Isolation and characterization of stem endophytic bacteria from weed plants for enhancing Vanadium tolerance in *Brassica napus*

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List of abbreviations

PGPB –	Plant growth promoting bacteria
ACC –	1-aminocyclopropane-1-carboxylate
IAA –	Indole-3-acetic acid
R2A –	Reasoners-2 agar
LB –	Luria-Bertani
ICP-OES –	Inductively coupled plasma optical emission spectrometry
YEM –	Yeast extract mannitol
EDTA –	Ethylenediaminetetraacetic acid
SOD –	Superoxide dismutase
APX –	Ascorbate peroxidase
TCA –	Trichloroacetic acid
TBA –	Thiobarbituric acid
TAE –	Tris-acetate-EDTA
NBT –	Nitro blue tetrazolium chloride
TEMED –	N, N, N, N'-Tetramethylethylenediamine
CAT –	Catalase
MDA –	Malondialdehyde
PCD –	Programmed cell death
ROS –	Reactive oxygen species
ARDRA –	Amplified Ribosomal DNA Restriction Analysis
SDS –	Sodium dodecyl sulfate

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Abstract

Bacterial endophytes are able to improve the growth of their hosts through a number of different mechanisms such as nutrient uptake regulation, plant hormone production and regulation, siderophore production and phosphate solubilisation. They have also been shown to be able to provide protection to plants against various abiotic stressors, through various means such as oxidative stress protection. The purpose of this study was therefore to isolate endophytic bacteria from the stems of different weeds, to characterize their ability to use some of the most important growth promoting mechanisms including the ability to produce IAA, siderophores and ACC deaminase, what effect they had on the nutrient uptake in their hosts and to determine to what extent they could promote growth in the roots, stems and leaves of *Brassica napus* plants. In addition to this the endophytes were tested to see to what extent they could protect *Brassica napus* from the negative effects of vanadium stress and how this affected the plant physiologically in terms of morphology, overall biomass, the plants nutrient profile, lipid peroxidation and levels of cell death. The effect of vanadium stress on the oxidative state of *Brassica napus* was also monitored by determining the levels of stress induced reactive oxygen species (ROS) and the corresponding antioxidants that are responsible for regulating these reactive oxygen species.

Six different endophytes (P1, P2, P3, P4, P5, P6) were isolated from different weed samples. Each endophyte was found to be able to significantly improve germination and growth in their host plant. Each isolate was able to improve the uptake of certain macronutrients and micronutrients in their respective hosts, while all of the isolates were shown to be capable of producing siderophores and ACC deaminase. One isolate had high levels of IAA production, with the remaining isolates producing small amounts of IAA. All isolates were also unable to solubilize phosphate.

The five best performing endophytes (P1, P2, P3, P5, P6) in the preliminary growth trials were used in the follow up vanadium stressed growth trials, with endophyte P4 being left out of the remaining experiments. All of the endophytes showed improvements in growth promotion in comparison to the control, with endophyte treated plants showing both increased growth and biomass in both the non-stressed and vanadium stressed treatments

of the vanadium stressed growth trial; however, the leaves of the vanadium stressed plants were significantly smaller than their non-stressed counterparts. When looking at the oxidative state it was found that vanadium stress caused a significant increase in the development of O_2^-, H_2O_2 and •OH in the control and in addition to this it was shown that treatment with endophytes was able to cause a significant decrease in the levels of stress induced H_2O_2 and •OH in all of the treatments and O_2^- for plants treated with endophyte P5. The noted change in the oxidative state of endophyte treated plants was attributed to an increase in the antioxidant activity of these plants, as it was found that endophyte treated plants showed a combination of increased activity for Superoxide dismutase, catalase and ascorbate peroxidase.

This study has shown that endophytic bacteria from plant stems can be used to improve crop growth and yield, while simultaneously producing more nutrient dense crops from the same amount of land. It has also determined that endophytes P1, P2, P3, P5 and P6 are able to successfully provide protection to crop plants from the harmful effects of exposure to vanadium stress. This has great potential for improving food security locally and around the world, by allowing those who cannot gain access to large amounts of food to take in more nutrients from the same amount of food. Furthermore, it also presents the opportunity to use endophyte treatments to grow crops on land that has been previously contaminated with certain heavy metals.

Chapter 1

Literature review

Introduction

Food security refers to the situation in which all the people in a country or across the globe have access to safe and nutritious food in a sufficient amount so that they are able to live normal and healthy lives (Schmidhuber & Tubiello, 2007). Currently food security is being negatively affected in a number of ways such as climate change, which negatively affects crop food production, due to more erratic climates and a decrease in soil quality (Schmidhuber & Tubiello, 2007). Furthermore, the increasing global population has placed a strain on the production capacity of the agricultural industry and requires the use of external inputs to provide improved growth capacity in the industry (Glick, 2014). These external inputs include the use of pesticides as well as fertilizers; however, this can be harmful to the environment, is not entirely sustainable, can lead to damage to viable life supporting water sources through contributing to eutrophication events and may contain trace amounts of metals that can accumulate and eventually pollute agricultural soils over time (Cordell et al., 2009; Glick, 2014). Among those factors affecting food security and crop yield, abiotic stressors collectively contribute to the largest loss in crop yield annually, causing nearly 50% of all crops grown worldwide to be lost (Alcázar et al., 2006). Among abiotic stressors pollution of agricultural soils by heavy metals is one of the more serious abiotic stressors as it routinely causes losses of between 25–80% in various cultivated crops annually (Eid et al., 2019; Wuana et al., 2011). In addition to this, these heavy metals are able to accumulate in substantial amounts in plant tissues to the point that they can easily exceed the levels that are toxic to humans, upon consumption of the affected crops (D'amore et al., 2005).

Heavy metal toxicity

The term heavy metals can be defined as metals that have densities above 5 g/cm^{-3}, display toxicity to biological organisms at low concentrations and cannot be biologically degraded leading to significant harm to the environment and many ecological niches (Ahemad, 2014; Gzik et al., 2003; Sarma, 2011). Heavy metals are naturally present in relatively low amounts in the soil; however, they are also exogenously introduced in much larger quantities into the environment as a result of anthropogenic activities such as mining, as run-off from the excessive use of fertilizers, in the production of batteries, in fossil fuel combustion and as by-products of the production of metal based products (Chibuike & Obiora, 2014; Sarma, 2011).

The result of these activities is that the levels of heavy metals found in certain soil and water sources are often much higher than the levels naturally present there, to the point that they can be extremely toxic (Clemens, 2006).

The cause of this toxicity is attributed to three different functional changes that heavy metals can initiate in plant tissues (Sharma & Dietz, 2009). This includes damaging cellular proteins by binding certain functional groups found in their structures, the disruption of cellular processes through the displacement of ions needed for the proper function of specific biomolecules involved in these processes and the production of reactive oxygen species (Ashfaque et al., 2016; Clemens, 2006; Gzik et al., 2003). The combination of these three factors causes a variety of morphological and ultrastructural changes to occur in plants, including leaf chlorosis and necrosis, decreased seed germination, loss of turgor pressure, molecular alterations in plant tissues, decreased nutrient uptake, the disruption of transpiration and ion transport (Gamalero et al., 2009; Sharma and Dubey, 2007). In light of this it is important to study the effect that different heavy metals have on crop plants, in order to assess the possible damages that pollution of agricultural soils with heavy metals can entail on the yield of important crops, as well as food security. One such heavy metal that is of particular importance in South Africa is the heavy metal vanadium, as South Africa possesses one of the largest sources of vanadium ores worldwide and through mining is one of the top producers of vanadium for the global market (Aihemaiti et al., 2018; Xiao et al., 2015).

Vanadium is an economically important but highly toxic metal

Vanadium is the 5th most abundant transitional metal in the earth's crust and has been estimated to be present at concentrations of 90mg kg^{-1} in soils globally (Aihemaiti et al., 2018; Larsson et al., 2013). Vanadium is found in various forms based on its oxidation state; however, among these the two most common occurring forms are vanadium (IV) and Vanadium (V), more commonly known as "vanadyl" and "vanadate" (Gzik et al., 2003; Larsson et al., 2013). It is not found as the pure version of elemental vanadium naturally; however, it is found bound to a large variety of parental ores such as vanadiferous magnetite. Vanadium is widely sought after for its use in the production of high strength steel and as such its parent ores are extensively mined in high quantities to obtain sufficient vanadium, which indirectly leads to large amounts of vanadium being introduced into the environment (Aihemaiti et al.,

2019; Akoumianaki-Ioannidou et al., 2016; Yang et al., 2017). This poses a problem in countries that have been extensively mining vanadium such as in South Africa, as the levels of contamination in these surrounding areas can pose problems for health of the soil as well as the wildlife and vegetation that inhabit it (Xiao et al., 2015). This problem is of concern as South Africa has been shown to have particularly high levels of vanadium contamination, in soils near mining areas, with levels reaching as high as 3600mg kg^{-1} in the most extreme cases of contamination (Aihemaiti et al., 2018; Xiao et al., 2015). High concentrations of vanadium can induce high levels of ROS production in plants and can result in the onset of lipid peroxidation, loss of membrane integrity and eventual cell death if left untreated (Imtiaz et al., 2015b; Vachirapatama et al., 2011). Vanadium contamination can, therefore, be considered an agricultural issue for crops grown in and around vanadium contaminated soils as it can lead to the reduction in the crop size, a loss of yield and ultimately a reduction in the food security of the nation as a result (Aihemaiti et al., 2018; Gzik et al., 2003).

Brassica napus L

The genus *Brassica* belonging to the family *Brassicaceae,* contains many economically important plant species that are grown either for their vegetables, such as cabbage *(Brassica juncea L.)* and turnip *(Brassica rapa L.)*, as sources of vegetable oil or as animal feed (Zhang et al., 2014). One of the more important species in the *Brassica* genus is the oil crop *Brassica Napus L.,* also called canola, which is an economically important source of vegetable oil, with it being the third biggest source of vegetable oil worldwide (Carruthers et al., 2017). Canola is important as an oil crop for a number of reasons, with it having a high seed oil content (40%-50%) and low levels of erucic acid (less than 2%) and glucosinates, which adversely affects the taste of the vegetable oil and the nutritional state and development of animals at high levels of intake (Rakow et al., 2003; Raymer, 2002; Sana et al., 2003). In addition to this, it is also considered a healthy alternative to other vegetable oils as it has the lowest saturated fat content among vegetable oil crops and is also high in unsaturated fats at levels of 60% to 65% monounsaturated fats, and 30% to 35% polyunsaturated fats (Raymer, 2002). *Brassica napus* has been previously shown to be susceptible to vanadium stress; however, it has not been determined what effects inoculation with endophytic bacteria might have on its growth and redox state under vanadium stress (Gokul et al., 2018).

Reactive oxygen species in plants

Reactive oxygen species (ROS) are a by-product of the normal activities of aerobic organisms, in cellular organelles such as chloroplasts, mitochondria and peroxisomes which are directly involved in activities related to strong electron flow (Choudhury et al., 2014). They are produced either when electrons present in these organelles leak onto O_2, as a by-product of the metabolic pathways present in cellular compartments or as a result of the exposure of the plant to abiotic and biotic stressors in their environment (Sharma et al., 2012). Reactive oxygen species are present in plants under both normal and stressed conditions; however, they play two entirely different roles depending on whether they are present in the former or the latter situation (Karuppanapandian et al., 2011; Sharma et al., 2012). Under the former conditions the production and scavenging of ROS are highly regulated, allowing for their utilization as signalling molecules that play roles in certain processes in plants (Choudhury et al., 2014). Once the production of ROS molecules exceeds the rate at which they are scavenged, the excess ROS starts to cause widespread damage by attacking many of the plants cellular macromolecules, leading to protein oxidation, lipid peroxidation, DNA mutations and enzyme inhibition, which ultimately causes cell death if the levels of ROS molecules cannot be brought under control (Apel & Hirt, 2004; Karuppanapandian et al., 2011; Mittler, 2002).

The production of ROS is known to be unavoidable as molecular oxygen (O_2) is missing two valence electrons, which have a parallel spin, resulting in O_2 only being able to randomly select one of two free electrons, to fill the position of the missing valence electrons (Gill & Tuteja, 2010). This selection can only be carried out one electron at a time which results in the formation of various ROS including superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (•OH) (Mittler et al., 2004; Sharma et al., 2012).

