic acids, urea and uric acid also go through ammonification. Ammonia and ammonium ions are then utilised by soil microbes and plants, either for assimilation or conversion into nitrite and nitrate (nitrification) thus forming an essential part of the nitrogen cycle and supply of usable nitrogen to plants and microbes (Shmaefsky, 1991). The ability to produce alkaline volatile compounds such as ammonia has also been suggested as a biocontrol strategy (Brimecombe

et al. 2001) as well as mechanism to avoid pH stress through the neutralizing of acidic environments (Cotter & Hill, 2003).

Out of the 9 isolates tested, 7 were positive for ammonia production (Table 3.2) to varying degrees. These results are supported by literature which states that production of ammonia in terrestrial environments is driven by soil microbes (Shmaefsky, 1991). The ability of these isolates to promote the cycling of nitrogen within soils may be beneficial both to plant growth and to the development of symbiotic relationships with mycorrhizal fungi and other soil microbes.

Detection of catalase production

Drought, salinity and temperature extremes are often interconnected and thus may result in similar cellular damage (Singh *et al*., 2011; Wang *et al*., 2003). Reactive oxygen species (ROS) such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxylradicals (OH) which cause lipid peroxidation of membranes (Sgherri*et al*., 2000) are produced during abiotic stresses and result in membrane damage. Naveed *et al*. (2014) found that the inoculation of *Burkholderia phytofirmans* strain PsJN and *Enterobacter* sp. FD17 onto maize plants reduced the H_2O_2-induced damage compared to control in both cultivars under drought stress. It was suggested that bacterial colonization augmented plant defence enzymes such as catalase, peroxidase, superoxide dismutase or phenolic compounds, to alleviate the oxidative damage elicited by drought (Naveed *et al*., 2014).

The ability of these isolates to reduce oxidative damage caused by superoxides such as H_2O_2 is another beneficial trait of PGPR (Kohler *et al*., 2008)**.** The catalase enzyme is produced by bacteria to mitigate the damage caused by H_2O_2 in cells as it reduces H_2O_2 into water and oxygen (Wang *et al*., 2009). For endophytic bacteria, this enzyme may help to alleviate oxidative stress both of the plants and bacterial cells (Wang *et al*., 2009). Isolates were tested for the ability to produce catalase and enzyme production was observed in varying degrees in all except one isolate (Table 3.2), indicating that they may posess mechanisms of adaptation

to harsh environments as well the ability to promote the cycling of nitrogen within the plant rhizosphere.

Summary of PGPR selection

The results from the screening of these isolates demonstrate that individual species or strains may possess a multitude of potentially advantageous or beneficial characteristics, however the distribution of traits across many different species emphasises the need for diversity and commensalism within microbial communities. The many methods of *in vitro* determination of PGPR traits enable researchers to select for bacterial strains with desirable functional capabilities which can then be evaluated for *in vivo* efficacy.

Molecular identification of selected PGPR isolates

Isolates were selected for application under greenhouse conditions on the basis of their promising expression of typical PGPR traits (Table 3.2). Identification of bacteria is important in order to determine their functional abilities, as well as to gain insight into their natural and evolved habitats and thus their potential application into studies such as these. Morphologic and microscopic examination of bacteria provides only basic and superficial descriptions of bacteria and thus molecular techniques are employed to identify and distinguish between species. The 16S rDNA gene sequence is universally distributed among bacteria and is often used for identification as it contains variation across phylogenetic lines yet contains segments which are conserved at the species, genus or kingdom level (Drancourt *et al*., 2000; Wilson *et al*., 1990). In this study the primers rP2 and fD1 were used (Table 2.1) to amplify the 16S gene segment through PCR due to their ability to target most eubacteria, thus being considered ‘universal’ bacterial primers (Weisburg *et al*., 1991). Nucleotide sequences can then be searched for similarity against the NCBI database using the BLAST query-matching programme. When using the 16S rRNA gene sequence for identification, similarity matches of 97-98% are considered sufficient for identification down to genus level and ≥99% is considered identification at species level (Drancourt *et al*., 2000). These

identities should then be further confirmed on the basis of Gram reactions, phenotypic and biochemical characteristics such as IAA production.

The sequences in this study aligned with 97-98% homology and were thus accepted to genus level (and tentatively to species level) as belonging to *Achromobacter xylosoxidans* strains A8 and C54 and *Klebsiella oxytoca* strain M1 (Table 3.3). The sequence identities obtained from the BLAST searches are supported by literature on the characteristics and expression of plant growth promoting traits in these bacterial genera.

Achromobacter

Achromobacter xylosoxidans is an aerobic, motile, gram-negative rod (Duggan *et al*., 1996). It is a member of a heterogenous group of oxidase-positive, non-fermentative gram-negative bacilli which includes other related organisms such as *Agrobacterium tumefaciens*. Previously, this organism was named *Alcaligenes denitrificans* subspecies *xylosoxidans*. *Achromobacter* species inhabit aquatic environments and have been known to be pathogenic in humans (Duggan *et al*., 1996**)** however it is also associated with plants and has been reported as a promoter of plant growth. *A. xylosoxidans* has previously been indicated as a potential PGPR due to its nitrogen fixing capability, ACC deaminase activity (Blaha *et al*. 2005) and salt tolerance under drought stress (Forchetti *et al*., 2007; Tilak *et al*., 2005).

Mayak *et al*. (2004) found that *A. piechaudii* ARV8, a PGPR strain which produces 1-aminocyclopropane-1-carboxylate (ACC) deaminase conferred tolerance to drought stress in *Capsicum annuum* L. (pepper) and *Solanum lycopersicum* L. (tomato) plants. Under drought stress conditions, the natural plant response is to reduce root and shoot growth through the regulatory activity of the plant hormone ethylene. ACC is the precursor of ethylene and degradation of this compound inhibits the stress response, allowing plants to maintain vegetative growth (Glick *et al*., 2007).

Sgroy *et al*. (2009) isolated an *A. xylosoxidans* strain 53B from the roots and rhizosphere soils of *Prosopis strombulifera* and found that their isolate was unable to solubilise phosphate or produce proteases but was able to fix nitrogen, exhibited ACC deaminase activity and phytohoromone production. *A. xylosoxidans* has also been isolated from the roots and culm of healthy wheat plants (Jha & Kumar, 2009) and Bertrand *et al*. (1999) reported the isolation of an *Achromobacter* sp. from oilseed rape which was reported to increase NO_3^- and K^+ influxes and H^+ efflux and affect root hair development, thereby increasing root and shoot dry weight (Bertrand *et al*., 1999). Certain strains have also been reported to degrade catechol, biphenyl, phenol, benzene and toluene (Ho *et al*., 2012) and it has been reported that *A. xylosoxidans* was able to help plants tolerate lethal concentrations of aromatic compounds and decrease catechol- and phenol-induced phytotoxicity (Ho *et al*., 2012).

Klebsiella

Klebsiella oxytoca, is a non-motile, aerobic and facultative anaerobic Gram-negative rod-shaped bacterium. It belongs to the genus *Klebsiella*, in the family Enterobacteriaceae and is closely related to *K. pneumonia* but can be distinguished by being indole-positive (Brisse *et al*., 2006). *Klebsiella* species have been shown to be present in the rhizosphere and exhibit PGP traits such as nitrogen fixation, IAA and siderophore production as well as antifungal activity (Sachdev *et al*., 2009).

K. pneumonia and other *Klebsiella* species been reported to colonize internal tissues in sweet potato cultivars, *Zea mays* and *Z. luxurians* as well as (Adachi *et al*., 2002; Palus *et al*., 1996) and field inoculation trials in maize have reportedly improved crop yield (Rosenblueth *et al*., 2004). *K. oxytoca* has also been isolated from the rhizosphere of *Aspidosperma polyneuron* and is known to produce IAA (Celloto *et al*., 2012) and *K. oxytoca* strain G-3 was also reported to be endophytic in *Typha australis* and exhibit PGPR activities such as nitrogenase activity, IAA production and P solubilisation when grown diazotrophically (Jha & Kumar, 2006).

K. oxytoca strain Rs-5 has been isolated from saline soils and found to relieve salt stress and promote cotton seedling growth (Wu *et al*., 2013). These effects were partly attributed to the promotion of plant absorption of N, P, K and Ca and decreasing the uptake of Na. This strain was however found to be sensitive to environmental factors such as pH and temperature, nutrient status and competition from indigenous microflora. A solution to this problem was presented in the form of encapsulation which increased survival of the inocula and thus their growth promoting activities (Wu *et al*., 2013).

The selection of these bacterial strains through the process of *in vitro* testing for traits common to PGPR are supported by reports of related species in plant growth studies, indicating that *in vitro* tests may accurately establish at least functional capabilities of bacterial species which then require further *in vivo* evaluation. Despite the diverse range of PGPR activities and promising potential as field applicants, both *K. oxytoca* and *A. xylosoxidans* has also been associated with human diseases and pathogenicity and so care must be taken to distinguish between environmental and clinical strains

Mycorrhizal isolation and identification

Mycorrhizal fungi exist in the soil as hyphae, vesicles and spores. Mycorrhizal spores are complex reproductive structures which are able to survive in soils under adverse conditions and initiate infection when they come into contact with the coleoptiles or roots of germinating seeds and plants under favourable conditions (Maia & Yana-Melo, 2001; Mohammadi *et al*., 2011).

These spores are easily obtainable from soils using quick and efficient extraction methods and can then be used for morphological and molecular identification as well as to propagate the fungus for further evaluation and culturing. In order to obtain inoculum consisting of mycorrhizal species adapted to drought and saline conditions, rhizosphere soils from the Kalahari desert and Cerebos Salt Pans were sampled. The Kalahari soils yielded roughly half the number of spores as the Salt Pan soils, however both contained abundant spores (Table

3.4) with diverse morphological characteristics. A variety of sizes and colours ranging from pale-yellow to ocre, gold, reddish-brown and brown-black were observed under the microscope. Many spores had subtending hyphae still attached and appeared singly or in grape-like clusters and some spores were irregularly shaped and ornamented. The vast range of spore morphologies indicates a variety of species present within the communities sampled.

Mycorrhizal fungi are obligate biotrophs (Mohammadi *et al*., 2011) and require an actively growing host plant in order to survive and propagate. The Salt Pans from which samples were taken were heavily vegetated and received a regular supply of water due to their proximity to the sea. This may explain the greater number of spores obtained from these soils compared to the Kalahari sample (Table 3.4). Lack of vascular plant cover is known to influence nutrient cycling and soil erosion (Thomas *et al*., 2013) and analysis of the two soil samples (Table 3.5) revealed differences in the physiochemical and nutrient status of the soils. The Kalahari soils contained several times more Potassium than the Salt Pan soils which may be attributed to the increased evaporation leading to accumulation of solutes at high levels within soils. The Salt Pan soils contained much higher levels of Magnesium (Mg) and Zinc (Zn) which may be attributed to the fact that Mg and Zn are found in sea water and concentrated in algae in relatively high concentrations (Wahbeh, 1985) and thus may have accumulated in the pans.

Lumini *et al*. (2010) found that small differences in physicochemical characteristics in soils from different agronomic practises are reflected in the composition and overlapping of similar AM fungal assemblages. In addition, the composition of the vegetative cover significantly influences the community composition of rhizosphere-associated organisms (Wardle *et al*., 2004). Environmental conditions, nutrient levels and vegetation cover collectively influence the microbial community composition (Wardle *et al*., 2004) and the effect of these differences can be elucidated in greater detail using high throughput techniques such as 454-Pyrosequencing.

