THE EFFECT OF *PSEUDOMONAS KOREENSIS* ON THE LEVEL OF DROUGHT TOLERANCE OF *HELIANTHUS ANNUUS*

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A Dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science

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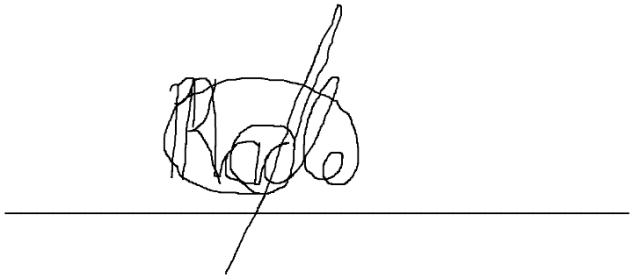
DECLARATION

I declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



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ABSTRACT

Drought stress is one of the major limitations to crop production worldwide and has been predicted to become more severe in the future due to global climate change. Research has often been focused on genetic engineering to improve the tolerance of plants to abiotic and biotic stresses. Plant growth-promoting rhizobacteria (PGPR) are an alternative mechanism to improve the tolerance of plants to many stresses and is crucial for developing and third world countries. In this study, *Helianthus annuus* was inoculated with *Pseudomonas koreensis* and subjected to drought stress. The germination and growth characteristics, leaf water content, leaf electrolyte leakage and leaf area, substrate water content, phenolic compounds and proline concentration, root bacterial counts, as well as recovery and regrowth, were compared between uninoculated and inoculated plants. In addition, the phosphatase activity, siderophore and indole-3-acetic acid (IAA) production, as well as growth at -0.73 MPa was compared between *P. koreensis* and *P. fluorescens*. It was found that inoculated plants were significantly taller plants and had a larger leaf area; and had significantly higher phenolic and proline concentration and a higher colonised root surface under drought stress. However, inoculation negatively affected germination and chlorophyll fluorescence. These plants also had a lower substrate water content under drought stress. *P. koreensis* outperformed *P. fluorescens* in all parameters studied, except for growth under osmotic stress. It can be concluded that *P. koreensis* generally improves the drought stress tolerance of *H. annuus*, however, further investigations are needed to determine the reasons for some of the negative effects.

ACKNOWLEDGEMENTS

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LIST OF ABREVIATIONS

ABA	Abscisic acid
ACC	1-Aminocyclopropane-1-carboxylate
ATP	Adenosine triphosphate
CAS	Chrome azurol S
CAT	Catalase
CI	Confidence interval
DF	Dworkin and Foster
DFOM	Deferoxamine mesylate
dH_2O	Deionised H_2O
DNA	Deoxyribonucleic acid
DW	Dry weight
e^-	Electron
EPS	Exopolysaccharides
ERD15	Early responsive to dehydration 15
ETR	electron transport rate
FC	Folin-Ciocalteu
FW	Fresh weight
GAE	Gallic acid equivalents
HDTMA	Hexadecyltrimethylammonium
HKT	Histidine kinase transporter
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
ISR	Induced systemic resistance

IST	Induced systemic tolerance
MDA	Malondialdehyde
MES	2-(N-morpholino)ethanesulfonic acid
MGT	Mean germination time
MS	Murashige and Skoog
NADPH	Nicotinamide adenine dinucleotide phosphate
NB	Nutrient broth
OD	Optical density
P5C	1-Pyrroline-5-carboxylic acid
PAL	Phenylalanine ammonia lyase
PEG	Polyethylene glycol
PEP	Phosphoenolpyruvate
PGPR	Plant growth-promoting rhizobacteria
pNP	*para*-Nitrophenyl
pNPP	*para*-Nitrophenyl phosphate
POD	Peroxidase
PSII	Photosystem II
RC	Relative conductivity
ROS	Reactive oxygen species
Rubisco	Ribulose-1,5-bisphosphate
RWC	Relative water content
SOD	Superoxide dismutase
SWC	Substrate water content
TSB	Tryptic soy broth

TW Turgor weight

VAM Vesicular arbuscular mycorrhizal

VOC Volatile organic compound

1 BACKGROUND

One of the major production limiting factors to agriculture is mild to severe drought conditions. This affects many African countries that are already stricken by poverty, disease, low levels of education and research input and it has been predicted that conditions such as drought will occur more regularly and have increased duration and severity due to global climate change (Smol 2012). Considering that agriculture plays a major role in alleviating poverty and malnutrition, it is imperative to ensure high levels of food production in the face of global climate change and a growing human population. Large-scale farmers can maintain high levels of crop production in adverse conditions with the use of irrigation and fertilization. However, these methods are often used in excess, adding to the water shortage problem and leading to the pollution of groundwater and river systems and are therefore not sustainable. On the other hand, small-scale farmers generally cannot afford the expensive equipment and chemicals needed to maintain high levels of crop production and are therefore at more risk in the face of global climate change. As the global climate changes, farmers will need to plant on lands that were once thought to be sub-standard (due to climatic and soil properties such as amount of available nutrients, soil composition, etc.) in order to meet the demand and this will have an effect on the quality of the produce.

Thus far, agricultural research has been mainly focused on the improvement of crop plants through breeding and genetic engineering to maintain growth and productivity in adverse conditions such as drought. However, it has been shown that a plant's evolutionary responses to drought are relatively weak compared with the adaptive responses brought about by other biota in a community such as soil microorganisms (Lau and Lennon 2012). Plant breeders have selected plant traits to increase yield, however, early plant breeding programs may have lost certain traits pertaining to drought tolerance and therefore, soil microorganisms are important in improving the tolerance of plants to drought stress. There is also an increasing demand worldwide for alternative 'green technologies' that rely less on synthetic chemical products and the use of expensive technology. Also, in places like the European Union, the use of genetically modified organisms is highly regulated (Levidow *et al.* 2000). The use of plant growth-promoting rhizobacteria (PGPR) is therefore a potential solution which offers the possibility of improving drought stress tolerance in plants through a cheap, natural and self-regulating system (Timmusk *et al.* 2013). PGPR also regulate the availability of nutrients and can transform a non-arable land into a fertile one.

2 LITERATURE REVIEW

2.1 The response of plants to drought stress

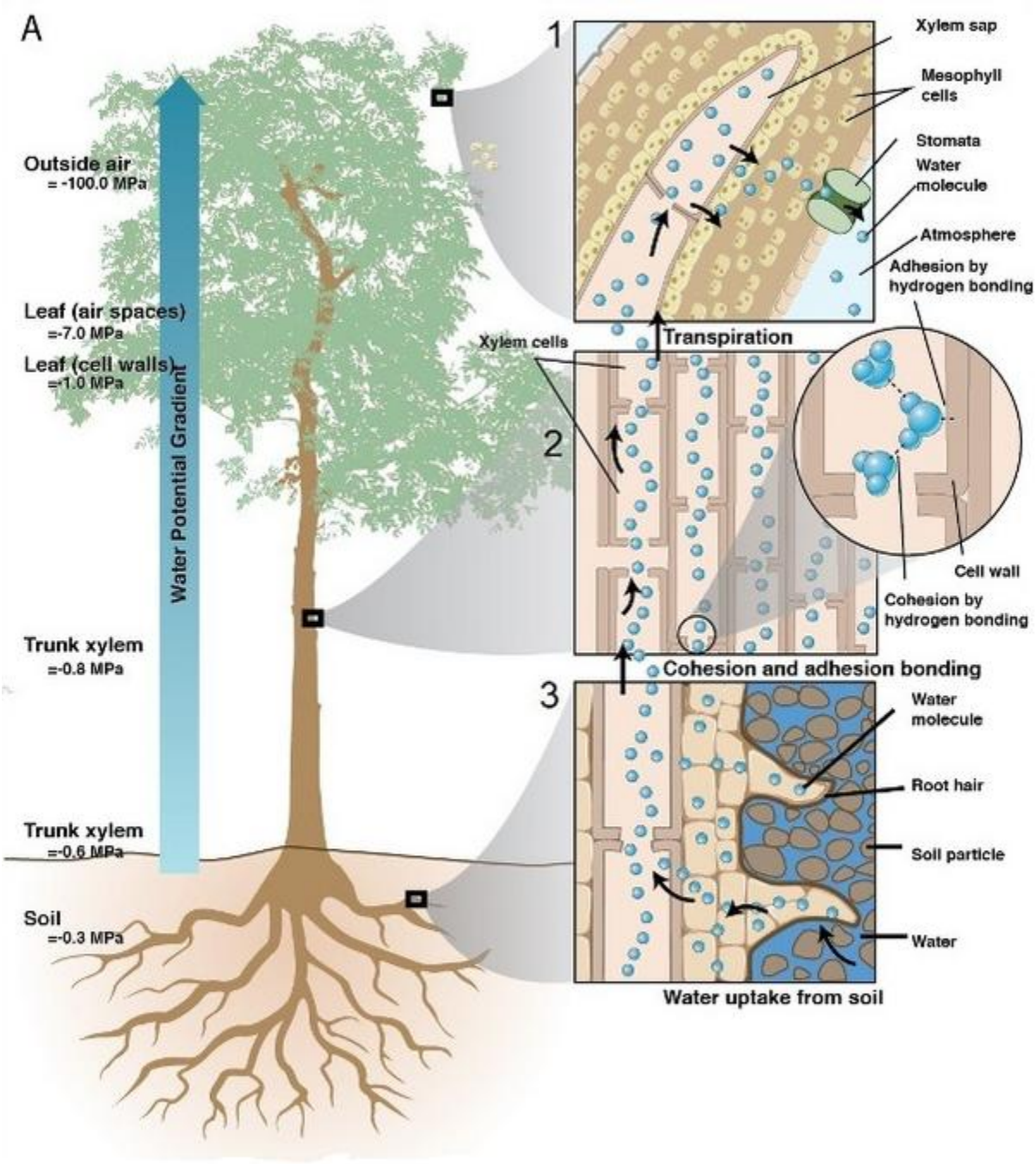
2.1.1 How water transport is affected by drought stress

Water is very important for plant survival, making up 80 to 90 % of a plant's weight. Plants are also very 'thirsty' organisms, with some large rainforest trees using nearly 1200 L of water per day (McElrone *et al.* 2013). However, it has been estimated that plants only utilize 5% of the water absorbed by the roots for cell expansion and plant growth (McElrone *et al.* 2013). The reason behind the discrepancy is that plants need to obtain CO_2 from the atmosphere in order to make sugars during photosynthesis that are essential to plant survival. Plants absorb CO_2 through apertures in their leaves called stomata (White 2012) and it is through these apertures that the majority of absorbed water evaporates. Therefore, plants need to balance the rates of transpiration and photosynthesis with the amount of water available to the plant in order to avoid a water deficit. However, these survival mechanisms are not necessarily good for plant productivity in an agronomic sense (Blum 2011a).

The flow of water through the plant begins with the absorption of water from the soil by the roots. Water must pass through several layers of cells before entering the root xylem (Figure 1.). These layers of cells include the epidermis, the cortex (composed of a few layers of cells), the endodermis, the pericycle and the xylem parenchyma. These cells have a higher resistance to water flow than the xylem and act as a filtration system, keeping out unwanted and harmful chemicals. The flow of water through cells can occur through the apoplast (cell walls), from cell-to-cell through plasmodesmata and from cell-to-cell across plasma membranes (McElrone *et al.* 2013, Blum 2011a). In the apoplastic pathway, the water comes to a barrier in the cell walls of the endodermis called the Casparian strip (Nagahashi *et al.* 1974). The strip is made of suberin and/or lignin (Blum 2011a) and is impermeable to water thus, forcing water into the cells of the endodermis and through the filtration system before it enters the stele (vascular tissue). The flow of water across cell membranes is through water specific channels called aquaporins (Carbrey and Agre 2009). And therefore, the abundance and activity of these channels can influence the rate of water flow.

Once in the xylem tissue, the water goes against relatively little resistance in these specialized water transport tissues. Xylem cells are tube shaped and are stacked end to end along the roots and stems of plants (McElrone *et al.* 2013). Water flows from the stem into the leaves via the petiole which then turns into the leaf midrib which progressively branches into minor veins that are embedded in the mesophyll tissue. The water then passes from the veins into the bundle

sheath cells and into the mesophyll cells where a small amount is used in photosynthesis. It is likely that the water flows via the apoplastic pathway to the cells in the sub-stomatal cavity where it evaporates from the open stomata into the atmosphere (Sack and Holbrook 2006).



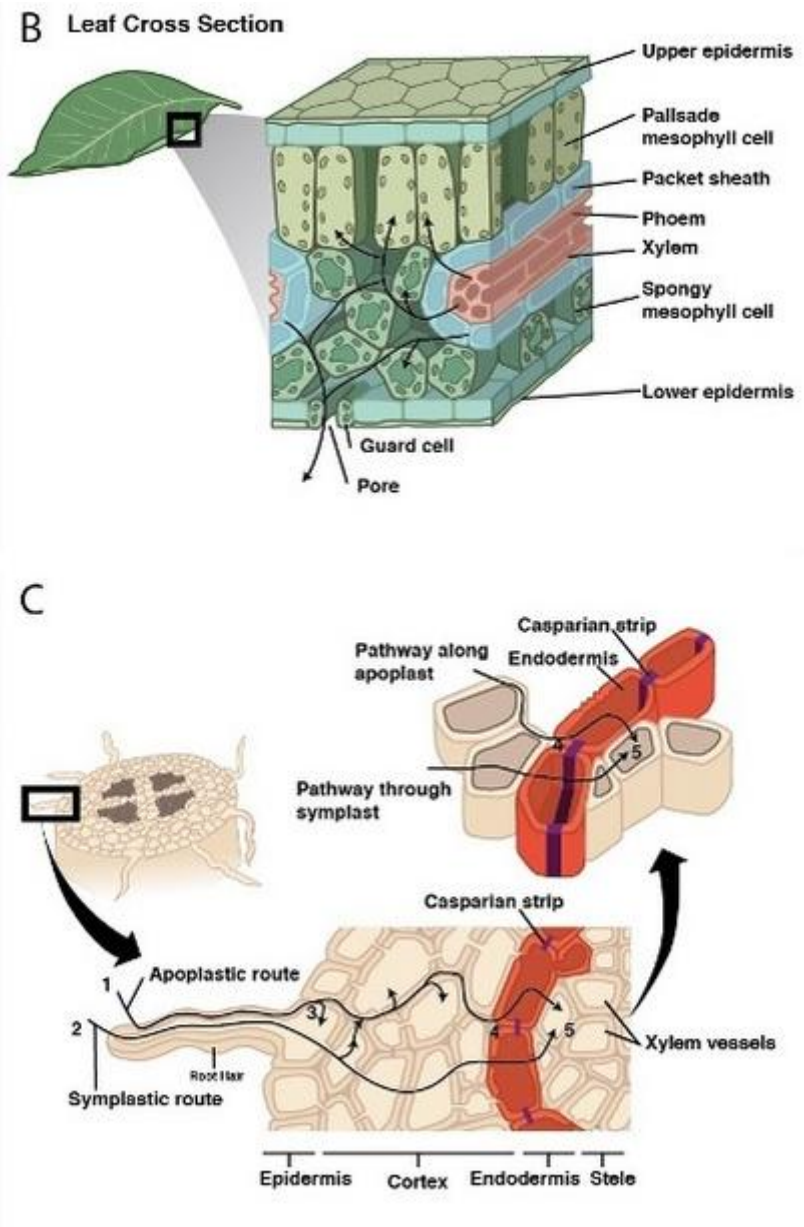


Figure 1. Pathways of water flow in a plant. (A) Water flow through a plant as a whole; (B) Water flow through a leaf cross section; (C) Water flow through a root cross section. From McElrone *et al.* 2013.

The movement of water through the plant, from the soil into the air, is a passive process driven by the differences in pressure and chemical potential gradients (McElrone *et al.* 2013). The majority of water is moved by the negative pressure that is created when the water evaporates from the leaves. When the water evaporates it creates a pulling force on the water molecules to replace the water molecules that have left the plant. Water throughout the plant is held together in columns by hydrogen bonding (Chavarria and dos Santos 2012) and therefore, the pull generated at the leaves results in water entering at the roots. Water also moves via osmosis and

this governs the movement of water between cells and various organelles within the plant. Also, when transpiration is limited or absent as is the case during many abiotic stresses, water enters the roots via osmosis.

A plant experiences water stress when the demand for water is not matched by the supply (Lisar *et al.* 2012, Blum 2011b), as is the case during periods of drought, in saline soils, or periods of high transpiration – the result of hot, dry conditions caused by global climate change. When the supply of water becomes limiting, plants initiate a variety of responses that decreases the water use of the plant to a level that is in equilibrium with the available water. These responses are dynamic and tailored to the severity of the water deficit.

One of the first responses of a plant when faced with drought stress is to close the stomata thus, decreasing the amount of water lost via transpiration. This is triggered by changes in the cell membranes in response to limited water availability. Low water content can disturb the association of membrane lipids and proteins (Lisar *et al.* 2012) resulting in a disruption of signalling and transport networks. Under normal conditions, membranes consist of a fluid lipid bilayer with proteins embedded among the lipid molecules or in close association with the polar heads of the lipid molecules (Akıncı and Lösel 2011). Under drought stress conditions, the fluidity of membranes is decreased thus, affecting the functioning of the membrane as a permeable barrier. The membranes are also damaged via oxidation. This leads to the leakage of important electrolytes out of the organelles or the cell as a whole, the major result being a decrease in photosynthetic output. Plants that are more tolerant of drought stress are able to maintain membrane fluidity and protect membranes against damage during periods of water deficit, and therefore, have a lower electrolyte leakage than less tolerant plants (Bajji *et al.* 2001).

2.1.2 The effect of drought stress on CO_2 uptake, stomata and photosynthesis

The opening and closing of the stomata is regulated by various environmental conditions, for example, light intensity, CO_2 concentration and air humidity (Blum 2011a). Situations such as high light levels, low CO_2 concentrations and high air humidity are perceived by sensors in the guard cells and the stomata open; while the opposite conditions result in the closure of the guard cells (Blum 2011a). The regulation of the stomata is also governed by hormone levels, particularly abscisic acid (ABA). ABA is generated by the roots (as well as in chloroplasts) in response to low soil water availability and is transported through the xylem and perceived by

ABA receptors in the guard cells resulting in the closure of the stomata (Blum 2011a, Davies *et al.* 2002). In this way, ABA serves as a warning to the plant at the start of drought stress and also gives an indication of the severity of the stress (Blum 2011a, Davies *et al.* 2002).

The opening and closing of the aperture of the stomata is driven by osmosis in the guard cells. Accumulation of potassium (K) and occasionally sugars in the guard cells reduces the water potential and thus, water in the surrounding tissues flows into the guard cells causing them to expand resulting in an opening of the stomatal aperture (Blum 2011a). The reverse is applicable to the closure of the aperture. CO_2 enters through open stomata and is subsequently used in photosynthesis.

Photosynthesis is the use of CO_2 from the atmosphere and the energy from light (absorbed by pigments such as chlorophyll) to create adenosine triphosphate (ATP; via photo-phosphorylation) and to synthesize carbohydrates, such as glucose and sucrose, which are then used in metabolism or storage. Water is an important component in photosynthesis as it supplies electrons (e^-) and H^+ ions for the generation of carbohydrates. Photosynthesis is an essential process for plant survival and is therefore well protected in times of abiotic stress, during which damage to the photosynthetic apparatus can occur thus, impinging on the survival of the plant. Plants will close their stomata during the periods of highest evaporation (around midday) and open them during periods of less intense evaporation which then allows the plant to optimize the amount of carbon (C) gained in relation to the amount of available water (Chaves *et al.* 2002, Cowan 1982, Jones 2013). The closure of the stomatal aperture also decreases the availability of CO_2 from the atmosphere and thus, photosynthesis is impaired causing an imbalance in the plant's metabolism. The decrease in CO_2 supply to Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) induces the adjustment of the photosynthetic machinery to utilize the available concentrations as well as to increase the dissipation of energy, especially when exposed to high light intensity and high temperatures (Chaves *et al.* 2002, Chaves *et al.* 2009). The diffusion of CO_2 is also reduced in the leaf mesophyll in plants under water and salinity stress (Chaves *et al.* 2009). The decreased rates of diffusion may be linked to the effects that leaf shrinkage has on the structure of the intercellular spaces (Lawlor and Cornic 2002), or to an altered biochemistry and/or to membrane permeability (Chaves *et al.* 2009). There are also mechanisms to down regulate the Calvin cycle during periods of drought stress, such as the deactivation of Rubisco and alterations leading to a decreased supply of ATP resulting in a decreased regeneration of Ribulose-1,5-bisphosphate (Chaves *et al.* 2009).

Often, when plants are faced with drought stress, high light intensity and heat stress are also present. This combination of stresses can cause damage to the thylakoid membranes and chloroplasts thus, affecting plant survival and productivity. There are various protective mechanisms that lower the rate of nicotinamide adenine dinucleotide phosphate (NADPH) production that would otherwise lead to the reduction (*i.e.* redox) of proteins and lipids. The general mode of action of the protective mechanisms against drought stress is to reduce the amount of incoming light energy by using accessory pigments, deactivating photosystem II (PSII) and other membrane proteins, or releasing the absorbed energy as heat (Chaves *et al.* 2009).

In plants that have C3 photosynthesis, such as sunflower or rice, at a low concentration of CO_2, Rubisco has a greater affinity for O_2 which results in decreased sugar production and does not generate ATP (Campbell *et al.* 2008). However, this so-called photorespiration does decrease the damage incurred under high light intensities at low CO_2 concentrations (Campbell *et al.* 2008). In plants that have C4 photosynthesis, such as maize or sorghum, where the light and dark reactions are spatially separated, *i.e.* in the mesophyll cells and the bundle sheath cells, respectively, the incoming CO_2 is converted to oxaloacetate by phosphoenolpyruvate (PEP) carboxylase in the mesophyll cells (Campbell *et al.* 2008). Oxaloacetate is then transported to the bundle sheath cells after being reacted to malate which then enters the Calvin cycle (Campbell *et al.* 2008). In this way, CO_2 is concentrated in the bundle sheath cells so that Rubisco binds to CO_2 instead of O_2 and therefore, the amount of photorespiration is decreased, photosynthetic output is sustained and the amount of damage during periods of drought stress, heat and high light intensity is reduced (Campbell *et al.* 2008). Plants with C4 photosynthesis are more tolerant of drought stress than C3 plants. However, this underlines the need to develop strategies to improve the drought stress tolerance of economically important C3 plants, such as sunflower.

2.1.3 Reactive Oxygen Species increases as a response to drought stress

Any condition that alters a plant's metabolism, such as drought, will result in an increased production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), the superoxide radical ($O_2\cdot^-$) and the hydroxyl radical ($OH\cdot$) (Gill and Tuteja 2010). ROS production occurs as a by-product of normal metabolism in the chloroplasts, mitochondria and peroxisomes (Foyer and Noctor 2005). For example, in the chloroplasts, oxygen can gain the e^- passing

through the photosystems thus, forming $O_2\cdot^-$ (Gill and Tuteja 2010). Through various reactions, $O_2\cdot^-$ develops into H_2O_2, OH· and other ROS. However, under steady-state conditions, the ROS is sufficiently quenched by numerous antioxidants (Foyer and Noctor 2005) including superoxide dismutase (SOD), catalase (CAT), ascorbic acid, glutathione and many phenolic compounds (Gill and Tuteja 2010).