Superoxide (O_2^-)

It is widely accepted that the first reactive oxygen species to be generated is the superoxide radical (O_2^-) as a result of the reduction of O_2, which predominantly occurs in the chloroplasts during photosynthesis (Gill & Tuteja, 2010). The production of O_2^- occurs across the cell at points containing electron transport chains, with around 1-2% of O_2 used for metabolic processes resulting in the formation of superoxide; however, it is also produced in much

larger quantities after the plant is exposed to stress (Dat et al., 2000; Gill & Tuteja, 2010). Among ROS, superoxide is only moderately reactive and although it can cause lipid peroxidation, reduce cytochrome C and can oxidize proteins containing Fe-S clusters, its short half-life of 2-4µs requires it to be present in large amounts across the cell to be highly toxic (Dat et al., 2000; Sharma et al., 2012). As a result of this its toxicity stems more from its conversion to the more toxic ROS hydrogen peroxide (H_2O_2). Hydrogen peroxide is formed when the plant tries to prevent the accumulation of sufficient O_2^- for it to reach highly toxic concentrations, through the intervention of the enzyme Superoxide dismutase (SOD), which acts by dismutating superoxide into H_2O_2 and water, resulting in a decrease in the level of intracellular O_2^- (Apel & Hirt, 2004; Sharma et al., 2012).

Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is a relatively more reactive ROS compared to O_2^-, due in part to its longer half-life of around 1ms and the fact that it is able to diffuse across membranes unlike O_2^-. (Mittler & Zilinskaz, 1991). This means that even though H_2O_2 is only able to diffuse short distances from its site of generation, its potential for damage is not localized to the cellular compartment in which it is produced. This ability to cross membranes and relatively stable nature allows H_2O_2 to act as a messenger for certain biological processes at lower concentrations and is also responsible for helping to induce tolerance against abiotic stressors, by helping to activate the plants antioxidant machinery (Møller et al., 2007; Neill et al., 2002; Yan et al., 2007). Conversely when H_2O_2 is present at relatively high concentrations it can be highly dangerous as it can oxidize, the thiol groups of enzymes, protein kinases and transcription factors and in sufficient quantities it can inactivate Fe-SOD and Cu/Zn-SOD enzymes (Dat et al., 2000; Halliwell & Gutteridge, 1989).

Hydroxyl radical (•OH)

When H_2O_2 accepts an electron from a reduced metal ion such as Fe^{3+}, in a process known as the Haber-Weiss reaction, it produces the Hydroxyl radical (•OH) (Dat et al., 2000). The hydroxyl radical is known to be the most reactive ROS, as it has an extremely high affinity for biological molecules with a rate of reaction nearing the rate of diffusion. This high affinity for biological molecules stems from the hydroxyl radical being the last molecule produced by the Haber-Weiss reaction, which causes it to only possesses a single electron that can react with

all biological molecules including DNA, proteins and lipids, which can lead to lipid peroxidation, DNA damage and eventually cell death (Foyer et al., 1997; Karuppanapandian et al., 2011; Manoharan et al., 2005) Its extremely short lifetime of around 1ns prevents it from diffusing far away from its source of production; however, this means that its damage is localized to the local cellular compartment (Halliwell, 2006; Møller et al., 2007) This localization of damage results in the extent of the damage being far more extensive than if it was dispersed (Halliwell, 2006; Møller et al., 2007). In addition to this, since •OH has no known enzymatic scavengers, it is of utmost importance that the cellular antioxidants APX, SOD and catalase are involved in scavenging any excess O_2^- and H_2O_2 before they can be converted into •OH (Apel & Hirt, 2004; Sharma et al., 2012).

Lipid Peroxidation

Lipid peroxidation is a highly damaging process which occurs when the levels of intracellular ROS exceed a certain concentration, above which they begin to damage the phospholipid linkages present in cell and organelle membranes (Han et al., 2009; Sharma & Dubey, 2005). The main target of ROS molecules are polyunsaturated fatty acids which upon degradation, release breakdown products, many of which are biologically active lipid-derived radicals, that can exacerbate the level of lipid peroxidation, by going on to further oxidize other polyunsaturated fatty acids (Sharma et al., 2012). As a result of its close link with ROS, lipid peroxidation is a widely accepted measure of the toxicity of stress induced ROS (Sharma et al., 2012). This is usually carried out by measuring the concentration of a molecule called malondialdehyde (MDA). MDA is one of the final breakdown products of lipid peroxidation, is highly toxic, can cause cross-linkages among cellular proteins and is able to react with proteins via their free amino groups (Gill & Tuteja, 2010). MDA can react with thiobarbituric acid to form a colourmetric compound called thiobarbituric acid reactive substances (TBARS) and it is through this reaction that levels of lipid peroxidation can be measured (Heath & Packer, 1968).

Antioxidant pathways in plants

In order to help reduce the detrimental effects caused by the build-up of intracellular ROS, plants have developed a number of different antioxidant pathways to help facilitate the regulation of ROS molecules through enzyme-based scavenging (Lee et al., 2007; Scandalios,

1993). Upon exposure to abiotic stressors, ROS levels can increase to the point that they can become highly detrimental to the plant and as such the upregulation of the above antioxidants are essential for protecting plants under these highly oxidative conditions (Sharma et al., 2012). The antioxidant system of the plant employs a number of different enzymes to achieve this purpose and among those present, the more prevalent antioxidant enzymes include Superoxide dismutase (SOD), Catalase and Ascorbate peroxidase (APX), which each have a distinct function and target of their antioxidant activities (Alscher et al., 2002; Gupta et al., 1993; Lee et al., 2007).

Superoxide dismutase (SOD)

Superoxide dismutase (SOD) are a family of enzymes that is responsible for converting the reactive oxygen species superoxide into H_2O_2 and water (Choudhury et al., 2014). It has been established as the first antioxidant enzyme that a plant will employ and it is found as three different and distinct forms dependent on what metal co-factor is needed to bind to its active site (Gupta et al., 1993). The three types of SODs are the Cu/Zn SODs, Mn SODs and Fe SODs, with the former being predominantly found in chloroplasts, Mn SODs being found in the peroxisomes and the latter being localized to the chloroplasts (Gupta et al., 1993). The difference in requirements for co-factors is thought to be as a result of selection of the most abundant metal in the environment, as a result of changes in the oxygen availability, at the time of each isozymes evolution (Alscher et al., 2002). Superoxide radicals have been shown to be incapable of crossing phospholipid membranes, which means that it is essential for SODs to be present in each location that O_2^- can be formed, making them highly ubiquitous throughout every cellular compartment within the cell (Alscher et al., 2002).The balance between SOD and Catalase is known to be essential for alleviating the effects of ROS induced stress, as this toxicity is induced by the collapse of the delicate balance between quantities of O_2^-, H_2O_2 and •OH present in the cellular environment (Apel & Hirt, 2004).

Catalase

Catalase is a haem-based tetrameric enzyme that catalyzes the dismutation of two H_2O_2 molecules into H_2O and O_2 (Du et al., 2008). It is present in all eukaryotic organisms and is mainly found in peroxisomes, where it scavenges H_2O_2 produced during photosynthesis and under oxidative stress (Sharma et al., 2012). Catalase is one of multiple different H_2O_2

scavenging enzymes present in plants; however, it is unique in that it does not require any reducing equivalent to degrade H_2O_2 such as in the case of APX with ascorbate (Luis et al, 2006). Catalase is one of the fastest acting biological enzymes and has a high specificity for H_2O_2, which makes it extremely important for helping plants to tolerate being under oxidative stress (Polidoros & Scandalios, 1999). It must be noted, however, that abiotic stressors can either upregulate or downregulate catalase activity depending on the duration and type of stress present (Han et al., 2009; Moussa & Abdel-Aziz, 2008; Sharma & Dubey, 2005) In most situations it is stressors such as heavy metals and drought, that reduce the rate of protein turnover, that affect the level of catalase activity (Chen et al., 2010; Hojati et al., 2010; Karuppanapandian and Manoharan, 2008). In cases involving these types of stressors, the efficiency of catalase will be much lower than usual and the plant will be vulnerable to ROS induced stress and thus the plant will have to rely on other H_2O_2 scavenging enzymes, such as ascorbate peroxidase (APX) in order to sufficiently protect the plant.

Ascorbate peroxidase (APX)

Ascorbate peroxidase is a hydrogen peroxide scavenging enzyme of the peroxidase class, that catalyzes the oxidation of ascorbate with H_2O_2, resulting in the formation of water and monodehydroascorbate (MDA). It is localized in the chloroplast, cytosol and mitochondria, where it plays the role as the predominant H_2O_2 scavenger in these compartments (Asada, 2006; Quan et al., 2008). APX has a higher affinity for H_2O_2 than catalase, as it will initiate scavenging activities in the µM range instead of in the mM range that catalase functions at (Gill & Tuteja, 2010). This means that APX is able to respond to an increase in H_2O_2 long before catalase does, making it far more efficient in responding to increases in ROS when the plant is under stress. A possible reason for why APX has such a high affinity for H_2O_2 is thought to be due to the chloroplasts being highly susceptible to ROS and can therefore be easily damaged, resulting in a loss of activity under low levels of hydrogen peroxide (Asada, 2006). It is therefore better to have an easily activated scavenging enzyme, as opposed to one that only functions when the target molecule is already present in high concentrations.

Taking into account the negative impact heavy metal stress plays on crop growth and yield as well as considering the adverse effects that fertilizers can have on the soil and the surrounding water sources, there is the need to use more environmentally friendly means to promote

growth as well as protect plants from the harmful effects of heavy metals, such as the utilization of microbes already present in the soil or inside plants.

There are many types of microorganisms which play some sort of role in a plant's life cycle with epiphytes and endophytes being the most important for plants (Diep et al., 2016). Epiphytes refer to those microorganisms which live on the external surfaces of plants whereas endophytes refer to fungi and bacteria that are able to colonise the internal tissues of a plant without causing any noticeable negative side effects to the plant itself (Ferrando et al., 2011). These endophytes have been found in all major plant tissues, in almost all previously studied plant species and it is thought that all plant species should contain endophytes (Martinez-Romero & Rosenbleuth, 2006).

It is well known that certain endophytes, through a variety of mechanisms, are able to promote growth in plants including the solubilisation of certain nutrients in the soil, the development of plant growth promoting phytohormones such as auxins, cytokinins and gibberellins, producing useful metabolites, producing antifungal and antibacterial compounds and by providing increased protection to the plant from different types of stressors (Antony et al., 2015).

Endophytic bacteria and their roles in plant growth

What are endophytes

The term endophyte refers to bacteria that live on the inside of plants without causing any noticeable disease symptoms (Doty & Khan, 2009). Bacterial endophytes have been shown to possess different mechanisms with which to promote the growth and development of plants (Doty & Khan, 2009). The most important of these mechanisms are the direct mechanisms, which can include the production of various plant growth regulators such as indole-3-acetic acid (IAA) and 1-aminocyclpropane-1-carboxylate (ACC) deaminase, as well as the improvement in the uptake of a wide range of nutrients required by the plants (del Carmen Orozco-Mosqueda et al., 2016).

How endophytes colonize plants

Endophytes have been shown to gain access to plants by colonizing the internal parts of the roots of the plant, which involves the endophytic bacteria first colonizing the root surface

before entering the plant through the roots (Clément et al., 2010). This helps to explain the relationship between bacterial endophytes and rhizosphere colonizing bacteria, which is to say that many facultative endophytic bacteria can also survive as rhizospheric bacteria (Clément et al., 2010). These endophytes find their host by electrotaxis, chemotaxis, or by an accidental encounter between the host plant and some object, animal or person carrying the endophyte. Once contact with the plant is made, the main entry point for the endophytic bacteria is thought to be through wounds on the roots, through root hairs or at epidermal junctions on the plant (de Faria & Sprent, 1988). Although wounds provide entry avenues for endophytes, they provide favourable conditions for the bacteria in the soil by allowing for plant exudates to leak into the soil, which acts as a nutrient source for the bacteria.

Even though the zone around the roots offers the easiest entry point for many endophytes, entry has also been shown to occur on aerial portions of plants (Sharrock et al., 1991). It was also suggested that certain fruit-based endophyte colonies may come about by entry through flowers (Sharrock et al., 1991). Endophytic penetration can also occur via natural openings on stem lenticels or the leaves (der Lelie et al., 2002; Sharrock et al., 1991).

