Isolation and characterization of plant growth promoting endophytic bacteria from *Medicago lupulina* for enhanced drought tolerance in Maize

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List of abbreviations

ACC	1- aminocyclopropane- 1- carboxylate
APX	Ascorbate peroxidase
CAT	Catalase
CAS	Chrome azurol S
DNA	Deoxyribonucleic acid
IAA	Indole- 3- acetic acid
ICP- OES	Inductively coupled plasma optical emission spectrometry
LB	Luria- Bertani
MDA	Malondialdehyde
NBT	Nitro blue tetrazolium chloride
OD	Optical density
PGPB	Plant growth promoting bacteria
PvP	Polyvinylpyrrrolidone
R2A	Reasoners-2 agar
mRNA	Messenger ribonucleic acid
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
ROS	Reactive oxygen species

RWC	Relative water content
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TBA	Thiobarbituric acid
TEMED	Tetramethylethylenediamine
YEM	Yeast extract mannitol

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Keywords

Antioxidant enzymes
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Phytohormones
Proline
Relative water content
Reactive oxygen species
Superoxide
Superoxide dismutase

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Abstract

Maize is one of the major staple foods in many African nations however, this crop is sensitive to environmental stressors. Drought is a major environmental stressor affecting maize production. Due to global warming, drought episodes are expected to increase in duration and frequency. Therefore, it is vital to improve maize drought tolerance thereby increasing food security. Many studies have looked at improving maize drought tolerance through genetic engineering techniques which has shown promising results, nonetheless GMOs have been under scrutiny due to public concerns regarding unknown side effects.

An alternative route into improving maize drought tolerance could be through the use of endophytes which are microbes that are able to promote plant growth. These endophytes have been documented to produce metabolites which are known to aid their plant host during stress. Therefore the aim of the study is to determine whether the endophytes are able to alleviate some of the strain induced by drought stress. In order to test the feasibility of this method, endophytes was extract from a plant host which exhibition perceived drought tolerance. These endophytes were then screened for plant growth promoting abilities such as IAA and siderophore production. After endophytes were screened for endophyte properties the maize growth trial could start.

During the trail, plants were grown under well-watered conditions treated with endophytes and other being untreated. After growth trial was completed plants were harvested and physiological changes between samples was measured. Aside from these measurements plant material underwent a number of biochemical test such as relative water content and proline content. In the relative water content water-deprived maize samples treated with endophytes had an increase in relative water content compared to untreated water-deprived. This improved in relative water content could be due to the increase in proline content which was observed in

endophyte treated samples, an increase in proline production has been documented to improve plants water retention capacity through its role as osmoregulation in some plants.

Plant material was also tested for ROS production and antioxidant activity. Plants under drought stress have been well document to have increase ROS production which can lead to oxidative damage. One of the ROS molecules tested was superoxide which had a 21.23% increase in its production in water-deprived untreated plants compared to the control plant while water-deprived treated plants were statically similar to control plants. The antioxidant enzyme responsible for the removal of superoxide is SOD which also observed a significant increase in activity in water-deprived endophyte treated plant samples.

These results demonstrated that these endophytes used in this study were able to alleviate some of the harmful effects of drought stress, however more research in this field is required to make this viable option.

Chapter 1: Literature review

1.1: Introduction

Water is a major limiting factor which affect plant growth, development and yield mainly in arid and semi-arid regions where plants are often exposed to periods of water deficit stress also known as drought stress. Drought is one of the major causes of crop loss worldwide, reducing average yields by up to 50% (Wang *et al*., 2003). In 2015 between January and December South Africa received the lowest recorded rainfall since the recording of rainfall started in 1904 (Funk *et al*., 2016). The frequency of droughts are expected to increase with global warming as temperatures rise and rainfall distribution changes in key traditional production areas. Global warming is the long term heating of the earth climate which have been observed since the pre-industrial period and is due to human activity such as the dependents on fossil fuels. Global warming is now generally considered to be underway by mainstream scientist and is projected to result in a long-term trend towards higher temperatures, greater evapotranspiration and an increased incidence of drought in specific regions (Hillel *et al.*, 2002).

African nations has been highlighted as being particularly vulnerable in the future largely due to its low adaptive capacity and its sensitivity to many of the projected changes (Solomons *et al*., 2007; Callaway *et al*., 2004). These climatic changes are taking place in the background of other developmental pressures such as poverty, fluctuating oil prices, and most notable food insecurity (FAO, 2006). Due to the large threat water deprivation poses the physiological mechanisms involved in cellular and whole plant responses to it has frequently been reviewed (Tuteja *et al*., 2007; Khan *et al*., 2008; Singh *et al*., 2008). A large number of physiological and biochemical changes take place in response to drought stress in various plant species. Changes in protein expression, accumulation and synthesis have been observed in many plant species as a result of drought stress during growth. It is well documented that drought stress damages a number of

metabolic and physiological processes in plants (Levitt *et al*., 1980). Drought stress leads to reduction in growth, content of chlorophyll pigments and water (Nayyar *et al*., 2006; Yang *et al*., 2006). Decrease in nutrient uptake by plants under drought stress conditions have also been observed, due to weakened active transport, reduced transpiration and membrane permeability which consequentially results in reduced root absorbing power (Tanguilig *et al*., 1987).

Most of the damaging effects of drought is associated with the photosynthetic process of the plant and numerous studies have shown that the reduction in photosynthetic activity under drought stress can be credited to both stomatal and non-stomatal limitations (Yordanov *et al*., 2003; Zlatev *et al*., 2004). One of the first responses to drought stress is stomatal closure, stomatal closure allows plants to limit the amount transpiration. This decrease transpiration however comes at a cost as it limits CO_2 absorption, which leads to a decreased photosynthetic activity (Yang *et al*., 2006).

1.2: Maize is essential for global food security

Cereal crops provide humankind with more nourishment than any other food class and almost half of the total caloric requirement (McIntosh *et al.,* 2016). Although there are a number of different cereal crops used as a food source rice, maize, and wheat are important human food sources as it accounts for 94% of all cereal consumed by humans. In combination with wheat and rice maize provides at least 30% of the food calories to more than 4.5 billion people in 94 developing countries (Shiferaw et al., 2011). This includes 900 million people living in poverty for whom maize is the preferred staple food. Maize crops is currently produced by 125 developing countries on nearly 100 million hectares and is among the three most widely grown crops in 75 of those countries (Tubiello *et al*., 2013). About 67% of the total maize production in the developing world comes from low and lower middle income countries; hence, maize plays an important role in the livelihoods of millions of poor farmers (Shiferaw et al., 2011).

The demand for maize in the developing world is said to double between now and 2050 (Rosegrant *et al*., 2009). Maize has been documented to be sensitive to drought stress (Farre *et al*., 2000). Early investigations into the sensitivity showed that genotypic differences occur in growth response of maize to drought stress. The characteristic differences in response to drought stress conditions have also been identified as an array of physiological and morphological characteristics which include stomatal activity, root development, osmotic adjustment, abscisic acid and proline levels increase in the whole plant (Li *et al*., 1998; Selmani *et al*., 2003). In order to improve the agricultural productivity within the water limited areas, it is imperative to ensure higher crop yields against drought stress.

1.3: Drought-induced oxidative stress in plants

Plant adaption to changing environmental conditions such as drought stress is essential for survival and growth. Drought stress is well documented to inhibit photosynthetic activity in as a result of an imbalance between light capture and its utilization (Foyer *et al*., 2000). However plants have evolved mechanisms which allow them to adapt and survive periods of water deficit, if not at the whole plant level then at some level or form of plant structure.

Plants ability to avoid or tolerate drought stress depends to the type of strategy adopted, although these are not mutually exclusive. A plants drought response is subject to the species inherited strategy but also on the duration and severity of the drought period. Under prolonged drought stress will inevitably result in oxidative damage due to the over production of reactive oxygen species. Reactive oxygen species (ROS) are the result of the partial reduction of atmospheric O_2. Cellular ROS have basically four forms, singlet oxygen ($^1O_2$), superoxide radical (O_2^-) hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-), each ROS have their own unique half-life and oxidizing potential. ROS molecules can be extremely reactive, particularly $^1O_2$ and OH^-, they can oxidize multiple cellular components (Bhattachrjee *et al*., 2005). Which can lead to unrestricted oxidation of the cellular components which will ultimately cause cell death.

1.3.1: Superoxide (O_2^-)

It widely documented that ROS molecules appear continuously during photosynthesis in the chloroplast partly due to reduction of O_2 molecule of their energy transfer. The production ROS molecules production is the inevitable result of aerobic respiration. When the terminal oxidase-cytochrome c oxidase and the alternative oxidase-react with O_2 it result in four electrons being transferred and H_2O being released (Halliwell *et al*., 2006).

Although O_2 can react with other components in the electron transfer chain, in incidents where only one electron is transferred and result in the production of O_2^-. Which is a moderately reactive ROS with a half- life of approximately 2-4 µs. It has document that O_2^- is usually the first ROS produced under stress (Elstner *et al*., 1987). In plant tissues usually 1 to 2% of the O_2 consumed is converted into O_2^-. The creation of O_2^- can lead to the production of other reactive ROS such as OH^- , which can cause membrane lipid peroxidation and weaken the cell (Bielski *et al*., 1983).

Additionally, O_2 is able to donate an electron to iron (Fe3þ) to produce a reduced form of iron which can then reduce H_2O_2 which is produced as a result of SOD led dismutation of O_2^- to OH^-. The reactions through which O_2^-, H_2O_2 and iron rapidly generates OH^- is called the Haber Weiss reaction, whereas the final step which involves the oxidation of Fe2þ by H_2O_2 is referred to as the Fenton's reaction (Gambarova *et al*., 2008).

1.3.2: Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide is produced by the univalent reduction of O_2^-. Hydrogen peroxide is moderately reactive ROS and has comparatively long half- life of 1 ms whereas other ROS such as O_2^-, OH^- and $^1O_2$ have much shorter half-life (Tewari *et al*., 2006). It has been well document that high levels of H_2O_2 in the plant cells can lead to oxidative stress. H_2O_2 can also inactivate enzymes by oxidizing their thiol groups.

Hydrogen Peroxide has a dual role in plants, at low concentrations it acts as a signal molecule which is involved in acclamatory signaling triggering tolerance to various stresses and at high concentrations it leads to programed cell death (Kariola *et al*., 2005). Hydrogen peroxide also act as an important regulator in a wide range of physiological processes such as photorespiration, senescence, photosynthesis, cell cycle, growth and development (Desikan *et al*., 2003). Hydrogen peroxide is generally recognized as a second messenger for signals generated by ROS due to its comparatively long life and high permeability across membranes (Quan *et al*., 2008).

1.3.3: Hydroxyl radicals (OH^-)

Hydroxyl radicals are among the most highly reactive ROS molecules. In the presence of suitable transitional metals such as Fe, OH^- can also be produced from H_2O_2 and O_2^- at ambient temperatures and neutral pH by the iron-catalyzed (Levitt *et al*., 1980). These OH^- is believe to be largely responsible for mediating oxygen toxicity in vivo. Hydroxyl radicals can possibly react with biological molecules like DNA, proteins and lipids, due to the absence of any enzymatic mechanism for the elimination of this highly reactive ROS. Thus excess production of OH^- can ultimately lead to cell death (Vranova *et al*., 2002)

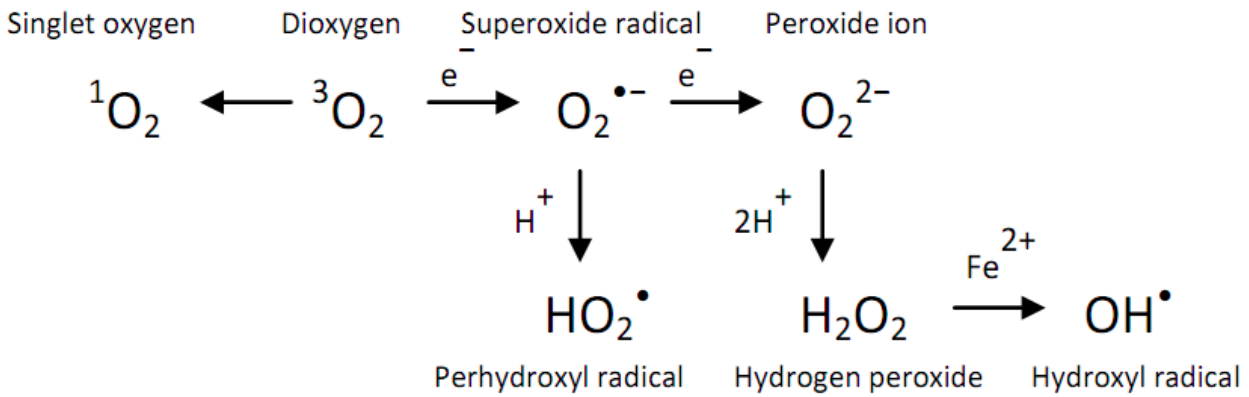

Figure 1.1: Progressive generation of ROS molecules in response of drought stress (Gilbert *et al*., 1999)

1.4.1 ROS and lipid peroxidation

Peroxidation of lipids is considered to be the most destructive process known to occur in every living organism. Damage to the membrane can be taken as a single parameter to determine the level of lipid destruction under various stresses such as drought. During lipid peroxidation polyunsaturated precursors are formed as byproducts which include small hydrocarbon fragments such as ketones and compounds related to them (Garg *et al*., 2009). Some of these small hydrocarbon fragments react with thiobarbituric acid (TBA) to form coloured products called thiobarbituric acid reactive substances (TBARS) (Heath *et al*., 1968). Lipid peroxidation in cellular and organelle membranes occurs when ROS levels above-threshold are reached, thereby it directly affecting normal cellular functioning and also exasperating the oxidative stress through production of lipid-derived radicals (Montillet *et al*., 2005).

Lipid peroxidation occurs in three distinct stages namely initiation, progression and termination steps. In the initiation step involves transition metal complexes such as Fe and Cu. However, H_2O_2 and O_2^- are able to initiate the reactions but as OH^- is sufficiently reactive, the initiation of lipid peroxidation in a membrane is initiated by the abstraction of a hydrogen atom, in an unsaturated fatty acyl chain of a polyunsaturated fatty acid residue, largely by OH^-. In an aerobic environment, lipid peroxyl radicals formed by the addition of oxygen to the fatty acid at the carbon-centered lipid radical. Once initiated, lipid peroxyl radicals are able further propagate the peroxidation chain reaction by abstracting a hydrogen atom from adjacent polyunsaturated fatty acid side chains. The subsequent lipid hydroperoxide can easily decay into several reactive species

including which include aldehydes (malonyldialdehyde), lipid alkoxyl radicals, alkanes, alcohols and lipid epoxides (Davies *et al*., 2001). Thus a single initiation event is able to generate multiple peroxide molecules by a chain reaction. Lipid peroxidation overall effects include decreased membrane fluidity; making it easier for phospholipids to exchange between the two halves of the bilayer. It also an increase the leakiness of the membrane to substances that normally do not cross it other than through specific channels and inactivating receptors, damage membrane proteins, ion channels and enzymes (Fam *et al*., 2003).

1.4.2: Protein oxidation

Protein oxidation is the covalent modification of a protein by ROS or byproducts of oxidative stress. Most types of protein oxidations are fundamentally irreversible, while a few sulfur containing amino acids are reversible (Ghezzi *et al*., 2003). The most widely used marker for protein oxidation is protein carbonylation (Job *et al*., 2005). Oxidation of a number of protein amino acids give free carbonyl groups which may inhibit or change their activities and increase its susceptibility towards proteolytic attack (Moller *et al*., 2007). Protein carbonylation can occur by direct oxidation of amino acid side chains (Shringarpure *et al*., 2002).

1.4.3: DNA damage

While plant genome is very stable its DNA might get damaged due to the exposure to abiotic and biotic stress factors which might damage the DNA (Tuteja *et al*., 2009). Damage endogenously generated is known as ‘‘spontaneous DNA damage” which is produced by reactive metabolites such OH^-, O_2^- and NO^-. High levels of ROS can cause damage to cell structures, nucleic acids, lipids and proteins (Valko *et al*., 2006). It has been shown that OH^- is most reactive and is able to damage to all components of the DNA molecule (Halliwell *et al*., 1999). ROS is capable of inducing damage to almost all cellular macromolecules including DNA which includes base deletion, pyrimidine dimers, cross- links, strand breaks and base modification, such as alkylation and oxidation (Wiseman *et al*., 1996; Tuteja *et al*., 2001). This DNA damage can result in numerous physiological effects such as decreased protein synthesis, cell membrane destruction and

damage to photosynthetic proteins, which ultimately affects the growth and development of the whole plant (Britt *et al*., 1999).

DNA damage can also result in seizure or induction of transcription, induction of signal transduction pathways, replication errors, cell membrane destruction and genomic instability. Lipid peroxidation and DNA damage independently have been showed to be the determining factor of seed viability loss. There is a number of mechanisms available to repair DNA damage both in the mitochondria and nucleus. These mechanisms consist of direct reversal of the damage and replacement of the base or the whole nucleotide (Cooke *et al.*, 2003).