When the equilibrium of production and scavenging of ROS is disturbed, for example under drought stress, the plant must increase the production of antioxidants so as to counter the damaging effects of ROS build-up. ROS damages proteins, lipids, carbohydrates and deoxyribonucleic acid (DNA) which, if left unchecked, will result in cell death (Gill and Tuteja 2010). A plant that can quickly respond to an increased production of ROS will incur less damage and will therefore, be more tolerant of drought stress. Initially, as the plant encounters drought stress, there is a spike in the levels of ROS. This spike acts as a signal for regulation of gene expression and therefore ROS in this regard can be considered as a secondary messenger. Superoxide is scavenged by the enzymatic antioxidant SOD which catalyses the dismutation of $O_2\cdot^-$ where one $O_2\cdot^-$ is reduced to H_2O_2 and another oxidized to O_2, therefore, the production of the highly reactive OH· and H_2O_2 is lowered. Superoxide is also scavenged by the non-enzymatic antioxidant ascorbic acid (Vitamin C). Plants that can effectively reduce the build-up of ROS during drought stress through the above mechanisms will incur health benefits as they will have high levels of antioxidants, such as phenolic compounds.

A compound is classified as 'phenolic' if it has at least one aromatic ring (C6) with one or more hydroxyl groups (Michalak 2006). Phenolic compounds are mainly synthesized from cinnamic acid which is formed from phenylalanine by L-phenylalanine ammonia-lyase (PAL) which is the branch point enzyme between primary (shikimate pathway) and secondary (phenylpropanoid) metabolism (Michalak 2006). Phenolic compounds are important for normal growth of plants and also act in the defence against biotic and abiotic stresses (Kähkönen *et al.* 1999). Polyphenolic compounds have an ideal structure to be a free radical-scavenger and have been shown to have a higher efficacy *in vitro* as an antioxidant than vitamins E and C (Rice-Evans *et al.* 1995, Rice-Evans *et al.* 1996). The antioxidant activities of phenolic compounds is mainly due to their redox properties which enable them to donate e^- and H^+ and also quench singlet O_2 (Kähkönen *et al.* 1999, Rice-Evans *et al.* 1997). These compounds are also metal-chelators, particularly for Fe and Cu, which inhibits transition metal-catalysed free radical formation (Hudson and Lewis 1983, Rice-Evans *et al.* 1995, Rice-Evans *et al.* 1997).

Biotic and abiotic stresses have a substantial effect on the levels of secondary metabolites in plants (Dixon and Paiva 1995). For example, there was a general increase in the phenolic content of drought stressed *Hypericum brasiliense* in both root and shoot samples, by 61% and 80%, respectively (de Abreu and Mazzafera 2005). The authors linked the increase in phenolics as a response to the generation of ROS. The increase in phenolics and the corresponding biosynthetic enzymes has been reported in other drought stressed plants (English-Loeb *et al.* 1997, Aguilar *et al.* 2000). Andre *et al.* (2009) reported that drought stress induced the upregulation of the phenolic biosynthesis genes in potato, however, the induction was cultivar specific. There are, however, several studies that report a decrease in phenolic content in plants under drought stress. Drought stressed *Rehmannia glutinosa* had a decrease of around 45% in the root phenolic content as compared with unstressed plants (Chung *et al.* 2006). The decrease was linked to a decrease in the activity of key biosynthetic enzymes. Phenolic compounds have also been reported to increase under other abiotic stresses, such as heavy metal stress, which also causes oxidative stress (Michalak 2006). The phenolics act as metal-chelators and scavengers of ROS during heavy metal stress in plants (Michalak 2006).

2.1.4 The effect of drought stress on plant growth in general

Plant leaf and stem growth is adversely affected by water deficit. The growth of cells relies, in part, on the turgor and extensibility of the cell wall (Blum 2011a). Under drought stress, there is a decrease in cell turgor as well as the loss of cell wall extensibility and hence, a decrease in growth. Cell division can only occur after a cell reaches a certain size (Blum 2011a, Granier and Tardieu 1999). Therefore, cell differentiation is also effected by drought stress. The increased concentration of ABA in the leaves and shoots of plants under drought stress also inhibits growth. Furthermore, there is an increase in leaf senescence in plants under drought stress (Blum 2011a). However, it has been found that the root-to-shoot ratio increases under drought stress (Blum 2011a). It has been found that the roots have a greater osmotic adjustment ability (particularly with proline and carbohydrates) compared with the leaves (Ober and Sharp 2007) which ensures water flow into the root. Furthermore, ABA signals generated in the roots under drought stress enhances root growth while arresting leaf and shoot growth (Blum 2011a). There is also an increased activity of cell wall expansion in root cells under water deficit (Wu *et al.* 1996) allowing the turgor driven expansion of the root cells under osmotic adjustment. All of these modifications increases the surface area of the plant's roots allowing the plant greater access to water.

Drought stress also affects the plant's ability to acquire nutrients. With the reduction in transpiration, photosynthesis and flow of water into the plant there will be a reduction in the amount of nutrients absorbed and the availability of the nutrients in the soil. There are also reductions in active transport and membrane permeability in drought stressed plants (Akıncı and Lösel 2011). There are differences in the reduced uptake of certain nutrients, for example, there is a decrease in the uptake of K, magnesium (Mg), calcium (Ca), iron (Fe), zinc (Zn), copper (Cu) as well as nitrogen (N) and phosphorous (P) in some crop plants (Akıncı and Lösel 2011). However, drought stressed maize showed an increased uptake of K and Ca (Tanguilig *et al.* 1987). Since K is involved in the osmotic adjustment of many plant species, the increased uptake is beneficial and may show increased tolerance in those plants. It has been repeatedly shown that the uptake of P in many species is decreased under drought stress (Akıncı and Lösel 2011). It is also apparent that older roots lose their absorptive functions in dried soil and therefore, nutrients are only taken in at the root tips (Akıncı and Lösel 2011).

2.1.5 Osmotic adjustment is a response of plants to drought stress

Under normal growth conditions, there is a negative water potential gradient, with the least negative potential being in the soil while through the plant the water potential become more and more negative from the roots, to the stems, to the leaves and the most negative water potential is found in the air (Figure 1.). In this way, water follows the negative water potential and flows through the plant into the air. In drought conditions, as the soil dries, the water potential in the soil becomes more negative disrupting the normal potential gradient. However, plants can actively change the water potential of the cells via osmotic adjustment in order to maintain the water potential gradient lower than that in the soil and thus, water flows from the soil into the plant. Plants lower the water potential of cells, for example in the leaf, by importing organic and inorganic solutes into the leaf cells which then dilute the water within the cells so that water from surrounding tissues flow into those cells (Blum 2011a). These solutes can include ions (Cl^-, K^+, etc.), sugars and sugar alcohols, as well as amino acids such as proline. Plants that are better equipped to maintain the negative water potential gradient during periods of drought will be more tolerant of drought stress than plants that are not as efficient in osmotic adjustment. The use of organic solutes (such as soluble sugars) in osmotic adjustment competes with plant growth, however, because cell expansion decreases at the onset of water deficit before photosynthesis is arrested, there is an initial abundance of organic solutes that can be used for osmotic adjustment which allows root growth and photosynthesis during drought

stress, albeit at a slower rate (Blum 2011a). Some of the solutes used in osmotic adjustment not only maintain the flow of water but also have various protective functions against the harmful effects of water deficit.

The increase in concentration of soluble sugars in both leaves and roots is strongly correlated with the attainment of tolerance to drought stress in plants (Hoekstra *et al.* 2001). Sugars such as sucrose, glucose and fructose increase during drought stress. The increase in sugars can be the result of the degradation of starch and also from *de novo* synthesis while the plant is still photosynthesising (Fisher and Höll 1991, Mohammadkani and Heidari 2008). The roles of soluble sugars in plants under drought stress include osmotic adjustment and as a substrate for the production of other organic protective molecules (Mohammadkani and Heidari 2008). Soluble sugars are also involved in energy production and in the stabilization of membranes (Hoekstra *et al.* 2001). Further, soluble sugars also act as signalling molecules (Koch 1996, Sheen *et al.* 1999, Smeekens 2000) bringing about other tolerance mechanisms to drought stress. Soluble sugars are also compatible solutes which means that they can accumulate to high concentrations within a cell without interfering with metabolism (Mohammadkani and Heidari 2008). Lastly, another mechanism by which soluble sugars may operate during drought stress also is by acting as water replacement molecules where the sugars form H-bonds to the polar groups of macromolecules such as proteins (thereby, maintaining their protein conformation and function), and phospholipids (by maintaining spacing and fluidity of the membrane) (Bryant *et al.* 2001).

Another important solute that accumulates during stress is the amino acid proline. The accumulation of proline is a ubiquitous response in algae, bacteria, animals and plants to water deficit (Raymond and Smirnoff 2002, Delauney and Verma 1993, Samaras *et al.* 1995) and it is widely acknowledged that it has a role in the acclimation of plants to water deficit (Raymond and Smirnoff 2002). Proline is synthesised from glutamate via two consecutive reductions involving pyrroline-5-carboxylate (P5C) synthetase and P5C reductase (Samaras *et al.* 1995, Verbruggen and Hermans 2008). Proline can also be synthesized from ornithine which is transaminated into P5C by ornithine-δ-aminotransferase which is located in the mitochondria (Verbruggen and Hermans 2008).

The accumulation of proline is a result of increased biosynthesis in the cytosol and in plasmids and slower oxidation in the mitochondria (Raymond and Smirnoff 2002, Samaras *et al.* 1995). Biosynthesis is increased and degradation is decreased upon dehydration of plant cells (*i.e.*

during drought stress) while the opposite occurs upon rehydration (Yoshiba *et al.* 1995, Verbruggen *et al.* 1996). The major protective roles of proline during drought stress include action as a compatible solute and the stabilization of macromolecules (Delauney and Verma 1993). Proline stabilizes macromolecules, such as proteins, by functioning as a molecular chaperone ensuring that the proteins do not denature and lose function, including many antioxidant enzymes (Verbruggen and Hermans 2008, Szabados and Savouré 2010). Proline also acts as a solubilizer, reducing the precipitation or aggregation of proteins during drought stress (Verbruggen and Hermans 2008). Other roles of proline during drought stress include the reduction of ROS production by reducing NADPH to $NADP^+$, thereby generating an e^- acceptor (Verbruggen and Hermans 2008). The accumulation of proline can also buffer cytosolic pH and balance cell redox status (Verbruggen and Hermans 2008) and proline can also act as a stress signal bringing about other adaptation and protection mechanisms (Verbruggen and Hermans 2008). After a period of stress, proline is a store of C and N that can be used for growth and repair (Raymond and Smirnoff 2002, Verbruggen and Hermans 2008). Overexpression of P5C synthase in tobacco led to the over accumulation of proline and enhanced growth under high levels of salt stress (Kishor *et al.* 1995). As mentioned, proline accumulation also occurs in the roots, with the highest concentration occurring at the root apex (containing meristem cells) where it lowers the water potential and allows root growth (Raymond and Smirnoff 2002).

While plants have many sophisticated mechanisms of coping with drought stress, these may not always be effective, depending on the intensity and duration of the stress. Furthermore, if a plant cannot cope with the severity of the stress, major reductions in productivity and crop failure could occur. And even after a drought stress event, a surviving plant's growth and reproduction will be effected. However, the responses of a plant to a particular stress may be enhanced by the interaction of the plant with free-living PGPR, as discussed below.

2.2 Plant growth-promoting rhizobacteria (PGPR) confer benefits to plants

PGPR colonize the rhizosphere of many plant species and have been shown to confer many benefits to plants. For instance, PGPR can reduce the plant's susceptibility to disease from pathogenic fungi, bacteria, viruses and nematodes (Kloepper *et al.* 2004) through the biocontrol of these pathogens and/or by the induced physical and chemical changes within the plant, *i.e.* 'induced systemic resistance' (ISR) (Annapurna *et al.* 2013). The PGPR are also able to induce

physical and chemical changes as well as alter gene expression that results in greater tolerance to abiotic stress, including heavy metal, cold, heat, salt and drought stress (Yang *et al.* 2009, Milošević *et al.* 2012). This has been termed by Yang *et al.* (2009) as 'induced systemic tolerance' (IST). Furthermore, PGPR enhance the uptake and availability of important nutrients such as P and Fe, can produce phytohormones related to growth promotion (e.g. indole-3-acetic acid – IAA) and can decrease the amount of growth inhibiting phytohormones like ABA and ethylene.

2.2.1 PGPR form biofilms on roots

Bacteria such as PGPR form a biofilm (an extracellular matrix) that provide a buffer against abiotic stress, for both the plant and the bacteria, and serves to permanently attach the bacteria to the surface of the plant roots (Bashan and Holguin 1997, Timmusk *et al.* 2005, Timmusk *et al.* 2011). Under drought conditions, Timmusk *et al.* (2011) found that wild barley root tips had a greater colonisation of *Bacillus* species and the bacteria formed a more cohesive biofilm than the wild barley under unstressed conditions, indicating that the biofilm is a protective mechanism induced by stress. The biofilm consists of a wide range of macromolecules, many of which have not yet been identified (Timmusk *et al.* 2013, Yang *et al.* 2009). In *B. subtilus*, the main components of the biofilm are sugars and proteins (Timmusk *et al.* 2013). The sugars, such as monosaccharides, oligosaccharides and polysaccharides, have water retention properties, for example, the exopolysaccharides (EPS) can retain 70 g of water for every gram of EPS (Vu *et al.* 2009, Chenu 1993, Zhang *et al.* 1998, Sutherland 2001). The biofilm also creates a microenvironment around the roots, where the biofilm forms a porous aggregation of soil particles, of both micro- and macroaggregates (Milošević *et al.* 2012, Timmusk *et al.* 2013). Microaggregates (< 250 µm) contain higher concentrations of bioavailable nutrients, however, anaerobic conditions and high water retention may occur if the proportion of microagreggates is too high (Milošević *et al.* 2012). Macroaggregates (> 250 µm) maintain the right balance between aerobic and anaerobic conditions and ensure the flow of water and nutrients to the plant (Milošević *et al.* 2012). Biofilms can therefore, provide a buffer from abiotic stresses, such as drought, by retaining water which then facilitates nutrient transfer (Timmusk *et al.* 2011, Timmusk and Nevo 2011, Timmusk *et al.* 2013). The biofilm can also contain phytohormones (ABA, gibberellic acid, cytokinins, and auxins) and enzymes, for N fixation, solubilisation of P and other nutrients, and for the production of siderophores (Yang *et al.* 2009, Dimkpa *et al.* 2009a, Conrath *et al.* 2006, Kim *et al.* 2012). It is through the biofilm

that the plant and bacteria exchange all signals and chemicals responsible for the improvement of drought stress tolerance (Timmusk *et al.* 2013).

2.2.2 Microbial P solubilisation

P is a macronutrient that is essential for plant growth and it is in the concentration range of 400 – 1200 $mg.kg^{-1}$ of soil (Rodríguez and Fraga 1999). However, soluble P in soil is in a very low concentration, 1 ppm or lower (Goldstein 1994). The largest reservoir of P is mineral P, such as apatite, hydroxyapatite and oxyapatite, however these forms are insoluble (Rodríguez and Fraga 1999). Mineral P can also be associated with hydrated oxides of aluminium (Al), manganese (Mn) and Fe, but these too are poorly soluble and assimilable (Rodríguez and Fraga 1999). Agricultural soils tend to have a high concentration of P due to the application of fertilizers (Richardson *et al.* 1994). However, the P becomes insoluble soon after application and is therefore unavailable to plants (Dey 1988). Organic P is the second major reserve of soil P and can account for 30 – 50% of total P in soils (Paul and Clark 1988). The majority of organic P is in the form of inositol phosphate (Dalal 1977, Anderson 1980, Harley and Smith 1985), which is a high molecular weight compound and must therefore, be converted into soluble ionic phosphate (Pi, HPO_4^{2-}, $H_2PO_4^-$) or low molecular weight organic phosphate before assimilation can occur (Goldstein 1994, Beever and Burns 1981). Microorganisms play a critical role in the P cycle and in making it available for absorption by plants (Rodríguez and Fraga 1999, Gyaneshwar *et al.* 2002).

Mineral P is primarily solubilized by the organic acids produced by soil microbes, in particular, bacteria (Banik and Dey 1982, Craven and Hayasaka 1982, Duff and Webley 1959, Halder *et al.* 1990, Leyval and Berthelin 1989, Salih *et al.* 1989, Sundara Rao and Sinha 1963). Proton substitution for Ca^{2+} results in the release of Pi from mineral phosphate (Goldstein 1994). Periplasmic oxidation of glucose to gluconic acid accounts for the principal organic acid used in phosphate solubilising *Pseudomonas* sp. (Goldstein 1994, Goldstein 1995, Illmer and Schinner 1992). Other organic acids include glycolic, oxalic, malonic and succinic acid (Rodríguez and Fraga 1999). Organic phosphate is hydrolysed to inorganic P, a process called mineralization, and is facilitated by phosphatase enzymes (Rodríguez and Fraga 1999). Some of these phosphatases work outside of the cell, either secreted in a soluble form or retained as membrane-bound proteins and are therefore scavenging enzymes (Rodríguez and Fraga 1999).

The phosphate solubilizing ability of some bacteria can enhance the nutrient status of the plants and reduce the adverse environmental effects of over fertilization. In one study, the yield of wheat supplied with only three quarters of the recommended amount of fertilizer and inoculated with a PGPR strain was comparable with the yield of uninoculated wheat supplied with 100% fertilizer (Shaharoona *et al.* 2008). Another study on tomato showed similar results, with the dry weight of greenhouse plants supplied with 75% fertilizer and two strains of PGPR was significantly heavier than that of the plants supplied with normal levels of fertilizer and no PGPR (Yang *et al.* 2009). Once transplanted into the field, the plants with a combination of PGPR and mycorrhizal fungi and only 50% fertilizer had a greater yield than the plants with 100% fertilizer and no microbes (Yang *et al.* 2009).

The drought resistance or yield of crops that were exposed to fluctuating levels of drought stress has been reported to increase with an increase in fertilization or improved plant nutrition (Arnon 1975, Begg and Turner 1976, Lahiri 1980, Viets 1972). Onion plants that were infected with vesicular-arbuscular mycorrhizal (VAM) fungi and exposed to drought stress had a higher concentration of P than the uninfected plants that were supplied with a P fertilizer and exposed to drought stress (Nelsen and Safir 1982). Visual inspection of the onions plants showed the infected plants to be dark green with no brown tips while the uninfected plants were pale green and had tip dieback (Nelsen and Safir 1982). Two factors limited the growth and survival of the uninfected and stressed plants – water and P nutrition, while for the infected plants water was the only factor therefore, these plants had improved growth and drought tolerance (Nelsen and Safir 1982). There is a reduced uptake of P when plants are stressed (Dunham and Nye 1976, Greenway *et al.* 1969) and there is a lower diffusion rate of P in soils with a low water content (Viets 1972). Therefore, drought stressed plants that are inoculated with microbes, such as PGPR, that have the ability to make P available to the plants will have a better P nutrition and a better chance of surviving the drought stress episode.

2.2.3 Microbial (bacterial) siderophores help plants take up Fe

Siderophores are low molecular weight Fe chelating compounds (specifically Fe^{3+}) produced by a wide variety of microbes under Fe-limiting conditions (Lankford and Byers 1973, Neilands 1981a). Siderophores are secreted out of the cell and bind to Fe^{3+} with a very high affinity and return through the cell membrane via specific receptors (Hilder 1984, Neilands

1981b, Neilands 1982). Fe is abundant in the soil, usually in the form of crystalline and amorphous Fe oxides and other Fe minerals (Alexander and Zuberer 1991). The dissolution of the Fe minerals releases two soluble forms of Fe, Fe^{2+} and Fe^{3+}, which can be assimilated by plants and microorganisms (Alexander and Zuberer 1991). However, in the presence of oxygen and a neutral soil pH, Fe^{2+} is rapidly oxidized to Fe^{3+} which then forms insoluble $Fe(OH)_3$ (Crosa 1989, Lindsay and Schwab 1982, Neilands 1981a, Neilands 1981b, Neilands 1995). Under these conditions, Fe becomes limiting which can lead to Fe deficiency, and since Fe is a macronutrient for microbes (Stintzi *et al.* 2000) siderophores are produced and secreted.

The majority of siderophores fall into three classes, the hydoxamates, the phenolates-catecholates, or a mixed class (Neilands 1981a, Saha *et al.* 2013). Siderophores secreted by PGPR can promote plant growth directly by increasing Fe nutrition (Crowley *et al.* 1991) or indirectly by limiting the growth of Fe dependant soil pathogens, such as *Fusarium oxysporum* and *Pythium ultimum* (Kloepper *et al.* 1980, Weller 2007, Sahu and Sindhu 2011). Siderophores also help in phytoremediation where they bind the heavy metals making them available to the plant and improving the phytoextraction capabilities of the plant (Glick 2003, Rajkumar *et al.* 2010). The Fe provided by siderophores also counteracts the harmful effects of heavy metals on plant growth (Dimkpa *et al.* 2009b).

Fe is a micronutrient for plants and is essential for photosynthesis, respiration, N fixation, DNA synthesis and hormone synthesis (Briat and Lobreaux 1997, Becana *et al.* 1998). Fe has a dual role in the production and quenching of ROS, Fe is involved in the Fenton reactions which generates the hydroxyl radical (OH·) and is also a component of the antioxidant enzymes ascorbate peroxidase (POD), CAT, quaiacol POD and ferro-SOD (Becana *et al.* 1998). Fe, therefore, plays an important role in ROS signalling, oxidative damage and quenching of ROS, all of which are important responses to drought stress.

2.2.4 Microbial (bacterial) IAA production and its effect on plant stress mechanisms

IAA is the most common phytohormone of the auxin class of hormones. IAA is produced by plants to regulate growth, however it is also produced by some rhizosphere bacteria. IAA synthesized by PGPR via the indolepyruvic pathway contributes to the plant's endogenous auxins and promotes plant growth, while IAA produced via the indoleacetamide pathway (pathogenic bacteria) causes plant tumours (Morris 1986, Patten and Glick 2002). PGPR produced IAA can stimulate root growth directly by plant cell elongation or cell division

(Campanoni and Nick 2005) or it can act indirectly by stimulating bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Li *et al.* 2000). Exogenous IAA stimulates ACC synthase in plants (Peck and Kende 1995) and the resulting increase in ACC stimulates bacterial ACC deaminase (Patten and Glick 2002) which then lowers the amount of root growth-inhibiting ethylene in the plant (Penrose and Glick 2003). Ethylene causes an increase in IAA synthesis in the root elongation zone which inhibits cell elongation (Růžička *et al.* 2007). The root growth promotion by bacterial IAA will result in a larger root surface area and greater number of root tips (Mantelin and Touraine 2004).

Bacterial IAA enhances the establishment of seedling roots increasing their anchorage in the soil and their ability to acquire water and nutrients (Patten and Glick 2002). Plants with a well-developed root system also have the greatest ability to take up water and therefore, have a greater tolerance of drought than plants with a poorly developed root system (Hurd 1974, Marulanda *et al.* 2009). Marulanda *et al.* (2009) showed that bacterial IAA improved the shoot and root biomass as well as the plant's water content in a drought stressed environment.