Endophytic bacteria have an advantage over bacteria that can only colonize plants epiphytically (der Lelie et al., 2002). Endophytes have this advantage over epiphytes because they are better protected from exposure to extreme temperatures, UV radiation and varying osmotic potentials by the internal tissues of the plant, allowing them to overcome these conditions which limit long term bacterial survival (der Lelie et al., 2002).

How do they promote plant growth

In general plant growth promoting endophytes usually have three different means by which to promote plant growth (Glick, 1995) either by synthesizing a number of different compounds for the plant to use (Zahir et al., 2003) through the facilitation of the uptake of certain nutrients from the soil (Çakmakçi et al., 2006) and the protection of the plants from pathogens and diseases (Ahmed et al., 2010).

The possible mechanisms of plant growth promotion are shown in figure 1 and usually include the ability to produce a vital plant growth regulating substance such as ACC deaminase which helps to reduce the levels of stress related ethylene in the roots of the plant thereby

improving root growth (Penrose & Glick, 2001), or the ability to produce important plant phytohormones such as the auxin, indole-3-acetic acid (IAA) (Patten & Glick, 2002). This can also include mechanisms that improve the plants nutrient uptake such as the ability to perform biological nitrogen fixation (Kennedy et al., 2004) or through the solubilization of nutrients such as mineral phosphates (Alvarez et al., 1996). The endophyte could also be capable of helping the plant to survive various biotic and abiotic stressors such as providing protection against phytopathogenic microorganisms (Glick, 1995) and by offering enhanced resistance to drought (Alvarez et al., 1996), high salinity levels and exposure to various heavy metals (Saleem et al., 2007).

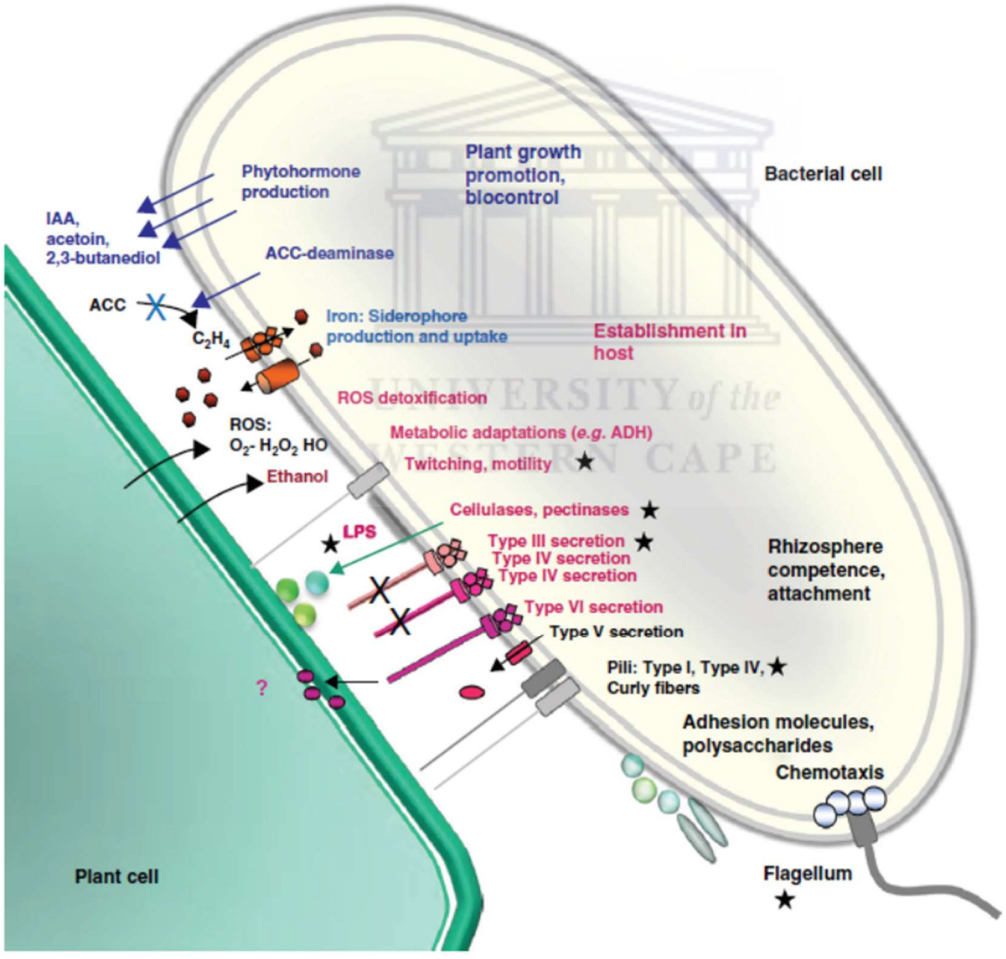

Figure 1: General mechanisms employed by bacterial endophytes to promote growth in plants (Hurek & Reinhold-Hurek, 2011).

Plant hormones

Endophytic bacteria may play a role in the promotion of plant growth through the development of a number of different plant hormones (Ahmed et al., 2010). These plant phytohormones act as plant growth regulators, as they are known to influence the physiological processes of plants when present at sufficient levels. There are five major plant hormones including ethylene, auxins, cytokinins, gibberellins and abscisic acid.

Ethylene in plants

Ethylene is one of the most important plant hormones, which plays a role in seed germination, leaf and flower senescence, the ripening of fruit, root nodulation as well as programmed cell death (Ecker & Guo, 2004). It is also known to be involved in a number of critical plant growth processes including the induction of stem and petiole growth, the formation of the apical hook in dicotyledonous plants and the general inhibition of uncontrolled stem growth in terrestrial plants (Ecker & Guo, 2004).

Apart from its role in the promotion of plant growth and organ formation, ethylene is also involved in higher plants in various ways as a result of the plant being exposed to abiotic and biotic stressors in the form of toxic metals, chemicals in the environment, extreme temperatures, drought stress, salinity stress, UV damage, predation by insects and other pests, pathogen damage and mechanical damage, such as being grazed upon (Abeles et al., 1992).

The result of a plants exposure to any one of these stressors causes the formation of two distinct peaks in the levels of “stress ethylene” within the plant (Hao et al. 2007). The first peak is thought to be responsible for initiating the transcription that encodes the plants protective and defensive proteins and involves the depletion of the available pool of the ethylene precursor, ACC present in the plant (Robison et al., 2001a). The second peak in the levels of ethylene occurs after the plant has synthesized more ACC in response to the stressor’s effects on the ACC level in the plant. It occurs a few days after exposure to the initial stressor, is usually much larger in magnitude than the first ethylene level peak and is responsible for activating a number of negative stress related events in the plant such as leaf chlorosis and senescence (Glick, 2014).

In this way it can be seen that if the formation of this second burst of ethylene within the plant can be decreased or stopped altogether, then it would have a positive effect on the survival of the plant during times of stress (Glick, 2014). As such looking for endophytes which possess such a trait would allow for the growth of plants under harsher growing conditions through exposure to and treatment with the endophyte which has this ability.

Plant growth regulators

ACC deaminase

The enzyme ACC deaminase is involved in the cleavage of ACC, the precursor of the hormone ethylene in plants thereby producing α-ketobutyrate as well as ammonia thereby reducing the quantity of endogenous ethylene synthesized by the plant (Gamalero & Glick, 2015). In the majority of higher plants, ethylene is synthesised from the molecule S-adenosyl-methionine by the enzyme ACC synthase, during normal plant growth and when the plant is exposed to a wide variety of different environmental stressors (Gamalero & Glick, 2015). As a result of its modulation of the plants ethylene levels, ACC deaminase is one of the main bacterial mechanisms improving plant growth under different stressed conditions, where the concentration of ethylene could reach levels that are inhibitory to plant growth (Gamalero & Glick, 2015).

ACC deaminase is a multimeric enzyme, found in the cytoplasm that utilizes pyridoxal phosphate as a coenzyme for its normal function. Its native size is estimated to be approximately 100 to 112kD, with a subunit of between 35 to 42kD (Hontzeas et al., 2004; Jacobson et al., 1994; Sheehy et al., 1991). Most bacteria with ACC deaminase contain a basal level of enzymatic activity; however, when even a small amount of ACC is present, even as low as 100nm, then the synthesis of ACC deaminase is induced (Gamalero & Glick, 2015). This indicates that ACC deaminase is transcriptionally activated by the presence of ACC (Gamalero & Glick, 2015).

Feedback Inhibition of stress Ethylene through IAA and ACC deaminase

Bacterial IAA as well as plant IAA is responsible for promoting the transcription of the ACC synthase enzyme in the plant, which is responsible for catalysing the conversion of ACC from S-adenosyl-methionine. Due to the increased levels of ACC, the ethylene levels inside a plant

is increased which if left unchecked would become growth inhibitory. Bacteria capable of producing relatively large amounts of IAA are often inhibitory to plant growth in this way; however, this may not occur because as plant ACC levels increase some root bound ACC is absorbed by the bacteria and is converted to α-ketobutyrate and ammonia through the action of ACC deaminase (Gamalero & Glick, 2015).

This causes the amount of ethylene produced by the plant to be reduced and this is why colonization of plants by bacteria that synthesize ACC deaminase can lead to plant growth promotion (Glick, 1995). Furthermore, it has also been shown that ACC deaminase containing endophytic bacteria can help protect against growth inhibition of a plant from a number of ethylene inducing stressors such as high salt levels, flooding, drought, heavy metals and other contaminants in the soil as well as from temperature extremes (del Carmen Orozco-Mosqueda et al., 2016).

Indole-3-acetic acid

The class of phytohormones called auxins are the most important class of plant hormones in terms of plant growth regulation. Indole-3-acetic acid also called IAA, is one of the most active plant auxins, which is responsible for stimulating cell elongation, cell division as well as cell differentiation (Cleland, 1990; Hagen, 1990). It is one of the best characterized phytohormones and it has been proven that many endophytes are able to produce IAA (Ahmed et al., 2010; Patten & Glick, 1996).

These bacteria use tryptophan to synthesize IAA, with some of the resulting IAA being taken up by the plant (Patten & Glick, 1996). The exogenous bacterial IAA along with the IAA produced in the plant, can regulate a number of the phases of plant development, such as seed germination, cell proliferation, cell elongation, the emergence of the lateral roots and the adventitious roots, xylem formation, vegetative growth in plants, plant responses to gravity and light as well as the development of fruit (Tsavkelova et al., 2006).

Production of the hormone IAA is present among many bacteria which are associated with plants, but studies have shown that the pathways used in phytopathogenic bacteria and beneficial bacteria differs, as IAA is produced from tryptophan through the indole acetamide (IAM) pathway in phytopathogenic bacteria and usually via the indole pyruvic acid pathway

in beneficial bacteria (Patten & Glick, 2002). This difference can therefore be used by the plant to direct the plants defensive mechanisms after the pathogen and away from the beneficial bacteria (Spaepen et al., 2007).

Tryptophan has been shown to be the major precursor for IAA biosynthesis pathways in bacteria and as a result of the discovery of the intermediates used for IAA production, it was found that five pathways which use tryptophan as a precursor for IAA production exists (Spaepen et al., 2007).

