Optimisation of approaches to study plant-microbe interactions under drought stress

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Signed on 24 Day of May, 2018 in Johannesburg

Declaration

I declare that this Dissertation is my own original and unaided work, and has not been submitted in any form for any degree or diploma at any other tertiary institution. Any research work conducted by other investigators has been duly acknowledged in the following text, as evidenced by citations and references.

The experimental work described in this dissertation was carried out in the School of Animal, Plant and Environmental Sciences, University of the Witwatersrand, Johannesburg, South Africa, under the supervision of Dr Kershree Padayachee (School of Animal, Plant and Environmental Sciences, University of the Witwatersrand) and Professor Karl Rumbold (School of Molecular and Cell Biology, University of the Witwatersrand).

Signature:  (Taralyn Moodley - 476326)

On the 24 day of May 2018 in Johannesburg, South Africa.

Dedication

For my parents and sister.

Acknowledgements

To my parents and sister for their love and encouragement. Thank you for always being an amazing support system. You mean more than the world to me and I owe every triumph and accomplishment to you entirely.

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ACC	1-Aminocyclopropane-1-carboxylate
ABA	Abscisic acid
ATP	Adenosine triphosphate
APX	Ascorbate peroxidases
AA	Ascorbic acid
CE-MS	Capillary electrophoresis-mass spectrometry
CO_2	Carbon dioxide
CAT	Catalases
cm	Centimeters
cm/week	Centimeters per week
CFU	Colony forming units
C	Control
CK	Cytokinins
°C	Degrees Celsius
dH2O	Distilled water
DWB	Dry weight basis
ET	Ethylene
FC	Folin-Ciocalteau
FWB	Fresh weight basis
GAE	Gallic acid equivalent
GC-MS	Gas chromatography-Mass spectrometry
GAS	General adaptation syndrome
GA	Gibberellins
$C_6H_{12}O_6$	Glucose
GR	Glutathione reductase
g	Gram
>	Greater than
≥	Greater than or equal to
Hz	Hertz
IAA	Indole-3-acetic acid
ISR	Induced systemic resistance
IST	Induced systemic tolerance

IS	Inoculated, stressed plants
I	Inoculated, unstressed plants
JA	Jasmonates
LEA	Late embryogenesis abundant proteins
<	Less than
≤	Less than or equal to
LC-MS	Liquid chromatography-mass spectrometry
MGT	Mean germination time
m	Meter
µg	Microgram
µl	Microlitre
µmole	Micromoles
mg	Milligrams
mL	Millilitre
mm	Millimeter
mM	Millimolar
M	Molar
MS	Murashige and Skoog nutrient medium
nm	Nanometers
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance spectroscopy
NB	Nutrient broth
%	Percentage
POD	Peroxides
PAL	Phenylalanine ammonia-lyase
PGPR	Plant growth promoting bacteria
±	Plus and /or minus
pH	Potential of hydrogen
PCD	Programmed cell death
REDOX	Reaction-oxidation reaction
ROS	Reactive oxygen species
RSD	Relative standard deviation

rpm	Revolutions per minute
NaCl	Sodium chloride
NaClO	Sodium hypochlorite
cm^2	Square centimeters
SOD	Superoxide dismutase
SE	Standard error
S	Stressed plants
UNFCCC	United Nations Framework Convention on Climate Change
VOCs	Volatile organic compounds

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Abstract

Food insecurity is a rising concern in the present era, with agriculture facing potentially severe limitations due to environmental changes. The greatest of these limitations, is the effect of drought induced water stress on crops. The use of plant growth promoting bacteria (PGPR) has however, been shown to significantly improve plant growth, productivity, and tolerance and resistance mechanisms. This study focussed on optimising approaches for the study of plant-microbe interactions under drought stressed and unstressed conditions. Physiological, biochemical and metabolomic approaches were optimised in this study. This was achieved by using *Helianthus annuus* and *Pseudomonas koreensis*. There were four sampling treatments which included: a control set of plants that was neither stressed nor inoculated (C), an inoculated, unstressed set (I), an inoculated and water stressed set (IS), and an uninoculated set, water stressed set (S). The physiological measurements conducted included height and leaf area, both of which were found to be significantly larger in the inoculated treatments. Biochemical analyses included ROS, phenolic acids and proline assays. Both phenolic acids and proline were significantly upregulated in inoculated plants, which was likely in response to a ROS spike. The uninoculated, stressed subset showed severe deterioration, with some plants dying. This result, in comparison with responses observed in stressed, inoculated plants further demonstrated the tolerance mechanisms elicited by the PGPR in plants under drought stress. The metabolite extraction and sampling technique optimised in this study, showed a high technical reproducibility for leaf and biofilm extraction methods. Leaf and biofilm metabolites appeared to have larger metabolite peak areas in inoculated plants. Metabolite extraction was however, less successful for root samples. It can be concluded that the overall health and drought tolerance of *H. annuus* plants were enhanced significantly, compared to uninoculated plants. The use of various experimental approaches and techniques in this study enabled a systems interpretation of plant-microbe interactions under drought stress. However, further studies are required for a better understanding of biochemical and metabolite pathways that are exclusively associated with tolerance mechanisms, which can be done using metabolite techniques optimized in this study.

Chapter One: Background and Literature Review

1.1 Background

The earth is a dynamic system and changes to this system have occurred since the beginning of time. These changes occur as a result of any consistent trend in the environment that affect the earth's system and its mechanisms (Schellnhuber *et al.* 1997, Vitousek, 1992, Vitousek, 1994). However, these fluctuations have substantially increased since the start of the industrial revolution due to anthropogenic influences, such as increased population growth, resulting in the current predicament, which is global change (Falkowski *et al.* 2000, Robin *et al.* 2013, Vitousek, 1992, Vitousek, 1994). Over the last 60 years, the population has grown from 2.5 billion people in 1950, to over 7 billion people currently and is expected to reach nearly 9.5 billion in 2050 (United States Census Bureau, 2014). The increased human population growth is unsustainable and to the detriment of resource and land use, which in time will likely result in natural constraints on said population (Robin *et al.* 2013, Vitousek, 1992).

Global change has been linked to increased temperature, changes in precipitation (resulting in flooding and droughts), rising sea levels, ocean acidification and increased atmospheric carbon dioxide. One of the main concerns regarding global change is its impact on the earth's hydrological cycle which can affect vegetation considerably (Tuba *et al.* 2007). Thus, contributing greatly to agricultural issues and increases in food insecurity which is of great concern for human well-being, especially with the rapid growth in population.

Agriculture plays an important role in mitigating poverty and hunger problems, especially on the African and Asian continents where agriculture serves as an important component in maintaining economic sustainability (Rijsberman, 2006). South Africa for example, has a dual agricultural economy which means that there are large-scale well developed, commercial farms as well as small-scale, less developed rural subsistence farms.

Food insecurity is expected to increase in the next half century, where several countries are likely to have a food deficient and approximately 80 million additional people worldwide are likely to be at risk of hunger due to effects of climate change on agriculture (Parry *et al.* 1999). The African continent alone is expected to have approximately 60 million additional people at risk of hunger (Parry *et al.* 1999). The United Nations Framework Convention on Climate Change (UNFCCC) secretary stated in an address delivered in February 2011 that: "On a global level, increasingly unpredictable weather patterns will lead

to falling agricultural production and higher food prices, leading to food insecurity" (FAO., 2011).

Water scarcity is one of the main environmental concerns for agriculture, due to drought-induced loss in crop yield being higher than other comparable environmental factors (Farooq *et al.* 2009, Singh, 2000, Skoric, 2009). Most crops are grown on soil that receive moisture from precipitation and irrigation systems (Rosegrant *et al.* 2009). Irrigated agricultural fields use approximately 70 % of the world's freshwater supply, this has resulted in increased overall agricultural yield in irrigated fields when compared to fields that are purely rain fed (Rosegrant, *et al.* 2009). However, the agricultural industry will be faced with many critical problems regarding acquisition of adequate water supply due to the current increase in demand for water as a consequence global climate change and increased population size.

Drought frequency, duration and severity has increased substantially over the years and is predicted to continue to increase in subsequent years, as a result of climate change (Allen *et.al* 2010, Chaves *et al.* 2002, Lisar *et al.* 2012, Sheffield *et al.* 2012). These environmental changes may directly affect the metabolic, physical and chemical composition of plants, thereby increasing the degree of stress placed on the plant and increasing the pressure for successful plant production, growth and survival. This, in turn, results in a critical problem for agriculture (Drake *et al.* 1997).

The establishment of adequate drought mitigation techniques is therefore, critical. Further, establishing techniques that will decrease detrimental effects to the environment through noninvasive means is cardinal for our progression into a more sustainable world. Plant growth promoting bacteria (PGPR) has been found to be highly beneficial to crop production and development in a noninvasive manner. The interactions of plants and microbes are still being understood, therefore the development of approaches to understand and study these interactions is very beneficial. However, to explore and exploit such biotic interactions, it is necessary to first understand each component independently, as outlined below.

1.2 Mechanisms of water movement in plants and photosynthesis

Water is the most important limiting and influential abiotic factor for plant growth and productivity due to it being a vital molecule in physiological processes and the transportation of nutrients through the plant (Bohnert and Jensen, 1996, Lisar *et al.* 2012, McElrone *et al.* 2013). Water comprises approximately 80-90% of the biomass of non-woody plants (Lisar *et al.* 2012). All water used by terrestrial plants is essentially absorbed from the soil by the roots and some root systems form symbiotic relationships with microbes to increase water and mineral absorption (McElrone *et al.* 2013). Roots have the ability to grow towards wet locations rather than dry locations and root growth is often stimulated toward wet areas, a biological phenomenon called hydrotropism (McElrone *et al.* 2013, Takahashi, 1994).

Woody species generally have large roots which extend to great lengths therefore allowing the plants to access water at lower depths, these usually occur in climates with high precipitation. It has been found that tree roots can extend to depths of 68 meters (m) and as far as 50 m wide (McElrone *et al.* 2013, Schenk and Jackson, 2002). Once water has been absorbed by the roots, it has to be transported to the xylem tissue. In order for this to occur, the water has to pass through other cell layers (the epidermis, cortex and endodermis), which have a great resistance to the flow of water, thus acting as a filtration system in the plant (McElrone *et al.* 2013). As the water moves toward the xylem, it has to travel through cell walls, membranes and inside cells. Aquaporins, which are water-specific protein channels, are present in cell membranes to influence and make the flow of water more efficient (McElrone *et al.* 2013). In the xylem, water can move relatively freely. The components found in the xylem include, water conducting elements: the tracheids and the vessels - the former being relatively smaller than the latter, fibers that provide support, and lastly parenchyma cells which store nutrients and maintain flow of water and nutrients (McElrone *et al.* 2013). The water is then transported from the xylem which is stacked along the stem and roots to the petiole and subsequently to the leaves of the plant. The water from the xylem then moves from the veins to the bundle sheath, then to the mesophyll cells where some of the water will be used for photosynthesis (McElrone *et al.* 2013). It is thought that water is transported via the apoplastic pathway to the stomatal cells where water is transpired to the atmosphere (McElrone *et al.* 2013, Sack and Holbrook, 2006).

Water movement in plants is governed by pressure and chemical potential gradients, and mainly by a negative pressure gradient that is generated by transpiration from leaves

(McElrone *et al.* 2013). Transpiration of water from the leaves of a plant is referred to as the cohesion-tension mechanism, because water is able to form water columns in the plant that are held together via hydrogen bonds which allow water to be transported effectively to all parts of a plants regardless of its size (McElrone *et al.* 2013). The Soil Plant Atmospheric Continuum occurs when the sun causes the breaking of the hydrogen bonds holding the water molecules together, thus causing evaporation and water molecules to be pulled to the surface through the water columns to replace evaporated molecules. The movement of water that is governed by its chemical potential gradient is osmosis which allows water to move between cells and controls movement of water through roots in the absence of transpiration in stressful conditions (McElrone *et al.* 2013). As mentioned above, a large amount of water is absorbed by a plant, although only a small fraction (5%) is utilized for growth and productivity and the rest is lost to the atmosphere through transpiration (Lisar *et al.* 2012, McElrone *et al.* 2013).

The extensive loss of water is intrinsically linked to plants assimilating carbon dioxide (CO_2) from the atmosphere, through tiny pores on the sides of leaves which are referred to as stomata. As CO_2 is assimilated, water is evaporated thus transpiration occurs (Lisar *et al.* 2012, McElrone, *et al.* 2013). There is a delicate balance between the amount of water that can be transpired and the rate of photosynthesis in plants (Lisar *et al.* 2012, McElrone, *et al.* 2013). The rate of transpiration can affect a plant's physiological and biochemical processes; therefore a plant's stomatal conductance has to be synchronized with the metabolism and environment of the plant in order to optimize water use efficiency and maintain productivity. This process is also essential for the process of photosynthesis.

Photosynthesis is the process that occurs in autotrophs by which they convert solar energy and carbon dioxide ($CO_{2)}$ from the atmosphere into chemical energy and it is vital for plant survival (Figure 1).

LIGHT

$$6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2$$

CHLOROPHYLL

Figure 1. Summary of photosynthetic reaction in plants

Photosynthesis fundamentally comprises of two processes; the light-dependent or light reactions and the light-independent or Calvin cycle (Campbell and Reece, 2011). The light-dependent reactions which occur in the thylakoid membranes of the chloroplast, absorb light energy via chlorophyll a, b and accessory pigments such as carotenoids, which then drives the transfer of electrons causes the water to split subsequently releasing oxygen as a byproduct (Campbell and Reece, 2011). The light energy is converted to chemical energy thus reducing nicotinamide adenine dinucleotide (NADP) to nicotinamide adenine dinucleotide phosphate (NADPH) by adding electrons and also generating adenosine triphosphate (ATP) through chemiosmosis. The second phase - the light-independent reactions which take place in the stroma, use the ATP and NADPH to reduce CO_2 and synthesize phosphates, starch, glucose ($C_6H_{12}O_6$), and other carbohydrates (Campbell and Reece, 2011). Due to photosynthesis being such an important process, plants have several mechanisms in place to protect the photosynthetic apparatus within cells, particularly under adverse environmental conditions such as limited water availability, which may result in the plants being stressed

1.3 Water stress in plants

1.3.1 The stress concept in plants

Stress is caused by a change to a systems equilibrium that causes a shift from its optimal state (Gaspar *et al.* 2002). The stress concept "General adaptation syndrome" or GAS was established by H. Selye in 1936, for mammalian organisms and states that different stresses result in similar adaptation responses (Lesham and Kuiper, 1996). Modification of the GAS concept to plant stress shows that every plant is exposed to variety of environmental conditions through its life cycle on different scales such as diurnal-nocturnal fluctuations or seasonal changes (Lesham and Kuiper, 1996).

The stress concept of plants was developed by Larcher in 1987 whom stated that "Every organism experiences stress, although the way in which it is expressed differs according to its level of organization, and stress is a selection factor as well as a driving force for improved resistance and adaptive evolution" (Lichtenthaler, 1996). The stress concept of plants was then expanded by Lichtenthaler in 1988 when he stated that there is a regeneration phase in plants and that stress can be divided into two components; eustress and distress (Lichtenthaler, 1996). Eustress is a stimulating stress that results in plant development, thus it

has a positive outcome, whereas distress is a severe, negative stress which results in damage of the plant, therefore it has a negative effect on development (Lichtenthaler, 1996). It is important to understand that the transition between eustress and distress is fluent and the stress tolerance of a plant is generally dependent on the species, growing conditions and buoyancy before exposure to the stress (Lichtenthaler, 1996). Lichtenthaler (1996) also proposed that all agents can be stressors which induce specific responses, though there is a difference between stressor-specific responses and non-specific general responses.

Both abiotic and biotic stress, placed on a plant system, will decrease the productivity of that system (Gaspar *et al.* 2002, Hong *et al.* 2016, Liljenberg, 1992). Abiotic stress refers to non-living, natural occurring stress, such as drought, flood or temperature stress. Biotic stress refers to stress brought about by living organisms, such as viruses, parasites or harmful insects. It is accepted in plant physiology that all abiotic or biotic stresses placed on a plant will reduce the plants productivity and growth, however it may also increase resistance to future stresses by acclimating to these conditions (Kaur and Gupta, 2005, Liljenberg, 1992). Acclimation to a specific stress is often indicated by new metabolites that improve the plants functioning while exposed to the stress (Lisar *et al.* 2012).

A plant is said to be in optimal condition in terms of its physiology and functioning with regard to its location before being exposed to a stress factor (Kranner, *et al.* 2010, Lichtenthaler, 1996). Exposure to stress will induce a cascade of physiological and biochemical responses to cope with the stress. This response usually occurs in four phases (Figure 2). These phases include the alarm, resistance, exhaustion and regeneration phases (Lichtenthaler, 1996).

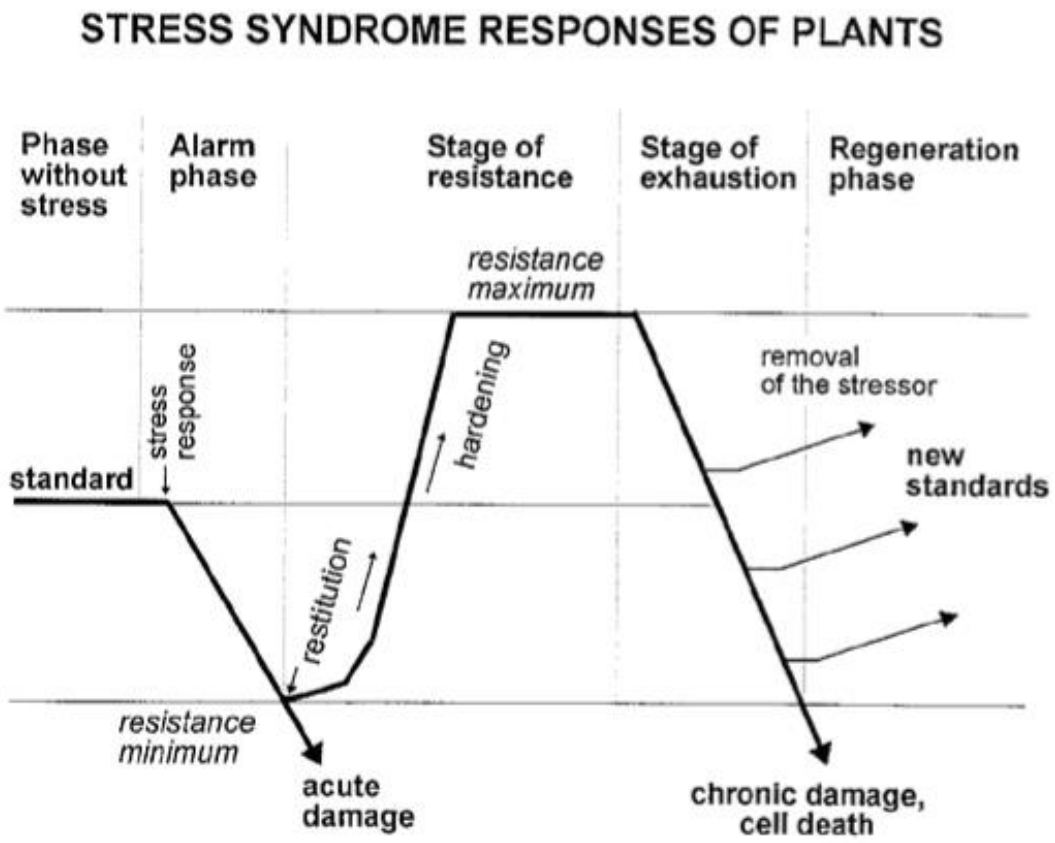

Figure 2. The general stress syndrome concept of the sequence of the phases and responses induced in plants when exposed to a stress (Taken from Lichtenthaler, 1996).

The alarm phase is the initial phase a plant goes through in response to a stress. This corresponds with the beginning of the stress and often results in a decrease in the plant's normal functioning which result in decreases in the vitality, photosynthetic and metabolic activities (Kranner, *et al.* 2010, Lichtenthaler, 1996). The alarm phase is critical because the type of stress factor that a plant is exposed to can determine the level of damage and how quickly a response can be generated by the plant. This, in turn, is dependent on the intensity and duration of the stress, i.e., whether it is an acute or chronic stress.

The second phase is the resistance or restitution phase. In the resistance phase, the plant develops acclimation strategies toward the new environmental condition or stress and begins the repair and hardening processes that allow the plant to develop new physiological standards (Lichtenthaler, 1996).

The third phase is referred to as the end phase or stage of exhaustion. This occurs when the stress is prolonged or very intense, thereby causing failure to repair and adapt and if

it does repair, it is often with several impairments (Kranner, *et al.* 2010, Lichtenthaler, 1996). When a plant is exposed to stress of high intensity or removal of the stress follows after it has been exposed for a long duration, death will most likely be the result. For example, if there was a prolonged drought period, it will lead to severe water stress and decreased transpiration in plants, thus leading to the inability of the plant to function optimally on a molecular level. Such a plant will subsequently have chronic damage or may die.

The regeneration phase is the fourth and final phase, where the plant will regenerate its physiological functioning. However, whether the regeneration is complete or partial depends, once again, on the intensity and duration of the stress (Kranner, *et al.* 2010, Lichtenthaler, 1996).

1.3.2 Effects of water stress on plants

Plants maintain a very specific equilibrium and any stress that causes a change to that equilibrium needs to be urgently responded to, in order to maintain homeostasis and prevent cell damage and plant death (Hong *et al.* 2016, Liljenberg, 1992). Water and salt stress are the most limiting stresses on agricultural production and crop yield in several countries (Bohnert and Jensen, 1996, Kaur and Gupta, 2005, Liljenberg, 1992, Lisar *et al.* 2012). Water stress occurs in plants either when the amount of available water is decreased or when transpiration rate is increased (Bray, 1997, Liljenberg, 1992, Lisar *et al.* 2012). Almost every process in a plant is affected directly or indirectly by water stress (Akinci and Losel, 2011). Some of the problems plants are faced with due to water deficit include functional impairment, disruption of membrane integrity, water potential and turgor and a decrease in the overall functioning. Further problems; include decreased productivity with regards to growth and reproducibility, increased solute concentrations in cytosol and extracellular matrices and reduced water use efficiency (Bray, 1997, Farooq, *et al.* 2009). The increased solute concentrations may result in growth inhibition, reproductive failure and decreased osmotic adjustment (Akinci and Losel, 2011, Lisar *et al.* 2012). Ultimately, prolonged water stress exposure leads to extreme dehydration and death of the plant (Liljenberg, 1992, Lisar *et al.* 2012).

1.3.3 Plant response to water stress

Plants have developed several adaptive tolerance mechanisms and responses to water stress which allow the plant to reduce water usage depending on water availability (Akinci and Losel, 2011, Kaur and Gupta, 2005, Lisar *et al.* 2012). These tolerance mechanisms and

acclimations do however require a great amount of energy. Due to reduced carbon assimilation and photosynthetic ability when stressed, energy is utilized from the plants stored carbohydrates, which raises agricultural concerns in terms of nutritional value and growth potential. Tolerance and resistance strategies do, however, differ depending on plant genotypes and may be influenced by other stresses placed on the plant (Chaves, *et al.* 2002, Lisar *et al.* 2012). In order for a plant to respond, the first step is recognition of the stress which allows for the triggering of specific biochemical responses (Bray, 1997). Reponses to water stress may be within seconds or within hours, such as a change in phosphorylation of a protein or change in gene expression respectively (Bray, 1997). The faster a plant responds to a stress the better acclimated it becomes to the stress – this acclimation leads to new metabolite production through changes in gene expression (Chaves, *et al.* 2002). However, the response is highly dependent on the level of stress that the plant is exposed to, i.e., the duration and intensity of the stress is important, it also depends on the plant itself and if it is capable of bringing about a swift response, based on its genotype and amount of signaling networks. The ability of a plant to tolerate water stress is dependent on several biochemical pathways, such as those that synthesize methylated metabolites and proteins (Bohnert and Jensen, 1996, Lisar *et al.* 2012). These molecules are able to control ion and water fluxes and aid the scavenging of oxygen radicals, which, if not regulated, may adversely affect plant survival. The stress response pathways thus allow the plant to retain water, protect photosynthetic apparatus and maintain the plants homeostasis in order to ensure survival (Bohnert and Jensen, 1996, Lisar *et al.* 2012).

1.3.3.1 Stomatal closure

One of the first responses to water deficit is a modification to stomatal conductance through stomatal closure, which allows the plant to reduce water loss through transpiration at the cost of decreased carbon assimilation by leaves, therefore affecting chloroplast light-harvesting and subsequently affecting photosynthetic operations in the plant (Akinci and Losel, 2011, Bohnert and Jensen, 1996, Chaves, *et al.* 2002, Kaur and Gupta, 2005, Lisar *et al.* 2012). Photosynthesis then decreases with decreased transpiration due to limitation of CO_2 absorption and less effective water-splitting reactions, and decreased leaf area and photosynthetic rate per leaf unit, therefore resulting in photoinhibition and changes in the carbon metabolism (Akinci and Losel, 2011, Bohnert and Jensen, 1996, Lisar *et al.* 2012). Nevertheless, it has been found that several biochemical changes are the result of protection of the photosynthetic apparatus to dehydration (Chaves, *et al.* 2002). Photosynthetic rate is

affected in both C_4 (such as corn, sugarcane and sorghum) and C_3 (such as rice, wheat and barley) species, though the effects are evidently greater in C_4 species (Farquhar and Sharkey, 1982, Lisar *et al.* 2012).

Stomatal closure as well as the expression of water deficit defence genes are more closely linked to soil moisture than leaf water status, due to the former being regulated by abscisic acid (ABA) signals (Bray, 1997, Chaves, *et al.* 2002, Lisar *et al.* 2012). ABA, a growth inhibiting hormone, increases in concentration in response to root dehydration and soil drying, it subsequently surrounds the guard cells which results in stomatal closure (Lisar *et al.* 2012). The accumulation of ABA causes increased root-to-shoot ratio under water stressed conditions in order to maintain osmotic pressure and increase water absorption (Chaves, *et al.* 2002, Lisar *et al.* 2012).

1.3.3.2 Carbohydrates

Plants may also respond to water stress by altering the carbohydrate concentrations in cells. Alterations to carbohydrate concentration (increase or decrease depending on the level of the stress), such as starches and sugars (including glucose, sucrose and fructose) may act as a metabolic signal in response to drought (Akinci and Losel, 2011). Plants may also be able to maintain a balance between sucrose synthesis and translocation which increases soluble sugar concentration in leaves (Akinci and Losel, 2011, Quick, *et al.* 1992). In general leaves of water stressed plants contain higher soluble sugar concentrations than unstressed plants. Studies have found that soluble sugar accumulation, amongst all other carbohydrates, contribute greatly to osmotic adjustment and osmoregulation under stress as a response known as osmoprotection (Akinci and Losel, 2011, Munns, *et al.* 1979, Quick, *et al.* 1992).