2.2.5 Other important PGPR mechanisms effect on plant stress mechanisms

The establishment of ISR and IST involves the mutual recognition of signals and the alignment of both plant and microbe responses (de Zelicourt *et al.* 2013). The symbiotic relationship between rhizobia or mycorrhiza and the plant share a mutual plant-signalling pathway which is triggered by microbial factors (Corradi and Bonfante 2012) and this pathway appears to be also triggered by other bacteria such as PGPR or by pathogenic microbes (de Zelicourt *et al.* 2013). Timmusk and Wagner (1999) have shown that *Paenibacillus polymyxa* altered the gene expression of *Arabidopsis thaliana* and thereby improved the drought stress tolerance of *A. thaliana*. The bacteria brought about the increased expression of the drought responsive gene Early Responsive to Dehydration 15 (ERD15). However, Kariola *et al.* (2006) found that a decreased expression of ERD15 increased the plant's tolerance to drought and freezing stress. ERD15 is involved with the sensitivity of plants to the multi-faceted ABA (Kariola *et al.* 2006). In a study of salt stressed *A. thaliana* inoculated with *B. subtilis*, the volatile organic compounds (VOC) released by the bacteria decreased the number of transcripts for histidine kinase transporter 1 (HKT1) in the plant's roots and therefore, decreased the uptake of Na^+ (Zhang *et al.* 2008). There are various PGPR that can confer ISR to plants. PGPR such as *P. putida*, *P. fluorescens*, *B. pumilus*, *Paenibacillus alvei* and *Chryseobacterium balustinum* are

known to enhance the plant's 'immune system' against pathogenic microbes and this has a negligible consequence for the growth and yield of the plants (Van Hulten *et al.* 2006).

There are some PGPR that can produce antioxidants which aid the plant in maintaining the homeostasis of ROS and therefore, counter the destruction that would occur with a build-up in ROS under stress conditions such as drought (described earlier). The enzyme CAT is one such antioxidant produced by PGPR. CAT catalyses the decomposition of H_2O_2 to oxygen and water thereby decreasing the amount of damage incurred during stress. Ordookani *et al.* (2010) found that the inoculation of tomato with PGPR enhanced the antioxidant status of the plant and therefore, its ability to tolerate abiotic stress.

PGPR can also increase water relations and osmotic adjustment in the plants. *Zea mays* co-inoculated with *Rhizobium* and *Pseudomonas* showed improved tolerance to salt stress; these plants had reduced electrolyte leakage and had higher leaf water contents compared with uninoculated plants (Bano and Fatima 2009, Creus *et al.* 2004, Karlidag *et al.* 2010).

2.3 A framework for our understanding of how PGPR moderate plant drought stress

While the overall effects of drought stress on plant growth are known (Lisar *et al.* 2012), much still remains unknown regarding the initial effect of the lack of water at the biochemical and molecular levels. A deeper understanding at that level is crucial for the complete insight into plant resistance mechanisms towards drought conditions (Lisar *et al.* 2012), which will ultimately enable accurate quantitative predictions about the plant's response to drought. Even though evidence shows that PGPRs can positively influence the growth and development of plants as well as the plant's response to both abiotic and biotic stress, the extent to which the plant-microbe interaction is responsible for the mitigation of abiotic stress is only beginning to be understood. There are strain specific interactions within a species which suggests that there are very precise and intricate association mechanisms that need to be identified. There are also a large number of metabolites involved, some of which have not yet been identified and therefore, their importance in the mitigation of stress is not clear (de Zelicourt *et al.* 2013). Therefore, a systems biology approach is needed to give a better understanding of the plant-microbe interactions under drought stress. This information will greatly benefit the design of self-regulating plant-microbe systems that can aid both large- and small-scale farmers by lowering the amount of fertilizer needed and lowering the amount of yield lost to drought and thus, help alleviate food insecurity in the face of global climate change.

2.4 Aims and Objectives

The first aim of this project was to compare the PGPR traits of two *Pseudomonads*, namely *P. fluorescens* and *P. koreensis*, in order to identify the better PGPR. The other aim was to use a systems approach to gain a deeper understanding of the physiology and biochemistry behind the drought stress response of *H. annuus* and to see how this response was altered by inoculation with the PGPR *P. koreensis*.

The objectives were to:

1. compare PGPR traits between *P. fluorescens* and *P. koreensis*, namely phosphatase activity, siderophore production, IAA production, and growth under osmotic stress;
2. identify the germination characteristics of *H. annuus* and to see how inoculation with *P. koreensis* changed these characteristics;
3. determine the drought stress response of *H. annuus*, in terms of various physiological and biochemical parameters, and to see how inoculation with *P. koreensis* affected this response. Certain physiological parameters were also compared with unstressed plants.

3 MATERIALS AND METHODS

3.1 Study species

H. annuus was chosen as the plant species of interest for this study. *H. annuus* was chosen because it is a rain-fed crop with C3 photosynthesis, therefore, drought is a larger threat to sunflower than, for example, vegetable crops which are irrigation-fed crops. *H. annuus*, part of the *Asteraceae* family and native to the Americas, is cultivated mainly for its oil but is also used as bird food or livestock feed. The plant also has some industrial applications in the production of biodiesel and phytoremediation. The sunflower seeds used in this study (AGSUN 8251) were obtained from Agricol, South Africa, and were pre-treated with a fungicide by the supplier (Figure 2.).

The PGPRs used in this study included *P. fluorescens* and *P. koreensis* (Figure 2.). From preliminary results, it was shown that *P. koreensis* outperformed *P. fluorescens*, a commercially available PGPR, in terms of phosphatase activity, IAA and siderophore production. Therefore, *P. koreensis* was used to inoculate the *H. annuus* plants in the experiment.

Due to unforeseen circumstances that resulted in the loss of all images recorded during the project, there are a limited number of photographs in the dissertation.

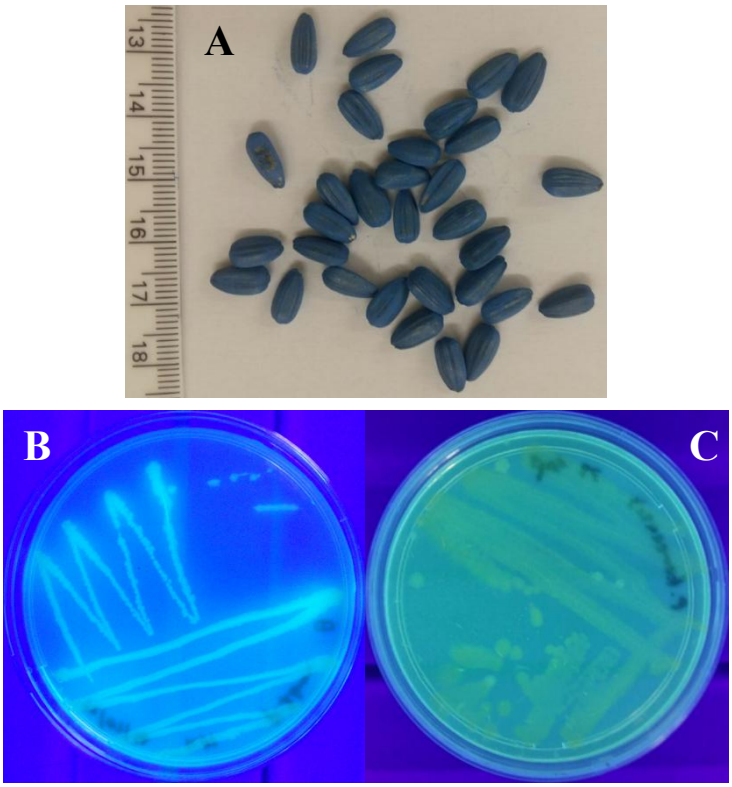


Figure 2. Study species used in the project. (A) *H. annuus* (AGSUN 8251) seeds; (B) *P. fluorescens* on King's Agar B under UV light; (C) *P. koreensis* on King's Agar B under UV light.

3.2 Bacterial studies

3.2.1 Bacterial isolation

P. fluorescens was isolated from a commercial source (Rizofos, MBFi, South Africa) containing a variety of *P. fluorescens* strains. Serial dilutions of the 1.0 x10^9 formulation were made with sterile Milli Q deionised H_2O (dH_2O) and then spread onto King's Agar B (Sigma, Germany) plates. The plates were incubated for 48 hours at 25 °C and then viewed under UV light to identify the colonies with the highest fluorescence. A single highly fluorescent colony was chosen and subcultured twice and then stored on Petri plates at 4 °C until needed. For long-term storage, glycerol stocks were made and stored at -80 °C.

P. koreensis was isolated from the rhizosphere of a mixed crop cultivation in the Free State, South Africa. The soil from the rhizosphere was gently teased from the plant's roots into sterile

50 mL conical tubes. Soil suspensions, 10% (w/v), were prepared in sterile dH_2O with sterile coarse silica granules and the tubes were vortexed. Dilutions of this suspension were made and then spread onto King's Agar B plates and incubated for 48 hours at 25 °C. Thereafter, the plates were screened for fluorescent colonies and those with substantial fluorescence were subcultured onto new plates and incubated as above for 24 hours. This step was repeated and then the plates were stored on Petri dishes at 4 °C until required. An isolate that was screened for PGPR ability and had shown promising results was identified via 16s rDNA sequencing as *P. koreensis*. For long-term storage, glycerol stocks were made and stored at -80 °C.

For 16s rDNA identification, 20 µL polymerase chain reactions (PCR) of the approximately 1.5 kb *16srRNA* gene was conducted using 0.4 mM 27F forward primer (5' AGA GTT TGA TCM TGG CTC AG 3') and 0.4 mM 1492R reverse primer (5' CGG TTA CCT TGT TAC GAC TT 3'), 0.02 U/µL Phusion high-fidelity DNA polymerase (Thermo Scientific), 3% (v/v) DMSO (Thermo Scientific), 200 µM dNTP mix (Fermentas) and 5x Phusion-HF buffer (Thermo Scientific), template at 1-5 ng/uL with nuclease-free water (Fermentas) to fill. Conditions entailed 1 cycle initial denaturation for 10 min at 98 °C; 35 cycles of denaturation for at 10 sec at 98 °C, annealing for 30 sec at 60 °C, elongation for 90 sec at 72 °C and a final extension of 10 min at 72 °C. A template-free reaction was included as negative control. Two micro litre aliquots of PCR products were checked using ethidium bromide agarose gel electrophoresis, using a 1.2 % (w/v) gel run at 80 V in TAE buffer. A Bio-rad gel Doc™ was used to visualise bands. A GeneRuler™ 1 kb plus DNA ladder (Thermo Scientific) was used for molecular size reference. Amplicon purification was not required for sequencing. Sanger Sequencing (5'-3' strand) was outsourced to Inqaba biotec ™. Sequence editing was achieved using Bioedit and subsequent identification was by using the EZ-taxon-e 16s rDNA database.

3.2.2 Culture conditions

The bacteria stored at 4 °C was first spread onto fresh King's Agar B plates and incubated at 25 °C for 48 hours. Thereafter, a single colony was transferred into 1 mL of Dworkin and Foster (DF) medium (Dworkin and Foster 1958) or modified M9 (MM9) medium (Alexander and Zuberer 1991) depending on which assay was being performed. For each assay, there were 12 samples of each bacteria and each assay was repeated three times. The bacteria were cultured in 1.5 mL microcentrifuge tubes stoppered with sterile cotton wool to allow gas permeation.

The DF medium (Appendix A) comprised of the following: KH_2PO_4 (4 g/L), Na_2HPO4 (6 g/L), $MgSO_4.7H_2O$ (0.2 g/L), glucose (2 g/L), gluconic acid (2 g/L), citric acid (2 g/L), $(NH_4)_2SO_4$ (2 g/L), and the trace salts $FeSO_4.7H_2O$ (1 mg/L), H_3BO_3 (10 µg/L), $MnSO_4.H_2O$ (11.19 µg/L), $ZnSO_4.7H_2O$ (124.6 µg/L), $CuSO_4.5H_2O$ (78.22 µg/L), and MoO_3 (10 µG/L). The media was adjusted to pH 7.2 with 10M NaOH before autoclaving at 121 °C and 100 kPa for 20 minutes.

The MM9 medium (Appendix B), which is modified to contain no Fe, composed of: KH_2PO_4 (0.3 g/L), NaCl (0.5 g/L), NH_4Cl (1.0 g/L), HEPES (23.83 g/L), glucose (2 g/L), mannitol (2 g/L), casamino acids (10% w/v) and the trace salts $MgSO_4.7H_2O$ (0.493 g/L), $CaCl_2.2H_2O$ (14.57 mg/L), $MnSO_4.H_2O$ (1.17 mg/L), H_3BO_3 (1.4 mg/L), $CuSO_4.5H_2O$ (40 µg/L), $ZnSO_4.7H_2O$ (1.2 mg/L), and $NaMoO_4.2H_2O$ (1.0 mg/L). The pH of this medium was adjusted to pH 6.8 with 50% KOH before autoclaving.

The MM9 medium was used in the siderophore assay, which measures the amount of Fe scavenging compounds, therefore, all glassware was acid-washed before use, to ensure that no metals (especially Fe) leached from the glassware into the medium during storage. This process involved filling the glassware with 25 % nitric acid for 24 hours, after which, the solution was removed, the glassware rinsed three times with dH_2O and allowed to dry. Once dry, the glassware was used in the preparation and storage of the MM9 medium.

3.2.3 Phosphatase activity

The method of Gügi *et al.* (1991) was used to determine phosphatase activity. A single colony from the freshly spread King's Agar B plates was transferred into 1 mL of DF medium in a 1.5 mL gas permeable microcentrifuge tube. The tubes were placed on an orbital shaker SPO 15-MP (Labcon, USA) at 200 rpm and incubated at 25 °C for 48 hours. The tubes were then centrifuged on a MiniSpin (Eppendorf, Germany) at 12000 rpm for 5 minutes and the supernatant was discarded. The pellet was resuspended in sterile 0.9% NaCl (0.8 mL) to an optical density (OD) 600 of 0.3-0.8. From this suspension, 50 µL was transferred to a new 1.5 mL microcentrifuge tube containing 500 µL 50 mM Tris-maleate buffer (pH 6.3 for acid phosphatase activity or pH 8.8 for alkaline phosphatase activity) and 50 µL of freshly prepared 0.12 M *para*-Nitrophenyl phosphate, pNPP (Sigma, Germany). The tubes were sealed, vortexed and then incubated at 30 °C in a water bath for 2 hours (Appendix C). Afterwards, the tubes were centrifuged as above. For each tube, in triplicate, 100 µL of the supernatant was transferred to a 96-well microtiter plate containing 100 µL of NaOH and mixed by pipetting

100 µL up and down three times. The absorbance of the resulting mixture was measured at 405 nm (Radio *et al.* 2006) on a Multiskan Go (Thermo Scientific, USA). For the blank, 50 µL of 0.9% NaCl was substituted for the supernatant followed by the same procedure described above, and this was used to zero the absorbance readings. The entire assay was repeated three times.

A standard curve of *para*-Nitrophenyl, *p*NP (Sigma, Germany), was obtained by preparing standards in the concentration range of 1 -10 mM, in 1 mM increments. For each standard solution, in triplicate, 100 µL was added to 100 µL of NaOH, the solution was mixed and the absorbance was measured at 405 nm. This was repeated three times. The absorbance readings from the bacterial cultures were related to the equation resulting from the standard curve and the results were represented as µM pNP/min/OD600.

3.2.4 Siderophore production

The method of Alexander and Zuberer (1991) was used to determine siderophore production. This assay required the use of a chrome azurol S (CAS) (Sigma, Germany) dye solution. This was prepared as follows: 21.9 mg of hexadecyltrimethylammonium bromide (HDTMA, Sigma, Germany) was dissolved in 25 mL dH_2O under low heat and constant stirring. In a separate vessel, 1.5 mL of 1 mM $FeCl_3.6H_2O$ (in 10 mM HCl) was vortexed with 7.5 mL of 2 mM CAS and added to the HDTMA. A third solution, namely, 9.26 g of 2-(*N*- morpholino)ethanesulfonic acid, MES (Sigma, Germany), dissolved in 50 mL of dH_2O and the pH adjusted to 5.6 with 50% (w/v) KOH, was mixed with the CAS/HDTMA solution and the volume made up to 100 mL. The resulting CAS solution was stored at 4 °C in a sealed sterile plastic 250 mL flask, and was stable for a few months (Alexander and Zuberer 1991).

A single colony from the freshly spread King's Agar B plates was transferred into 1 mL of MM9 medium (modified to contain no Fe) in a 1.5 mL gas permeable microcentrifuge tube. The tubes were placed on an orbital shaker at 200 rpm and incubated at 25 °C for 48 hours. The tubes were removed from the incubator, vortexed briefly and the OD540 was measured. The tubes were then centrifuged at 12000 rpm for 5 minutes. For each tube, in triplicate, 100 µL of CAS solution was added to 100 µL of supernatant in a 96-well microtiter plate. A 100 µL volume was pipetted up and down three times in order to ensure proper mixing. The 96-well microtiter plates were sealed and incubated at room temperature for 3.5 hours. Afterwards, the absorbance was measured at 630 nm. The absorbance readings were blanked with a solution

of 1.5 mM deferoxamine mesylate (DFOM, Sigma, Germany) and CAS solution (1:1), incubated as above. A reference solution containing 100 µL sterile MM9 and 100 µL CAS solution was prepared and incubated as above. The entire assay was repeated three times. The absorbance readings of the culture supernatant was divided by the absorbance at 630 nm of the reference solution (A_{ref}) and then related to the standard curve. The results were represented as µM DFOM/OD540.

A standard curve of A_{630}/A_{ref} was prepared using freshly prepared 20 – 120 µM DFOM (in 10 µM increments) dissolved in MM9 in place of the culture supernatant and incubated as above. This was repeated three times.

3.2.5 IAA production

The method of Patten and Glick (2002) was used to determine IAA production. A single colony from the freshly spread King's Agar B plates was transferred into 1 mL of DF medium in a 1.5 mL gas permeable microcentrifuge tube. The tubes were placed on an orbital shaker at 200 rpm and incubated at 25 °C for 24 hours. From this culture, 5 µL was transferred into 1.5 mL gas permeable microcentrifuge tubes containing 1 mL of DF medium supplemented with 500 µg/mL DL-tryptophan (Sigma, Germany). The tubes were then incubated as above for 48 hours. The OD600 was measured after the incubation and the tubes were centrifuged at 12 000 rpm for 5 minutes. Afterwards, in triplicate, 50 µL of the supernatant was transferred to a 96-well microtiter plate. To the supernatant, 200 µL of Salkowski's reagent (150 mL H_2SO_4, 250 mL dH_2O and 7.5 mL of 0.5 M $FeCl_3.6H_2O$) was added. This was mixed by pipetting 175 µL of the mixture up and down three times. The 96-well plate was sealed and incubated at room temperature for 20 minutes. Afterwards, the absorbance at 535 nm was measured. Zero absorbance was measured with sterile DF medium containing 500 µg/mL DL-tryptophan in place of the culture supernatant and incubated as above. The entire assay was repeated three times.

A standard curve of IAA (Sigma, Germany) was prepared in the range of 80 – 300 µg/mL, in 20 µg/mL increments, by dissolving IAA (2 mg/mL stock solution) in sterile DF medium and diluting to the desired concentrations. The standard solutions were used in place of the culture supernatants as above and the absorbance at 535 nm was measured. The absorbance from the bacterial cultures was related to the standard curve and IAA production was represented as µg/mL IAA/OD600.

3.2.6 Growth at -0.73 MPa

The method of Sandya *et al.* (2009) was used to determine growth at -0.73 MPa. A single colony from the freshly spread King's Agar B plates was transferred into 100 mL of Tryptic Soy Broth (TSB) and incubated overnight at 25 °C at 200 rpm. The following day, in triplicate, 1 mL of the overnight culture was transferred into 100 mL of fresh TSB (control) and 100 mL of TSB containing 25% polyethylene glycol 6000 (PEG6000), for a water potential of -0.73 MPa (Michel and Kaufmann 1973), and incubated at 25 °C for six days. On each day, the OD600 was measured using sterile media as a blank.

3.3 Plant studies

3.3.1 Sterilisation, Inoculation and Germination

The seeds had been previously treated (by the supplier) with a fungicide which had to be removed prior to further treatment. This involved placing 60 seeds in a 50 mL conical tube with 40 mL dH_2O on a rotary shaker at 200 rpm for 20 minutes. The seeds were then shaken in 70% ethanol for 1.5 minutes, after which they were shaken in 40 mL of 3.5% sodium hypochlorite (NaClO), with a drop of Tween 20, for 30 minutes. The seeds were then rinsed six times with dH_2O (+/- 40 mL). In the case of the treatment, seeds were placed into 40 mL of inoculant (single *P. koreensis* colony in 100 mL of nutrient broth (NB) incubated at 25 °C and 200 rpm for two days) and shaken for 10 minutes. This step was omitted for the control. The seeds were then placed in a Petri dish containing three sheets of sterile filter paper (two below the seeds and one on top of them). There were 15 seeds per Petri dish and each was given 6 mL of sterile dH_2O. The Petri dishes were placed in a growth room at 25 °C with a 14 hours light/10 hours dark photoperiod. Germination was checked daily for four days, with germination only positively recorded if the radicle was longer than 2 mm (Tobe *et al.* 2000). The seeds were supplied with fresh sterile dH_2O on the second day. The germination data was used to calculate the following values:

- Germination percentage (%), and
- The mean germination time (MGT = $\sum nT / \sum n$); where n = number of newly germinated seeds at time T, T = days from the beginning of the test, and $\sum$n = final germination (Mavi *et al.* 2010).

The seeds that had germinated after four days were used in the main drought stress experiment.

3.3.2 Experimental set-up

There were two main experiments in this study: unstressed plants and drought stressed plants (Figure 3.). In each experiment there was a control (uninoculated seedlings) and a treatment (inoculated seedlings). In the drought stressed experiments, there were 10 seedlings each in the control and treatment and the experiment was repeated 3 times (*i.e.* 30 seedlings in total). All 3 replicates ran concurrently and 10 seedlings were randomly selected for each replicate. There were an extra 10 seedlings for the recovery and regrowth study (detailed below), there were no repeats for this study. In the unstressed experiment, there were 15 seedlings in each and the experiment was not repeated. For a single seedling in the drought stressed experiment, the following parameters were measured: plant height, number of nodes, leaf relative water content (RWC), substrate water content, leaf electrolyte leakage, chlorophyll fluorescence, proline content, and total phenolic acid content. In contrast, the following was measured in each seedling in the unstressed experiments: leaf area, leaf RWC, leaf electrolyte leakage, and chlorophyll fluorescence.

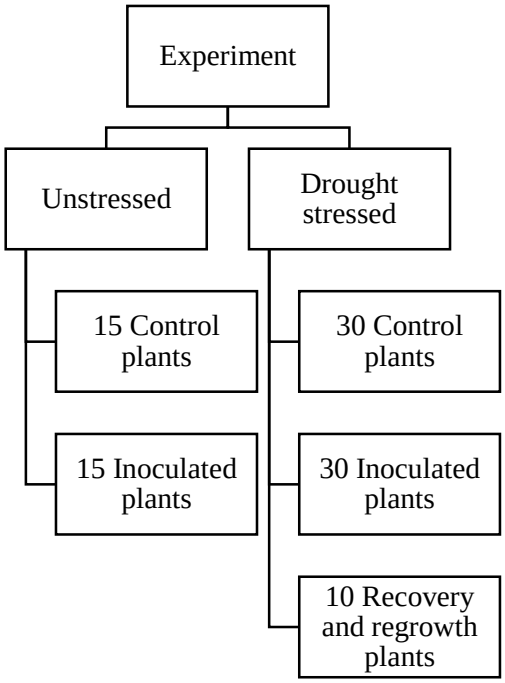


Figure 3. Flow chart for experiment set-up.