Molecular identification of fungal assemblages

Identification of AM fungal species based on descriptions of morphology and ontogeny of their spores has been the standard approach and is widely used to elucidate AM community

composition (Davidson *et al*., 2012; Johnson *et al*., 1991; Robinson-Boyer *et al*., 2009). However, identification of mycorrhizal fungi based solely on observation and description of morphological characteristics observed under a microscope can be difficult and may be inaccurate if the observer is not experienced in the technique (Abbott & Robson, 1991; Redecker & Raab, 2006). Using spores as the foundation of the identification process is considered to be a flawed approach, as sporulation is known to be a seasonal occurrence which is dependent on the physiological status and identities of both the AM fungus and the host plant (Mangan *et al*., 2010; Sanders, 2004). Trap cultures using a single host plant species have been used to induce sporulation for identification purposes, but the resulting AM community is expected to differ from the natural composition as certain species may not sporulate in the absence of their natural host (Davidson *et al*., 2012; Jansa *et al*., 2002; Liu & Wang, 2003; Oehl *et al*., 2004).

Identification of AM fungi from mixed assemblages found in field soils can be complicated by species that produce spores with alternative forms or whose morphology alters when associated with different plant hosts (Abbott & Robson, 1991; Lackie *et al*., 1987; Morton & Redecker, 2001). The *Archaeospora* include species which form atypical *Acaulospora*-like spores from sporiferous saccules and some of these species are also dimorphic, forming spores alike to those produced by *Glomus* species (Morton & Redecker, 2001). In addition, separation of spores into species can be confounded by the simple design of spores in some *Glomus* species which is said to mask divergence at a molecular level (Morton & Redecker, 2001). For example, the *Paraglomaceae* are reported to contain two species that produce spores which are said to be indistinguishable from those of the *Glomus* species (Morton & Redecker, 2001).

Due to these difficulties, AM fungi cannot confidently and accurately be identified based on spore morphology alone (Abbott & Robson, 1991) and morphological examination of AM fungal root-colonizing structures allows only superficial identification to family level (Merryweather & Fitter, 1998). Identification based solely on morphology may possibly result in misidentification and underestimation of species richness and diversity and thus molecular methods have been increasingly employed to achieve species-level or isolate-level discrimination (Redecker, 2002; Redecker & Raab, 2006). For example, Redecker *et al*. (2007) analysed signature sequences in the 18S ribosomal subunit in order to distinguish between species within the *Glomus* and *Diversispora* genera. This approach was chosen due

to the reported "scarcity of morphological characteristics on the light microscope level" which could be used for identification (Redecker *et al*., 2007).

High-throughput molecular techniques such as 454-Pyrosequencing have enabled researchers to delve to new depths of detail within the information gained from environmental samples. 454-Pyrosequencing was employed in this study to further quantify and describe the diversity within the AM fungal assemblages associated with plants growing under adverse conditions such as those present in the Kalahari desert and Cerebos Salt Pans. Pyrosequencing achieves a depth of coverage that is unmatched by traditional methods and has elevated the number of recovered taxa several-fold and increased rare-species recovery (Buée *et al*., 2009; Jumpponen & Jones, 2009; Jumpponen *et al*., 2010; Öpik *et al*., 2009; Tedersoo *et al*., 2010). Although pyrosequencing resolves species diversity in greater depth than traditional methods, the commonly used and highly conserved SSU gene is reported to provide little species-level discrimination and lead to the bunching of closely related phylotypes and genera (Tedersoo *et al*., 2010).

The use of 454-Pyrosequencing in this study revealed a depth of species richness and diversity far greater than would have been obtained through microscopy alone. The Kalahari and Salt Pan soils each yielded in excess of 5000 unique sequences which were assigned to 324 and 398 OTU's respectively (Figure 3.10; Table 3.6). The slight differences in physiochemical parameters between the two soil samples were reflected in the proportions of OTU's assigned to the different fungal genera. The two sample sites hosted a similar proportion of OTU's within the *Glomus* genera (33-34%) but had significantly different proportions of the A*mbispora* and *Paraglomus*. The Salt Pan community hosted *Ambispora* species which comprised 37% of the total number of OTU's with the *Paraglomus* accounting for 22%. On the other hand, the Kalahari site was dominated by the *Paraglomus* (46%) with the *Ambispora* representing only 16%.

Microscopic identification based on spore morphology may have observed the presence of the five genera but is unlikely to have detected the differences in community composition. For example, the reported difficulty in distinguishing spores of *Paraglomaceae* from those of *Glomus* (Morton & Redecker, 2001) may have resulted in gross overestimation of the

proportion of *Glomus* species within each sample. The differences in community composition revealed through pyrosequencing may also infer something about the environmental preferences of the different genera, perhaps related to the soil, vegetative and climatic characteristics of the two sites (Lumini *et al*., 2010). The *Archaeospora*, *Redeckera* and *Geosiphon* genera represented the rare components in both sites at less than 1% of the total species composition. It is the presence of these rare genera which are most often overlooked and may have been less easily cultured in trap culture as indicated by their rarity within the samples. These results indicate the usefulness of techniques such as pyrosequencing, which despite the difficulty in obtaining species identities, provides insight into mycorrhizal diversity in different environments independently of lab culturing or sampling techniques.

Assessment of mycorrhizal colonization of plant roots

The mycorrhizal propagules obtained from the sample soils and enriched in trap cultures were inoculated into soils planted with a local cultivar of *Zea mays* and grown for 7 weeks with or without drought stress. In order to assess whether the propagules had initiated infection and established themselves within the root tissues, samples of roots were cleared, stained and examined under a light microscope. Colonization of root tissues was confirmed by the presence of both intra- and extra-radical fungal structures as well as an abundance of spores (Figure 3.12).

Drought stress has been reported (Zhu *et al*., 2012) to decrease mycorrhizal colonization, a result which is perhaps indicative of damage to the structures or activity through drought stress. Simpson and Daft (1990) found that AM fungal sporulation was reduced by drought stress and that this may reflect upon the ability of the particular isolates to withstand drought stress and thus their potential as field inocula. Sporulation could affect the long-term survival and competitive ability of the inocula introduced into the field and thus for use as a plant aid it should be unaffected by drought stress (Simpson & Daft, 1990).

Mycorrhizal colonization was present in all treatments, including the non-inoculated controls

and the level of colonization was not affected by the presence or absence of water (Figure 3.12). The level of colonisation in AM-inoculated plants was very high (80-100%) which indicates successful mycorrhizal establishment. The roots examined in this study contained abundant spores (data not shown), indicating that the inoculated AM fungal assemblage was adept at colonization and sporulation during drought. The intensity of colonisation combined with the lack of growth response detected in the biomass data suggests that although the fungi may have colonized efficiently and perhaps gained carbon from the host plant, the relationship does not appear to be reciprocal and colonization by the AM fungi may have been neutral rather than mutualistic.

Wind and soil movement are reportedly the primary mechanisms by which mycorrhizal fungi are dispersed (Tommerup & Carter, 1982; Warner *et al*., 1987) and this may explain the presence of mycorrhizal fungi and colonization in un-inoculated plants. Duryea & Dougherty (1991) reported that airborne spores may be responsible for substantial natural infection in nurseries located near mycorrhizal vegetation and thus it may be possible that this baseline level of colonization was due to spore dispersal by wind movement within the growth tunnel and splashing during watering of plants. It may also be possible that infectious mycorrhizal propagules survived the soil sterilization process and established a low level of colonization within plants (Figure 3.13).

If one accepts as a baseline the level of colonization observed in the control treatment (and thus assumed to be present in all treatments), it can be seen that there is still a substantial amount of colonization in the bacteria-only treatments (Figure 3.13). The level of colonization appears to increase with the presence of water and is significantly higher in treatment B2 (colonized with *Klebsiella oxytoca* strain M1) than the drought stressed plant in the same treatment. The same effect is evident to a lesser extent in the *Achromobacter xylosoxidans* A8 colonized plants. It is possible then that there was a mycorrhizal helper bacteria effect on plant root colonization.

Plant Biomass Measurements

Referring to Figure 3.14, the well-watered maize plants generally produced higher root biomasses than the drought stressed plants, although these differences were only significant in the two *Achromobacter* treatments and the mycorrhizal treatment. No significant response to inoculation with mycorrhizas or PGPR isolates was observed within either of the soil moisture treatment groups in comparison to the un-inoculated control plants.

The imposition of drought stress on plants was severe and resulted in immediate decline of photosynthetic activity in maize plants (Figure 3.15) and this photosynthetic decline was expected to reflect in the root and shoot data. Referring to Figure 3.14A it can be seen that all the drought stressed plants had similar root dry weights, irrespective of treatment, thus it can be confidently concluded that none of the treatments promoted root growth under drought stress. It can also be seen drought stress had no significant negative effect on the root weight of the either the co-inoculated treatments (Myc + B) or on the control plants (C) as they all had very similar dry weights. This may indicate that maize plants invest more in above-ground biomass under well-watered conditions and thus drought stress was not reflected in root biomass. However, it is interesting to note that the two *Achromobacter* and AM treatment groups exhibited significantly higher root biomass under well-watered conditions compared to their drought stressed counterparts.

These results may possibly reflect growth promotion under well-watered but not under drought conditions, a result which does not support our hypothesis but may perhaps be of interest in other studies where drought is not the stress to be alleviated. It may be that mycorrhizas adapted to conditions in the Salt Pans were adapted to well-watered or even flooding conditions instead of the intended drought tolerance. It may also be an artefact of the use of trap cultures to enrich mycorrhizal cultures for inoculum production. These trap cultures are grown under well-watered conditions in order to sustain growth of the host plant for maximum allocation of carbon to mycorrhizal fungi however these conditions may have inadvertently amplified a greater proportion of mycorrhizas which flourish under well watered conditions than those which were adapted to drought.

The effect of drought stress on shoot growth (Figure 3.14B) was uniform and well-watered plants had significantly higher shoot biomass than drought stressed plants, however no significant differences were observed between any of the treatments subjected to drought or well-watered conditions. In Figure 3.14C, it can be seen that there were no significant differences in total biomass of drought plants, irrespective of treatments and that the trends observed in Figure 3.14A are repeated in the total biomass data. Figure 3.14D depicts the root-to-shoot ratio which is designed to demonstrate any differences in above-and below-ground resource allocation between control and inoculated plants. There did not appear to be any significant difference in resource allocation.

It has been said that crude biomass measurements may not be sensitive or subtle enough to capture the effects of AM colonization and drought stress on plant physiology (Hetrick *et al*., 1987). For example, Zhu *et al*. (2012) found that differences of plant height, shoot dry weight, root dry weight and total dry weight were not significant between mycorrhizal and non-mycorrhizal plants. They did however find significant differences in physiological parameters (stomatal conductance, water use efficiency, activity of PSII) but these changes did not translate into increased biomass. For this reason, plant gaseous exchange measurements were taken throughout the duration of the trial to determine whether the inoculants exerted any effect on plant physiology at any stage of the trial.

Plant photosynthetic capacity

In Figure 3.15, the dynamics of the plant photosynthetic rate are plotted along the time-course of the drought trial. The trial ran for 7 weeks, over which 12 sets of measurements were taken, each spanning two consecutive days of measuring. For the sake of clarity, the treatments were grouped under panel A and B according to drought stress (A) or well-watered conditions (B) as well as by inoculant where the top row depicts dual-inoculants, the second row bacterial inoculants only, and the bottom row depicting the mycorrhizal and control treatments.

As can be seen from Figure 3.15, drought stress resulted in the uniform, immediate and rapid decline of photosynthetic rate in all treatments. There were no significant differences observed between any of the treatments and the control groups, confirming the conclusion from the biomass data that the microorganism inoculants were unable to stimulate any changes in the photosynthetic capacity of this cultivar of maize plants. This trend was mirrored in the well-watered plant set where all plants maintained a similar photosynthetic rate for the duration of the experiment.