1.5.1: Plant responses to drought stress

Plants responses to drought stress are very complex which include involve both deleterious and adaptive changes. While early responses to drought stress usually aid the plant to survive for a period of time the acclimation can aid in prolonged exposure. The acclimation of a plant to drought is indicated by the accumulation of certain new metabolites which are associated with the improved plant functioning under drought stress (Pinhero *et al*., 2001). Under continuous oxidative attack on plants during drought stress plant have number of enzymatic and non-enzymatic antioxidant defenses to lessen the effects of oxidative stress.

1.5.2: Superoxide dismutase (SOD)

Metallo-enzyme SOD is known as the most effective intracellular enzymatic antioxidant that is abundant in all aerobic organisms and subcellular sections which is prone to ROS mediated oxidative stress. It has been well-documented that various environmental stresses can lead to increased generation of ROS and SOD had been proposed to be significant in plant stress tolerant by been the first in line of defense against the toxic effects of increased levels of ROS. SODs does this by removing O_2^- by catalyzing its dismutation, the one O_2^- that is reduced to H_2O_2 and another oxidized to O_2. SODs removes O_2^- which decreases the risk of OH^- formation through the metal catalyzed Haber Weiss type reaction.

This catalyzed dismutation reaction has a 10,000 fold faster rate than spontaneous dismutation. SODs are classified according their metal cofactors into three known types which can either be copper/zinc (Cu/Zn-SOD), the iron (Fe-SOD) and the manganese (Mn-SOD), which are localized in different cellular compartments (Mittler *et al*., 2002). The Mn-SOD is located in the mitochondria of eukaryotic cells and in peroxisomes (del Rio *et al*., 2002). While some Cu/Zn-SOD isozymes have been found in the cytosolic fractions and in chloroplasts of higher plants (del Rio *et al*., 2003). The Fe-SOD isozymes are frequently not detected in plants but are commonly associated with the chloroplast compartment when present (Ferreira *et al*., 2002) .The Cu/Zn-SOD is sensitive to both inhibitors; Mn-SOD is resistant to both inhibitors whereas Fe-SOD is sensitive to H_2O_2 and resistant to KCN. The upregulation of SODs has showed to be implicated in opposing oxidative stress as a result of biotic and abiotic stress and have a key role in the survival of plants (Alscher *et al*., 2002).

1.5.3: Ascorbate peroxidase (APX)

APX is thought to have the most essential role in scavenging ROS and protecting cells in higher plants, algae, and other organisms. APX is involved in scavenging of H_2O_2 in water-water and ASH-GSH cycles and utilizes ASH as the electron donor. The APX family consists of at least five different isoforms including thylakoid (tAPX) and glyoxisome membrane forms (gmAPX), as well as chloroplast stromal soluble form (sAPX), cytosolic form (cAPX) (Noctor *et al*., 1998). APX may play a more important role in the management of ROS during stress as it has a higher affinity for H_2O_2 than catalase and Guaiacol Peroxidase it. During different stress conditions heightened expression of APX in plants has been reported. It was put forward that the overproduction of APX increased the POD activity which reinforces the ROS scavenging system and leads to oxidative stress tolerance (Sarowar *et al*., 2005). Transgenic plants in which APX was overexpression conferred increased abiotic stress tolerance.

1.5.4: Catalase (CAT)

Catalases (CATs) are tetrameric heme containing enzymes with the ability to directly dismutate H_2O_2 into O_2 and H_2O and thus is crucial for ROS detoxification during stressed conditions (Garg *et al.*, 2009). CAT has one of the highest turnover rates as one molecule of CAT can convert 6 million molecules of H_2O_2 to O_2 and H_2O per minute. Making CAT vital in the removal of H_2O_2 produced in the cell through reactions such as the b-oxidation of fatty acids, purine catabolism and photorespiration.

Many studies have been carried to extensively catalog CAT isozymes in higher plants. Maize has 3 isoforms namely CAT1, CAT2 and CAT3, which are found on separate chromosomes and are differentially expressed and also independently regulated (Scandalias *et al.*, 1990). CAT1 and CAT2 are found in peroxisomes and cytosol, while CAT3 is localized mitochondria. The CAT isozymes have been documented to be regulated spatially and temporally and may respond differentially to light (Skaden *et al.*, 1995; Nagamiya *et al.*, 2007). It has also been shown that separately from its reaction with H_2O_2, it also react with a few hydroperoxides such as methyl hydrogen peroxide (MeOOH) (Ali *et al.*, 2006).

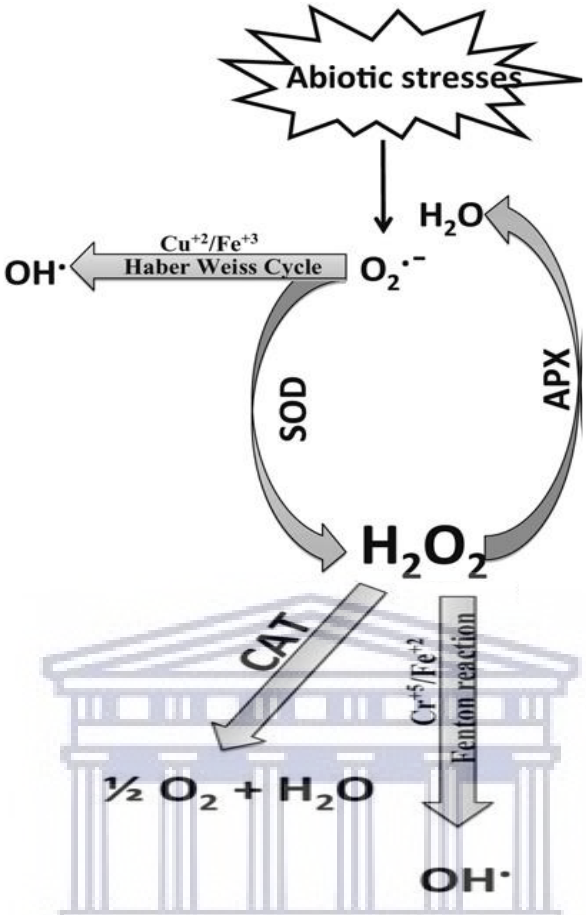

Figure 1.2: Antioxidants defence mechanism in response to drought (Gill *et al*., 2010)

Therefore is critical to development strategies to enhance crop production and make them more drought tolerant in order to secure South Africa's food security, one such approach is research into endophytes bacteria.

1.6.1: Endophytes

Endophytes are bacteria which occupy the internal area of plants such as the stems, leaves and roots. Endophytic bacteria which are found in the roots of plants (rhizosphere) are referred to rhizobacteria. Some of these plant associated bacteria have been found to have the ability to facilitate plant growth and expansion both indirectly and directly, therefore these microbes have been termed as Plant Growth Promoting Bacteria (PGPB). Direct growth promotion may refer to getting assists from bacteria in the uptake of essential nutrients and minerals while indirect plant growth promotion can be by preventing phytopathogens which allows plant to proliferate. These PGPB also have applications in the improvement of phytoremediation, which is the use of plant to clean up soil (Glick *et al*., 1995).

Bacteria in soil specifically have the capability to use arrange of different substances as nutrient sources and to grow rapidly. Although a large number of these bacteria are distributed within the soil, they regularly adhere to particles in soil and have interactions with the roots of plants. It is not usual for the abundance of bacteria in the rhizosphere to be much larger than that found in the soil (Lynch *et al*., 1990), thus increasing the probability of finding novel PGPB.

1.6.2: Plant microbe interactions

The interaction between the root of a plant and soil bacteria can either be regarded as being neutral, beneficial or harmful for the plant, and occasionally the effects of a specific bacterial strain might differ as the conditions change in the soil (Lynch *et al*., 1990). For instance, bacteria which are able to aid growth of plant through fixation of nitrogen would not be beneficial if the soil already contains large quantities of nitrogen through the addiction nitrogen fertilizer. Correspondingly, bacteria which are able to aid growth through diminishing the inhibitory effects of a number of environmental stresses, which can either biotic or abiotic, are not likely to have a considerable effect on plant if the conditions in the soil are ideal (Glick *et al*., 2007).

In conditions which are not optimal for plant these bacteria have great scientific value as biofertilizers. Biofertilizers can be described as any substance that contains living microorganisms once added to seed, surface of plant, or soil will colonizes interior of plant or the rhizosphere and promotes growth through enhancing the availability or uptake of essential nutrients to the host plant (Wu *et al*., 2005).

1.7.1 Importance of nutrient uptake

Mineral elements such as nitrogen (N), phosphorus (P), magnesium (Mg), calcium, iron (Fe), manganese (Mn), and zinc (Zn), amongst others have important role in all living organisms. An appropriate balance of all these nutrients is needed at each phase of plant development to attain maximum yield. Plants require large amounts of N and P that are vital nutrients because they are building blocks for important biological molecules while they need only small quantities of micronutrients, such as Zn, Fe, and Boron. Among all the vital nutrients, N and P are the most limiting factors for agricultural production, making it essential to add large amounts of fertilizers to soil each year to increase crop yield (Ma *et al*., 2011).

1.7.2: Nitrogen fixation

Nitrogen is required for the cellular synthesis of chlorophyll, enzymes, proteins, RNA and DNA, thus it is vital in development of plants and production of feed and food. The biological fixation of nitrogen happen primarily through symbiotic relationship between N fixing microbes and legumes of plant that are able to transform atmospheric N into ammonia with the enzyme nitrogenase (Shiferaw *et al*., 2004). Biological fixation of nitrogen is responsible for 65% of the nitrogen presently used in agriculture, and will carry on playing a significant role in a sustainable crop production system (Matiru *et al*., 2004).

1.7.3: Phosphate solubilisation

Phosphorus (P) is an essential nutrient for growth and development of plants. The solubility of phosphate is regularly the limiting nutrient in the increase of biomass in natural ecosystems and can solely be taken up in monobasic or dibasic soluble forms by plants. An increase in heavy metals within the soil inhibits P uptake which can cause plant development and growth being obstructed (Glass *et al*., 1989; Zaidi *et al*., 2006).

A large number of metal-resistant PGPB under metal stressed conditions will either convert these insoluble P into their available forms through, chelation, acidification, and release of organic acids and exchange reactions or through the mineralization of organic phosphates by discharging extracellular phosphatases (Chung *et al*., 2005). The increase in phosphate availability through the injection of phosphate-solubilizing bacteria has been done in pot experiments and in field conditions thus increasing the amount of limited resources (Pal *et al*., 1998; Zaida *et al*., 2003).

1.7.4: Increase iron uptake through siderophore production

Iron is a necessary co-factor in a number of enzymatic reactions in plants and therefore it an important nutrient for almost all organisms. Iron exists in the aerobic condition and mainly found in ferric state. In this state it reacts to produce very insoluble oxyhydroxides and hydroxides which cannot be used by microorganisms and plants. Therefore, plants are unable to take up adequate quantities of iron and additionally harmful heavy metals that amassed in plant tissues can result in alterations in several important growth processes and can have negative influence on iron nutrition.

To obtain appropriate iron, bacteria produce siderophore is which can bind to Fe with a high affinity to solubilize this metal for its efficient uptake. For instance *Poaceae* is able to release phyto-siderophores to improve Fe uptake, however microbial siderophores normally has a larger affinity for iron than phytosiderophores. In these conditions, the siderophore produced PGPB can offer a biological system which is proficient in chelating Fe 3^{+} and thus making Fe available for uptake the plant (Rajkumar *et al*., 2010) .The roots of plants might then be able to take in Fe from siderophores Fe complexes feasibly through the “chelate degradation and release of iron, the

direct uptake of a ligand exchange reaction or siderophore Fe complexes". Examples of the enhanced uptake of iron in plants with simultaneous promotion of plant growth as a result of inoculation by PGPB have been document in studies such as Barzanti and colleagues in 2007. Bacterial IAA synthesis is also promoted through siderophores through decreasing the harmful influence of heavy metals by chelation reactions (Dimkpa *et al*., 2008)

1.7.5: Zinc solubilization

Zinc is essential micronutrient required by animals and plants. Zinc is essential in many processes of life in plants such as nitrogen metabolism, photosynthesis, resistance against biotic and abiotic stresses (Alloway *et al*., 2004). Zinc also plays an important role in protein synthesis, membrane integrity and plant resistance against diseases and increases the level of antioxidant enzymes and chlorophyll within plant tissues (Gurmani *et al.,* 2012) (Sbartai *et al.,* 2011).

Furthermore, Zn is essential as a co-factor for the activity of more than 300 enzymes (Mccall *et al.,* 2000). Zn is also required for the creation of a number of phytohormones such as auxin, absicic acid, gibberellins and cytokinins and its deficiency can result in the impairment of growth of the plant. Thus its deficiency in plants can seriously affects numerous essential processes within plants (Imran *et al*., 2014; Younas *et al*., 2014). Some microorganisms are able solubilize zinc from inorganic and organic forms in soil which increases the availability of Zn for plants.

1.8.1: Plant hormones

Ethylene is one of the most well- known plant growth hormones, is produced endogenously by nearly all plants and stimulates plant cell elongation and proliferation (Saleem *et al*., 2007). Ethylene is composed two carbon and four hydrogen atoms (C_2H_4). Ethylene has effects on a wide arrange of difference processes within the life cycle of the plant such as organ senescence, seed germination, fruit ripening and stress responses (Figure 1.3). Over a number of years great strides has been made in understanding mechanics by which this phytohormone operates and most of this study used *Arabidopsis thaliana* as a model plant.

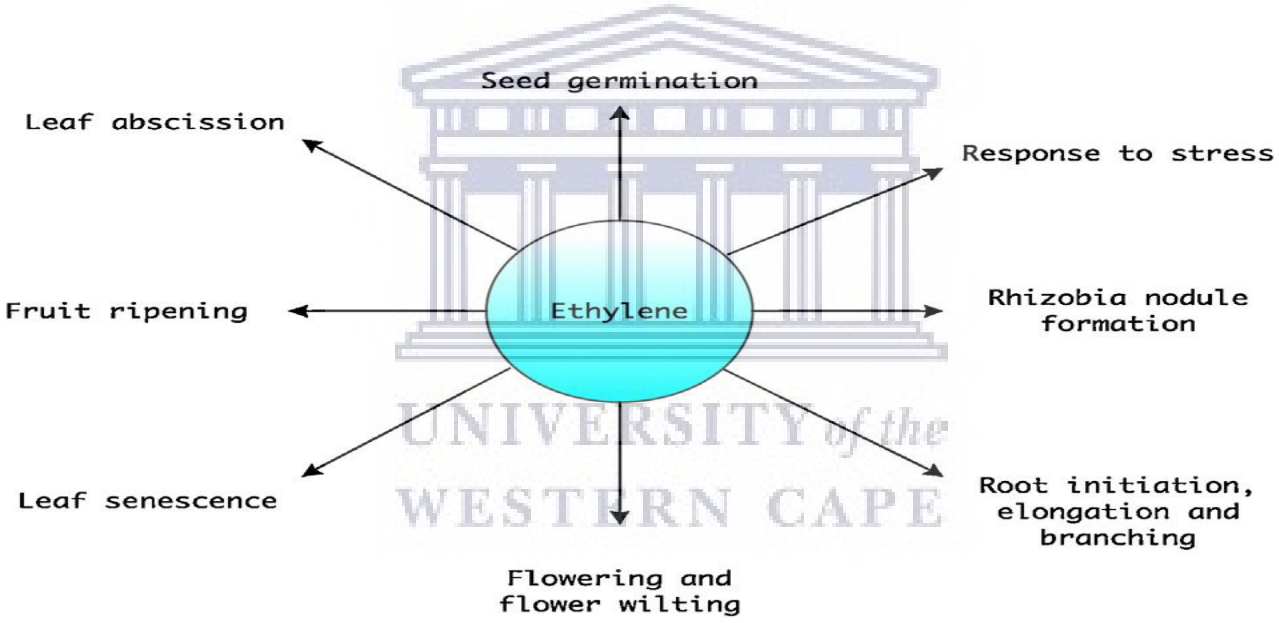

Figure 1.3: The phytohormone ethylene roles in plants (Glick *et al*., 2014).

Ethylene has a major part in provoking a number of physiological changes within plants at molecular level; however when it is overproduced it can lead the inhibition of lateral root growth, root hair formation, and root elongation. Nevertheless, bacteria are able to relieve the stress of ethylene overproduction in plants through the enzymatic hydrolysis of 1-aminocyclopropane- 1-carboxylic acid (ACC) (Mayak *et al*., 2004). Phytohormones which are produced by plant associated bacteria, comprising of, cytokinins, gibberellins and indole-3- acetic acid (IAA), which regularly increase germination rates, protect plants against abiotic (such as drought)and biotic (such as pathogens) stress, promote growth, and reproduction (Glick *et al*., 2007).