1.3.3.3 Proteins

Water stress may also result in the inhibition of cell division and expansion therefore, there is a reduction in protein synthesis (Akinci and Losel, 2011). Proteins decrease in plant leaves due to suppressed synthesis under water stress conditions; this is more apparent in C_3 species. However, some types of stress-induced proteins may be synthesized under conditions of water stress. These proteins can be differentiated into the following groups: dehydrins, late embryogenesis abundant (LEA) proteins, Rab (G-protein) proteins and storage proteins (Akinci and Losel, 2011). The production of such proteins is a result of the alteration of gene expression that may occur in some plants under long periods of water stress (Lisar *et al.* 2012). LEA proteins are the most synthesised proteins in comparison with other stress

molecules such as ABA and proteases, as they play an important role in increasing osmoprotectants (Akinci and Losel, 2011, Goyal *et al.* 2005 Lisar *et al.* 2012). LEA proteins take on a protective function in plants under stress conditions due to their hydrophilic properties (Goyal *et al.* 2005). There is also a substantial increase in synthesis of dehydrins and proline in response to an increase in reactive oxygen species (ROS) under water stress (Akinci and Losel, 2011).

1.3.3.4 Lipids

Lipids serve as the main non-protein components in plant membranes. The composition and state of the lipid bilayer is important for the functioning and structural properties of membrane proteins which are embedded in it (Akinci and Losel, 2011). Approximately 70% and 80% of total proteins and lipids respectively in leaf tissue are present in the chloroplast, thus changes to the chloroplast membrane result in changes to total concentrations of these molecules (Akinci and Losel, 2011). In general, studies show that water stress results in decrease in phospholipid and glycolipid concentrations and increase in triacylglycerol concentration in plants (Akinci and Losel, 2011). In a study conducted on sunflower seedlings by Navari-Izza *et al.* (1993), it was found that there was a decrease in total lipids (24%), phospholipids (31%) and glycolipids and a substantial increase in triacylglycerol concentration and free sterols (Akinci and Losel, 2011). The study also found that there was no alteration to fatty acid content.

1.3.3.5 Mineral and nutrient uptake

Apart from the regulation of select categories of biomolecules, plants under conditions of water stress, also have mechanisms for the regulation of mineral and nutrient uptake. As a result of reduced water absorption, mineral and nutrient uptake decreases. This decrease disrupts ion homeostasis and there may be a reduced uptake of several nutrients and minerals from the soil, however, nutrient uptake concentrations vary between different plants (Lisar *et al.* 2012). Some studies show that increases in nitrogen, potassium, calcium, magnesium and sodium concentrations, and decreases in phosphorous and iron concentrations have been found in water stressed plants (Akinci and Losel, 2011). However, several other studies show decreased uptake of potassium, calcium, magnesium, chlorine, phosphorus, zinc and iron concentrations (Akinci and Losel, 2011). This indicates that alterations to nutrient and mineral concentration are dependent on the species.

Calcium, nitrogen and potassium are essential nutrients in maintaining the plants functionality. Calcium maintains structural and functional integrity of the plant, nitrogen metabolism is critical for plants growth and productivity, and potassium is essential for maintaining water relation, osmotic adjustments and drought resistance (Lisar *et al.* 2012). Water stress generally decreases calcium in leaves though it increases content in roots (Lisar *et al.* 2012). Potassium concentration generally also decreases thereby decreasing the plants overall resistance to water stress (Lisar *et al.* 2012).

1.3.3.6 Phenolic acids

Phenolic acids are plant secondary metabolites that generally upregulates and accumulates as a protective measure in plants under abiotic and biotic stress conditions due to their antioxidant properties (Akula and Ravishankar, 2011, Chalkers-Scott and Fuchigami, 1989, Dixon and Paiva, 1995, Kähkönen *et al.* 1999, Padayachee *et al.* 2008). One of the most prominent phenolic acids groups are the phenylpropanoids which are antioxidant compounds that increase and act as radical scavengers in response to an accumulation of reactive oxygen species (ROS), which cause oxidative stress within a plants system (Akula and Ravishankar, 2011, Dixon and Paiva, 1995, Kähkönen *et al.* 1999, Padayachee *et al.* 2008, Pennycooke *et al.* 2005).

Cinnamic acid give rise to phenylpropanoids amongst other antioxidants (Dixon and Paiva, 1995, Solecka, 1997). Cinnamic acid is produced from phenylalanine through phenylalanine ammonia-lyase (PAL) which is the branch point enzyme between primary and secondary metabolism (Dixon and Paiva, 1995, Harborne, 1888, Kähkönen *et al.* 1999, Michalak, 2006, Solecka, 1997).

Dixon and Paiva (1995) outlined that many less complex phenylpropanoids are derived from cinnamate through hydroxylation, methylation and dehydration reactions. Several of these reactions can cause great structural diversity in phenylpropanoids (Dixon and Paiva, 1995). The structure of these compounds allows them to be highly efficient at scavenging free radicles under oxidative stress (Rice-Evans *et al.* 1995). Phenolic acids have a high *in vitro* antioxidant efficiency and thus have an exalted capacity *in vivo* (Dixon and Paiva, 1995).

There is a very delicate balance between ROS radicals and scavenging antioxidants which maintains a plant's homeostasis (Michalak, 2006, Pennycooke *et al.* 2005). ROS build up is extremely detrimental to the external and internal structures of plants (Michalak, 2006);

the faster a plant is able to respond to a ROS accumulation by means of antioxidant generation, the better its chance of survival.

1.3.3.7 Reactive oxygen species

ROS also referred to as free radicals, play an important role in plants under both stressed and unstressed conditions (Bowler and Fluhr 2000, Foyer and Noctor 2005). It plays a vital function by maintaining homeostasis within a plant system. ROS is critical to a plant's general developmental and functioning processes when a system is in equilibrium (Foyer and Noctor 2005, Mittler 2002, Risenga *et al.*2013). Plants can maintain this equilibrium by balancing ROS producing and ROS scavenging molecules (Foyer and Noctor 2005, Mittler *et al.* 2004, Mittler 2002, Van Breusegem and Dat 2006). ROS production is accumulated in response to any change in a plants metabolic system due to stress, thus causing deviations to the plants reduction-oxidation reaction (REDOX) potential (Bowler and Fluhr 2000, Foyer and Noctor 2005).

Oxygen becomes superoxide by gaining electrons in chloroplasts that are passing through the plants photosystems and then converted to other types of ROS through various reactions (Lisar *et al.* 2012). Hydroxyl radicals, superoxide, nitric oxide radicals and singlet oxygen are classified at ROS radicals, and fluctuate as a response to disequilibrium brought about by biotic and abiotic stress-induced changes (Beckett *et al.* 2005, Bowler and Fluhr 2000, Foyer and Noctor 2005, Gaspar *et al.* 2002, Gill and Tuteja 2010, Han and Lee 2005a, Lisar *et al.* 2012).

ROS essentially has two key roles with regard to plant stress. The first role invokes a signal to the plant to bring about a response to a stress and the second role triggers a spike in ROS that signals programmed cell death (PCD), a process that is determined by the intensity and duration of the stress (Apel and Hirt, 2004, Foyer and Noctor, 2005, Gaspar *et al.* 2002). Programmed cell death has been linked to very large ROS spikes, which is extremely toxic to the system (Apel and Hirt, 2004, Foyer and Noctor, 2005, Risenga *et al.* 2013). PCD often occurs in response to both extreme abiotic and biotic stresses, and is a highly energy reliant process (Foyer and Noctor, 2005, Risenga *et al.* 2013). ROS is responsible for many activities within a plant's system, and maintaining an equilibrium is critical for survival and the prevention of injury, as any alteration to the system may drastically influence the plant (Lisar *et al.* 2012, Mittler, *et al.* 2004, Mittler, 2002).

Plants utilize two methods to handle ROS accumulation, these include avoidance and prevention strategies against stress (Nakabayashi and Saito, 2015). These methods are carried out through enzymatic and non-enzymatic processes such as the accumulation of antioxidants (Nakabayashi and Saito, 2015). All plant systems produce ROS scavenging compounds or antioxidants. These can be both enzymatic or enzymatic scavengers of ROS and non-enzymatic or non-enzymatic scavengers of ROS (Lisar *et al.* 2012). Examples include enzymatic scavengers such as superoxide dismutase (SOD), peroxides (POD), ascorbate peroxidases (APX) and glutathione reductase (GR), catalases (CAT), and non-enzymatic scavengers such as glutathione, tocopherols, ascorbic acid (AA), carotenoids, hydroxycinnamates, flavonoids and anthocyanins (Ahmad, *et al.* 2009, Lisar *et al.* 2012, Nakabayashi and Saito, 2015). In addition, increases in production of osmolytes such as proline (described earlier) may also aid in the quenching of ROS compounds (Lisar *et al.* 2012, Szabados and Savoure, 2010).

In general, the quicker a plant responds to the ROS increase, the better it can acclimate to the stress and minimize damage. If ROS is not effectively quenched in time and the spike is sustained, it may lead to several deleterious consequences for the plant, such as denaturation of macromolecules such as carbohydrates and proteins, affects biochemical processes and may result PCD (Foyer and Noctor, 2005, Gill and Tuteja, 2010, Lisar *et al.* 2012).

1.3.3.8 Proline

In addition to the already mentioned stress biomolecules, proline is another important amino acid in plants that commonly accumulates as a response to abiotic stress conditions (Raymond and Smirnoff, 2002). Proline is unique from other amino acids due to its cyclic structure (Williamson, 1994). It plays many roles in stress response, such as its function as an osmoregulator and ROS scavenger. Proline molecules have a very rigid, strong structure therefore they cannot donate hydrogen to other molecules, however they are known to promote stability in protein cell structures, the maintenance of REDOX homeostasis and in the signalling cascade that may be triggered to bring about responses to tolerate stress (Ansary *et al.* 2012, Ashraf and Foolad 2007, Szabados and Savoure 2010). Proline may also impart a hydrating effect to drought-stressed plants due to its ability to bind to hydrophobic surfaces, converting these to hydrophilic molecules (Stein *et al.* 2011, Tantau *et al.* 2004). Furthermore, being a source of nitrogen and carbon, proline is vital to a plants metabolism under stress conditions by supporting recovery and repair.

Plants have developed many tolerance and resistance mechanisms to cope with water stress, however some of these mechanisms decrease in effectiveness with increased stresses. This may become increasingly challenging as the effects of climate change manifest. For instances, both abiotic and biotic stresses have increased in recent years in terms of intensity and extent, such as prolonged periods of drought (Allen, *et al* 2010, Chaves, *et al.* 2002, Lisar *et al.* 2012). As a result, there has also been increased attention payed to the various ways in which the effects of such stresses can be mitigated. In particular, significant attention has been given to natural mechanisms of increasing stress tolerance and resistance, such as the potential benefit of the use of plant growth promoting bacteria (Van Loon, 2007). The plant-microbe dynamic is further discussed below.

1.4 Plant-microbe interactions

Over recent years the study of plant-microbe interactions has become an exciting topic. Several studies have focused on both the beneficial and harmful relationships between several species of plants and microbes, especially for agronomic utilization. The study of plant-microbe interactions has broadened scientists' understanding in several fields, such as metabolite, biochemical and hormone studies, and virulence and resistance mechanisms in pathogens and plants respectively (Figueiredo, *et al.* 2010, Grover *et al.* 2011, Stacey and Keen, 1995). Plant-microbe interactions can be manipulated in order to increase resistance and tolerance to both abiotic and biotic stresses, expand medicinal knowledge for human well-being and improve our overall understanding of these interactions at a biological level (Figueiredo *et al.* 2010, Stacey and Keen, 1995). Understanding how plant-microbe interactions have been studied in the past is important for the expansion of this field, with special focus placed at a biochemical level. Plants can produce compounds that acts as signals for certain microorganisms. Diffusible signals are also present that are exchanged in plant-microbe interactions that control the gene expression between both organisms (Stacey and Keen, 1995).

1.4.1 Plant growth promoting rhizobacteria (PGPR)

Microbes provide important services to several environmental processes. There are three main types of microbe interaction: beneficial, neutral and harmful. This study focused primarily on beneficial plant-microbe interactions brought about by plant growth promoting rhizobacteria (PGPR). In general, non-pathogenic soil borne microbes have the ability to

enhance plant growth and this is accomplished by beneficial services provided by the bacteria to the plant which include production of antibiotics and hormones that stimulate growth, pathogen and predator resistance and improved acquisition of water, nutrients and minerals (Figueiredo *et al.* 2010, Grover *et al.* 2011, Van Loon, 2007). Several plant species are dependent on microbial associations for survival and acclimation to environmental stress conditions (De Zelicourt, *et al.* 2013, Figueiredo *et al.* 2010, Grover *et al.* 2011). Microbes provide several benefits to the plants through various mechanisms, such as, enhanced root respiration, reduced transpiration by modifying stomatal conductance and improved seed germination (De Zelicourt, *et al.* 2013, Grover *et al.* 2011). Plants require several nutrients and minerals, but adequate absorption of these from the soil is not always effective thus PGPR actively help plants acquire these nutrients and minerals efficiently (Figueiredo *et al.* 2010). Due to the many benefit provided by PGPR to plants, such as optimizing plant productivity and growth, PGPR may contribute sustainable agricultural practices (Figueiredo *et al.* 2010, Van Loon, 2007). PGPR can decrease the need for chemical fertilizers by making use of biofertilizers. Biofertilizers are products which contain living microorganisms, which improve plant growth, nutrient absorption and biocontrol mechanisms (Figueiredo *et al.* 2010).

PGPR are bacteria associated with the roots of plants and is a current solution that has been implemented to mitigate stress placed on plants (De Zelicourt, *et al.* 2013, Figueiredo, *et al.* 2010, Van Loon, 2007, Yang, *et al.* 2009). The most well studied genera of PGPR include *Pseudomonas* and *Bacillus* species, which have been found to improve plant growth, productivity and stress adaptation in many plant species (Figueiredo *et al.* 2010). The bacteria and plants form a beneficial plant-microbe mutualistic relationship which requires recognition and response from both parties (De Zelicourt *et al.* 2013).

The PGPR survive by utilizing nutrients released by the host and secretes metabolites into the rhizosphere (Van Loon, 2007). The rhizobacteria often form biofilm structures around roots of host plants and this allows the bacteria to survive and stay in place (Costerton *et al.* 1995, Timmusk *et al.* 2005, Timmusk *et al.* 2011). Biofilms are attached to root surfaces and are made up of rigid structures of bacterial cell communities which are surrounded by an extracellular matrix which contributes to structural stability and contains a variety of macromolecules (Allison *et al.* 1998, Costerton *et al.* 1995, Timmusk *et al.* 2005, Timmusk *et al.* 2013). These biofilm structures bring about enhanced metabolic, biochemical and physiological abilities within the community, such as heightened antimicrobial resistance

skills, that may not have been likely in a single bacterial organism/cell (Allison *et al.* 1998). The structure of the biofilm is very dense, which helps reduce any loss of nutrients and elements to the surroundings, thus increasing these concentration around the roots, which can positively influence plant productivity and well-being (Timmusk *et al.* 2013). The main components of the biofilm are proteins and sugars which help improve tolerance against stresses (Costerton *et al.* 1995, Timmusk *et al.* 2011, Timmusk *et al.* 2013).

The use of PGPR to improve growth and productivity of crops is effective as an environmentally and economically friendly means that may help decrease production-limitation faced by farmers, due to harsh environmental changes (Grover *et al.* 2011). It has been found that many PGPR strains are specific to certain plant types, and improve overall plant well-being successfully only in those plants (Figueiredo *et al.* 2010, Timmusk *et al.* 2013) Several studies have already been conducted which have found enhanced growth and productivity in plants that were inoculated with PGPR strains, compared to uninoculated plants (Figueiredo *et al.* 2010). In a study conducted by Figueiredo *et.al.* (2008) *Phaseolus vulgaris* L.; a species of bean, were co-inoculated with *Rhizobium tropici* and two strains of *Pseudomonas polymyxa* and this resulted in a greater number of nodules, larger shoot and root growth and taller plants in comparison to plants inoculated with just one strain of *P. polymyxa*. Cheng *et.al.* (2007) found that canola plants inoculated with a wild-type of *Pseudomonas putida* grew significantly taller than uninoculated plants under salt stress conditions. Additionally, in another study conducted by Kloepper *et.al.* (1980), there was a significant increase of 144% in potato, radish and sugar beet crops, which were inoculated with strains of *Pseudomonas fluorescens* and *Pseudomonas putida*.

PGPR elicits physical and chemical changes in plants that are referred to as induced systemic resistance (ISR) and induced systemic tolerance (IST), which enhance responses to biotic and abiotic stresses faced by the plant, respectively (Figueiredo *et al.* 2010, De Zelicourt, *et al.* 2013, Van Loon, 2007, Yang, *et al.* 2009). ISR largely relates to a decrease in the number of diseased plants and a reduced susceptibility to disease (Figueiredo *et al.* 2010, Van Loon, 2007, Yang, *et al.* 2009). Studies have shown that ISR enhanced resistance overlaps with pathogen induced systemic acquired resistance (SAR) and are dependent on the signaling compounds jasmonic acid and salicylic acid respectively (Van Loon, 2007). IST confers tolerance against abiotic stresses such as water, temperature and salt stresses. It has been found that IST is more successful with a range of bacterial strains (Yang, *et al.* 2009). Several studies have found that PGPR strains have substantially improved drought tolerance

in inoculated plants (Table 1). The use of PGPR strains to alleviate water stress is currently of extreme importance, with the growing food security crisis (Grover *et al.* 2011, Timmusk *et al.* 2013).

It has been found that PGPR assists in the degradation of aminocyclopropane-1-carboxylic acid (ACC), which is the precursor to ethylene, by bacterial ACC deaminase which protects the plant from various abiotic stresses, such as water stress (Figueiredo *et al.* 2010, Grover *et al.* 2011, Timmusk *et al.* 2013, Van Loon, 2007, Yang, *et al.* 2009). Ethylene is the hormone involved in the ripening of fruit, opening of flowers and shedding of leaves, though it is also secreted under stressful conditions in order to regulate plant homeostasis, however the consequence is inhibition of root and shoot growth (Figueiredo *et al.* 2010, Grover *et al.* 2011, Lisar *et al.* 2012, Yang, *et al.* 2009). The ACC secreted by the plant enters the rhizobacteria where it is broken down by ACC-deaminase thereafter the products ammonia and alpha-ketobutyrate are released by the bacteria (Van Loon, 2007). ACC-deaminase is said to be a signaling compound that can bring about stress responses and tolerance in plants, therefore the upregulation of this compound is highly beneficial to the plant under stressed conditions (Timmusk *et al.* 2013). The PGPR helps improve homeostasis within the plant and roots, thus decreasing damage from water stress (Timmusk *et al.* 2013).

Table 1. Summary of selected plant-microbe studies that have found an enhanced drought tolerance in plants inoculated with various PGPR strains.

Plant scientific name	**Common name**	**PGPR strain**	**Reference**
Solanum lycopersicum L. *Capsicum annuum* L.	Tomato Pepper	*Achromobacter piechaudii*	Mayak *et al.* 2004
Arabidopsis thaliana	Mouse-ear cress	*Paenibacillus polymyxa B2*	Timmusk *et al.* 2013
Arabidopsis thaliana	Mouse-ear cress	*Paenibacillus polymyxa*	Timmusk and Wagner, 1999
Zea mays L.	Maize	*Burkholderia phytofirmans PsJN Enterobacter sp. FD17*	Naveed *et al.* 2014

The PGPR can excrete volatile organic compounds (VOCs) which can alter the plants' gene expression – bringing about induced systemic tolerance (IST) and resistance (ISR). Mechanisms of plant growth promotion by PGPR include nitrogen fixation, efficient nutrient uptake from soil, production of plant hormones and metabolites that act as signaling compounds to other bacterial colonies in the soil and host (Grover *et al.* 2011, Van Loon, 2007). When plants are under abiotic stress such as drought or salt stress, IST is elicited. The levels of cytokinin secretions are decreased which then causes an increase ABA accumulation, thus resulting in stomatal closure which helps prevent excessive water loss to transpiration (Figueiredo *et al.* 2010, Van Loon, 2007). ABA production, which is a common response to water stress, has been linked to increased proline generation. Proline, commonly accumulates under stress conditions has been found to be upregulated far more in plants inoculated with PGPR strains than in uninoculated plants, under various stresses. Several studies have found that proline concentrations are higher in inoculated plants compared to uninoculated plants under stress conditions (Table 2).

Table 2. Summarized compilation of plant-microbe studies that have found an increase in proline concentration and stress tolerance in various plant species.

Plant scientific name	Common name	PGPR strain	Stress	Reference
Raphanus sativus L.	Radish	*B. subtilis* *P. fluorescence*	Salinity	Mohamed and Gomua, 2012
Ociumum basilicm L.	Basil	*Various strains*	Water	Heidari *et al.* 2011
Glycine max L.Merrill	Soybean	*Various strains*	Salinity	Kumari *et al.* 2005
Zea mays L.	Maize	*P. fluorescence*	Water	Ansary *et al.* 2012
Lactuca sativa L.	Lettuce	*Pseudomonas mendocina*	Salinity	Kohler *et al.* 2009
Solanum tuberosum L.	Potato	*Various strains*	Abiotic stress	Gururani *et al.* 2012
Zea mays L.	*Maize*	*Rhizobium and Pseudomonas spp*	Salinity	Bano and Fatima, 2009

ROS formation can be detrimental to a plants membranes, cells and results in metabolic damage, as discussed in detail earlier (Lisar *et al.* 2012). Thus, the enhanced production of catalase and antioxidants proves to be extremely important for the survival of the plant under stressful conditions. PGPR causes an increase in antioxidants and enzymes such as catalase in order to counteract the oxidative stress (Figueiredo *et al.* 2010, Lisar *et al.* 2012, Sandhya *et al.* 2010, Singh *et al.* 2002). The upregulation of phenolic acids is a fundamental response in plants to a spike in ROS compounds. There is a fine equilibrium that is maintained between these antioxidant and oxidant compounds which preserve a plants homeostatic state. During stressed conditions, oxidative molecules do increase, thus triggering an increase in phenolic acids which are able to quench and scavenge the free radicals. In a study conducted by Singh *et.al.* (2002), it was found that *P. fluorescence* and *P. aeruginosa* caused an increase in phenolic acid compounds in inoculated pea plants that were

stressed. It is well known that many *Pseudomonas* spp. are able to improve plant stress tolerance mechanisms. In another study by Logananthan *et.al*. (2014) it was found that tomato plant which were inoculated with *Bacillus subtilis* for the prevention of leaf wilting, had significantly higher levels of phenolic compounds than the uninoculated set. Maize crops that were inoculated with *Pseudomonas* spp. had elevated levels of phenolic acids which could be attributed to the enhanced quenching of ROS radicals (Sandhya *et al.* 2010).

PGPR also excrete auxins such as indole-3-acetic-acid (IAA) into the soil which are upregulated into the roots and shoots, therefore enhancing growth by increasing root surface area and enabling greater nutrient and water absorption (Van Loon, 2007, Yang, *et al.* 2009). The increase in root growth may be beneficial in aiding plants against pathogens whilst improving overall abiotic stress tolerance by allowing absorption of water and nutrients from deeper in the ground (Figueiredo *et al.* 2010, Van Loon, 2007, Yang, *et al.* 2009).

The use of PGPR to confer ISR and IST to plants can enable both small and large-scale farmers to reduce chemical fertilizer and irrigation thereby reducing expenses and prevent excess runoff of chemical fertilizers into water systems. This will allow them to use a "greener", more sustainable method, through the use of biofertilizers containing PGPR that will also benefit them economically.

1.4.2 The future of plant-microbe interaction studies

As outlined above, PGPR elicit many benefits to plants under both stressed and non-stressed condition by improving ISR and IST, thereby, improving plant physiology and biochemical response. Physiological benefits include increased overall growth, production and yield, and biochemical benefits such as the upregulation of ABA, proline, VOCs, IAA and antioxidants such as phenolic acids.

However, there is still much to understand about these interactions and there is still a great deal that is unknown. The best way forward are systems approaches that utilize various integrated techniques and tools, which is what was focused on in this project. These techniques can include biochemical, physiological, genomic, proteomic, transcriptomic and metabolomic tools to name a few. Metabolomics is a relatively new study which is a fast becoming a very important tool for systems biology, as it can broaden our understanding of many other omics approaches (Hong *et al.* 2016, Tugizimana *et al.* 2012). For that reason, metabolomics may prove to be a very insightful tool in improving our understanding of plant-

microbe interactions under various environmental conditions, by allowing the determination of specific metabolite pathways that may elicit tolerance and resistance mechanisms.

1.5 Metabolomics

Metabolomics as described by Fienh (2002) is the link between genotypes and phenotypes. Metabolomics is essentially the comprehensive quantitative and qualitative analyses of complex metabolite mixtures present in and around growing cells or extract of an organism at a specific time in production (Fukusaki and Kobayashi, 2005, Garcia *et al.* 2008, Hall *et al.* 2002, Mashego *et al.* 2007, Tugizimana *et al.* 2012). Metabolomic analyses provides a "snapshot" into the biological system studied, meaning that it depicts the metabolites that are present at a precise time and under specific environment situations and provides information about various genetic pathways (Hong *et al.* 2016, Tugizimana *et al.* 2012). In the same manner that a set of genes synthesized by an organism constitute its genome and a set of proteins constitute its proteome, a set of metabolites that has been synthesized by an organism subsequently constitute its metabolome (Fiehn, 2002). Essentially metabolomics can be concerned with the phenotype in the same way that proteomics and transcriptomics are concerned genetic information (Figure 3) (Fukusaki and Kobayashi, 2005).

