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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 therefore M6 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 severe abiotic stresses and hinders the plant normal growth and development. Maize plants under drought stress showed a significant 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 decreases 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<sub>2</sub> 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<sub>2</sub> 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 µg.ml<sup>-1</sup>) 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 µg.ml<sup>-1</sup> 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).

## Chapter 4:

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



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## 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).

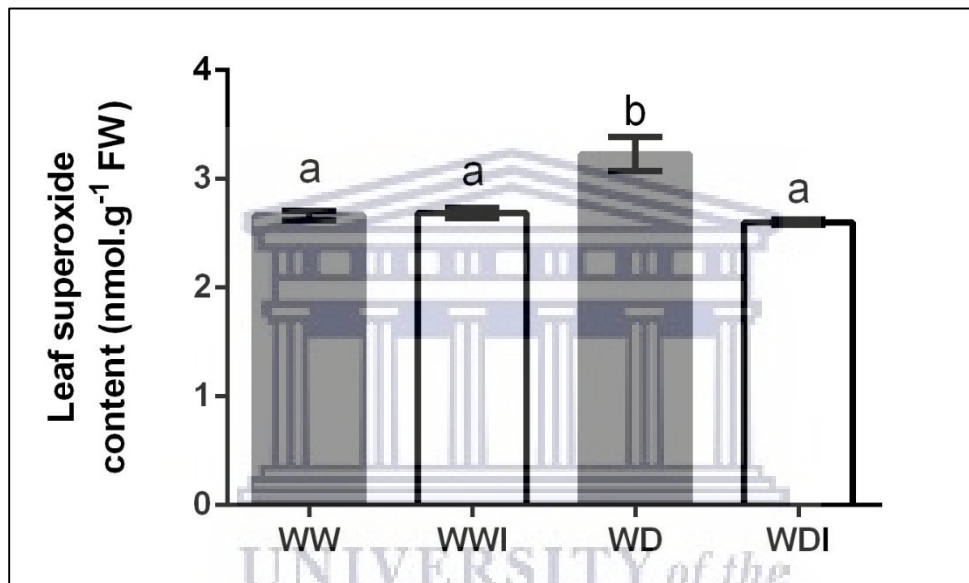


Figure 4.1.1: Superoxide production in Maize plants grown under water- deprived conditions and influence of endophytes there on. Control plants which were not under effects of drought stress which were untreated (WW) and treated (WWI). Plants which were under the effects of drought stress which were 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  $\pm$  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).