Figure 3.15C depicts the combined data from the drought and well-watered plants, illustrating that the water deficit imposed was severe enough to alter plant photosynthesis and cause the decline and subsequent suspension of photosynthesis until water was supplied again. These conditions were imposed in order to imitate the nature of drought in South Africa, where heavy rainfall is followed by months of drought and soil moisture is depleted rapidly by evaporation (O'Keeffe *et al*. 1992; Richard *et al*., 2001).

Figures 3.16 and 3.17 depict the effects of drought on stomatal conductance and transpiration of maize plants. The trends observed in Figure 3.15 were mirrored in Figures 3.16 and 3.17, reiterating the conclusion that the microorganism inoculants applied in this trial were unable to stimulate any effects on plant physiology either under drought stress or well-watered conditions.

General discussion

The rhizosphere is a dynamic environment which is characterised by the reciprocal cycling and exchange of nutrients between soil, plants, microorganisms and which is highly variable depending on plant species, soil type, biogeochemical variables and environmental conditions (Bowen & Rovira, 1999; Gamalero *et al*., 2004).

The application of AM fungi and PGPR for the growth promotion of a range of host plants

under both well-watered and drought conditions has been reported with varying degrees of success (Allen & Boosalis, 1983; Augé *et al*., 2001; Hetrick *et al*., 1984; Hetrick *et al*., 1987; Marulanda *et al*., 2003; Simpson & Daft, 1990). Many authors have reported on the advantages conferred to maize plants colonized with a variety of AM fungi and PGPR with regard to drought tolerance and improved yield under a range of environmental conditions (Boomsma & Vyn, 2008; Hao *et al*., 2008; Liu *et al*., 2000; Marulanda *et al*., 2010; Naveed *et al*., 2014; Ortas & Akpinar, 2011; Simpson & Daft, 1990). However, in this study application of a mixed assemblage of AM fungi and three strains of potential PGPR did not increase the drought resistance of the host maize plants, either in terms of sustaining growth under drought stress, improving recove**r**y from wilting or inducing more efficient use of water.

The physiology of drought stress tolerance is highly varied and the contribution of AM fungi and PGPR symbiosis to plant drought tolerance results from a combination of physical, nutritional, physiological, and cellular effects (Pinior *et al*., 2005; Tian *et al*., 2012) and is thus expected to be highly variable depending on a complex range of environmental conditions. Neutral or negative responses to symbioses have been reported (Grant *et al*., 2005; Hetrick *et al*., 1984; Hetrick *et al*., 1987; Li *et al*., 2005; Simpson & Daft, 1990; Stewart *et al*., 2005) and are thought to be caused by differences in compatibility between the partners in the symbiosis, attributed to genetic variation and ecological specificity of the microorganisms and host plants (An *et al*. 2010; Liu *et al*. 2000; Tian *et al*., 2012). Many of the effects observed are actually caused by the regulation (whether up or down) of certain uptake or metabolic pathways and thus it is evident that a successful symbiosis is determined by the compatibility between both the individual AM fungi, PGPR and plant genomes. Considering the enormous genetic variability of these organisms, the formation of an efficient symbiosis should not be taken for granted (Jakobsen *et al*., 2002; Smyth *et al*., 2011).

There are a multitude of reasons why interactions between microorganism inoculants and host plants may not yield the desired and anticipated beneficial effects. Briefly, results such as these have been attributed to:

Genetic incompatibility between AM fungi and host plant

AM fungi were traditionally thought to be non-specific (Sanders, 2002) but observations of apparent reciprocal relationships based on feedback mechanisms between AM fungi and host plant species (Bever *et al*., 2002) and the variation in responses of different plant species to individual AM isolates have supported the argument that these interactions are somewhat specific (Streitwolf-Engel *et al*., 1997). Plant genetics are thought to account for a significant proportion of the factors which contribute to AM fungi responsiveness, making the symbiosis unpredictable in that the exact genetic mechanisms dictating the relationships are unknown (Chu *et al*., 2013). Responsiveness of a given species is variable with the identity of the AM fungi and certain maize hybrids have shown a reduction in nutrient uptake efficiency when associated with some species of mycorrhizal symbiont (Smith *et al*., 2010). For example, *Glomus etunicatum* has been shown to colonize maize roots extensively but cause a negative response in host plant physiology whereas *G. mosseae* colonized roots less extensively but stimulated a positive response (Hao *et al*., 2008; Ortas, 2012; Sangabriel-Conde, 2014). Results such as these stress the specificity of plant-microbe relations where each cultivar or host plant will interact differently with varying strains and species and as such, laboratory tests can only predict to a limited extent the efficacy of such relationships *in vivo*.

Reduced mycorrhizal dependency due to modern selection and breeding processes

In commercial agriculture, it is assumed that most cultivars have been selected for maximum production under high-input agrochemical regimes (Evans, 1980), thereby reducing inherent mycorrhizal dependency and suppressing the symbiosis, whereas local cultivars have been selected under reduced- or zero-input regimes (Hess *et al*., 2005) and as such are expected to have a higher degree of dependency (Kaeppler *et al*., 2000; Martinez & Johnson, 2010; Rao *et al*., 1990; Sangabriel-Conde *et al*., 2014; Tawaraya, 2003). Observed differences in mycorrhizal growth responses between old and modern maize genotypes thus suggest that the variability may be a result of changes in the relationship between maize and AM fungi during the breeding or evolutionary processes (Chu *et al*., 2013; Hetrick *et al*., 1992). Chu *et al*. (2013) observed that AM symbiosis with old genotypes selected under low-P conditions resulted in superior growth promotion in low-P soils and that the symbiosis with the modern

genotype, selected under high-P conditions, was favoured by high-P levels. Similarly, Sangabriel-Conde *et al*. (2014) found that increased P soil concentration reduced AM dependency in three native maize cultivars and one hybrid cultivar. The hybrid showed a significant decrease in dependency and it was suggested that under greater P availability, the plant is capable of obtaining nutrients through its roots alone. These results indicate perhaps that one needs to know the conditions under which the test host plant was selected in order to gauge whether it may benefit from mycorrhizal inoculation under the desired field or test conditions.

Estimation of colonization unrelated to efficiency of symbiosis

Although commonly used as an indicator of AM fungal symbiosis, quantification of the frequency and intensity of fungal infection in plant roots after Trypan Blue staining is considered by some to be an inadequate indicator of AM efficiency (Vierheilig & Ocampo, 1989; Vivas *et al*., 2003). Alternatives to this method are vital staining techniques which measure the activity of enzymes such as succinate dehydrogenase an alkaline phosphatase due to their involvement in active fungal metabolic processes (Tisserant *et al*., 1993; Vásquez *et al*., 2000; Vivas *et al*., 2003).These techniques are able to determine with greater accuracy whether the observed colonization is actually facilitating an active symbiosis, characterized by the reciprocal exchange of nutrients.

A number of studies have reported that AM colonization does not result in any increases in plant growth or total P content, and that AM plants may actually be smaller than the non-AM controls (Smith *et al*., 2010). Naveed *et al*. (2014) tested 5 endophytes for growth promotion of various maize cultivars and found that although highly colonized by all the endophyte strains, growth promotion in one of the cultivars was only correlated with colonization to a limited extent. Responses to AM colonization therefore vary along a continuum from highly effective to ineffective or even detrimental, indicating a high level of "functional diversity" in AM symbioses with different host plants and cultivars (Jakobsen *et al*., 2002; Smith *et al*., 2010).

Results such as these support the notion that the confirmation of AM activity through assessment of plant root colonization alone is considered by some to be insufficient as the distribution and biomass of extra-radical hyphae are considered to be more important than

root colonization in the activity of mycorrhizas on plant growth promotion due to their role in acquisition of poorly soluble nutrients and root-inaccessible water from the soil (Finlay, 2005, 2008; Horton & Bruns, 2001; Maldonado-Mendoza *et al*., 2001). Length of hyphae formed in soil is not necessarily related to the extent of root colonization and thus both parameters should be assessed (Abbott & Robson, 1985).These are all factors which should be taken into consideration for further evaluation of AM fungal-host plant interactions and efficiency.

Soil nutrients and characteristics

The key characteristic of symbiosis- the *reciprocal* exchange of resources (habitat, nutrients and water)-may exhibit altered expression in different environments and combinations of host plant and fungal isolate (Singh *et al*., 2011). The interaction may shift from mutualism to commensalism or even parasitism. In phosphorus-limited soils, mycorrhizal plants may depend entirely upon the symbiosis to provide P nutrition, however, as P content in soils increases, so the developmental differences between AM and non-AM plants tend to be eliminated (Sangabriel-Conde *et al*., 2014; Smith & Read, 2008). In fertile agricultural soils, colonization by mycorrhizal fungi may actually reduce crop productivity as the fungus assimilates more carbon from the plant than that which is being additionally fixed due to the symbiosis (Boomsma & Vyn, 2008).

Nyaga *et al*. (2014) tested the ability of a mixed inoculum of indigenous mycorrhizas to improve the growth of maize and bean plants under field conditions and 3 different soil amendment practices. The soil amendments consisted of two different commercial fertilizers (Triple Super Phosphate plus Calcium Ammonium Nitrate and MANUVO) as well as cow manure. Their investigation aimed to establish whether indigenous mycorrhizas which had been amplified in trap culture could promote a plant growth response when applied alongside existing agricultural methods used by farmers. Their results found that AM fungi alone were unable to promote plant growth compared to the controls. The fungi were however able to stimulate plant growth when combined with TSP + CAN, suggesting that the baseline phosphate levels were extremely low, rendering the AM fungi incapable of stimulating growth and that with the addition of fertilizer, the levels of available phosphate were increased but were perhaps insoluble and thus the activity of AM fungi was able to increase the plant growth. These results encourage the incorporation of AM inoculants into

agricultural fertilizer regimes but do not advocate their use as the sole nutritional aid. Instead, they suggest perhaps that the gradual lowering of external fertilizer input may stimulate the desired symbiosis and produce better results over time, especially in soils which are heavily nutrient depleted.

It is difficult to separate nutritional effects from direct effects on water transport as the nutritional advantage becomes more pronounced as soil dries. The supply of poorly soluble minerals such as P will become more limited, making the hyphal contribution more important (Finlay, 2008). Although nutrient levels were controlled in this study and were intended to be P-limited in order to stimulate mycorrhizal dependency, no response was observed which may indicate either that the plant was not dependent of mycorrhizas for nutrition or perhaps that the inherent amount of P available in the growth medium was depleted prior to the imposed drought and thus was unavailable for access by mycorrhizas under drought stress.

In vitro selection of PGPR

Many researchers have reported inconsistent results when plants have been inoculated with microorganisms selected under axenic conditions (Bowen & Rovira, 1999; Marulanda *et al*., 2009). The response of plants to bacterial inoculation with PGPR varies depending on the bacterial and plant species, soil type, inoculant density and environmental conditions and thus cannot be accurately predicted by measurements *in vitro* (Bashan, 1998; Smyth *et al*., 2011). Growth and productivity of select traits by PGPR is dependent on the organic acids, sugar, amino acids, minerals, enzymes and other components of host plant root exudates as well as on a specific set of conditions such as temperature, substrate and moisture content which are evidently highly variable under field conditions (Deelip *et al*., 1998; Sayyed *et al*., 2010).

Through review of literature, it has become apparent that the common *in vitro* tests used to select potential PGPR are only useful to a certain extent and often do not provide realistic substrates or relevant culturing conditions to make them directly applicable in field conditions (Finlay, 2005, 2008; Smyth *et al*., 2011). Isolates are often only screened for a limited number of characteristics produced *in vitro* under laboratory conditions and

subsequently prove ineffective under non-sterile field conditions (Barret *et al*., 2011; Smyth *et al*., 2011). It is possible that the growth responses induced by effective bacterial species may actually be induced by production of compounds or enzymes other than those tested for *in vitro* or possibly that the effects are an indirect result of interactions with other soil microorganisms (Smyth *et al*., 2011).