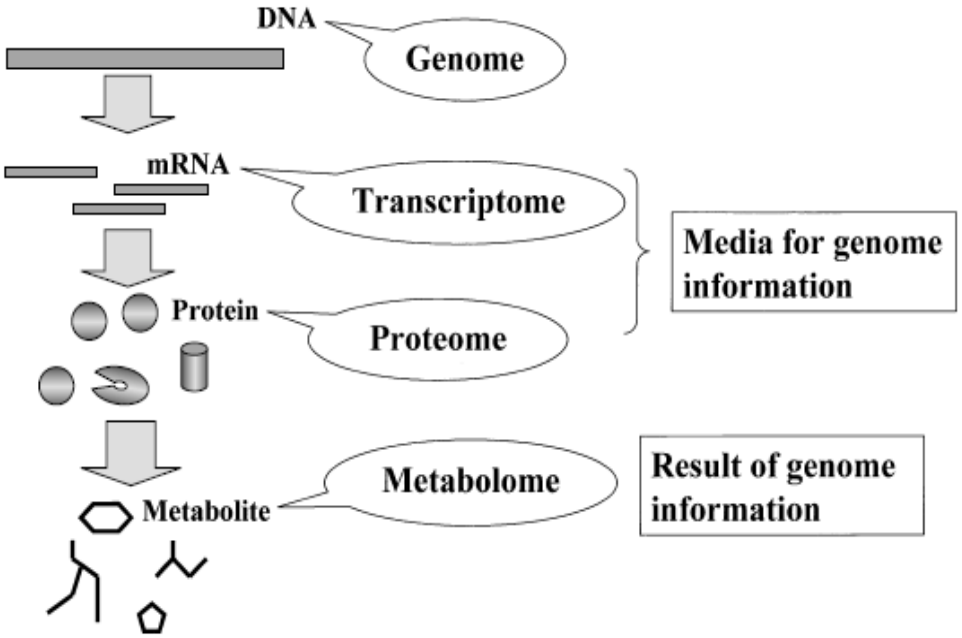

Figure 3. The role of metabolomics in functional genomics (Taken from Fukusaki and Kobayashi, 2005).

Metabolites are the end products of cellular processes (Fiehn, 2002). They are tiny molecules that maintain homeostasis within a system through distinctive interactions (Hong *et al.* 2016). Understanding how these metabolite levels fluctuate in response to biotic and abiotic changes will be vital for further understanding of adaptation and mitigation strategies to stresses that affect plant growth and production in a holistic manner. Metabolomics complements other genomic approaches, such as genomics and proteomics, thus providing means to improve metabolic engineering and biotechnology (Hong *et al.* 2016). Metabolomics is regarded as the newest science in functional genomics and metabolome analyses are relatively recent due to the advancement in chromatography and spectrometry technologies (Hall *et al.* 2002, Hong *et al.* 2016, Fukusaki and Kobayashi, 2005, Garcia *et al.* 2008).

There are a few widely applied technologies used for metabolite studies, these include gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance spectroscopy (NMR) and more recently capillary electrophoresis-mass spectrometry (CE-MS) (Fukusaki and Kobayashi, 2005, Mashego *et al.* 2007, Osorio *et al.* 2012, Tugizimana *et al.* 2012). Metabolomic research is very novel and has led to the advancement of a society and journal (Mashego *et al.* 2007).

1.5.1 Plant metabolomics

Plants metabolomics have rapidly become increasingly important in functional genomics, abiotic and biotic stresses and food security studies (Biais, *et al.* 2012, Hall, 2007, Hong *et al.* 2016, Tugizimana *et al.*2012). Hong *et.al.* (2016) stated that increasing plant metabolite studies may improve our knowledge of plant systems and help improve quality and production of agricultural crops. Plants synthesize specific metabolites under different conditions, such as stressed (biotic and abiotic) and unstressed conditions (Crozier *et al.* 2006, Nakabayashi and Saito, 2015). There is a large variety of known and unknown metabolites associated with plant growth and functioning. Plant metabolites can be broadly separated into primary and secondary metabolites (Crozier *et al.*2006, Hong *et al.* 2016). Primary metabolites are associated with a plants growth and developments and have unified, specific formations throughout the plant kingdom, whereas secondary metabolites are critical for survival under stress conditions, as protective response mechanisms and vary in structure depending on the plant (Akula and Ravishankar, 2011, Crozier *et al.*2006, Hong *et al.* 2016). The metabolites extracted from plants are multifarious, therefore many factors need be considered for successful extraction methods (Tugizimana *et al.*2012).

In terms of plant sciences, metabolomics may be more accurate and useful when used in concurrence with other omics sciences due to it being extremely difficult to essentially unpack the variety of stresses imposed on plants (Fukusaki and Kobayashi, 2005). Several plants have already been genetically sequenced, therefore using the genome information along with metabolomic techniques will increase our plant science understanding in an integrated manner (Hong *et al.* 2016). Even though understanding other omics sciences may be useful and convenient, metabolomics does not require genome information (Fukusaki and Kobayashi, 2005). For example, genomes have yet to be sequenced for several commercial crops, such as wheat, but metabolomic analysis can still be conducted for these species (Fukusaki and Kobayashi, 2005).

Metabolomics is regarded one of the best tools to analyze genetically modified, transgenic and mutant plants. Understanding the relationship between metabolites and corresponding genes in transgenic plants is at the forefront of research for many biotechnology companies who aim to commercially grow and sell these plants (Fukusaki and Kobayashi, 2005, Ward *et al.* 2007). It is apparent that several stresses imposed on plants via environmental changes, physiological strains, mutation etc., may result in slight changes to the metabolome (Fukusaki and Kobayashi, 2005, Hong *et al.* 2016). Plant metabolites are often family-specific and likely play a role in plants survival mechanisms in different environmental conditions (Fiehn, 2002). Therefore, metabolomic analyses can be used to unpack plant metabolites and their phenotypes in relation to their physiology and growth in response to stress (Fukusaki and Kobayashi, 2005, Ward *et al.* 2007). When extracting metabolites from plants, the main factors that need to be considered are the development of the plants over time and the environmental stress factors that the plants may have been exposed to (Ward *et al.* 2007).

1.5.2 Microbial metabolomics

Microbial metabolomics, like plant metabolomics, has fast become a riveting field of research due to its wide range of applicability especially in the biotechnology field, which uses several microorganisms. These microorganisms are generally genetically modified in order improve specific biochemical strains depending of bio-product, in comparison to the natural microbe (Mashego *et al.* 2007). In a review paper by Mashego *et.al*. (2007), it is stated that several biotechnology products that are derived from microbes are secondary products therefore they do not directly, but rather indirectly affect the primary metabolism of the organism. Due to the role these microbes hold in the plant system, it is critical to have a

very clear understanding of plant metabolism in order for effective metabolic engineering for biotechnology products (Mashego *et.al*. 2007). This is where microbial metabolomics comes into play, even though the science is still in early stages of development and is very novel.

Generally, two levels of metabolites need to be understood; intracellular and extracellular, with regard to the quantification of microbial metabolomics networks and there is a lot of research being focused on understanding *in vivo* regulation of these networks (Mashego *et.al*. 2007). There has not been a universal quenching or extraction protocol for microbial metabolomics though it is known that instant quenching is critical in order for there to be no electrolyte leakage during the process (Garcia *et.al*. 2008, Mashego *et.al*. 2007). Though as stated by Mashego *et.al*. (2007) if leaking does occur, those metabolites need to be quantifiable.

1.5.3 Challenges for inter-kingdom metabolomics

There are many complications related to establishing techniques for metabolite extraction and quantification *in vivo* and almost every step has room for experimental error, which proves to be rather challenging (Fukusaki and Kobayashi, 2005, Hong *et al.* 2016, Mashego *et al.* 2007). Establishing a comprehensive overview of metabolic compositions is very complex and requires a variety of steps in order to gain an optimal extraction (Fiehn, 2002, Hall *et al.* 2002). Metabolomic analysis requires cautious consideration of methods for preparation of samples, extractions and data acquisition (Biais *et al.* 2012, Fiehn, 2002). In order for successful metabolomics analyses to be conducted the use of known and reliable experimental techniques and procedures need to be considered (Mashego *et al.* 2007). These conventions include microbial cultivation, biomass sampling, and isolating and extracting metabolites procedures (Mashego *et al.* 2007). The analysis should be a simple, reproducible setup. Since the success of metabolomic studies is dependent on several variables, the occurrence of several challenges is inevitable in metabolomic studies, however the field is one that is constantly growing and improving, therefore recent advancements may decrease these challenges (Hong *et al.* 2016).

1.5.3.1 Experimental design: sample generation and analyses approaches

A general overview of all common metabolic activity sampling procedures was outlined by Mashego *et.al*. (2007). The initial step is the separation of cells from culture supernatant which is done using rapid sampling from bioreactors followed by instant quenching of metabolic activity (Mashego *et al.* 2007). Quenching is often done by changing

temperatures to extreme values or applying an extreme pH to the sample (Mashego *et al.* 2007). After quenching, cells are separated from the medium using a centrifuge and then permeabilized using organic solvents to extract the metabolites inside the cells (Mashego *et al.* 2007). The solvents are subsequently evaporated using a vacuum and the residual is resuspended in an ultra-pure water and is centrifuged again, then the supernatant is stored in low temperature until analysis (Mashego *et al.* 2007). There are a few necessary requirements that need to be met for metabolite extraction that were outlined by Mashego *et.al*. (2007). 1. Quenching should instantly freeze cellular activity. 2. Membranes should not be damaged during quenching procedure in order to prevent intercellular metabolite leakage. 3. Extraction should extract as many metabolites as possible. 4. The extraction should not cause any modification to metabolites that may cause them to be unrecognizable. 5. The method of extraction needs to be congruent with analysis methods.

For accurate metabolomic analysis to be achieved, the following steps need to be followed (Figure 4): Plant cultivation, sampling, extraction, derivatization, separation and quantification of metabolites, data matrix conversion and data mining (Fukusaki and Kobayashi, 2005).

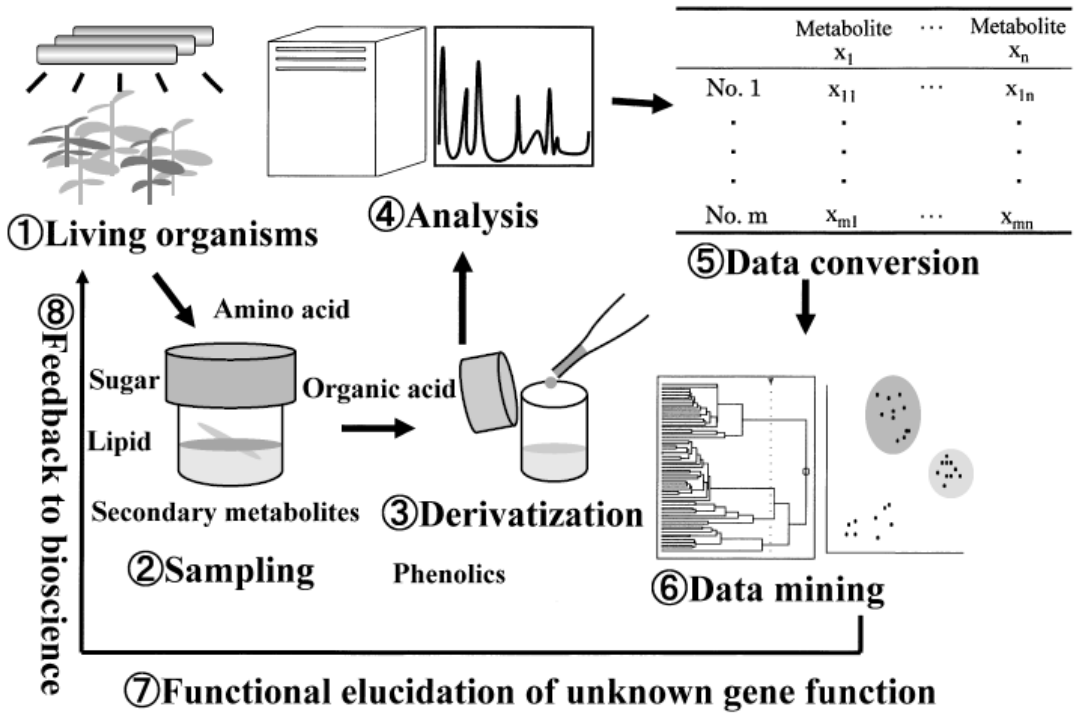

Figure 4. Schematic of metabolomic workflow methods (Taken from Fukusaki and Kobayashi, 2005).

In a review paper on plant metabolomics, Fukusaki and Kobayashi (2005) listed several possible experimental errors in different steps of the procedure. These steps have been summarized below:

Plant cultivation: variation among samples, especially when cultivating plants and microorganisms, even when conducted in controlled environments may result in slight alterations to the metabolome. This proves to be a great problem in metabolomics. Usually plants are cultivated in soil and this may lead to water stress. It is preferable to rather cultivate crops in a large-scale plantation with large-volume growth chambers, although this may not be possible, if cultivation takes place in small-volume growth chambers, it is important to rotate pots periodically. Soil-less agriculture is the best method for growing plants in terms of being able to completely control water and nutrient availability and extract high resolution metabolites.

Sampling: sampling needs to be done very carefully to avoid error – the growth stage, time and area of sampling needs to be controlled. Post-harvested material needs to be cared for and preparation of material is dependent on the type of analysis.

Extraction: extraction should include a wide array of metabolites and each metabolite needs to be categorized depending on whether they are hydrophilic, hydrophobic, small molecules or large molecules etc. A ball mill can be used instead of a blender to homogenously crush material more effectively in the extraction step.

Derivatization and pretreatment: derivatization is the chemical modification of compounds in order to make new compounds that are better suited for certain chromatography analyses. GC-MS analysis can only be used for volatile compounds therefore compounds have to be derivatized. Therefore, derivatization of metabolites is done depending on the equipment used. For example, hydrophilic metabolites need to be derivatized for GC-MS analyses. It is important to take into account the derivatizing conditions such as reagent and reaction conditions. Mass spectrometry is used for both quantification and qualitative qualification procedures, however any contamination such as that of the ionization source will reduce efficiency of the mass spectrometer. This subsequently leads to ion suppression which can occur in all mass spectrometry analyses. The most effective solution is to allow optimum time separation by means of chromatography prior to mass spectrometry.

Separation and quantification: the former is the most important operation in metabolomic analyses. Metabolome data need to be categorized depending on 'resolution' and 'quantification' performance. Separation can affect both. Resolution performance is the possible number of metabolites that can be separated. In terms of resolution CE-MS is superior, followed by GC-MS and then LC-MS. Quantitative performance depends on a range of linearity of the metabolite variables in each analytical system (Beer-Lambert Law). As mentioned earlier ion suppression occurs in mass spectrometry which decreases quantitative reproducibility.

Data conversion: multivariate analyses such as principal component analysis (PCA) and hierarchical cluster analysis (HCA) are used to evaluate metabolic data. Firstly, conversion of analogue data to a digital matrix data table is required. All matrix data tables need to be in the same format to be assessed using multivariate analyses. All data from GC-MS and LC-MS need to be corrected by appropriate preprocesses which includes noise reduction, baseline correction, resolution enhancement and normalization.

Although there is a high risk of experimental error in metabolomic studies, it is a very useful tool and will substantially improve our knowledge in various fields, including plant, microbe and interkingdom studies.

1.6 Conceptualization of research

1.6.1 Rationale

A precarious situation presently is finding ways to mitigate the predicted food insecurity and malnutrition in coming decades. As mentioned earlier, water is the greatest limiting factor on agriculture and crop yield therefore this project will be focused primarily on water stress. Thus, methods to improve agricultural production in response to changing conditions, especially increased drought occurrences need to be implemented. The ability of plants to withstand effects of drought is vital for economic stability. We are aware of the benefits gained by plants through plant-microbe interactions and several studies have shown the benefits of PGPR on plant growth and production. However, understanding the physiology, biochemistry and metabolomics of these interactions using systems approaches still fairly new. There are several known and unknown metabolites involved in these interactions and their involvement in tolerance and resistance mechanisms are not yet entirely understood. Certain plant growth promoting bacteria are specific to certain plant species.

This study focused on the interplay between plants and microbes by optimizing different approaches to study these interactions, thus bridging the gap between various techniques. This was achieved through studies on the interaction between *Helianthus Annuus* L. (Sunflower) and *Pseudomonas koreensis* (PGPR) under drought stressed and unstressed condition.

1.6.2. *Helianthus annuus* L. – Sunflower

Helianthus annuus commonly referred to sunflower, is a member of the Asteraceae family and is native to North America. It is characteristic for the flower to turn its head to face the sun, hence giving the sunflower its name (Beard, 1981). Once the sunflower head is in full bloom it only faces east (Beard, 1981). There are approximately 100 known species of sunflower. There is evidence that the crop was cultivated as far back as 3000 B.C. in New Mexico and Arizona by Native Americans (Beard, 1981). There are ornamental and crop varieties of the sunflower plant. The former has a branched stem with many heads, whereas the latter has one long stem, with a single head and large seeds (Beard 1981).

Globally, it is estimated that sunflower production covers ~ 21 million hectares of land across 60 countries (Jan *et al.* 2006). It is the fourth largest oilseed crop in the world. As an oilseed it is utilized as an important commercial crop in many countries due to it being a nutritional source of vegetable oil (Ahmad *et al.* 2009, Beard, 1981, Huffman *et al.* 2006). There is a great interest in oilseed crops due to the shortage of foods that are high in protein in many countries as a result of the high cost animal based food. The oil from sunflower plants is of very high quality in terms of nutritional value, is easily refined and is utilized as edible oil and in margarines (Huffman *et al.* 2006). The emulsions of oilseeds have also been utilized in several food industries to enhance flavor, colour and retain moisture in products (Huffman *et al.* 2006). Oilseed proteins have been used to maintain freshness, aerate products and in confectionary goods, thus playing a vital role in the food industry. Sunflower meal is also used as animal feed due to its high protein value and low cost.

The sunflower plant it is highly susceptible to drought stress conditions (Ahmad *et al.* 2009). Drought stress affects many physiological and biochemical parameters in a sunflowers growth and development, as well as the plants germination potential. Over recent years, sunflower cultivation has expanded to areas with less than ideal environmental and climatic conditions, which results in decreased production and global yield (Jan *et al.* 2006). In these areas which decrease cultivation capacity, adaptability of the crops is an important challenge

for farmers. Jan *et al.* (2006) stated that in order for sunflower to remain an economically viable global crop, a multidisciplinary approach needs to be integrated into sunflower cultivation programs.

1.6.3 *Pseudomonas koreensis*

As mentioned earlier, the *Pseudomonas* genus is one of the most studies PGPR species, and strains from this genus have been used in many plant-microbe studies. However, the *Pseudomonas* genus was considered highly heterogenous in the late 1800s, with a wide variety of species under it, though this heterogeneity was elucidated after many phenotypic and genotypic studies were conducted in the 1980s and 1990s (Kwon *et al.* 2003). *Pseudomonas* strains are often found in agricultural soils around the world and have been found improve plant growth, productivity, stress tolerance and resistance, and yields under various stress and unstressed conditions (Kwon *et al.* 2003).

Pseudomonas koreensis was the PGPR strain utilized in this study. *P. koreensis*, a Gram negative bacterial strain was originally isolated from agricultural soils in Korea (Kwon *et al.* 2003, Lin *et al.* 2016). *P. koreensis* has been found to remediate heavy metal continuation in soil for mining site remediation (Babu *et al.* 2015), produce biosurfactants (Toribo *et al.* 2011), act as a biocontrol agent eliciting increased resistance again pathogens (Liu *et al.* 2014, Rafikova *et al.* 2016) and improve disease tolerance in tomato plants (Hultberg *et al.* 2010a and Hultberg *et al.* 2010b).

P. koreensis is a relatively newly discovered *Pseudomonas* species, therefore analyses using this strain are novel, especially for plant-microbe interaction studies. The understanding of how plant-microbe interactions through systems approaches will help advance our knowledge in this area and enhance our ability to create technologies based on this information. For example, this information can be used to better the production of microbial inoculants that can be used in agricultural biotechnology as natural fertilizers and pesticides.

1.7 Aim and objectives

The aim of this project was to optimize physiological, biochemical and metabolomic techniques to study sunflower-microbe interactions under drought stress.

The objectives of this project were to:

1. establish a repeatable growth regime for sunflower plants under controlled conditions;

2. compare physiological parameters (height and leaf area) between PGPR inoculated and uninoculated plants under drought stressed and unstressed condition;

3. compare biochemical factors (total phenolic acids, proline and ROS) between PGPR inoculated and uninoculated plants under drought stressed and unstressed conditions;

4. optimize a metabolite sampling method for the extraction of metabolites form plant leaves, roots and biofilm layer;

5. determine the technical reproducibility of the optimized metabolite method.

Chapter Two: Methods and Materials

2.1 Summary of methodology

All experiments carried out in this study was to assess and compare *P. koreensis* inoculated plants against uninoculated plants. The study further placed focus on how these two groups tolerated and resisted drought induced stress. The seeds were initially surface sterilized, then germinated in Petri dishes. The seeds that successfully germinated were transferred into pots containing vermiculite and were grown for four weeks in a growth chamber. The growth chamber was set at 25 °C and at a 14-hour light/10-hour dark photoperiod. Plants were watered daily and supplemented with Murashige and Skoog nutrient medium (MS) twice a week. Bacteria inoculated plants were inoculated twice in the growth period. After the four-week growth period, stressed plants were subjected to an induced drought stress for 10 days, whereby water and MS were withheld. The unstressed plants were grown under the normal growth conditions that were followed during the four-week growth period. The heights of all plants were measured weekly and once again at the end of the 10 day drought stress period. With the aim of calculating leaf area, the leaf width and length were measured for every plant at the end of the four-week growth period. The experiments were done in triplicate and each biological replicate consisted of 48 plants which were divided into 4 sampling subsets; 1. Control – uninoculated and unstressed (C), 2. Bacteria inoculated and unstressed (I), 3. Uninoculated and stressed (S) and 4. Bacteria inoculated and stressed (IS) plants (Figure 5). This meant that there were 12 plants in each sampling subset. Due to growth room constraints, 48 plants were the largest sample size possible.

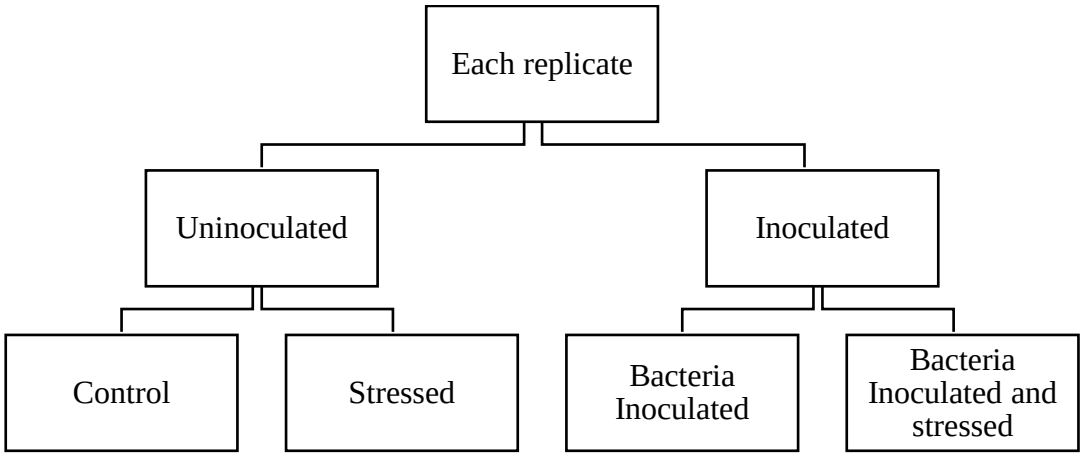

Figure 5. Summarized breakdown of sampling subsets for each experimental replicate.

Each subcategory was then further divided into three categories (four plants each), for the various experiments and assessments that were carried out (Figure 6). The first category of plants was utilized for all experiments that made use of leaf material (Reactive oxygen species, phenolic acid, proline assays, and for the optimisation of leaf metabolite extraction). The first, second and third fully developed leaved were used for these assays. The second category of plants were utilized for experiments that made use of root material (root microbial counts and for the optimisation of root metabolite extraction). Lastly, the third category of plants was utilized for the biofilm metabolite extraction method that was optimized in this study. Once all laboratory analyses were concluded, the results were transferred digital formats and analysed statistically.

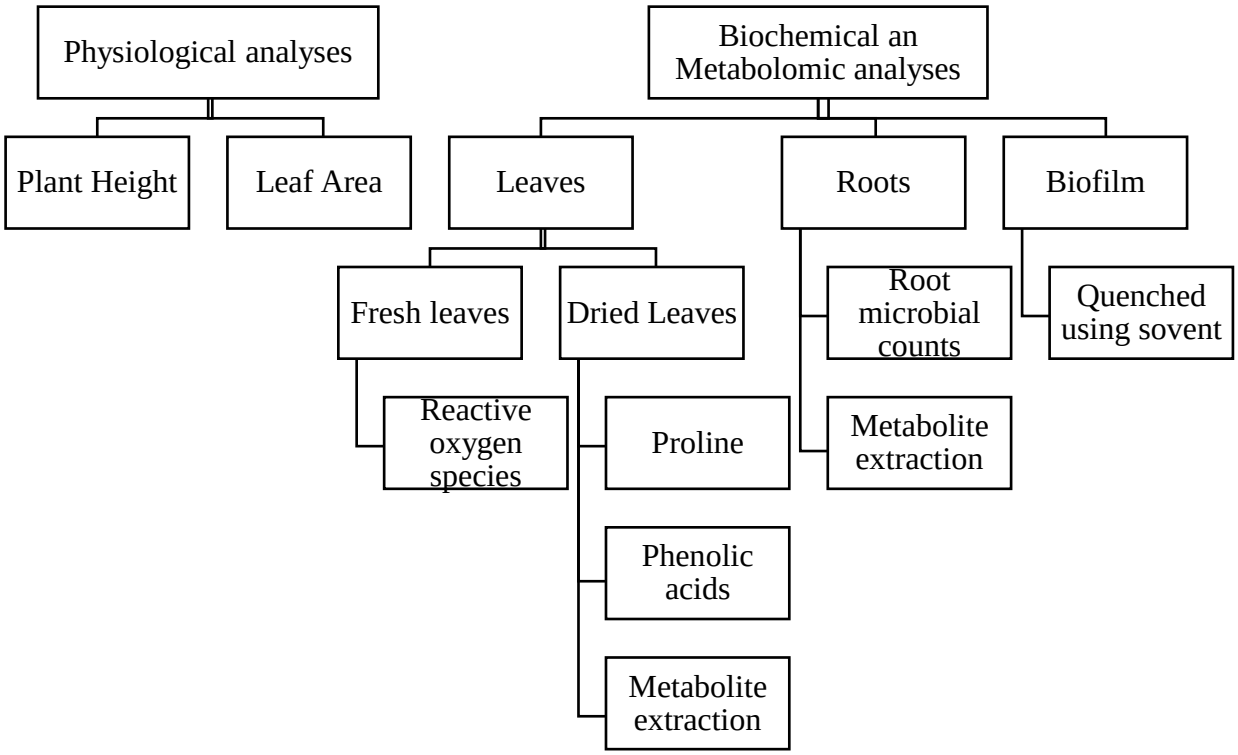

Figure 6. Summarized breakdown of experiments and evaluations carried out for each of the sampling subsets, i.e., physiological evaluations were carried out for all plants, and biochemical and metabolomic analyses were carried out for each of the subdivided sampling categories (4 plants in each category).