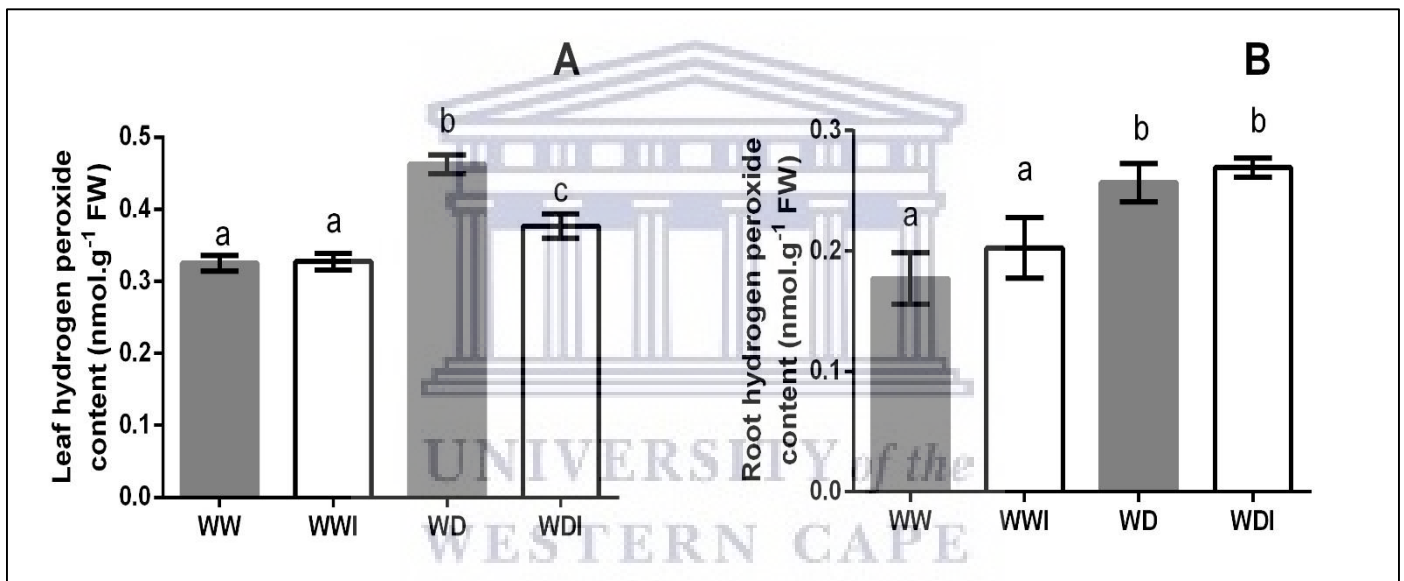


Figure 4.1.2: Hydrogen peroxide production in Maize plants grown under water- deprived conditions and influence of endophytes there on. Control plants which were not under effects of drought stress which were untreated (WW) and treated (WWI). Plants which were under the effects of drought stress which 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  $\pm$  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%.