For example, Smyth *et al*. (2011) tested 15 bacterial strains of *Acinetobacter*, *Bacillus*, *Micrococcus*, *Pseudomonas* and *Stenotrophomonas*, isolated from the rhizosphere or phyllosphere of cereals, for a number of typical plant growth promoting traits such as auxin, siderophore, ACC-deaminase, hydrogen cyanide and protease production as well as mineral solubilisation. They then evaluated the effects of bacterial inoculation upon the growth of spring wheat plants grown in non-sterile agricultural soil under greenhouse conditions. It was observed that despite the expression of multiple beneficial traits by many of the isolates, only one bacterial strain, *Pseudomonas fluorescens* was able to significantly increase the growth of wheat plants.

The strain of *P. fluorescens* which had the growth promotion effect was in fact unable to produce IAA *in vitro*. On the other hand, the *Micrococcus luteus* strain which produced the highest levels of auxin *in vitro* and had positive growth effects on coleoptiles in germination tests was unable to stimulate a growth response under greenhouse conditions. Similarly, Smyth *et al*. (2011) found no correlation between the ability to produce ACC-deaminase and plant growth promotion. In addition, none of the species which stimulated growth responses were able to solubilise phosphate, and the species which were able to solubilise phosphate *in vitro* actually caused a slight negative effect on plant growth. It was thus concluded that *in vitro* testing of PGP traits is not robustly indicative of the ability of bacteria to affect the growth or yield of plants at maturity and that there is no simple or verified method of confidently selecting bacteria able to efficiently promote plant growth.

Similarly, Upadhyay *et al*. (2011) found no correlation between *in vitro* IAA production and significant root, shoot or total biomass stimulation. They suggested that this finding may be because plant growth promotion relies not only upon the ability of the bacteria to produce stimulants but also upon their ability to effectively colonize the rhizosphere, produce osmoprotectants and withstand changes in the ion balance in the soil environment (Upadhyay *et al*., 2011).

Inadequate colonization and survival

Populations of bacterial inoculum are known to decrease over time (Bakker *et al*., 1987; Frey-Klett *et al*., 1997; Chiarini *et al*., 1998; Gamalero *et al*., 2002) a phenomenon which has been attributed to both biotic (predation, competition, root exudation) and abiotic (temperature, pH, soil texture and mixture) factors (Van Veen *et al*., 1997). Furthermore, traits such as the ability to utilize root exudates as energy sources, synthesize amino acids and vitamins as well as motility and the ability to firmly adhere to roots have also been identified as factors in rhizospheric competence and are important for the efficient colonization of plant roots and the establishment of introduced bacterial species (Gamalero *et al*., 2004; Lugtenberg & Dekkers, 1999). The heterogeneity of soil represents a huge challenge to the successful application of PGPR as it represents an unpredictable growth medium, even on a small scale (Bashan, 1998; van Elsas & van Overbeek, 1993).

A trait which is difficult to assay *in vitro*, inoculated bacteria are sometimes unable to compete successfully with indigenous microorganisms and secure a niche under non-sterile conditions, as is the case under field conditions (Naveed *et al*., 2014). Reduced survival in the rhizosphere would of course affect efficacy as PGPR and survival could be highly variable depending on the environmental conditions and the intrinsic ability of each species to withstand and recover from stress (Smyth *et al*., 2011).

As soils dry and the matric water potential is reduced, restricted diffusion of substrates to bacteria will become a determining factor in their ability to maintain osmoregulatory adjustment (McAneney *et al*., 1982). Although soil water potential can be controlled to a certain extent, soil texture is an important variable which has the potential to affect the survival and efficiency of colonization by bacteria (Fuhrmann *et al*., 1986; Mahler & Wollum, 1986). As soils dry, solutes naturally present in soils may alter soil water potential in different ways and bacteria able to deal with NaCl- or PEG-induced drought may not necessarily cope as well with the variety of solutes found in soils (Busse & Bottomley, 1989; Singleton *et al*., 1982).

In addition to the selection for typical PGPR traits such as phosphate solubilisation, indole and siderophore production, Naveed *et al*. (2014) tested for aggregation ability, biofilm formation and exopolysaccharide production. Exopolysaccharides have been shown to help protect bacteria from dessication, predation and the activity of certain antibiotics (Staudt *et al*., 2012). Motility, or chemotaxis, has also been shown to be important in successful root colonization as bacteria are able to mobilize towards nourishing root exudates (Bhattacharjee *et al*., 2012; Danhorn & Fuqua, 2007). These are traits which may affect bacterial dispersal, adsorption to roots and survival in plant environment and should thus be included in the selection process of future studies on PGPR. The provision of an environment (even temporarily) in which the bacteria are initially able to multiply and establish themselves is thus considered key factor in inoculant formulation (Bashan, 1998) and is something which could be improved upon in further studies. Colonization and survival of inoculated bacteria was not assessed in this study and may have provided further insight into the lack of growth responses observed.

In addition, bacterial species which show high growth promoting potential *in vitro* may also act as plant pathogens *in vivo* e.g. *Pseudomonas aeruginosa* (Rahme *et al*., 1995), a factor which needs careful attention when developing strategies for bacterial plant growth promotion (Egamberdieva *et al*., 2008).

Nutrient availability

The metabolic and enzymatic pathways which determine the expression and activity of PGPR traits are complex and highly specific with regards to substrate and environmental conditions and thus may result in disparities between *in vitro* and *in vivo* PGPR activity. For example, Sayyed *et al*. (2010) found that disaccharides (fructose, xylose, maltose and lactose) and monosaccharides (glucose) were able to support growth of *Alcaligenes faecalis* while only polysaccharides (starch) were able to support both growth and siderophore production. Similarly, lactic acid supported growth whilst succinic acid encouraged siderophore production and methionine, histidine, tyrosine and proline enhanced siderophore production where cysteine and histidine only supported growth. Sayyed *et al*. (2010) also found that pH and temperature had significant effects on siderophore production, thus emphasizing the key role that environmental conditions play in the ability of PGPR to perform optimally. These

results suggest that the perceived 'inability' of bacterial isolates to promote the growth of plant *in vivo* may be due to the absence of specific nutrients and substrates necessary for production or expression of desirable traits.

Experimental design

It has been suggested that experimental design parameter such as pot size and inoculum distribution may affect the results obtained by pot trial. The possibility of trial plants becoming root bound is well recognized problem and many studies have shown that growth is reduced by smaller pot sizes (Peterson *et al*., 1984; Robbins & Pharr, 1988; Townend & Dickinson, 1995; Whitfield *et al*., 1996). Pot size has been shown to affect a number of physiological processes including nutrient efficiency and photosynthetic rate, however the relationship between pot size and physiological effects have not been consistent (Ray & Sinclair, 1998). Pot size may potentially influence the dynamics of resource allocation and mycorrhizal symbiosis by restricting root proliferation and extraradical hyphae (Abbott & Robson, 1991; Audet & Charest, 2010; Koide, 1991)

The intensity of colonization observed in inoculated plants indicated that the inoculation process was successful, however the size of the pots used may have factored into the observed results. Audet and Charest (2010) reported that pot size is a key experimental factor and that when limiting, can significantly reduce plant growth and AM colonization. The "rootable" volume (Audet & Charest, 2010) of soil directly determines the total nutrient supply and can potentially limit the plant growth potential without necessarily causing noticeable symptoms of nutrient deficiency (Ray & Sinclair, 1998). AM symbiosis is primarily beneficial due to the increased capacity for nutrient and water uptake and when the volume of soil available for exploration and proliferation is limited, this benefit is attenuated (Audet & Charest, 2010; Bååth & Hayman, 1984). Audet and Charest (2010) found that pot size significantly affected the ability of AM colonization to promote plant growth. AM colonized plants grown in large pots had biomasses similar to their well-watered counterparts whereas no such differences were observed in plants grown in small pots.

Maize plants develop large root systems which extend approximately 1.5 m laterally and 2 m down into the soil in order to access ground water reserves (du Plessis, 2015). It is possible in our study that pot size reduced the ability of mycorrhizal colonization to improve the growth

of maize plants during drought stress. The plants were grown for 7 weeks and when the root bundles were removed it was observed that all plants were root bound. If pot size had been a limiting factor, the proliferation of roots and mycorrhizal hyphae would have been severely restricted and therefore negated any water uptake effects which may have been induced by AM colonization (Simpson & Daft, 1990). This effect may have been eliminated by using larger pots, but this was impractical due to logistical limitations. The other alternative would have been to run a shorter trial and use a subset of plants to monitor root growth and morphology. Pot size was however excluded as a factor in the results obtained due to the fact that no treatment effects on any gas exchange parameters were observed even in the early stages when plants were smaller and thus before root-binding could occur.

Length and severity of drought

Upon review of the literature, it has become apparent that the method and severity of the drought imposed in this study was different to most other studies. For example, Zhu *et al*. (2012) reported improved drought tolerance of maize under a 'drought' treatment where soil moisture was maintained at 50% field capacity. In our study, 50 % field capacity would have meant maintenance of soil water potential at 12%, a value which was surpassed in the first week of the initial drought and within two days during the second drought. Similarly, Naveed *et al*. (2014) found that inoculation with *Burkholderia phytofirmans* and *Enterobacter sp*. FD17 increased the growth of two maize species (Mazurka and Kaleo) significantly increased the photosynthetic rate by up to 75% under drought stress. Stomatal conductance, transpiration, vapour pressure deficit in leaves (VpdL) as well as chlorophyll content were all significantly increased by inoculation with the two PGPR strains. In this study, drought was imposed as maintenance of soil moisture content at 12-13%.

Saia *et al*. (2014) found that AM symbiosis increased productivity of clover under drought stress, which was classified as "rain-fed" only whereas the well-watered plants were given extra water. Their drought treatment plot had a soil moisture content of 19.4% compared to the control plot at 25.4%. Marulanda *et al*. (2009) assayed the effect of PGPR in promoting growth of *Trifolium repens* under drought stress. In their study, water was supplied at field capacity for 2 weeks after sowing and then drought was imposed at 75% of field capacity

(approximately 12% soil water content, estimated at 0.17 MPa).

In our study, drought was initially imposed as 50 ml water applied to the soil surface every second day and the subsequent drought then imposed more gently as 250 ml water every second day. Soil water potential was monitored but was not controlled or held at any specific water potential. This is in contrast to most studies, a consideration which should perhaps be included in future studies.

A study in which the methodology was similar to this one, Simpson and Daft (1990) inoculated maize with 6 different AM fungal species as well as a combined AM fungal treatment. Their plants were grown in sterilized sea sand supplemented with granular rock phosphate (11% P, 2.25 g per 10 Kg sand). During establishment, plants were fertilized bi-weekly with Hoagland's nutrient solution without the phosphate ion. Once drought was imposed, plants were given 30 ml double strength nutrient solution bi-weekly and no additional water. Plants in the drought treatment were watered only when wilting (at least half the total number of leaves rolled). This was continued for 28 days, giving between 2-5 wilting cycles per plant. Mycorrhizal colonization did not affect dry weights of plants and the control plants were actually amongst the largest plants in each treatment, even though contamination by AM fungi was low (mean 6.6%). No mycorrhizal species resulted in a significant weight gain compared to controls in any treatment.

These results emphasize that any benefits obtained from AM symbiosis under drought conditions may depend on root and water distribution in the soil and the duration and severity of drought stress (Simpson & Daft, 1990). The main recognized benefit of mycorrhizal symbiosis consists of improved access to limited soil resources-mainly phosphorus and water (Saia *et al*., 2014) and it is thus possible that in our trial, having controlled for nutrient deficiency, the only effect which remained to be seen was the uptake of water by mycorrhizal hyphae. These results suggest perhaps that the drought imposed was too severe to allow the effect of AM fungi and PGPR to be exerted and it is possible that these organisms were damaged by the severity of the drought. In that case the mycorrhizal colonization observed would have established prior to the imposition of drought stress and not during drought

stress. High vesicle abundance (Figure 3.12) indicates that mycorrhizas were under nutrient stress, as the presence of vesicles indicates a stage of preparation for fungi entering senescence as they are generally storage structures and at a later stage, reproductive structures (Nyaga *et al*., 2014; Verbruggen & Kiers, 2010).