2.2 Study species

The plant species chosen for this study was *Helianthus annuus*, commonly known as sunflower. This crop was chosen due to its utilization as an important oilseed and source of protein in many countries, including South Africa (Huffman, *et al.* 2006). The seeds (AGSUN 8251) that were used in this study were obtained from Agricol, South Africa. The seeds were pre-treated with a mild fungicide by the supplier (Figure 7). The fungicide that was used to treat the seeds was a surface coating fungicide, however the exact variety of fungicide was not disclosed by the supplier. The seed were between 8 to 10 mm in length and 4 to 5 mm in width.

Figure 7. *Helianthus annuus* seeds (AGSUN 8251) used in the project.

The microbial species chosen for this study was *Pseudomonas koreensis* (Figure 8), which is a plant growth promoting bacteria, PGPR. This strain of *Pseudomonas* was chosen due to it outperforming the commercially available *Pseudomonas fluorescence* in several biochemical and microbial assays that were conducted in a previous study, which included phosphatase activity, IAA and siderophore production. The *P. koreensis* used in this study were taken from previously made glycerol stocks which were stored at – 80 ° C, and were originally isolated from the rhizosphere of a crop cultivation from the Free State Province, South Africa.

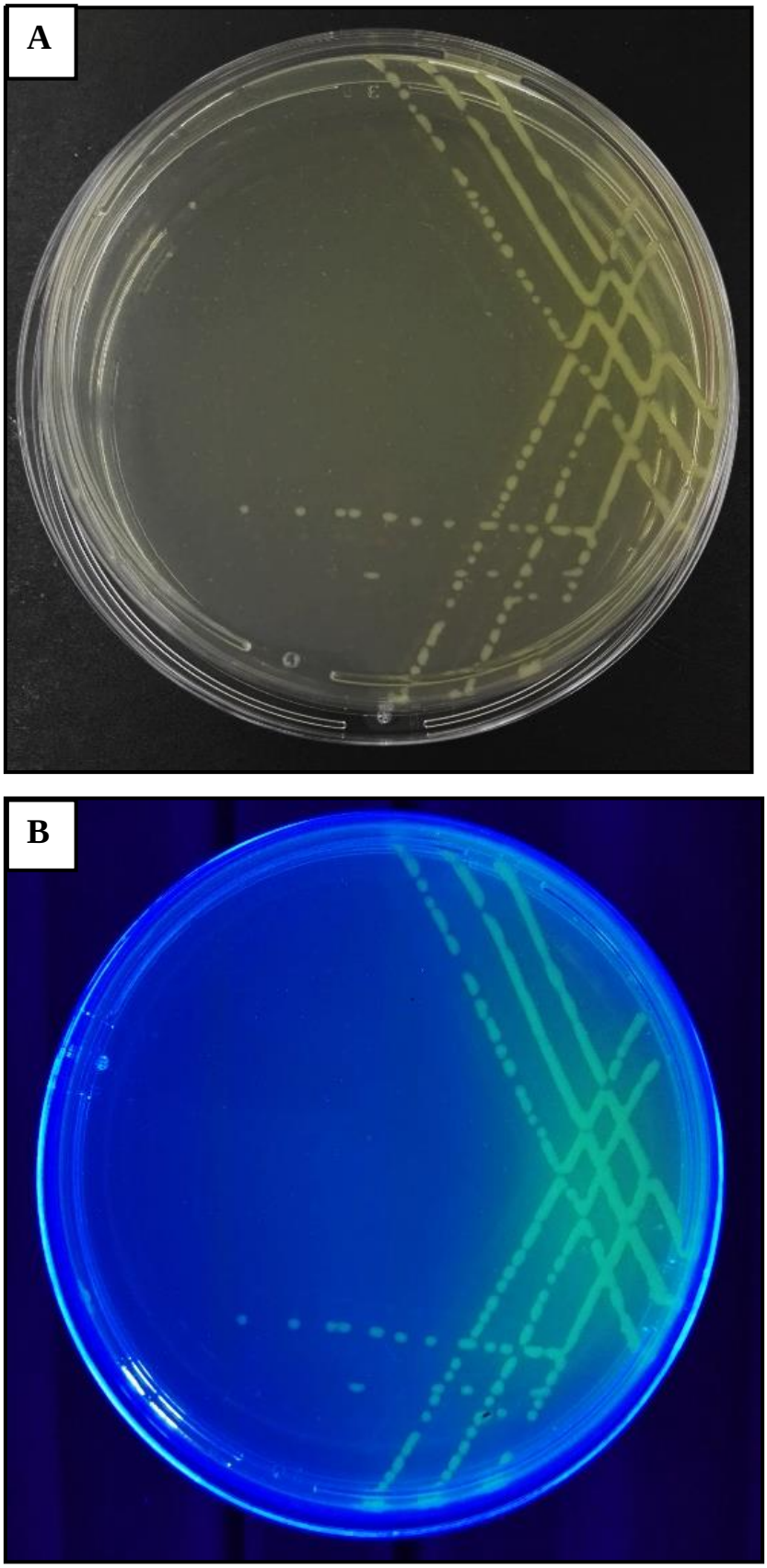

Figure 8. *Pseudomonas koreensis* plated on King Agar B (Sigma-Aldrich ®) under white light (A) and under ultra violet light (B)

2.3 Seed preparation and germination

The seeds were surface sterilized to remove most of the fungicide that had been used to treat the seeds by the supplier. A preliminary experiment was carried out to test two surface sterilization methods, to find which method would enable a greater germination percentage. The first method involved placing 40 seeds in a Falcon 50 mL conical tube with 40 mL sterile distilled water (dH_2O) and allowing the tube to shake on a rotary shaker (MRC, TS-400P, Britain) for 20 minutes at 200 revolutions per minute (rpm). The seeds were drained and shaken again for 90 seconds in 40 mL of 70% ethanol, thereafter they were drained again and shaken in 40 mL of 3.5% sodium hypochlorite (NaClO), with one drop of Tween 20 for a further 30 minutes. The seeds were then rinsed six times using dH_2O to remove any chemical residue. The second method involved placing 40 seeds in a Falcon 50 mL conical tube with 40 mL of sterile dH_2O and allowing the tube to shake on a shaker for 20 minutes at 200 rpm. The seeds were drained and shaken in 40 mL of 3.5% NaClO, with one drop of Tween 20 for a further 30 minutes. The seeds were then rinsed six times using dH_2O to remove any chemical residue. Therefore, the second method omitted the use of ethanol.

The rinsed seeds were placed in Petri dishes which contained two sheets of sterilized filter paper below the seeds and one sheet above the seeds. Ten seeds were placed in each Petri dish and 6 mL of sterile dH_2O was pipetted over the top sheet of filter paper. The Petri dishes were sealed using parafilm and placed in a 25 °C growth room with a 14 hour light/ 10 hour dark photoperiod. The seeds were checked daily and 3 mL of sterile dH_2O was supplied to the seeds after two days. Germination percentage (%) and mean germination time (MGT) were calculated. Germination percentage was calculated by using the equation given below:

$$\textbf{Germination } \% = \frac{\mathbf{A}}{\mathbf{B}} \times \mathbf{100}$$

where,

A = number of seeds that have germinated and

B = total number of seeds that were placed on the Petri dish for germination.

The MGT was calculated using the methods presented by Mavi *et al.* (2010),

$$\mathbf{MGT} = \frac{\Sigma \mathbf{nT}}{\Sigma \mathbf{n}}$$

Where,

T = time in days from the start of the test

n = number of germinated seeds (2 mm radicle), at time T at 25 °C

Σn = final germination at the end of the test

Method two for seed preparation was utilized in this study for surface rinsing, as it yielded a higher germination percentage. The seeds that germinated successfully were used in the drought stress experiment that began on the fifth day of germination.

2.4 Bacterial growth and preparing of inoculant

The bacteria were obtained from bacterial stocks made during a previous study conducted on *P. koreensis* and stored at -80 °C. Bacteria were scraped from the stocks and streaked on a specific *Pseudomonas* agar base, King Agar B (Sigma-Aldrich ®). The plates were incubated for 48 hours at 25 °C to attain single bacterial colonies. A bacterial inoculant was used in the drought stress experiment to inoculate the treatment plants with *P. koreensis*. To prepare the bacterial inoculant, a single colony was then taken from an agar plate and placed in 450 mL of sterile Nutrient Broth (NB) media (Sigma-Aldrich ®) which was then shaken at 200 rpm on a rotary shaker for 48 hours at 25 °C. The bacterial concentration of the inoculant used to inoculate bacteria inoculated plants in the experiment was approximated before each inoculation. This was done by adding 100 µL of the inoculant in 400 µL of dH_2O. The solution was vortexed for 10 seconds at 40 Hz (Velp Scientifica, Zx3, Italy). Eight serial dilutions were made. 100 µL of each dilution (individually) was pipetted on to King Agar B plants and spread using a hockey stick spreader. The plates were grown for 48 hours at 25 °C in a growth chamber.

2.5 Plant growth conditions and regime

2.5.1 Experimental conditions and set up

A preliminary experiment was carried out to establish the growth regime which was based off the work done by another student in a previous experiment which focussed on drought stressed sunflower crops. All plants were grown in an indoor growth facility at the University of the Witwatersrand, Johannesburg. The growth room was set at 25 °C and at a

14-hour light/10-hour dark photoperiod. Temperature and humidity were checked weekly using eye buttons throughout the experiments.

The experiment was done in triplicate and each replicate consisted of 48 plants which were separated into 4 sampling subsets: 1. Control which were neither inoculated or stressed (C), 2. Bacteria inoculated and unstressed (I), 3. Stressed and uninoculated (S) and 4. Bacteria inoculated and stressed (IS) plants, which each contained 12 plants (Table 1). The seedlings were planted on day five of germination. All plants were grown for a four-week period after which, a 10-day induced drought stress period was initiated for the drought stressed subsets of plants (S and IS), by withholding water. The unstressed subsets (C and I) were provided with water as per the usual growth regime (Appendix 1).

Table 3. Experimental set up showing sampling subsets used in each experimental replicate of the presented study

Sampling subset	Abbreviation used throughout this study	Description
Control – Uninoculated and unstressed	C	Drought stress absent. Bacterial inoculation absent.
Bacteria inoculated and unstressed	I	Drought stress absent. Bacterial inoculation present
Uninoculated and stressed	S	Drought stress present. Bacterial inoculation absent.
Bacteria inoculated and stressed	IS	Drought stress present. Bacterial inoculation present

2.5.2 Growth regime

All material, (plastics pots, plastic drainage trays, filter paper, vermiculite, forceps, tap water), used in the planting procedure were sterilized by autoclaving for 20 minutes at 121 °C. Approximately 200 grams (g) of vermiculite (Mandoval Vermiculite, South Africa) was placed into 15 cm plastic pots, with one sheet of filter paper below the vermiculite to block the drainage holes at the bottom of the pots. Each pot had an 18 cm drainage tray underneath it. The vermiculite was dampened with 250 mL of autoclaved tap water. The germinated seeds were placed 1 cm below the surface and covered with vermiculite. The

vermiculite was then firmly patted down using sterile forceps. Another 100 mL of water was then provided to the seeds.

The plants were watered daily. For the first week and second week of growth 100 mL of autoclaved tap water was provided, which was then increased to 200 mL for the third and fourth weeks and to 300 mL for the 10 days after 4 weeks of growth (this only applied to the non-stressed plants). The plants received 20 mL of Murashige and Skoog (MS) medium (Sigma-Aldrich ®). MS medium was supplied at a pH of 5.8 (Murashige and Skoog, 1962) twice a week, starting from the first day that the germinated seeds were potted. To inoculate the seedlings that were components of subsets I and IS, 10 mL of the bacterial inoculant was pipetted around the plants on the first day of planting. The plants were further supplemented with an additional 10 mL of the inoculant two weeks after planting. It was calculated (method elaborated above), that each bacteria-inoculated plant was provided with ~ 4.4×10^{12} colony forming units (CFU) per the 10 mL inoculant. During the ten-day stress period, the drought stressed plants were not provided with MS medium and water. The non-stressed plants continued to be treated under the normal growth regime. Due the growth chamber being in proximity to other growth chambers which were running several biocontrol experiments, a mild fungicide and mild pesticide (Table 2) were used as a cross contamination prevention measure and were sprayed on plants alternating each week. A detailed growth regime schedule is tabulated in Appendix 1.

Table 4. Cross-contamination preventative measures utilized in the growth regime of sunflower plants assessed in the current study.

	Name	**Active ingredient**	**Dosage/1 L water**	**Company**
Fungicide	Bravo®	Chlorothalonil	1.6 mL	Syngenta Crop Protection, New South Wales
Pesticide	Malasol®	Mercaptothion (Organophosphate)	1.25 mL	Efekto, South Africa

2.6 Physiological analyses

2.6.1 Plant height

All plants were measured weekly from the part of the stem that was in line with border of the pot, to the tip of the plant, using a tape measure (Figure 9) Heights were recorded over the 4-week growth period and once on the final day on sampling after stress period.

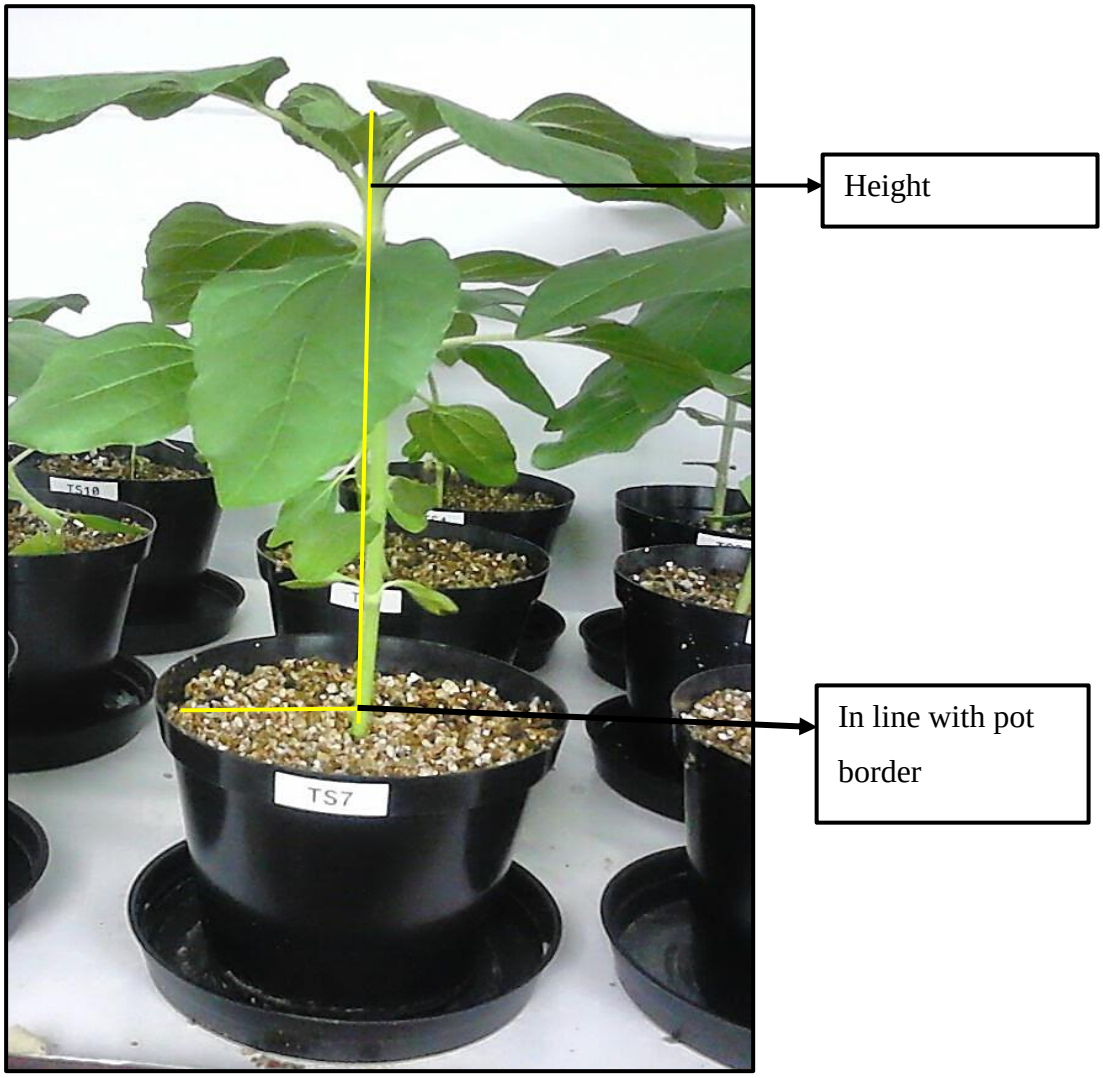

Figure 9. Height of plant as measured in this study.

2.6.2 Leaf area

Leaf measurement (length and width at the longest part) were done on the first fully extended leaves (Figure 10). Leaf area was calculated using a specialized equation developed by Rouphael *et al.* 2007, for sunflower leaves. We required a simple, effective and non-destructive means of measuring leaf area. Image analysis was not an option as there were too many leaf samples.

The equation was chosen due to the high coefficient of determination (r^2 = 0.978) and low mean square error value found in the study conducted by Rouphael *et al.* (2007).

$$\mathbf{LA = a + bW^2}$$

Where:

LA = leaf area

a = constant

b = fitted coefficient

W = width at widest part of leaf

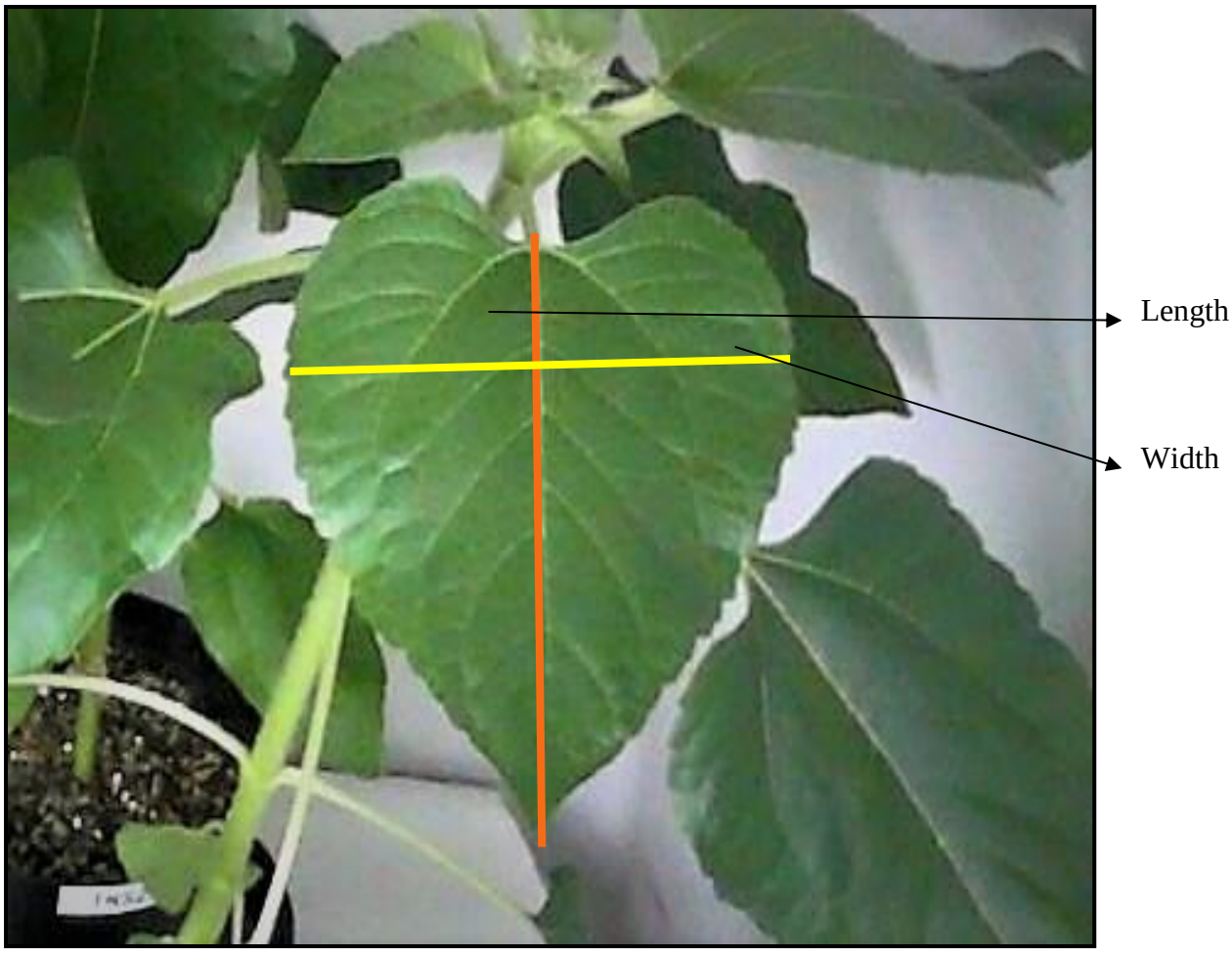

Figure 10. Leaf length and width as measured in this study.

2.7 Sampling and preparation of plants

After the growth regime was completed the plants were sampled. Leaves, roots as well as the entire plant were sampled. For each replicate, the four subsets were further broken

down into three sample categories which each consisted of four plants each. The first sample category was used for leaves; the second sample was used for roots and the third sample was used for the optimized metabolite extraction of the biofilm (Figure 11).

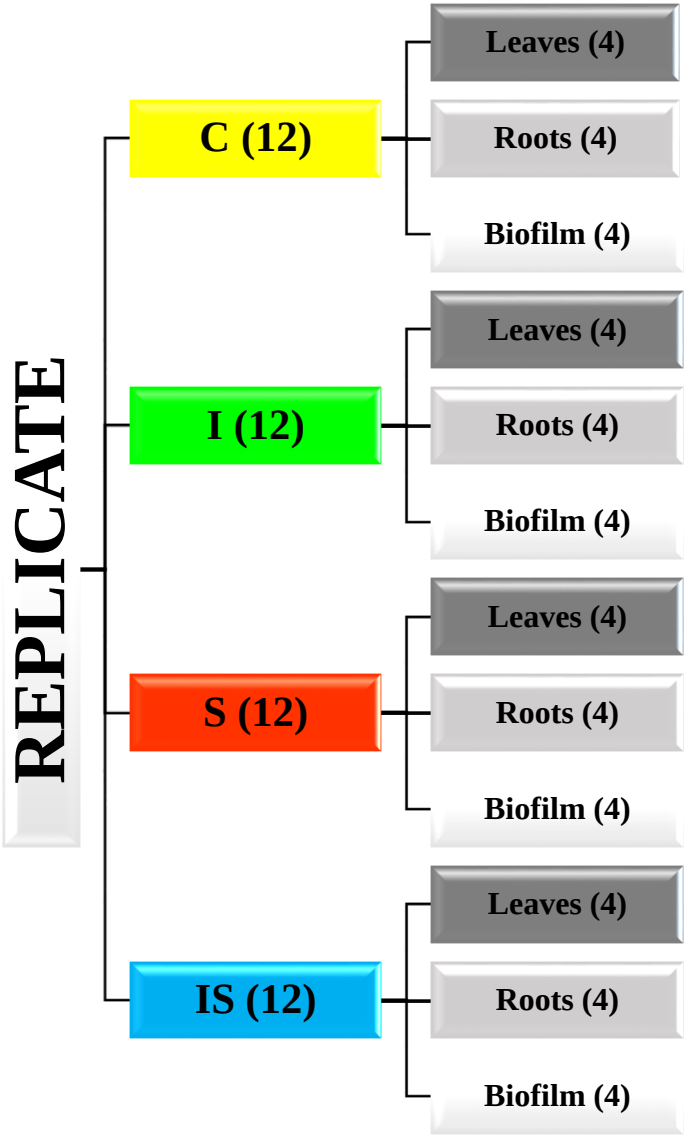

Figure 11. Diagrammatic representation of the sampling divisions for each replicate. Each replicate consisted of four subsets which comprised 12 plants each; control (C), bacteria inoculated and unstressed (I), Uninoculated and stressed (S) and bacteria inoculated and stressed (IS); and were further divided into three sampling categories. Each category; leaves, roots and biofilm; each encompassing four plants. Growth conditions for all replicates: 25 °C and 14-hour light/10-hour dark photoperiod.

The first, second and third fully developed leaves were cut for biochemical assays and metabolite optimization analyses. The position of the leaves was kept consistent for the different assays and experiments to avoid discrepancy and to keep measurements uniform.

Takabayashi *et al.* 1994, detail the differences in composition at different parts of a leaf. The first set of fully developed leaves were utilised for the proline and phenolic compound assays, the second set of fully developed leaves were used for the metabolite extraction and analyses, and the third set of fully developed leaves were utilised for the reactive oxygen species (ROS) assays. The leaves were cut using a sterile scalpel blade at the point below the petiole at the base of the leaf.

The first and second fully developed leaves were placed in separate, well labelled, zip lock bags and then into a Dewar which contained liquid Nitrogen within 30 seconds of cutting. The third set of fully developed leaves was used for the ROS assay which had to be done immediately on the day of sampling by following the method explained below. Roots were sampled by cutting the entire root system from ±1 cm below the vermiculite surface. Most of the vermiculite was shaken off gently. Approximately one gram of root sample was placed into a 50 mL Falcon conical tube to be used for root bacterial counts, as explained below. The rest of the root sample was placed into zip lock bags and then into a Dewar containing liquid Nitrogen with 1 minute of sampling, for metabolite analyses.

The root and leaf samples were then stored at -80 °C and subsequently freeze-dried (SP Scientific Benchtop Pro 9L-85, USA) to remove all moisture present in the samples. The dried leaf matter was ground using a liquid nitrogen, a mortar and a pestle. The samples were then stored at -20 °C until needed for their respective metabolomic and biochemical assays. Samples were however, not stored for more than a month.