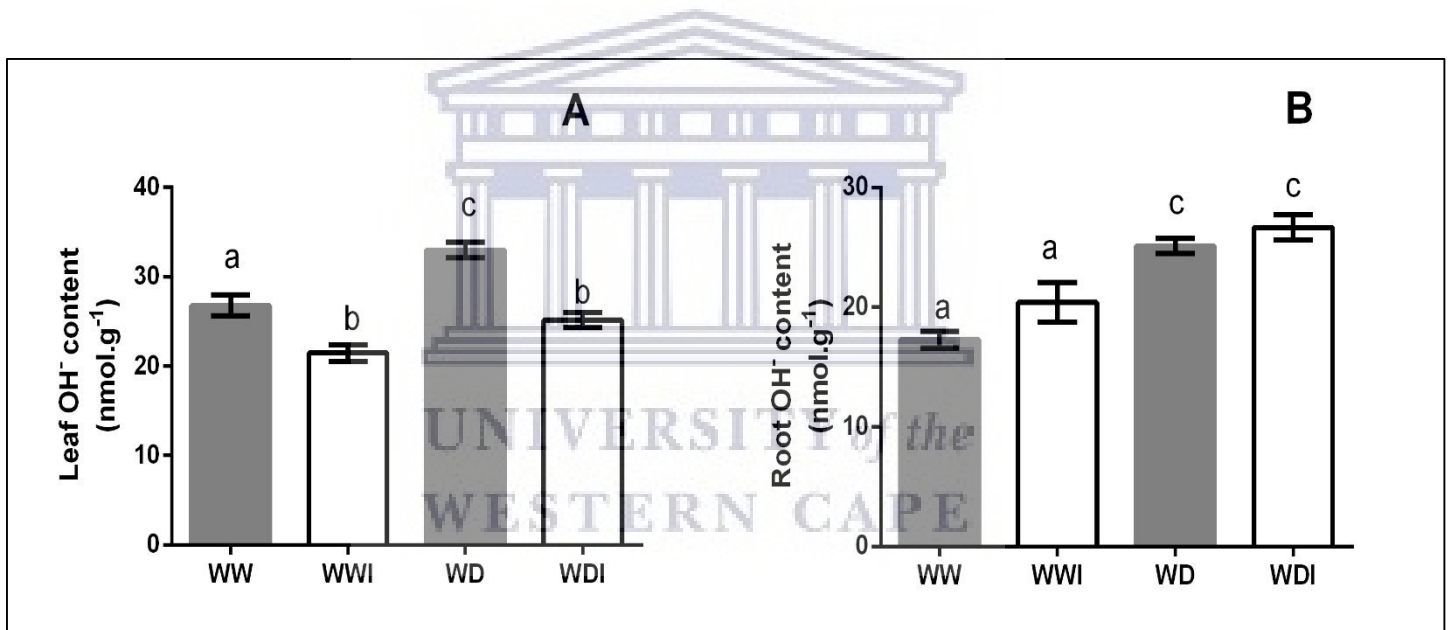


Figure 4.1.3: Hydroxyl radical production in Maize plants grown under water- deprived conditions and influence of endophytes there on Control plants which were not under effects of drought stress which were untreated (WW) and treated (WWI). Plants which were under the effects of drought stress which 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  $\pm$  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).

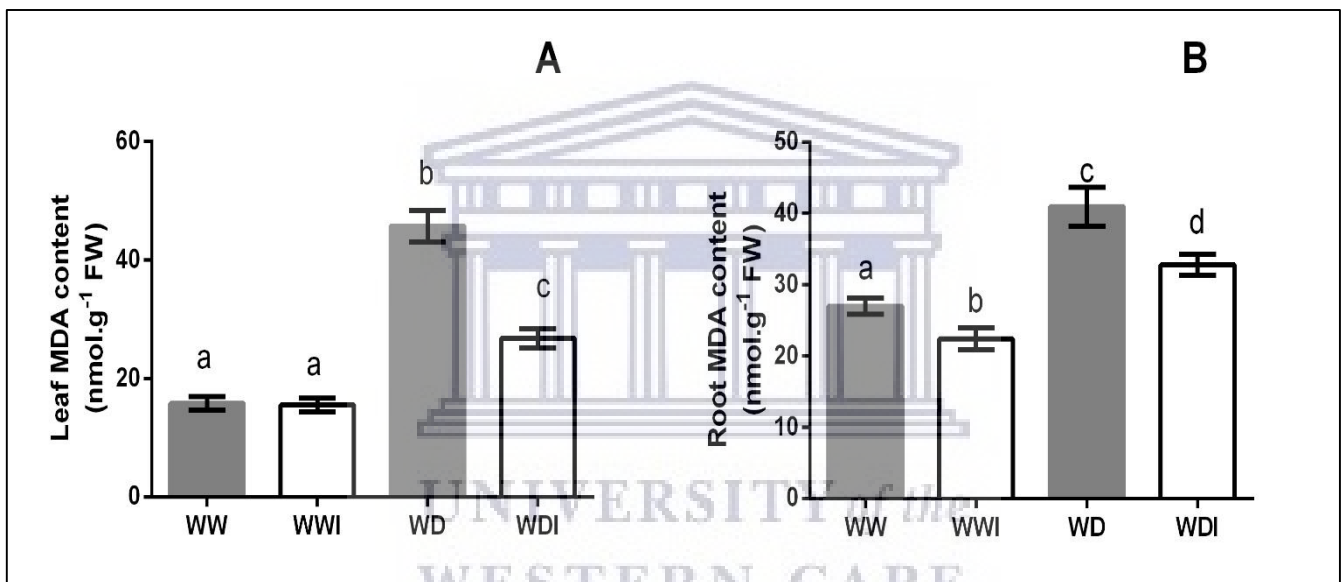


Figure 4.1.4: Lipid peroxidation of maize grown under well-watered and water-deprived conditions and the influence of endophytes there on. Control plants which were not under effects of drought stress which were untreated (WW) and treated (WWI). Plants which were under the effects of drought stress which 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  $\pm$  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).