3.3.3 Plant growing conditions

After 4 days from the start of germination, the seeds that had germinated were transferred to plastic pots (15 cm) with drip trays (18 cm) underneath. Each pot had a sterile filter paper disk (90 mm) to cover the drainage holes and were filled with +/- 200 g (Precisa 3100C,

Switzerland) of autoclaved medium-grade vermiculite (Mandoval Vermiculite, South Africa). The pots, water trays, filter paper and vermiculite were autoclaved at 121 °C for 20 minutes. Germinated seeds were placed in the centre of the pot with the top of the seeds flush with the level of the sterile vermiculite. The vermiculite was then patted down around the seed with sterile forceps. Once the seeds were planted in pots, the time was recorded as day 0.

Once potted, samples were watered using an automated drip irrigation system (Orbit 94162, USA). To minimise contamination, sterile filters (0.2 µm) were installed onto the ends of the drip irrigation pipes. The irrigation was initially run for 15 minutes (at a rate of 60 mL/minute) to ensure the substrate was sufficiently wet. The watering cycle was then set to water for 1 minute daily at 06:00 am (as the lights turned on) and increased to 2 minutes after 2 weeks and 3 minutes at 4 weeks (the latter was applicable to the unstressed seedlings only). The seedlings were also supplied with 20 ml of 100% Murashige and Skoog (MS) medium (Highveld Chemicals, South Africa) at a pH of 5.8 (Murashige and Skoog 1962) twice a week, commencing on the first day they were transferred to the pots. The combination of vermiculite and MS medium was chosen because vermiculite is inert and the use of MS medium will have ensured constant concentrations between treatments, unlike if soil was to be used as a substrate, in which case there would be variation in the amount of nutrients, which could in turn, influence the interaction between the seedlings and bacteria between replicates. The treated seedlings (*i.e.* the inoculated seedlings), were supplied with 10 ml of inoculant (a single colony of *P. koreensis* in 450 ml of NB, grown for 2 days at 25 °C and 200 rpm) on the day of planting and then again at 2 weeks. The seedlings were subsequently transferred and maintained in a growth room at 25 °C with a 14 hours light/10 hours dark photoperiod.

In the case of the drought stressed seedlings, the seedlings were treated in a similar manner until four weeks, after which the drought stress regime described below was imposed. In the case of the unstressed seedlings, the seedlings grew for 4 weeks plus 10 days, under well-watered conditions. For the drought stressed experiments, during the initial four weeks of growth under well-watered conditions, plant height and number of nodes was measured once a week. The plant height was measured from the surface of the vermiculite to the tops of the seedlings with a mini tape measure that was bent to the shape of the stem in order to obtain the most accurate results.

3.3.4 Drought stress regime

A preliminary investigation was conducted in order to determine an appropriate drought stress regime. To achieve this, 15 uninoculated and 15 inoculated seedlings were planted and grown for 4 weeks (as described earlier), after which the daily irrigation to the seedlings was stopped and the water available to the seedlings slowly decreased. Following cessation of watering, chlorophyll fluorescence (Opti-Sciences OS1p, USA; Appendix D) measurements were recorded twice a day, in the morning (10:00) and afternoon (15:00), every day. For consistency, only the two leaves from the first fully expanded set of leaves were measured. Daily chlorophyll fluorescence measurements continued until a dip in the Fv/Fm values was detected, after which, water was resupplied to the seedlings. Chlorophyll fluorescence measurements were continued the day after watering was resumed in order to determine whether the Fv/Fm values had reverted to "normal" values (Appendix E). In addition to chlorophyll fluorescence, visual observations were also recorded on a daily basis to determine the time till the seedling reached a severe state of drought. However, this measurement did not give an accurate enough description of the drought stress status of the seedling, *i.e.* the drop in Fv/Fm values was noticed when the seedlings were already in a severe state of drought stress and did not recover. Therefore, it was decided that the number of days to withhold water would be based on visual observations between the 7-12th day of cessation of watering. Preliminary experiments led to the conclusion that 10 days from the time irrigation was stopped would be the optimal duration for sampling.

Therefore, from the preliminary investigation, it was decided that the following drought stress regime would be implemented: 4 weeks of post-germination growth (as described above) after which the irrigation to the seedlings was stopped. The water available to the seedlings then slowly decreased due to evapotranspiration. Ten days afterwards, samples for the physiological and biochemical analyses were taken.

3.3.5 Sampling

There is variation in the composition of leaves at different regions of the plant (Takabayashi *et al.* 1994), *i.e.* top, middle, bottom. Therefore, samples were consistently taken from the first and second fully expanded leaves of the seedling in order to keep the characteristics measured constant.

3.3.6 Physiological analyses

a. *Leaf relative water content (RWC)* (Zygielbaum *et al.* 2009)

Two leaf disks (0.28 cm^2) were taken from one leaf of the first fully expanded set of leaves on each seedling. The leaf disks were quickly placed into pre-weighed 1.5 mL microcentrifuge tubes and closed. The tubes were then weighed (Precisa 92SM-202A-DR, Switzerland) and the fresh weight (FW) of the leaf disks was calculated by determining the difference between the full and empty tubes. One millilitre of dH_2O was then added to the tubes and these were refrigerated in darkness at 4 °C for 15 hours. Afterwards, the leaf disks were removed from the tubes, patted dry with tissue paper and then weighed again, giving the full turgor weight (TW). The leaf disks were then placed into new tubes and placed open into a drying oven at 105 °C for 24 hours. Silica drying beads were placed into the oven at the same time. Once removed from the oven, the tubes were sealed and then weighed. After being weighed, the leaf disks were discarded and the empty vials were re-weighed. The difference between the full and empty tubes gave the dry weight (DW) of the leaf disks. The RWC was calculated as follows:

RWC (%) = [(FW-DW)/(TW-DW)]*100

where: FW was the fresh weight of leaf disks; TW was the turgor weight after being submerged in dH_2O in darkness and at 4 °C for 15 h; DW was the dry weight of the leaf disks after being in a drying oven at 105 °C for 24 h.

b. *Leaf electrolyte leakage*

The electrolyte leakage of the leaves was determined by quantifying the electrical conductance of leaf cell leachate. Two leaf disks (0.28 cm^2) were washed with deionized dH_2O and then transferred into glass test tubes containing 5 ml of deionized dH_2O. The test tubes were incubated at 25 °C for 24 hours with 14 hours of light and 10 hours of darkness. The conductance was measured on a CM 100-2 conductivity meter (Reid and Associates, USA). On the first day after the overnight incubation, 1 ml of the deionized dH_2O containing leachate was placed into the wells and the conductivity was measured (C_1). The test tubes were then incubated at 100 °C for 1 hour in order to rupture the cell membranes and allow complete ion leakage. The test

tubes were then incubated again as described above. On the following day, the conductivity was measured for the heat-killed samples (C_2). Electrolyte leakage was then expressed as relative conductivity (RC) which was calculated as follows:

$RC = C_1/C_2$

where: C_1 was the conductivity measured after the first incubation; C_2 was the conductivity measured after the second day.

c. *Leaf area*

In the unstressed experiments only, the leaf area of the uninoculated seedlings was compared with the leaf area of the inoculated seedlings. The first set of fully expanded leaves (2 leaves per seedling) were removed from the seedlings and photographs were taken, including a scale. The leaf area was determined with the freeware program imageJ (http://imagej.nih.gov/ij/download.html).

d. *Substrate water content*

In the drought stressed experiments only, the substrate water content at the end of the drought stress regime (*i.e.* 10 days without water) was compared between uninoculated and inoculated seedlings. After the root mass was removed and sampled, the remaining vermiculite in the pot was mixed to homogenize the sample. Thereafter, a +/- 20 g sample was placed into a pre-weighed foil packet (Chyo JP_2-160, Japan). The packets containing the root samples (*i.e.* full packets) were weighed, and the difference between the full and empty packets gave the FW of the vermiculite sample. The full foil packets were then placed into a drying oven at 105 °C for 48 hours with silica beads. Afterwards, the packets were weighed again, giving the DW of the substrate. The weight of the packets was constant to 72 hours in the drying oven, showing that 48 hours was sufficient in drying out the substrate. The substrate water content (SWC) was calculated on a wet mass basis as follows:

SWC = [(FW-DW)/FW] * 100

where: FW was the fresh weight of the substrate; DW was the weight of the substrate after 48 h in a drying oven at 105 °C.

3.3.7 Biochemical analyses

a. *Sampling*

Leaf and root samples were taken for the biochemical analyses. For leaf samples, the first and second set of fully expanded leaves (4 leaves in total) were removed from the seedling with a scalpel blade. The cut was made just below the point where the petiole joined the base of the leaf blade. Isolated leaves were placed into zipper storage bags (two leaves per bag) and then placed into a Dewar containing liquid N.

For the root samples, the roots were cut below the stem (+/- 1.5 cm below the surface of the vermiculite). As much of the vermiculite particles as possible was removed by gently shaking the root mass, after which a sample was cut off and placed in a zip lock bag. This was then placed in the Dewar containing liquid N. The remaining above 'ground' biomass was also placed into a zip lock bag. After six seedlings were sampled in this way, the zip lock bags containing the seedling samples were sealed and then placed into a -80 °C freezer. Unfortunately, the root samples contained too much vermiculite which could have potentially interfered with the mass calculation in the assays, therefore, it was decided not to analyse the root samples for proline or total phenolic acids.

b. Sample preparation

After being stored at -80 °C, the leaf samples were freeze-dried (SP Scientific, Benchtop Pro 9L EL-85, USA) and then stored at -20 °C until needed. The freeze-dried leaf samples were ground with liquid N in a pestle and mortar until a homogeneous powder was obtained. In order to have enough plant material for both the proline and the total phenolics assay, the first and second set of fully expanded leaves were combined.

c. *Total phenolic compounds* (Tabart *et al.* 2007, Waterman and Mole 1994)

0.2 g of leaf material was mixed with 25 mL of 95% ethanol. The mixture was placed on an orbital shaker (MRC, TS-400P, Britain) in a cold room (4 °C) at 200 rpm for three hours. The mixture was then centrifuged (ThermoFisher Scientific, Sorvall RC-6 plus, USA) at 4000 rpm for 15 minutes. The supernatant was then filtered to remove any residual suspensions. The filtrate was set aside in the cold room while the pellet was suspended in 10 mL of 95% ethanol and placed on the shaker for 30 minutes and

then centrifuged and filtered as above. The filtrates were combined and then used in the colorimetric quantification of phenolic compounds.

The total concentration of phenolic compounds was determined according to the Folin-Ciocalteu (FC) method. From the sample filtrate, 1 mL was used in the assay. In a 15 mL conical tube, 1 mL of filtrate, 1 mL 95% ethanol, 5 mL dH_2O and 0.5 mL of 50% FC reagent (Promark Chemicals, South Africa) were added and the tube was vortexed for +/- 5 seconds. After 5 minutes at room temperature, 1 mL 5% Na_2CO_3 was added and the tubes were vortexed. The tubes were then placed in the dark and incubated at room temperature for one hour. Afterwards, the tubes were briefly vortexed and then 1 mL was transferred to a plastic cuvette and the absorbance at 760 nm was measured (Thermo Scientific Hελios γ, USA).

The gallic acid (Sigma, Germany) standard curve was generated using a range of concentrations from 20 – 100 µg/mL in 20 µg/mL increments. A 10 mg/mL stock solution was prepared by initially dissolving the gallic acid in 1 mL 95% ethanol and then dH_2O was added until the required volume. From the stock solution, serial dilutions were made in the 20-100 µg/mL range. The standard curve was prepared as above, with 1 mL of the standard used in place of the sample filtrate. The concentration of phenolic compounds in the plant samples was calculated using the standard curve and represented as gallic acid equivalent (GAE) expressed in units of mg GAE/g of dry weight leaves. The formula used was:

[(µg/mL x 35 mL)/g sample]/1000

where: µg/mL is the value obtained from the standard curve equation; 35 mL is the total volume of 95% ethanol used to extract the phenolic compounds; g sample is the dry weight of the ground leaf sample used in the assay.

d. *Proline* (Bates *et al.* 1973, Sun *et al.* 2007)

In a 15 mL conical tube, 0.2 g (Appendix F) of leaf powder (one sample per seedling) plus 10 mL of 3% (w/v) sulfosalicylic acid (Sigma, Germany) were added and the tube was vortexed (Velp Scientifica, Zx^3, Italy) at 40 Hz for two minutes. The tubes were then placed in a dry heating block (Gemmyco, DB-006E, Taiwan) at 100 °C for 10 minutes. They were subsequently cooled in water (+/- 22 °C) for 5 minutes. The tubes were then centrifuged at 3000 rpm for 10 minutes (Beckman Coulter Allegra, X-30, USA) and the supernatant was filtered (Munktell Ahlstrom, 3HW 90 mm, Sweden). To

the filtrate (two sub-samples per seedling), the acid-ninhydrin reagent (1.25 g ninhydrin, 30 ml glacial acetic acid, and 20 ml 6M phosphoric acid) and glacial acetic acid were added in a 1:1:1 ratio. The mixture was briefly vortexed and then placed in the dry heating block at 100 °C for one hour. Afterwards, the tubes were cooled on ice and once sufficiently cooled, 4 mL of toluene (or more, if after 4 mL was added and mixed, there was still proline-ninhydrin in the reaction phase) was added. The mixture was vortexed for 30 seconds after which the toluene phase was aspirated, diluted as needed, allowed to warm to room temperature and then the absorbance was measured at 520 nm (PG Instruments, T60U, Britain), with toluene as a blank.

A standard concentration curve was generated using L-proline (Sigma, Germany) in the following concentrations: 5, 10, 15, and 20 µg/ml. The amount of proline in the seedling samples was calculated as follows:

µmole Proline/g dry weight = [(µg proline/ml) x ml toluene)/115.5 µg/µmole] / (g sample)

where: (µg proline/ml) is the value obtained from the standard curve equation; mL toluene is the amount of toluene added; 115.5 µg/µmole is the molecular weight of proline; (g sample) is the dry weight of the plant sample.

Glass cuvettes had to be used as the toluene melted the plastic cuvettes. Preparation of the glass cuvettes was as follows: initially these were washed in warm water with a small amount of dishwashing liquid. They were then rinsed thoroughly with tap water, patted with tissue paper to remove the large drops of water and then toluene was added to the cuvettes. The cuvettes were shaken and then rinsed with copious amounts of tap water, patted dry and finally rinsed with deionized dH_2O. The cuvettes were then put into a drying oven (+/-50 °C). Just prior to use, the external glass was wiped with tissue containing 70% propanol and wiped dry with a cloth to ensure a clean, fibreless surface.

3.3.8 Root bacterial counts

In order to determine whether or not sterility was maintained throughout the experiments, the amount of microbes on the uninoculated seedlings was compared with the inoculated seedlings, in both the unstressed and drought stressed experiments. The method of Sandhya *et al.* (2009) was used to enumerate the amount of microbes present on the root surface. This was achieved

by placing approximately 1 g of the root sample (as free from vermiculite as possible) into a 50 mL conical tube, followed by the addition of 10 mL solution of 0.9% NaCl with 0.05% Tween 20. The resulting contents of the tubes were vortexed for 2 minutes at 40 Hz. A sample of the liquid was plated onto King's B Agar and incubated at 25 °C for 48 hours. Where possible bacterial counts were taken.

3.3.9 Recovery and regrowth

This parameter was only conducted in the drought stressed experiments. At the predetermined sampling date for the drought stressed seedlings, a set of 10 seedlings was left to endure further drought stress. The seedlings were left without water until there was a discernible dip in the chlorophyll fluorescence of the seedlings. Thereafter, water was resupplied to the seedlings and the normal fertilizing schedule was resumed. Plant height and number of nodes were measured two weeks later, at which point the above ground biomass was harvested and placed into a drying oven at 80 °C for 4 days. The dry weight of the samples was then recorded.

3.4 Data analysis

All data was analysed using Statistica 12 (StatSoft, version 12.5.192.0). Initially the data was checked for outliers. Once all outliers were removed, the data was tested for normality. The data was then tested for differences with either a t-test or the Mann-Whitney U test, depending on the normality of the data. To compare data between the unstressed and stressed experiments, ANOVAs and Tukey HSD post-hoc tests were used. Data was represented as Mean ± SE. Statistically significant differences in tests with only two means (*i.e.* t-tests) were represented on the graphs in the form of asterisks as follows: * = $P \leq 0.05$; ** = $P \leq 0.01$; *** $P \leq 0.001$; and **** = $P \leq 0.0001$. In the ANOVA tests, statistically significant differences were represented on graphs with the use of letters. Means that share a letter are not significantly different at $P \leq 0.05$, while those that do not share a letter are significantly different at $P \leq 0.05$.

4 RESULTS

4.1 Bacterial studies

4.1.1 Phosphatase activity

In the alkaline phosphatase assay, the reaction mixtures of *P. fluorescens* and *P. koreensis* had an A_{405} value, on average, of 0.016 ± 0.001 and 0.011 ± 0.001, respectively. This value is below the range in which the Beer-Lambert law is linear (*i.e.* 0.1 – 0.9) therefore, the results obtained from using the A_{405} values would be inaccurate.

In the acid phosphatase activity assay, *P. koreensis* had, on average, 7.7 µM pNP/min/OD600 (95% Confidence Interval (CI): 5.2 – 10.3) higher phosphatase activity than *P. fluorescens* (Figure 4; t = -3.176, df = 65, P = 0.002).

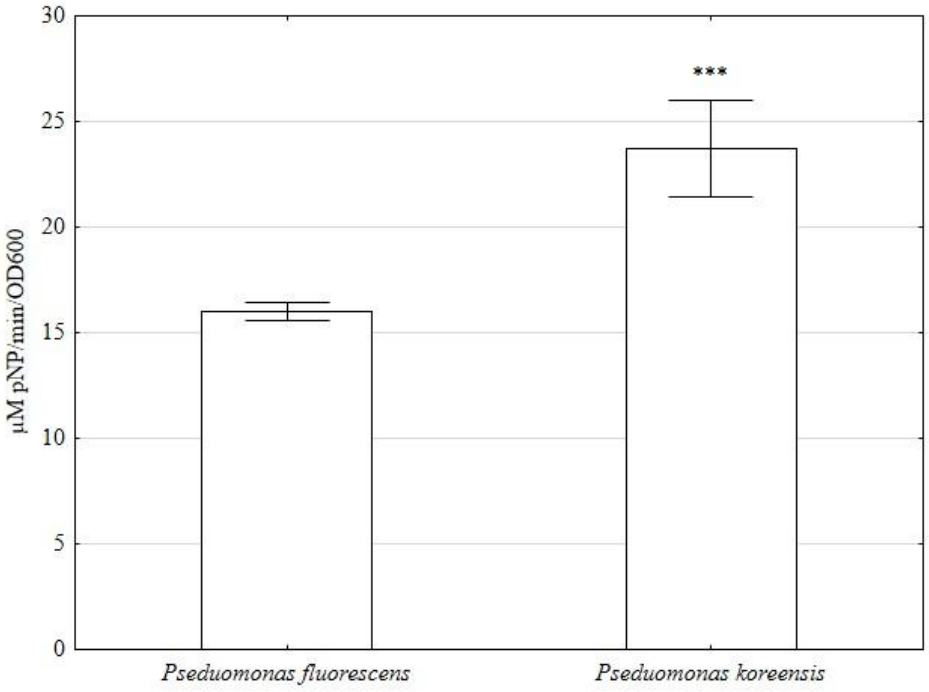


Figure 4. Average (mean ± SE) acid phosphatase activity between *P. fluorescens* and *P. koreensis* as shown by the amount of *para*-Nitrophenyl (pNP) produced via the cleaving of phosphate from *para*-Nitroplenyl phosphate (pNPP) by the phosphatase enzymes at a pH of 6.3. *P. koreensis* had a higher acid phosphatase activity than *P. fluorescens* (t = -3.176, df = 65, P = 0.002).

4.1.2 Siderophore production

In the siderophore assay, *P. koreensis* produced, on average, 20.9 µM DFOM/OD540 (95% CI: 17.4 – 24.1) more siderophores (as a DFOM equivalent) than *P. fluorescens* (Figure 5.; t = -10.148, df = 67, $P < 0.001$).

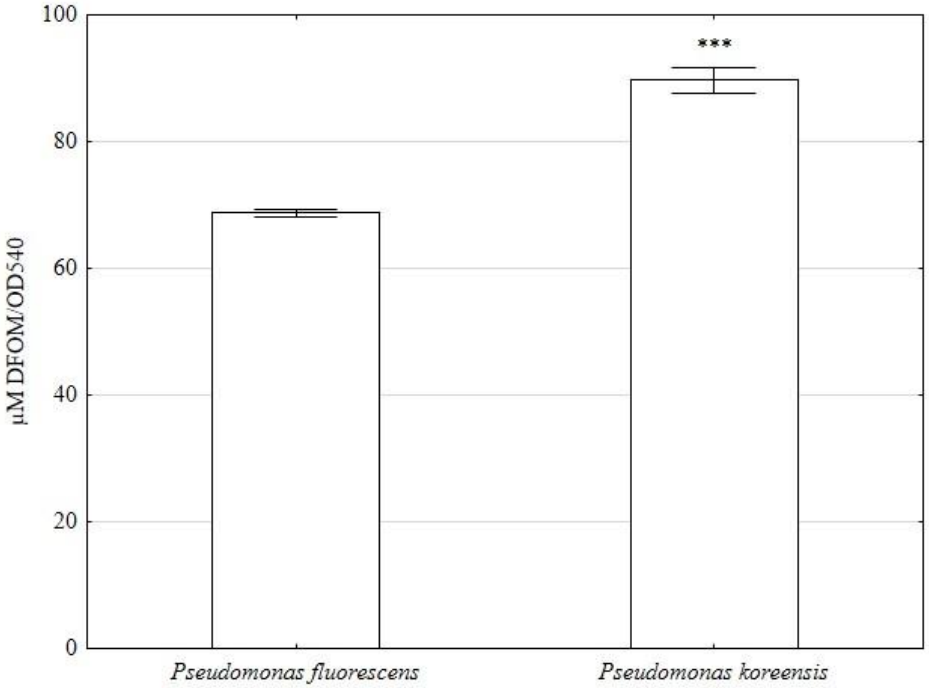


Figure 5. Average (mean ± SE) siderophore production between *P. fluorescens* and *P. koreensis* as shown by the equivalent of deferoxamine mesylate (DFOM), an iron chelator. *P. koreensis* produced a higher amount of siderophores than *P. fluorescens* (t = -10.148, df = 67, $P < 0.001$).

4.1.3 IAA production

In the IAA assay, *P. koreensis* produced 134.8 µg/mL/OD600 (95% CI: 118.1 – 151.5) more IAA than *P. fluorescens* (Figure 6; t = -26.177, df = 68, $P < 0.0001$).