Interpretation of symbiosis outcomes

The intricate and complex relationship between plant hosts and mycorrhizal fungi involves so many inter-linked factors that attempts to determine exactly why a given symbiosis does or does not result in a 'positive' outcome are often in vain. Recent research has however suggested that just because a given plant-host combination does not result in a measurable increase in plant growth does not mean that the symbiosis is not active.

Most experiments involving a comparison between AM and NM plants growing under low-P conditions result in a large increase in plant growth because the activities of the fungal symbionts in absorbing and transferring nutrients found in the soil relieve the nutrient deficiencies which inhibit plant growth (Smith *et al*., 2010). However, an increasing number of studies have reported that AM colonization does not result in increased plant growth or total P accumulation and that sometimes the AM symbiosis appears to exert a negative effect on plant growth (Johnson *et al*., 1997; Smith *et al*., 2009; Smith *et al*., 2010). Responsiveness to AM symbiosis may vary widely within a single plant species according to the identity of the fungus and range from strongly positive to neutral or even negative, indicating significant "functional diversity" within these relationships (Jakobsen *et al*., 2002; Smith *et al*., 2010).

P uptake in AM plants may be accomplished via two pathways, namely the 'direct uptake pathway' through roots and root hairs and the 'AM pathway', in which P absorbed by external hyphae is transferred to structures inside the root cells (Smith *et al*., 2010). Traditionally, it was believed that these two pathways were interchangeable and acted additively to provide P to the plant but that view has since been brought into question (Smith *et al*., 2010). Investigation into plant-fungus symbioses using compartmentalized growth

chambers where radioactive P is only available to external mycelium has illustrated that the mycorrhizal P uptake pathway is highly active in plants, even those in which no increase in growth or tissue P accumulation was observed and were thus considered "unresponsive" (Hetrick *et al*., 1996; Ravnskov & Jakobsen, 1995; Smith *et al*., 2003, 2004; 2010; Zhu *et al*., 2003). The absence of a direct growth response or increase in P accumulation in some instances has led to the term "hidden P uptake", a phenomenon which may have important implications for the interpretation of AM symbiosis outcomes (Smith *et al*., 2010).

One such example of these hidden effects which may affect the interpretation of the outcomes of symbiosis is a study by Sangabriel-Conde *et al*. (2014). Their experiment aimed to investigate the differences in mycorrhizal responsiveness between native landraces and modern maize varieties inoculated with mixed AM species. Amongst other observations, it was noted that one native landrace in particular exhibited high levels of accumulated P in its shoots, increased root volume and was highly colonized, but that it exhibited low levels of mycorrhizal dependency (the requirement of mycorrhizal symbiosis to achieve maximum growth at a given level of fertility) and thus that it may be a naturally 'fitter' plant with a range of mechanisms to assimilate P in both the presence and absence of mycorrhizal symbionts (Sangabriel-Conde *et al*., 2014). In the standard method of comparison between AM and non-AM plants, the lack of mycorrhizal dependency may lead one to conclude that the plant variety is "unresponsive" to mycorrhization, but the P accumulation and increased root volume indicate that under different or adverse conditions, the symbiosis may confer and advantage to the plant which was not immediately evident during this study.

Another example is a study by Zhu *et al*. (2012), where maize plants were infected with *G. etunicatum* and a positive response observed, as measured by photosynthetic parameters such as net photosynthetic rate, transpiration rate, stomatal conductance, WUE and RWC. However, differences in plant height, shoot dry weight, root dry weight and total dry weight were not significant between AM and non-AM treatments and thus the effects of the symbiosis did not translate into the desirable growth response. Zhu *et al*. (2012) suggested that the lack of growth response could be attributed to the 'carbon drain effect" or dysfunction of maize metabolism (Zhu *et al*., 2012). Although the effect of this symbiosis

may not have directly translated into increased growth compared to the control, under different climatic conditions the increased photosynthetic rate may have proved advantageous-an effect which may only become clear under extensive testing. These so called 'hidden' effects of the AM symbiosis indicate that achieving a successful outcome is requires the identification of a plant host-fungus symbiosis which is tailored to and tested under the specific set of conditions under which the plants will be grown.

Chu *et al*. (2013) evaluated four maize genotypes (HMY, ZD2, ND108 and NE15) for mycorrhizal responsiveness to *G. intraradices* at five different levels of P-fertilization (4, 9, 18, 36 and 60 mg P kg^{-1}). The results of their experiments revealed 3 patterns of responses in terms of shoot growth: the response was positive if the soil P content was low, but negative if the soil Olsen-P content was high (HMY and ND108); the response was neutral regardless of soil P content (ZD2); and the response was positive regardless of soil P content (NE15). In the study, all the genotypes showed the same growth patterns in response to the different levels of P when they were not colonized by AMF whilst the growth patterns of AM plants different greatly in response to P-fertilization (Chu *et al*., 2013).

The operation of the AM pathway results in an increased expression of genes for individual transporters for orthophosphate (Pi) in root cortical cells which are strongly up-regulated in AM plants, regardless of plant responsiveness (Bucher, 2006; Poulsen *et al*., 2005). In many cases of non-responsive symbioses (as well as responsive symbioses), measurement of the P contribution of both pathways has shown that the majority of plant P enters via the AM pathway and thus that the direct pathway makes a minor contribution in AM plants (Li *et al*., 2006; Poulsen *et al*., 2005; Smith *et al*., 2003, 2004, 2010).

Smith *et al*. (2010) summarized three possible explanations for the reduced activity within the direct P uptake pathway in AM plants. The first is the possible depletion of P in the zones surrounding the root cortical cells and the subsequent inactivity of the direct pathway. The second is the possibility of reduced expression and activity of the orthophosphate transporters in the direct pathway. The third explanation put forward is the downstream regulation of transporter synthesis and activity.

Sawers *et al*. (2008) suggested that differences in mycorrhizal responsiveness in modern cereal crops may be linked to their improved ability to access and assimilate P without the aid of mycorrhizal fungi. Chu *et al*. (2013) observed that the uptake of P in shoots per unit of root length increased with increasing P in non-mycorrhizal plants whereas P-uptake in mycorrhizal plants was inhibited with increasing P. The effective 'replacement' of the direct pathway with the AM pathway is advantageous under P-deficient conditions but in some instances can result in the effective 'inactivation' of both the direct and AM pathways under high-P conditions which may explain the results observed by Chu *et al*. (2013). Regardless of the exact mechanism which dictates the AM-plant's response to different levels of P, it is clear that even though some plant varieties such as ZD2 do not respond positively to AM symbiosis irrespective of P levels, varieties such as NE15 show great potential as an AM crop plant and require further testing under a range of conditions.

AM-induced reductions in plant growth

The obligately symbiotic nature of the mycorrhizal fungus means that the outcomes of symbioses are often summed up as a net balance equation where net cost of C supplied to fungus is balanced against the net benefit of extra P supplied (Smith *et al*., 2010). The occurrence of negative plant growth responses, or 'growth depressions' (Smith *et al*., 2010) is often attributed to the net cost of C exceeding net benefit, however this explanation is only tenable in situations where there is extensive root colonization (Smith *et al*., 2010). This effect was demonstrated by Valentine *et al*. (2001) where cucumber plants in symbiosis with AM fungi produced 19 % lower biomass yield in comparison to their non-AM counterparts when grown in a full-strength nutrient solution. This effect was reversed when plants were grown under P-limited conditions, with the AM plants producing 66 % more biomass than the non-AM plants (Grant *et al*., 2005). However, these growth depressions have been observed in studies where there was very low internal root colonization and in some cases very little external mycelium in the soil, thus suggesting that the cost-benefit equation is perhaps only a superficial explanation and that the mechanisms and drivers of these interactions are far more complicated than previously thought (Smith *et al*., 2010).

It is possible that the observed growth 'depressions' or absence of growth responses are caused by an inadequate supply of P due to the reduced contribution from the direct pathway

coupled with a low P input from the fungus due to low internal colonization and hyphal proliferation within the soil (Smith *et al*., 2010). Some researchers (Koide and Schreiner, 1992) have suggested that these observed growth depressions result from a combination of factors associated with the inherent artificiality of pot trials. Factors such as low light availability which affect photosynthesis and thus C supply may suffice to explain growth depressions in cases of extensive root colonization, however the theory lacks applicability where there is low colonization (Smith *et al*., 2010).

Growth depressions in AM plants may also be linked to drought tolerance strategies. In water limited environments, a large plant size may actually prove to be disadvantageous (unless reproductive fitness is directly linked to vegetative biomass) due to their higher water requirement (Smith et al., 2010). It follows then that AM plants which appear 'unresponsive' or exhibit growth depressions will require less water and if nutrient uptake is maintained by AM fungal hyphae which are more adept at extracting nutrients from dry soils, these plants may actually survive longer than their larger counterparts (Smith *et al*., 2010).

Li *et al*., (2006, 2008) observed that wheat plants in symbiosis with AM fungi exhibited growth depressions during the initial growth stages but that the developmental differences disappeared at maturity. The smaller AM plants were reported to use less water (compared to non-AM controls) during growth but produced higher grain yields after being supplied with supplementary P. These results suggest that the general preoccupation of agriculturalists with larger plant size may be somewhat misguided and the advantages of smaller plant size being overlooked (Smith *et al*., 2010).

Water use efficiency (WUE) has often been used as a tool to assess the effects of AM symbiosis on drought tolerance and establishment of plant species (Smith *et al*., 2010) and both negative and positive responses have been observed in about equal numbers (Augé, 2001; Smith *et al*., 2010). Querejeta *et al*. (2006) observed differing WUE outcomes when inoculating two different plant species with a single fungal strain and two inoculation regimes. WUE in *Olea europea* was increased by inoculation with AM fungi and results varied with single and multiple inoculations whereas *Rhamnus lycoides* was not affected by inoculation with AM fungi, regardless of regime (Smith *et al*., 2010; Querejeta *et al*., 2006). The observed differences between species and inoculation regimes emphasize the 'functional

diversity' inherent to AM symbioses which needs to be considered when planning experiments and evaluating the outcomes of these trials (Smith *et al*., 2010).

Simpson and Daft (1990) tested maize (*Zea mays*) and sorghum (*Sorghum bicolor*) for mycorrhizal responsiveness to inoculation with *Glomus clarum, Glomus monosporum, Acaulospora sp., Glomus epigeum, Acaulospora morroweae, Glomus constrictum* under water stressed and well-watered conditions. The only fungal species which had a positive effect on plant growth was *G. clarum* and colonization by some fungal species significantly reduced host plant weight. In the maize plants, *A. morroweae, Acaulospora sp., G. monosporum* and *G. constrictum* all resulted in a growth depression compared to the un-inoculated control under water-stressed conditions.

Their study found no significant interactions between mycorrhizal inoculation and watering treatments in such aspects of plant growth as dry weight, plant height or leaf number. Root colonization percentages ranged from 30.5-54 % in maize and 29-39 % in sorghum plants, however colonization was in no way correlated to growth responses in host plants. VAM treatment significantly affected plant dry weight and WUE in sorghum but not in maize. No fungal inoculum gave a significant weight gain compared to controls in either plant species but the study found that in both species, inoculation with *G. clarum* produced significantly heavier plants of both host species in comparison to other fungal species. Simpson and Daft (1990) concluded that any benefits from VAM colonization under drought conditions may depend on the root and water distribution within soil and the duration of drought stress. WUE in plants in their experiment was closely correlated with plant size and it was suggested that plants which may have benefitted in growth from AM symbiosis may use more water and eventually suffer more from water limitation than smaller, uninfected plants (Simpson & Daft, 1990).