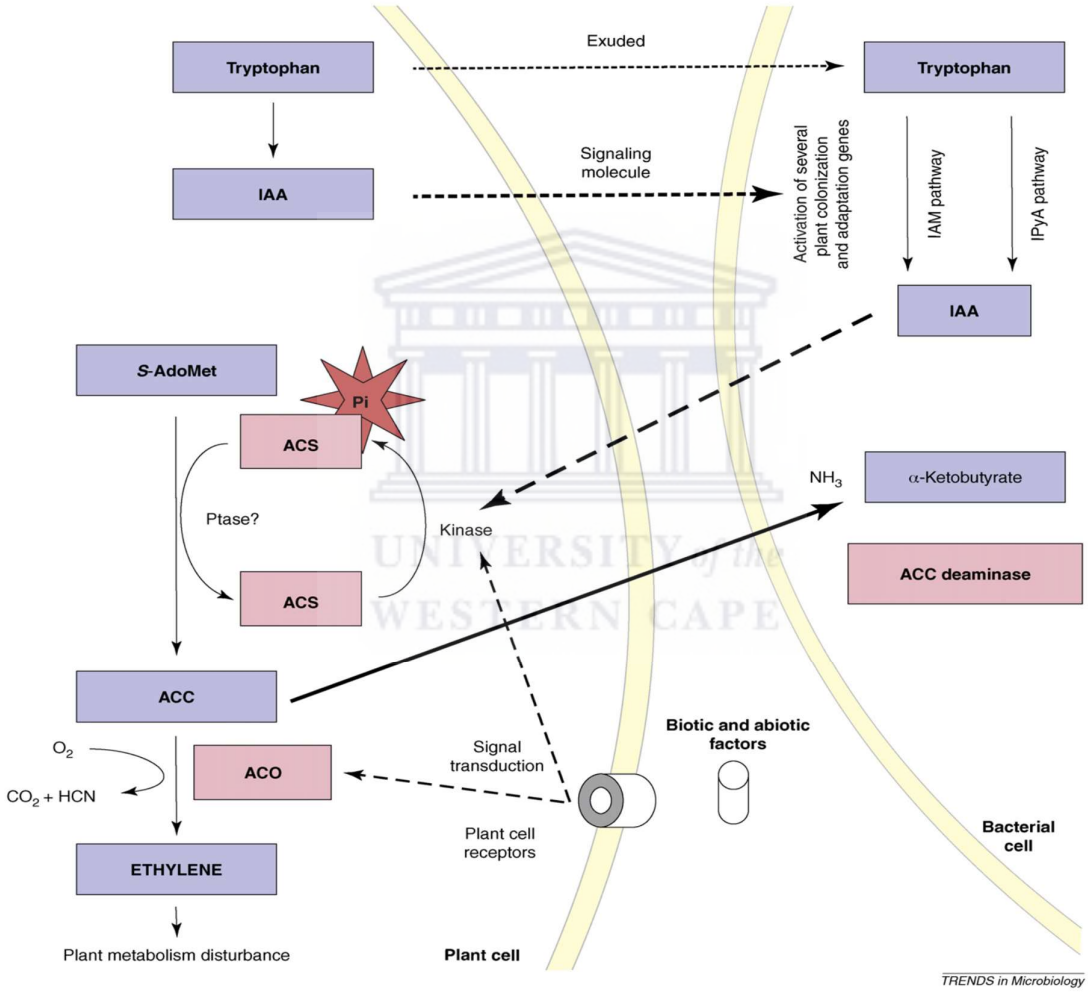

Figure 2: Figure showing mechanisms used by bacterial endophytes in their modulation of plant ethylene levels through the use of IAA and ACC deaminase (Elsas et al., 2008).

These five pathways include the indole-3-acetamide pathway, the indole-3-pyruvate pathway, the tryptamine pathway, the tryptophan side-chain oxidase pathway and the indole-3-acetonitrile pathway (Spaepen et al., 2007).

Through the use of these different pathways' endophytes are able to produce beneficial IAA for the plant and in doing so allow for the improved growth of the plant's roots leading to increased nutrient uptake by the plant. This increase in nutrients therefore means that plants containing IAA producing endophytes should have improved growth in most soil environments and will have a higher chance of survival in soils containing low levels of nutrients (Patten & Glick, 2002).

Nutrient uptake improvement

One of the more important mechanisms used in the promotion of plant growth by endophytic bacteria is the facilitation of the increased uptake of nutrients by the plant, including the essential macronutrients and micronutrients that are required by the plant to survive and grow (Maathuis, 2009). The macronutrients are required in relatively large quantities by plants and include the elements nitrogen, phosphorous, calcium, magnesium, sulphur and potassium (Maathuis, 2009). Although they are found in large quantities in plants, these elements are usually found in low concentrations in soils and are difficult for plants to assimilate as they have to be accumulated over concentration gradients (Maathuis, 2009). The term micronutrients refers to essential plant nutrients that are required in trace amounts by plants and includes the elements chloride, copper, boron, iron, manganese, zinc, nickel and molybdenum (Hansch & Mendel, 2009). These elements are almost exclusively metal based elements, which are found as metal ions in soil (Hansch & Mendel, 2009). The micronutrients are often in sufficient supply in fertile soils for the requirements of plants and as such the major benefit of plant growth promoting bacteria in nutrient uptake improvement relies on the improvement of the availability of macronutrients in the soil (Dawson & Hilton, 2011). This, however does not mean that the improvement of micronutrient uptake into plants will have no beneficial effects on the plant.

Phosphate solubilization

Phosphorous is the second most important macronutrient required for plant growth after nitrogen and although phosphorous is available in high levels in the soil, the majority of it is in an insoluble form which is biologically unavailable for plants to use (Araújo et al., 2004). In fact, only around 0.1% of phosphorous is in the correct form for plants to use and even with the use of phosphorous containing fertilizers there is often insufficient phosphorous for plants to grow correctly as the phosphorous in soil is very quickly mineralized in large amounts into insoluble phosphate (Taurian et al., 2010). A possible solution to this problem relies on the use of endophytes which are able to solubilize phosphate in order to increase the amount of available phosphorous in the soil for plants to utilize (Dowling et al., 2015).

The mechanism behind the solubilisation of organic phosphates was found to rely on the production of enzymes such as phytases, phosphonatases and C-P lyases (Dowling et al., 2015). In the solubilisation of insoluble mineral phosphates, endophytes create a number of organic acids in order to turn the insoluble phosphate compounds into soluble dibasic ($HPO4^{-2}$) and monobasic ($H_2PO_4^-$) ions which are more easily taken up by plants (Taurian et al., 2010).

The use of these endophytes on crop plants could therefore lead to an increase in the uptake of phosphate by the plants, leading to improved crop growth and crop yield (Taurian et al., 2010). This should also allow for the reduction of the use of phosphate containing fertilizers by up to 50% annually, which would also be beneficial to the environment, while leading to a decrease in the levels of minerals leaching from soil thereby helping to prevent issues of eutrophication in water systems (Dowling et al., 2015).

Siderophore production

Iron is an important nutrient required by microorganisms for their growth and survival, with endophytic bacteria not being an exception. It is usually present in the form of Fe^{3+} molecules in the aerobic environment in which endophytes live; however, this is not a very biologically active form of iron for most bacteria, as it is highly insoluble and as such adaptations are required for these bacteria to access the iron present in the molecules (Ma et al., 2010). The most common adaptation usually takes the form of the production of siderophores, by these endophytes, which are relatively low-molecular weight iron-chelating compounds which are

able to bind to the Fe^{3+} molecules in the environment, forming complexes with those molecules. The formation of the siderophore-Fe^{3+} complex causes an increase in the level of solubility of the Fe^{3+} molecules, resulting in it becoming accessible to the endophytes that need it (Ma et al., 2010). This means they are usually produced by microorganisms and plants under conditions of extreme iron deficiency as a means of increasing the level of available iron (Cabirol et al., 2007). Siderophores usually form complexes with $Fe3^{+}$ in a 1:1 ratio and upon being taken up by the microbial cell membrane, are converted to Fe^{2+}, which is then separated from the siderophore and released into the cell. The siderophore can then either be recycled to be used once again by the endophyte or is destroyed after its use (Ma et al., 2010).

Siderophores are found in three major forms based on the acid moiety used in the metabolic pathway by which it is produced and these include catecholates, hydroxymates and α-carboxylates (Cabirol et al., 2007). The catecholate siderophore producing organisms consist of both fungi and bacteria, the hydroxymates are only produced by bacteria and the α-carboxylates are produced almost exclusively by fungal zygomycetes (Cabirol et al., 2007).

Another benefit of siderophores is that it can help prevent the growth and spread of phytopathogens which helps to facilitate plant growth (Castignetti & Smarrelli, 1986). This involves the secreted siderophore molecules binding most of the Fe^{3+} that is available in the environment thereby preventing any pathogens in the vicinity from proliferating because of a lack of iron for the pathogens to use (Buysens et al., 1994; O'Sullivan & O'Gara, 1992). This also benefits plants because they are not usually harmed by the depletion of local reserves of iron caused by endophyte produced siderophores and furthermore a number of plants are able to bind the iron-siderophore complex, release the iron from the siderophore thereby allowing it to be used by the plant (der Lelie et al., 2002).

Indirect mechanisms of plant growth promotion

The majority of endophytic bacteria promote plant growth through the above mentioned direct plant growth promoting mechanisms; however, there are also a number of indirect mechanism by which endophytic bacteria promote plant growth including bioremediation of

the soil in which the plant grows and by providing protection of plants against harmful pathogens (Bashan & De-Bashan, 2005).

Phytoremediation and microbe enhanced phytoremediation

The continued effect of an industrialized economy around the world has led to increased levels of harmful anthropogenic chemicals and heavy metals into the environment (Gerhardt et al., 2009). These harmful compounds include pesticides, solvents, heavy metals, salts, polycyclic aromatic hydrocarbons and petroleum hydrocarbons and have been increasingly connected to increased levels of harmful diseases in humans, as well as to decreasing the quality of the soil in the ecosystem which can lead to poor crop yields and an overall stunted plant growth.

Phytoremediation, which is the use of plants to aid in the in-situ removal of contaminants, has thus been increasing receiving attention around the world as a means of counteracting the harmful effects of harmful chemicals and pollutants on the environment (Ma et al., 2011). Along with this many bacterial species have been shown to possess contaminant degrading mechanisms and these have been shown to be of beneficial use in the breakdown of harmful organic chemicals in a process called bacterial bioremediation (Gerhardt et al., 2009).

This has led to a new and developing field in which the phytoremediation effects of plants have been combined with the bioremediation effects of bacteria in order to produce a more efficient and beneficial form of remediation (Gerhardt et al., 2009). This could therefore be used to improve soil conditions on agricultural land, by making them healthier and can therefore be considered a means of indirect plant growth promotion which can have beneficial effects on crop production and yields.

Justification for the use of endophytes

In the past agricultural practices and methods of the promotion of crop plant growth has predominantly been based on the use of external inputs such as pesticides and fertilizers in order to increase crop yields and also to control soil borne diseases. The problem with this; however, lies in the fact that these products can cause serious environmental and health damage caused by their excessive use in agriculture (Damalas & Eleftherohorinos, 2011).

Therefore, worldwide agricultural practices are moving towards more sustainable and environmentally friendly approaches (Gamalero & Glick, 2015).

When this is coupled with the prediction that the global population is expected to reach about 10 billion in around 50 years, then it becomes apparent that new environmentally friendly approaches need to be looked at in order to improve the agricultural capacity that is currently available to feed the ever increasing population (Glick, 2014). One such approach is the use of plant growth promoting bacteria in order to improve the growth of agricultural crops in a more sustainable and biologically friendly manner (Glick, 2014). In addition to this, these endophytes also possess mechanisms of promoting tolerance towards abiotic stressors, allowing for the growth of crops in soils that were previously too inhospitable for them to be grown in. One such use of endophytes to alleviate abiotic stressors, comes in the form of their ability to protect plants from the harmful effects of heavy metal stress. This has been shown to be a promising option for plant growth promotion in plants under various types of heavy metals; however, this has been carried out on more commonly found heavy metal such as cadmium and copper, with much less attention being paid to metals such as vanadium which has been more sparsely studied. We would thus like to investigate what effect vanadium would have on *Brassica napus L* treated with endophytic bacteria and whether the use of these endophytic bacteria could help protect their hosts from vanadium stress

Chapter 2

Materials and methods

2.1 List of reagents used in the study

1-Aminocyclopropane-1-carboxylic acid -	Sigma Aldrich
2-Deoxy-D-Ribose -	Sigma Aldrich
2-Thiobarbituric acid -	Sigma Aldrich
Agar -	Sigma Aldrich
Ammonium sulphate -	Sigma Aldrich
Boric acid -	Sigma Aldrich
Bradford dye reagent 1X -	Bio-Rad
Brassica napus seeds (AV Garnet) -	Agricol
Calcium chloride -	Sigma Aldrich
Calcium phosphate -	Sigma Aldrich
Chromeazurol S -	Sigma Aldrich
Cobalt chloride -	Sigma Aldrich
Copper sulfate -	Sigma Aldrich
Dextrose -	Sigma Aldrich
Dipotassium hydrogenphosphate -	Sigma Aldrich
Dipotassium phosphate -	Sigma Aldrich
Evans blue -	Sigma Aldrich
Ethanol 200 proof -	Sigma Aldrich
Ethylenediaminetetraacetic acid (EDTA) -	Sigma Aldrich
Ferrous sulphate -	Sigma Aldrich
Glacial acetic acid -	Sigma Aldrich
Hydrogen peroxide -	Sigma Aldrich
Indole-3-acetic acid -	Sigma Aldrich
Iron sulphate -	Sigma Aldrich