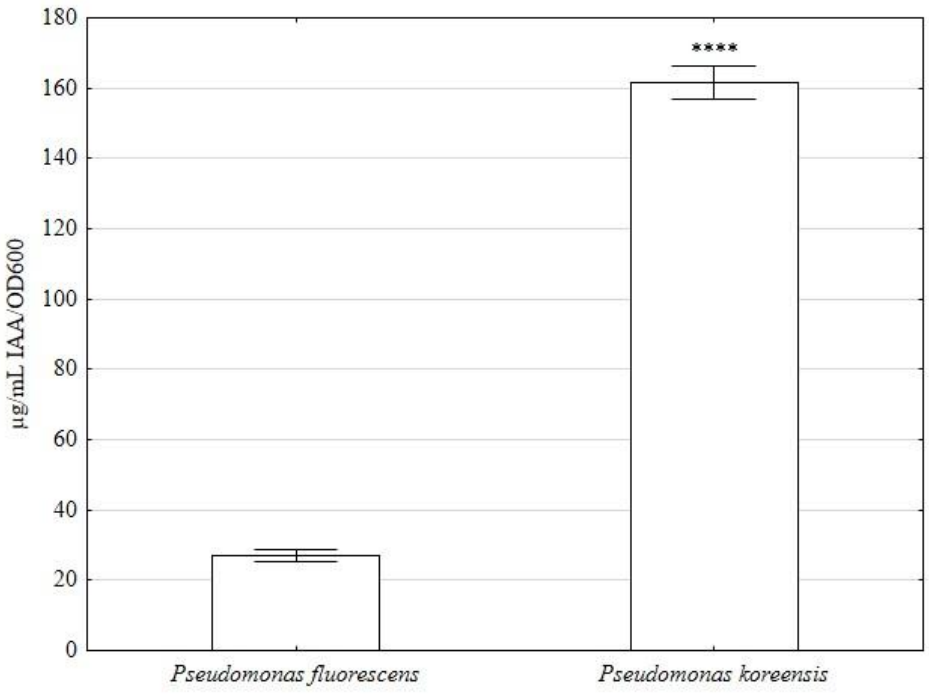


Figure 6. Average (mean ± SE) production of indole-3-acetic acid (IAA) between *P. fluorescens* and *P. koreensis*. *P. koreensis* produced a significantly larger amount of IAA than *P. fluorescens* (t = -26.177, df = 68, $P < 0.0001$).

4.1.4 Growth at -0.73 MPa

There was no evidence of a difference in the OD of cultures of *P. fluorescens* and *P. koreensis* grown in TSB with 42mM polyethylene glycol (PEG) in the first three days; however, after six days, *P. fluorescens* had an OD600 of 1.7x higher than that of *P. koreensis* ($F(1,7)= 151.790$, $P = 0.0002$) as a result of the OD600 of *P. koreensis* decreasing from day three to day six (Figure 7.). Under normal (unstressed) conditions, namely the growth of the bacteria in plain TSB, a similar pattern was observed (Figure 8.; $F(1,7)= 91.44$, $P = 0.0248$). On the third day of incubation, the unstressed cultures of both *P. fluorescens* and *P. koreensis* had an optical density twice as high as that of the cultures grown in TSB with PEG (Figure 9.; $F(1,3)= 1219.24$, $P = 0.0002$).

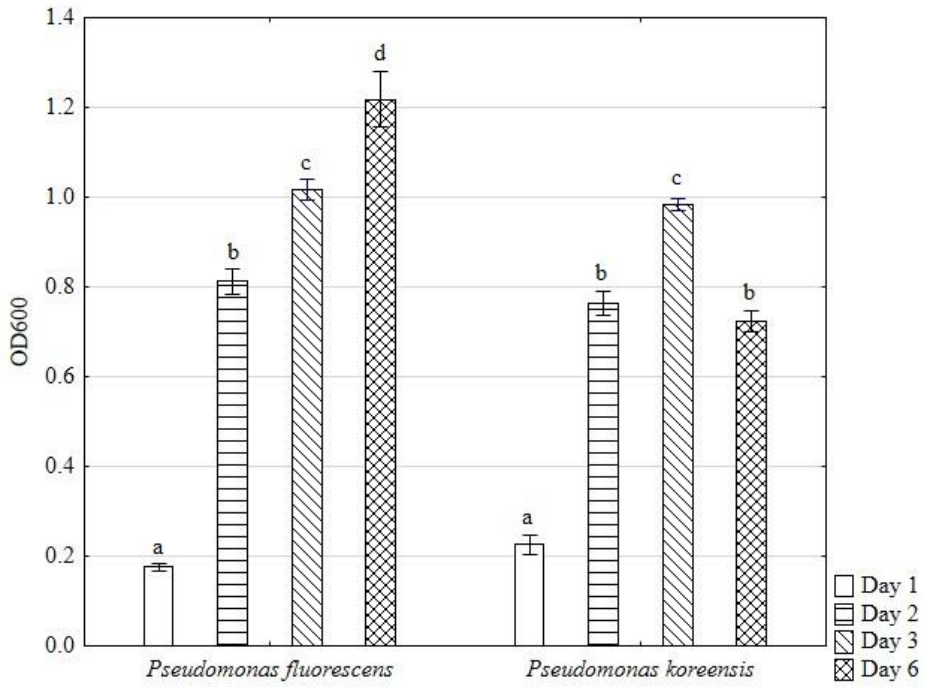


Figure 7. Average (mean ± SE) optical densities (OD) of *P. fluorescens* and *P. koreensis* grown in tryptic soy broth (TSB) supplemented with 42 mM polyethylene glycol (PEG) in order to induce water stressed conditions. *P. fluorescens* only had a higher OD600 than *P. koreensis* on the sixth day ($F(1,7)= 151.790$, $P = 0.0002$).

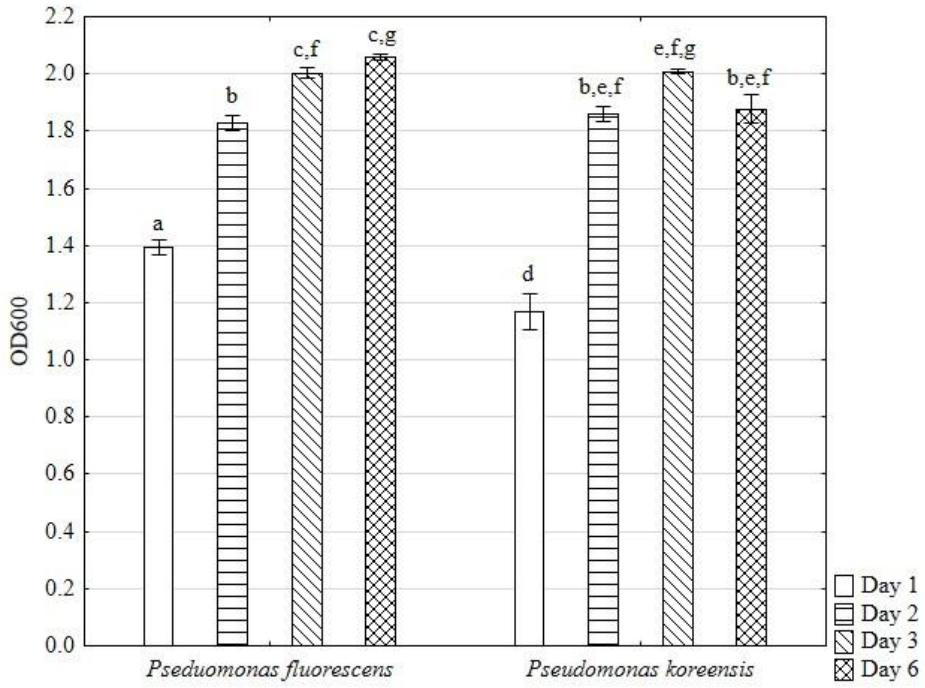


Figure 8. Average (mean ± SE) optical densities (OD) of *P. fluorescens* and *P. koreensis* grown in tryptic soy broth (TSB) under unstressed conditions. *P. fluorescens* only had a higher OD600 than *P. koreensis* on the sixth day ($F(1,7)= 91.44$, $P = 0.0248$).

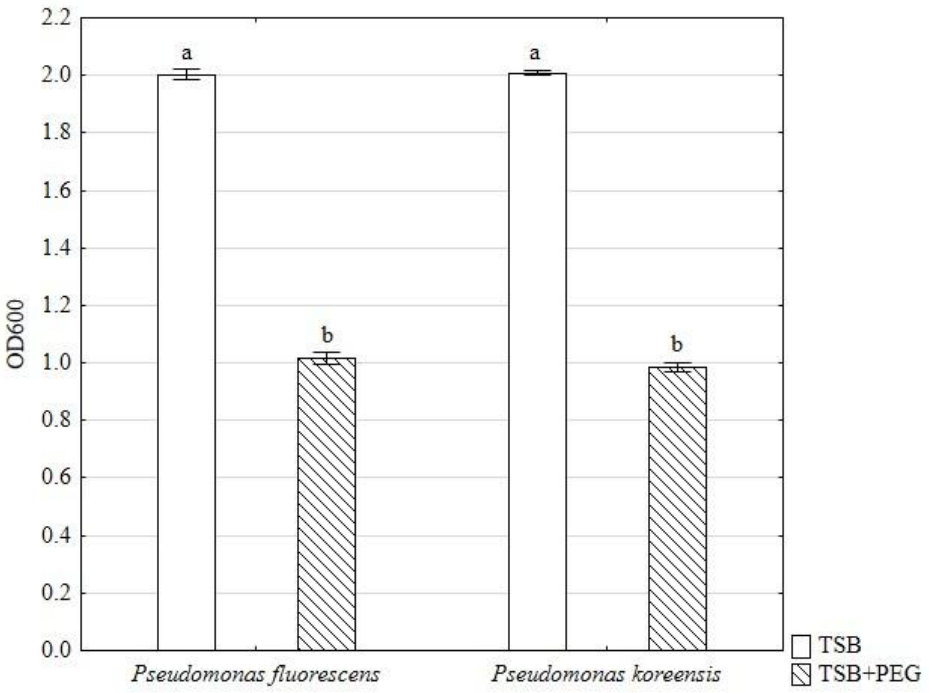


Figure 9. Average (mean ± SE) optical densities (OD) of *P. fluorescens* and *P. koreensis* on the third day of incubation at 25 °C and 160 rpm in unstressed Tryptic Soy Broth (TSB) and TSB supplemented with 42 mM polyethylene glycol (TSB+PEG) to induce water stress. Unstressed cultures had a significantly higher OD600 than the stressed cultures ($F(1,3)= 1219.24$, $P = 0.0002$).

4.2 Plant studies

4.2.1 Sterilisation, Inoculation and Germination

Sterilisation

There was no evidence that the use of NaClO in varying concentrations had any effect on the germination of sunflower seeds (Table 1.; $F(1,3)= 0.667$, $P = 0.6151$). However, the use of NaClO in increasing concentration did decrease the amount of contaminated sunflower seeds (Table 1.; $F(1,3)= 22.333$, $P = 0.0058$).

Table 1. Germination and contamination percentages of *Helianthus annuus* seeds treated with sodium hypochlorite (NaClO) in varying concentrations. Mean ± SE.

Concentration of NaClO	**Germination (%)**	**Contamination (%)**
0 %	100[a]	100[a]
1 %	90 ± 10[a]	40 ± 20[b]
2 %	90 ± 10[a]	0[c]
3.5 %	100[a]	0[c]

*Values in the same column that share the same letters are not significantly different at P = 0.05

Inoculation

Inoculation of the sunflower seeds with *P. koreensis* before they had germinated had an adverse effect to the germination of the seeds (Table 2.). However, the seeds that were not inoculated, had excellent germination characteristics (Table 2.). It was therefore decided, that for the main experiments, the plants designated as the treatment plants would be inoculated only after the seeds had germinated.

Table 2. Germination characteristics of *Helianthus annuus* seeds either uninoculated or inoculated with *Pseudomonas koreensis*. MGT = Mean Germination Time.

Group	**Final germination %**	**MGT**
Uninoculated	100	2.2
Inoculated	0	N/A

4.2.2 Recovery and Regrowth

The uninoculated plants endured 13 days without water, until they had an average Fv/Fm value of 0.697 ± 0.077, before the normal watering regime resumed. However, there was only an 11% survival rate. The inoculated plants endured 14 days without water, until they had an average Fv/Fm value of 0.421 ± 0.101, before the normal watering regime resumed. However, there was only a 20% survival rate. After two weeks of recovery and regrowth for the surviving plants, there was no evidence of a difference in the dry biomass between the uninoculated and the inoculated plants (Figure 10.; t = -1.724, df = 14, P = 0.107).

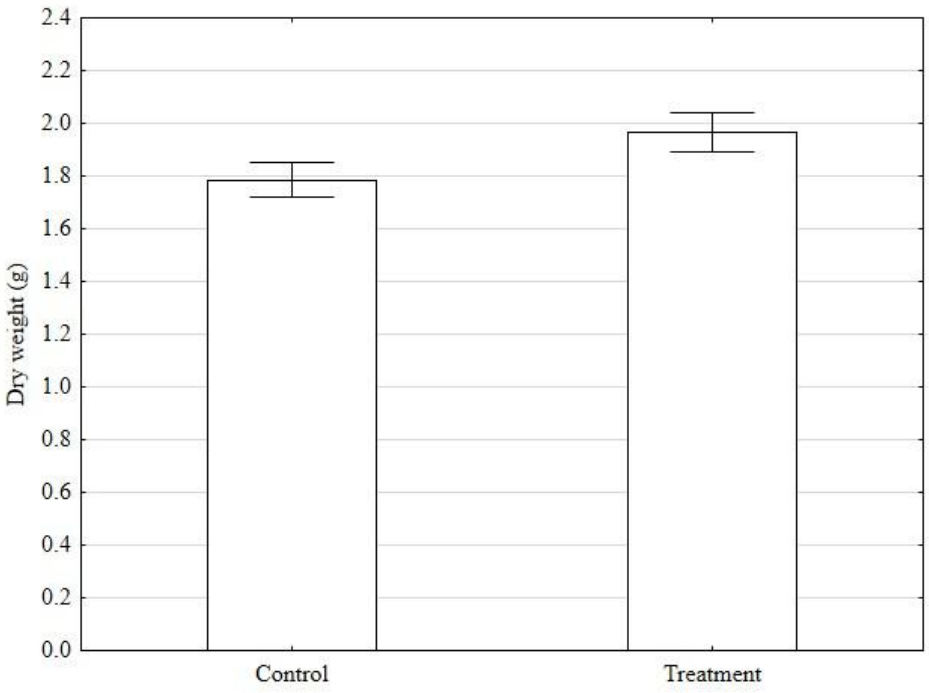


Figure 10. Average (mean ± SE) dry biomass of uninoculated and inoculated plants after two weeks of recovery after 13 and 14 days, respectively, without water. There was no evidence of a difference between the uninoculated and the inoculated plants (t = -1.724, df = 14, P = 0.107).

4.2.3 Root bacterial counts

Unstressed plants experiment

The inoculated plant roots had, on average, 2.5 x10^6 cfu/g root (95% CI: 1.4 – 3.6 x10^6) more microbial colonies than that of the uninoculated plants (Figure 11; t = -2.38, df = 28, P = 0.024). For the inoculated plants, the total microbial count consisted entirely of bacterial colonies, while the uninoculated plants contained 0.273 ± 0.144 x10^6 cfu/g root of fungal colonies (Figure 12.).

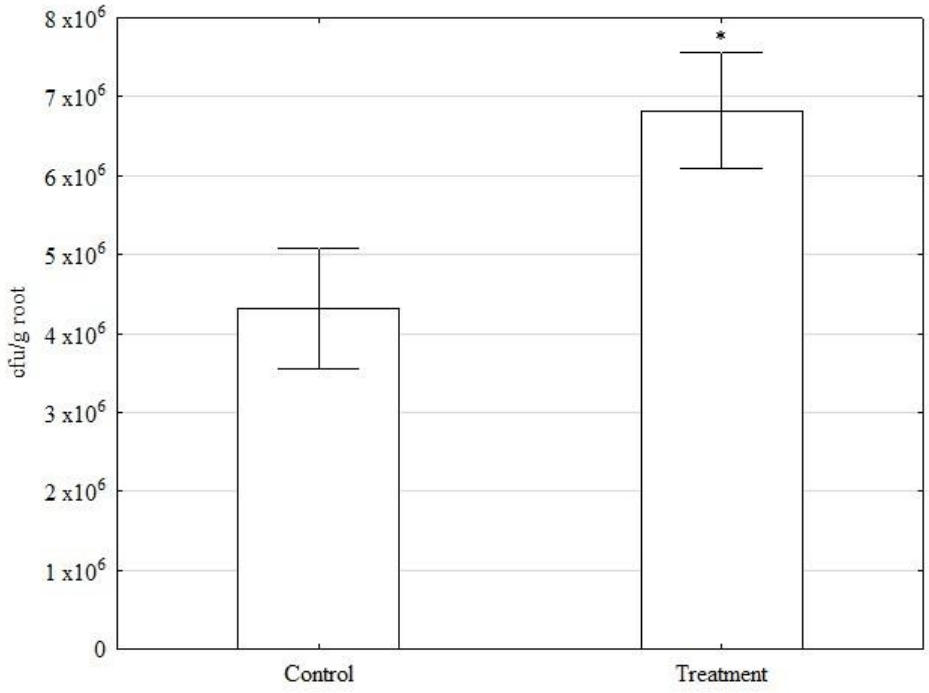


Figure 11. Average (mean ± SE) root microbial counts of inoculated and uninoculated plants after 5+ weeks of growth under unstressed conditions. The inoculated plants had a higher microbial count than the uninoculated plants (t = -2.38, df = 28, P = 0.024).

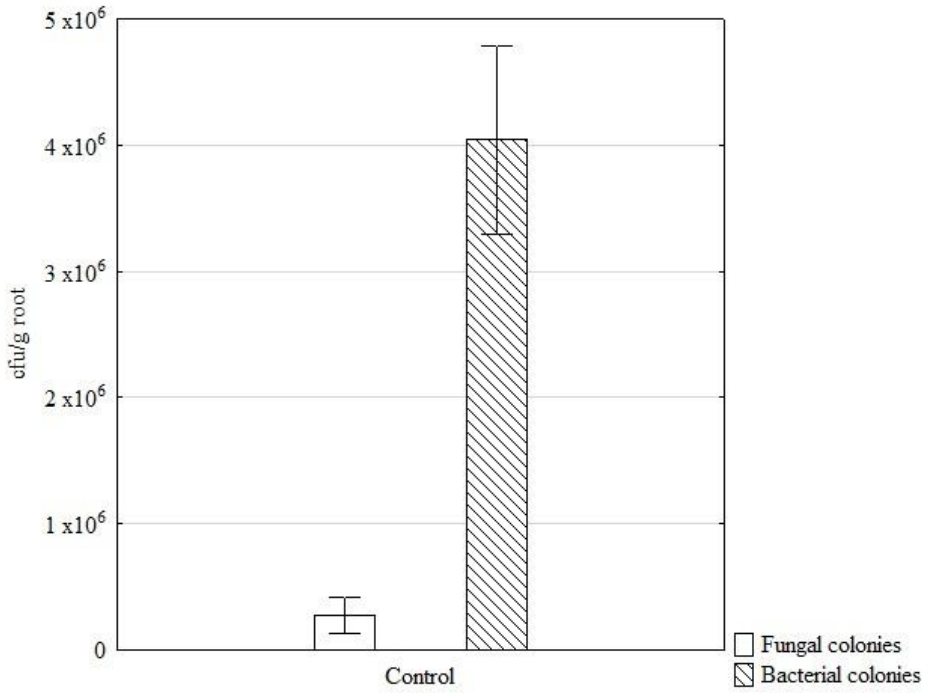


Figure 12. Average (mean ± SE) root microbial counts of uninoculated unstressed plants showing a low proportion of fungal colonies as compared with bacterial colonies.

Drought stressed experiment

Both the uninoculated and the inoculated plants had a large presence of microbes on the plant roots. However, the majority of the inoculated plants' microbial count were *P. koreensis*, with a count of 6.1 x10^8 cfu/g root, and a count of 0.3 x10^8 cfu/g root of other microbes; while the uninoculated plants had no identifiable *P. koreensis* colonies and there were only two fluorescent colonies found.

4.2.4 Plant growth data

In the weekly plant height measurements, the inoculated set of plants had a growth rate of 4.3 ± 0.7 cm/week; however there was no evidence that this was higher than the 3.9 ± 0.4 cm/week growth rate of the uninoculated plants (t = -0.532, df = 6, P = 0.6134). However, the inoculated plants had consistently taller plants than the uninoculated plants, and after four weeks of growth, the inoculated plants were 1.8 cm (95% CI: 1.4 – 2.2) taller than the uninoculated plants (Figure 13.; F(1,7) = 472.37, P < 0.0001). There was no evidence of a difference in the number of nodes between the uninoculated and the inoculated seedlings (F(1,7) = 4879.4, P > 0.05).

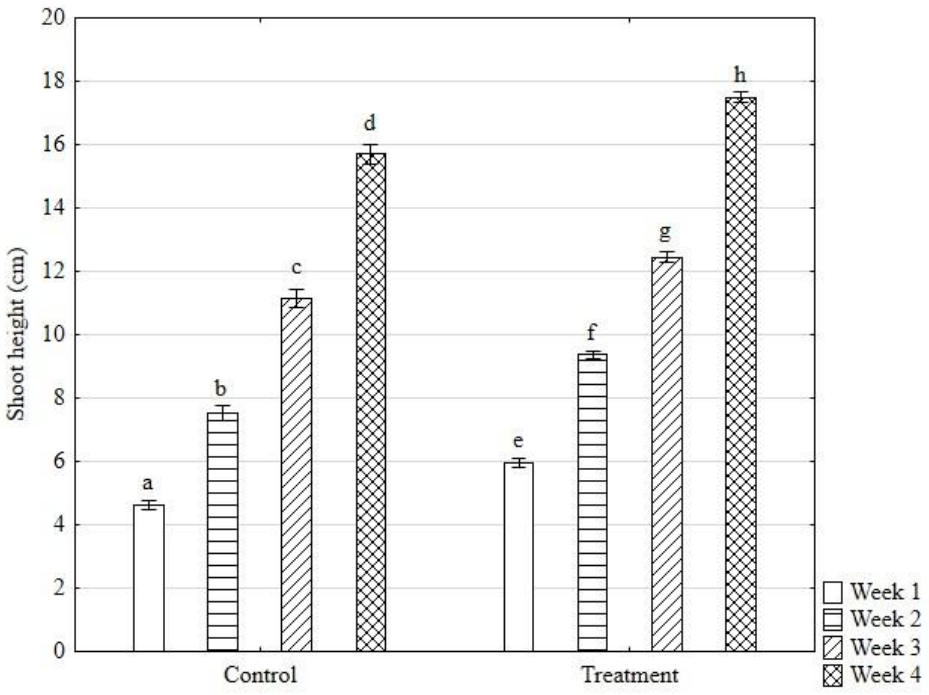


Figure 13. Average (mean ± SE) plant height of uninoculated and inoculated plants during the first four weeks of growth. The inoculated plants were consistently taller than the uninoculated plants (F(1,7)= 472.37, P < 0.0001).

4.2.5 Unstressed leaf area

In the unstressed experiment, the total leaf area after 5+ weeks of the inoculated plants was, on average, 18.9 cm^2 (95% CI: 11.5 – 26.3) larger than that of the uninoculated plants (Figure 14.; t = -2.756, df = 28, P = 0.01).