In a study which illustrates the specificity of relationships between host plant species and fungal identity, Pringle and Bever (2008) tested a range of species (*Allium vineale L., Anthoxanthum odoratum L., Cerastium glomeratum Thuillier, Plantago lanceolata L., Rumex acetosella L.,* and *Veronica arvensis L.*) for mycorrhizal responsiveness under laboratory conditions and then field conditions. Five species of AM fungi (*Acaulospora morrowiae, Archaeospora trappei, Gigaspora gigantea; Glomus sp., Scutellospora pellucida*) were

applied individually to each plant species and compared to an uninoculated control. Their results illustrated a broad diversity of responses of each plant to the different fungal species.

For example, *Anthoxanthum* plants exhibited a growth depression when associated with *G. gigantea* whereas the other plant species exhibited neutral responses. The *Allium* plants exhibited neutral responses to all fungal species with the exception of *A. trappei*, which stimulated a large increase in shoot weight. Similarly, *Rumex* plants exhibited neutral responses to all species with exception to *Glomus sp*. which induced a significant growth depression. The study by Pringle and Bever (2008) concluded that laboratory results were broadly comparable to field study results and that plant-fungus combinations which show promise in the laboratory are more likely to be successful in the field, however these results highlight the necessity of experimental trials which test the target plant species against a wide range of mycorrhizal species in order to identify the optimal combinations. The study also highlights the importance of thorough and extensive testing before concluding that a plant species is "unresponsive" to mycorrhization as the observed outcomes may be due to a number of factors such a fungal identity, soil characteristics, plant growth stage and many more.

Stewart *et al*. (2005) performed a field study to assess the potential of mycorrhiza to improve the growth and reproduction of strawberry plants under high P fertility. Their inocula consisted of either *Glomus intraradices* or a mixed inoculum (*G. intraradices*, *Glomus mosseae*, and *Glomus etunicatum*) and host plants were five different cultivars of commercial strawberries (Chambly, Glooscap, Joliette, Kent and Sweet Charlie). Their study aimed to investigate whether, despite the general observation that high levels of phosphorus in soil inhibit the beneficial activities of mycorrhizas, they could improve the growth of commercial strawberry cultivars grown in soil with excessively high P.

Their study found that colonization by both *G. intraradices* and the mixed inoculum resulted in no significant increase in root biomass per plant compared to the control in all cultivars except 'Joliette', in which plants colonized by the mixed inoculum had a greater root biomass. In the 'Kent' variety, colonization with mycorrhiza resulted in a significant depression of root biomass where control plants had an average of 20 grams more root material than mycorrhizal plants. The number of daughter plants per mother plant was increased significantly in the 'Glooscap' variety colonized by *G. intraradices*, whereas all

other varieties showed either neutral or negative responses to mycorrhizal colonization (both single and mixed inoculum).

These results once again emphasize the diversity within plant cultivars and their responses to mycorrhizal colonization. It also raises questions about the manner in which we quantify a "positive response" as the 'Glooscap' variety did not benefit from the symbiosis in root biomass but showed a reproductive advantage, whereas the Joliette variety had significantly more root biomass when colonized by the mixed inoculum, but did exhibited a reduction in reproductive fitness. The increase in root biomass may be considered a successful outcome in areas where water supply is inconsistent or soils are compacted, whereas the increase in reproductive fitness may be advantageous under different conditions, for example where water supply is secure but financial resources are limited or under time constraints.

It may therefore be wise to conduct studies such as these over longer periods of time which span multiple successive generations and observe the effects of altered resource allocation on total plant fitness and reproductive capacity. The unique set of conditions present in each laboratory, greenhouse or farm means that no single, generalized combination of host plant-fungi can be prescribed for a successful outcome, but rather has to be defined by trial and error.

Conclusion

In order to create sustainable relationships between the host plant and the microbial inoculants, the ecosystem functioning and the interaction between host plants and microorganisms must be fully understood as positive results are heavily dependent upon the suitable selection of microorganisms (Marulanda *et al*., 2009; Vivas *et al*., 2006). The *in vitro* testing performed in this study revealed that bacteria isolated from drought and saline environments possessed many interesting and valuable PGPR traits and were moderately drought tolerant. With regard to the application of microorganisms such as AM fungi and PGPR to a selected host plant, the results observed in this and other studies have

demonstrated that the nature of symbiotic relationships between host plants and microorganisms is infinitely complex and unpredictable. The *in vitro* selection processes provide a basic description of functional diversity within bacterial communities and species but are unable to confidently identify species which will predictably promote the growth of plants under drought stress. In addition, there will always be a large discrepancy between the laboratory conditions under which the isolates are screened and those under which they are expected to live and produce the desired effects on plant growth promotion. Similarly, the specificity of the relationship between AM fungal assemblages and host plants means that the introduction of AM species into an artificial growth environment with the selected host is unpredictable and is thus only confirmed through experimental trials such as these. It seems perhaps that in order to effectively incorporate mycorrhizas into agricultural practices, tailored site-, host plant cultivar- and fungal inoculum-specific trials would have to be run and the results used to develop strategies for individual farms.

In future studies, the process of selecting adapted organisms may possibly be further enhanced by planting the selected host plant in a field soil such as is used in the local rural agricultural practices and allowing the natural assemblages to colonize the rhizosphere under drought conditions. Isolation and enrichment from these soils may then present assemblages of organisms which are known to establish symbioses with the desired host under the target conditions and the subsequent enrichment and re-application of the most promising isolates may possibly yield superior results compared to the introduction of exotic species. Additionally, the design of the greenhouse or pot trial should ensure that conditions replicate as closely as possible the field conditions under which the symbiosis is to establish in the future. These considerations include pot size, plant age, watering regime and method of assessing plant drought responses.

Maize is known to be highly responsive to mycorrhizal colonization and as such, the exploitation and optimization of the natural symbiosis may make a meaningful contribution to rural agriculture if the issues of compatibility can be overcome.

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Appendices

A: Preparation of Standard Curve for estimation of IAA

Salkowski Reagent: 2 ml 0.5M $FeCl_3$; 49 ml dH_2O; 49 ml 70% $HClO_4$.

Standard Curve: Standards were prepared as described below using Tryptophan Broth (Merck, catalogue no. 1106940500) supplemented with 0, 5, 10, 20, 50, and 100 µg/ml IAA .

Step 1: 10 mg IAA was added to 10 ml Acetone. This was the 1000 µg/ml stock.

Step 2: 1 ml of 1000 µg/ml stock was added to 9 ml TB. This was the 100 µg/ml standard.

Step 3: 5 ml of 100 µg/ml standard was added to 5 ml TB. This was the 50 µg/ml standard.

Step 4: 1 ml of 100 µg/ml standard was added to 9 ml TB. This was the 10 µg/ml standard.

Step 5: 2 ml of 100 µg/ml standard was added to 8 ml TB. This was the 20 µg/ml standard.

Step 6: 1 ml of 50 µg/ml standard was added to 9 ml TB. This was the 5 µg/ml standard.

Step 7: 2 ml of Salkowski reagent was transferred into glass vials labelled with each standard.

Step 8: 1 ml of each standard was then added to the vials, including a control of pure TB.

Step 9: Vials were incubated at room temperature for 25 min and the colour development measured spectrophotometrically at 530 nm.

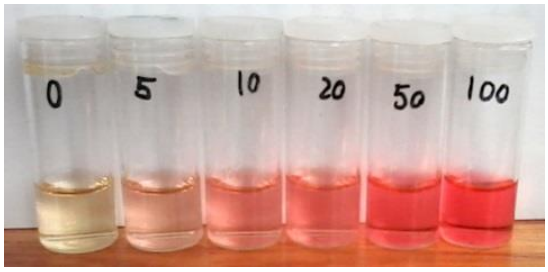

Figure **1**: Colour development observed in IAA standards (0-100 µg/ml) after 25 minutes incubation at room temperature.

Step 10: The absorbance readings were then correlated with the standards to create a standard curve (Figure **2**).

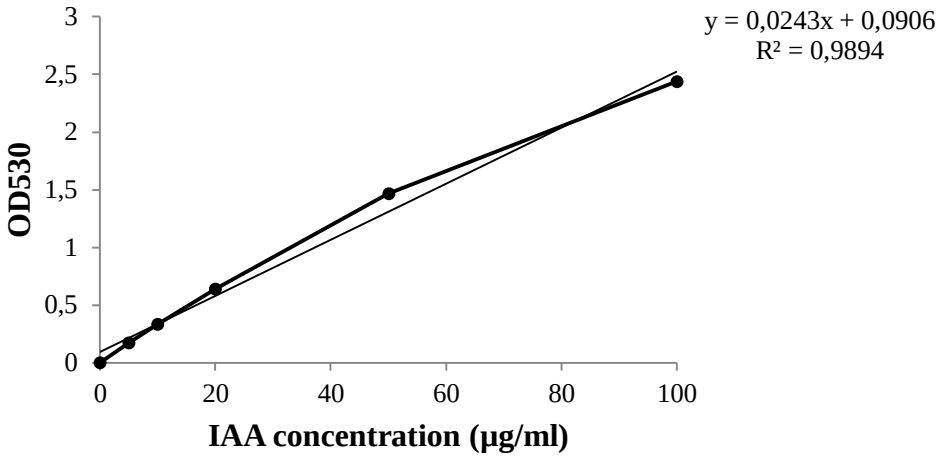

Figure 2: Standard curve prepared from known standards of IAA in Tryptone Broth.

Step 11: The equation (y = 0,0243x + 0,0906) provided by the correlation graph (Figure **2**) was then used to calculate approximate concentrations of IAA in culture supernatant (Table **1**)

Table 1: IAA Standards and values returned by equation R^2=0,9894.

IAA concentration (µg/ml)	**OD_{530}**	**IAA calculation**
0	0	-3,7284
5	0,173	3,390947
10	0,3337	10,00412
20	0,6394	22,58436
50	1,4662	56,60905
100	2,4362	96,52675

B: Phosphate Solubilisation agar

Table **2**: Composition of Pikovskaya's Agar (HiMedia) modified to include Bromophenol blue to aid visibility.

Ingredients	g/L
Yeast extract	0.5
Glucose	10.0
Calcium phosphate	5.0
Ammonium sulphate	0.5
Potassium chloride	0.2
Magnesium sulphate	0.1
Manganese sulphate	0.0001
Ferrous sulphate	0.0001
Agar	15.0 - 17.0
Bromophenol blue	0.1

All ingredients were dispensed into a flask and ddH2O added up to 1L. Mixture was then autoclaved at 121 °C for 15 min and allowed to cool. Agar was swirled gently while dispensing into petri dishes in order to prevent settling of un-dissolved components.

C: CAS agar for detection of siderophores

Protocol (Milagres *et al*., 1999):

Step 1: 60.5 mg of Chrome Azurol S was added to 50 ml ddH_2O.

Step 2: 10 ml Fe^{3+} solution (1mM $FeCl_3.6H_2O$, 10mM HCl) was prepared.

1mM $FeCl^{3+}.6H_2O$: 0.003 g $FeCl_3.6H_2O$ in 10ml ddH_2O.

10mM HCl: 10µl 32% HCl.

Step 3: 72.9 mg HDTMA was dissolved in 40ml H_2O. Under stirring, reagents from steps 1 and 2 were added to HDTMA mixture.

Note: This mixture should turn dark blue in colour. If it appears purple/brown add very small amounts of $FeCl_3$ under stirring until the colour turns blue.

Step 4: 750ml ddH_2O, 15g agar and 30.24 g of PIPES were dispensed into a 1L flask.