1.8.2: Indole-3-acetic acid (IAA)

Charles Darwin propose in 1880 that the growth of some plant is regulated by "a matter which effects from one part of the plant to another" and sometime later this matter was coined auxin ("from the Greek 'auxein' which means to grow"). IAA is one of the most frequently studied phytohormones and is produced in the shoot of plants then is transported to the root tips basipetally. This auxin is related with cell division and elongation donates to plant defence system development and growth. It was later discovered that microorganisms such as bacteria and fungi are able to synthesize IAA. (Rashotte *et al*., 2000; Navarro *et al*., 2006).

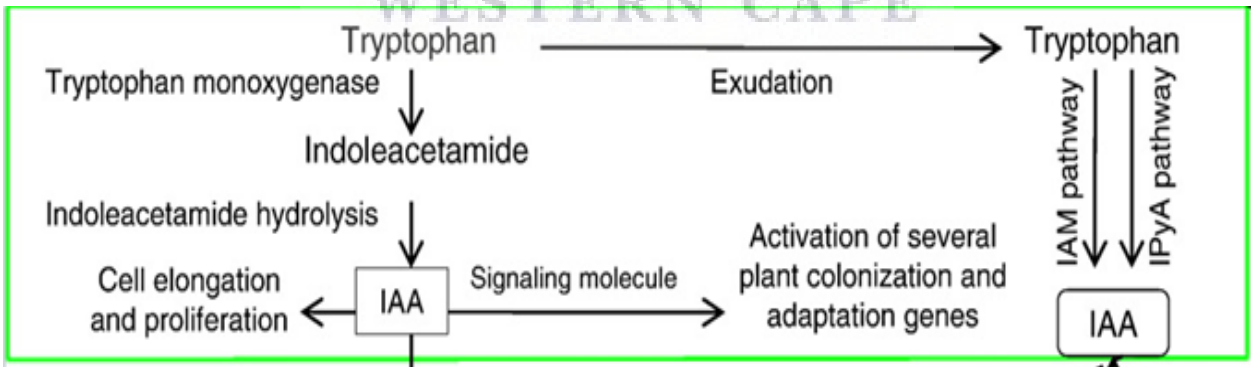

Figure 1.4: Figure of IAA in the plant and enzymes involved (Ma *et al*., 2011)

Through studies into bacterial IAA synthesis pathways a number of them have been identified. It was observed that the bacterial and plants IAA biosynthesis pathways share a large amount of similarity. Tryptophan has been found to be the main precursor in most IAA biosynthesis pathway in bacteria. Additionally, plant and microbe interactions can be determined through knowing which IAA biosynthesis pathway they use, for example, pathogenic bacteria largely uses the indole-3-acetamide (IAM) pathway while advantageous plant-associated bacteria synthesize IAA through the indole- 3-pyruvate (IPyA) pathway, while (Glick *et al*., 1996).

1.9.1: Justification

Zea mays L. is one of the major cereal crops in the world however this crucial food crop is subjected to an array of abiotic and biotic environmental stressors. Maize has been documented to be particularly susceptible to drought which is a major abiotic environmental stressor. With the ongoing influence of global warming droughts are expected to increase in numbers and duration. This increase in drought frequency may lead to the production of maize being reduced and endangering the food security of regions which rely on maize as a main food source. Therefore it is of vital importance improve maize ability to tolerate drought. A number of studies have looked into use of transgenic methods to improve drought tolerance in maize which has showed promise. Another approach is the use endophytes, endophytes have been well documented promote plants growth through facilitating nutrient uptake, production of phytohormones and other metabolites. Endophytes may also be able to reduce some of the damage caused during drought through phytohormone regulation and production of metabolites which aid plant in maintain osmotic pressure such as proline. However, research into this field is still on going.

1.9.2: Aim and objectives

The focus of this study is to understand the effect endophytes may have on the biochemical and physiological characteristics of *Zea mays L*. under the influence of drought stress. The aim of this study is to improved Zea *mays L*. drought tolerance through treatment with endophytic bacterial consortium. In order to achieve this goal endophytes will be extracted from plants with proposed drought tolerance. Endophytes for use in consortium will be selected based on endophyte characteristics such IAA, siderophore production and identified through 16S rDNA sequencing. The endophytic consortium will then be used to treat maize plants under well-watered and water-deprived conditions which will be compared to maize grown under well-watered and water-deprived conditions without the consortium. After the growth trial, plant physiological characteristics such as weights and lengths will be recorded. The samples relative water content will also be assessed to determine what impact endophytes had on the water status. The cell death will also be tested to determine whether the endophytes will be able to reduce the

damages caused by drought. Aside from the physiological tests, samples will also undergo a number of assays to measure ROS production inside plant tissue. ROS production increases during stress and lead to oxidative damage at high levels. In order to measure the amount of damage ROS molecules caused, MDA content will also be assayed since it is a by-product of oxidative damage and will indicate whether the endophytes was able to alleviate some of oxidative damage. Plants have number of self-defence strategies in place to handle the production of ROS such as antioxidant enzymes. The antioxidant enzyme activity will also be assayed to test whether the endophytes will able to reduce the oxidative stress by increasing antioxidant activity. The information obtained from this study will advance knowledge on the use of endophytes in order to increase drought tolerance of crop plants.

Chapter 2: Materials and Methods

List of chemicals and suppliers

(3- (4, 5- Dimethylthiazol- 2- yl)-2, 5- Diphenyltetrazollium Bromide)	Sigma- Aldrich
1, 4- Piperazinediethanesulfonic acid	Sigma- Aldrich
2- Thiobarbituric acid	Sigma- Aldrich
2-Deoxy- D- Ribose	Sigma- Aldrich
Acetone	Sigma- Aldrich
Agar	Sigma- Aldrich
Ammonium chloride	Sigma- Aldrich
Ammonium sulphate	Sigma- Aldrich
Boric acid	Sigma- Aldrich
Calcium chloride	Sigma- Aldrich
Calcium phosphate	Sigma- Aldrich
Casamino acid	Sigma- Aldrich
Copper (ll) sulfate	Sigma- Aldrich
Dextrose	Sigma- Aldrich
Dipotassium hydrogen phosphate	Sigma- Aldrich
Dipotassium phosphate	Sigma- Aldrich
Ethanol 200 proof	Sigma- Aldrich
Ethylenediaminetetraacetic acid	Sigma- Aldrich
Evans blue reagent	Sigma- Aldrich
Ferrous sulphate	Sigma- Aldrich
Glacial acetic	Sigma- Aldrich
Glucose	Sigma- Aldrich

Hexadecyltrimethylammonium bromide	Sigma- Aldrich
Hydrochloric acid	Sigma- Aldrich
Hydrogen peroxide	Sigma- Aldrich
Indole acetic acid	Sigma- Aldrich
L- Ascorbic acid	Sigma- Aldrich
Liquid nitrogen	UWC Che. dep.
Luria broth	Sigma- Aldrich
Magnesium sulfate	Sigma- Aldrich
Magnesium sulfate monohydrate	Sigma- Aldrich
Magnesium sulphate	Sigma- Aldrich
Magnesium sulphate	Sigma- Aldrich
Maize seeds	Sigma- Aldrich
Manganese (ll) sulfate	Sigma- Aldrich
Manganese sulphate	Sigma- Aldrich
Mannitol	Sigma- Aldrich
Molybdic acid sodium salt dehydrate	Sigma- Aldrich
Monopotassium phosphate	Sigma- Aldrich
Nitric acid	Sigma- Aldrich
Nitro blue tetrazolium chloride monohydrate	Sigma- Aldrich
PCR reagents	New England Biolabs
Perchloric acid	Sigma- Aldrich

Phosphate buffer	Sigma- Aldrich
Potassium chloride	Sigma- Aldrich
Potassium cyanide	Sigma- Aldrich
Potassium hydroxide	Sigma- Aldrich
Potassium iodide	Sigma- Aldrich
Potassium Phosphate	Sigma- Aldrich
Proline standard	Sigma- Aldrich
Pro-mix soil	Builders Warehouse
R2A agar	Sigma- Aldrich
Riboflavin	Sigma- Aldrich
Sodium chloride	Sigma- Aldrich
Sodium chloride	Sigma- Aldrich
Sodium Dodecyl	Sigma- Aldrich
Sodium permanganate	Sigma- Aldrich
Trichloroacetic acid	Sigma- Aldrich
Tris (hydroxymethyl) aminomethane hydrochloride	Sigma- Aldrich
Tryptophan	Sigma- Aldrich
Yeast extract	Sigma- Aldrich
Zinc sulfate heptahydrate	Sigma- Aldrich

2.1: Plant sample collection and Endophytes isolation

The endophytes were isolated from *Medicago lupulina* plant. The plant was collection at coordinates 33.986093, 18.1617309 in arid soil. Surface sterilization was carried out on the leaf and root material to remove any epiphytic bacteria. Surface sterilization was performed by rinsing the samples with 70% ethanol for 1 min then 2.5% sodium hypochlorite for 1 min followed by 3 washes with sterile dH_2O, and the last wash was plated on R2A agar to test sterility.

The surface sterilized leave and root material were grind separately in a sterile solution of 0.9% NaCl into a homogenous solution. The solutions were collected in greiner tubes and a serial dilution was preform which was plate onto R2A agar. Afterward isolates on the R2A agar were selected and streaked out in order to acquire singe colonies.

2.2.1: Phosphate Solubilization

This test was carried through the use of Pikovskaya Agar which was prepared through a modified method described by Gupta *et al* (1994). Each isolate was spot inoculated on to a pikovskaya agar plate. The plates consisted of dextrose 1%, calcium phosphate 0.5%, ammonium sulphate 0.05%, yeast extract 0.05%, potassium chloride 0.200 g, magnesium sulphate 0.01%, manganese sulphate 0.00001%, ferrous sulphate 0.00001% and 1.5%agar. Plates were then incubated at 30°C for 7 days. The formation of a halo around the colony is considered a positive result.

2.2.2: Zinc Solubilization

This method is modification of the method described by Saravanan *et al* (2004) for determining zinc solubilizing ability. The zinc solubilization plates were prepared by combing 0.5 g sodium chloride, 0.3 g monopotassium phosphate and 1.0 g ammonium chloride, 2 g glucose, 493 mg magnesium sulfate· heptahydrate, 2 g mannitol, 11 mg calcium chloride, 1.17 mg manganese(ll) sulfate, 0.4 mg copper sulfate pentahydrate, 1.4 mg boric acid, 1.0 g sodium molybdate dehydrate and 1.2 mg zinc sulfate heptahydrate. The mixture pH was adjusted with 50% KOH to 6.8 and H_2O was added to make a final volume of 800 ml. Afterwards 15 g of agar added to the mixture and was autoclaved, solution was kept at 50°C until it was poured in petri-dishes.

2.2.3: Indole acetic acid (IAA)

This method is modification of the method described by Idris *et al* (2007) for determining IAA content. The production IAA of each isolate was measured using colorimetric assay. Isolates were first grown up for 5 days in Yeast Extract Mannitol (YEM) and YEM with 0.1% tryptophan at 30°C. YEM media consisted of Yeast extract 0.1%, Magnesium sulphate 0.02%, Mannitol 1%, Sodium chloride 0.01% and Dipotassium phosphate 0.05% however Dipotassium phosphate was autoclaved separately to avoid the formation of sediment in the media.

Afterward the culture was centrifuged at 13000 x g for 10 min. Then 1 ml supernatant was mixed with 2 ml of Salkowski reagent (35% $HClO_4$ and 0.25 M $FeCl_3$) inside a cuvette and incubated at room temperature for 30 min. The optical density was then read at 530 nm and recorded. The standard curve was constructed using YEM and a known concentration of IAA ranging from 0-1000 µl/ml and also read at 530 nm and used to determine IAA concentration.

2.2.4: Siderophore plate assay

This test was carried through the use of Chrome azurol S (CAS) agar which was prepared with a modified method described by Alexander *et al* (1991). CAS agar consists of four solutions which were prepared and sterilized separately. Solution 1 was Fe-CAS indicator which was prepared by mixing 10 ml of 1 mM $FeCl_3 \cdot 6H_2O$ in 10 mM HCl and adding 50ml of 1.21 mg/ml CAS. The reagents were then slowly added to each other with constant stirring and 1.82 mg/ml HDTMA was added. The resulting blue mixture was autoclaved and cool down to 50°C. Solution 2 was a buffer solution which was prepared by dissolving 30.24 g of PIPES in 750 ml of salt solution which contain 0.5 NaCl, 0.3 g KH_2PO_4 and 1.0 g NH_4Cl. The pH of the buffer was adjusted with 50% KOH to 6.8 and H_2O was added to make a final volume of 800ml. Before the solution was autoclaved 15 g of agar was added and solution was kept at 50°C.

The 3rd solution consisted of 2 g glucose, 493 mg magnesium sulfate· heptahydrate, 2 g mannitol, 11 mg calcium chloride, 1.17 mg manganese(II) sulfate, 0.4 mg copper sulfate pentahydrate, 1.4 mg boric acid, 1.0 g sodium molybdate dehydrate and 1.2 mg zinc sulfate heptahydrate. Solution 3 was then autoclaved and kept at 50°C, then added to solution 2 along with 30 ml of filter sterilized 10% weight to volume casamino acid which is solution 4. Solution 1 was added last with sufficient stirring to avoid forming bubbles. The mixture was then added to clean petri-dishes and left to solidify. A single colony was spot inoculated onto a CAS agar plate and incubated at 30°C for 7 days. The formation of an orange halo around the colony is considered a positive result.

2.2.5: DNA extraction and identification

DNA was extracted using adaption boiling lysis method described by Zhy *et al* (2005). The endophytes were grown overnight in 20 ml of LB broth. Then 2 ml of culture was transferred to an Eppendorf tube and centrifuged at 12000 x g for 5 minutes. The supernatant was then decanted and the pellet resuspended in 200µl TE buffer. The suspension was boiled on a heating block from 15 minutes at 90^{o}C. Afterwards tubes was cooled on ice and centrifuged at 12000 x g for 5 minutes. Then 100 µl of the supernatant was transferred to a new tube.

The 16S rRNA gene was amplified through PCR using the genomic DNA extracted from isolates. Primers E9F (5'- GAG TTT GAT CCT GGC TCA- 3') (Farelly *et al*., 1995) and U510R (5'- GGT TAC CTT ACG T- 3') (Reysenbach *et al*., 1995), were used to amplify the V3-V4 region of the 16S rRNA gene. The PCR was carried in 25 µl reaction which consisted of 2.5 µl DNA, 0.2µM forward primer, 0.2 µM reverse primer, 1 X New England Biolabs Ampliqon 2 X Taq mastermix and was made up 25 µl with nucleases free water. PCR products were then sent away for sequencing at the University of Stellenbosch. The sequences was analysed using the algorithms EzbioCloud.

2.3.1: Growth trials

The isolates were grown in 25 ml of LB broth inside a 50 ml greiner tube overnight until an OD of 1 was obtained. Maize seeds were surface sterilized, then imbibed in LB broth containing consortium of isolates while the control seeds were imbibe in only LB broth for 30 minutes. The seeds were separated into four group namely well-watered (WW), water deprived, (WD) well-watered inoculated (WWI) and water deprived inoculated (WDI). There was 9 plants grown per treatment to provide enough plant material for later test. Maize seeds were planted in 5 liter pots with a Promix 90/10 (Cocopeat/Perlite) soil which was dried previously. Well-watered pots was watered once a week, while water deprived pots received 40% less water to simulate drought. Plants were grown till there were visible signs of leave rolling was observed.

2.3.2: Harvesting of plant material

Plant were carefully removed from their pots to determine their lengths and weights of respective plants. Some of the plants were separated for use in determination of the relative water content of plant respectively. Some of the plant material were also separated for use in cell death and superoxide assays. The rest of the plants were crushed up using mortar and pestle in liquid nitrogen. After the plant material was finely crushed it was transferred into 50ml greiner and store at - 80°C for later used.

2.3.3: Relative Water Content assay

Relative water content was determined using a modified method describe by Sade *et al* (2015). A 5 cm long piece of fresh leave material cut of each plant sample and weighted to determine fresh weight (FW). After each piece of leave material was then submerged in water for 8 hours. After the 8 hours the leaf material is place of towel and the excess water removes, then the leaves are weight again to determine turgid weigh (TW). The leaf samples were then stored at 60^{o}C for 48 hours and weighted again to determine the dry weight (DW). The FW, TW and DW was used to determine relative water content.

2.3.4: Inductively Couple Plasma (ICP) spectrometry

First 200 mg of plant material from each experimental sample was weighed out and transferred into 2 ml Eppendorf tube. Then 1 ml of 65% Nitric acid was transferred to the tube and mixed. Tubes were then sealed and placed on a heating block for 3 hours at 94°C. The 1 ml of sample was then mixed with 9 ml of 2% nitric acid using a syringe and filtered into a 15 ml greiner tube and ICP-OES analyses were done on the samples (Beauchemin *et al*., 2010).