L-ascorbic acid -	Sigma Aldrich
Luria-Bertani (LB) broth -	Sigma Aldrich
Methionine -	Sigma Aldrich
Magnesium sulphate -	Sigma Aldrich
Manganese sulphate -	Sigma Aldrich
Mannitol -	Sigma Aldrich
Monopotassium phosphate -	Sigma Aldrich
Nitric acid (65%) -	Sigma Aldrich
Nitro blue tetrazolium chloride monohydrate -	Sigma Aldrich
Perchloric acid (70%) -	Sigma Aldrich
Potassium chloride -	Sigma Aldrich
Potassium cyanide -	Sigma Aldrich
Potassium iodide -	Sigma Aldrich
Potassium phosphate -	Sigma Aldrich
Potting soil -	Stodels
Reasoner's 2A Agar -	Sigma Aldrich
Riboflavin -	Sigma Aldrich
Silica sand -	Cape Silica
Sodium acetate -	Sigma Aldrich
Sodium Chloride -	Sigma Aldrich
Sodium dodecyl sulfate -	Bio-Rad
Sodium hydroxide -	Sigma Aldrich
Sodium hypochlorite -	Sigma Aldrich
Sodium metavanadate -	Sigma Aldrich
Sodium molybdate -	Sigma Aldrich

Sodium phosphate dibasic - Sigma Aldrich

Trichloroacetic acid 99 % - Sigma Aldrich

tris(hydroxymethyl)aminomethane - Sigma Aldrich

Tryptophan - Sigma Aldrich

Yeast extract - Sigma Aldrich

Zinc sulfate - Sigma Aldrich

Table 1: List of equipment

Instrument	Model	Company
Centrifuge	5415D	Eppendorf
ICP-OES	Varian Vista Pro	Varian
Incubator Quincy Lab economy digital	Incubator Quincy Lab economy digital	Incubator Quincy Lab economy digital
Spectrophotometer (Cuvette)	Ultrospec 2	Biochrom
Spectrophotometer (Microtitre plate reader)	POLARstar	Omega
Thermocycler	GeneAmp PCR system 9700	Applied biosystems
Camera	80D digital camera	Canon
Gel viewing system	Enduro gel documentation system	Labnet international

2.2 Collection of plants and surface sterilization

Five different weed samples were collected from the UWC campus grounds (33°56´06´´S, 18°37´41´´E), the roots and leaves were then removed from each plant. The plant stems were then surface sterilized using 2% sodium hypochlorite, 70% ethanol and 3 water washes, with the final water wash being plated on Reasoner's 2A agar (R2A), to check for proper surface sterilization. These plates were then grown at 37°C for 7 days.

2.3 Endophyte extraction and plating

Surface sterilized weed stem samples were ground up in 0.9% NaCl using a sterile mortar and pestle. Each ground up sample was incubated in 0.9% NaCl for 5 hours allowing for sufficient endophytic growth. Incubated cultures were used to make nine 10-fold dilutions up to 10^{-9},

with each dilution being plated on R2A agar at 37°C for 7 days. Endophytes were purified by streaking onto R2A agar which was then incubated for 7 days at 37°C.

2.4.1 Plant growth promotion trials (No heavy metal stress)

The purified isolates, given the names P1-P6, were used to prepare cultures in LB broth and were incubated at 30^{o}C with shaking until the optical density (OD) was in the range of between 0.4 – 0.6. *Brassica napus* seeds (Agricol) were surface sterilized and imbibed in separate endophyte cultures for 30 minutes, with a control being prepared using pure sterilized LB broth in place of endophyte culture. Inoculated seeds were planted in a pre-watered soil mixture consisting of 1:1:1 soil: fine silica sand: coarse silica sand. The planted seeds were treated with 2ml of the endophyte culture per seed, with the control being treated with 2ml of LB per seed. The plants were set up in a completely random design and grown under a 26/19 ◦C day/night temperature cycle with a 16/8 h light/dark cycle, at a photosynthetic photon flux density of 300 µmol photons.m^{-2} s^{-1} (during the day phase). The germination rates were recorded over the 1st 10 days, with results being recorded on the 4th, 5th, 6th and 10th days of growth. Each plant was watered every 3 days using deionised water for the duration of the growth period of 46 days, while plants were checked regularly to prevent overwatering.

2.4.2 Harvesting and grinding

Endophyte treated and control-untreated plants were grown for 46 days, until the plants were at the correct stage for harvesting. Plants were carefully removed from the pots to prevent damage to the stems or leaves and the roots were removed from the samples to be used in the study to prevent erroneous data interpretation as a result of damage caused during the separation of the roots from the soil. Three samples of each plant were taken and separated into stem and leaf samples and placed in foil packets of known weights in order to determine the plant fresh weights. Once the fresh weights were known, plants were then incubated at 50°C for 48 hours, following which the dry weight of each sample was determined. Photographs of the growth results, of a randomly selected representative of each sample, were captured using a Canon 80D digital camera (lens; Canon EF-S 10–18mmf/ 4.5–5.6 IS STM)

The remainder of the plant samples for each endophyte treatment was taken and ground up in a sterile mortar and pestle, using liquid nitrogen to maintain plant nutrient content integrity. The ground up plant material was then stored at -80°C for further analysis via ICP-OES.

2.5 Inductively couple plasma optical emission spectrometry (ICP-OES)

In preparation for ICP-OES analysis, 200mg of each ground up plant sample, was weighed out in triplicate and placed in separate eppendorf tubes. Samples were immersed in 1ml of 65% nitric acid, with the lids properly secured with parafilm prior to digestion, to prevent loss of material during the digestion. Digestion of the samples was carried out at 95°C for 3 hours in a heating block and then placed on ice to cool for 15 minutes. Following cooling samples were diluted 10-fold to a final volume of 10ml in a 1:9 ratio of 65% nitric acid:2% nitric acid and syringe filtered through a 0.45µm filter. The samples were then sent for ICP-OES analysis (Gokul et al., 2018).

2.6 Phosphate solubilisation test

Single colonies of each isolate along with an *E. coli Krx* control was plated onto Pikovskayas phosphate agar plates (Agar 1.5%, ferrous sulphate 0.00001%, Manganese sulphate 0.00001%, Magnesium sulphate 0.01%, Potassium chloride 0.02%, Ammonium sulphate 0.05%, Calcium phosphate 0.5%, dextrose 1%, Yeast extract 0.05%) and grown for 7 days at 37°C. Colonies were observed for halos as a sign of phosphate solubilisation capability after the 7 day growing period (Rao & Sinha, 1963).

2.7 Indole-3-acetic acid (IAA) assay

Indole-3-acetic acid production was determined through the use of a colorimetric assay in which each isolate was grown in both Yeast extract mannitol (YEM) broth (Mannitol 1%, Yeast extract 0.1%, dipotassium phosphate 0.05%, sodium chloride 0.01%, magnesium sulphate 0.02%) to which 0.1% tryptophan was added and YEM broth without the addition of tryptophan. The cultures were grown for 5 days at 30°C with shaking. After 5 days of growth the cultures were centrifuged for 15 minutes at 13000 rpm and the supernatants were taken and mixed with Salkowski reagent (0.25M $FeCl_3$, 35% perchloric acid) in a 1:2 ratio, supernatant: Salkowski reagent. This was left to incubate for 30 minutes at room temperature in the dark in order to ensure proper reactivity of the Salkowski reagent with the endophyte

samples. Samples were read at 530nm in a spectrophotometer and the absorbances were compared to a pre-prepared standard made using IAA diluted in YEM to concentrations that ranged from 5 µg/ml to 100µg/ml (Gordon & Weber, 1951).

2.8 ACC deaminase assay

The level of ACC deaminase activity was assessed by firstly growing each of the isolates in LB broth at 30^{o}C for 48 hours. Isolates were diluted 1:10 using 0.1M $MgSO_4$ and 22µl of the diluted culture along with 122µl of DF minimal medium (1.36% KH_2PO_4, 2.13% Na_2HPO_4, 0.2% $MgSO_4$. $7H_2O$, 0.7% $CaCl_2.H_2O$, 0.2% $FeSO_4.7H_2O$, 0.04% $CuSO_4.H_2O$, 0.02% $MnSO_4.H_2O$, 0.02% $ZnSO_4.7H_2O$, 0.003% H_3BO_3, 0.007% CoCl. $6H_2O$, 0.004% $Na_2MoO_4.2H_2O$, 5mM ACC, 1.0% glucose) and was placed in 9 wells for each isolate (3 rows, 3 columns per row) in a 96 well microtitre plate. Each row per sample was given a different treatment, with the addition of 15µl of 0.1M $MgSO_4$ to the first row, 15µl of 0.1M $(NH_4)_2SO_4$ to the second row and 15µl of 3µM ACC being added to third row. A control was also prepared by adding 122µl DF media and 22µl of 0.1M $MgSO_4$ into three wells on the same 96 well plate. Absorbance readings were taken at an OD of 550nm (FLUOstar Omega microtitre plate reader; BMG labtech) at 0 hours, 24 hours, 48 hours, 72 hours, 96 hours and 120 hours. (Arshad et al., 2007).

2.9 Siderophore production test

Chrome azurol S (CAS) agar plates were prepared according to the method described by Alexander and Zuberer (1991). Each isolate was spot inoculated onto separate CAS plates along with controls known for siderophore production. The colonies were then grown at 37°C for 7 days and were inspected for a clear halo surrounding the colony as an indicator of siderophore production.

2.10.1 Bacterial DNA extraction via boiling lysis

A modified version of the boiling lysis method proposed by Junior et al. (2016) was used for bacterial DNA extraction. Cultures of each isolate was prepared in LB broth and grown for 72 hours at 30^{o}C, following which 2ml of each isolate was centrifuged at 13000 rpm for 5 minutes. The supernatants were then discarded and the pellets were suspended in 200µl of TE buffer before being subjected to boiling for 15 minutes. The Eppendorf tubes were then placed on ice for 15 minutes and centrifuged at 13000 rpm for 5 minutes. The supernatant containing DNA was transferred to a new Eppendorf tube and stored at -20^{o}C for further use.

2.10.2 PCR amplification of isolated bacterial DNA

The amplification of bacterial 16s DNA was carried out using the 16s primers E9F (5'-GAG TTT GAT CCT GGC TCA-3') (Hansen et al., 1998) and U151OR (5'-GGT TAC CTT GTT ACG T -3') (Reysenbach and Pace, 1995) in order to amplify a 1501bp section of the bacterial 16s gene. The PCR reaction was carried out in a 25µl reaction, containing 2.5µl DNA, 0.2µM forward and reverse primer, 1X New England Biolabs Ampliqon 2X Taq mastermix and the reaction was made up to 25µl using nuclease free water. The PCR reaction was run for 30 cycles according to the following parameters, initial denaturation at 95^{o}C for 5 minutes; cyclic denaturation at 95^{o}C 30 seconds, annealing at 55^{o}C for 30 seconds, extension at 68^{o}C for 90 seconds; and a final extension at 68^{o}C for 5 minutes (GeneAmp PCR system 9700; Applied biosystems). The PCR products were run on a 1% agarose gel in 1X TAE (20 mM Tris, 10 mM sodium acetate and 0.5 mM EDTA [pH 8.0]) at 80V for 90 minutes to ensure the correct size of the products and then the products were sent to the Central Analytical Facility of Stellenbosch University for 16s DNA sequencing. The sequence data was then compared to other 16s DNA sequences with a BLAST analysis.

2.10.3 Amplified Ribosomal DNA Restriction Analysis (ARDRA) of bacterial 16s DNA

Amplified 16s DNA PCR products as obtained in section 2.10.2 were digested in a digestion mix containing 12µl PCR product, 5U of *RSAI*, 2µl 10X restriction enzyme buffer, 0.2µl of BSA and the reaction was made up to a final volume of 20µl with nuclease free water. The reaction mixtures were digested for 3 hours at 37^{o}C. The digested samples (12µL) were added to 3µl of (gel red + orange G) DNA stain and then run on a 3% agarose gel and electrophoresed at 100 V in 1X TAE buffer. Visualization of DNA bands was done with the Enduro gel dock system and compared to the GeneRuler 100bp DNA ladder.