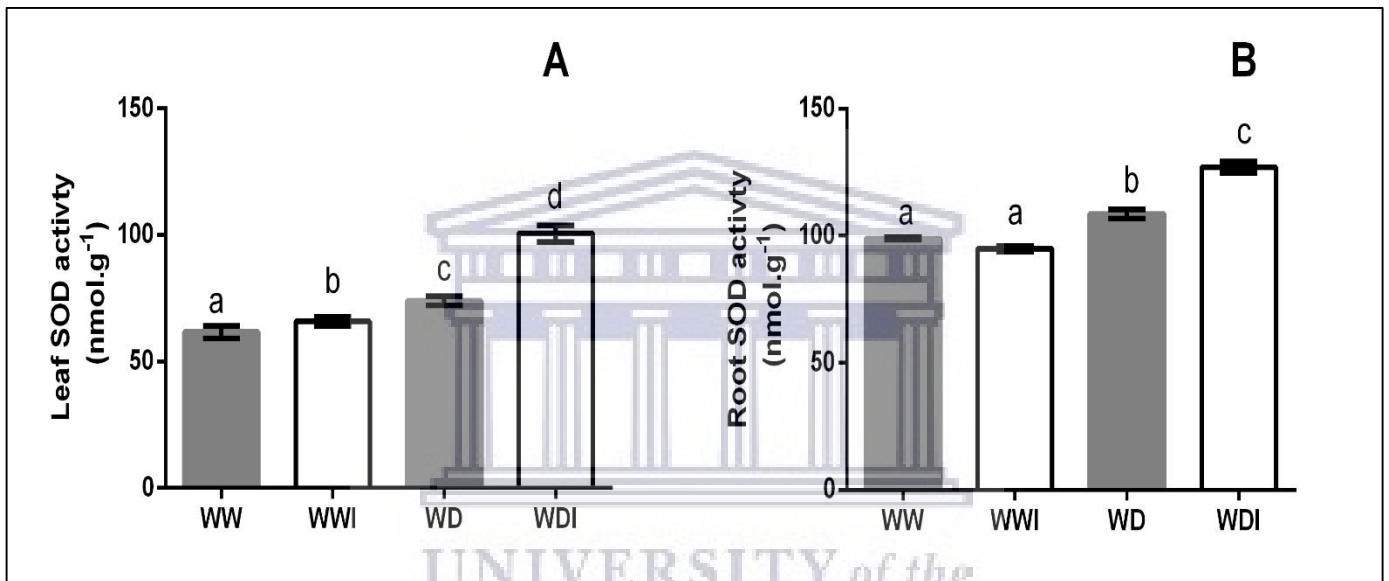


Figure 4.2.1: Superoxide dismutase activity of maize grown under well- watered and water- deprived conditions and the influence of endophytes there on. Control plants which were not under effects of drought stress which were untreated (WW) and treated (WWI). Plants which were under the effects of drought 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  $\pm$  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).