2.3.5 Evans blue assay (Cell death)

This method is modification of the Cell death assay used in Sanevas *et al* (2007).First 0.25% Evans blue solution (w/v) was prepared and 1 ml of this solution was then aliquoted into a clean 2 ml Eppendorf tube. Afterwards a 1 cm^3 square was cut from the fresh leaf material and submerged in the tubes containing the 1 ml of Evan blue solution. In order perform the assay on the roots 2 cm in length from the tip of root was cut and submerged in Evan blue solution. The samples was then incubation at room temperature for an hour in the dark. Then the samples were rinsed with dH_2O in order to remove the Evans blue solution, then samples were left in water overnight. The water in the tubes was then decanted and 1 ml of 1% of SDS (w/v) solution was added. The samples was crushed and then incubated at 65 ^0C on a heating block for an hour. After heating samples were centrifuged at 13000 x g for 5 minutes to pellet the plant material and then the supernatant was obtained. Then 200 µl of the supernatant was added to a microtiter plate and read at a wavelength of 600nm of a spectrometer.

2.4.1: Superoxide content determination

A solution containing 10 mM KCN (to inhibit Cu/Zn SODs), 10 mM H_2O_2 (to inhibit Mn and Cu/Zn SODs), 2% SDS (inhibit Mn and Fe SODs) and 80 µM NBT was prepared and made up to a volume of 800 µl with 50 µM Potassium Phosphate at pH 7.0 in a clean Eppendorf tube. Then eight 1 cm^3 squares were cut from the fresh leaf material and carefully submerged in the solution, this was done in triplicate. For the root samples 4 cm cuts were made from the tip of the root and submerged in the above prepared solution, this was done in triplicate. Then the plant material was incubated in the solution for 20 minutes at room temperature.

After incubation the plant material were crush in their tubes using miniature pestle. The tubes was then centrifuged at 13000 x g for 5 minutes to pellet the material. After centrifugation the supernatant was transferred to a clean Eppendorf. Once supernatant was obtained free of plant material 200 µl of it was added a well on a microtiter plate in triplicate. The microtiter plate was read at 600 nm and with the use of the extinction coeffient of 12.8 mM cm^{-1} the superoxide content was determined. This was a modification of the method used in by Russo *et al* (2008) in their determination of superoxide content.

2.4.2: Trichloroacetic acid (TCA) extraction and MDA

A modification of the method use by Zhang *et al* (2009) in the quantification of MDA levels in their samples. The TCA extract was obtain by weighing 100 mg of frozen ground up plant material and adding 500µl 6% TCA (w/v) to it. The mixture was then briefly vortexed and centrifuged at 13000 x g for 10 minutes. Afterwards the supernatant was then transferred to a need tube. The TCA extract of the samples was then thawed on ice and 200 µl of it was added to clean Eppendorf tube 300 µl of 0.5% TBA (w/v). The mixture was then boiled at 90^0C for 20 minutes and cooled on ice for 10 minutes. After samples cooled down the tubes were centrifuged at 13000 x g for 5 minutes. Then 150 µl supernatant was loaded on to microtiter plate and read at 532 nm and 600 nm on spectrophotometer.

2.4.3: Hydroxyl radical content assay

A modification of the proposed by Halliwell *et al* (1987) in the determination of OH^- concentration. In order to assess OH^- concentration 50mg of frozen ground leaf material was added to 10 mM phosphate buffer containing 15 mM 2-Deoxy-D-Ribose in a 1 ml solution. Then the samples were incubated at 37°C for 2 hours on a heating box. Once the incubation was completed 0.7 ml of the sample was add to a solution with 3 ml of 0.5% TBA (w/v) which was made up in 5 mM NaOH with 1 ml glacial acetic acid. The reaction mixture was briefly vortexed and incubated in a hot water bath at 100°C for 30 minutes. After samples cooled down the tubes

were centrifuged at 13000 x g for 5 minutes. Then 150 µl supernatant was loaded on to microtiter plate and read at 532 nm and 600 nm on spectrophotometer.

2.4.4: Hydrogen peroxide content determination

This method is a modification of the method used by Velikova *et al* (2000) in their determination of H_20_2 content. The standard was prepared by making dilutions of H_2O_2 to the following concertation 0 nM, 5000 nM, 10000 nM, 15000 nM, 20000 nM and 25000 nM by diluting with dH_2O. The standard was then loaded on to a microtiter plate in triplicate. The TCA extract of the samples was then thwart on ice and 50 µl of the TCA extract was added into their respective well on the microtiter plate. Then to the standard and samples wells 1.25 mM dipotassium hydrogen phosphate (K_2HPO_4) and 250 mM potassium iodide (KI) was added. After all the reagent were added to the appropriate wells the microtiter was incubated on a shaker at room temperature for 20 minutes. The samples were then read at a wavelength of 390 nm on a spectrometer.

2.4.5: Protein extraction

The protein was extracted using a modified method from Marcozzi *et al* (1991). First 100 mg of frozen ground up leaf and root material was weighted in to separate 2 ml Eppendorf tubes in triplicate of each sample respectively (work in liquid nitrogen). Then 0.5 ml protein extraction buffer (0.004 M Phosphate buffer, 1 mM EDTA and 5% PvP (w/v) was added to added to one of the three from each sample, the tubes was then vortexed briefly. The tubes were then centrifuged at 12000 x g for 5 minutes. The supernatant was then transferred to the second tube containing 100 mg of the same material. The previous step was then repeated for the second and third tubes. The supernatant of the final tubes of each samples was then taken out transferred into a clean 2 ml Eppendorf tube respectively. The protein samples were then quantified using a Bradford assay to their respective protein concentrations. The tubes were store at -20^0C for later use.

2.4.6: Proline content assay

This method is a modification of the method used by Bates *et al* (1973) to determine proline content. Proline was extracted from mixing 50 mg of fresh plant material with 1 ml of ethanol: water (40:60 v/v) and resulting mixture was left over night at 4°C, then centrifuged at 14000 x g for 5 min. The previously stated step was repeated on the pellet and the supernatant was pooled for further analysing.

Then 100 µl reaction mix is added to 50 µl of proline extract and sealed inside the tube, then the mixture was heated to 95 °C for 20 mins. The standard was prepared using 50 µl of 0.04, 0.1, 0.2, 0.4 and 1 mM proline standard which prepared in 70:30 ethanol: water (v/v). After heating sealed tubes were cooled at room temperature and centrifuged at 2500 x g for 1 min. Then 100 µl of the mixture was added to a microplate well in triplicated and read at 520 nm.

2.5.1: Ascorbate peroxidase activity

This was a modification of method used by Nakano *et al* (1980) in their determination of ascorbate peroxidase activity. The frozen protein sample which concentration was determined was aliquoted into Eppendorf tube and incubated for 5 minutes in 2 mM ascorbate. After incubation 10 µl of protein sample was loaded on to a well in a microtiter plate with 71.34 mM K_2HPO_4 and 0.36 mM ascorbate. In order to determine the ascorbate peroxide activity 0.714 mM H_2O_2 was added to the well prior to staring the reading at 290 nm on the spectrophotometer to start the reaction.

2.5.2: Catalase activity determination

This was a modification of method used by Aebi (1984) in their determination of catalase activity. The 1 ml reaction mixture was prepared adding 0.5 mM EDTA, 100 mM K_2HPO_4 and 20 µl of extracted protein sample in a 1 ml cuvette. Before reading was taken 1 mM H_2O_2 was added to the reaction mixture and then wavelength was recorded at 240 nm. The absorbance decreases

over time due the dissociation of H_2O_2 and the speed of this decrease is together with extinction coeffient of 39.4 $mM.cm^{-1}$ was used to determine the level of catalase activity.

2.5.3: Superoxide dismutase activity assay

This was a modification of method used by Freitas *et al* (2017) in their determination of superoxide dismutase activity. The plant protein sample was diluted to a concentration of 1 mg/ml and then 10 µl of each protein sample was loaded into a 96 well microtiter plate in triplicate. To each well containing sample 0.1 mM Nitrotrezolium blue chloride (NBT), 20 mM phosphate buffer, 0.005 mM riboflavin and 0.1 mM EDTA was added then made up to 200 µl with distilled water. For the blank there was no protein added .The samples were them incubated on a light box at room temperature for 20 minutes, after incubation samples were read at 560 nm on a spectrophotometer

2.5.4 Statistical analysis

Statistical analysis was performed using the Tukey-Kramer test, where significance was represented by a $P< 0.05$. These test and graphs were produce in Prism.

Chapter 3: Characterization of endophytic bacteria and plant physiological changes under drought stress

3.1: Results

After the extraction endophytes were plated on R2A media and streaked to obtain single colonies. The isolates were then tested for growth promoting characteristics such phosphate solubilization, zinc solubilization, siderophore production and IAA production. The endophytes with the best growth promoting capabilities were chosen for a consortium. The 9 isolates chosen had the best activity. Plants were grown till they displayed signs of drought stress such as leaf rolling which occurred at the 48 day mark. Afterward biochemical tests were carried on the plant material to determine whether the endophytes was able to reduce some the harmful effects of drought stress.

3.2.1: Endophytes growth promoting characteristics analysis

Out of the 9 endophytes chose for use in the consortium 5 tested positive for phosphate solubilization on pikovskaya agar. With M1 and M8 having the highest activities indicated by the size of their halos. While a number isolates grew without the formation of a halo such M2.

Table 1: Phosphate solubilization activities of 9 endophytes isolated from *Medicago lupulina*

Isolate	Phosphate solubilization	Colony diameter (cm)	Diameter of halo (cm)
M1	+	1.17 a	2.26 a
M2	-	0.64 b	
M3	+	1.23 a	1.65 b
M4	+	1.37 c	1.98 b
M5	-	0.95 b	
M6	-	3.86 d	
M7	-	1.76 e	
M8	+	1.25 a	3.05 c
M9	+	0.75 b	0.86 d

The presence of an activity is indicated by "+" and the absence is indicated by "-" for the phosphate solubilization. Values above which have common letters are not significantly different, ($p \leq 0.05$) as determined by $P < 0.05$. Values are means ± S.E (N=3).

Zinc solubilization plate assay revealed that 7 endophytes tested positive for this activity. With M6 and M5 having the highest activity on the plates. While M1 and M2 merely grew without the formation of a halo after the growth period ended.

Table 2: Zinc solubilization activities of 9 endophytes isolated from *Medicago lupulina*

Isolate	Zinc solubilization	Colony diameter (cm)	Diameter of halo (cm)
M1	-	1.26 a	
M2	-	1.23 a	
M3	+	0.33 b	1.34 a
M4	+	0.25 b	1.24 a
M5	+	2.32 c	2.65 b
M6	+	2.13 c	2.36 b
M7	+	1.32 a	1.57 a
M8	+	1.56 a	2.14 d
M9	+	1.34 a	1.53 a

The presence of an activity is indicated by "+" and the absence is indicated by "-" for the Zinc solubilization. Values above which have common letters are not significantly different, ($p \leq 0.05$) as determined by $P < 0.05$. Values are means ± S.E (N=3).

Siderophore activity assay indicated that 7 of the endophytes tested positive. While M3 and M4 have the best activity with halos of 4.75 cm and 4.02 cm in diameter respectively. With M8 and M9 being the only isolates not to produce a halo.

Table 3: Siderophore production activities of 9 endophytes isolated from *Medicago lupulina*

Isolate	Siderophore production	Colony diameter (cm)	Diameter of halo (cm)
M1	+	2.05 a	3.62 a
M2	+	2.21 b	3.75 a
M3	+	2.53 b	4.75 b
M4	+	2.16 a	4.02 b
M5	+	2.12 a	2.66 c
M6	+	2.81 b	3.02 d
M7	+	1.31 c	1.53 e
M8	-	2.02 a	
M9	-	0.51 d	

The presence of an activity is indicated by "+" and the absence is indicated by "-" for the siderophore production. Values above which have common letters are not significantly different, ($p \leq 0.05$) as determined by $P < 0.05$. Values are means ± S.E (N=3).

Almost all endophytes showed a degree of IAA production with M2 being the exception. The endophytes with the heights was M7 with 44.20 µg/ml and M8 with 35.71 µg/ml respectively. While M2 was the only isolate unable to produce IAA after growth trial was completed.

Table 4: Indole-3-acetic acid production (µg/ml) of each isolated endophyte

Endophyte	M1	M2	M3	M4	M5	M6	M7	M8	M9
Concentration in µg/ml	11.32 a	0.00 b	15.10 c	5.34 d	20.87 e	22.93 e	44.20 f	35.71 f	2.35 d

Values above which have common letters are not significantly different, ($p \leq 0.05$) as determined by $P < 0.05$. Values are means ± S.E (N=3).

During sequencing it was observed that only 5 endophytes were amplified during PCR. While M4, M5, M7 and M8 did not achieve amplification and therefore was not able to be sequenced.

Table 5: 16S rDNA analysis of endophytes

Endophyte	Closest match on EzbioCloud database (Accession no.)	No. bases sequenced	% identity
M1	*Bacillus wiedmannii strain FSL W8- 0169*	1227	84%
M2	*Bacillus mycoides strain NBRC 101228*	1115	97%
M3	*Chryseobacterium arachidis strain 91A- 593*	1131	92%
M4	No amplification		
M5	No amplification		
M6	*Pantoea agglomerans strain ATCC 27155*	1067	96%
M7	No amplification		
M8	No amplification		
M9	*Curtobacterium flaccumfaciens strain LMG 3645*	1137	95%

3.4.1: Plant physiological changes

It was observed that in terms of shoot length and weight that WW was statistically similar to WWI, while WDI was statistically similar to WW in terms of shoot length and shoot weight. However WD showed a decreases of shoot length of 32.31% (Fig 3.1.1A) and a decrease of 63.83% in shoot weight (Fig 3.1.1B) compared to WW.

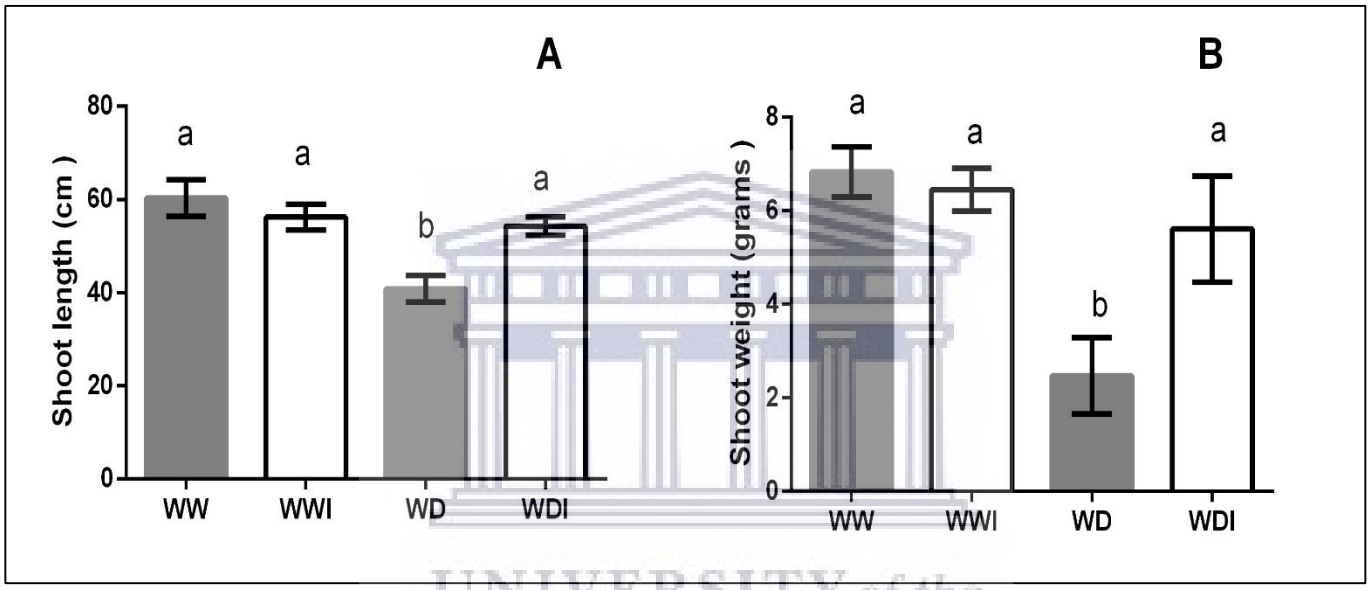

Figure 3.1.1: Shoot physiology of Maize cultivars grown under well-watered and water-deprived conditions. The plants were for 48 days in greenhouse under control conditions. Control plants which were not under effects of drought stress which were untreated (WW) and treated (WWI). Plants which were under drought stress which were untreated (WD) and treated (WDI). Values above the columns which have common letters are not significantly different, ($p \leq 0.05$) as determined by $P < 0.05$ (Tukey-Kramer test). Values are means ± S.E (N=3).

It was observed that WW and WWI were statistically similar in terms of root length and weight. It was also observed that WD was statistically similar to WW in terms of root length while WDI observed an increase of 19.41% (Fig 3.1.2A) compared to WW. However a reduction was observed in root weight of water-deprived samples with a decrease of 61.68% in WD and 51.16% in WD (Fig 3.1.2B).