Approximately 12 ml 50% NaOH was slowly added until pH reached 6.8. Total volume was brought up to 900ml using ddH_2O .

Step 5: Mixtures were separately autoclaved at 121 °C for 15 min and allowed to cool. Once agar solution reached pouring temperature, blue dye solution was added by slowly pouring against the glass side of flask. Mixture was mixed well by swirling (do not shake) and dispensed into petri dishes.

D: Composition of nutrient solution applied to plants

Table **3**: Composition of nutrient solution (Smith *et al.*, 1983), modified to contain less phosphorus. The final solution was comprised of 200 ml 'Macro A', 200 ml 'Macro B' and 100 ml 'Micro C' diluted in 4.5 L water.

Ingredient	**g/L**	**Solution**
NH_4NO_3	8.43	Macro A
KNO_3	2.28	
$Mg(NO_3)6H_2O$	4.94	
$Ca(NO_3)4H_2O$	16.78	
$NaH_2PO_4H_2O$	4.138	Macro B
K_2SO_4	6.62	
Na_2SO_4	0.6	
NaCl	0.33	
H_3B0_3	0.1288	Micro C
$CuCl2H_2O$	0.00484	
$MnCl_24H_2O$	0.0811	
$(NH_4)Mo_7O_{24}$	0.00083	
$ZnSO_4$	0.0506	
Ferric citrate	0.01	

E: Use of psychrometry to evaluate the water potentials of growth media

Standards used to calibrate psychrometer were prepared with laboratory grade NaCl in $ddH_2O$to formulate known water potentials (Lang, 1967).

The method of Halverson and Firestone (2000) was adapted to use LB as the basal media and attempted to cover a similar range of water potentials

Table 4: Water potential of growth medium as determined by Dew Point Psychrometry (Srivastava, G. C. 2009)

Solution	Average µV	Bars (- 0.75 µV/ bar	MPa (10 bar / MPa)
10 g/L PEG	9,17	-12,22	-1,22
130 g/L PEG	10,50	-14,00	-1,40
150 g/L PEG	10,83	-14,44	-1,44
262 g/L PEG	15,00	-20,00	-2,00
330 g/L PEG	19,33	-25,78	-2,58
400 g/L PEG	23,33	-31,11	-3,11
LB	8,00	-10,67	-1,07
NB	6,00	-8,00	-0,80

F: Soil-specific Calibration

Note: This document was extracted directly from the user manual and was followed precisely (Internet 3, 1999).

Whalley, and White, Knight, Zeggelin and Topp have shown that there is a simple linear relationship beween the complex refractive index (which is equivalent to $\sqrt{\varepsilon}$), and volumetric water content, θ, of the form:

$$\sqrt{\varepsilon} = a_0 + a_1 \cdot \theta \qquad \textbf{[3]}$$

Since the relationship between *ThetaProbe* output and $\sqrt{\varepsilon}$ is already known, it is only necessary to determine the two coefficients, a_0 and a_1. We suggest you use the following protocol:

Step 1: Collect a sample of damp soil, disturbing it as little as possible so that it is at the same density as *in situ*.
Insert the *ThetaProbe* into the sample and measure the probe output, $\mathbf{V}_w$.
Use equation [1] or [2] to calculate $\sqrt{\varepsilon_w}$.
Weigh the damp sample, (W_w), and measure its volume (L).

Step 2: Oven-dry the sample, insert the *ThetaProbe* into the dry soil ($\theta = 0$), and

measure the probe output, V_0.
Weigh the dry sample, (W_0). Use equation [1] or [2] to calculate $\sqrt{\varepsilon_0}$.
This equals a_0. It will usually have a value between 1.0 and 2.0.

Step 3: Calculate the volumetric water content θw of the original sample:

$\theta_w = (W_w - W_0) / L$

Step 4: Then

$a_1 = (\sqrt{\varepsilon_w} - \sqrt{\varepsilon_0}) / \theta_w$ **[4]**

It will usually have a value between 7.6 and 8.6.

Step 5: By inverting equation [3], and substituting from equation [2], the water content determined from a calibrated *ThetaProbe* will then be:

$\theta = [1.1 + 4.44V] - a_0 / a_1$ **[5]**

The corresponding equation using the polynomial relationship is:

$\theta = [1.07 + 6.4V^2 + 4.7V^3] / a_1$ **[6]**

Using this relationship (rather than the linear form) will enable the T*hetaProbe* to achieve full accuracy over the full specified range, particularly for wetter soils with $0.5 < \theta < 0.6$.

G. Pyrosequencing data curation: Mothur Log File

Windows version

Running 64Bit Version

mothur v.1.33.3

Last updated: 4/4/2014

by

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http://www.mothur.org

When using, please cite:

Schloss, P.D., *et al*., Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol, 2009. 75(23):7537-41.

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

Type 'quit()' to exit program

Interactive Mode

mothur > summary.seqs(fasta=MID1.fasta)

Using 1 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	35	35	0	3	1
2.5%-tile:	1	81	81	0	3	263
25%-tile:	1	524	524	0	5	2629
Median: 1	538	538	0	6	5257	
75%-tile:	1	555	555	0	6	7885
97.5%-tile:	1	565	565	0	6	10250
Maximum:	1	840	840	5	11	10512
Mean: 1	479.909	479.909	0.0269216		5.34713	
# of Seqs:	10512					

Output File Names:

MID1.summary

mothur > summary.seqs(fasta=MID2.fasta)

Using 1 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	35	35	0	3	1
2.5%-tile:	1	46	46	0	3	301
25%-tile:	1	522	522	0	5	3004
Median: 1	541	541	0	6	6007	
75%-tile:	1	555	555	0	6	9010
97.5%-tile:	1	562	562	0	6	11713
Maximum:	1	818	818	3	10	12013
Mean: 1	465.133	465.133	0.017814		5.36885	
# of Seqs:	12013					

Output File Names:

MID2.summary

mothur > trim.seqs(fasta=MID1.fasta, minlength=500, maxhomop=7, maxambig=0)

Using 1 processors.

1000

2000

3000

4000

5000

6000

7000

8000

9000

10000

10512

Output File Names:

MID1.trim.fasta

MID1.scrap.fasta

mothur > trim.seqs(fasta=MID2.fasta, minlength=500, maxhomop=7, maxambig=0)

Using 1 processors.

1000

2000

3000

4000

5000

6000

7000

8000

9000

10000

11000

12000

12013

Output File Names:

MID2.trim.fasta

MID2.scrap.fasta

mothur > summary.seqs(fasta=MID1.trim.fasta)

Using 1 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs

Minimum:	1	500	500	0	3	1
2.5%-tile:	1	523	523	0	5	202
25%-tile:	1	534	534	0	5	2018
Median: 1	552	552	0	6	4036	
75%-tile:	1	557	557	0	6	6054
97.5%-tile:	1	565	565	0	6	7870
Maximum:	1	759	759	0	7	8071
Mean: 1	546.311	546.311	0	5.64825		
# of Seqs:	8071					

Output File Names:

MID1.trim.summary

mothur > summary.seqs(fasta=MID2.trim.fasta)

Using 1 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	500	500	0	4	1
2.5%-tile:	1	521	521	0	5	229
25%-tile:	1	535	535	0	5	2285
Median: 1	549	549	0	6	4570	
75%-tile:	1	556	556	0	6	6854
97.5%-tile:	1	562	562	0	6	8910
Maximum:	1	636	636	0	7	9138
Mean: 1	545.327	545.327	0	5.70486		
# of Seqs:	9138					

Output File Names:

MID2.trim.summary

mothur > make.group(fasta=MID1.trim.fasta, groups=Coega)

Output File Names: MID1.trim.groups

mothur > make.group(fasta=MID1.trim.fasta, groups=MID1)

Output File Names: MID1.trim.groups

mothur > make.group(fasta=MID2.trim.fasta, groups=MID2)

Output File Names: MID2.trim.groups

mothur > merge.files)input=MID1.trim.fasta-MID2.trim.fasta, output=MERGE.trim.fasta)

[ERROR]: You are missing (

Invalid.

mothur > merge.files(input=MID1.trim.fasta-MID2.trim.fasta, output=MERGE.trim.fasta)

Output File Names:

MERGE.trim.fasta

mothur > merge.files(input=MID1.trim.groups-MID2.trim.groups, output=MERGE.trim.groups)

Output File Names:

MERGE.trim.groups

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

Using 1 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	500	500	0	3	1
2.5%-tile:	1	522	522	0	5	431
25%-tile:	1	534	534	0	5	4303
Median: 1	550	550	0	6	8605	
75%-tile:	1	556	556	0	6	12907
97.5%-tile:	1	564	564	0	6	16779
Maximum:	1	759	759	0	7	17209
Mean: 1	545.789	545.789	0	5.67831		
# of Seqs:	17209					

Output File Names:

MERGE.trim.summary

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

1000 622

2000 1171

3000 1723

4000 2298

5000 2885

6000 3470

7000 4107

8000 4772

9000 5306

10000 5766

11000 6217

12000 6675

13000 7157

14000 7634

15000 8146

16000 8717

17000 9343

17209 9478

Output File Names:

MERGE.trim.names

MERGE.trim.unique.fasta

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

Using 1 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	500	500	0	3	1
2.5%-tile:	1	522	522	0	5	431
25%-tile:	1	534	534	0	5	4303
Median: 1	550	550	0	6	8605	
75%-tile:	1	556	556	0	6	12907
97.5%-tile:	1	564	564	0	6	16779
Maximum:	1	759	759	0	7	17209
Mean: 1	545.789	545.789	0	5.67831		

# of unique seqs: 9478

total # of seqs: 17209

Output File Names:

MERGE.trim.unique.summary

mothur > align.seqs(fasta=MERGE.trim.unique.fasta, reference=silva.eukarya.fasta, flip=T)

Using 1 processors.

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

It took 3 to read 1238 sequences.

Aligning sequences from MERGE.trim.unique.fasta ...

Some of you sequences generated alignments that eliminated too many bases, a list is provided in MERGE.trim.unique.flip.accnos. If the reverse compliment proved to be better it was reported.

It took 295 secs to align 9478 sequences.

Output File Names:

MERGE.trim.unique.align

MERGE.trim.unique.align.report

MERGE.trim.unique.flip.accnos

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

Unable to open MERGE.trim.unique.align.fasta

Using 1 processors.

[ERROR]: did not complete summary.seqs.

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

Using 1 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1044	1044	1	0	1	1
2.5%-tile:	10303	26786	521	0	5	431
25%-tile:	10351	26864	534	0	5	4303
Median: 10351	26991	550	0	6	8605	

75%-tile:	10366	26991	556	0	6	12907
97.5%-tile:	13883	26996	564	0	6	16779
Maximum:	43113	43116	759	0	7	17209
Mean:	11281.6	27015.3	540.124	0	5.64623	

# of unique seqs: 9478

total # of seqs: 17209

Output File Names:

MERGE.trim.unique.summary

mothur > screen.seqs(fasta=MERGE.trim.unique.align, name=MERGE.trim.names, group=MERGE.trim.groups, end=26864, optimize=start, criteria=95)

Using 1 processors.

Optimizing start to 13144.

Output File Names:

MERGE.trim.unique.good.align

MERGE.trim.unique.bad.accnos

MERGE.trim.good.names

MERGE.trim.good.groups

It took 54 secs to screen 9478 sequences.

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

Using 1 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	8511	26864	517	0	5	1
2.5%-tile:	10303	26864	534	0	5	339

25%-tile:	10351	26988	546	0	6	3381
Median: 10364	26991	554	0	6	6761	
75%-tile:	11887	26991	557	0	6	10141
97.5%-tile:	13144	26996	565	0	6	13183
Maximum:	13144	31129	759	0	7	13520
Mean: 11041.2	26979.6	550.982	0	5.7963		
# of unique seqs:	7497					
total # of seqs:	13520					

Output File Names:

MERGE.trim.unique.good.summary

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

Using 1 processors.