The third category of sample plants were utilized for a metabolite optimization technique, developed during this study to extract the bacterial biofilm layer surrounding the roots. The entire root system of the plant was quenched using 250 mLs of a solution made up of isopropanol, acetonitrile and water in a ratio of 3:3:2, which was poured directly into the pot, being sure to go around the entire stem. Below the pot a sterile funnel which flowed into a beaker, was placed. Quenching took a few minutes during which time approximately two thirds of the solution passed through the plant and into the beaker. In order to maintain the cold temperature of the solution during the quenching process, the beaker and funnel were placed in a box containing dry ice (Figure 12). The solution was transferred into a 250 mL centrifuge bottle and centrifuged (ThermoFisher Scientific, Sorvall RC-6 plus, USA) at 4800 rpm for 10 minutes. The 80% supernatant was carefully transferred into two 50 mL Falcon conical tubes and placed into a Dewar containing liquid nitrogen then into storage at -80 °C.

The solutions were then freeze dried (SP Scientific Benchtop Pro 9L-85, USA) for 8 hours before metabolite extraction.

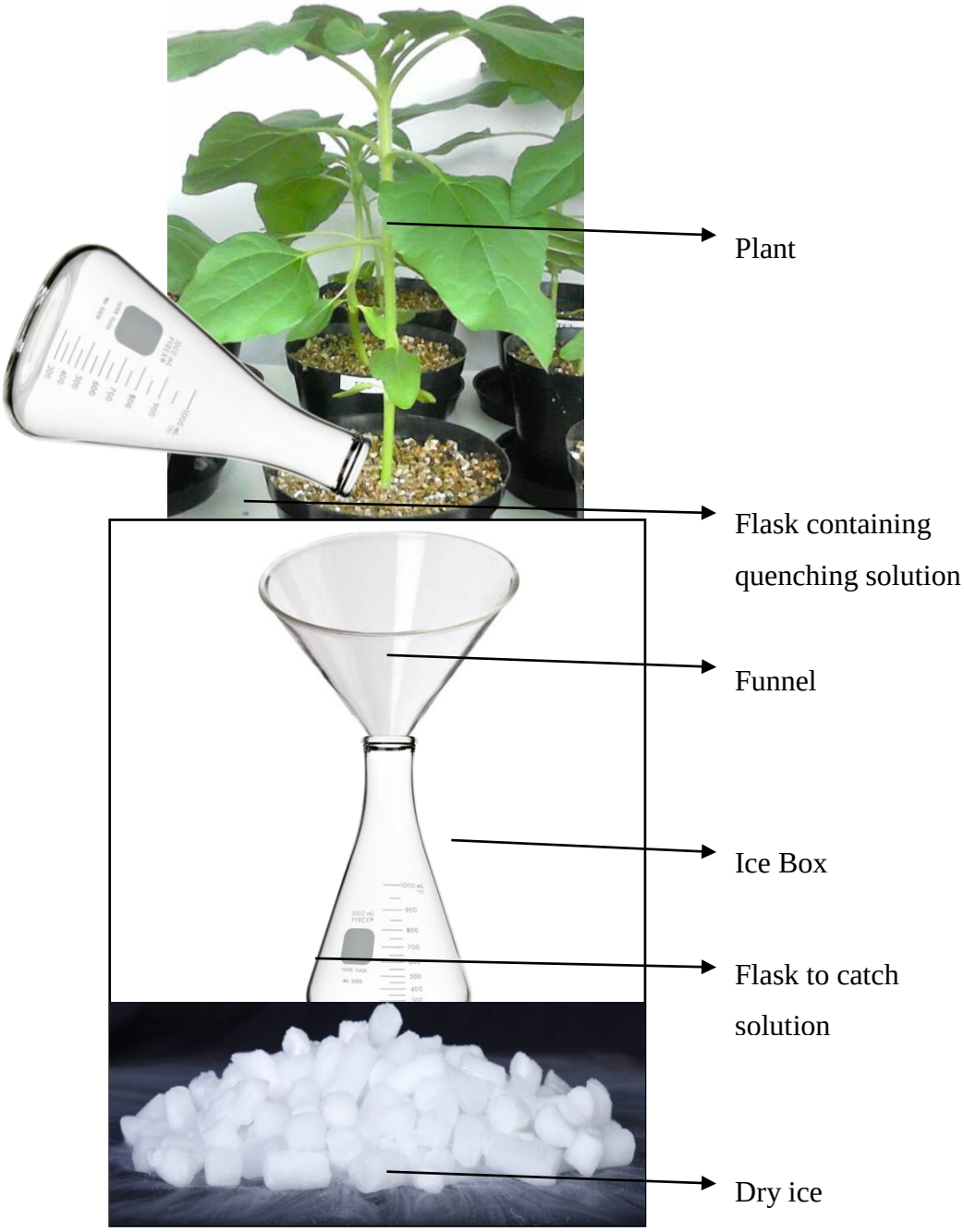

Figure 12. Diagrammatic representation of the biofilm quenching process optimization and utilized in this study. The pot of each plant used for this process was placed on the opening of a sterile funnel, which passed into a flask that was submerged in an ice box containing dry ice. 250 mL of a chilled quenching solution made up of acetonitrile/isopropanol and water (3:3:2) was slowly poured into the pot. The solution that passed through the pot and funnel, into the flask was transferred to centrifuge bottled and utilized for the biofilm metabolite extraction.

2.8 Root bacterial counts

Root bacterial counts were done in order to assess if any contamination had occurred during the experiments. To do this, root bacterial counts were carried out for both inoculated and uninoculated plant samples using a method adapted from Sandhya *et al.* (2009).

Approximately one gram of the root sample was shaken loose from as much vermiculite as possible. Thereafter, the root sample was placed in a 50 mL Falcon conical tube with 10 mL 9% sodium chloride (NaCl) and 0.05% Tween 20. The mixture was vortexed at 40 Hertz (Hz) for two minutes. One mL of the solution was then spread onto a King B Agar plate and incubated for 48 hours at 25 °C. Using a plate count method, bacterial colonies were counted where possible.

2.9 Biochemical analyses

2.9.1 Reactive oxygen species (extracellular superoxide)

To calculate the concentration level of extracellular superoxide produced, the third pair of fully developed leaves (from the top of the plant), were used immediately after cutting. The method (adapted from Beckett *et al.* 2003; Misra and Fridovich, 1972) is based on oxidation of epinephrine to adrenochrome which is instigated by NADH, and subsequently estimated spectrophotometrically (Misra and Fridovich, 1972). Therefore, it is important to note that the value calculated in this assay reflects the extracellular concentration of superoxide levels that were released into the external environment of the sampled leaves, in the form of adrenochrome. After cutting, the leaves were immediately placed in a 50 mL Falcon conical tube containing 15 mL of 1 mM epinephrine (Sigma-Aldrich ®), which had been adjusted to pH 7 using 1 M sodium hydroxide (NaOH). The tubes were placed on an orbital shaker (MRC, TS-400P, Britain) set at 120 rpm, in the dark for 15 minutes, at 25 °C. After incubation, the spectrophotometric absorbance of the adrenochrome representative of the extracellular superoxide produced was read at 480 nm (Helio Thermo Scientific Spectrophotometer, USA). The adrenochrome molar extinction coefficient of 4020 M^{-1} cm^{-1} was used to calculate the level of extracellular superoxide produced and released into the external environment of the sampled leaves.

2.9.2 Phenolic compounds

The extraction of phenolic compounds was achieved using a method adapted from Tabart *et al.* 2007; Torti *et al.* 1995; Waterman and Mole 1994.

The process involved mixing 0.1 g of homogenized ground leaf material (taken from the first pair of fully developed leaves), with 12.5 mL of 95% ethanol, in a 15 mL Falcon conical tube. The mixture was there placed on an orbital shaker (MRC, TS-400P, Britain) at 200 rpm, in a cold room set at -4 °C for three hours. Thereafter, the mixture was centrifuged at 4000 rpm for 15 minutes (ThermoFisher Scientific, Sorvall RC-6 plus, USA). The supernatant was carefully removed and filtered using a syringe and filter, to exclude any residual constituents, and stored in the cold room. The pellet was then resuspended in 5 mL 95% ethanol and centrifuged once again at 4000 rpm for 15 minutes. The supernatant was removed and filtered, then combined with the first filtrate. Using the combination of both filtrates, the spectrophotometric quantification of phenolic compounds was done (Helio Thermo Scientific Spectrophotometer, USA).

The Folin-Ciocalteau (FC) method was utilized to determine the total concentration of phenolics present, represented as a gallic acid equivalent (GAE), which is widely used for the accurate determination of phenolic compounds in several plant species (Waterman and Mole 1994). A GAE standard curve was generated by using gallic acid (Sigma-Aldrich ®) to form a concentration range of 20 – 100 µg/mL in 20 µg/mL increments. A 10 mg/mL stock solution was prepared by dissolving 10 mg of gallic acid in 1 mL of 95% ethanol, thereafter adding dH_2O to make up the volume required. Dilutions were made using the stock solution and a standard curve was prepared.

In a 15 mL Falcon conical tube, 1 mL of the combined filtrate, 1 mL of 95% ethanol, 5 mL dH_2O and 0.5 mL 50% FC reagent (Sigma-Aldrich ®), were vortexed at 40 Hz (Velp Scientifica, Zx3, Italy) for 30 seconds. The solution was then left to stand for 25 minutes at room temperature, after which 1 mL of 5% sodium carbonate (Na_2CO_3) was added and vortexed for 20 seconds. The tubes were then incubated at room temperature, in the dark, for one hour. Post incubation, 1 mL of the solution was transferred to a plastic cuvette for the spectrophotometric absorbance reading at 760 nm (Helio Thermo Scientific Spectrophotometer, USA). Absorbance was read in triplicate for each leaf sample. Using the standard curve generated, the approximate concentrations of phenolic compounds could be calculated and represented as GAE. The formula used was:

$$\mathbf{mgGAEg^{-1}of\ dry\ weight\ of\ leaves} = \frac{\mathbf{\mu g/mL\ x\ 35\ mL}}{\mathbf{g\ sample}} \div \mathbf{1000}$$

Where:

µg/mL = value attained from standard curve equation

35 mL = total volume of 95% ethanol used for extraction

g sample = dry weight of the ground leaf sample

2.9.3 Proline

The method adapted from Sun *et al.* 2006; and Bates *et al.* 1973 was used for the extraction of proline. 0.1 g of homogenized ground leaf material (taken form the first pair of fully developed leaves), was added to a 15 mL Falcon conical tube containing 10 mL of 3% sulfosalicylic acid (Sigma-Aldrich®). The tubes were immediately vortexed at 40 Hz (Velp Scientifica, Zx3, Italy) for 90 seconds and then placed on a dry heating block (Gemmyco,

DB-006E, Taiwan) for 10 minutes at 100 °C. The tubes were cooled in a water bath set at 22 °C for 5 minutes, then centrifuged (ThermoFisher Scientific, Sorvall RC-6 plus, USA) at 4000 rpm for 15 minutes. The supernatant was removed and filtered (Munktell Ahlstrom, 3HW 90 mm, Sweden). The filtrate was divided into two 2 mL sub-samples per plant, which was mixed with 2 mL freshly prepared ninhydrin acid reagent and 2 mL glacial acetic acid in the ratio of 1:1:1. To prepare the ninhydrin acid reagent 1.25 g of ninhydrin (Sigma-Aldrich ®), was mixed with 30 mL glacial acetic acid and 20 mL 6 M phosphoric acid). The solution was vortexed for 20 seconds and then placed on a dry heating block for one hour at 100 °C. The reaction was stopped by dipping the tubes in ice water. Once cooled, 4 mL toluene was added and vortexed for 30 seconds. The tubes were left to warm to room temperature after which, spectrophotometric absorbance was read at 520 nm. Dilutions were made as required.

Spectrophotometric absorbance was read in triplicate for each sample (Helio Thermo Scientific Spectrophotometer, USA). Toluene was utilized as a blank. Toluene was incompatible with plastic cuvettes, as it melted them, therefore glass cuvettes had to be used for the proline assays.

A standard concentration curve was established using L-proline (Sigma-Aldrich ®) for the range of 5 – 30 µg/ml in 5 µg/ml increments. To calculate the concentration of proline in each plant sample, the following equation was used:

$$\frac{\textbf{µmole Proline}}{\textbf{g dry weight}} = \frac{\textbf{µg proline/ml} \times \textbf{ml toluene}}{\textbf{115.5 µg/µmole}} \div \textbf{g sample}$$

Where:

µg proline/ml = value attained from the standard curve equation

mL toluene = total amount of toluene added

115.5 µg/µmole = molecular weight of proline

g sample = dry weight of ground leaf sample

2.10 Optimization of metabolomics analyses

Metabolomic analyses were conducted according to a method adapted from Fiehn *et.al.* (2008). It is important to note that these analyses were conducted in order to optimize suitable techniques that can be utilized for future plant-microbe system studies. To reiterate, these analyses focussed on the methods carried out, from the growing of plants, sampling, derivatization and GC-MS analyses, in order to assess the technical reproducibility of the method. Actual metabolite quantification was not the focus therefore no biological reproducibility was tested. Thus, intensive statistics for quantification of metabolites were not conducted. By assessing technical variation through the use of relative standard deviation; repeatability and accuracy of the technique utilized for non-targeted metabolomics was assessed, in order to optimize the techniques utilized for future studies. All metabolomic analyses were conducted using stressed plants (S and IS) from experimental replicate three only.

2.10.1 Leaf and root sample preparation and extraction processing

2.10.1.1 Leaf samples preparation

For the extraction of metabolites in the plant leaf samples, the first pair of fully developed leaves were used. Approximately 20 mg (correct to two decimal places) of the homogenized ground leaf material was used for the metabolite analyses for each sample. Three leaf pairs form three different plants grown in the same experimental replicate were used. Each leaf pair was divided into three further leaf subsamples thus making a total of nine samples. Each sample was subsequently injected three times (GC-MS) as described further below, therefore a total of 27 metabolite profiles were gained for leaf samples (Figure 13). A high replication number in terms of samples allowed the assessment of reproducibility of the method utilized.

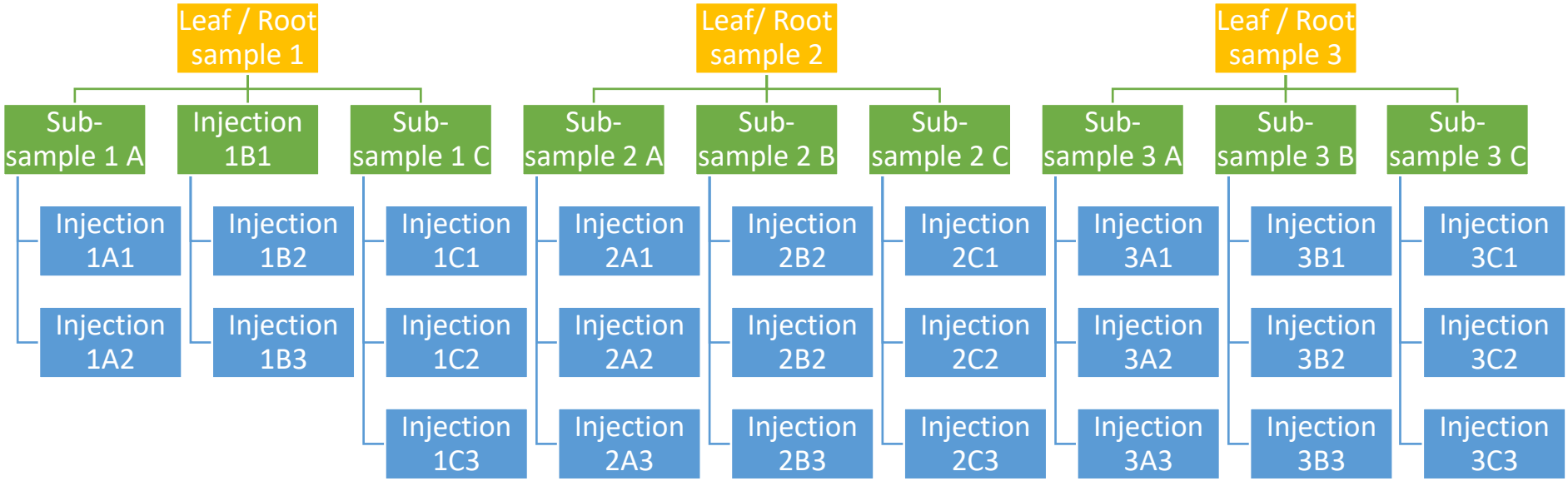

Figure 13. Diagrammatic breakdown of samples and subsamples as used for metabolite analyses.

2.10.1.2 Root sample preparation

The root samples contained a large amount of vermiculate which had to be carefully shaken off before snap freezing using liquid nitrogen (Figure 14).

Figure 14. Roots of plant study species intertwined in vermiculite.

Even after shaking, there was still a considerable amount of vermiculite attached to the root samples. To remove the surfeit vermiculite from the root samples, the roots and vermiculite were separated carefully by hand. Thereafter, the samples were ground very lightly, using a pestle and mortar then sieved. This process was repeated several times to remove excess vermiculite. It is important to note that this technique was adapted for these samples and there was still the likelihood of vermiculite particles present in the samples (Figure 15). Once the final sample (Figure 15J) was attained, approximately 20 mg (correct to two decimal places) of the homogenized ground root material was used for the metabolite analyses for each sample. Three root extracts from three different plants grown in the same

experimental replicate were used. Each root extract was divided into three further root subsamples thus making a total of nine samples. Each subsample was subsequently injected three times, therefore a total of 27 metabolite profiles were gained for root samples (Figure 13). A high replication number within samples allowed the assessment of reproducibility of the method utilized.

2.10.1.3 Extraction method for leaf and root samples

The extract solvent was made up of isopropanol, acetonitrile and water in the ratio of 3:3:2. The solvent was degassed by passing a steady stream of nitrogen through it for 15 minutes. The solvent was then chilled to -20 °C for four hours' prior extraction. One mL of the solvent was added to 20 mg of the ground plant tissue (leaf or root) in a 2 mL Eppendorf test tube. The mixture was subsequently vortexed (Velp Scientifica, Zx3, Italy) for 10 seconds at 40 Hz, then shaken (MRC, TS-400P, Britain) for 5 minutes at 4 °C, to promote the extraction of metabolites and precipitate proteins present. The solution was then centrifuged at 12800 g for 2 minutes (ThermoFisher Scientific, Sorvall RC-6 plus, USA) and 90 % of the supernatant was carefully removed. The supernatant was subjected to liquid nitrogen to freeze it and was then placed in a freeze drier (SP Scientific Benchtop Pro 9L-85, USA) for 8 hours. The residue was then resuspended to fractionate complex lipids by adding 500 µL of 50% aqueous acetonitrile to the tube. The solution was centrifuged once again at 12800 g for 2 minutes. 90% of the supernatant was transferred to a 1.5 mL Eppendorf tube and subjected to liquid nitrogen and drying as done before. The dried extracts could be stored at -80 °C for up to 4 weeks, but in this study, extracts were derivatized for GC-MS analyses immediately.

2.10.1.4 Biofilm extraction optimization

The extraction of biofilm metabolites was done by using the solution attained from the quenching technique outlined previously. Once the solution had been freeze dried the extraction methods used for the plant tissue samples was utilized, excluding the addition of the solvent, as the plants were already quenched using the same solvent.

Three inoculated and stressed (IS), and three uninoculated and stressed (C) plants were used for this extraction. Each sample was injected in triplicate.

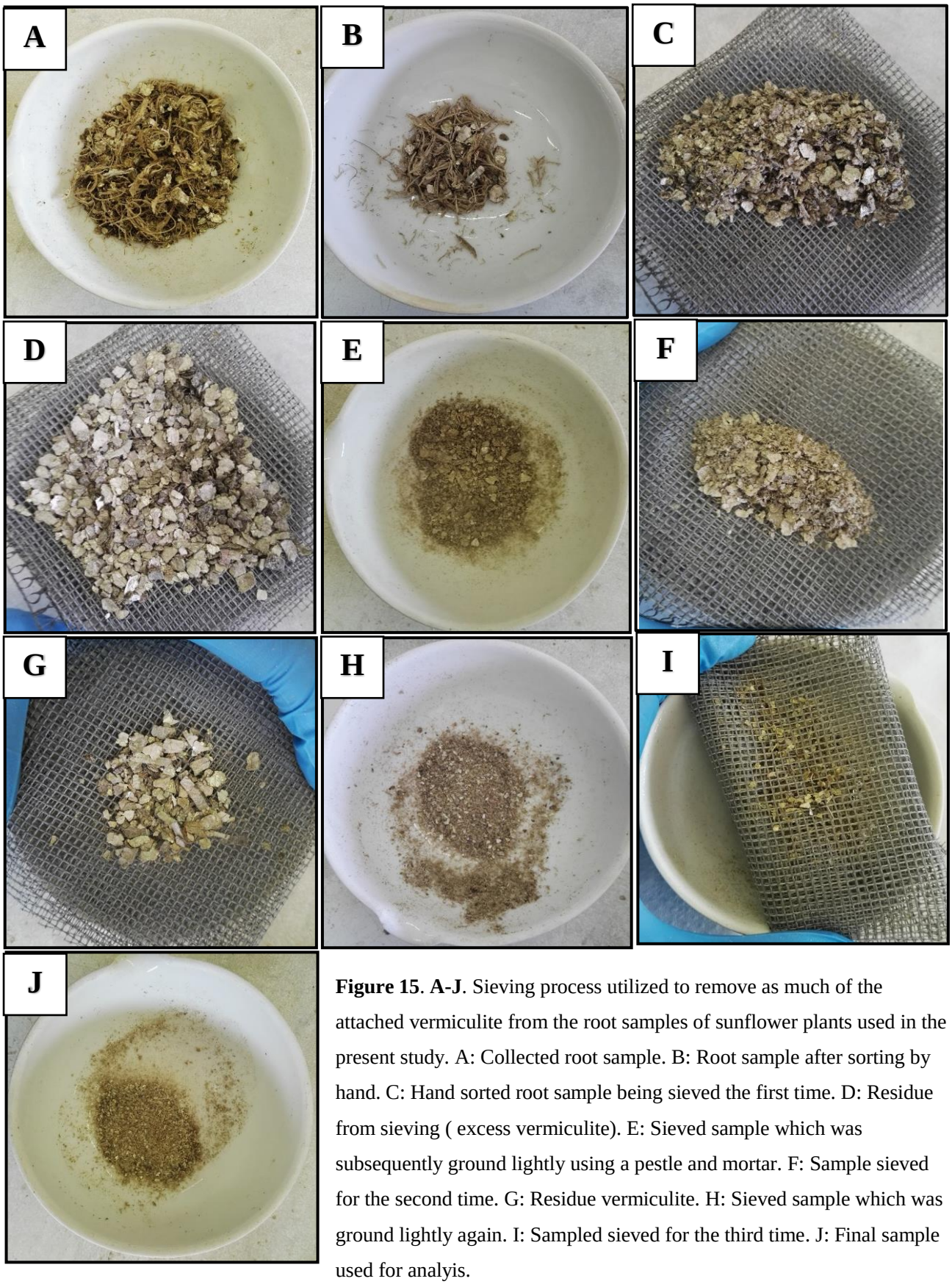

Figure 15. **A-J**. Sieving process utilized to remove as much of the attached vermiculite from the root samples of sunflower plants used in the present study. A: Collected root sample. B: Root sample after sorting by hand. C: Hand sorted root sample being sieved the first time. D: Residue from sieving (excess vermiculite). E: Sieved sample which was subsequently ground lightly using a pestle and mortar. F: Sample sieved for the second time. G: Residue vermiculite. H: Sieved sample which was ground lightly again. I: Sampled sieved for the third time. J: Final sample used for analyis.

2.10.2 Gas chromatography – mass spectrometry sample preparation

Three subsamples were prepared from each sample thus producing nine leaf and nine root samples as detailed above, to better assess accuracy and reproducibility. Two μL of an internal retention index mixture made up of fatty acids (Superlco ®) was added to the dried extracts. 10 μL of 20 mg mL^{-1} freshly made solution of 98% pure methoxyamine hydrochloride (Sigma-Aldrich ®) in pyridine was added then shaken for 90 minutes at 30 °C. Thereafter, 90 μL of N-methyl-N-trimethylsilyltrifluoroacetamide (Sigma-Aldrich ®) was added and shaken for 30 minutes at 37 °C. Once the reaction was complete, the mixture was transferred into 2 mL clear glass auto-sampler vial (Agilent Technologies) with the addition of a 250 μL pulled point conical glass micro insert, due to very small volumes. The vial was then closed using a screw on cap with a silicone septa (Agillent Technologies).

2.10.3 Instrumentation and methods used

2.10.3.1 Auto-injection methods

The gas chromatography-mass spectrometry analysis was conducted at the School of Chemistry, at the University of the Witwatersrand, Johannesburg. The analyses for all samples were performed on a Leco GCxGC-TOF low resolution mass spectrophotometer. The sampled were injected in the form of a liquid injection. One μL of the sample was injected in the injection port with a Topaz liner, split single taper gooseneck without wool (4mm × 6.5 × 78.5) using a Gerstel Multi-Purpose auto sampler. Each sample was injected in triplicate with blanks between each replicate. Three blanks were run between each sample and one blank between each subsample). The injector was washed three times with 10 μL ethanol and methanol between injections. The liner was changed after every sample (14 injections including blanks) injections.

2.10.3.2 Gas chromatography methods

A BPX-5 column of length 29.261 m out of 30 m was used. The internal diameter of the column was 250 μm and the film thickness was 0.25 μm, and 360 °C was the maximum temperature utilized. The front inlet type was split/splitless with the activation of split mode in the split ratio of 10:1. The carrier gas used was 99.99% pure Helium. There was a constant flow at a rate of 1.5 mL/min. The front inlet temperature was at 280 °C, while the oven temperature was at 50 °C which was maintained for one minute before being increased at 20 °C/min until 250 °C as reached and maintained for three minutes. The transfer line temperature was maintained at 250 °C.

2.10.3.3 Mass Spectrometry Methods

There was a solvent acquisition delay of 4.8 minutes. A mass range of 35 o 459 *m/z* with an acquisition rate of 20 spectra/ second, acquisition voltage at 1445 eV and ionization energy of -70 eV was utilized. The ion source temperature was at 250 °C.