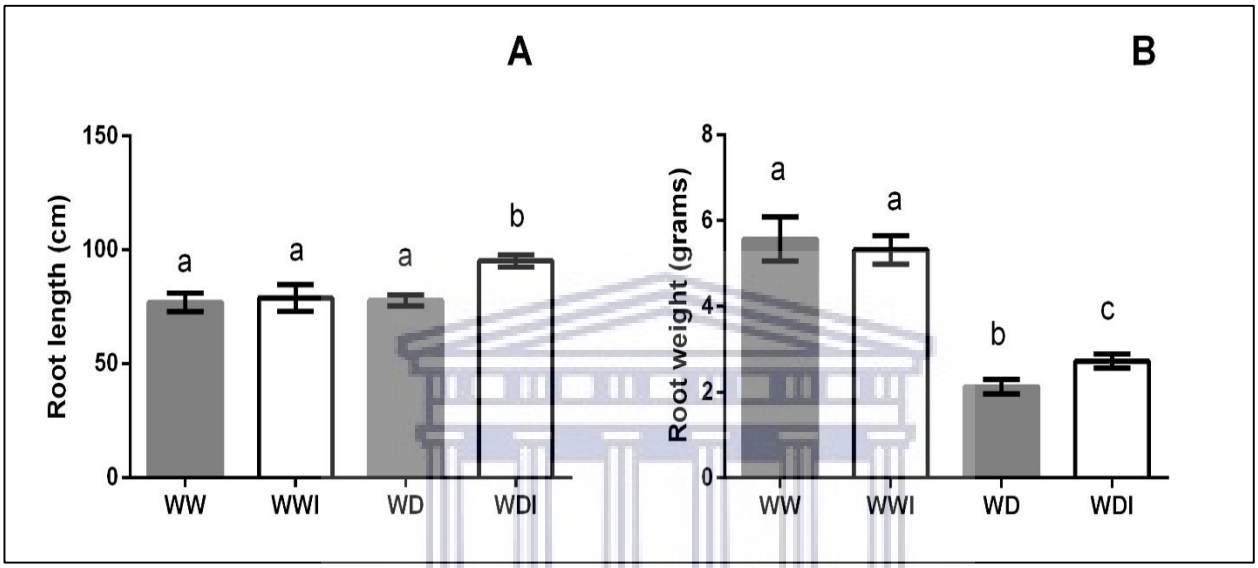

Figure 3.1.2: The effect of drought stress on the root physiology of Maize cultivars when treated with endophyte consortium. The plants in this study were for 48 days in greenhouse under control conditions. Control plants which were not under effects of drought stress which were untreated (WW) and treated (WWI). Plants which were under the effects of drought stress which were untreated (WD) and treated (WDI). Values above the columns which have common letters are not significantly different, ($p \leq 0.05$) as determined by $P < 0.05$ (Tukey-Kramer test). Values are means ± S.E (N=3).

3.4.2: Relative water content of Maize plants after growth trial

The RWC of the maize plants was tested when the growth trial was completed and was carried in triplet. The RWC of the control with stress WW was statistically similar to WWI. Whereas WD under drought conditions had a decrease in RWC of 10.25%. While WDI which was also grown under water-deprived conditions only had a decreased of 6.55% compared to well-watered plants (Fig 3.2).

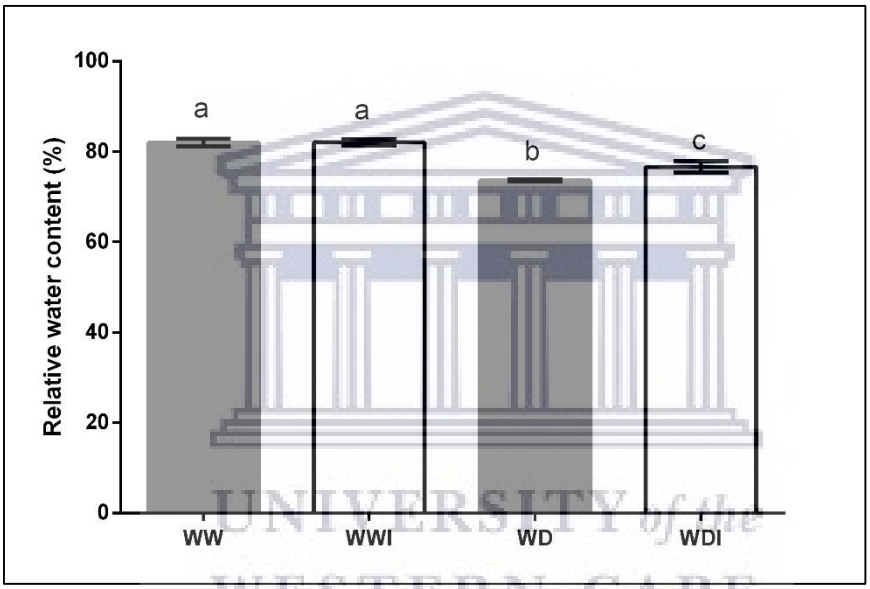

Figure 3.2: RWC of well-watered and water- deprived Maize and the effects of endophytes there on. Control plants which were not under effects of drought stress which were untreated (WW) and treated (WWI). Plants which were under the effects of drought stress which were untreated (WD) and treated (WDI). Values above the columns which have common letters are not significantly different, ($p \leq 0.05$) as determined by $P< 0.05$ (Tukey-Kramer test). Values are means ± S.E (N=3).

3.4.3: Proline content determination of maize samples

The proline content was determine in WW, WWI, WD and WDI in both leaves and roots. An increase in proline content was observed in WWI leaves of 59.56% compared to WW. There was also an increase in WD of 47.10% compared to the well-watered control, while WDI had the highest increase of 86.49% (Fig 3.3A). In the roots proline content of WW and WD was not significantly different. Whereas the inoculated samples showed a notable increase of 288.98% in WWI and 266.113% in WDI compared to WW (Fig 3.3B).

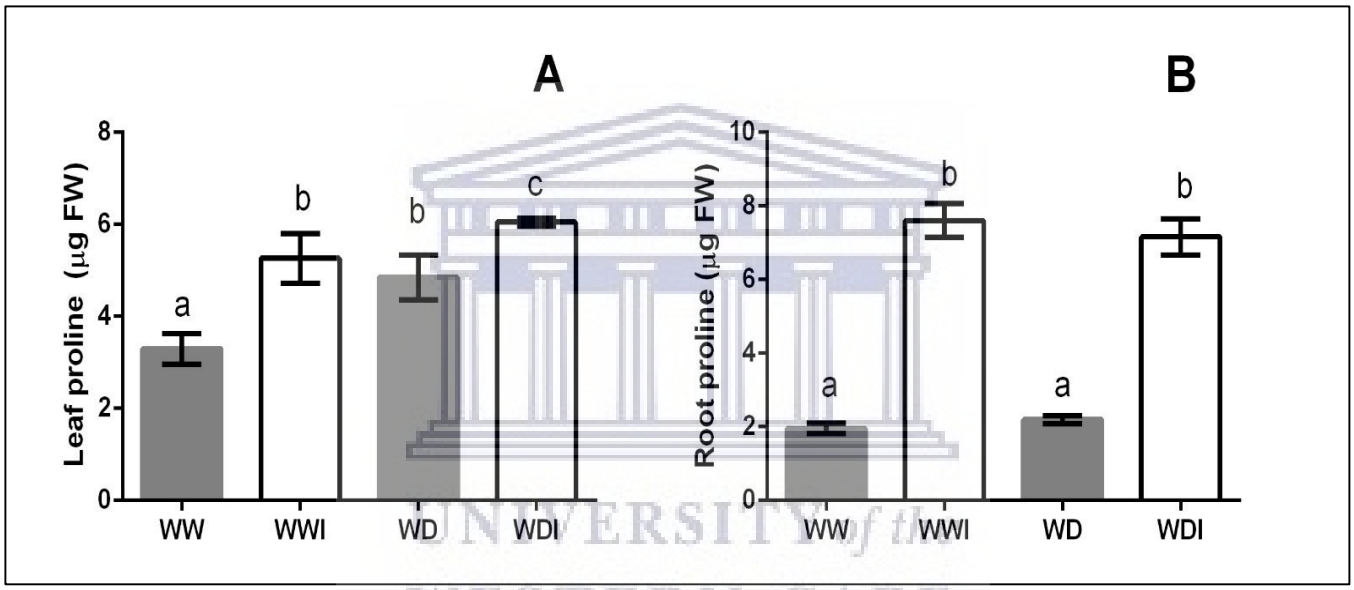

Figure 3.3: Proline of Maize under well- watered water- deprived conditions and the effect of endophytes consortium there on. Control plants which were not under effects of drought stress which were untreated (WW) and treated (WWI). Plants which were under the effects of drought stress which were untreated (WD) and treated (WDI). Values above the columns which have common letters are not significantly different, ($p \leq 0.05$) as determined by $P < 0.05$ (Tukey-Kramer test). Values are means ± S.E (N=3).

3.4.4: ICP analysis of plant material growth trial

The nutrient content in the leaves after the growth trial was analysed through ICP. Ca levels decrease slightly in comparison to the control WW. While K content in WW, WWI and WDI were statistically similar for WD went down by 20.43%. Mg and Cu levels remain constant across the different samples. Zinc levels decreased by 14.71% in WD while the rest did not differ significantly. While P levels decreased in stressed samples by 24.14 % in WD and 26.06 % in WDI respectively. The Mn levels was not significantly different amongst WW, WWI and WDI however, WD decreased by 30.77%. Fe levels increased by 11.54% in WWI 17.86% in WDI, however a decrease was observed in WD of 28.26%.

Table 6: Leaves macro and micronutrient composition

Element	WW	WWI	WD	WDI
Ca	48.93 a	39.89 b	42.56 b	40.03 b
K	1224.97 a	1215.40 a	974.75 b	1296.90a
Mg	26.67 a	24.95 a	22.50 a	24.83 a
Cu	0.26 a	0.21 a	0.23 a	0.24 a
Zn	0.68 a	0.65 a	0.58 b	0.67 a
P	72.84 a	66.30 a	55.25 b	53.86 b
Mn	0.26 a	0.22 a	0.18 b	0.24 a
Fe	0.46 a	0.52 b	0.33 c	0.56 b

Control plants which were not under effects of drought stress which were untreated (WW) and treated (WWI). Plants which were under the effects of drought stress which were untreated (WD) and treated (WDI). Values are means ± S.E (N=3).Values above which have common letters are not significantly different, ($p \leq 0.05$) as determined by $P < 0.05$ (Tukey-Kramer test). Values are means ± S.E (N=3).

The nutrient content in the roots after the growth trial was analysed through ICP. Ca levels increased in inoculated samples by 75.6% in WWI and 75.21% in WDI, while WD was not significantly different from WW the control. An increase in the levels of K was also observed in inoculated samples which increased by 34.87% in WWI and 38.12% in WDI respectively, while WD decreased by 2.04% compared to WW.WW and WD Mg content were not significantly different while inoculated sample increased by 38.75% in WWI and WDI having an increase of 60.88%. The Cu levels increased in WWI by 35% and WDI by 77.5%, while WD decreased by 10% compared to WW.

Zinc levels decreased by 36.04% in WD while inoculated samples WWI increased by 39.64% and WDI increased by 23.42% compared to WW. The P levels in WW were not significantly different from WD whereas endophyte treated plants had an increase of 54.14% in WWI and 61.17% in WDI. Mn increased in WWI by 127.66% and 117.02% in WDI compared to WW, while WW and WD were not significantly different from each other. Fe levels increased by 30.31% in WWI and by 47.35% in WDI, however a decrease was observed in WD of 23.52% compared to WW.

Table 7: Roots macro and micronutrient composition

Element	WW	WWI	WD	WDI
Ca	120.48 a	211.56 b	128.84 a	211.09 b
K	694.04 a	936.04 b	679.85 c	958.59 b
Mg	38.04 a	52.78 b	40.13 a	60.88 c
Cu	0.40 a	0.54 b	0.36 b	0.71 c
Zn	1.11 a	1.55 b	0.71 c	1.37 b
P	63.33 a	97.62 b	63.83 a	102.07 b
Mn	0.47 a	1.07 b	0.57 a	1.02 b
Fe	20.32 a	26.48 b	15.54 c	29.94 b

Control plants which were not under effects of drought stress which were untreated (WW) and treated (WWI). Plants which were under the effects of drought stress which were untreated (WD) and treated (WDI). Values are means ± S.E (N=3).Values above which have common letters are not significantly different, ($p \leq 0.05$) as determined by $P < 0.05$ (Tukey-Kramer test). Values are means ± S.E (N=3)

3.4.5: Cell death in Maize

After cell death was determined it was observed that WW and WWI were statistically similar to each other. Whereas WD the water-deprived samples experienced an increase in cell death of 169.7%. While WDI was observed to be statistically similar to the WW controls (Fig 3.4).

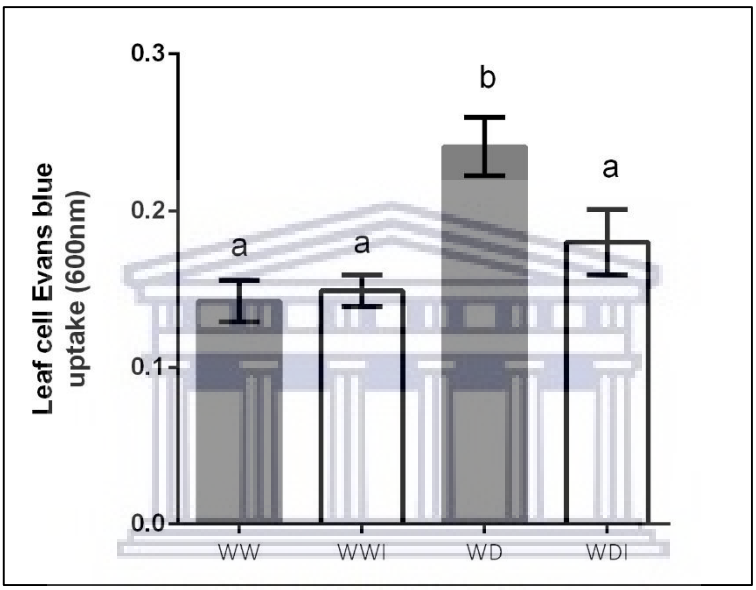

Figure 3.4: The effect of Drought on the cell death of Maize plants and Maize plants treated endophyte consortium. Control plants which were not under effects of drought stress which were untreated (WW) and treated (WWI). Plants which were under the effects of drought stress which were untreated (WD) and treated (WDI). Values above the columns which have common letters are not significantly different, ($p \leq 0.05$) as determined by $P< 0.05$ (Tukey-Kramer test). Values are means ± S.E (N=3).

Discussion

In this study the effect of plant growth promoting bacteria on the physiology and morphology of maize under the influence of drought stress was investigated. To determine the affect PGPBs would have on these plants under the influence of drought stress experiments such as relative water content, cell viability and proline production was carried out. Biochemical assays were also carried out on the endophytes to determine what growth promoting characteristics they possess such as phosphate and zinc solubilization.

The endophytes were extracted from *Medicago lupulina* which is a short-lived perennial herb which a wild type native and which can be found across South America, Europe, Asia and Africa. Whiles it was concluded by Whyte in 1953 that *Medicago lupulina* is not drought resistant Molina in 1970 reported that *Medicago lupulina* is drought resistant in South America. This different in response to drought could be due to the of influence microbial communities which occupy these respective regions. In our study the endophytes were extracted from the *Medicago lupulina* plant which was found in an arid region in the middle of summer which indicated that the plant might have improved drought tolerance and thus being a good sample for testing. The isolates 16S rRNA gene was amplified and sent away for sequencing and analysed to find their identity or closest relation. A number of isolates were shown to be related to documented endophytic bacteria, such as M1 which was found to be closely related to *Bacillus wiedmannii strain FSL W8-0169* and M2 which was closely related to *Bacillus mycoides* strains. *Bacillus* species is a vital resource in the unearthing of novel endophyte strains since it has emerged as an efficient and a safe alternative to crop management practices. Endophytic *Bacillus* species have been shown to provide plants with a number of benefits such as protection from phytopathogens, eliciting resistance and promoting growth (Ongena *et al*., 2008; Pérez-García *et al*., 2011). This relatedness to *Bacillus* species may indicate that these isolates may have other positive characteristics which are associated with this species.

M3 was found to be closely related to the genus of Chryseobacterium which comprises species that are typically yellow rod and nonmotile. Chryseobacterium species are abundant in water, soil and also been isolated from plant rhizospheres. Some of these microbes are involved in

biocontrol and plant growth promotion. The genome analysis also revealed related plant growth promotion such as phosphate solubilization, IAA, siderophore production and environmental stress adaption (Jeong *et al*., 2017).

M6 was revealed to be closely related to *Pantoea agglomerans* which is considered to be ubiquitous in the environment, its most commonly found in soil, water, plants and occasionally humans. Members of this species such as *P. agglomerans CPA-2* has been documented to provide anti-disease properties in plants while *P. agglomerans strain EH31* was shown to produce antibiotics which inhibit the causal agent of fire blight in vitro (Wright *et al*., 2001). While *P. agglomerans NAS206* isolated from wheat played a role in regulation of water content in the rhizosphere of wheat by improving aggregation and stabilization of root-adhering soil (Amella *et al*., 1998) and thereforeM6 might provide similar benefits to plants tested in this study.