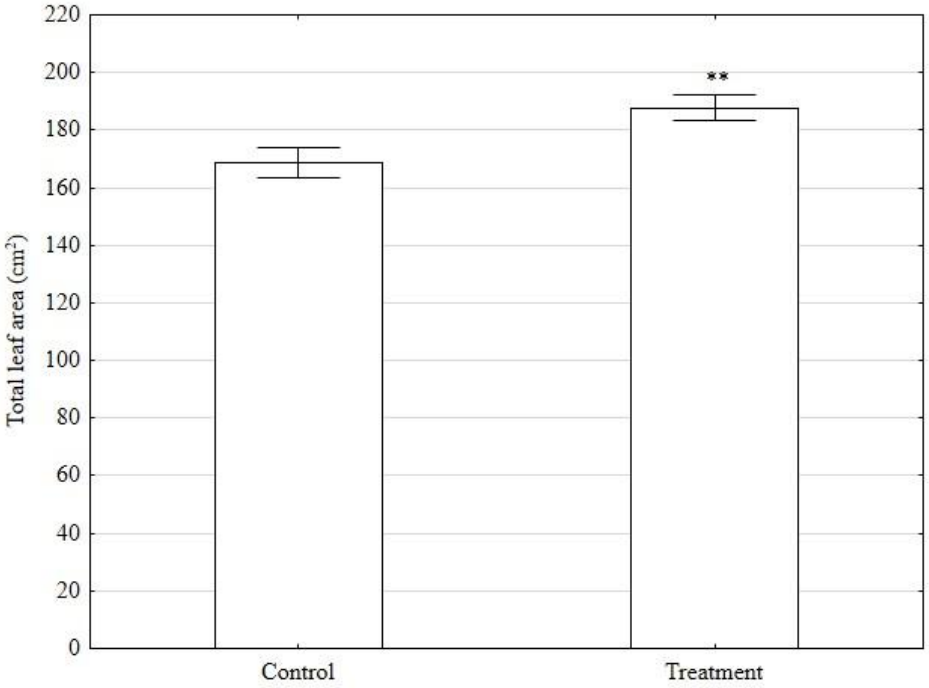


Figure 14. Average (mean ± SE) leaf area of the first fully expanded set of leaves in the unstressed experiment. The inoculated plants had a larger leaf area than the uninoculated plants (t = -2.756, df = 28, P = 0.01).

4.2.6 Leaf RWC

There was no evidence that the leaf water content, in both the drought stressed experiment and the unstressed experiment, was different between the uninoculated and the inoculated plants (F(1,3)= 51.568, P = 0.907 and P = 0.470, respectively). However, between the stressed and unstressed experiments, the stressed plants had 32.6 % (95% CI: 25.8 – 39.4) less leaf water content in the uninoculated plants and 30.0 % (95% CI: 24.7 – 35.4) in the inoculated plants (Figure 15.; F(1,3)= 51.568, P = 0.0002).

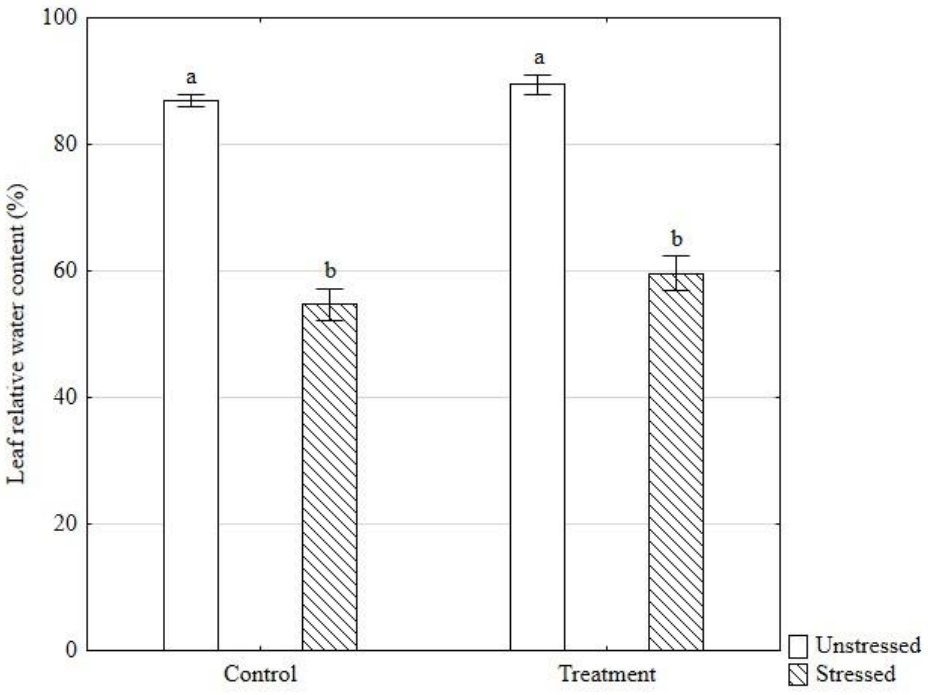


Figure 15. Average (mean ± SE) relative leaf water content of uninoculated and inoculated plants in both the unstressed and drought stressed experiments. There was no difference between the uninoculated and inoculated plants in both the unstressed and the drought stressed experiments (F(1,3)= 51.568, *P* = 0.907 and *P* = 0.470, respectively). There was a difference between the unstressed and the drought stressed plants in both the uninoculated and inoculated treatments (F(1,3)= 51.568, *P* = 0.0002).

4.2.7 Leaf electrolyte leakage

There was no evidence of a significant difference in the electrolyte leakage between the uninoculated and inoculated plants in the unstressed experiment (F(1,3)= 9.943, *P* = 0.9814). There was also no evidence of a difference between the unstressed and stressed plants in the uninoculated plants (F(1,3)= 9.943, *P* = 0.0910) or that there was a difference between the uninoculated and the inoculated plants in the drought stressed experiment (F(1,3)= 9.943, *P* = 0.1506). However, the drought stressed inoculated plants had a relative conductivity of 15.7 % (95% CI: 11.2 – 20.2) higher than the unstressed inoculated plants (Figure 16.; F(1,3)= 9.943, *P* = 0.0002).

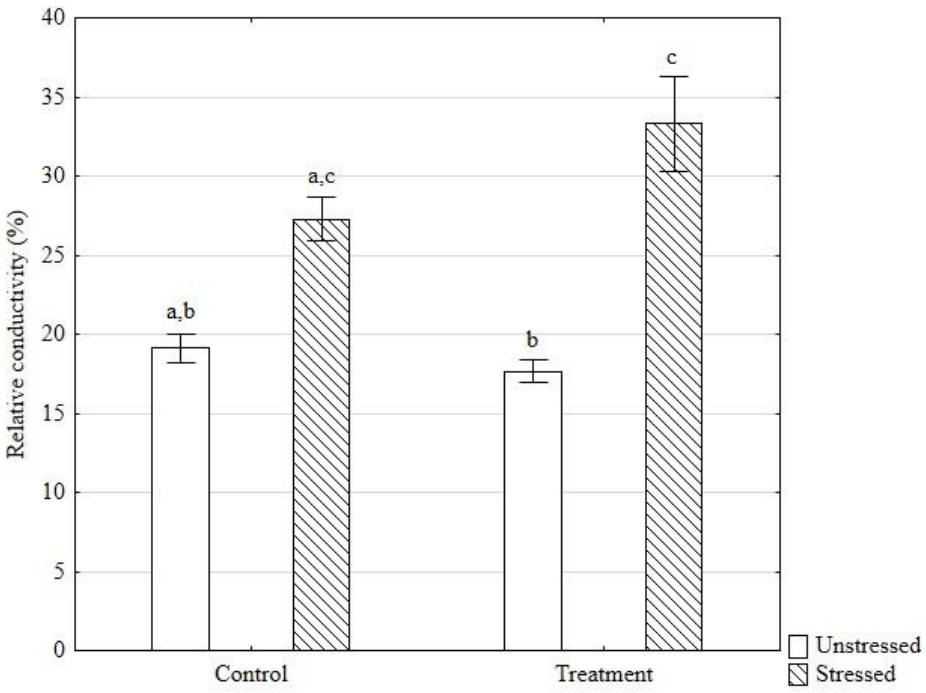


Figure 16. Average (mean ± SE) relative conductivity measurements of leaf cell leachates of both uninoculated and inoculated plants in unstressed and drought stressed experiments. The drought stressed inoculated plants had a higher electrolyte leakage than the unstressed inoculated plants ($F(1,3)= 9.943$, $P = 0.0002$). In contrast, there was no difference between the drought stressed and unstressed uninoculated plants ($F(1,3)= 9.943$, $P = 0.0910$).

4.2.8 Chlorophyll fluorescence

In the drought stressed experiment, the inoculated plants had, on average, a Fv/Fm value of 0.007 (95% CI: 0.005 – 0.010) lower than that of the uninoculated plants (Figure 17.; $F(1,3)= 5.0$, $P = 0.0027$). Apart from this finding, there was no evidence of a difference in the Fv/Fm values in uninoculated plants, inoculated plants or between unstressed inoculated plants and uninoculated plants (both unstressed and stressed).

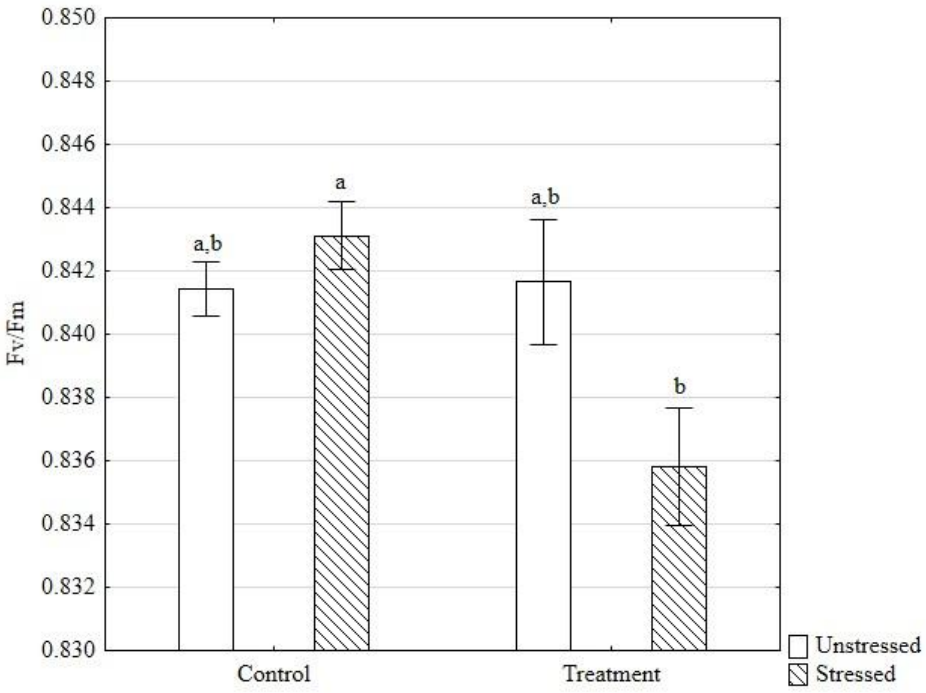


Figure 17. Average (mean ± SE) Fv/Fm values of both uninoculated and inoculated plants in unstressed and drought stressed experiments. The Fv/Fm values of inoculated drought stressed plants were significantly lower than that of the uninoculated drought stressed plants ($F(1,3)= 5.0$, $P = 0.0027$).

4.2.9 Substrate water content

In the drought stressed experiment, the inoculated plants had a substrate water content of 3.1 % (95% CI: 1.9 – 4.3) lower than that of the uninoculated plants (Figure 18.; $U = 249.0$, $n_1 = 29$, $n_2 = 28$, $P = 0.01$).

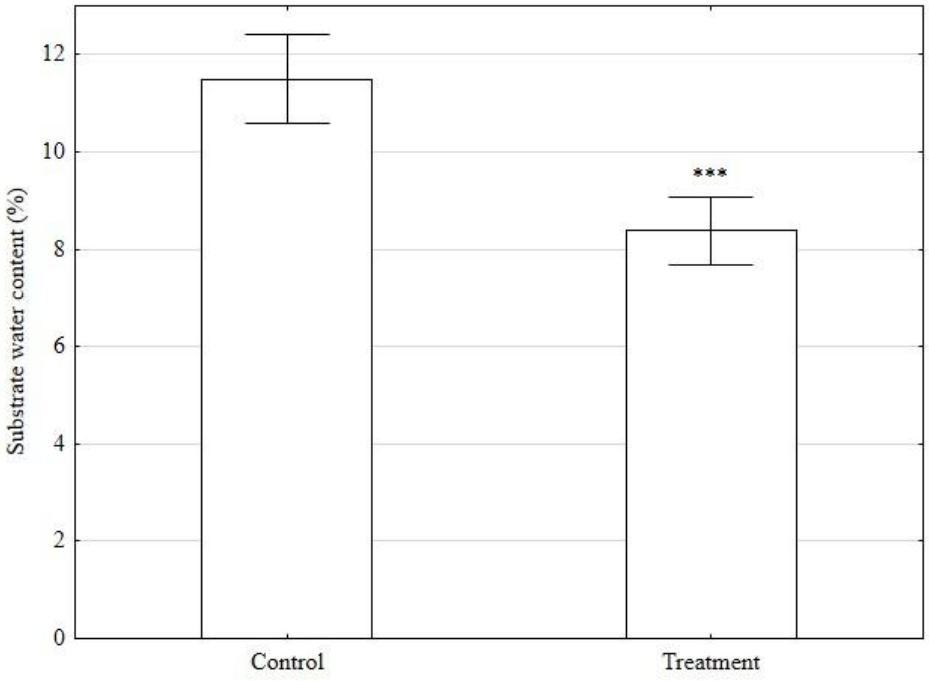


Figure 18. Average (mean ±SE) substrate water content of the uninoculated and inoculated plants in the drought stressed experiment. The inoculated plants had a significantly lower substrate water content than the uninoculated plants (U = 249.0, n_1 = 29, n_2 = 28, P = 0.01).

4.2.10 Total phenolic compounds

In the drought stressed experiment, the inoculated plants had 1.32 mg/g (95% CI: 0.99 – 1.64) more phenolic compounds (as GAE equivalents) than the uninoculated plants (Figure 19.; t = -4.784, df = 48, P < 0.0001).

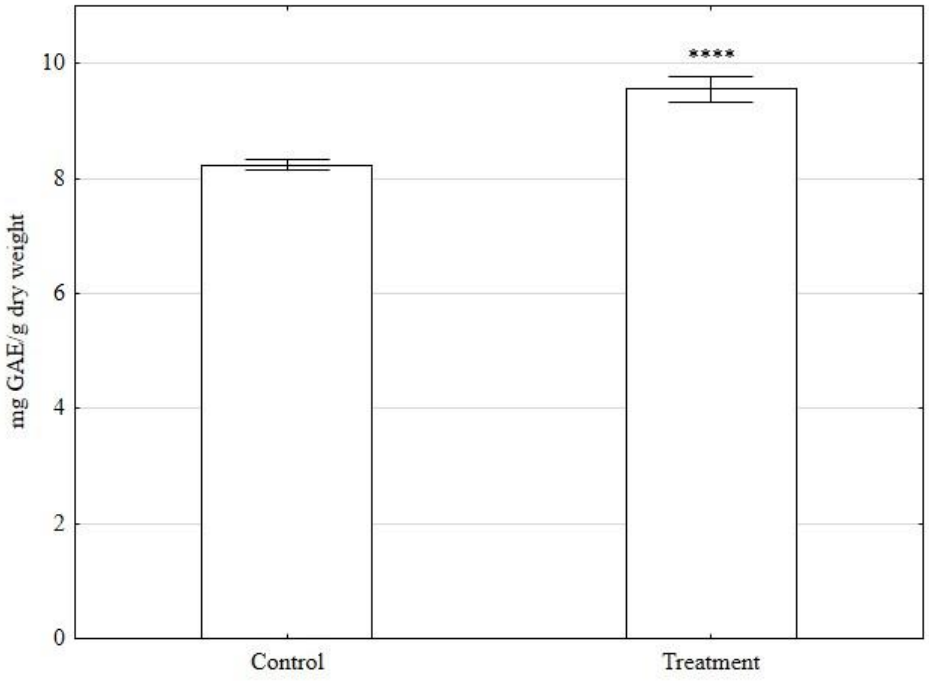


Figure 19. Average (mean ± SE) total phenolic compounds, expressed as gallic acid equivalents (GAE), in leaf tissue of both uninoculated and inoculated plants in the drought stressed experiment. The inoculated plants had more phenolic compounds than the uninoculated plants (t = -4.784, df = 48, P < 0.0001).

4.2.11 Proline

In the drought stressed experiment, the inoculated plants had 10.24 µmol/g (95% CI: 6.09 – 14.40) more proline than the uninoculated plants (Figure 20.; U = 126.0, n_1 = 18, n_2 = 27, P = 0.007).

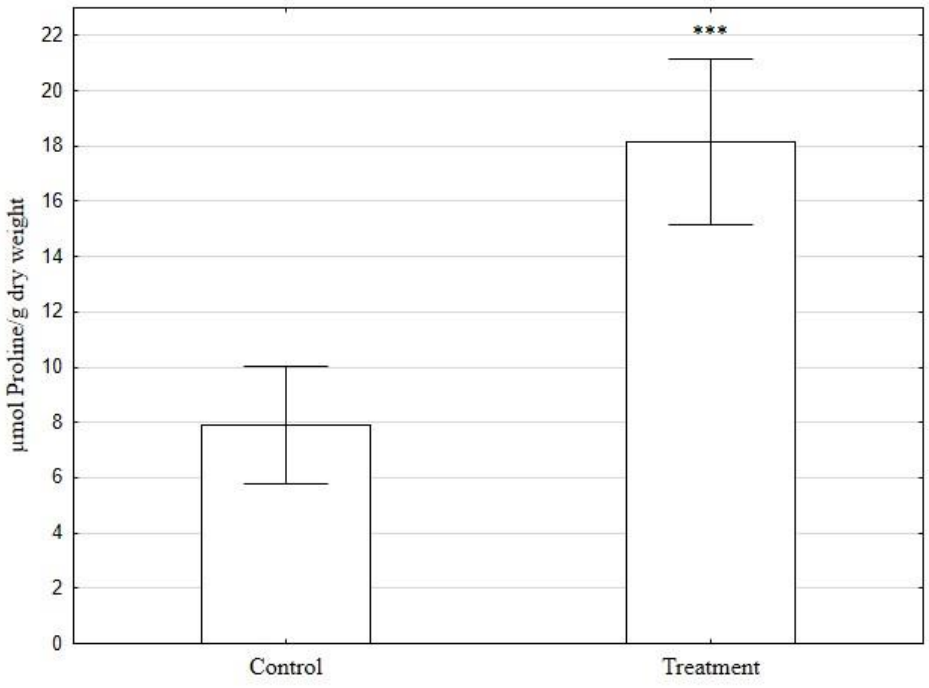


Figure 20. Average (mean ± SE) proline content in leaf tissue of both uninoculated and inoculated plants in the drought stressed experiment. The inoculated plants had a higher concentration of proline than the uninoculated plants (U = 126.0, n_1 = 18, n_2 = 27, P = 0.007).

5 DISCUSSION

5.1 Bacterial studies

5.1.1 Phosphatase activity

P is a plant macronutrient and is important for plant growth. However, the majority of P in soil is insoluble and is made available to plants through the action of soil microorganisms. Plants that are deficient in P are also less tolerant of drought stress (Nelsen and Safir 1982), therefore, the presence of soil microorganisms, such as *P. koreensis*, is beneficial not only to the growth of plants but also the plant's tolerance to drought.

Sunflower plants that are deficient in P have a lower leaf area and number of leaves, decreased shoot growth and a decreased carbohydrate utilization (Hemalatha *et al.* 2013). When sunflower plants are supplied with ample P, the plants have an increased head diameter, an increased number of seeds per head as well as an increase in seed weight (Hemalatha *et al.* 2013). All of these factors contribute to an increase in yield and profit for the farmer.

In the present study however, the alkaline phosphatase assay failed to produce measurable results. This could be due to the alkaline phosphatase enzymes being down regulated by the concentration of inorganic P (Rodríguez and Fraga 1999, Thaller *et al.* 1995, Gügi *et al.* 1991). It has been found that the *P. fluorescens* strain, MF3, has these phosphate-repressible phosphatases (Thaller *et al.* 1995, Gügi *et al.* 1991). Since the growth medium used in the present study had P in the form of $H_2PO_4^-$ and HPO_4^{2-}, the alkaline phosphatases of the bacteria were most likely repressed. Moreover, the pH of most soils range from acidic to neutral and therefore, acid phosphatases should play the key role in making P available to plants (Rodríguez and Fraga 1999).

In the acid phosphatase assay, *P. koreensis* had a higher enzyme activity than *P. fluorescens* suggesting that *P. koreensis* would mineralize more inorganic P from organic phosphate sources in the soil than *P. fluorescens*. Therefore, it was expected that *P. koreensis* would enhance the P nutrition of plants more than *P. fluorescens* and the plants would be better able to survive drought stress (Nelsen and Safir 1982). However, the acid phosphatase activity in the present study is probably lower than the potential maximal activity of the phosphatase enzymes due to the maximum expression of acid phosphatases occurring at a growth temperature of 17.5 °C (Burini *et al.* 1994). In the present study, a growth temperature of 25 °C was chosen to keep the results comparable with the plant experiments.

For future work, it would be interesting to compare the inorganic P solubilizing abilities of *P. koreensis* and *P. fluorescens* by, for example, measuring the amount of gluconic acid produced (or the activity glucose dehydrogenase) or measuring the drop in pH of P starved media. Also, the concentration of P in sunflower plants inoculated with *P. koreensis* and undergoing drought stress can be compared with uninoculated plants in order to determine whether or not P nutrition plays a role in the drought stress tolerance of sunflower.

5.1.2 Siderophores

Siderophores are important Fe-chelating compounds secreted by soil microbes in Fe-limiting environments (e.g. calcareous soils). The siderophores produced by soil microbes, such as *P. koreensis*, not only make Fe available to plants, which increases the growth and drought tolerance of the plants, but also decreases the amount of Fe available to soil pathogens thus, providing a bio-fungicide service to the plants (Kloepper *et al.* 1980, Weller 2007, Sahu and Sindhu 2011).

Fe deficiency in sunflower resulted in chlorosis and a decreased respiration rate (Glenister 1944). Sunflower plants grown in Fe-limiting conditions also had a decreased POD activity which resulted in an accumulation of H_2O_2 and thus, exposed the plants to oxidative stress (Ranieri *et al.* 2001).

In the present study, *P. koreensis* produced a higher concentration of siderophores than *P. fluorescens* which suggested that *P. koreensis* would solubilize more Fe^{3+} than *P. fluorescens*. Hence, plants inoculated with *P. koreensis* would have better Fe nutrition. Plants with a higher availability of Fe would have improved ROS signalling and quenching capabilities (Briat and Lobréaux 1997, Becana *et al.* 1998), which will better allow the plant to tolerate drought stress by lowering the amount of oxidative stress. However, the amount of siderophores produced *in vitro* does not necessarily reflect the amount produced in nature as the production of siderophores depends of the availability of organic and inorganic nutrients (Teintze and Leong 1981, Neilands 1984).

Another important observation was that the concentration of siderophores produced by the both of the bacteria in this study was lower than the concentrations reported by Alexander and Zuberer (1991), the authors of the paper describing the method used in this study. Those authors reported siderophore concentrations ranging from 134 – 238 µM DFOM equivalent for *P. fluorescens* and 176 – 204 µM DFOM equivalent for *P. putida* (Alexander and Zuberer 1991), while in this study, the concentration ranged from 68 – 70 µM DFOM equivalent for *P. fluorescens* and 86 – 94 µM DFOM equivalent for *P. koreensis*. In that paper, the authors showed that the production of siderophores lagged behind the exponential phase of cell growth while substantial amounts of siderophores were produced in the stationary phase, the stationary phase being the phase of maximum siderophore production (Alexander an Zuberer 1991). In the present study, the amount of inoculum added to the MM9 medium could have delayed the culture reaching the exponential and stationary phases and it is therefore possible that at the time of sampling, the cultures were only in the early stationary phase. If a culture is in the early stationary phase, the accumulation of secondary metabolites such as siderophores would be low. Another possibility is that the strain of *P. fluorescens* and *P. koreensis* used in this study did not have the genetic capability to produce a large amount of siderophores compared with other strains and species (Jung *et al.* 2006, Meyer 2000, Wilson *et al.* 2006).