2.11.1 Plant growth trial (Vanadium stress)

The Isolates P1, P2, P3, P5 and P6 were inoculated in LB broth and grown at 30^{o}C until each of the cultures had reached the same OD in the range of between 0.4-0.6. *Brassica napus* seeds were surface sterilized and imbibed in separate purified endophyte cultures for 30 minutes each, with a control being prepared using pure sterilized LB broth. Once the seeds were imbibed, 12 seeds were planted, 3 per pot, for each endophyte and the control in the

vanadium free experiment, along with a further 12 for each of the vanadium treated samples. The seeds were planted in a soil mixture of 1:2, soil: silica sand that had been pre-watered with 80ml water for the vanadium free plants and 80ml of 350µM sodium metavanadate solution for the vanadium treated plants. The seeds were treated with 2ml of the endophyte culture per seed post planting, with the control being treated with 2ml of LB per seed. Each vanadium free plant was treated with 80ml of water and each vanadium treated plant was treated with 80ml of 350 µM sodium metavanadate solution every 3 days, for the duration of the growth period of 46 days. The plants were set up in a completely random design and grown under a 26/19^{o}C day/night temperature cycle with a 16/8 h light/dark cycle, at a photosynthetic photon flux density of 300 µmol photons.m^{-2} s^{-1} (during the day phase).

2.11.2 Harvesting and grinding

Plants were carefully removed from the pots to prevent damage to the stems or leaves and the roots were removed from the samples to prevent erroneous data interpretation as a result of damage caused during the separation of the roots from the soil. Three leaf samples of each plant was taken from the 2nd, 3rd and 4th trifoliate and were placed in foil packets of known weights in order to determine the plant fresh weights. Once the fresh weights were known, leaf samples were then incubated at 50°C for 48 hours, following which the dry weight of each sample was determined. Photographs of the 2nd, 3rd and 4th trifoliate of a randomly selected representative for each sample was captured using a Canon 80D digital camera (lens; Canon EF-S 10–18mmf/ 4.5–5.6 IS STM).

The remainder of the plant samples for each endophyte treatment was taken and ground up in a sterile mortar and pestle, using liquid nitrogen to maintain plant nutrient content integrity. The ground up leaf and root material were then stored at -80°C for further analysis via ICP-OES.

2.12 Evans blue assay (cell death)

A modified method of Sanevas et al. (2007) was used for the cell death assay. A 0.25% (w/v) Evans blue solution was prepared and 1 ml of the solution was then aliquoted into Eppendorf tubes. A 1cm^3 block was excised from fresh leaf material an inserted into an Eppendorf containing the Evans blue solution. The samples were then incubated for 1 hour at room

temperature in the Evans blue solution. After the incubation period the Evans blue was rinsed from the samples. These samples were then incubated in water overnight. The water was decanted and 1 ml of a 1% (w/v) SDS solution was added to the sample. The samples were crushed in the SDS solution and incubated at 65°C on a heating block for 1 hour. After incubation the samples were centrifuged to pellet the plant material and obtain the supernatant. The supernatants were then added to a 96 well microtitre plate and read at a wavelength of 600nm on a spectrophotometer.

2.13 A Spectrophotometric assay for superoxide content determination

A modified version of the method proposed by Russo et al. (2008) was used to determine superoxide content. An Eppendorf tube containing 10 mM KCN (to inhibit Cu/Zn SODs), 10 mM H_2O_2 (to inhibit Mn and Cu/Zn SODs), 2% SDS (to inhibit Mn and Fe SODs) and 80 µM NBT was prepared, the solution in the tube was then made up to a volume of 800 µl using a solution of 50 mM potassium phosphate (pH 7.0). Eight 1 cm3 squares were cut from fresh leaf material and carefully inserted into the above prepared solution in the Eppendorf tube. The root samples were prepared by making 4 cm cuttings from the tip of the root and inserted into a tube with the above prepared solution. This was repeated in triplicate for both the leaves and roots. The plant material was then incubated for 20 minutes within the solution. Once the incubation was completed the plant material was crushed using a miniature pestle. The tube was then centrifuged at 13000 x *g* for 5 minutes to pellet the crushed plant material and the supernatant was removed carefully and added to a clean Eppendorf tube. Once the supernatant (sample) was free of plant material it was loaded onto the microtitre plate by adding 200 µl into a well. This process was repeated for untreated as well as treated plant samples. The samples were then read at a wavelength of 600 nm and a calculation taking into consideration the extinction coefficient of 12.8mM. cm^{-1} was used to determine the superoxide. The intensity of the blue colour produced by the reaction was an indication of superoxide levels.

2.14 A Spectrophotometric assay for hydrogen peroxide content determination

A modified version of the method proposed by Velikova et al. (2000) was followed to determine H_2O_2 content in the plant material. The standards for this assay (0 nM, 5000 nM, 10000 nM, 15000 nM, 20000 nM, 25000 nM, 30000nM, 35000nM, 40000nM, 45000nM,

50000nM, 55000nM, 60000nM, 65000nM and 70000nM) were prepared by diluting an appropriate volume of H_2O_2 in distilled water. The standards were then loaded in triplicate onto a microtitre plate. Samples were prepared by using TCA extraction on frozen ground plant material (prepared in section 2.11.2). Fifty microliters of the TCA extraction was added onto the plate. To the samples as well as the standards 1.25 mM dipotassium hydrogenphosphate (K_2HPO_4) and 250 mM potassium iodide (KI) was added. Once all the reagents were added to the appropriate wells, the plate was incubated on a shaker for 20 minutes at room temperature. The samples were then read at a wavelength of 390 nm.

2.15 A spectrophotometer assay to determine hydroxyl ion concentration

The method proposed by Halliwell et al. (1987) was used to determine Hydroxyl ion concentration. Frozen ground leaf material (50mg) was homogenized in a 1ml solution consisting of 10mM phosphate buffer containing 15mM 2-Deoxy-D-Ribose. The samples were then incubated at 37°C for 2 hours. After the incubation was completed, 0.7ml of the sample was taken and added to a reaction mixture containing 3ml of 0.5% (w/v) TBA solution made up in 5mM NaOH and 1ml glacial acetic acid. The samples were then briefly vortexed and then heated at 100°C for 30 minutes. After the incubation the samples were chilled on ice for 5 minutes and then centrifuged at 10000 x *g* for 5 minutes. The supernatants of each sample were then plated in triplicate on a 96 well microtitre plate and read at 532nm and 600nm, with the extinction coefficient of $155mM^{-1}. cm^{-1}$. being used to determine the Hydroxyl ion concentration from the results.

2.16 A spectrophotometric assay to determine lipid peroxidation by quantifying MDA

A modified version of the method proposed by Zhang et al. (2007) was used for quantifying the levels of MDA in samples. The assay was carried out by weighing out 100mg of frozen ground plant material per sample and this was added into separate 2ml eppendorf tubes. To each eppendorf tube, 5 volumes of 6% (w/v) trichloroacetic acid (TCA) was added, following which the eppendorf tubes were centrifuged at 13000 x *g* for 10 minutes. Following the centrifugation, 200µl of the supernatant was added to a clean eppendorf tube containing 300µl of 0.5% TBA. The samples were then boiled at 90°C for 20 minutes and chilled on ice for 10 minutes. A final centrifugation step was then carried out at 13000 x *g* for 5 minutes.

The supernatant for each sample was loaded on a 96 well microtitre plate and the absorbance readings were taken at 532nm and 600nm.

2.17 Protein extraction

Protein extraction was done in triplicate from untreated as well as vanadium treated plant leaf tissue by adding 100 mg of frozen ground leaf material to three individual Eppendorf tubes (three tubes per plant sample). Protein extraction buffer (0.5 ml) [0.004 M phosphate buffer, 1 mM EDTA and 5% (w/v) PvP] was then added to one of the three tubes. The homogenate in the tube was then further mixed using a vortex. After the mixture had been adequately mixed, the plant material was then pelleted in a centrifuge at 12000 x *g* for 5 minutes. The supernatant was then removed and transferred in to the second tube containing another 100 mg leaf material. The previous steps were then repeated for the second and third tube. The supernatant was then removed from the third tube and inserted into a clean Eppendorf tube. The protein concentrations were then quantified using a Bradford assay. Thereafter, the protein samples were stored at -20°C.

Antioxidant spectrophotometric assays

2.18 Spectrophotometric assay for catalase activity determination

A modified version of the method proposed by Aebi (1984) was used to determine catalase activity. A 1ml reaction mixture was prepared, in triplicate per sample, in a cuvette containing 0.5mM EDTA, 100mM K_2HPO_4 (pH 7.0), and 20µl of the experimental sample's protein extract. Prior to recording the absorption reading of each sample, 1mM H_2O_2 was added to the reaction mixture and the absorbance was recorded at 240nm. The principle behind the assay relies on the decrease in absorbance due to the dissociation of H_2O_2 and an extinction coefficient of 39.4 $mM^{-1}.cm^{-1}$ was used to determine the level of catalase activity based on the above-mentioned decrease.

2.19 A Kinetic spectrophotometric assay to determine total ascorbate peroxidase activity

Ascorbate peroxidase activity was determined according to the method proposed by Gokul et al. (2016). Frozen plant protein material of a known concentration was aliquoted into eppendorf tubes and incubated with 2mM ascorbate for 5 minutes. After the incubation 10µl of the protein sample was loaded in triplicate into 3 separate cuvettes and 71.34 mM K_2HPO_4

and 0.36mM ascorbate was added to each sample. The reaction required to determine the ascorbate peroxidase activity was started by adding 0.714mM H_2O_2 to each sample immediately prior to reading them at 290nm on a spectrophotometer.

2.20 Spectrophotometric assay to determine total Superoxide dismutase activity

Superoxide dismutase activity was determined according to the method proposed by Stewert and Bewley (1980). Plant protein samples of a known concentration were diluted to 1mg/ml and then 10µl of each sample was loaded in triplicate in a 96 well microtitre plate. To each sample, 20mM phosphate buffer, 0.1mM Nitrotrezolium blue chloride (NBT), 10mM methionine, 0.005mM riboflavin and 0.1mM EDTA before making it up to 200µl with distilled water. The microtitre plate was then incubated on a light box for 20 minutes at room temperature, following which the samples were read at 560nm on a spectrophotometer.

2.21 Data interpretation and statistical analysis

The translocation factor (TF) was estimated as follows: TF=concentration of metal found in leaf/Concentration of metal found in root. All statistical data was analysed using one –way analysis of variance (ANOVA) and tested for statistical significance using the GraphPAD Prism 6 software via the Tukey- Kramer test at a 5% level of significance.

Chapter 3

Plant growth promoting effects of endophytic bacteria on *Brassica napus* physiology

3.1 Plant growth promotion trials

3.1.1 Seed germination rates

Table 2: Table showing the germination percentages of endophyte and control treated plants.

Treatment	Day 4	Day 5	Day 6	Day 10
Control	(11%)	(55.5%)	(66.7%)	(66.7%)
P1	(22%)	(88.8%)	(100%)	(100%)
P2	(0%)	(66.7%)	(88.8%)	(88.8%)
P3	(0%)	(55.5%)	(55.5%)	(77.7%)
P4	(0%)	(55.5%)	(88.8%)	(88.8%)
P5	(22%)	(66.7%)	(88.8%)	(88.8%)
P6	(0%)	(44.4%)	(66.7%)	(77.7%)

The effects of inoculation with endophytic bacteria (P1, P2, P3, P4, P5, and P6) on germination rates in *Brassica napus* was recorded over 10 days, with almost all of the endophyte treated plants having higher germination percentages, after 6 days, when compared to the control treatments. The P1 strain had a perfect germination rate of 100% after 6 days of growth, with the remaining strains also showing definitive improvements in germination. Plants treated with endophytes P2, P3, P4, P5 and P6 had germination rates of 88.8%, 77.7%, 88.8%, 88.8% and 77.7% respectively when compared to the germination rate of the control plants which reached 66.7% after 10 days.