The sequencing results of M9 found that it was closely related to *Curtobacterium flaccumfaciens* which has been identified as endophytes in citrus plants. *Curtobacterium flaccumfaciens* endophytes are able to reduce the symptoms of Citrus variegated chlorosis caused by *Xylella fastidiosain* a phytopathogens in citrus plants (Lacava *et al*., 2007). Four of the endophytes did not achieve amplification during PCR progress after a number troubleshooting attempts. This may be due to these bacteria being from a rare biospheres which cannot be identified if the employed primers are not applicable to them. Even though many 16S rRNA genes had been collected in databases, the real bacterial world in environmental samples will still be invisible under the current protocol for 16S rDNA detection. Therefore a primer independent method may be needed to identify these endophytes.

Drought is one of the most serve abiotic stresses and hinders the plant normal growth and development. Maize plants under drought stress showed a signification decrease in shoot length and weight while maize plants under the same water-deprived state but treated with a consortium of bacteria showed no significant differences from the WW and WWI. An increase in root to shoot ratio under drought conditions is normally related to ABA content of roots and shoots (Sharp *et al*., 2002; Manivannan *et al*., 2007)

In terms of root length there were no major deceases observed, however WDI had a slight increase. This result was also observed in a study carried out by Sacks and Colleagues in 1997 where the authors concluded that water deficits in maize and wheat did not result in significant decreases in root length. Whereas in root weight a significant decrease was observed in WD. In studies it has been observed that root dry weight decreases under mild and severe water stress in *Populus* species (Wullschleger *et al*., 2005). While WD roots showed to be suffering under the effects of drought stress, WDI was shown to progress better. The more developed a plant root system the better its ability is to uptake water is and thereby maintain osmotic pressure. Roots also have the critical importance for plants because they are the primary detectors and sensors of drought stress. In a study conducted by Nejad and colleagues in 2010 the authors tested the effects of drought on root development in maize and observed that the roots of maize plants becomes elongated under mild drought stress in order to explore the soil to increase water uptake whereas, under severe drought stress root length is reduced. In another study conducted by Ali and colleagues in 2011 they observed a significant decreases in maize fresh and dry weight under severe drought stress which correlates with the results observed in our study. The requirements of photosynthesis and energy are reduced in leaves due to reduced leaf area by leaf rolling or curling under mild drought stress. Photosynthetic assimilates from leaves are directed toward roots for their elongation to increase the water uptake (Taiz *et al*., 2006).

Several studies have shown the positive effects of endophytic bacteria inoculation in plants, e.g. sugarcane (*Saccharum* spp.) which lead to increased contribution of biological nitrogen fixation to promote root development, increased biomass and productivity (Oliveira *et al*., 2003); soybean [*Glycine max (L.) Merr*], with endophytic bacteria capable of inhibiting the growth and sporulation of pathogenic fungi (Assumpção *et al*., 2009); tomato (*Lycopersicum esculentum L.*), with bacteria increasing plant height, leaf area, leaf number, together with fresh and dry plant weight (Barreti *et al*., 2008).

Relative water contents act as integrative index for estimation of drought tolerance. Under drought stress stomata are closed minimizing transpiration which leads to reduction in CO_2 accumulation which can result in decreased RWC. The relative water contents and water potential is reduced due to increase leaf temperature which reduces transpiration cooling (Siddique *et al*., 2001). It can be easily perceived that plant water status is dependent on stomatal activity (Anjum *et al*., 2011). Despite of being an efficient water user maize is severely affected by drought stress due to hypersensitivity against water deficiency in its developmental stages. This hypersensitivity in its developmental stages starts from germination to harvest maturity and stages including seedling establishment, vegetative growth and development and reproductive growth stages (Parvin *et al* 2015). When the RWC of plant was tested it was observed that there was significant decreased in WD RWC when compare to the controls while WDI showed to have improved RWC compare to WD. This increase in WDI RWC could be due to an increase in proline content which have been well-documented to be osmoregulation during drought (Delauney *et al*., 1993).

Proline is thought to play a cardinal role as an osmoregulatory solute in plants subject to hyperosmotic stresses such as drought. The accumulation of proline may be a general adaption to unfavorable environmental conditions and have been reported to increase in response a number of stresses such as low temperature, nutrient deficiency, heavy metals and high acidity. The accumulation of proline was first observed in wilted plant tissue by Kemble and MacPherson in 1954 on rye grass. The most compelling evidence that proline accumulation is an integral part of plant cell adaption to hyperosmotic stress comes from studies done on osmotolerant bacteria. Proline was shown to be an osmoprotectant in bacteria, when proline was over-expressed in *Escherichia coli* mutants and it clearly exhibited increased osmotolerance (Csonka *et al*., 1989). Increase in proline levels have also been observed in pollen and seeds in order to protect cellular structures during dehydration (Lehmann *et al*., 2010). In our study, bacteria inoculated plants there was a significant increase in proline content compared to the untreated controls. This increase in proline content could explain the increase in RWC for water deprived samples treated with the bacterial consortium. The increase in proline observed could be acting as an

osmoprotectant and increasing osmotolerance. The accumulation of proline allows for osmotic adjustment which results in water retention and avoidance of dehydration (Blume *et al*., 2005).

In order to measure the nutrient content of each plant sample, Inductively Couple Plasma (ICP) spectrometry was performed. Drought stress have been well-document to decrease nutrient uptake (Farooq *et al*., 2009) thus it was important to determine what effect the endophytes would have on nutrient uptake and regulation. A general increase in essential macro and micro nutrients was observed in plants treated with the endophyte consortium. In leave samples, WW and WWI were mostly statistically similar, with the expectation of Ca which decreased and Fe which increased. While in WD decreases were observed in all nutrients with the exception of Cu which remained constant over the treatment period. In root samples, WWI shown an increase in macro and micronutrient when compared to WW. While WD samples had decreases in a number of nutrients but Ca, K, Mg and Mn were similar to WW. WDI shown uptake in macro and micronutrients similar to WWI. These results indicates that the endophytes facilitated nutrient uptake in maize plants. Microbes such as *Bacillus* spp which is known endophyte have the capacity to extract nutrients from other soil microbes by causing nutrient leakage from their cells. This leakage enables them to access nutrients which are contained in the soil microbial community and carry those nutrients back to the host plant (White *et al*., 2019). In addition endophytes have other mechanism to assist the plant in nutrient uptake such solubilization of insoluble nutrients into a soluble form for plants.

One of these nutrients is phosphate which is important for the growth of the plant, however phosphate often found in an insoluble form. A number of endophytes have been shown to facilitate the uptake of phosphate and are known as phosphate solubilizing microorganisms (PSM). PSM are abundant in soils and can be readily isolated from a plant's rhizosphere (Kucey *et al.,* 1983). In this study, the isolates were tested for phosphate solubilization properties on pikovskaya agar plates. A number of isolates were able to produce halo which is an indicator of phosphate solubility. This may account for the increase phosphate in the ICP analysis. There have been a number of studies done which use PSM as inoculants to increase phosphate availability

in soils such as in the study by Illmer and colleagues in 1995, where the inoculum were able to increase phosphate uptake in plants.

As previously stated Fe is an essential nutrient for plant and is required for chlorophyll synthesis and in enzymes for electron transfer. Thus an increase in Fe content will allow plants to absorb more light therefore carry out photosynthesis with higher efficiency. Generally Fe uptake among plants was improved in inoculum treated plants which can enhance biomass due to increased chlorophyll synthesis. This increase in iron content could be due to the production of siderophores. The siderophore producing capabilities of the endophytes were tested on Cas agar plates and most endophytes were able to produce siderophores in our study. The siderophores increases the availability of Fe in soil and allows the plant to increase the uptake of Fe. Aside from increasing Fe uptake, siderophores produced by PGPB also protect the plant from phytopathogens by chelating Fe in the soil surrounding the roots and consequently decreasing its availability to phytopathogens that are dependent on available Fe in soils (Miethke *et al.*, 2007; Rajkumar *et al*., 2010).

Another essential nutrient is Zn which is involved in a number of metabolic activities through inducing the activities of carbonic hydrogenase and anhydrase. Thus, a deficiency in Zn can cause plant growth to be inhibited (Sharma *et al*., 2011). Therefore, it is an essential nutrient in crops and it was upregulated in all endophyte treated plants. This increase in Zn content could be attributed to Zn solubilization activity observed on the plate assays. Indicating that the endophytes were able to enhance Zn uptake through the ability to solubilize Zn. Fungi have been extensively studied for solubilization of insoluble Zn compounds both in vitro and in vivo (Gadd *et al*., 2007; White *et al*., 1997). However, only some bacterial species of the genera such as *Acinetobacter*, *Bacillus*, *Gluconacetobacter*, and *Pseudomonas* have been reported to possess this ability (Simmine *et al*., 1998; Fasim *et al*., 2002; Sachdev *et al*; 2010 and Saravanan *et al*., 2007).

Other notable increases in elements such as Potassium (K), Calcium (Ca) and Manganese (Mn) were observed in our study. Potassium uptake levels in the roots was significantly increased in endophyte treated plants. Potassium is an essential element in a number of important

physiological processes such as maintenance of turgescence, activation of enzymes and photosynthesis. Therefore, a deficiency in K can result in a major reduction in photosynthetic CO_2 fixation and diminish the partitioning and utilization of photosynthates. Potassium also assist plants in biotic and abiotic stress responses. Calcium levels also increased in treated plants and is a multifunctional micronutrient in the makeup of plants. Calcium plays a critical role in the permeability and structure of cell membranes or cell walls. Therefore, a deficiency in Ca cause weakened stem structures. Another nutrient which was significantly increased in the roots of endophyte treated plants was Mn. Manganese is a crucial element in photosynthesis and is used in the control of a number of oxidation-reduction systems. Therefore, decreases in the levels of Mn will lead to lower photosynthesis efficiency, which will cause plant growth to be stunted (Leśniewizs *et al*., 2005).

PGPB have been reported to produce many phytohormones such as auxins, ethylene, gibberellins and cytokinins which can readily stimulate germination, growth and reproduction (Taghavi *et al*., 2009). IAA also contributes to plant growth and the defence system as well as in plant development (Navarro *et al*., 2006). A number of isolates obtained in this study showed to IAA production which can increase growth and root elongation. Xie and colleagues in 1996 evaluated the capacity of IAA producing PGPB to stimulate root elongation in canola seedlings which were under gnotobiotic conditions. The researchers observed that the wild-type *P. putida GR12-2* which produces low levels of IAA (around 2 $\mu g.ml^{-1}$) is able to promote root elongation of up to 3 fold while the mutant *P. putida GR12-2/aux1* which produced high levels of IAA around 8.2 $\mu g.ml^{-1}$ inhibited canola root elongation which may be due to ethylene stress . The researches attributed this inhibition of root elongation by the mutant *P. putida GR12-2/aux1* to the production of ACC by ACC synthase. Since ACC is the precursor in the biosynthesis of ethylene and when ethylene is overproduced it can lead to the inhibition of lateral root growth and root elongation (Mayak *et al*., 2004).

Apoptosis is a fundamental part of plant ontogenesis, it is controlled by cellular oxidative status, phytohormones and DNA methylation. The generation of ROS molecules during drought is a common occurrence and heightens the plants cellular oxidative status. The cell death assay

measures cell viability through the use of Evans blue reagent. Evans blue reagent is absorbed to the cell wall of non-viable cells and level of absorbance can be measured. Maize samples grown under normal well-water conditions (WW and WWI) showed no statistically significant differences. The addition of the endophyte consortium to WWI samples did not result in an increase in cell death which may indicate a symbiotic relationship. While WD plants shown a significant increase in cell death which can be result of oxidative stress from drought conditions. It have been well-documented that during drought ROS is produced, ROS can damage proteins, chlorophyll, lipids , DNA, and other important macromolecules, thereby fatally affecting plant metabolism, growth and yield (Sairam *et al*., 2004).

Researchers have found that overproduction of superoxide anions induced by salt stress lead to programmed cell death (PCD) in primary roots of wheat (Ling *et al.,* 2009). Cell death have also been reported to occur in response to a number of factors such as heat shock (Fan and Xing 2004), leaf senescence (Munne-Bosch *et al*., 2004), and water stress in *Arabidopsis* root tips (Duan *et al.,* 2010). In our study, WDI plants were statistically similar to WW and WWI, which indicates that the bacterial consortium was able to decrease the effects of oxidative stress thereby reducing PCD. This reduction of cell death could be due to the activity of antioxidant enzymes. The increase in antioxidant defense have been reported to contribute towards delaying the start of leaf senescence in wheat (Srivalli *et al*., 2009).

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Chapter 4:

Drought stress induces oxidative stress and reactive oxygen species scavenging pathways in Maize

4.1: Results

4.1.1. Superoxide content

When the leaf superoxide was determined it was observed that WW and WWI were not significantly different. While the water-deprived sample WD experienced an increase of 21.23% compared to WW. The WDI which were grown under the water-deprived conditions was observed to have statistically similar superoxide levels to the well-water samples (Fig 4.1.1).

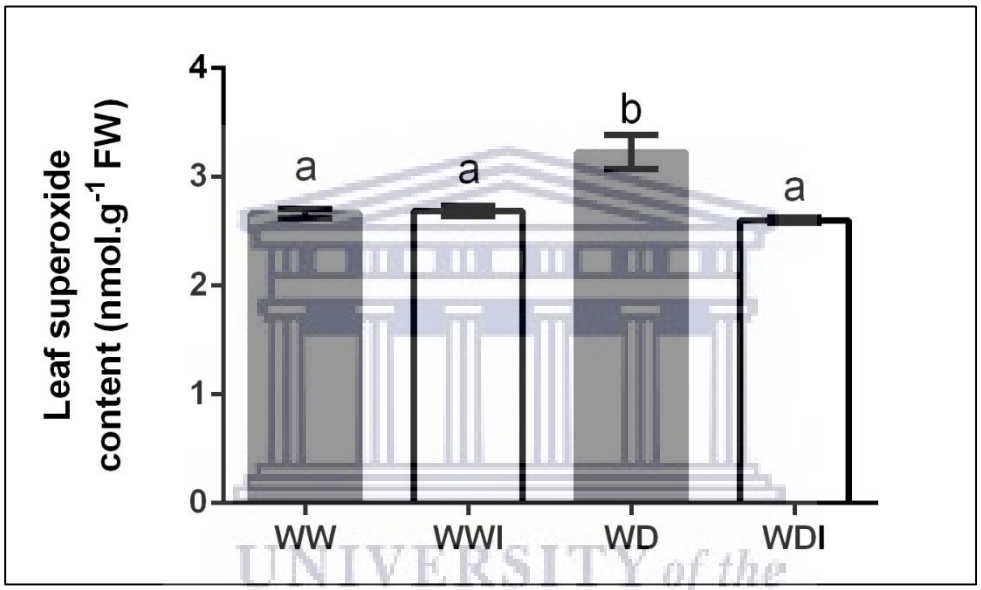

Figure 4.1.1: Superoxide production in Maize plants grown under water- deprived conditions and influence of endophytes there on. Control plants which were not under effects of drought stress which were untreated (WW) and treated (WWI). Plants which were under the effects of drought stress which were untreated (WD) and treated (WDI). Values above the columns which have common letters are not significantly different, ($p \leq 0.05$) as determined by $P< 0.05$ (Tukey-Kramer test). Values are means ± S.E (N=3)

4.1.2. Hydrogen peroxide content determination

In the measurement of hydrogen peroxide production in the leaves WW and WWI were shown not to be significantly different from each other. WD had an increase of hydrogen peroxide production of 42.3%, whereas WDI only had an increase of 15.89% compared to WW (Fig 4.2B). While in roots the WW and WWI hydrogen peroxide production were not significantly different. In the water- deprived conditions there was a notable increase from well- water samples, with WD having an increase of 44.98 % and WDI an increase of 52.12 % (Fig 4.2B).