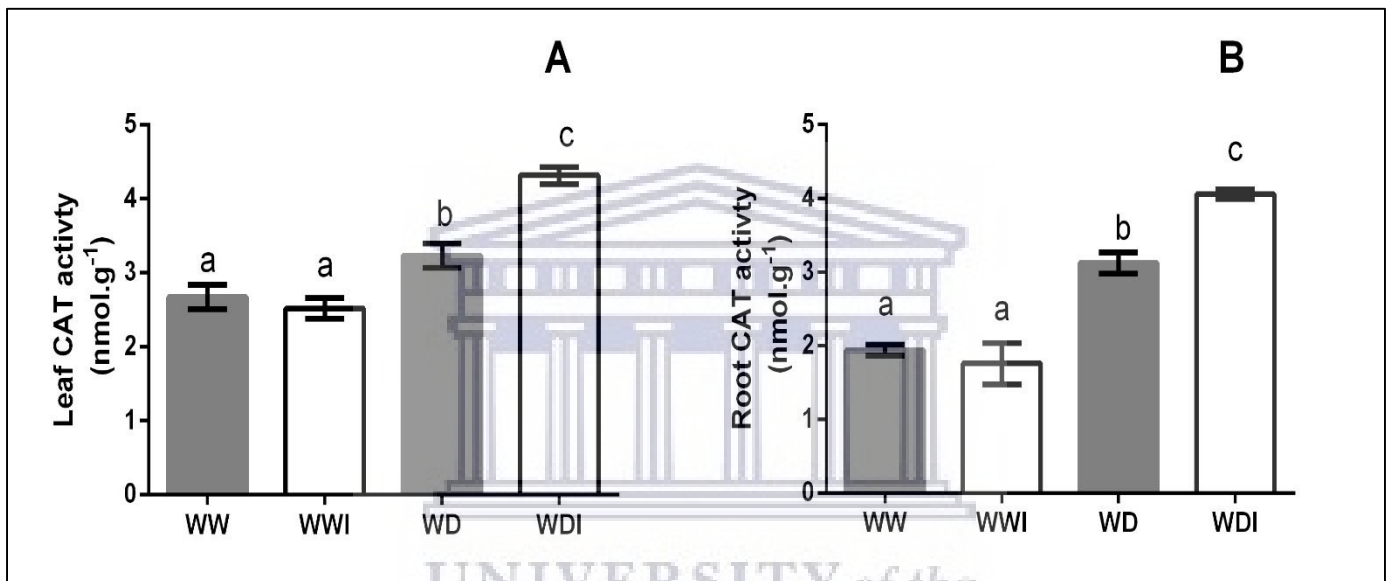


Figure 4.2.2: Catalase activity of maize grown under well- watered and water- deprived conditions and the influence of endophytes there on Control plants which were not under effects of drought stress which were untreated (WW) and treated (WWI). The water-deprived samples were untreated (WD) and treated (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  $\pm$  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).