2.10.3.4 Data processing

The base-line offset of one which was just above the noise wat utilized, with a peak width of 4 seconds and a signal to noise ratio of 100. The library search mode was normal and forward. The number of library hits was 10, while the minimum molecular weight allowed was 35 and maximum molecular weight was 450. The mass threshold was 5% and the minimum similarity before name was assigned was 50%. The library used was Replib and mainlb from NIST. ChromaTof software (LECO ChromaTOF®, 2017) was used for data normalization. The system was operated by Mr. Thapelo Mbhele.

Eight metabolites were analyzed in the leaf samples (D-xylose, L-5-oxoproline, D-arabinose, pipecolic acid, L-threitol, L-leucine, L-valine and L-alanine). Six metabolites were analyzed in the root samples (D-xylose, L-5-oxoproline, D-arabinose, L-leucine, L-valine and glycerol). Three metabolites were analyzed from the biofilm extraction samples (D-xylose, D-arabinose and glycerol).

2.11 Data analyses

All statistical analyses were carried out using R statistical software version 3.3.1 (R Core Team, 2016) and Statistica Software version 12.5 (StatSoft Inc, 2016). The data was tested for outliers which were subsequently removed. The data was then tested for normality using Shapiro-Wilk normality tests. Data precision was reported using standard error (SE).

T-tests were utilized to compare plant height of all sampling subsets after the ten-day stressed/unstressed period, leaf area, leaf length and leaf width of inoculated and uninoculated experiments. A repeated measures ANOVA and Tukey HSD post-hoc tests were ran to compare the height of plants over the four week growth period, between inoculated and uninoculated plants. One-way ANOVAs and Tukey HSD post-hoc tests were run to compare all biochemical analyses (ROS, Phenolic acids, Proline).

For metabolite analyses, relative standard deviation percentages were calculated between each injection within each leaf sample and between the all amalgamated injections

of each leaf sample. Average peak areas were also compared between inoculated and uninoculated stressed samples for metabolites analysed as mentioned above.

Chapter three: Germination and Physiology - Results and Discussion

3.1. Results

3.1.1 Seed preparation and germination

From preliminary experiments (performed in triplicate), method two (described in Chapter 2) was chosen to prepare the seeds. There was a 100% germination and the mean germination time (MGT) was calculated to be, on average, 2.5 days for the preliminary replicates, as well as for every replicate in the actual study (Figure 16). Method one did not yield the same return or consistent results, with an approximate germination percentage of 80% and a MGT of 3.0 days. There was no fungal or bacterial contamination of the seeds following either method, thus the method chosen was based purely on germination success.

Figure 16. Germinated seeds (100% germination) at the end of 5-day germination period which were ready to be transferred into pots for the drought stress experiments.

3.1.2 Root microbial counts

The bacteria inoculated plants that were not stressed had a significantly larger amount colony forming units (CFU) than all other sampling subsets (*F (3,16) = 15.684, P = 0.00005*). The bacteria inoculated subset also had on average 4.2×10^6 CFU/g, 5.8×10^6 CFU/g and 2.6×10^6 CFU/g more microbial colonies than the control, stressed, and bacteria stressed subsets respectively (95% CI: $1.93 – 3.72 \times 10^6$).

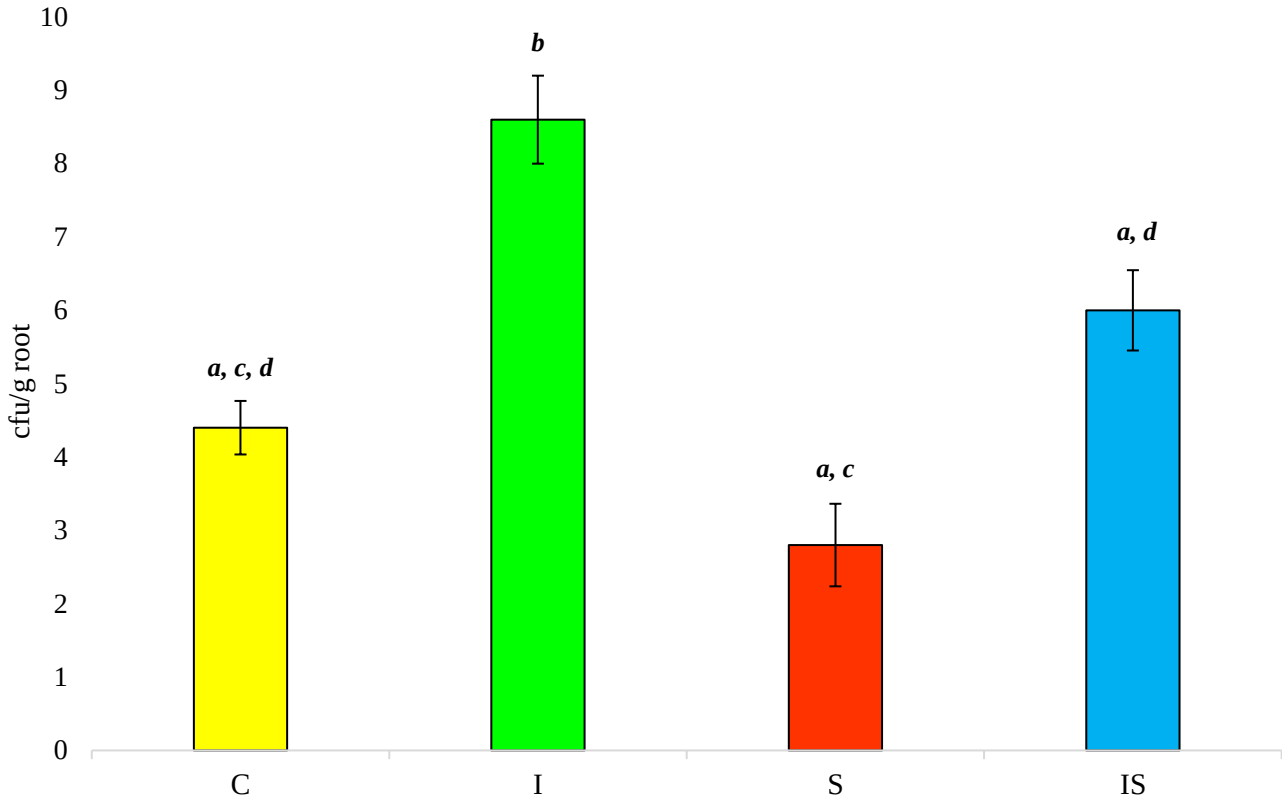

Figure 17. Average number of colony forming microbial units present in sampling subsets. Counts were done at the end of the 4 weeks under normal growth conditions and a further 10-day induced drought stress period (stressed plants), and 4 weeks + 10 days under normal growth conditions (unstressed plants). Vertical bars represent mean ± SE. Lower case italicized letters represent significant difference between treatments (*F (3,16) = 15.684, P = 0.00005*).

3.1.3 Physiological analyses

3.1.3.1 Plant height

There was no significant difference in plant growth rate between uninoculated plants which had a growth rate of 7.3 ± 0.14 cm/week and inoculated plants which had a growth rate of 8.07 ± 0.11 cm/week, for the first three weeks (*F (3,426) = 3.2912, P = 0.02061*). In the fourth week of growth the inoculated plants were significantly taller (32.38 ± 0.45 cm) than the uninoculated plants (29.2 ± 0.57 cm). However, it was visually observed that the inoculated plants were consistently taller than the uninoculated plants throughout the growth period of each replicate (Figure 18).

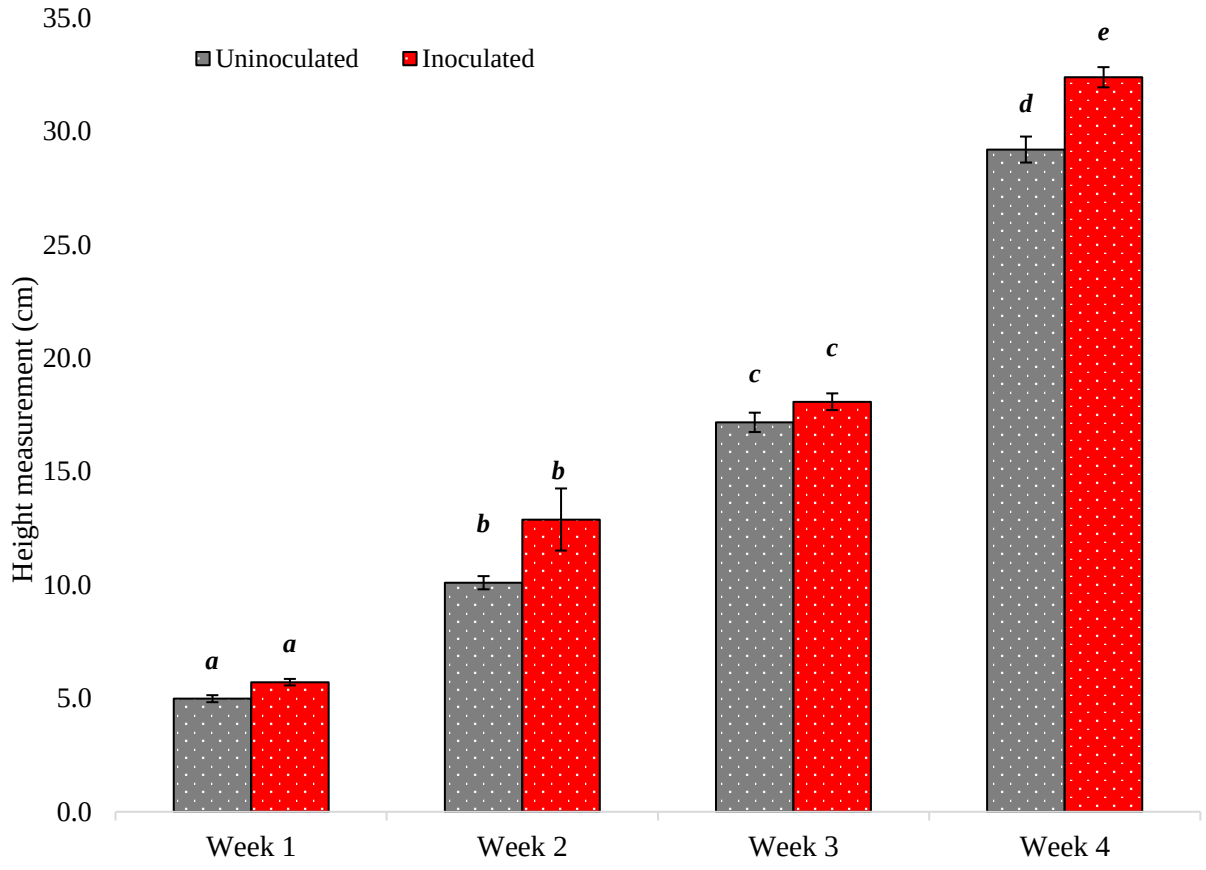

Figure 18. Average height of both uninoculated and inoculated plants during the first four weeks of vegetative growth before water stressed conditions. Vertical bars represent mean ± SE. Lower case italicized letters represent significant difference between treatments. There was no significant difference between the two treatments until the fourth week of growth (*F (3,426) = 3.2912, P = 0.02061*).

The plant height measurements taken after the 10-day drought stress period indicated that the inoculated, unstressed plants (I), and were significantly taller (53.6 ± 0.5 cm) than the other subsets. The control subset (C) was taller (46.9 ± 1.1 cm) than both stressed subsets (S and IS). There was, however, no significant difference between the two stressed subsets (S and IS) (*F (3,140) = 42.350, P = 0.0000*).

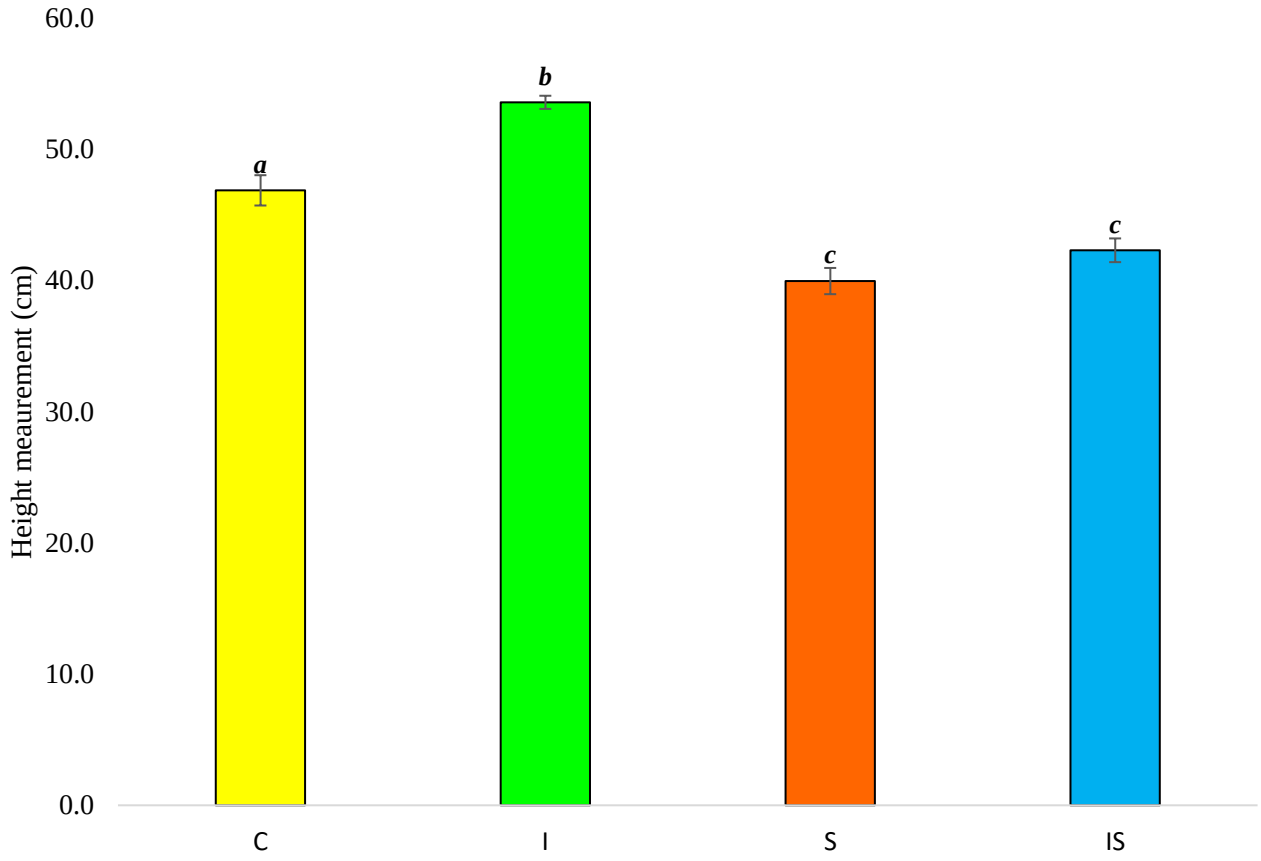

Figure 19. Average height of different plant treatment subsets at the end of the 10-day induced drought stress period (S and IS), and 10 days of normal growth conditions (C and S). Vertical bars represent mean ± SE. Lower case italicized letters represent significant difference between treatments. There was no significant difference between the stressed subsets (*F (3,140) = 42.350, P = 0.0000*).

3.1.3.2 Leaf area

The calculated leaf areas of inoculated plants (51.12 ± 1.31 cm^2) were on average larger than that of the uninoculated plants (42.24 ± 0.97 cm^2), and there was a significant difference between the leaf areas of the two treatments (*t = -5.439, df = 131, p < 0.0001*) (Figure 20).

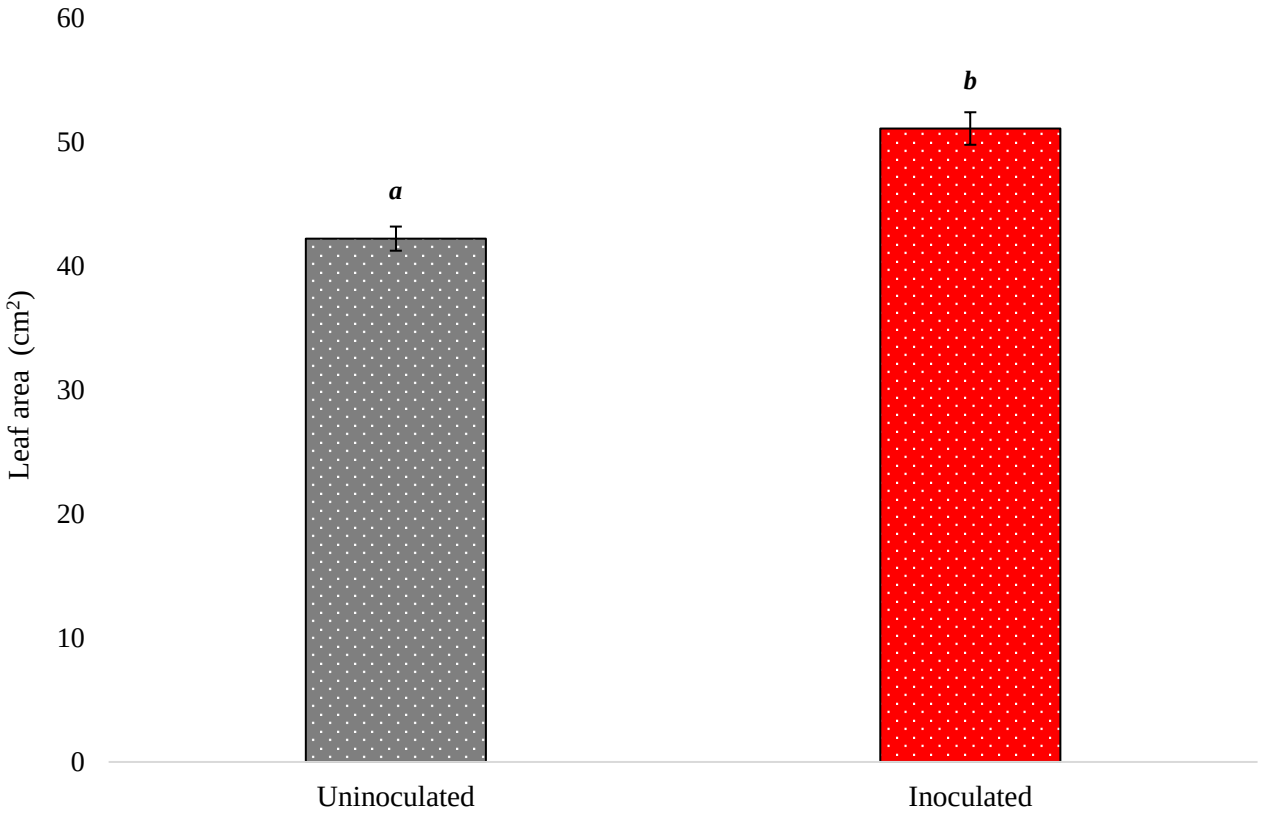

Figure 20. Average leaf area of both uninoculated and inoculated plants after four weeks of vegetative growth before water stressed conditions. Vertical bars represent mean ± SE. Lower case italicized letters represent significant difference between treatments (*t = -5.439, df = 131, p < 0.0001*).

Individual analyses comparing leaf width between treatments (*t = -5.259, df = 134, p < 0.0001*) and leaf length between treatments (*t = -5.049, df = 134, p < 0.0001*) also indicated that the inoculated plants performed better (Figures 21 and 22).

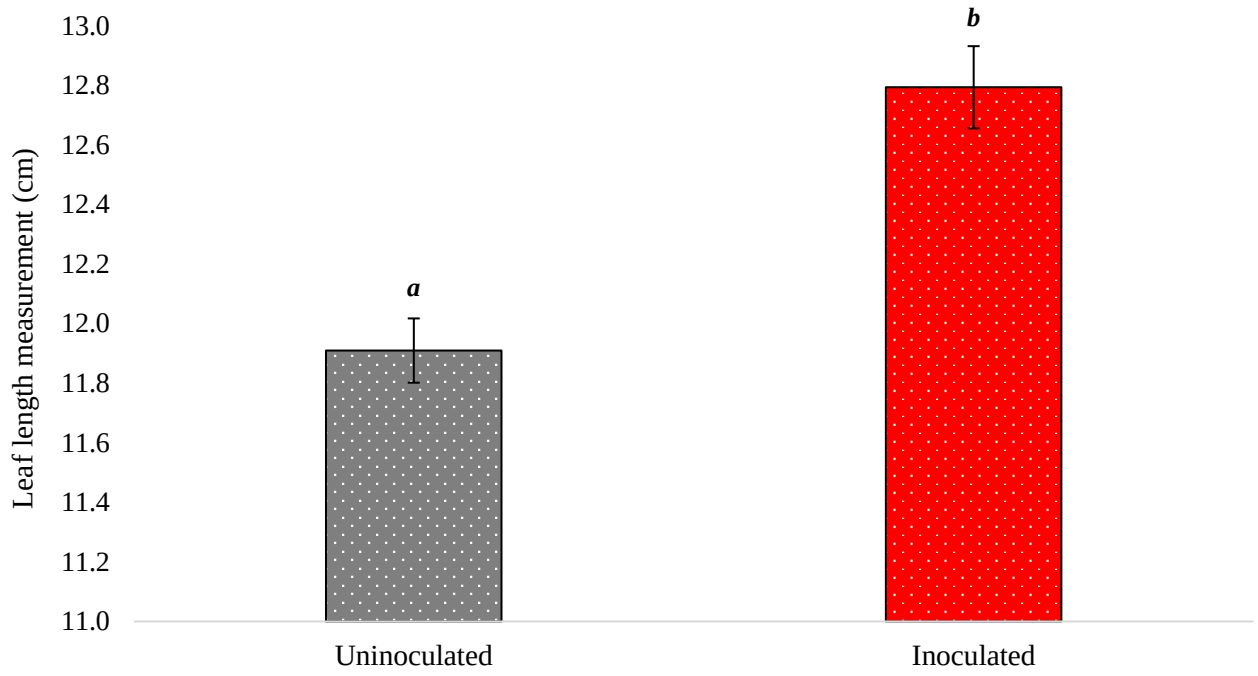

Figure 21. Average leaf length of both uninoculated and inoculated plants after four weeks of vegetative growth before water stressed conditions. Vertical bars represent mean ± SE. Lower case italicized letters represent significant difference between treatments (*t = -5.049, df = 134, p < 0.0001*).

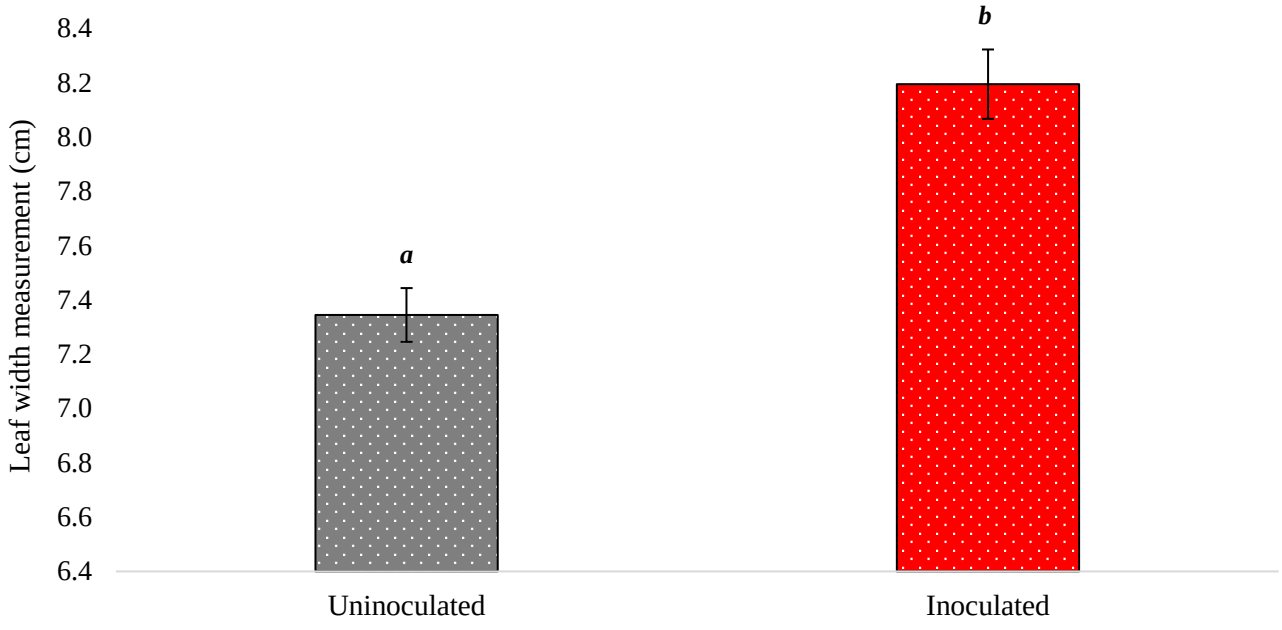

Figure 22. Average leaf width of both uninoculated and inoculated plants after four weeks of vegetative growth before water stressed conditions. Vertical bars represent mean ± SE. Lower case italicized letters represent significant difference between treatments (*t = -5.259, df = 134, p < 0.0001*).

3.2 Discussion

3.2.1 Seed preparation and germination

The mean germination time (MGT) for the seeds used in both the preliminary and study experiments was 2.5 days, using method two. This was lower than the MGT calculated for method one (3.0 days) and was perhaps due to the 70% ethanol inhibiting germination at a certain level. However, using method two which excluded the ethanol component, 100% germination was seen in all experimental replicates after a five-day germination period. The MGT calculated in the present study was slightly higher than that seen in a study conducted on a sunflower cultivar – Sanbro, by Kaya *et.al*. (2006), which reported a MGT of 1.87 days and a germination percentage of 98.6%, and in another study by Kaya and Day (2008) on two other cultivars – Muson and Sirena, which reported MGT's of 2.10 and 2.43 days respectively. The MGT in this study was however, lower than the 4.68 days reported by Gül *et.al*. (2017) for the sunflower cultivar – Corum. The wide range of MGT times reported in various studies indicate that different cultivars of sunflower take different amounts of time to germinate

For future studies, it would be beneficial to compare the MGT and germination percentages of various sunflower cultivars used for agricultural purposes. It would also be useful to determine MGT and germination percentage at different concentrations of ethanol, using method one from the germination protocol to evaluate the effect ethanol has on germination.

3.2.2 Root microbe colonization

Plant growth promoting bacteria (PGPR) are terrestrial exogenous bacteria, which colonize an inoculated plants rhizosphere, usually outcompeting other microorganisms. Many strains have been found and introduced into agricultural soils as they form mutualistic relationships with the plant. Effective PGPR colonisation can improve the plants overall resistance and tolerance mechanisms to stress thus improving a plants development (Babalola, 2010, Bhattacharyya and Jha, 2012).