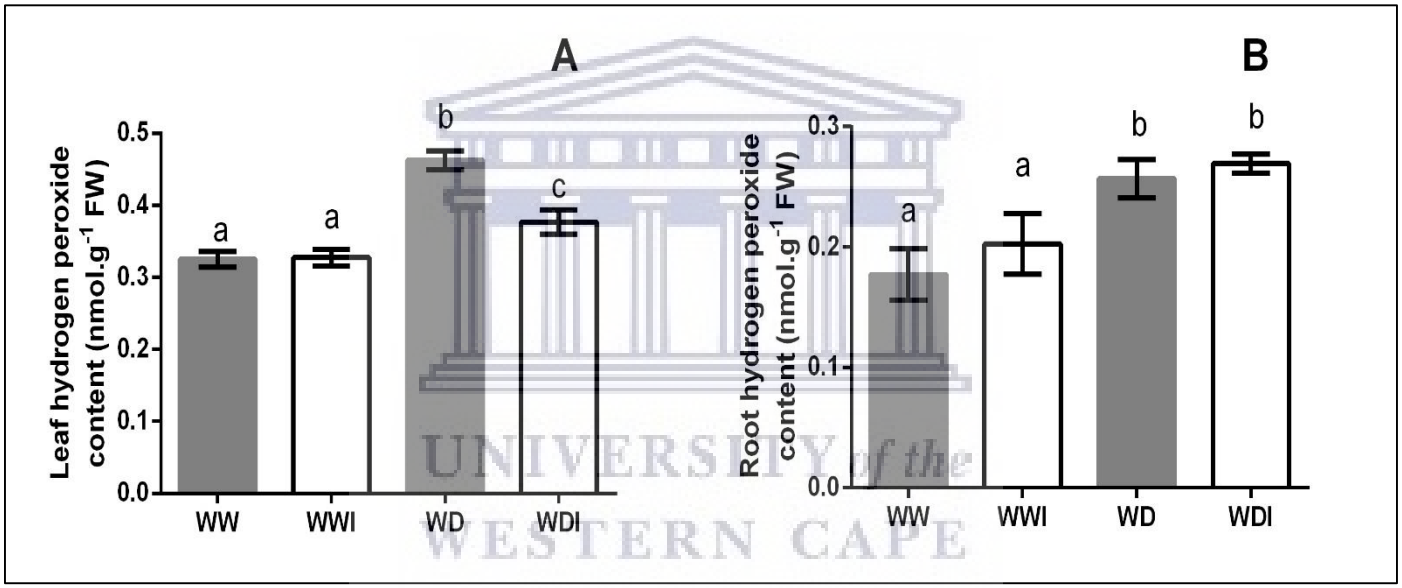

Figure 4.1.2: Hydrogen peroxide production in Maize plants grown under water- deprived conditions and influence of endophytes there on. Control plants which were not under effects of drought stress which were untreated (WW) and treated (WWI). Plants which were under the effects of drought stress which were untreated (WD) and treated (WDI). Values above the columns which have common letters are not significantly different, ($p \leq 0.05$) as determined by $P< 0.05$ (Tukey-Kramer Test). Values are means ± S.E (N=3).

4.1.3: Hydroxyl radical production under water deprived conditions

Hydroxyl radical production in the leaf decreased in WWI compared to WW by 19.85%. While WD which was grown under water-deprived conditions had increase in hydroxyl radical production of 23.15% and WDI had a decrease of 6.13%. The roots WW and WWI hydroxyl radical production was not significantly different. However there was an increase in hydroxyl radical content in water- deprived roots with WD having an increase of 45.31% and WDI having an increase of 54.26%.

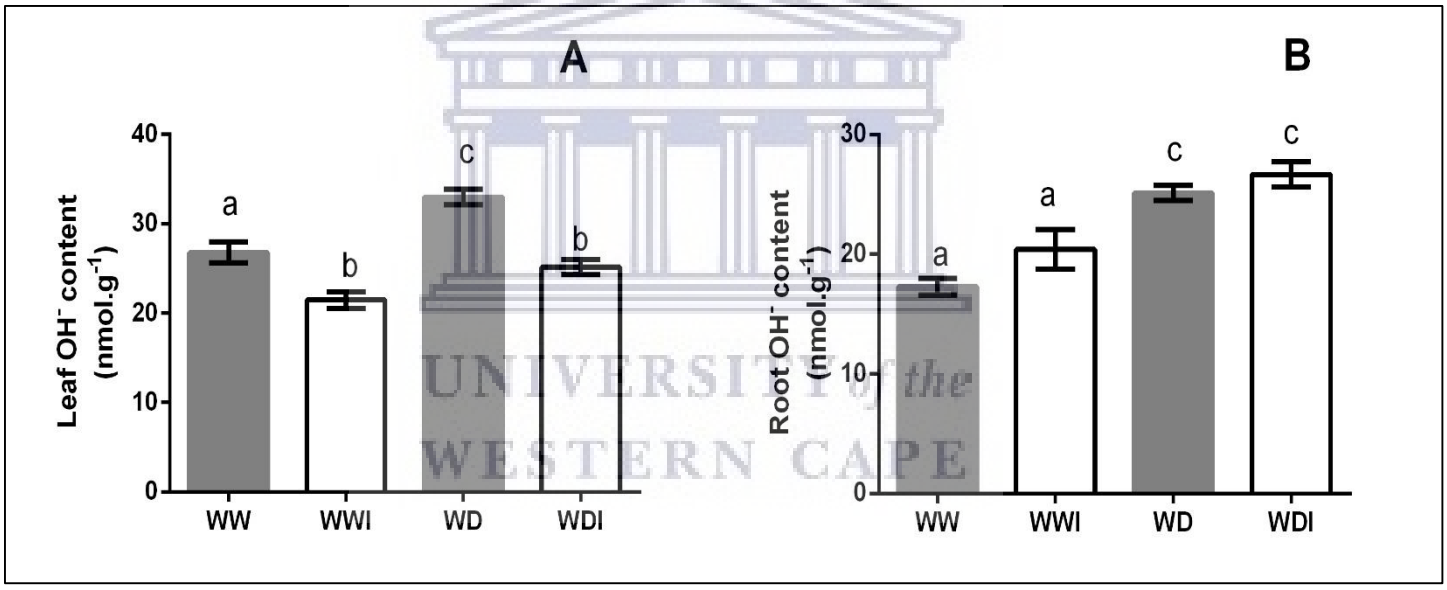

Figure 4.1.3: Hydroxyl radical production in Maize plants grown under water- deprived conditions and influence of endophytes there on Control plants which were not under effects of drought stress which were untreated (WW) and treated (WWI). Plants which were under the effects of drought stress which were untreated (WD) and treated (WDI). Values above the columns which have common letters are not significantly different, ($p \leq 0.05$) as determined by $P < 0.05$ (Tukey-Kramer test). Values are means ± S.E (N=3).

4.1.4. Lipid peroxidation content

The MDA production in the leaf showed that WW and WWI were statistically similar. In the water-deprived leaf MDA levels increased significantly with WD experiencing an increase of 189.92% and WDI having a 69.62% increase compared to WW (Fig 4.1.4A). In the roots WWI had a slight decrease in MDA production compared to WW of 17.17%. While in the water-deprived root samples MDA levels increased with WD having an increase of 51.63% and WDI having an increase of 21.43% compared to WW (Fig 4.1.4B).

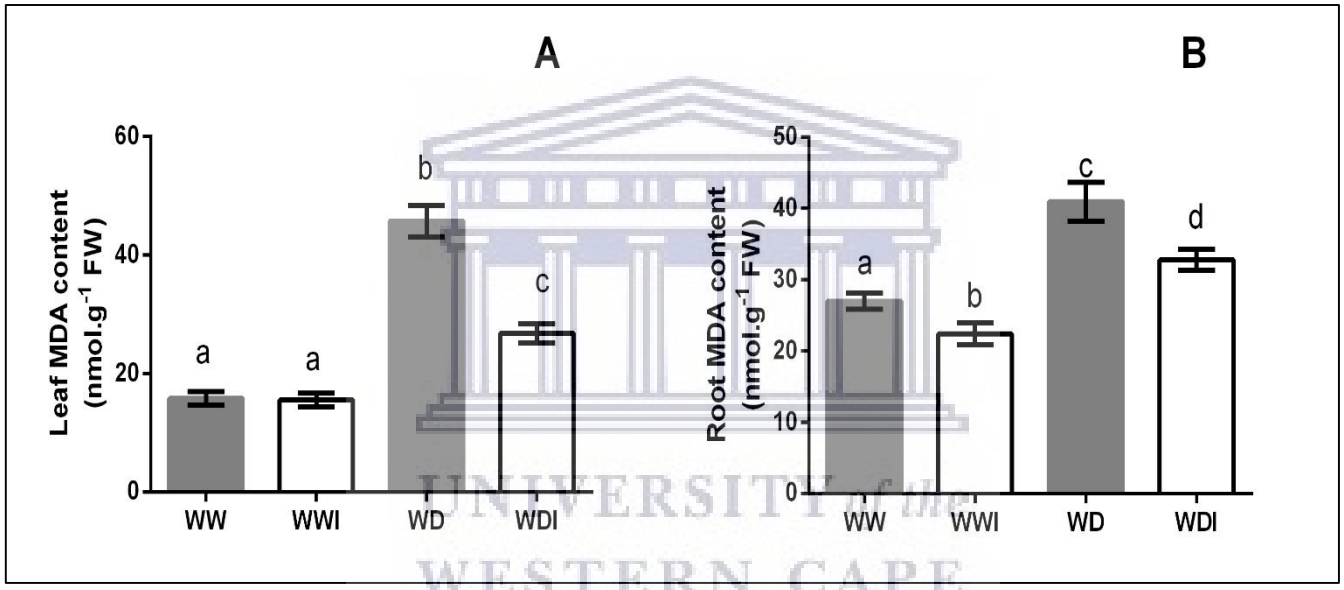

Figure 4.1.4: Lipid peroxidation of maize grown under well- watered and water- deprived conditions and the influence of endophytes there on. Control plants which were not under effects of drought stress which were untreated (WW) and treated (WWI). Plants which were under the effects of drought stress which were untreated (WD) and treated (WDI). Values above the columns which have common letters are not significantly different, ($p \leq 0.05$) as determined by $P < 0.05$ (Tukey-Kramer test). Values are means ± S.E (N=3).

4.2.1: Superoxide dismutase activity under drought stress

Superoxide dismutase activity in WWI had a slight increase of 6.78% compare to WW in the leaves. In the water- deprived sample a notable increase was observed in SOD activity, WD had an increase of 19.93% and WDI having larger increase of 63.07% (Fig 4.2.1A). In the roots WW and WWI had statistically similar SOD activity. However, water-deprived roots had an increase in SOD activity of 9.81% in WD and WDI having an increase of 28.38% compare to WW (Fig 4.2.1B).

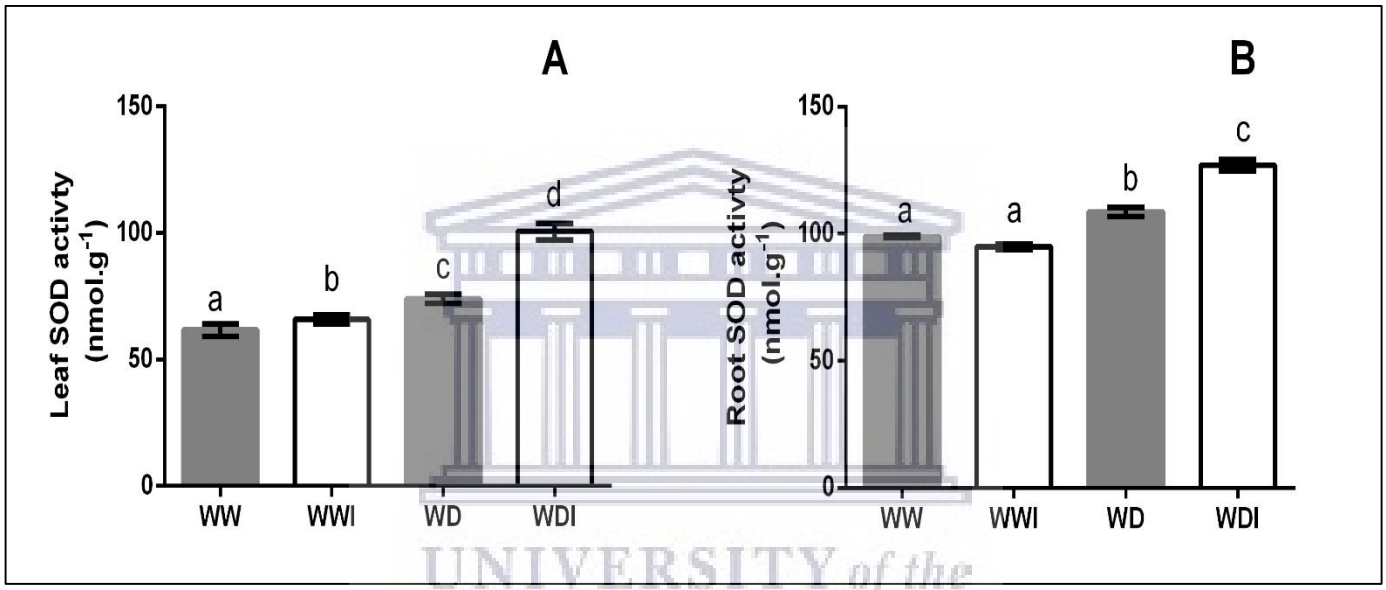

Figure 4.2.1: Superoxide dismutase activity of maize grown under well- watered and water- deprived conditions and the influence of endophytes there on. Control plants which were not under effects of drought stress which were untreated (WW) and treated (WWI). Plants which were under the effects of drought stress which were untreated (WD) and treated (WDI). Values above the columns which have common letters are not significantly different, ($p \leq 0.05$) as determined by $P< 0.05$ (Tukey-Kramer test). Values are means ± S.E (N=3).

4.2.2: Catalase assay under drought stress

The CAT activity assay results in leaves showed that CAT activity in WW and WWI were not significantly different. While WD had an increase of 20.73% and WDI had a much larger increase of 61.46% compared to WW (Fig 4.2.2A). In the roots CAT activity of WW and WWI were also not significantly different. Furthermore, in roots WD had an increase of 60.97% while WDI had increase of 109.16% compared WW control (Fig 4.2.2B).

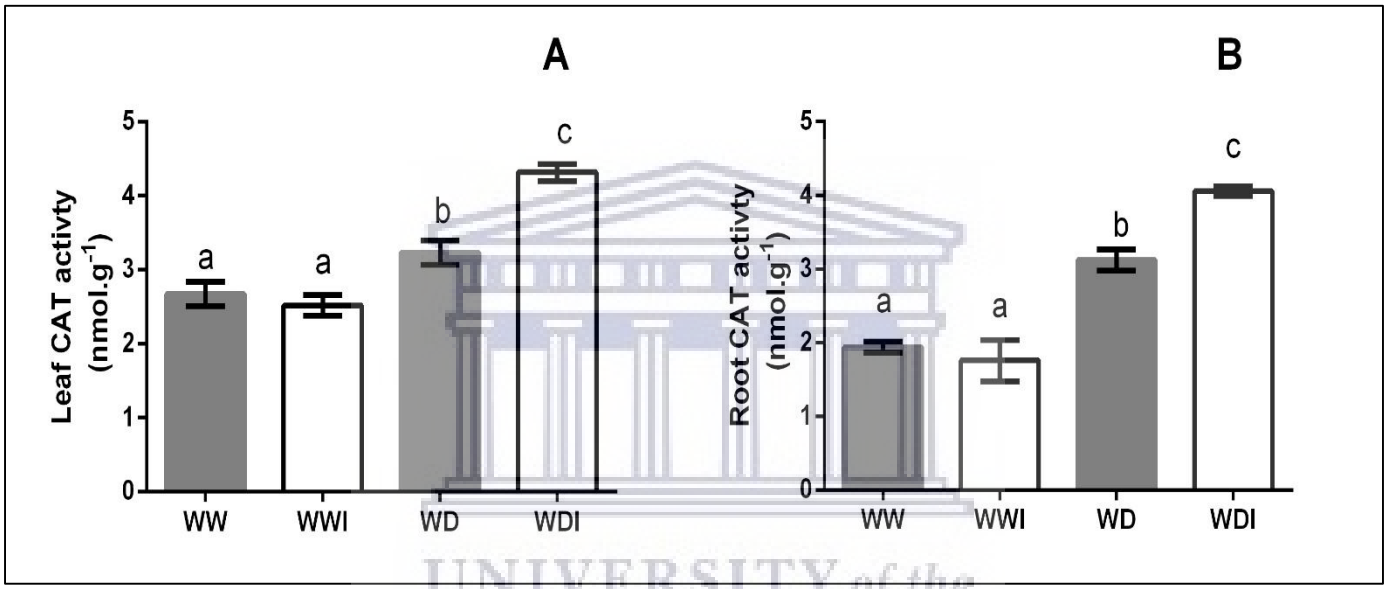

Figure 4.2.2: Catalase activity of maize grown under well- watered and water- deprived conditions and the influence of endophytes there on Control plants which were not under effects of drought stress which were untreated (WW) and treated (WWI). The water-deprived samples were untreated (WD) and treated (WDI). Values above the columns which have common letters are not significantly different, ($p \leq 0.05$) as determined by $P < 0.05$ (Tukey-Kramer test). Values are means ± S.E (N=3).

4.2.3: Ascorbate peroxidase activity in Maize drought condition

The APX activity in leaves showed that WW and WWI was statistically similar, while an increase in APX activity was observed in water-deprived sample in the leaves. WD had an increase of 59.79% and WDI had an increase of 101.88% compare to WW (Fig 4.2.3A). In the roots WWI had an increase of 37.11% of WWI in APX activity. There was also an increase in APX activity in water-deprived samples with WD having an increase of 73.08% and WDI having an increase of 104% (Fig 4.2.3B).