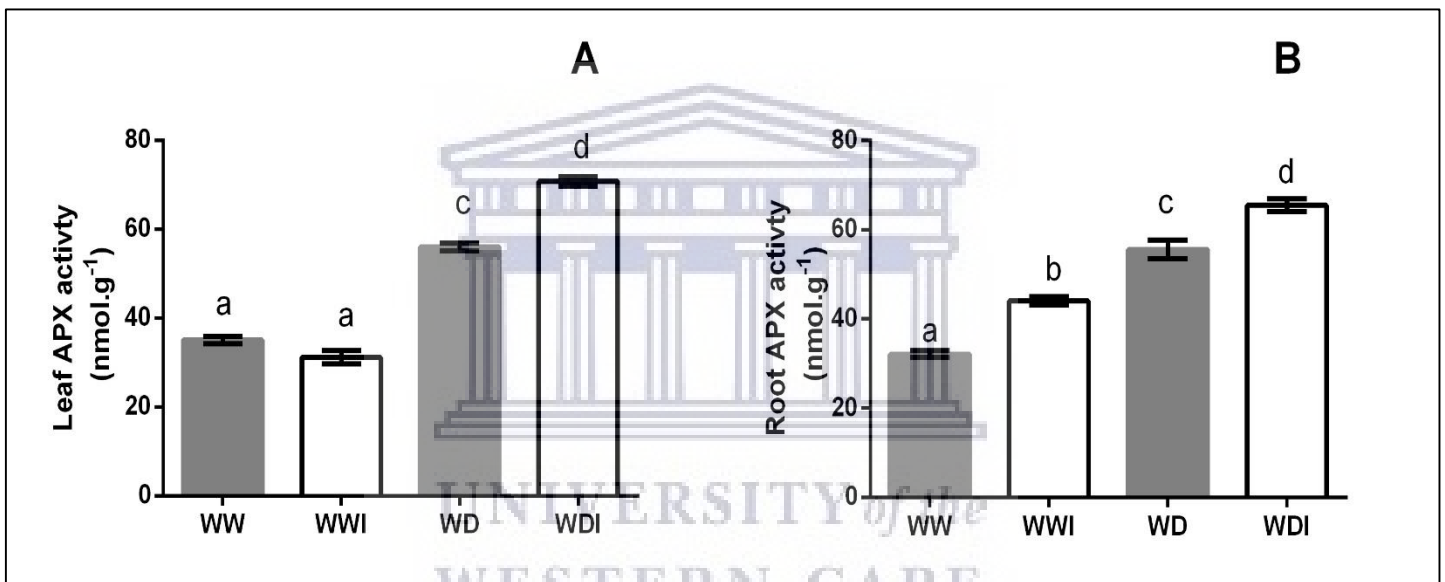


Figure 4.2.3: Adsorbate peroxidase activity of maize grown under well- watered and water- deprived conditions and the influence of endophytes there on. Control plants which were not under effects of drought stress which were untreated (WW) and treated (WWI). Plants which were under the effects of drought 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  $\pm$  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 acclimation, 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  $\beta$ -oxidation of fatty acids and photorespiration as well as by SOD enzymatic activity. CAT activity is decrease by high levels of  $\text{CO}_2$ , the levels of  $\text{CO}_2$  decreases under drought stress when the stomata closes. The stomata closes to reduce loss of water however it also result in limited  $\text{CO}_2$  uptake thus activating 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 close 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.

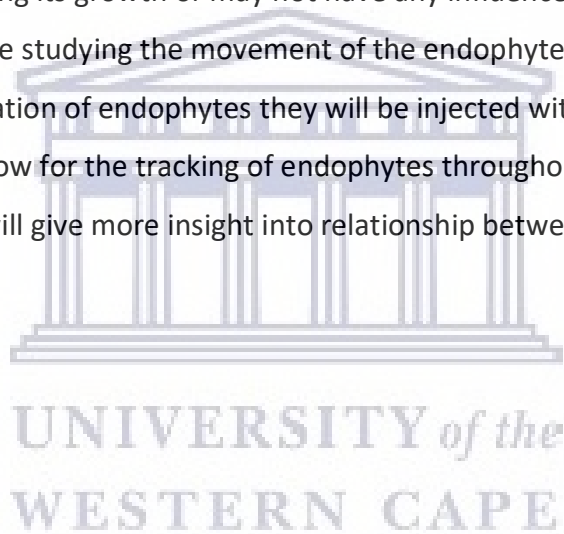
The production of ROS molecules have been well documented to increase during drought and leads to lipid peroxidation. It was observed that the ROS molecules had a significant increase in the WD samples for both leaves and roots, while in WDI these increase levels were significantly reduced. ROS production 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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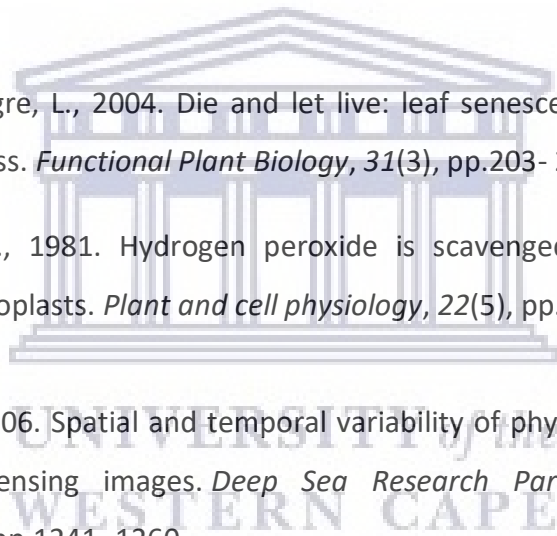
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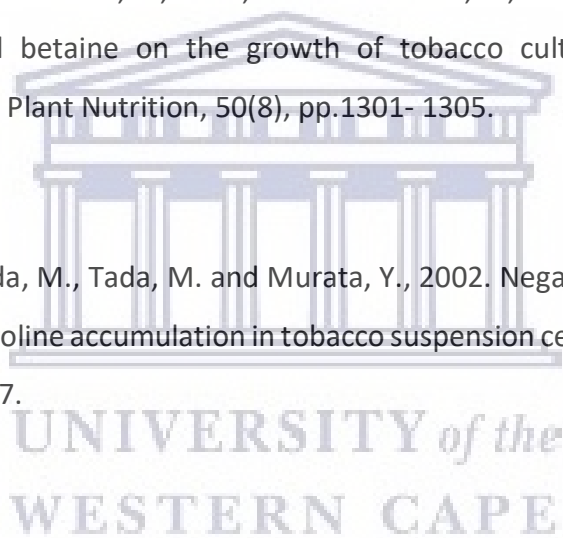
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