3.1.2 Comparative growth of plants

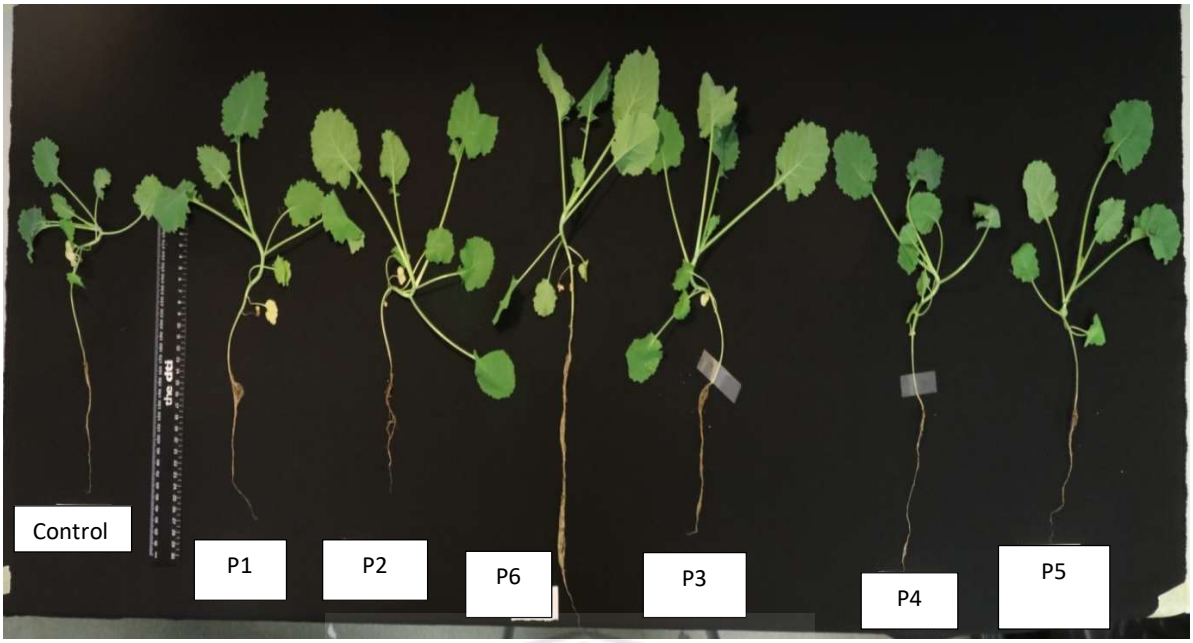

Figure 3: Showing growth of plants treated with different treatments. Plants were treated with six endophyte treatments (P1, P2, P3, P4, P5, and P6) and grown for 46 days prior to harvest

Endophyte treated plants displayed definite improvements in growth over the control, with longer stems and bigger leaves than the control plant. The P6 strain had the most significant improvement in growth with significantly larger root growth compared to the other treatments as well as having leaves present in a larger quantity than compared to the other plants. Endophyte strains P1, P2 and P3 also displayed improved growth compared to the control, with more rigid stems and larger roots compared to the control.

3.1.3 Biomass of leaves and stems

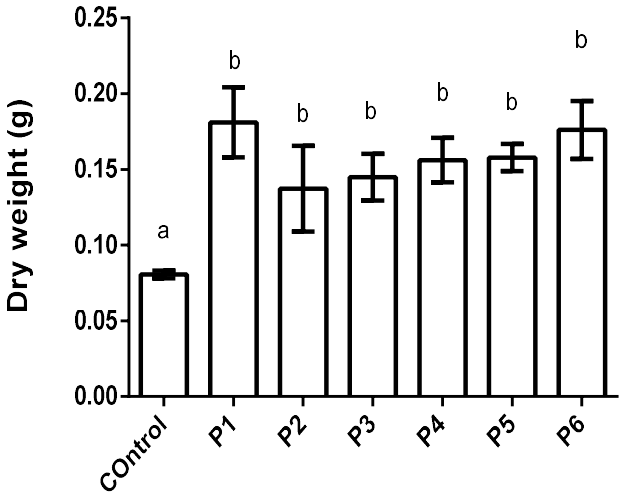

Figure 4.1: Dry weights of leaves of *Brassica napus* plants treated with different endophyte strains. Dry weights were taken after 46 days of growth. The different letters above each column indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=3).

The effect of inoculation with endophytes (P1, P2, P3, P4, P5, and P6) on leaf biomass in *Brassica napus* indicates a significant improvement among all isolate treated plants when compared to the control. Isolate P2 caused the smallest increase of 67%, while P1 caused the largest increase in comparison to the control at 75%. The increase among all of the leaf weights corresponds with the larger leaves observed in the comparative growth results.

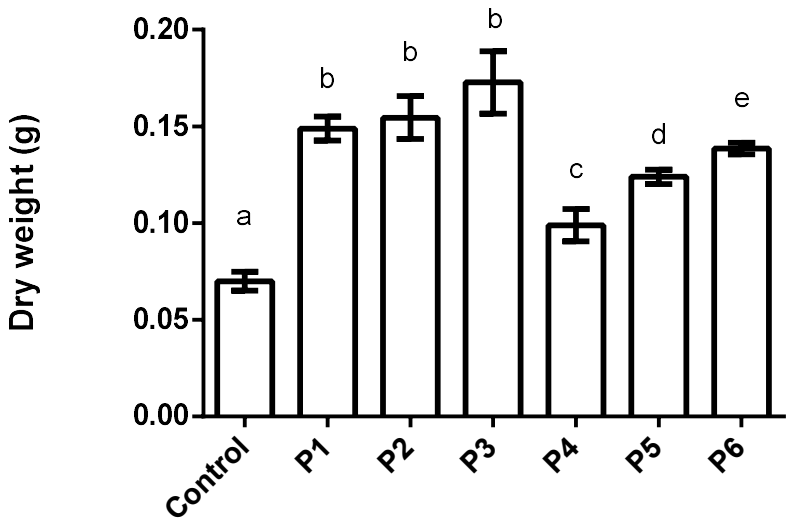

Figure 4.2: Dry weights of stems of *Brassica napus* plants treated with different endophyte strains. Dry weights were taken after 46 days of growth. The different letters above each column indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=3).

All isolates showed improvements in stem biomass after 46 days of growth, with isolates P1, P2 and P3 having statistically similar levels of growth in comparison with the control, with increases of 53%, 55% and 60% respectively. Plants treated with isolates P4, P5 and P6 also displayed significantly improved levels of stem biomass, with increases of 29%, 44% and 49% respectively in comparison to the control.

3.2 Inductively couple plasma optical emission spectrometry

Table 3: Showing comparative nutrient levels measured in control and experimental plants (mg/g) using ICP-OES.

Element	Control	P1	P2	P3	P4	P5	P6
K	3.5480^{a}	4.4430^{b}	4.7120^{c}	4.2270^{d}	4.4280^{b}	3.5880^{a}	3.8910^{e}
Ca	1.3690^{a}	1.7330^{b}	1.4700^{a}	1.4050^{a}	1.4020^{a}	1.6600^{b}	1.4540^{c}
Mg	0.2300^{a}	0.3110^{b}	0.2960^{c}	0.2920^{c}	0.2920^{c}	0.3200^{a}	0.3340^{c}
P	0.3330^{a}	0.4180^{b}	0.3600^{a}	0.3130^{c}	0.2997^{c}	0.3610^{a}	0.3040^{c}
Cu	0.0021^{a}	0.0021^{a}	0.0029^{b}	0.0035^{c}	0.0034^{c}	0.0027^{b}	0.0033^{c}
Mn	0.0070^{a}	0.0150^{b}	0.0090^{a}	0.0070^{a}	0.0040^{a}	0.0170^{b}	0.0090^{a}
Fe	0.0160^{a}	0.0240^{b}	0.0370^{c}	0.0240^{b}	0.0080^{d}	0.0370^{c}	0.0580^{e}
Zn	0.0120^{a}	0.0170^{b}	0.0160^{c}	0.0140^{a}	0.0140^{a}	0.0180^{b}	0.0360^{d}

The different letters above each number indicates statistically significant differences between means at P< 0.05 (Tukey-Kramer test), with statistical differences being compared per row. The colour scale indicates increased or decreased uptake in nutrients compared to the control, with green indicating an increase in nutrients and red indicating a decrease in nutrient content. The macronutrients and micronutrients were indicated with different coloured symbols, with the blue symbols indicating the macronutrients tested for and the green symbols indicating the micronutrients tested for.

The nutrient profile (whole plant) of the endophyte treated plants determined through ICP analysis, indicated a marked increase for all of the isolates for the nutrient's calcium, magnesium, copper and zinc, when they were compared to the control. There were 5 noticeable decreases in nutrient levels compared to the control plant, with P3 having a 6% decrease for its phosphorous content, P4 having a 10%, 34% and 53% decrease in phosphorous, manganese and iron nutrient content respectively and an 8% decrease in the phosphorous content of P6. All other nutrients tested for, among all isolates showed levels much higher than that found in the control plant, with isolate P1 produced the most consistent increase in nutrient uptake improvement, with an increase of at least 20% among all nutrients other than copper and increases of 55%, 31% and 33% for manganese, iron and zinc respectively.

3.3 Phosphate solubilisation plate experiment

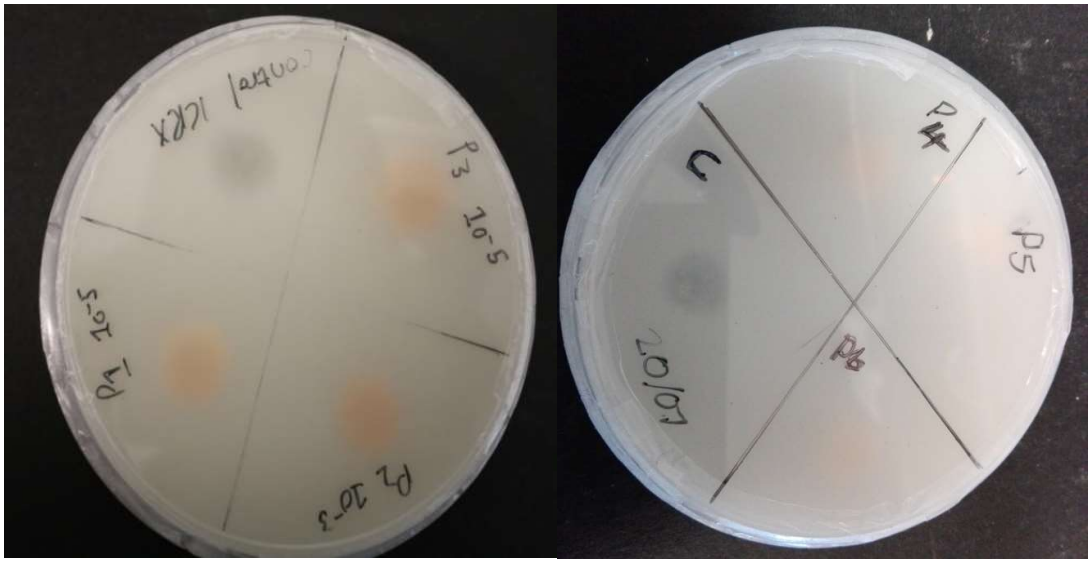

Figure 5: Image showing endophyte isolates P1-P6 plated on pikovskayas agar used for phosphate solubilisation test. Isolates were plated on pikovskaya agar and grown for 5 days at 37°C. A positive result for phosphate solubilization is indicated by a zone of clearing around the isolate.

Phosphate solubilisation was carried out on Pikovskaya agar plates and the results indicated that none of the endophyte strains were able to solubilize phosphate, as indicated by the lack of halo around any of the isolates and the corresponding halo showing a positive result around the *E. coli Krx* control.

3.4 Indole-3-acetic acid (IAA) assay

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

Endophyte strain	P1	P2	P3	P4	P5	P6
Concentration (µg/ml)	0.58^{a}	0.75^{b}	45.53^{c}	0.55^{d}	0.51^{e}	0.07^{f}

The letters above each number indicates which numbers are statistically significant, with those with the same number indicating a lack of statistical significance.

When testing for the production of indole-3-acetic-acid it was found that although all of the isolates were capable of IAA production, only isolate P3 was capable of a high level of IAA production, with all of the remaining isolates having levels of IAA below 1µg/ml.