For future work, it would be interesting to determine the role of siderophores in the Fe nutrition of drought stressed sunflower plants and to investigate what effect siderophores have on the Fe

dependant antioxidant enzymes CAT, ascorbate POD, guaiacol POD and ferro-SOD in drought stressed sunflower plants. Perhaps these could be determined in field trials with soils that have a neutral pH.

5.1.3 IAA production

Given that IAA, an auxin, regulates the growth of plant roots, the fact that *P. koreensis* produced significantly more IAA than *P. fluorescens* suggested that *P. koreensis* would better promote the growth of inoculated plant's roots and improve the plant's tolerance to drought stress (Hurd 1974, Marulanda *et al.* 2009) than *P. fluorescens*. The amount of IAA produced by *P. koreensis* was also greater than the 68.3 ± 2.2 µg/mL/OD600 produced by *P. putida* GR12-2, reported in another study (Patten and Glick 2002). Interestingly, those authors measured IAA production by high performance liquid chromatography (HPLC) as well as spectrophotometrically using the Salkowski's reagent and found that the amount of IAA detected by HPLC was around twice the amount detected using the Salkowski's reagent (Patten and Glick 2002). It should also be noted that indolepyruvic acid, which is a product of the catalysis of tryptophan, also reacts with the Salkowski's reagent although to a lesser degree than IAA (Patten and Glick 2002). It is therefore likely that the amount of IAA produced by *P. koreensis* and assayed via HPLC may be higher than the amount reported here.

High concentrations (in the micromolar range) of IAA stimulates the growth of lateral and adventitious roots, however, the growth of primary roots are stimulated by low IAA concentrations (in the pico- to nanomolar range) and inhibited by high IAA concentrations, likely via auxin-induced ethylene production (Alvarez *et al*. 1989, Meuwly and Pilet 1991, Peck and Kende 1995, Pilet and Saugy 1987). Plants inoculated with mutants that overproduce IAA were reported to have a root growth-inhibiting effect (Loper *et al.* 1982, Sarwar and Kremer 1995, Xie *et al.* 1996). The high IAA production by *P. putida* GR12-2 and an overproducing mutant stimulated the growth of many short (< 1 mm) adventitious roots on mung bean cuttings (Mayak *et al.* 1997, Patten and Glick 2002). An IAA-deficient mutant of *P. putida* GR12-2 stimulated the formation of less roots in mung bean cuttings and the roots were on average longer than the plants inoculated with the wild-type bacterium (Patten and Glick 2002). The result of having shorter primary roots and more lateral roots is a lower root surface area and therefore, a lower ability to absorb water and nutrients and hence, a lower tolerance of drought stress (Hurd 1974, Patten and Glick 2002).

However, the production of IAA *in vitro* does not necessarily reflect the amount of IAA produced by the bacteria *in vivo*. Therefore, to determine if sunflower plants are negatively affected by the potentially high production of IAA by *P. koreensis*, the length and biomass of the sunflower's roots needs to be measured. In the present study, an attempt was made to measure the root biomass, however, the roots had a large proportion of vermiculite particles that could not be removed and which could potentially skew the results. Another contributing factor to whether or not the amount of IAA produced by *P. koreensis* has an adverse effect on plant roots is the activity of ACC deaminase. A high ACC deaminase activity would lower the amount of auxin-induced ethylene in primary roots and therefore, no inhibition of root elongation would occur at higher IAA concentrations (Růžička *et al.* 2007). It is therefore necessary to measure the ACC deaminase activity of *P. koreensis* as well as the concentration of ethylene in the plant's roots. Also attempted in the present study, was the measurement of ACC deaminase (Appendix G), however, there were complications with the assay and ultimately, the enzyme activity could not be measured.

5.1.4 Growth at -0.73 MPa

P. fluorescens had a higher OD600 value than that of *P. koreensis* after six days of growth in TSB with 25% PEG, which suggests that *P. fluorescens* is better equipped to withstand water stress for a longer period than *P. koreensis* and therefore, it can grow to a higher density of cells. The decrease in the OD of *P. koreensis* from day three to day six indicates that the culture was in the decline phase of growth (Monod 1949). In this phase, the bacterial cells start lysing resulting in fewer cells able to scatter light and hence, a lower OD. Sandhya *et al.* (2009) showed that *P. putida* increased the production of EPS with increasing levels of water stress (from -0.05 to -0.73 MPa) which indicates that EPS production occurs as a response to stress (Roberson and Firestone 1992). EPS can create a microenvironment that holds water and desiccates slower than the surrounding microenvironment therefore, the bacteria are protected from drying and changes in water potential (Hepper 1975, Wilkinson 1958). EPS are also implicated in the formation of micro- and macroaggregates of soil which can improve the flow of water and nutrients into the plant and thus, improve biomass and drought tolerance (Alami *et al.* 2000, Milošević *et al.* 2012, Sandhya *et al.* 2009).

Bacteria also produce compatible solutes called osmolytes in response to water stress, such as amino acids – glutamine, glutamate and proline, and sugars – sucrose and trehalose (Crowe

and Crowe 1992, Csonka 1989, Potts 1994). These osmolytes lower the water potential in the cytoplasm and also maintain cell turgor thus, preventing cell damage (Sandhya *et al.* 2010a). The osmolytes can also act as free-radical scavengers or chemical chaperones by directly stabilizing membranes and proteins (Diamant *et al.* 2001) and thus, protect bacteria during water stress. Under stressful conditions, energy is used for these protective mechanisms (Räsänen *et al.* 2004) and therefore, the growth of the culture is affected resulting in a lower density of cells than in unstressed conditions. The production of osmolytes is also a response of drought tolerant plants to water stress (Hoekstra *et al.* 2001).

For future work, it would be interesting to measure the amount of EPS (and what sugars make up the EPS), amino acids and sugars (Sandya *et al.* 2010) these bacteria produce under unstressed and stressed (-0.73 MPa) conditions in order to obtain a better understanding as to how these bacteria can tolerate water stress. Also, it is necessary to measure the phosphatase activity (or mineral phosphate solubilisation) and siderophore and IAA production under stressed conditions as this will give the researcher a better idea of the tolerance enhancing effects of PGPR under water stress. Sandhya *et al.* (2010a) found that the strains which produced large amounts of EPS and osmolytes under water stress also expressed better plant growth-promoting traits than the strains that produce low amounts of EPS and osmolytes.

5.2 Plant studies

5.2.1 Sterilisation, Inoculation and Germination

Sterilisation

The seeds had to be surface sterilised to ensure that there were no other microorganisms present that could potentially influence the interaction between *P. koreensis* and *H.* annuus. The use of NaClO in varying concentrations did not affect the germination of *H. annuus*; it did, however, lower the amount of contaminated seeds which suggests that it was an ideal surface sterilant for *H. annuus*. Since there was no difference between the 2% and 3.5% NaClO, the 3.5% was chosen as the concentration for the certainty that the seeds would be sterile before germination.

Inoculation

Inoculation with *P. koreensis* and *P. fluorescens* prior to germination had a deleterious effect on the germination of *H. annuus* seeds (Appendix H), which suggests that there was a flaw in

the method of inoculation. Cassan *et al.* (2009) found that inoculation of both soybean and maize with PGPR improved the final germination %, the hypocotyl/coleoptile and radicle length, and the root and shoot length and dry biomass in seedlings. Gholami *et al.* (2009) also found that inoculation with PGPR, many of which were *Pseudomonas*, improved the germination of maize seeds. Perhaps, in the present study, the time spent submerged in liquid from the start of sterilisation to the end of inoculation was too long (+/- 60 minutes) resulting in anoxic conditions inside the seed. Sunflower seeds are nondormant seeds with a large oil content and whose germination is completely inhibited when the oxygen concentration is close to 2% (Corbineau and Come 1995). It was therefore, decided to inoculate the plants post-germination for the remainder of the study.

Germination

The uninoculated seeds had a MGT of 2.2, which means that on average it took 2.2 days for the majority of *H. annuus* seeds to germinate. The MGT was slightly longer than the 1.87 days reported by Kaya *et al.* (2006) for *H. annuus* seeds, however, those authors also report a 98.6 % germination which is lower than the 100% germination observed in this study. Further, the MGT of sunflower is longer than the 0.74 and 0.99 reported by Kondra *et al.* (1983) for *Brassica campestris* and *B. napus* (Rapeseed), with 70% and 91% germination respectively.

For future studies, it would be interesting to determine why pre-germination inoculation with *P. koreensis* affected germination and to find an alternative method of inoculation that does not interfere with the germination characteristics of *H. annuus*. If this can be determined, another study would be to look at the effect of negative water potentials on the germination characteristics of *H. annuus* and to determine whether or not inoculation with *P. koreensis* can improve these characteristics.

5.2.2 Recovery and Regrowth

Drought stress negatively affects the growth of plants during and after the stress event (Mittler and Zilinskas 1994). Whereas a drought event cannot be avoided, agricultural practices can be developed to lessen the effect of drought stress on the growth of a plant and improve the recovery of plants from a stress event. However, for sunflower in the present study, the number

of days without water, specifically in the recovery and regrowth experiment, was too long and exposed the plants to a severe and prolonged drought stress that the plants could not survive. If this experiment were to be repeated, closer attention should be paid to the drop in Fv/Fm values and the number of days without water reduced so that the majority of plants survive and comparisons can be made.

5.2.3 Root bacterial counts

Unstressed plants experiment

In the unstressed experiment, the inoculated plants had significantly more bacterial colonies/g root than the uninoculated plants and the majority, of these colonies were visually identified as *P.* koreensis. In contrast, for the control plants, no colonies could be identified as *P. koreensis*. Therefore, in the unstressed experiment the difference of 18.9 cm^2 in leaf area between the uninoculated and inoculated plants can be attributed to the presence of *P. koreensis*. Also, *P. koreensis* appeared to act as an effective biocontrol agent as there were no other microbes present in the root suspensions. However, if *P. koreensis* also outcompetes other beneficial bacteria and fungi, this could be a problem in field conditions or where seed treatments are made with other beneficial bacteria and fungi. The root bacterial counts of the inoculated plants compared well with the 6 $x10^6$ cfu of *P. putida* reported by Sandhya *et al.* (2009), however the uninoculated plants has a much larger microbial count than the 3 $x10^1$ cfu reported by the same authors.

Drought stressed experiment

In the drought stressed experiment, the inoculated plants had a much higher count in the drought stressed experiment than in the unstressed experiment which suggests that *P. koreensis* can tolerate drought conditions in the rhizosphere very well and may even thrive in drought stressed conditions. This is supported by the growth data from osmotic stress experiments. However, under drought stressed conditions, the biocontrol ability of *P. koreensis* was lessened as there were a 3 $x10^7$ cfu/g root of other microbes present in the root suspensions. Unfortunately, a proper microbial count could not be determined in the uninoculated plants as the serial dilutions used had too many colonies to count. However, a large number of microbes were noted based on a visual observation. The root bacterial counts of the inoculated plants

were 1.1 x 10^3 cfu/g root larger than the 5 x10^5 cfu/g root of *P. putida* reported by Sandhya *et al.* (2009) which suggests that *P. koreensis* can tolerate drought conditions in the rhizosphere better than the drought tolerant *P. putida*.

In both the unstressed and drought stressed experiments, the microbial count in the uninoculated plants was very high and this may have lessened the drought tolerance improving effect of *P. koreensis* as the microbes present by the control plants' roots may have made these plants more drought tolerant. Therefore, it is necessary in future studies, to improve on the sterile conditions in the growth chambers and the water supplied to the plants.

Other future studies could include experiments to determine the biocontrol abilities of *P. koreensis* by adding known concentrations of pathogenic microbes to media containing *P. koreensis* and to determine the final numbers of pathogenic microbes after a set period. This could then be compared with the same experiment but under -0.73 MPa. It would also be beneficial to determine how *P. koreensis* reacts to the presence of other beneficial microbes. Finally, it would also be interesting to enumerate *P. koreensis* in the different zones of soil, namely the bulk soil, the root-adhering soil and the rhizoplane (Sandhya *et al.* 2009) to determine where the majority of *P. koreensis* colonies exist. This can be supported by examination of the root surfaces with electron microscopy.

5.2.4 Plant growth data

Many authors report an increase in plant height when inoculated with a PGPR (Saharan 2011, Adesemoye *et al.* 2008, Ashrafuzzaman *et al.* 2009). This is thought to be a consequence of the action of plant growth regulating hormones produced by these bacteria, for example, IAA, which can increase the growth of roots thereby providing the plant with access to a larger volume of soil and thus, more nutrients. Also the bacterial siderophores and phosphatase enzymes can make more soil nutrients available to the plant and thus, enhancing plant growth. Further evidence of the beneficial effects of PGPR was presented by Gholami *et al.* (2009), who observed a 14.3 – 21.7 % increase in plant height of maize seedlings inoculated with various PGPR including many *Pseudomonas* species. The height of canola and tomato was also improved by the inoculation with *Azotobacter paspoli* (Abbass and Okon 1993), however, the authors also state that the there is an optimal inoculum size in order for a positive effect on plant growth. Conversely, the height of rice was either only slightly improved or not improved

at all with inoculation of various PGPRs (Biswas *et al.* 2000) even though the plants were grown in soil.

In the present study, inoculation with *P. koreensis* had only a weak effect on seedling height in the first four weeks and no effect on the number of nodes, which can probably be attributed to the way the experiment was set up, *i.e.* having an inert substrate with a nutrient solution where all the nutrients are available to the plant did not allow for the bacteria to improve the height of the plants.

For future studies, to properly measure the effects of *P. koreensis* on plant growth characteristics, the plants should be grown in soil rather than vermiculite. Ii is likely that this approach will show a marked difference between uninoculated and inoculated plants. Also, because *P. koreensis* inhabits the rhizosphere and produces plant hormones such as IAA, the focus should be directed more to root growth than shoot growth and parameters such as root biomass, length and surface area should be measured.

5.2.5 Unstressed leaf area

Another measure of plant growth promotion is the size of the plant's leaves. A plant, such as sunflower, with a larger leaf area, can intercept more light energy than a plant of the same species with smaller leaves. The larger amount of energy being received by the plant will enhance the growth rate as well as the stores of carbohydrates that the plant will utilise during a stress such as drought. In the present study, an attempt was made to compare the leaf area of uninoculated and inoculated plants that had undergone drought stress, however, the leaf curling of the drought stressed plants to too severe and therefore, the entire leaf area could not be measured. It was then decided that the leaf area would only be measured and compared between the two treatments in the unstressed experiment.

The leaf area of the inoculated plants was larger than that of uninoculated plants. A larger leaf area is typically not an adaptation to drought stress (Morgan 1984) as a larger leaf area means that there is a larger area to loose water (Wright and Smith 1983). In severe drought stress, plants respond to the loss of water by reducing leaf area, for example, leaf rolling (Begg 1980, O'Toole and Cruz 1980). Therefore, the larger leaf area in sunflower brought about by the inoculation with *P. koreensis* may have rendered the plants less drought tolerant. However, a larger leaf area is beneficial during periods without stress as there is an increase in the photosynthetic rate which, in turn, increases plant biomass and yield (Karami *et al.* 1980,

Wright *et al.* 1983). The increase in photosynthesis would allow an increase in the amount of sugars exuded by the plant's roots and therefore, increase the amount of bacteria on the roots (supported by the root microbial counts). This could increase the beneficial effect of the bacteria, *i.e.* higher concentration of chemicals, greater enzyme activity, and should therefore, increase the tolerance of the plant to drought stress.

Plants may adapt to an increased leaf area by increasing the leaf angle which therefore, decreases the amount of effective leaf area to intercept radiation during the hottest time of the day thus, decreasing the transpiration rate and water loss. Hence, it would be interesting to determine whether or not sunflower plants demonstrate this strategy to decrease water loss and to determine the effect of inoculation with *P. koreensis* on the ability of sunflower to reduce the effective leaf area during drought stress. Another potential parameter to measure is the rolling index, *i.e.* the ratio of the rolled leaf width to the maximum leaf width, to determine whether or not inoculated sunflower can effectively reduce water loss during drought stress.

5.2.6 Leaf RWC

The water content of the leaves of a drought stressed plant gives an indication of how well the plant can tolerate the lack of water surrounding the roots. In the present study, the finding that inoculation had no effect on the RWC of stressed sunflower leaves is contrary to many reported studies (Creus *et al.* 1997, Karlidag *et al.* 2013, Naveed *et al.* 2014), which state that inoculated water stressed plants have higher leaf RWC values than uninoculated plants. A drought stressed plant with a higher leaf water content would be more tolerant of drought stress than a plant with a lower leaf water content. Karlidag *et al.* (2010) found that there was, on average a 14% increase in the RWC of strawberry plants under salt stress inoculated with a variety of PGPR. However, del Mar Alguacil *et al.* (2009) reported that *P. mendocina* did not improve the leaf RWC of drought stressed lettuce seedlings. This indicates that sometimes there is a plant-microbe specific interaction with regards to leaf water content and perhaps this was the case with sunflower and *P. koreensis*.

5.2.7 Leaf electrolyte leakage

Another indication of drought stress tolerance is the effectiveness of cell membranes to retain their structure and functioning, *i.e.* to keep important electrolytes within the cell while the amount of water surrounding the membrane is decreased. The fact that inoculation had no effect

on the electrolyte leakage in both the unstressed and stressed experiments suggests that *P. koreensis* had no influence on membrane permeability and membrane damage related to the leakage of electrolytes. The finding is similar to that reported by several researchers (Bano and Fatima 2009, Shukla and Agarwal 2012). Bano and Fatima (2009) found that when two cultivars of maize were inoculated with *Pseudomonas* and subjected to salt stress, there were no differences in the electrolyte leakage as compared with uninoculated salt stressed plants. The authors also found that the different cultivars of maize had different responses to inoculation and salt stress. The Agaiti 2002 cultivar that was inoculated with *Rhizobium* and exposed to salt stress had electrolyte leakage values that were no different from uninoculated salt stressed plants, while the AV 4001 cultivar had a lower electrolyte leakage than the uninoculated plants (Bano and Fatima 2009). The same pattern was found when both cultivars were inoculated with a combination of *Rhizobium* and *Pseudomonas* and exposed to salt stress. Similar results were found by Shukla and Agarwal (2012), where some PGPR were able to decrease the electrolyte leakage of salt stressed *Arachis hypogaea* while others had no effect. These studies suggest that there is a specific plant-microbe interaction that is different with different species of plants and bacteria and even between different cultivars and strains. However, there are also several studies that report lower electrolyte leakage in inoculated drought stressed plants (Bano and Fatima 2009, Sandhya *et al.* 2010b, Shukla and Agarwal 2012, Vardharajula *et al.* 2011). Sandhya *et al.* (2010b) found that drought stressed maize had increased electrolyte leakage as compared with unstressed plants, however, inoculation with a variety of PGPRs decreased the electrolyte leakage over the uninoculated drought stressed plants. Several studies have also found that in unstressed conditions, inoculated plants have lower electrolyte leakage than uninoculated plants (Sandhya *et al.* 2010b, Shukla and Agarwal 2012).

In the studies that found that the electrolyte leakage of drought stressed plants was decreased in inoculated plants (Li-Ping *et al.* 2006, Sandhya *et al.* 2010b, Vardharajula *et al.* 2011), it was also found that electrolyte leakage was correlated with the activity of antioxidant enzymes such as SOD, POD and CAT, indicating that membrane injury is a result of oxidative damage. Generally, drought stress induces a decrease in the activity of antioxidant enzymes which leads to an increase in lipid peroxidation resulting in a higher membrane permeability (Chen and Dai 1994, del Longo *et al.* 1993, Qu *et al.* 1996, Li-Ping *et al.* 2006). However, plants inoculated with PGPR and subjected to drought stress have higher enzyme activity and therefore, less membrane damage (Sandhya *et al.* 2010b, Shukla and Agarwal 2012, Vardharajula *et al.* 201).

Malondialdehyde (MDA) is produced as a result of the decomposition of polyunsaturated fatty acids of membranes (Heath and Packer 1968), therefore, the presence of MDA in a plant sample reflects the extent of the stress induced as well as peroxidative damage by ROS (Jain *et al.* 2001). Therefore, it would be interesting to measure the MDA content and activity of SOD, POD and CAT, in drought stressed sunflower and compare this to inoculated drought stressed sunflower in order to gain a better understanding as to why *P. koreensis* had no effect on membrane permeability.

5.2.8 Chlorophyll fluorescence

Chlorophyll fluorescence is a measure of the performance of PSII and therefore, relates to the activity of photosynthesis which, if maintained during periods of drought stress, could suggest tolerance to drought. The Fv/Fm measurement is the maximum quantum yield of PSII if all PSII centres were open (Maxwell and Johnson 2000). From the results in the present study, *i.e.* the inoculated and drought stressed sunflower plants had lower Fv/Fm values than the uninoculated drought stressed plants; this suggests that inoculation affected the maximum PSII efficiency of drought stressed sunflower. However, the Fv/Fm values of the drought stressed inoculated plants were still within the optimal or 'healthy' range (Björkman and Demmig 1987, Johnson *et al.* 1993, Maxwell and Johnson 2000) which suggests that inoculation did not severely affect the photosynthetic performance of drought stressed sunflower in this study. The differences observed could be due to sample variation. Rincón *et al.* (2008) found that drought stressed *Pinus halepensis* inoculated with *P. fluorescens* Aur6 had higher Fv/Fm values than uninoculated drought stressed *P. halepensis*. However, inoculation had no effect on the photochemical efficiency of PSII in drought stressed *Quercus coccifera* plants (Rincón *et al.* 2008). This, once again, indicates that there are plant-microbe interactions that are specific to certain species of bacteria and plants.

The measurement of chlorophyll fluorescence is not however, an ideal measurement of photosynthesis in drought stressed plants and this is shown in the present study as there were no differences in the unstressed and stressed Fv/Fm values in either the uninoculated or the inoculated plants. However, the measurement was taken because of what was available to the researcher at the time that the experiments were conducted. A more sensitive measurement of photosynthesis in drought stress experiment is Y(II) which reflects the amount of energy used in photochemistry by PSII under steady state photosynthetic lighting conditions. It would

therefore, be of interest to observe any differences in Y(II) brought about by inoculation in drought stressed sunflower. Y(II) is also directly related to e^- transport rate (ETR).

5.2.9 Substrate water content

The water content of the substrate a plant is grown in gives an indication of the level of drought stress that the plant is exposed to. The inoculated drought stressed plants had a lower substrate water content than the uninoculated plants which suggests that the inoculation caused sunflower plants to use more water. The reason for this could be linked to the potentially high IAA production by *P. koreensis*. The higher concentration of IAA in the inoculated plants would enhance root growth and therefore, these plants would have an increased surface area from which to draw water out from the substrate. However, root biomass data would be necessary to confirm this. Another possibility is that the larger leaf area in inoculated plants allowed for these plants to use more water.