PGPR has been utilized as a natural replacement to chemical fertilizers, however understanding colonization potential is very important in agricultural settings. Better bacterial colonization potential brings about a more sustainable and functioning agricultural setting (Babalola, 2010, Bhattacharyya and Jha, 2012). It has been found that there are large discrepancies in experiments done in laboratory settings versus field settings, due to

variability in the latter (Lucy *et al.* 2004). Soil properties are often different in different environments and may alter depending on environmental changes, such as weather. Poor soil conditions are unfavourable to bacteria which may lead to an unsuccessful rhizosphere colonization (Frommel *et al.* 1993, Lucy *et al.* 2004). Frommel *et.al.* (1993) found that in poor soil condition the bacteria are unable to colonize the roots. The optimization of agricultural crop soil is very important if the industry is wanting to effectively move forward with this method.

In this study, we found that both bacterial subsets had higher concentrations of colony forming bacterial units than the uninoculated subsets. The inoculated, unstressed subset had the highest number of bacteria CFUs, which showed that under ideal conditions the bacteria can flourish. However, in nature, conditions are more variable than controlled laboratory settings, therefore it would not be reliable to assume that the same success would be true in a field setting. Although, it can be hypothesized that if a plant is inoculated with copious volumes of specific PGPR strains in field settings, those bacteria will flourish.

For future studies, it would be interesting to inoculate plants field experiments in various geological locations to better understand colonization potential. Thus, allowing us to better understand colonization potential in a variety of soil profiles. It would also be useful to try two or more strains at once, to find which strains can potentially work mutually together and which may dominate over other strains.

3.2.3 Physiological analyses

Regarding both growth measures, height and leaf area, there was a significant difference between inoculated and uninoculated plants, whereby the latter had a higher growth rate. This observation is supported by several studies (Gupta *et al.* 2017, Heidari *et al.* 2011, Rodriguez and Fraga, 1999) that found that many PGPR strains have favourable impacts on a plant's growth and development in stressed conditions, and many plant species have benefitted from the inoculation of PGPR strains. It has been found that several PGPR species have increased growth and yield in many crops, such as wheat, maize, rice, cotton and pepper to name a few (Gupta *et al.* 2017). There is also much supporting information in the literature suggesting that plant growth and survival is maintained in stress conditions by PGPR due to the upregulation growth hormones, nitrogen fixation, protection against pathogenic microorganisms through siderophore production and mineralization of nutrients to name a few (Heidari *et al.* 2011). PGPR promote plant growth using direct and indirect

mechanisms which improve the plant's tolerance and resistance strategies (Rodriguez and Fraga, 1999). The direct growth promoting mechanisms include nitrogen fixation, synthesis of phytohormones and enzymes, and mineralization of organic phosphates to increase availability of phosphorous; whereas, indirect growth promoting mechanisms include the synthesis of siderophores and antibiotics to protect the plant against pathogens (Rodriguez and Fraga, 1999). Growth data obtained in the present study corresponds with this known information.

3.2.3.1 Plant height

Hall *et.al*. (1995) found that length of root and shoot were increased in tomato, lettuce, canola and wheat crops that were treated with a strain of *Pseudomonas putida* under gnotobiotic conditions. Glick *et.al*. (1997) also found that canola seedlings inoculated with *P. putida* had greater growth than uninoculated plants, in stressed conditions. The increase in root mass is often the result of an increase in IAA levels caused by the bacteria.

In the present study it was found that the inoculated plants performed much better than the uninoculated plants, during the four-week growth period prior the induced drought stress period. Plant height in inoculated plants were however significantly difference to the uninoculated plants only in the fourth week of growth. This is likely due to the both groups being supplemented with Murashige and Skoog (MS) media which provided the both sets of plants with all required nutrients, thus requiring very little assistance from the *P. koreensis*.

It has been found the PGPR help plants grow in stress conditions, by mitigating the effects of the stress. This of course, depends on the level of intensity and duration of the stress (Lichtenhaler, 1996). In a study conducted by Mayak *et.al.* (2004), growth was not hindered in drought stressed tomato seedlings, that were inculcated with the ACC deaminase PGPR *Achromobacter piechaudii*, and continued to grow well after watering was reintroduced. Maize crop seedling that were inoculated with a diverse array of PGPR, some of which were *Pseudomonas* species, showed a significant increase in plant height, in a study conducted by Gholami *et.al.* (2009). It was found that overall plant growth *Lactuca sativa* L. (lettuce) plants inoculated with two strains of PGPR was significantly increased, and was likely caused by the plants increased nutrient uptake ability and decreased toxic ion uptake (Han and Lee, 2005). *Phaseolus vulgaris* L (common bean) plants that were co-inoculated with two strains of *Paenibacillus polymyxa* and *Rhizobium tropici*, under drought stress, were observed to have an improved plant height, and shoot dry weight (Figueiredo *et al.* 2008).

Cheng *et.al.* (2007) found that canola plants inoculated with *P. putida* grew significantly better, in comparison to the uninoculated group exposed under salt stressed conditions. They found that the growth of the group that was inoculated and stressed, closely mimicked the control with which were not inoculated or stressed, which suggests that the bacterial strain contained ACC deaminase which decreased ethylene synthesis that would have been heightened by induced stress. Therefore, indicating that the PGPR helped maintain a "normal" growth system, by improving the plants tolerance against the stress applied. Babu *et.al.* (2015) found that *Miscanthus sinensis* plants that were inoculated with *P. koreensis* to remediate heavy metal contamination, displayed increased tolerance, as well as improved growth in terms of plant height and leaf size.

However, in the drought stressed 10-day experiment of the present study, there was a distinguishable increase in plant height in the inoculated, unstressed subset (I), when compared to both stressed subsets. This correlated with the literature presented, thus it is likely that *P. koreensis* encouraged the regulation of certain growth hormones in the inoculated plants and/or increased the plants nutrient uptake ability. There was a small visible difference between the stressed subsets, IS and S, the former being taller, however, statistically there was no difference. These results, could again be attributed to the availability of nutrients from prior supplementation, or the setup of the experiment, which was highly controlled.

3.2.3.2 Leaf area

Leaf area is an important measure for plant growth, and it is well known that the larger a leaf in terms of surface area, the greater its photosynthetic potential due to the plants' ability to absorb a greater amount of light and produce more food. It has been observed that sunflower plants generally have large leaf areas. However, it is also acknowledged that bigger leaf areas are not suitable for drought stress, as it negatively impacts plant tolerance mechanisms, due to a higher water loss in larger leaves. Thus, a common mechanism that plants elicit to tolerate water stress, is decreased leaf size which in turn decreases stomatal conductance, thus reducing water potential and water loss, which also results in decreased photosynthetic ability (Morgan, 1984).

Rawson and Constable (1980) found that leaf area in non-irrigated sunflower plants were smaller than plants that were irrigated. Yegappan *et al.* (1982) found that sunflower leaf area was reduced under drought stress, at various developmental stages. In another study

focused on sorghum (*Sorghum bicolor* L), water stressed plants had significantly decreased leaf areas (McCree and Davis, 1974). The above examples, highlight the tolerance mechanism in plants, whereby leaf area is reduced under drought stressed conditions.

Given that PGPR has been proven to increase a plants' growth, including the plants leaf size (Bashan *et al.* 2004), it may be problematic when inoculated plants are exposed to drought stress. It would be expected that the increase in leaf area may be disadvantageous to a plants survival under stress conditions. However, due to PGPR having the ability to promote stress tolerance in plants, it is likely that the plant would not be disadvantaged because it would not endure the level of stress an uninoculated plant would endure under the same conditions.

Bashan *et al.* (2004) found that leaf size increased in plants inoculated with *Azospirillum*, which is often used as a natural fertilizer in agricultural settings. Gholami *et al.* (2009) found that maize plants inoculated with various strains of PGPR including *P. putida* and *P. fluorescens* had a significant increase in leaf area. This suggests that the bacterial strains used in the mentioned studies had an invigorating effect on the development and growth of the plant.

The results presented in the literature supported the findings in the present study for all measurements (leaf area, width and length). The inoculated plants were significantly larger than the uninoculated plants. This increase in leaf area would prove very beneficial to the plants under non-stressed conditions, allowing the plant to increase its' photosynthetic capacities and growth. It is important to note that leaf area was only calculated at the end of the four-week growth period, prior the induced drought stress period, due to difficulty of obtaining accurate measurements after the induced stress period.

Future studies can focus on the growth parameters of inoculated sunflower plants that are grown in greenhouse and/or field conditions. This would improve our understanding of growth of crops under natural stressed conditions using PGPR. Often, in natural environments there is not only one applied stress. Therefore, it would be worthwhile to observe how PGPR benefits the plant when exposed to various stresses. Plant leaf area can be monitored weekly, to improve observation accuracy. It would also be useful to measure root length and mass, due to the bacteria inhibiting the rhizosphere of the plant.

Chapter Four: Biochemical Analyses - Results and Discussion

4.1 Results

4.1.1 Phenolic acids

Phenolic acid levels were not significantly different between the C, I, and IS subsets. However, there was a significant difference between the three above mentioned subsets and S which had an average phenolic acid concentration of 4.71 ± 0.55 mg GAE/g leaf (Dry weight basis (DWB)), (*F (3,38) = 6.7468, P = 0.00093*).

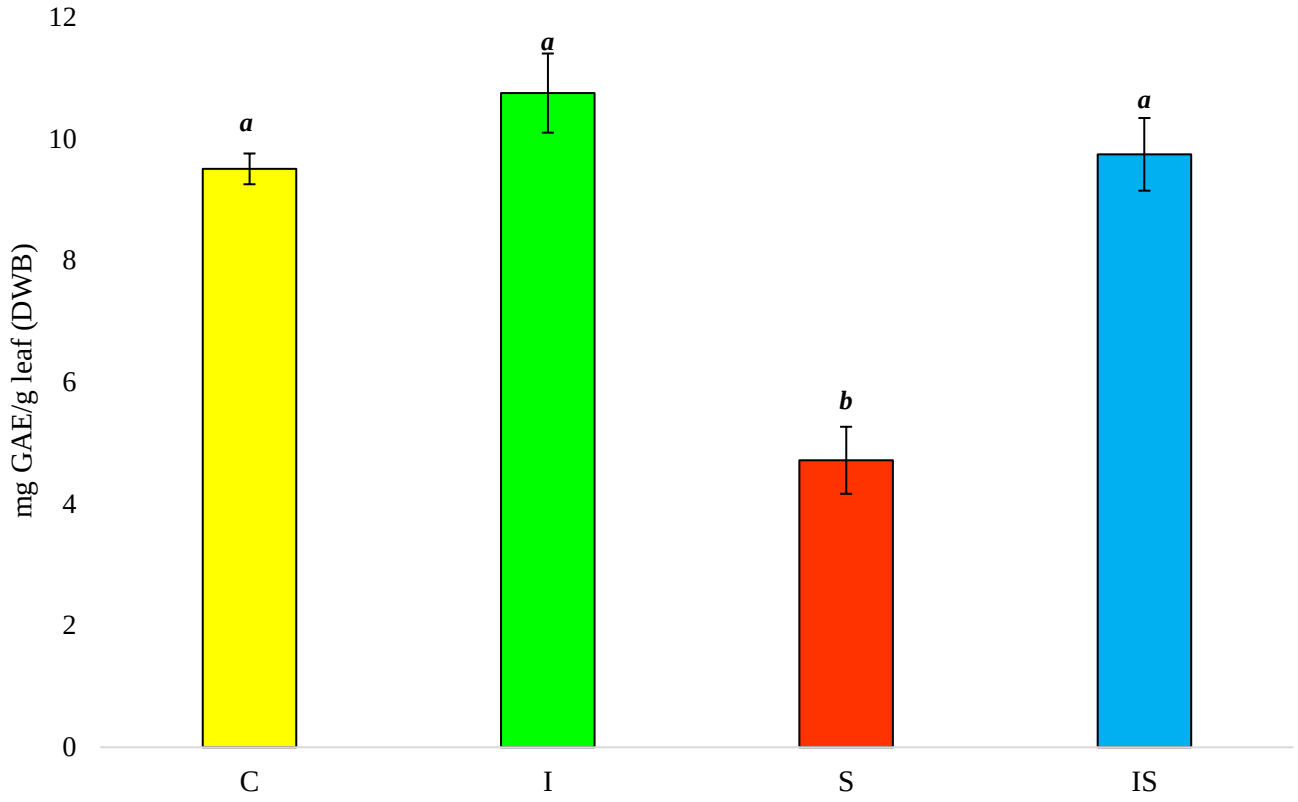

Figure 23. Average levels of total phenolic acid compounds, expressed as a Gallic acid equivalents (GAE), in leaf material between different plant treatment subsets at the end of the 10-day induced drought stress period (S and IS), and 10 days of normal growth conditions (C and I). Vertical bars represent mean ± SE. Lower case italicized letters represent significant difference between treatments. There was no significant difference between the treatment subsets (*F (3,38) = 6.7468, P = 0.00093*)

4.1.2 Extracellular reactive oxygen species

Reactive oxygen species levels were not significantly different between the C, I, and IS subsets and like with the phenolic acid levels, there was a significant difference between the three above mentioned subsets and S (*F (3,28) = 3.6268, P = 0.02499*)

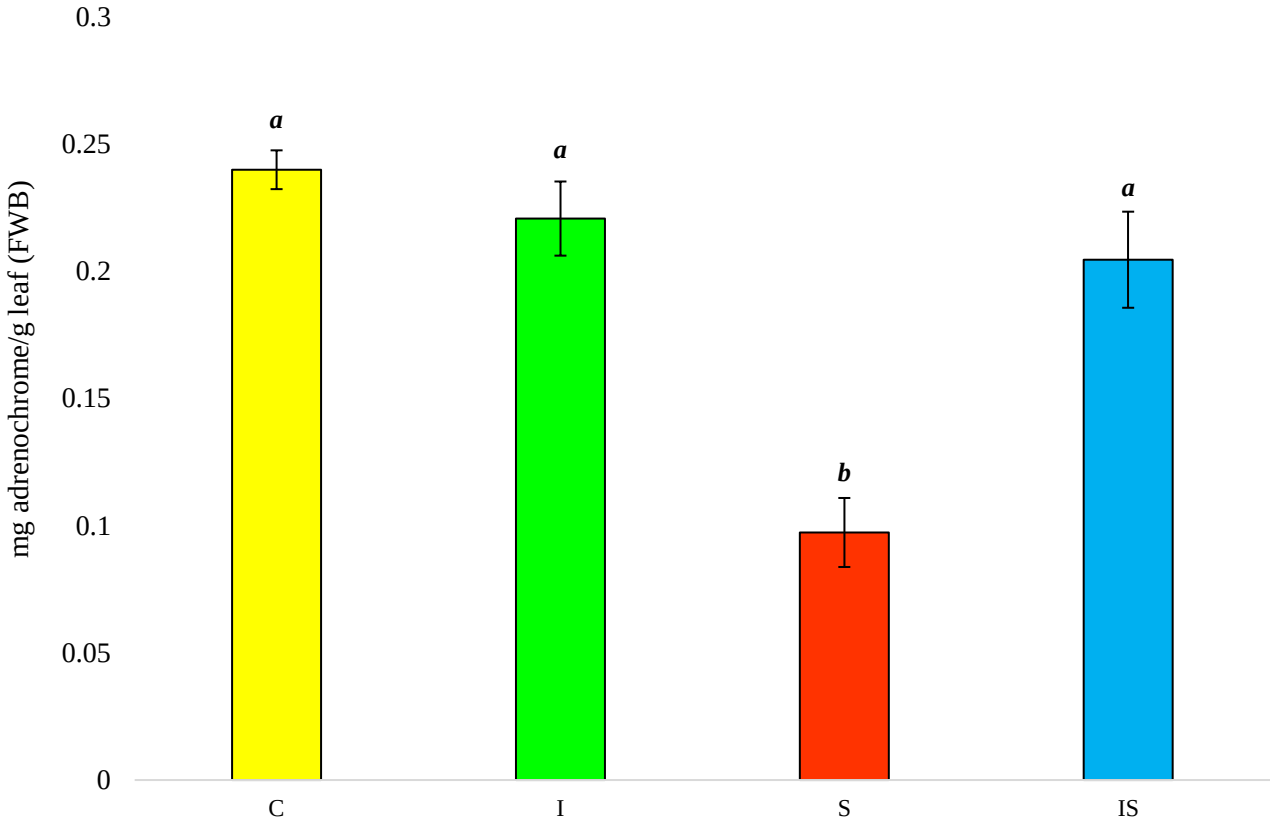

Figure 24. Average levels of extracellular reactive oxygen species, expressed as adrenochrome per gram of leaf material, between different plant treatment subsets. Concentrations worked out at the end of the 4 weeks under normal growth conditions and a further 10-day induced drought stress period (S and IS), and 4 weeks + 10 days under normal growth conditions (C and I). Vertical bars represent mean ± SE. Lower case italicized letters represent significant difference between treatments (*F (3,28) = 3.6268, P = 0.02499*).

4.1.3 Proline

Proline levels were the highest in the IS subset which was calculated to be 11.12 ± 0.70 µmole proline/g leaf (DWB), which was significantly different from both uninoculated subsets (C and S). C had the lowest proline concentration of 4.92 ± 0.54 µmole proline/g leaf (DWB), (*F (3,40) = 9.9139, P = 0.00005*).

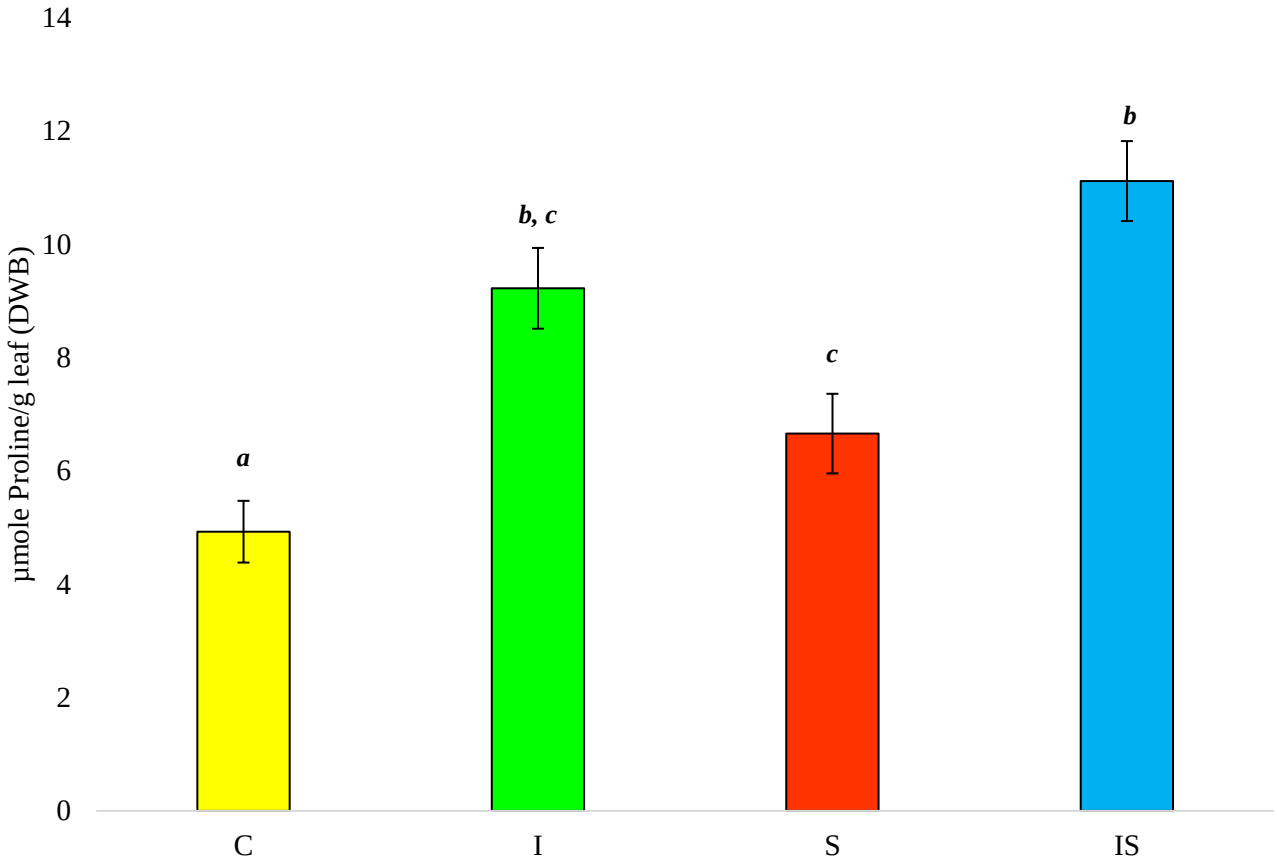

Figure 25. Average levels of proline content per gram of leaf material, between different plant treatment subsets. Concentrations were worked out at the end of the 4 weeks under normal growth conditions and a further 10-day induced drought stress period (S and IS), and 4 weeks + 10 days under normal growth conditions (C and I). Vertical bars represent mean ± SE. Lower case italicized letters represent significant difference between treatments (*F (3,40) = 9.9139, P = 0.00005*)

4.2 Discussion

Plants have developed several adaptation strategies and responses to drought stress, to reduce the damage caused to the plants system. The effectiveness of these strategies is dependent on the intensity and duration of the stress applied, as well as the plants functional type. Biochemical pathways play an important role in a plants ability to tolerate water stress. These pathways are critical for a plants survival. Literature shows that the inoculation of plants with PGPR strains, has been found to improve the regulation of these biochemical

mechanisms and pathways in several plants species (Lavania *et al.* 2006, Sandhya *et al.* 2010, Singh *et al.* 2002, Szabados and Savoure, 2010).

4.2.1 Phenolic acids

Plant secondary metabolites such as phenolic acids are known to accumulate in plants that are exposed to stress conditions, due to their antioxidant properties. The accumulation of phenolic acids is a commonly occurring, generalized stress response in plants (Chalker-Scott and Fuchigami, 1989). In stress conditions, these phenolic compounds accumulate and act as radical quenchers against reactive oxygen species (ROS), to decrease damage to a plants cells (Dixon and Paiva, 1995, English-Loeb *et al.* 1997, Michalak, 2006, Padayachee *et al.* 2008, Pennycooke *et al.* 2005). Larson (1988) found that in drought stressed willow plants, there was an increase in phenolic compounds. However, a study by Chung *et.al.* (2006) found that water stressed *Rehmannia glutinosa* had lower concentrations of phenolic acids than unstressed plants and the decrease was attributed to a decline in other stress response enzymes. Due to ROS and other biosynthetic enzymes being released at different times under stress, it is likely that the concentration of phenolic acids can spike and decrease with the plants' requirement to quench antioxidants.

There is a lot of evidence suggesting that ethylene production and phenolic compound accumulation are directly associated. Literature suggest that some phenolic compounds lead to the production of ethylene, whilst others inhibit it (Chalker-Scott and Fuchigami, 1989). Ethylene accumulation is common in stressed plants and causing the impediment of a plants growth. Phenolic compounds were increased in Cottonwood (Kimmerer, 1988) and poplar (Balwin and Schultz, 1983) plant leaves under stressed conditions. However, in both studies the neighbouring unstressed plants also showed an increase in phenolic levels. This increase suggests that an airborne signal, was emitted to the other plants as a "warning" (Chalker-Scott and Fuchigami, 1989). This type of "warning" is not uncommon to plants, and it has been observed that volatile organic compounds, like ethylene, are emitted from stressed plants as a defence and signalling mechanism under stressed conditions, to alert or prepare neighbouring plants of the stress (Lee and Seo, 2014, Ueda *et al.* 2012). Therefore, unstressed plants automatically release defence enzymes, such as phenolic compounds as a tolerance mechanism, even if no stress is applied to the plant. This communication between plants may have caused the increased phenolic compound levels that was found in unstressed plants in

the present study, indicating that the unstressed plants may have increased drought tolerance as a preventative measure.

Studies have shown that PGPR may enhance the levels of phenolic compounds under stressed conditions, however most studies have focussed on biotic rather than abiotic stress. Lavania *et al.* (2006) conducted a study on betelvine (*Piper betle* L) by inoculating plants with *Serratia marcescens* for growth promotion and biological control against foot and root rot. They found that treated plants expressed higher levels of phenolic acids over untreated plants, which was comparable to another study conducted by Singh *et al.* (2002) which found the same results in *Erysiphe pisi* infected pea (*Pisum sativum*) plants that were inoculated with *P. fluorescence* and *P. aerugnosa*. The *Pseudomonas* strains were highly effective in improving the pathogenic stress resistance in the pea plants (Singh *et al.* 2002). It was also found that cinnamic acid with is converted from phenylalamnine was higher in PGPR inoculated plants, suggesting that the PGPR increased PAL in response to the stress (Singh *et al.* 2002). Tomato plants inoculated with *Bacillus subtilis* to prevent wilting, showed significantly enhanced levels of phenylalamnine ammonialyase and phenolic compounds in comparison to the control group (Loganathan *et al.* 2014).

Babu *et al.* (2015) found that *M. sinensis* plants inoculated with *P. koreensis* produced high levels of antioxidant enzymes as a protective mechanism. Increased antioxidant levels are expected in plants that have been inoculated with PGPR to tolerate stress (Sandhya *et al.* 2010). However, in a study conducted by Kahkonen *et al.* (1999), they did not find any association between antioxidant ability and the accumulation of phenolic acids. This suggests that antioxidant ability depend on the form of the phenolic acid as well as the when it was released, thus changing the antioxidant competencies of the enzyme. The time of accumulation may play an important role in the plants tolerance and resistance mechanisms and the intensity of the stress may affect how well the radical scavenging compounds can perform.

The literature presented correlated with the findings of the present study. There was a high concentration of phenolic acids in all plants except the stressed, uninoculated subset (S). It is possible that this group reached a stage of exhaustion (Lichtenthaler, 1996) and therefore were unable to recover from the stress. The other subsets, including the unstressed groups, exhibited high levels of phenolic acids. The high levels of phenolic acids found in the unstressed subsets (C and I) could be attributed to plant “communication”, and likely

generated as a precautional response. The phenolic acid concentration in the unstressed, inoculated subset (I) was higher than the other groups suggesting that the bacteria did play a significant role in the increase.