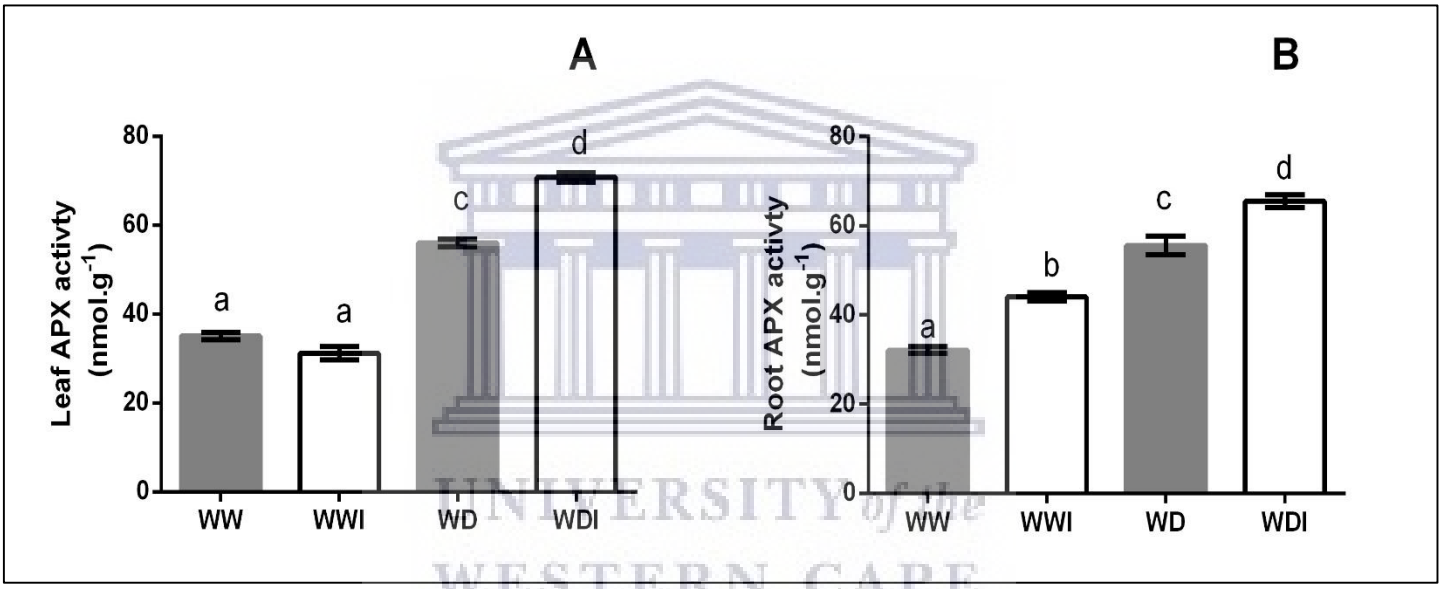

Figure 4.2.3: Adsorbate peroxidase activity of maize grown under well- watered and water- deprived conditions and the influence of endophytes there on. Control plants which were not under effects of drought stress which were untreated (WW) and treated (WWI). Plants which were under the effects of drought stress which were untreated (WD) and treated (WDI). Values above the columns which have common letters are not significantly different, ($p \leq 0.05$) as determined by $P< 0.05$ (Tukey-Kramer test). Values are means ± S.E (N=3).

Discussion

Environmental stresses prompts the formation of reactive oxygen species in plant cells which include superoxide, hydroperoxyl radicals, hydrogen peroxide and hydroxyl radical (Smirnoff *et al*., 1993). These ROS produced within plant cells and tissues can cause oxidative damage to plant proteins, nucleic acids, and membranes unlike biotic stress where biotic burst is part of a defense response that frequently triggers programmed cell death (Mittler *et al*., 2002). Besides providing growth promotion in plants, PGPB may also be able to provide oxidative stress elevation during drought.

Superoxide is produced in both stressed and unstressed cells under normal conditions. Plants have developed defence system against ROS acclamation, which involves limiting its production as well as its removal. Under normal condition the production and removal of superoxide are in balance, however under stressed conditions the production of superoxide far exceeds its removal (Alshcer *et al*., 1993). Superoxide content in WW and WWI were statistically similar which indicates that endophytes did not induce an increase in superoxide response. While the stressed plants WD had a significant increase in superoxide content which was to be expect from a plant during oxidative stress. Whereas WDI superoxide content was statistically similar to the controls which indicates that the endophyte consortium was able to mediate the removal of superoxide content. This might be due to the endophytes increasing the activity of antioxidant enzymes such as SOD.

In addition to being toxic to the plant hydrogen peroxide is also regarded as a signalling molecule and regulator of expression of some genes. When the hydrogen peroxide content was tested, there was a significant decrease in hydrogen peroxide production in the treated water-deprived compared to untreated water-deprived. While hydroxyl radical which is the most potent ROS however possess one of the shortest half-lives this same trend was observed of decreased ROS molecules in endophyte treated plant under drought stress. This indicates that endophytes may be triggering an antioxidant response in the plant. In similar studies, when exposing plants to stress, mock uninoculated plant tissues lost their greenness indicating increased ROS activity while the inoculated tissues remained green. They concluded that endophytes may be assisting

the plants to cope with drought stress by either efficiently scavenging ROS or preventing ROS production under drought stress (Creus *et al.*, 2004; Kohler *et al.*, 2008).

In another similar study they analysed q-PCR data and it showed that bacteria at the early stages of colonization caused upregulated transcript levels of ROS-degrading genes such as superoxide dismutase. The upregulation of these genes may also reduce oxidative damage to plants by pathogens. *Piriformospora indica* which is an endophytic fungus has been shown to induce abiotic stress tolerance in many plants. *P. indica* originally isolated from a desert has shown to confer drought tolerance to Arabidopsis by priming early and high expression of drought stress-related genes was observed. Endophytes in this study may be using a similar mechanism to mediated drought stress in the maize leaves (White *et al.*, 2019).

However when observing the hydrogen peroxide and hydroxyl radical content in the roots it appeared that the consortium treated plants had statistically similar concentrations to the untreated plants. This could be due a number of factors such as endophytes not being able to initiate a high enough antioxidant response to reduces ROS levels in the roots. However, it appears superficially that it did not result in root development being stunted but the damage can only be analysed by looking at the MDA content. This high levels of hydrogen peroxidase and hydroxyl radical in roots could also play a role in signaling. Even though many studies have looked into signaling capabilities of hydrogen peroxide not much is known about hydroxyl radical signaling. Hydroxyl radicals has been found to play fundamental and positive roles in development and adaptation. Mittler and Berkowitz in 2001 took a look beyond hydrogen peroxide signaling and found that hydroxyl radicals is a potent effector in calcium homeostasis (Demidchik *et al.*, 2003; Foreman *et al.*,2003; Zepeda-Jazo *et al.*, 2011; Laohavisit *et al.*, 2012) and stress signaling (Chung *et al.*, 2008; Laohavisit *et al.*, 2013). Hydroxyl radical acts positively in reproduction, germination, and growth (Schopfer *et al.*, 2002; Müller *et al.*, 2009; Duan *et al.*, 2014; Smirnova *et al.*, 2014), whilst also playing a part in cell death (Demidchik *et al.*, 2010). Thus, hydroxyl radicals in our study may play a signaling role in the roots.

Oxidative stress induced by drought often lead to the lipid membrane peroxidation. The MDA content is often used as an indicator of lipid peroxidation in plant tissue, resulting from oxidative stresses. MDA is one of the most well-known byproducts of lipid peroxidation, thus it is commonly used to measure cell damage. In the leaves the MDA content between WW and WWI was not significantly different. While WD had a significant increased indicating that the plant was experiencing lipid peroxidation and WDI which was treated with endophytes had a decrease in MDA production compare to WD. This same trend of increase tolerance was also observed in roots.

Even though the hydroxyl radical and hydrogen peroxide content of WDI roots were similar to WD it did not result in a similar MDA content response. This could be due to high levels of proline which was observed in inoculated plants. The accumulation of proline can reduce membrane damage and reduce MDA levels when the plant is tolerating drought stress. Proline is involve in numerous physiological functions and is synthesized when plants experienced stress. Proline is involved in membrane peroxidation prevention associated group as osmotic adjustment (Ashraf *et al*., 2007), osmoprotection (Kishor *et al*., 1995; Okuma *et al*., 2002), and protection of macromolecules from denaturation (Okuma *et al*., 2000), free radical scavenger activity (Shao *et al*., 2008), and inhibition of programmed cell death (Sivakuma *et al*., 2000). The accumulation of proline and contribution to avert lipid peroxidation varies among species (Lemos *et al*., 2011). This mediation of lipid peroxide in our study may due the elevated proline levels

Plants have developed complex sets of responses to the inevitable accumulation of ROS molecules which are known to lead to lipid membrane peroxidation and cell death. In order to protect plants from these toxic oxygen intermediates plant cells and its organelles employ antioxidant defense systems. There have been a great deal of research to understand cellular antioxidant machinery and the importance for protection against various stresses (Tuteja *et al*., 2007; Khan *et al*., 2008; Singh *et al*., 2008)

Superoxide dismutases are metallo-proteins which catalyze the dismutation of the superoxide radical to molecular oxygen and hydrogen peroxide. Since superoxide is one of the first ROS molecules produced during oxidative stress SOD is regarded as the first line of defense. When SOD activity was measured in the leaves of WWI we observed a slight increase over WW. While in the root WW and WWI were statistically similar. There was a significant increase in SOD activity in stressed plants which was expected since the plant are trying to regulate the superoxide radicals. However WDI showed the highest SOD activity in both leaves and roots. This increase in SOD activity in WDI could be the reason there was significant decreases in superoxide content in the WDI samples. In a similar study done on endophytic bacterium *Enterobacter sp. 638* which was isolated from stems of poplar trees was shown to have genes that encode for several superoxide dismutases including *SOD A*, *SOD B* and *SOD C.* This isolate was also shown to possess genes for catalases, hydroperoxide reductases, hydroperoxide reductases and thiol peroxidase, which were reported to aid in stress elevation. Bacterial SODs have an important role in their survival in the rhizosphere as it facilitates the removal of free radicals (Wang *et al*., 2007). These Bacterial SODs may have the ability to remove the free radicals produced during abiotic stress conditions in both the rhizosphere and inside plant tissues.

Another important antioxidant enzyme is CAT which converts hydrogen peroxide into water in the peroxisomes. Hydrogen peroxide is produced from β-oxidation of fatty acids and photorespiration as well as by SOD enzymatic activity. CAT activity is decrease by high levels of CO_2, the levels of CO_2 decreases under drought stress when the stomata closes. The stomata closes to reduce loss of water however it also result in limited CO_2 uptake thus activing CAT. In this study we observed that there was significant increases in CAT activity in water-deprived samples however WDI had the highest activity. This increase in CAT activity in the treated samples could explain the decrease in hydrogen peroxide levels in the leaves. However, the main hydrogen peroxide detoxification enzyme is APX (Asada *et al*., 1992). APX uses AsA as a specific electron donor in order to reduce hydrogen peroxide to water. APX also plays a role in ROS scavenging in cytosol, mitochondria and peroxisomes (Asada *et al*., 1999; Mittler *et al.*, 2004; Noctor and Foyer, 1998; Shigeoka *et al.*, 2002). APX activity generally increases along with

other ROS scavenging enzymes activities such as CAT and SOD, which was observed in this study. The APX activity also had a significant increase in the WDI samples which assisted in alleviating leaf hydrogen peroxide.

In study carried out by Gururani and colleagues in 2012 they observed that mRNA expression of SOD and APX in endophyte treated plants grown under stressed conditions increased significantly when compared with that in the untreated stressed plants. They also observed an increase in mRNA expression levels of genes encoding for other anti-oxidative enzymes such as CAT, dehydroascorbate reductase and Glutathione reductase also increased in the bacteria treated plants. These result was supported by semi-quantitative RT-PCR results where the expression of the anti-oxidative pathway genes was more evident in the inoculated plants than in the uninoculated plants. In the end they concluded that the inoculations with rhizobacteria strains protected *S. tuberosum* against abiotic stresses such as drought, salinity and heavy-metal toxicity. The increased plant tolerance to these stresses correlated with the increased expression of APX, SOD, CAT, DHAR, and GR, proposing that theses bacteria triggered abiotic stress related defense pathways in plants under stressed conditions. This observation was confirmed by looking at relative mRNA expression levels as determined by RT-PCR and real-time PCR.

In a similar study carried out on Chinese cabbage (*Brassica rapa*) treated with *P. indica* and treated with polyethylene glycol to mimic drought stress, *P. indica* treated plants exhibited upregulation of antioxidant enzymes such as peroxidases, catalases, and superoxide dismutases in leaves within 24 hours. Also the expression of drought tolerance genes *DREB2A*, *CBL1*, *RD29A* and *ANAC072* were upregulated in leaves of *P. indica* treated plants (Sun *et al*., 2010). The endophytes in our study may be triggering a similar transcriptional response to drought stress however this can only be determined by further investigation.

Conclusion and future work

This study it was observed that maize plant treated with the endophytic consortium had improved drought tolerance. The endophytes were extracted from *Medicago lupulina* which showed signs of drought tolerance and thereby making it a suitable plant to find endophytes for this study. A number of isolates were extracted from the host plant however only 9 isolates were selected for use in the consortium. These 9 endophytes were selected based on growth promotion characteristics which were tested on plate and biochemical assays. These biochemical assays included test for phosphate solubility, zinc solubility, IAA and siderophore production.

These endophytes were then sequenced to determine their identity or their closes relation. While some endophytes were able to be sequenced even though they had low coverage others were not able to achieve amplification. This lack of amplification may be due to endophytes requiring a primer independent sequencing method. Results indicated that endophyte treated water-deprived plants had generally improvement in shoot and root weight compared to untreated water-deprived. This indicates that the endophytes were able to recuperate some losses in biomass while the plant remained under stress. This improved biomass also resulted in improved nutrient profiles in the endophyte treated plants which is important as maize is a vital food for many people. This improved nutrient profile was observed in the ICP-OES experiment where a number of essential nutrients increased in treated samples. The plant ability to take up nutrients is dependent on the amount of water in the root as it help move nutrient from soil to the plants stems and leaves. The relative water content of water-deprived samples decreased however endophyte treated water-deprived plant had improved over the untreated water-deprived plants, and this may be due proline regulation. Plants treated with endophytes had increased production in proline, and increased proline production have been well documented to provide a number of positive characteristics to plants under drought stress. Increase cell death is one of the first signs of a plant not being able to overcome stress such as drought. The endophytes were also able to reduced cell death in water-deprived plants indicating that the microbes were able to lessen the effects of drought stress.

The production of ROS molecules have been well documented to increase during drought and leads to lipid peroxidation. It was observed that the ROS molecules had a significant increase in the WD samples for both leaves and roots, while in WDI these increase levels were significantly reduced. ROS production in roots of water-deprived samples, showed that WD and WDI were statistically similar. Lipid peroxidation was determined by calculating MDA content as it is a byproduct of lipid peroxidation. The MDA content was observed to decrease in endophyte treated water-deprived samples compared to untreated water-deprived samples. Indicating that endophytes was able to reduce the effects of lipid peroxidation, which is one of the most damaging effects of drought stress.

The plant have evolved to counteract the harmful effect of elevated ROS production through a number of mechanisms such as the use of antioxidant enzymes. A significant increase in activity was observed in CAT, APX and SOD in endophyte treated samples. This increased antioxidant activity could account for the decrease in ROS production observed in leaves however this increased activity in roots did not result in reduced ROS production. While WDI roots had similar ROS production compared to WD roots, WDI did not have similar levels of lipid peroxidation. WDI roots therefore may rely on alternative methods to reduce lipid peroxidation such as the production of proline. Aside from prolines osmotic regulatory capabilities it has also been documented to reduce the effects of lipid peroxidation. There might still be other means by which endophytes might be reducing lipid peroxidation and thus require further study.

This study has shown that treatment of plants with endophytic bacteria is a feasible alternative to genetic manipulation in order to enhance crop plants to drought stress. However, in order for PGPB to become a viable option more research in this field needs to be carried out.

Future work includes the use of alternative and more accurate DNA analyse tools to successfully identify endophytes. While some endophytes were sequenced there were errors observed in the sequenced which require further analysing. Other tests will also be carried out on plant material such as in gel assays to observe the different antioxidant isoforms produced during drought stress. Once these isoforms are isolated they can be analysed using proteomics analysing tools.

Field trials could also be carried out to determine whether endophyte consortium will have a similar effect on plants shown in field conditions. In a field trial plants are exposed to more variables such as the presence of the soil native rhizospheric bacteria and it is unknown what type of interaction will occur. The rhizospheric bacteria may out compete the endophyte consortium thereby inhibiting its growth or may not have any influence on the consortium. Future work will also include studying the movement of the endophytes after treatment. In order to study the translocation of endophytes they will be injected with carbon or gold nanoparticles which will allow for the tracking of endophytes throughout their life cycles of the plant. These experiments will give more insight into relationship between endophytes and host.

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