If the substrate water content gives an indication of the level of drought stress exposed to a plant, then it would seem that the inoculated plants were exposed to a slightly more severe drought stress than the uninoculated plants. Considering this, the inoculated *H. annuus* had similar leaf water contents and electrolyte leakage values to the uninoculated plants but were exposed to a larger water deficit, suggesting that *P. koreensis* did improve the drought tolerance of sunflower plants. However, future studies, where the level of drought stress is quantified, *i.e.* with the use of PEG, may show greater differences in the physiological parameters measured in this study. In order to determine if inoculation results in sunflower plants using more water under drought stress, measurements of stomatal conductance and transpiration rates should be taken.

5.2.10 Total phenolic compounds

Phenolic compounds are utilised as accessory pigments and antioxidants during drought stress and therefore, help a plant tolerate a water deficit. Inoculation with *P. koreensis* enhanced the phenolic content of drought stressed sunflower leaves which suggests that *P. koreensis* upregulates the phenolic compound biosynthesis pathway, presumably in response to the accumulation of ROS (de Abreu and Mazzafera 2005, Michalak 2006). There is not an abundance of studies that have investigated the effect of inoculation with PGPR on the levels of phenolic compounds or the regulation of the biosynthesis pathway in water stressed plants.

However, it has been found that there is a general increase in the total phenolic content of pea roots when inoculated with *P. aeruginosa* or *P. fluorescens* (Singh *et al.* 2002). Betelvine inoculated with *Serratia marcescens* also had an increased PAL activity in both leaves and roots (Lavania *et al.* 2006) and Jaleel *et al.* (2009) reported that there was no difference in the total phenolic content in the roots or leaves of *Catharanthus roseus* inoculated with *P. fluorescens*, although there was a slight increase (1 mg/g FW) in the stem – an increase similar to the results of the present study.

It has also been shown that plants inoculated with a PGPR and subjected to a biotic stress have increased levels of phenolics over control plants and over inoculated unstressed plants (Singh *et al.* 2002, Lavania *et al.* 2006). Additionally, it has been found that plants inoculated with PGPR and subjected to drought stress have an increased antioxidant status (Han and Lee 2005, Kohler *et al.* 2008, Sandhya *et al.* 2010b). It is well known that legume plants exude flavonoids from the roots which leads to the secretion of nodulation factors by rhizobia and ultimately the formation of nodules (Dardanelli *et al.* 2010). In this way, the flavonoids are used as a signalling molecule that sets up a symbiotic relationship between the plant and the microorganism. Flavonoids, as well as other phenolic compounds exuded by plant roots, may represent a source of C for rhizosphere microorganisms and PGPR have been shown to elicit changes in the pattern of flavonoids secreted under normal conditions and under salt stress (Dardanelli *et al.* 2010, Shaw *et al.* 2006).

Total phenolic content is measured by the Folin-Ciocalteu procedure. However, this procedure does not fully quantify all phenolic compounds in the sample as there are potential interferences with sugars and ascorbic acids (Kähkönen *et al.* 1999, Singleton and Rossi 1965). Also, Kähkönen *et al.* (1999) did not find a correlation between total phenolic content and antioxidant ability, therefore, a high phenolic content does not necessarily mean a high antioxidant potential. The state that the phenolic compound is in affects its antioxidant ability, *i.e.* flavonoids usually occur as glycosides which makes them water soluble enabling storage in the vacuole, however, in this form they are less reactive towards free radicals (Rice-Evans *et al.* 1997).

Future studies could focus on the determination of phenolic content from the stems and roots of drought stressed sunflower plants and comparison of this data with inoculated drought stressed plants. This will give a deeper understanding of where *P. koreensis* has the greatest effect on the phenolic content of drought stressed sunflower – the leaves, stems or roots.

Another interesting study would be to isolate the phenolic compounds exuded by sunflower roots and to determine whether or not *P. koreensis* utilizes phenolic compounds as a source of C. Also, the exuded phenolics under normal conditions could be identified and quantified and compared with drought stressed exudations and also compared with inoculated plants. This will determine whether or not the pattern of exuded phenolics is changed by drought stress or inoculation.

5.2.11 Proline

Proline production is a ubiquitous response of many organisms to water deficit. It acts as a compatible solute, a store of C and N, it stabilises macromolecules, reduces ROS build-up, and acts as a stress signal bring about response mechanisms in order to tolerate drought stress (Szabados and Savouré 2010). In the present study, the fact that the inoculated drought stressed plants accumulated more proline in the leaves than the uninoculated plants, suggests that *P. koreensis* influenced the metabolism and/or catabolism of proline in drought stressed sunflower. It has been found previously that drought stressed sunflower has an increased proline content in the leaves compared with unstressed plants (Cechin *et al.* 2006). The latter authors also found that proline levels returned to unstressed values only 24 hours after rewatering drought stressed plants (Cechin *et al.* 2006). Manivannan *et al.* (2007) also found that drought stressed sunflower had an increased accumulation of proline, with the highest accumulation occurring in the roots. The authors attributed the accumulation of proline to an increased activity of γ-glutamyl kinase and a decreased activity of proline oxidase (Manivannan *et al.* 2007). A high level of proline during stress allows for the regulation of negative water potentials. In addition, the proline also stabilizes membrane proteins and enzymes, scavenges ROS, maintains redox homeostasis and reduces photoinhibition and damage to the photosynthetic apparatus (Delauney and Verma 1993, Hare and Cress 1997, Szabados and Savouré 2010, Kumari *et al.* 2015). After stress, the degradation of accumulated proline distributes e^- to the respiratory chain and therefore, subsidises the energy supply when growth recommences (Hare and Cress 1997, Kishor *et al.* 1995).

The increased proline in drought stressed plants has been shown to be enhanced by inoculation with PGPR. Heidari *et al.* (2011) found that drought stressed basil inoculated with a variety of different PGPR had an increased proline content over uninoculated plants. Under normal conditions, inoculated soybean plants had a similar proline content to uninoculated plants

(Kumari *et al.* 2015). However, under salt stress conditions, the inoculated plants had a greater accumulation of proline than the uninoculated plants, with the highest accumulation occurring in the roots (Kumari *et al.* 2015). The authors showed that the increase in proline can be attributed to the overexpression of the P5CS gene caused by the inoculation with PGPR (Kumari *et al.* 2015). P5CS is a rate-limiting enzyme in the biosynthesis of proline (Delauney and Verma 1993, Szabados and Savouré 2010). Biosynthesis of proline is controlled by two P5CS genes, one is a housekeeping gene while the other is a stress specific P5CS isoform (Szabados and Savouré 2010). In *Arabidopsis*, *P5CS1* is induced by osmotic and salt stress and is activated by an ABA-dependant and ABA-insensitive pathway and H_2O_2 derived signals (Yoshiba *et al.* 1995, Savouré *et al.* 1997). Therefore, it is possible that PGPR enhance the production of proline via regulating ABA-dependant and ABA-independent pathways and by also influencing ROS signals. However, the direct mechanism of increased proline biosynthesis in water stressed plants inoculated with PGPR has not been elucidated. PGPR also exude osmolytes, such as proline, which acts synergistically with plant-produced proline (Kaushal and Wani 2016, Paul and Nair 2008).

For future studies, it would be interesting to determine the proline content of drought stressed sunflower in the stems and roots and compare that with the data in the present study and also with inoculated plants. If the proline content of the roots in inoculated plants is higher than that of the stems and leaves, it would suggest a maintenance of water potential ensuring water flow into the roots and lessening the drought stress damage in the plant (Porcel *et al.* 2004).

6 UNDERSTANDING THE INTERPLAY BETWEEN *P. KOREENSIS* AND *H. ANNUUS*

The symbiotic interaction between rhizosphere bacteria and host plants is complex and multi-faceted thus, making it difficult to uncover the mechanisms that make the interaction beneficial. In the present study, a systematic approach was undertaken to study how the drought stress response of *H. annuus* was influenced by *P. koreensis*. The drought stress response of *H. annuus* could be influenced by *P. koreensis* in the following ways, as observed in this study.

The phosphatase activity of *P. koreensis* would increase the amount of available P for *H. annuus*. P is a very important nutrient for plants as it is present in nucleic acids which regulates

protein synthesis. Therefore, P is instrumental in bringing about the plethora of responses to tolerate drought stress and *H. annuus* with better P nutrition, *i.e.* inoculated with *P. koreensis*, would be better able to tolerate drought than if the plant was not inoculated with *P. koreensis*.

Fe is also an important micronutrient for plants, required for photosynthesis, respiration, N fixation, DNA synthesis and hormone synthesis. Fe also plays a role in ROS signalling and quenching. Therefore, the increased amount of available Fe to *H. annuus*, as brought about by the siderophores secreted by *P. koreensis*, could have improved the drought stress response as follows: the increased photosynthesis would allow for a greater store of reserves that become imperative during drought stress as a source of energy to bring about response mechanisms such as an increase in phenolic compounds and proline. It was found in the present study that the concentration of both phenolic compounds and proline was increased in drought stressed *H. annuus* inoculated by *P. koreensis* and this could be due to the increased amount of Fe available to these plants. The improved ROS signalling and quenching capabilities as a result of increased Fe availability from siderophores, of *H. annuus* inoculated with *P. koreensis* would have improved the drought tolerance of the plant. It is important to note however, that ROS have a duel role in plants; they can be a stress signal which brings about a response to tolerate that stress, however, large amounts of ROS can lead to plant death. Therefore, a balance between ROS production and quenching is imperative in tolerating drought stress and the ability of *H. annuus* to maintain this balance should be improved with the inoculation of *P. koreensis*.

Another important interaction could be the effect of the IAA produced by *P. koreensis*. IAA would improve the drought tolerance of *H. annuus* because IAA promotes root growth through cell division and cell elongation, as well as having an interplay with the activity of ACC deaminase – the IAA stimulates ACC deaminase which in turn decreases the concentration of the growth inhibiting hormone ethylene. *H. annuus* inoculated with *P. koreensis* should have larger roots than those plants without *P. koreensis* and would therefore, have a greater access to water. The plant would also be able to better detect the soil moisture content as a larger volume of soil would be occupied by the plants roots and could therefore, be able to trigger an earlier response to the drought stress than if the roots were smaller, *i.e.* without *P. koreensis*. The increased root surface area could also have increased the PGPR effect of *P. koreensis* as there would be more space for the bacteria to inoculate thus, increasing the concentration of bacteria on the plant. *H. annuus* with a larger root system would secrete more sugar into the rhizosphere thus, sustaining and enhancing the *P. koreensis* population.

Inoculation of *H. annuus* with *P. koreensis* also increased the concentration of phenolic compounds and proline in the leaves of drought stressed plants, most likely via increased PAL activity and P5CS, respectively. Both of these secondary metabolites are an important drought tolerance response and is the main mechanism by which *P. koreensis* improves the drought tolerance of *H. annuus*, as uncovered by this study.

7 CONCLUSION

P. koreensis outperformed *P. fluorescens* on all aspects studied, *i.e.* phosphatase activity, siderophore and IAA production. However, *P. fluorescens* slightly outperformed *P. koreensis* when grown under osmotic stress. Regardless, *P. koreensis* seemed like the better bacterium to inoculate *H. annuus* plants with. Inoculated plants were slightly larger in height and had a larger leaf area than uninoculated plants. In the drought stressed plants, no differences were found in the leaf water content, electrolyte leakage and chlorophyll fluorescence between uninoculated and inoculated plants. However, the substrate water content of inoculated plants was lower than that of uninoculated plants, showing that the inoculated plants were exposed to a greater level of water stress than the uninoculated plants. The inoculated drought stressed plants had a higher concentration of phenolic compounds and proline in the leaves than the uninoculated plants. Therefore, in conclusion, inoculation of *H. annuus* with *P. koreensis* improved the drought tolerance of *H. annuus* by increasing the concentration of drought tolerance important biochemicals, namely phenolic compounds and proline.

8 APPENDICES

APPENDIX A

Dworkin and Foster (DF) medium preparation (Penrose and Glick 2003, Dworkin and Foster 1958)

Trace salts

Solution 1:

- H_3BO_3 – 0.01 g,
- $MnSO_4.H_2O$ – 0.01119 g
- $ZnSO_4.7H_2O$ – 0.1246 g
- $CuSO_4.5H_2O$ – 0.07822 g
- MoO_3 – 0.01 g

Dissolve in 100 mL deionised dH_2O. Autoclave. Store in fridge until needed.

Note: Add one component at a time, ensure chemical is completely dissolved before adding the next one. Heat solution to dissolve $CuSO_4$/MoO_3

Solution 2:

- $FeSO_4$ – 0.1 g

Dissolve in 10 mL sterile deionised dH_2O. Store in fridge until needed.

Solution 3:

- Glucose – 36.032 g

Dissolve in 100 mL warm (50 °C) deionised dH_2O for a final concentration of 2 M. Filter sterilize. Store in fridge until needed.

Major salts

Solution 4:

- KH_2PO_4 – 4 g
- Na_2HPO_4 – 6 g
- $MgSO_4.7H_2O$ – 0.2 g
- Gluconic acid – 2 g
- Citric acid – 2g
- $(NH_4)_2SO_4$ – 2 g
- Solution 1 – 0.1 mL
- Solution 2 – 0.1 mL

Dissolve in 995 mL deionised dH_2O. Adjust to pH 7.2 with 10 M NaOH. Autoclave for no longer than 20 minutes. After autoclaving, cool medium to 50 °C and aseptically add 5 mL of Solution 3. Store DF medium in fridge until needed.

APPENDIX B

Modified M9 (MM9) medium preparation (Alexander and Zuberer 1991)

Trace salts

Solution 1:

- $CuSO_4.5H_2O$ – 0.01 g

Dissolve in 250 mL deionised dH_2O. Store in fridge until needed.

Solution 2:

- Glucose – 20 g
- Mannitol – 20 g
- $MgSO_4.7H_2O$ – 4.93 g
- $CaCl_2.2H_2O$ – 0.1457 g
- $MnSO_4.H_2O$ – 0.0117 g
- H_3BO_3 – 0.014 g
- $ZnSO_4.7H_2O$ – 0.012 g
- $NaMoO_4.2H_2O$ – 0.01 g
- Solution 1 – 1 mL

Dissolve in 1 L deionised dH_2O. Autoclave. Store in fridge until needed.

Solution 3:

- Casamino acids – 30 g

Dissolve in 100 mL deionised dH_2O. Autoclave. Store in fridge until needed.

Major salts

Solution 4:

- KH_2PO_4 – 0.3 g
- NaCl – 0.5 g
- NH_4Cl – 1.0 g
- PIPES buffer – 30.24 g OR HEPES buffer – 23.83 g

Dissolve in 700 mL deionised dH_2O. Adjust to pH 6.8 with 50% KOH. Make volume up to 800 mL. Autoclave

Once all solutions are prepared, heat the solutions to 50 °C then aseptically add 100 mL of Solution 2 and 10 mL of Solution 3 to Solution 4. Then make up the volume of Solution 4 to 1 L with sterile deionised dH_2O. Store the MM9 medium in the fridge until needed.

APPENDIX C

Phosphatase activity: 30 minute incubation

Table 3. Phosphatase activity of *Pseudomonas fluorescens* and *P. koreensis* with a 30 minute incubation time. A_{405} is the absorbance at 405 nm. Mean ± SD.

	Acidic phosphatase		**Alkaline phosphatase**	
Bacteria	**A_{405}**	**µM/min/OD600**	**A_{405}**	**µM/min/OD600**
Pseudomonas fluorescens	0.098 ± 0.012	-18.747 ± 5.925	-0.002 ± 0.003	-64.206 ± 4.659
Pseudomonas koreensis	0.101 ± 0.017	-22.763 ±10.688	0.006 ± 0.002	-82.152 ± 3.362

The absorbance values of the phosphatase enzyme reaction product (*p*NP) were, on average, below that which is considered to be an accurate, trustworthy reading (*i.e.* between 0.1 – 0.9). Therefore, it was decided that the original incubation time of 30 minutes (as was used in the original paper from which the researcher obtained the method) was too short for the entire substrate to be hydrolysed by the phosphatase enzymes. An incubation time of two hours was then used thereafter.

APPENDIX D

Chlorophyll fluorescence: fluorometer settings and clamp placement

Settings

The following settings were based on the 'Auto-detect' feature of the OS1p:

Table 4. Fluorometer (OS1p) settings for Fv/Fm measurement

Modulation intensity	10%
Detector gain	1
Saturation intensity	100%
Far red	Off
Flash width	0.8 seconds
Flash zoom	Off

Clamp placement

Figure 21. Dark adaptation clamp placement on sunflower leaf. Leaves were dark adapted for 1 hour.

APPENDIX E

Water stress regime: chlorophyll fluorescence measurements

Uninoculated plants

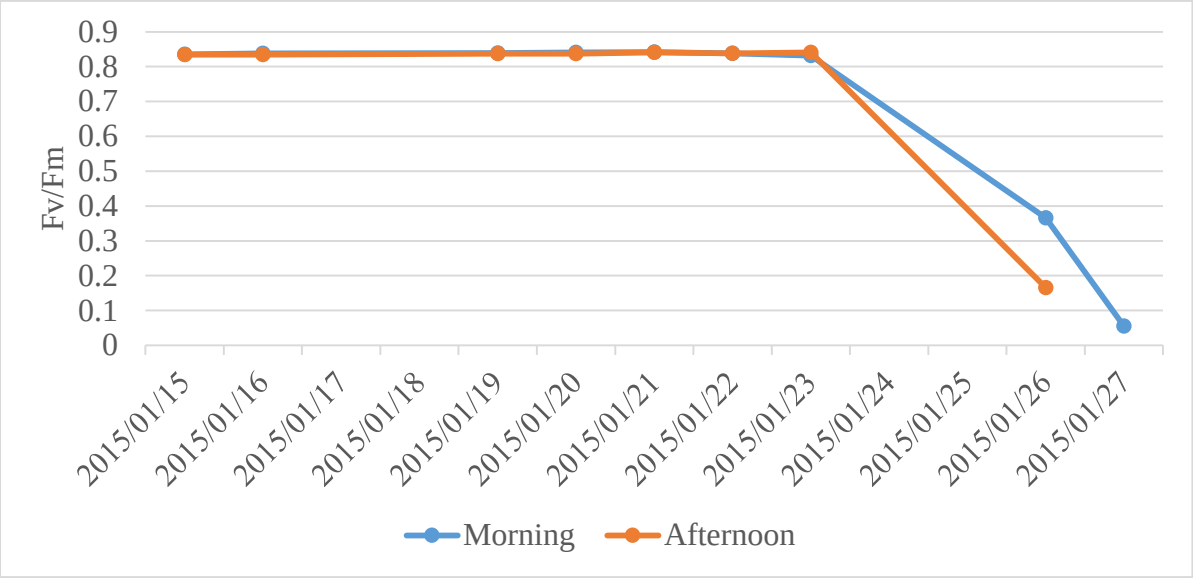


Figure 22. Fv/Fm values of uninoculated sunflower plants in the morning and afternoon during a period of decreasing substrate water content.

Inoculated plants

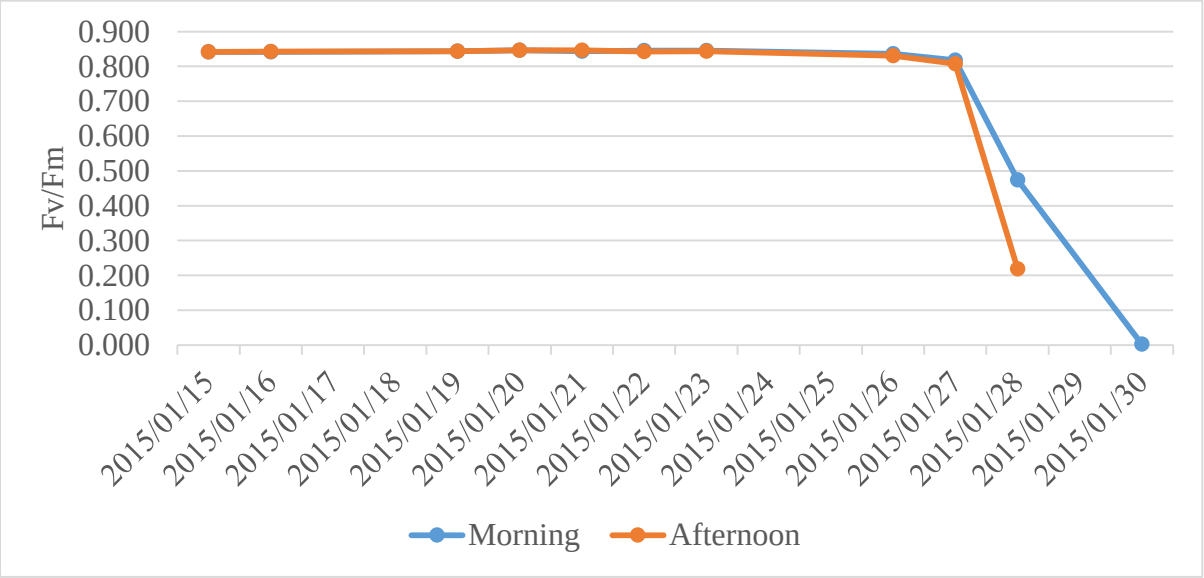


Figure 23. Fv/Fm values of inoculated sunflower plants in the morning and afternoon during a period of decreasing substrate water content.

APPENDIX F

Proline sample weights

The original proline assay required the use of 0.5 g leaf samples. However, to ensure there was sufficient leaf material for both the proline and phenolic compound assay lower sample weights were tested to determine whether or not there would be a difference in the final proline concentration of the leaf sample.

Table 5. Proline concentration from sunflower leaf samples of different weights. Mean ± SD.

Sample weight	**Proline concentration (µmol/g DW)**
0.5 g	2.10 ± 0.10^{a}
0.2 g	2.20 ± 0.20^{a}
0.1 g	2.49 ± 0.06^{b}

*Values that share the same letters are not significant at P = 0.05

There was no evidence that the proline concentration in the leaf samples were different for the 0.5 g and 0.2 g samples, however, the 0.1 g samples had a higher concentration of proline (Table 5.). Therefore, it was concluded that the use of 0.2 g leaf samples would not significantly alter the outcome of the accuracy of the proline assay and a 0.2 g sample was thereafter used.

APPENDIX G

ACC deaminase

The absorbance values obtained after being ‘blanked’ with the reagents and the culture supernatant were unreliable. The reason behind the unreliable results is believed to be caused by ACC deaminase activity occurring in the culture blank, breaking down the substrate and therefore, the abnormally high readings. The discrepancies in the results obtained between the first and second replicate further indicate problems with the assay. Due to time constraints, further investigation into the ACC deaminase assay was ceased.

APPENDIX H

Sunflower germination: inoculation with *Pseudomonas fluorescens*

Table 6. Number of germinated seeds of sunflower inoculated pre-germination with *Pseudomonas fluorescens*. Total number of seeds = 45.

Day 1	**Day 2**	**Day 5**	**Day 12**
0	2	5	9

The above data show the poor germination characteristics (20% final germination) of sunflower when inoculated pre-germination with *Pseudomonas fluorescens*. This experiment was conducted to determine whether *P. koreensis* negatively affected the germination of sunflower or rather the inoculation technique negatively affected the germination of sunflower. Although the germination characteristics of sunflower inoculated with *P. fluorescens* is better than that with *P. koreensis* (0% final germination), however, the germination % of sunflower inoculated with *P. fluorescens* is significantly lower than that without inoculation (100%). It was therefore concluded, that the inoculation technique affected the germination of sunflower and thus, the plants were inoculated post-germination. Plants inoculated in this way were healthy and did not show any signs of disease or damage.

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