Future studies could focus on extraction of phenolic acids from both the roots and the stems of inoculated and uninoculated plants. Most studies have indicated that concentration differs at different locations on the plants system. This may be due caused by the duration and intensity of the stress applied, also on the time that the stress was applied. It would also be useful to identify the type of phenolic acids involved.

4.2.2 Extracellular reactive oxygen species

ROS radicals play an important role in plants under stressed conditions, due to any change to a plants metabolic system encouraging its production (Bowler and Fluhr, 2000). Plants can maintain a steady state of homeostasis by balancing ROS producing and ROS scavenging molecules, due to the accumulation of ROS being notoriously toxic (Apel and Hirt, 2004, Foyer and Noctor, 2005, Mittler, 2002, Mittler *et al.* 2004, Van Breusegem and Dat, 2006). The system is in a steady state until there is a change, usually attributed to a stress, upon which both ROS scavenging and producing molecules are upregulated. Oxidative stress is deteriorating to a plants internal and external structure, causing membrane, organ and biomolecular damage that may lead to the inevitable death of the plant (Arora *et al.* 2002, Gaspar *et al.* 2002, Gill and Tuteja, 2010). The faster a plant can remove the build-up of ROS, the lesser the damage to the plant and the better its chances of survival. A superior antioxidant system can respond to oxidative stress quickly, subsequently improving a plants stress tolerance and resistance mechanisms (Arora *et al.* 2002, Gill and Tuteja, 2010, Han and Lee, 2005a).

The production of ROS in a plant system plays a dual role with regard to stress responses. The first role is the purposeful accumulation of ROS to signal a plant to bring about a response to a stress, and is often generated in amounts that can be quenched and balanced by antioxidants such as phenolic compounds (Apel and Hirt, 2004, Gaspar *et al.* 2002). The ability for a plant to decrease and eliminate ROS accumulation through the generation of antioxidants is critical for its survival, especially under stress conditions. It has been found that drought tolerant plants have developed mechanisms whereby they produce high levels of antioxidant enzymes, to reduce damage by scavenging ROS quickly (Gaspar *et al.* 2002). Abreu and Mazzafera (2005) found that ROS increases brought about by drought

stress cause a substantial increase in phenolic acids. The second role is often brought about by a copious spike in ROS to signal plant death after a very stressful event. This profuse accumulation ROS radicals is a well-known trigger of programmed cell death (Apel and Hirt 2004, Foyer and Noctor 2005, Risenga *et al.*2 013). In these cases, the plant would have reached a stage of exhaustion from the stress.

The literature presented supports what was found in the present study with the stressed, uninoculated subset of plants. Most plants in this subset either did not survive the induced drought period or showed severe deterioration suggesting that the stress period of 10 days was too long. This serves as a possible explanation for the very low levels of phenolic acids and ROS in the stressed, uninoculated subset. The plants in this subset were possibly at a stage of exhaustion. These findings also correlated to the stress syndrome responses of plants as proposed by Lichtenhaler (1996). The concept suggests, that if a plant reaches a stage of exhaustion after maximum resistance, it will likely die or endure chronic damage thus removing any regeneration ability.

Studies have found that the inoculation of plants using PGPR strains, increase phenolic content thus improving the plants ability to counteract oxidative build up in stressful conditions. Ruiz-Lozano *et.al.* (2001) found that mycorrhizal lettuce showed increased levels of superoxide dismutase under drought stress conditions, which improved the plants tolerance. In another study, conducted by Sandhya *et.al.* (2010), it was found that maize crops inoculated with *Pseudomonas* species, boasted high antioxidant levels, which could be attributed to increased quenching of ROS molecules. Gururani *et.al.* (2012) found that PGPR helped upregulate ROS scavenging antioxidants and increase ethylene biosynthesis, thus improving abiotic stress tolerance in *Solanum tubeosum*.

In the present study, both inoculated subsets were found to have lower ROS concentrations than the control subset, suggesting that the PGPR upregulated antioxidant levels within the plants system, thereby decreasing the levels of ROS. This further suggested that the inoculation of plants with PGPR maintains an equilibrium within the plants system, thus decreasing the plants need for the accumulation of ROS.

There is a great deal of information that is unknown still about the role ROS plays, especially in PGPR inoculated plants. Future studies could focus on quantifying ROS at different stages during induced drought stress period, and after recovery to assess

regeneration capability. It would also be useful to decrease the drought stress period to prevent the plants from reaching a stage of exhaustion.

4.2.3 Proline

Proline commonly accumulates under stress conditions and plays an important part in the adaptation of plants to water stress conditions (Raymond and Smirnoff, 2002, Szabados and Savoure, 2010).

It has been found that sunflower plants exposed to drought stress conditions usually exhibit high proline levels, whereas unstressed plants display low levels (Cechin *et al.* 2006, Manivanna *et.a.*, 2007, Ünyayar *et al.* 2004). In the present study, the control group displayed significantly lower proline levels in comparison to all other subsets.

Studies have shown that proline accumulates more in plants that have been inoculated with PGPR, especially under stress conditions. The increased production of proline in PGPR inoculated plants may be attributed to the upregulation of abscisic acid (ABA) pathways (dependent and independent). ABA commonly increases in water stress conditions. Usually, an increase in ABA results in increase in plant root growth, which has been found to occur in sunflower plants. A larger root system allows the plant to access more water and nutrients. P5CS is activated by an ABA pathway, and is responsible for the biosynthesis and overexpression of proline in stress circumstances (Szabados and Savoure, 2010). There are two P5CS genes linked o proline production. One of the strains is required for the general maintenance of homeostasis and the other is required to produce proline in stress conditions.

PGPR improves the plants tolerance by rapidly upregulating tolerance enzymes and mechanisms. Several studies have found that proline concentrations higher in inoculated plants compared to uninoculated plants in stress conditions (Ansary *et al.* 2012, Gururani *et al.* 2012, Heidari *et al.* 2011, Kumari *et al.* 2005, Mohamed and Gomua, 2012,). In all the mentioned studies, it was also found that the respective plants showed increased stress tolerance in comparison to uninoculated plants. The increased production of proline improves the plants tolerance by regulating relative water content in leaves, increasing uptake of potassium ions, decreasing electrolyte leakage and osmotic potential (Ansary *et al.* 2012, Bano and Fatima, 2009).

The findings in the present study correlated with that in the literature. Both inoculated subsets (I and IS) exhibited higher proline levels than the uninoculated subsets (C and S). There was no significant difference between the two inoculated subsets, suggesting that the stressed subset was able to maintain a relatively normal internal environment. The PGPR helped regulate a steady state of homeostasis within the plant, thereby increasing its proline concentration and drought tolerance.

Future studies could include the extraction of proline form the roots and shoots. It would also be interesting to quantify the ABA levels, as well other plant hormones such as auxins (IAA), cytokinins (CK), ethylene (ET), gibberellins (GA), and jasmonates (JA). This would give us a clearer picture of the biosynthesis of proline in PGPR inoculated plants, under stressed conditions.

Chapter Five: Metabolomic Optimization and Analyses - Results and Discussion

5.1 Results

This aspect of the study focussed on the technical reproducibility within samples, as calculated by relative standard deviation (RSD).

Technical reproducibility within leaf samples, as calculated by RSD was generally high for repeated injections of each leaf subsample of both uninoculated and inoculated, stressed samples (Tables 5 and 6). RSDs were lower than 10 % for all leaf subsamples, excluding three uninoculated subsamples (*L-alanine S1C1-S1C3, S2A1-S2A3; and L-5-oxoproline S2C1-S2C3*) and four inoculated subsamples (*L-alanine IS1A1-IS1A3, IS1C1-IS1C3, IS3B1-IS3B3; L-valine IS2B1-IS2B3*). Technical reproducibility between each set of combined leaf samples (*S1, S2, S3, IS1, IS2, IS3*) had calculated RSDs lower than 20%.

Technical reproducibility in root samples, as calculated by relative standard deviation (RSD) were not very consistent for repeated injections of each leaf subsample (Tables 7 and 8). The range extended from 0% < RSD < 20% for subsample injections in both uninoculated and inoculated sample sets. RSDs calculated for combined root samples (*S1, S2, S3, IS1, IS2, IS3*) also yielded inconsistent results as many sample RSDs were calculated to be greater than 20%. In the inoculated sample set there were several subsamples whereby the necessary metabolite peaks were not picked up by the machine (IS1B1-IS1B3, IS2C1-IS2C3, IS3B1-IS3B3, IS3C1-IS3C3).

Reproducibility in the biofilm extraction technique samples were relatively high for all samples in both inoculated and uninoculated sample sets (Tables 9 and 10). In the uninoculated sample set S1 yielded a RSD of 12.24% for D-arabinose and in the inoculated sample set IS3 yielded a RSD of 15.82% for glycerol. These were the only two samples that did not yield RSD's lower than 10%.

Table 5. Relative standard deviations (RSD) of eight metabolites analysed from the uninoculated, stressed subset (S) of leaves. RSD is given for each leaf subsample (between injections) and for the total leaf sample (including all subsamples and injections of each leaf). Total leaf sample RSD values are represented in bold font. 10% ≤ RSD > 15 % is highlighted in blue. 15% ≤ RSD > 20 % is highlighted in green.

Sample / Metabolite RSD %	**S1A1 - A3**	**S1B1 - B3**	**S1C1 - C3**	**S1**	**S2A1 – A3**	**S2B1 - B3**	**S2C1 - C3**	**S2**	**S3A1 - A3**	**S3B1 - B3**	**S3C1 - C3**	**S3**
D-xylose	6.74	2.29	2.59	**6.48**	8.14	6.39	5.07	**10.97**	2.94	3.83	2.28	**4.87**
L-5-oxoproline	7.48	4.41	7.79	**10.26**	8.25	7.16	14.49	**9.89**	1.79	3.33	3.55	**5.40**
D-arabinose	8.17	2.89	0.99	**18.29**	3.84	3.37	1.47	**12.12**	5.27	5.51	1.82	**15.26**
Pipecolic acid	8.00	5.90	6.45	**8.17**	3.59	2.77	5.40	**5.09**	8.49	2.42	3.40	**9.98**
L-threitol	4.06	4.20	1.90	**9.33**	4.55	1.32	4.97	**4.15**	4.05	2.36	1.75	**7.15**
L-leucine	4.99	7.43	7.06	**14.13**	2.13	7.04	8.56	**10.99**	8.59	7.19	9.24	**13.73**
L-valine	4.61	2.79	9.30	**9.02**	2.63	7.73	3.24	**11.69**	7.24	8.40	9.92	**11.05**
L-alanine	5.32	9.74	14.53	**17.66**	13.12	1.38	9.35	**10.49**	6.76	9.30	5.20	**16.05**

Table 6. Relative standard deviations (RSD) of eight metabolites analysed from the inoculated, stressed subset (IS) of leaves. RSD is given for each leaf subsample (between injections) and for the total leaf sample (including all subsamples and injections of each leaf). Total leaf sample RSD values are represented in bold font. 10% ≤ RSD > 15 % is highlighted in blue. 15% ≤ RSD > 20 % is highlighted in green.

Sample / Metabolite RSD %	**IS1A1 - A3**	**IS1B1 - B3**	**IS1C1 - C3**	**IS1**	**IS2A1 - A3**	**IS2B1 - B3**	**IS2C1 - C3**	**IS2**	**IS3A1 - A3**	**IS3B1 - B3**	**IS3C1 - C3**	**IS3**
D-xylose	2.71	6.75	3.40	**9.37**	4.23	5.31	9.57	**10.27**	3.12	3.89	3.05	**9.32**
L-5-oxoproline	9.80	9.19	7.35	**9.95**	1.23	8.23	7.69	**8.77**	0.77	7.63	0.26	**15.46**
D-arabinose	1.92	0.69	0.50	**8.79**	2.75	3.06	1.85	**2.50**	3.24	1.51	1.66	**3.54**
Pipecolic acid	1.55	5.46	2.64	**14.40**	6.04	3.56	2.62	**10.04**	6.40	2.08	7.95	**11.98**
L-threitol	4.06	4.20	1.90	**9.33**	4.55	1.32	4.97	**4.15**	4.05	2.36	1.75	**7.15**
L-leucine	6.54	6.76	1.90	**9.41**	8.25	6.79	7.78	**6.88**	7.90	4.35	2.48	**7.63**
L-valine	4.35	1.85	7.06	**7.37**	4.36	13.52	6.06	**14.89**	7.21	5.69	8.77	**9.40**
L-alanine	10.78	7.64	10.79	**15.36**	6.51	8.41	2.61	**5.83**	7.21	11.15	6.20	**13.79**

Table 7. Relative standard deviations (RSD) of six metabolites analysed from the uninoculated, stressed subset (S) of roots. RSD is given for each root subsample (between injections) and for the total root sample (including all subsamples and injections of each leaf). Total root sample RSD values are represented in bold font. 10% ≤ RSD > 15 % is highlighted in blue. 15% ≤ RSD > 20 % is highlighted in green. RSD ≥ 20 % is highlighted in yellow.

Sample / Metabolite RSD %	**S1A1 - A3**	**S1B1 - B3**	**S1C1 - C3**	**S1**	**S2A1 - A2**	**S2B1 - B3**	**S2C1 - C3**	**S2**	**S3A1 - A3**	**S3B1 - B3**	**S3C1 - C3**	**S3**
D-xylose	2.67	1.28	0.10	**19.66**	2.44	1.94	0.99	**9.89**	0.50	3.20	0.79	**3.89**
L-5-Oxoproline	16.81	7.43	9.56	**23.69**	8.61	14.47	11.88	**20.30**	7.74	8.51	11.78	**45.30**
D-arabinose	11.59	10.20	11.30	**43.59**	2.27	10.24	9.12	**25.64**	3.61	11.60	4.13	**22.31**
L-leucine	9.31	7.99	5.32	**27.64**	7.89	9.39	9.54	**26.28**	13.91	12.97	13.25	**40.49**
L-valine	13.94	3.34	17.30	**36.52**	9.76	9.11	4.27	**20.57**	7.31	5.36	2.17	**19.61**
Glycerol	7.98	0.38	0.49	**32.33**	16.34	1.79	0.29	**14.79**	1.66	1.31	0.79	**7.80**

Table 8. Relative standard deviations (RSD) of eight metabolites analysed from the inoculated, stressed subset (IS) of roots. RSD is given for each root subsample (between injections) and for the total root sample (including all subsamples and injections of each leaf). Total root sample RSD values are represented in bold font. 10% ≤ RSD > 15 % is highlighted in blue. 15% ≤ RSD > 20 % is highlighted in green. RSD ≥ 20 % is highlighted in yellow.

Sample / Metabolite RSD %	**IS1A1 - A3**	**IS1B1 - B3**	**IS1C1 - C3**	**IS1**	**IS2A1 - A3**	**IS2B1 - B3**	**IS2C1 - C3**	**IS2**	**IS3A1 - A3**	**IS3B1 - B3**	**IS3C1 - C3**	**IS3**
D-xylose	3.89		1.50	**67.40**	4.08	15.08		**31.84**	6.47			**6.47**
L-5-Oxoproline	9.95		7.58	**7.94**	11.26	12.47		**40.91**	6.26			**6.26**
D-arabinose	4.71		6.77	**47.54**	9.65	10.53		**54.32**	13.67			**13.67**
L-leucine	4.22		5.72	**9.74**	9.95			**9.95**	8.87			**8.87**
L-valine	12.70		10.84	**57.60**	1.49			**1.49**	3.74			**3.74**
Glycerol	4.31		1.85	**26.29**	3.00	7.33		**37.78**	5.54			**5.54**

Table 9. Relative standard deviations (RSD) of three metabolites analysed from the uninoculated, stressed subset (S) using the optimized biofilm metabolite extraction technique. RSD is given for each sample (between injections). 10% ≤ RSD > 15 % is highlighted in blue. 15% ≤ RSD > 20 % is highlighted in green.

Sample/ Metabolite RSD %	**S1**	**S2**	**S3**
D-xylose	4.31	1.80	0.93
D-arabinose	12.24	5.85	6.89
Glycerol	4.04	2.00	5.53

Table 10. Relative standard deviations (RSD) of three metabolites analysed from the inoculated, stressed subset (IS) using the optimized biofilm metabolite extraction technique. RSD is given for each sample (between injections). 10% ≤ RSD > 15 % is highlighted in blue. 15% ≤ RSD > 20 % is highlighted in green.

Sample/ Metabolite RSD %	**IS1**	**IS2**	**IS3**
D-xylose	0.68	3.04	8.32
D-arabinose	8.42	3.31	8.74
Glycerol	2.43	0.64	15.82

Peak areas of all metabolites analysed in leaf and biofilm extraction samples were larger in bacteria inoculated plants compared to uninoculated plants (Tables 11 and 13). Root samples yielded inconsistent result as many of the inoculated samples peaks were not accurately picked up during the GC-MS sampling process (Table).

Table 11. Comparison of average peak areas of eight metabolites analysed in uninoculated and inoculated stressed plant leaves. Averages were calculated by including all leaf samples and injections.

Metabolite	**Uninoculated**	**Inoculated**
D-Xylose	9591447	12151837
L-5-Oxoproline	11565223	14275820
D-Arabinose	134161225	190248417
Pipecolic acid	16353243	17077699
L-Threitol	198902716	273209540
L-Leucine	2466871	6975776
L-Valine	4917301	13196452
L-Alanine	4251715	9918173

Table 12. Comparison of average peak areas of six metabolites analysed in uninoculated and inoculated stressed plant roots. Averages were calculated by including all root samples and injections.

Metabolite	**Uninoculated**	**Inoculated**
D-Xylose	148487486	68151026
L-5-Oxoproline	1158826	1003139
D-Arabinose	13318469	11241549
L-Leucine	3192097	444198
L-Valine	1776924	1089315
Glycerol	125337580	86928671

Table 13. Comparison of average peak areas of three metabolites analysed in uninoculated and inoculated stressed plants using the optimized biofilm metabolite extraction technique.

Metabolite	**Uninoculated**	**Inoculated**
D-Xylose	12640756	161602519
D-Arabinose	7937649	33771816
Glycerol	66232932	98793132

5.2 Discussion

Plant metabolomics has broadened our understanding of plant physiology, biochemistry and behaviour under various conditions (Biais, *et al.* 2012, Hong *et al.* 2016, Tugizimana *et al.* 2012). Plants are known to synthesise specific metabolites under specific conditions (Crozier *et al.* 2006, Hong *et al.* 2016). Metabolic fluctuations occur within plants under stressed conditions. Understanding how these metabolites fluctuate can improve crop production, by breeding plants with specific biomarkers that will enhance stress tolerance (Hong *et al.* 2016). The optimization of metabolomic approaches and integration with other omics techniques may better our overall understanding of these metabolites and the pathways that they are utilized by plants to respond to stress (Hong *et al.* 2016). Furthermore, our understanding of how these responses are enhanced with the use of PGPR species will significantly benefit crop science. However, the scope of this study did not allow for deeper exploration of the biological significance of these interactions beyond simple interpretation of overall trends which showed the influence of the PGPR on key metabolites associated with drought stress tolerance.

It is well known that many challenges, errors and complications can occur in metabolomic extraction studies, due to it being relatively new in comparison to proteomic or genomic studies. There is room for error at every stage of the analyses, therefore caution needs to be taken in order to gain optimal results (Fiehn, 2002, Fukusaki and Kobayashi, 2005, Hong *et al.* 2016, Mashego *et al.* 2007, Parsons *et al.* 2009, Tugizimana *et al.* 2012). Variation can occur by both technical and biological variation within metabolite data sets (Parsons *et al.* 2009).

As much as accuracy is important, reproducibility of techniques is also critical. The use of relative standard deviations (RSDs) are commonly used for assessing technical and biological variation. This method is beneficial for optimizing metabolite extraction techniques. Variation can occur by both technical and biological variation within metabolite data sets (Parsons *et al.* 2009). Parsons *et al.* (2009) stated technical variation within datasets may range from between 1.6 – 20.6% and biological variation may range between 7.2% and 58.4% depending of the species.

In the present study, focus was on optimizing a sampling method for metabolite analyses for leaf, root and biofilm extractions from plant samples. As mentioned in chapter 2, quantification of metabolites was not the emphasis. Attention was placed on the technical

reproducibility of methods from the sampling of biological material to the actual metabolite analyses. Therefore, no effort was made to interpret the present data for biological significance.

In the leaf samples, all RSDs calculated between injections of each subsample and between subsamples of each leaf were lower than 20%, for both inoculated and uninoculated samples. This suggests that there was a high technical reproducibility with the method utilized. Future studies could include the sampling of leaves at different growth stages, to quantify specific targeted metabolites at the different stages for both stressed and unstressed plant sets.

In the root samples, there were several inconsistencies within samples. Several of the inoculated samples were not picked up which is likely due to an injection error. Technical error was relatively low within subsample injections; however the inconsistencies were greater between subsamples. Therefore, there was a lower reproducibility between subsamples. This may be the result of the root sampling technique utilized. The roots were covered with a copious amount of vermiculite, which had to be manually removed through several stages of grinding and sieving (as detailed in Chapter 2). It is highly possible that vermiculite particles were still present in samples and sample integrity was deteriorated. It is also very likely that some of the samples prepared for GC-MS analyses did not boast enough root material and rather contained more vermiculite. A more appropriate method of sampling root material to retain only the root needs to be optimized to decrease error. A possible suggestion is the use of another growth medium that can be easily separated from the roots. Vermiculite was chosen for this study due to it having a neutral pH and is sterile. It also has the ability to retain water and nutrients for the plant, it improves plant water drainage abilities and maintains an aerated environment. A possible medium that could be considered in perlite.

For the biofilm extraction optimization technique, RSDs for the three metabolites focussed on were less than 15% indicating very little technical error. Further studies would need to be done using the sampling method which was the focus for this part. In order to isolate the metabolites in the biofilm, it is recommended that the growth medium is also sampled and analysed, thus subtracting these metabolites from the solvent sample. Biochemical assays may be utilized to isolate compounds in the biofilm matrix, thereafter specific metabolites may be targeted using the optimized metabolite technique to further validate the technique. Biofilm extraction is a novel concept and may advance our knowledge

in this area. Both targeted and untargeted metabolomics, for quantitative and qualitative studies.

All peak areas in analyses metabolites for both the leaf and biofilm extractions were larger in the inoculated plants compared to the uninoculated plants. This was expected, as many of the uninoculated and stressed plants were severely damaged or died due to the intensity of stress induced. The inoculated and stressed plants performed significantly better, as seen in both the physiological and biochemical results. Peak areas of metabolites extracted from root samples were not consistent with the leaf and biofilm results, which is likely due to the sample errors and inaccuracies. Future studies could include targeted metabolomics and the inclusive of standards for quantification of specific stress-related metabolites.

Chapter six: Conclusion

Plant-microbe interactions are complex, dynamic and very important, especially in terms of crop agriculture, and it is of specific interest in biotechnology for biocontrol and biofertilizer advancements. As illustrated in this dissertation, several studies have highlighted the magnitude of importance microbes play, predominantly plant growth promoting bacteria (PGPR), in plant-microbe interactions under various environmental conditions. PGPR have also been shown to improve overall plant health, well-being, and stress tolerance and resistance mechanisms under both abiotic and biotic stress conditions.

This study focused on optimizing a variety of approaches for the study of plant-microbe interactions under drought stressed and unstressed conditions. A reproducible germination, growth and drought regime was established which can be adapted to other plant species depending on specific growth cycles. The findings in this study also correlated with findings in the literature (discussed earlier) in terms of the benefits attained by plants due to PGPR species.

In the present study, *P. koreensis* inoculated sunflower plants outperformed uninoculated plants in both drought stressed and unstressed conditions. In terms of physiological results, the inoculated plants were taller in height and had larger leaf areas than uninoculated plants. The increased growth in the unstressed, inoculated plants could be attributed to PGPR improving the plants overall nutrient and element uptake. The growth in terms of height was also affected to a lesser extent within the stressed, inoculated plants, which was likely due to bacterial ACC-deaminase which may have signaled quick stress responses within the plants that brought about higher drought tolerance.

It was also found that the PGPR elicited increased biochemical responses in inoculated plants. Both proline and phenolic acid levels were significantly higher in inoculated plants and reactive oxygen species levels were lower in both inoculated sample sets compared to the control sample set, which suggested that the concentrations of antioxidant compounds were increased, possibly as a protective mechanism to prevent oxidative stress, dehydration and programmed cell death. The optimized metabolomics technique also found that peak areas of metabolites assessed within the inoculated plant samples were much larger than uninoculated plant samples. However, the metabolomic analyses focused primarily on technical reproducibility; actual quantification of metabolites was not done. The optimized metabolite technique proved to have a high technical

reproducibility in the leaf and biofilm extraction samples. The root samples were less successful, which was likely due to the excess amount of vermiculite surrounding the roots.

Summarized recommendations for future studies:

1. The use of a different soil medium such as perlite, which may make root analyses more attainable. Thereafter, various root physiological (mass, length, etc.), biochemical (IAA, phenolic acids, proline etc.,) and metabolite analyses can be successfully conducted.
2. Sampling can be conducted throughout the drought stress period to establish the exact time certain biochemicals such as phenolic acids, ROS and proline are released in response to stress at various stages.
3. Assess the biological reproducibility of the optimized metabolite extraction technique.
4. Using the optimized metabolite technique, investigate the interplay of specific metabolite biomarkers and pathways between stressed and unstressed plants. Including pathways related to physiological and biochemical responses, thus integrating these fields in a more advantageous manner.

In conclusion, the systems study of plant-microbe interactions is fairly novel and beneficial to the future of sustainable agricultural success and food security mitigation, and integrating various study approaches promotes better understanding of the complexities of these interactions.

Chapter seven: References

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Chapter 8: Appendices

Appendix 1

Table 14. Optimized growth regime for the entire plant growth period, including drought stress period for each experimental replicate.

Day/ Week	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
0	**GERMINATE SEEDS**				**PLANTING** W/ I/ MS/ P	W	W
1	W/ MS	W	W	W	W/ MS/ F	W	W
2	W/ MS	W	W	W	W/ I/ MS/ P	W	W
3	W/ MS	W	W	W	W/ MS/ F	W	W
4	W/ MS	W	W	W	W/ MS/ P	W	W
					DP (S & IS)	(C & I)	(C & I)
5	W/ MS (C & I)	W (C & I)	W (C & I)	W (C & I)	W/ MS (C & I)	W (C & I)	W (C & I)
6	**SAMPLING**						

Where:

MS = Murashige and Skoog (MS) medium
I = Bacterial inoculant
F = Fertilizer
P = Pesticide
W = Autoclaved tap water
DP = Start of 10 day drought period for stressed plants (S and IS)