Creating Filter...

Running Filter...

Length of filtered alignment: 1006

Number of columns removed: 48994

Length of the original alignment: 50000

Number of sequences used to construct filter: 7497

Output File Names:

MERGE.filter

MERGE.trim.unique.good.filter.fasta

mothur > unique.seqs(fasta=MERGE.trim.unique.good.filter.fasta, name=MERGE.trim.good.names)

1000 860

2000 1707

3000 2551

4000 3392

5000 4198

6000 5047

7000 5911

7497 6349

Output File Names:

MERGE.trim.unique.good.filter.names

MERGE.trim.unique.good.filter.unique.fasta

mothur > pre.cluster(fasta=MERGE.trim.unique.good.filter.unique.fasta, name=MERGE.trim.unique.good.filter.names, group=MERGE.trim.good.groups, diffs=3)

Using 1 processors.

Processing group MID1:

3285 1743 1542

Total number of sequences before pre.cluster was 3285.

pre.cluster removed 1542 sequences.

It took 2 secs to cluster 3285 sequences.

Processing group MID2:

3329 1686 1643

Total number of sequences before pre.cluster was 3329.

pre.cluster removed 1643 sequences.

It took 2 secs to cluster 3329 sequences.

/**/

Running command: unique.seqs(fasta=MERGE.trim.unique.good.filter.unique.precluster.fasta, name=MERGE.trim.unique.good.filter.unique.precluster.names)

1000 1000

2000 1968

3000 2960

3429 3387

Output File Names:

MERGE.trim.unique.good.filter.unique.precluster.unique.names

MERGE.trim.unique.good.filter.unique.precluster.unique.fasta

/**/

It took 5 secs to run pre.cluster.

Output File Names:

MERGE.trim.unique.good.filter.unique.precluster.fasta

MERGE.trim.unique.good.filter.unique.precluster.names

MERGE.trim.unique.good.filter.unique.precluster.MID1.map

MERGE.trim.unique.good.filter.unique.precluster.MID2.map

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

Using 1 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	1002	493	0	5	1
2.5%-tile:	1	1006	518	0	5	339
25%-tile:	1	1006	520	0	6	3381

Median:	1	1006	521	0	6	6761
75%-tile:	1	1006	522	0	6	10141
97.5%-tile:	1	1006	525	0	6	13183
Maximum:	4	1006	560	0	7	13520
Mean:	1.00044	1006	520.942	0	5.78706	

# of unique seqs: 3387

total # of seqs: 13520

Output File Names:

MERGE.trim.unique.good.filter.unique.precluster.summary

mothur > chimera.uchime(fasta=MERGE.trim.unique.good.filter.unique.precluster.fasta, name=MERGE.trim.unique.good.filter.unique.precluster.names, group=MERGE.trim.good.groups))

Unable to open MERGE.trim.good.groups). It will be disregarded.

[ERROR]: no valid group files.

[ERROR]: The number of groupfiles does not match the number of fastafiles, please correct.

Using 1 processors.

[ERROR]: did not complete chimera.uchime.

mothur > chimera.uchime(fasta=MERGE.trim.unique.good.filter.unique.precluster.fasta, name=MERGE.trim.unique.good.filter.unique.precluster.names, group=MERGE.trim.good.groups)

Using 1 processors.

uchime by Robert C. Edgar

http://drive5.com/uchime

This code is donated to the public domain.

Checking sequences from MERGE.trim.unique.good.filter.unique.precluster.fasta ...

It took 80 secs to check 1743 sequences from group MID1.

It took 101 secs to check 1686 sequences from group MID2.

It took 183 secs to check 3429 sequences. 1200 chimeras were found.

The number of sequences checked may be larger than the number of unique sequences because some sequences are found in several samples.

Output File Names:

MERGE.trim.unique.good.filter.unique.precluster.uchime.chimeras

MERGE.trim.unique.good.filter.unique.precluster.uchime.accnos

mothur > remove.seqs(accnos=MERGE.trim.unique.good.filter.unique.precluster.uchime.accnos, fasta=MERGE.trim.unique.good.filter.unique.precluster.fasta, name=MERGE.trim.unique.good.filter.unique.precluster.names, group=MERGE.trim.good.groups)

Removed 2227 sequences from your name file.

Removed 1200 sequences from your fasta file.

Removed 2227 sequences from your group file.

Output File Names:

MERGE.trim.unique.good.filter.unique.precluster.pick.names

MERGE.trim.unique.good.filter.unique.precluster.pick.fasta

MERGE.trim.good.pick.groups

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

Using 1 processors.

	Start	End	NBases	Ambigs	Polymer	NumSeqs
Minimum:	1	1002	493	0	5	1
2.5%-tile:	1	1006	518	0	5	283
25%-tile:	1	1006	520	0	5	2824

Median: 1	1006	521	0	6	5647	
75%-tile:	1	1006	522	0	6	8470
97.5%-tile:	1	1006	526	0	6	11011
Maximum:	4	1006	560	0	7	11293
Mean: 1.00053	1006	520.914	0	5.74887		
# of unique seqs:	2187					
total # of seqs:	11293					

Output File Names:

MERGE.trim.unique.good.filter.unique.precluster.pick.summary

mothur > system(cope MERGE.trim.unique.good.filter.unique.precluster.pick.names FINAL.names)

'cope' is not recognized as an internal or external command,

operable program or batch file.

mothur > system(copy MERGE.trim.unique.good.filter.unique.precluster.pick.names FINAL.names)

1 file(s) copied.

mothur > system(copy MERGE.trim.unique.good.filter.unique.precluster.pick.fasta FINAL.fasta)

1 file(s) copied.

mothur > system(copy MERGE.trim.good.pick.groups FINAL.groups)

1 file(s) copied.

mothur > dist.seqs(fasta=FINAL.fasta, cutoff=0.15)

Using 1 processors.

Output File Names:

FINAL.dist

It took 58 to calculate the distances for 2187 sequences.

mothur > cluster(column=FINAL.dist, name=FINAL.names)

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

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

****

changed cutoff to 0.061571

Output File Names:

FINAL.an.sabund

FINAL.an.rabund

FINAL.an.list

It took 7 seconds to cluster

mothur > make.shared(list=FINAL.an.list, group=FINAL.groups, label=0.03)

0.03

Output File Names:

FINAL.an.shared

FINAL.an.MID1.rabund

FINAL.an.MID2.rabund

mothur > remove.rare(shared=FINAL.an.shared, nseqs=1, bygroup=t)

0.03

Output File Names:

FINAL.an.0.03.pick.shared

mothur > count.groups(FINAL.groups)

FINAL.groups is not a valid parameter.

The valid parameters are: shared, group, count, accnos, groups, inputdir, and outputdir.

Using FINAL.an.0.03.pick.shared as input file for the shared parameter.

[ERROR]: did not complete count.groups.

mothur > count.groups(FINAL.an.shared)

FINAL.an.shared is not a valid parameter.

The valid parameters are: shared, group, count, accnos, groups, inputdir, and outputdir.

Using FINAL.an.0.03.pick.shared as input file for the shared parameter.

[ERROR]: did not complete count.groups.

mothur > count.groups(FINAL.an.0.03.pick.shared)

FINAL.an.0.03.pick.shared is not a valid parameter.

The valid parameters are: shared, group, count, accnos, groups, inputdir, and outputdir.

Using FINAL.an.0.03.pick.shared as input file for the shared parameter.

[ERROR]: did not complete count.groups.

mothur > count.groups(MERGE.trim.good.pick.groups)

MERGE.trim.good.pick.groups is not a valid parameter.

The valid parameters are: shared, group, count, accnos, groups, inputdir, and outputdir.

Using FINAL.an.0.03.pick.shared as input file for the shared parameter.

[ERROR]: did not complete count.groups.

mothur > count.groups(FINAL.groups)

FINAL.groups is not a valid parameter.

The valid parameters are: shared, group, count, accnos, groups, inputdir, and outputdir.

Using FINAL.an.0.03.pick.shared as input file for the shared parameter.

[ERROR]: did not complete count.groups.

mothur > count.groups(FINAL.an.0.03.pick.shared)

FINAL.an.0.03.pick.shared is not a valid parameter.

The valid parameters are: shared, group, count, accnos, groups, inputdir, and outputdir.

Using FINAL.an.0.03.pick.shared as input file for the shared parameter.

[ERROR]: did not complete count.groups.

mothur > count.groups(shared=FINAL.an.0.03.pick.shared)

MID1 contains 5016.

MID2 contains 5795.

Total seqs: 10811.

Output File Names:

count.summary

mothur > sub.sample(shared=FINAL.an.0.03.pick.shared, size=5016)

Sampling 5016 from each group.

0.03

Output File Names:

FINAL.an.0.03.pick.0.03.subsample.shared

mothur > collect.single(shared=FINAL.an.0.03.pick.subsample.shared, calc=chao-invsimpson, freq=5)

Unable to open FINAL.an.0.03.pick.subsample.shared

Using FINAL.an.0.03.pick.0.03.subsample.shared as input file for the shared parameter.

[ERROR]: did not complete collect.single.

mothur > collect.single(shared=FINAL.an.0.03.pick.0.03.subsample.shared, calc=chao-invsimpson, freq=5)

Processing group MID1

0.03

Processing group MID2

0.03

Output File Names:

FINAL.an.0.03.pick.0.03.subsample.MID1.chao

FINAL.an.0.03.pick.0.03.subsample.MID1.invsimpson

FINAL.an.0.03.pick.0.03.subsample.MID2.chao

FINAL.an.0.03.pick.0.03.subsample.MID2.invsimpson

mothur > venn(groups=MID1.trim.groups-MID2.trim.groups)

Using FINAL.an.0.03.pick.0.03.subsample.shared as input file for the shared parameter.

MID1.trim.groups is not a valid group, and will be disregarded.

MID2.trim.groups is not a valid group, and will be disregarded.

You provided no valid groups. I will run the command using all the groups in your file.

0.03

Output File Names:

FINAL.an.0.03.pick.0.03.subsample.0.03.sharedsobs.MID1-MID2.svg

FINAL.an.0.03.pick.0.03.subsample.0.03.sharedsobs.MID1-MID2.sharedotus

mothur > get.oturep(column=FINAL.dist, name=FINAL.names, list=FINAL.an.list, fasta=FINAL.fasta, sorted=group, groups=FINAL.groups)

You must provide a groupfile or have a count file with group info to sort by group. I will not sort.

You must provide a groupfile to use groups.

[ERROR]: did not complete get.oturep.

mothur > get.oturep(column=FINAL.dist, name=FINAL.names, list=FINAL.an.list, fasta=FINAL.fasta, sorted=group, group=FINAL.groups)

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

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

****

unique 2187

0.01 1576

0.02 891

0.03 593

0.04 421

0.05 311

0.06 238

Output File Names:

FINAL.an.unique.rep.names

FINAL.an.0.01.rep.names

FINAL.an.0.02.rep.names

FINAL.an.0.03.rep.names

FINAL.an.0.04.rep.names

FINAL.an.0.05.rep.names

FINAL.an.0.06.rep.names

FINAL.an.0.01.rep.fasta

FINAL.an.0.02.rep.fasta

FINAL.an.0.03.rep.fasta

FINAL.an.0.04.rep.fasta

FINAL.an.0.05.rep.fasta

FINAL.an.0.06.rep.fasta

FINAL.an.unique.rep.fasta

mothur > count.seqs(name=FINAL.an.0.03.rep.names, group=FINAL.groups)

Using 1 processors.

It took 0 secs to create a table for 11293 sequences.

Total number of sequences: 11293

Output File Names:

FINAL.an.0.03.rep.count_table