3.5 Siderophore production plate experiment

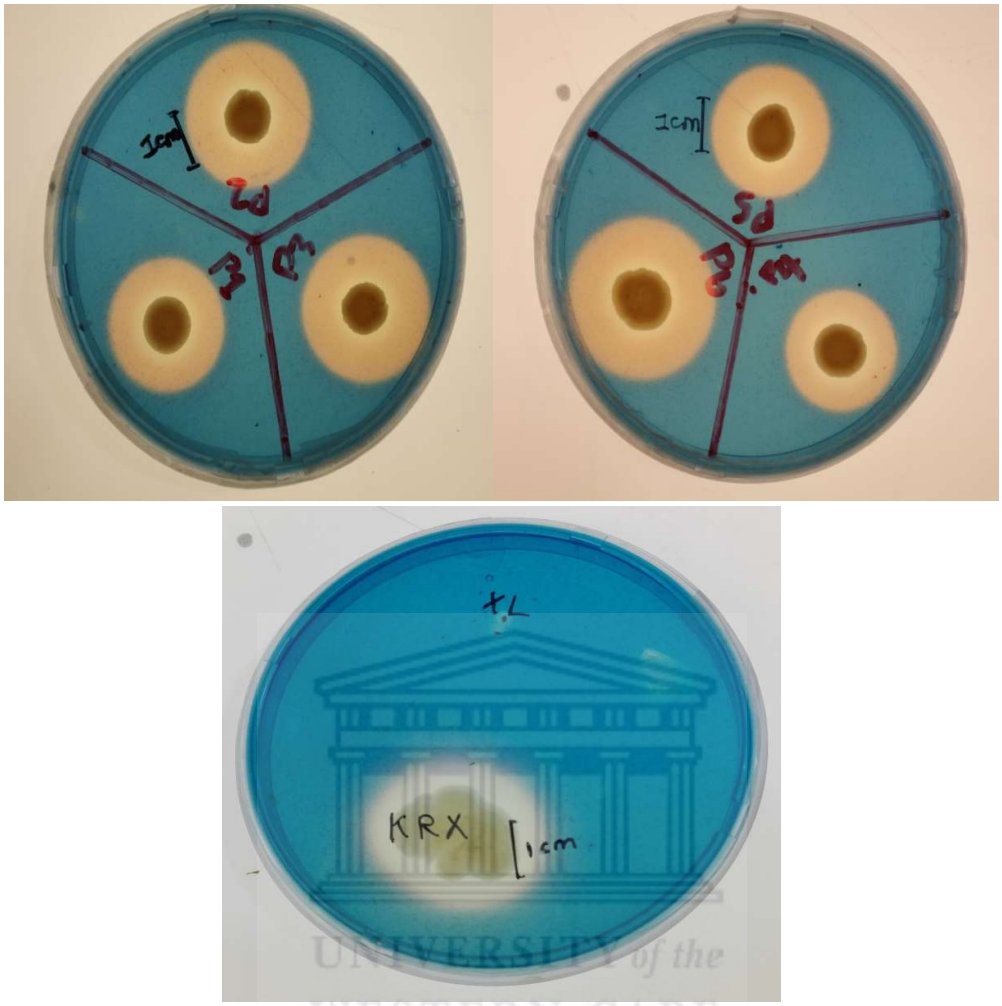

Figure 6: Showing siderophore activity of endophyte strains P1-P6 on Cas media plates. The zone of clearing around each colony indicates the presence of siderophore production, with the size of the clearing being directly proportional to the level of siderophore production.

All of the isolates were found to possess siderophore production capabilities, as determined by the zone of clearing around colonies P1-P6. Two *E. coli* strains, namely *E. coli XL gold and E. coli Krx* were used as controls respectively. The extent of siderophore production was determined by taking the diameter of the halo produced and subtracting it from the diameter of size of the colony. In terms of siderophore production based on the above mentioned measurement system, the isolates levels of activity were, P1 with 12mm, P2 with 14mm, P3 with 17mm, P4 with 11mm, P5 with 13mm and P6 with 17mm. Isolates P3 and P6 showed the

greatest level of siderophore production, while isolate P4 had the lowest activity level of siderophore production.

3.6 ACC deaminase assay

Table 5: ACC deaminase activity of each isolated endophyte strain.

Strain	ACC deaminase activity
P1	+
P2	+
P3	+
P4	+
P5	+
P6	+

The level of ACC deaminase activity was determined by comparison of growth of each isolate on $(NH_4)_2SO_4$, $MgSO_4$ and ACC.The comparison of the growth of each isolate in ACC to the other two additives after 5 days was used as an indicator of ACC deaminase activity. Those isolates that had growth that was similar or higher than the growth in $MgSO_4$ and $(NH_4)_2SO_4$ were said to have ACC deaminase activity.

All of the isolates were shown to be capable of ACC deaminase production as can be seen in figures 5-10, where the growth on media containing ACC among all isolates was found to be similar or higher than the growth on media containing $MgSO_4$ or $(NH_4)_2SO_4$. This result indicates that the isolates were able to cleave ACC into ammonia and α-ketobutyrate.

3.7 Discussion

3.7.1 Germination percentages

It was determined that all of the isolates were able to improve germination percentage. The increase in percentage was least significant in isolates P3 and P6, while P1 gave the most significant increase in germination percentage with all seeds germinating successfully. The definitive mechanisms behind which each endophyte was able to improve the germination rate of the plant seeds is not known; however, a number of different mechanisms could play a role. One of these mechanisms relies on the increased uptake of different nutrients into the plant, such as in the case of zinc which plays a major role in the proper development of seeds (Hansch & Mendel, 2009). It was also shown that those seeds that were deficient in zinc suffered from delayed germination compared to seeds not deficient in zinc (Hansch & Mendel, 2009). This means that since all of the endophyte treated plants showed improved

levels of zinc compared to the control, it could help to explain the increases levels of germination noted among these plants (Table 2). The control and regulation of the different plant hormones is also able to play a role in the onset and promotion of seed germination, with cytokinins, IAA, gibberelins and ethylene all contributing towards releasing the seed from its dormant state, thereby promoting germination (Liu et al., 2013). Many endophytes are both capable of producing the phytohormones IAA and cytokinins and can also play a role in the regulation of these hormones (Asaf et al*.,* 2017). This points to the possibility that the increase in germination noted in *Brassica napus* treated with the isolated endophytes could be attributed to endophytically produced IAA (Table 4).

3.7.2 Comparative growth of plants

When the growth of endophyte treated plants was compared to that of the non-treated control (figure 3), it was found that all of the treated plants displayed increased stem length, while also having bigger leaves in comparison to the control treatment.

This could be contributed to a number of mechanisms that each isolate used to promote growth in their respective host plants. One such mechanism was observed in canola seeds treated with endophytic bacteria, capable of producing both ACC deaminase and IAA which induced significant improvements in root growth in comparison to the control untreated plants (Antony et al*.,* 2015). This could help explain the improved growth in roots (Figure 3) in all of the endophyte treated strains in this study, as all the strains were able to produce ACC deaminase as well as produce IAA. A Similar improvement in plant growth was also noted among plants treated with a number of different bacterial species such as Acetobacter, Azotobacter, Azospirillum and Bacillus species (de-Bashan & Bashan et al*.,* 2005). In the study these improvements were contributed to a number of different mechanisms including phytohormone production, phosphate solubilisation, inhibition of plant ethylene production and enhanced nutrient uptake (de-Bashan & Bashan et al*.,* 2005). From this it can be determined that the growth in the isolate treated plants could be attributed to the isolates possessing a combination of the above-mentioned mechanisms for plant growth promotion.

3.7.3 Plant biomass

It has been demonstrated in different cases that plant biomass is positively impacted by the addition of plant growth promoting bacteria to the plant, with field experiments conducted on pomegranate yielding an increase in biomass of the treated pomegranate plants (Adesomoye & Kloepper, 2009). In this experiment the increase in biomass was thought to be as a result of the increase in the uptake of a number of nutrients by the plant including Ca, Mg, P, K and Cu (Adesomoye & Kloepper, 2009). This could explain the increase seen in the dry weights found among the stems and leaves of the endophyte treated plants, as the isolated strains were proven to be capable of nutrient upregulation according to the ICP-OES results (table 3), with all of the isolates showing enhanced nutrient uptake for K, Ca, Mg and Zn and most of the isolates showing upregulation in P, Mn, Cu and Fe.

A similar result was found when plants were inoculated with certain strains of plant growth promoting bacteria, which resulted in increases in the resulting biomass of the treated plants, in cases where the plant was grown in both normal soil conditions, as well as fertilizer treated soil (Nain et al*.,* 2012). This indicates that the nutrient condition of the soil can differ; however, the addition of beneficial plant growth promoting bacteria to the plant should still improve the biomass of the plant. The increase in biomass seen in the endophyte treated plants in this study could therefore be attributed to the effects of the inoculated endophytes, as this was the only notable difference between the control and treated plants.

The effect of endophytes on plants does not only involve an overall increase in biomass and plant size but can also include situations in which an increase in the weight or size occurs in either the roots, stem or leaves or in any two of these plant tissues, while the remaining tissues shows no significant improvement when compared to the control (Kadir et al*.,* 2008). This corresponds to the results found in this study where all of the isolates have a higher biomass than that of the control plant for both their leaves and stems; however, when looking at the morphology it can be seen that not all of the endophyte treated plants produced noticeably larger roots.

3.7.4 Inductively coupled plasma optical emission spectrometry (ICP-OES)

The upregulation of macronutrients and micronutrients in each of the treated plants as determined by ICP-OES is supported by the results in figure 3 where all of the endophyte treated plants have improved physiological growth compared to the control plants. The contribution of both macronutrients and micronutrients towards the improvement of growth within a plant is due to the varying roles which each nutrient has in the plant. This includes roles in photosynthesis, nitrogen assimilation, proper mitochondrial function, phytohormone production, protection from reactive oxygen species, production of proteins, production of nucleotides and in the formation of the plant structure (Maathuis, 2009; Hansch & Mendel, 2009). Although this is not the full range of functions carried out by macro and micronutrients in plants, it indicates the extent to which these nutrients are involved in the proper growth and function of plants. This also relies on the proper balance and availability of each nutrient inside of the plant, with a deficiency in any of these essential nutrients leading to the development of negative morphological phenotypes and a decrease in the overall growth of the plant, thus maintaining or improving the availability of nutrients for the plant to use should benefit its overall growth (Alatorre-Cobos et al.,2013).

The improvement in the level of nutrients among endophyte treated plants has been previously noted where the addition of plant growth promoting bacteria resulted in the improvement of the levels of both macronutrients and micronutrients in the host plant (Nain et al., 2012). This increase in nutrients was also found to have a positive correlation to the increase in size of the plant, a correlation which has also been noted in this study, with endophyte treated plants displaying significant increases in the uptake of both macronutrients and micronutrients (Table 3).

It has been determined that iron plays an important role in the development and metabolism of plants, as those plants that are deficient in iron have been shown to undergo chlorosis in the leaves (Hell & Stephan, 2013). The result of this usually leads to loss of the plant or a decrease in growth due to reduced function and hence the increased uptake of iron in isolate treated plants P1, P2, P3, P5 and P6 could be part of the reason for the improved growth observed in these treatments (Hell & Stephan, 2013). This correlates with the production of siderophores (Figure 6) by all of the isolates as this could lead to the improvement in the

levels of iron seen in the plant. Isolate P4, however was not shown to improve the uptake of iron present in the plant and was actually found to decrease the iron levels in comparison to the control plant. This could be as a result of the endophyte producing siderophores to increase the amount of available Fe^{3+}; however, this would then be taken up by the bacterium to be used for its metabolic processes.

The observed increase in phosphate levels among the ICP-OES results of strains P1, P2, and P5 does not coincide with the phosphate solubilisation results (figure 5); however, this could be due to there being more free phosphorous present in the soil environment, as it has been found that one of the more important reasons why phosphorous is not readily available to plants is because of its high reactivity with metals such as iron, aluminium and calcium (Adesomoye & Kloepper, 2009). This high reactivity causes between 75% and 90% of the phosphorous in the soil to be adsorbed or precipitated by these metal complexes (Adesomoye & Kloepper, 2009). This means that the facilitation of the improved uptake and assimilation of both iron and calcium by the plant, due to action from the endophyte, should have led to there being less of these elements to bind the phosphorous molecules in the environment. There could thus be more unbound phosphorous in the environment allowing for the improved uptake of phosphorous by the plant, even though the endophytic isolates were not able to solubilize phosphate. It should also be noted that the improvement in the size of the roots seen in the endophyte treated plants (Figure 3) , could be responsible for the increase in phosphorous levels among plants treated with isolates P1, P2 and P5, as it was found that deeper and larger roots can help plants scavenge for nutrients even in soils which are deficient in nutrients (Nain et al*.,* 2012).

Plants treated with endophytes P2, P3, P4, P5 and P6 showed increased levels of copper uptake in comparison to the control plants, with endophytes P3, P4 and P6 having the most significant effect on copper uptake (Table 3). Copper has been shown to be essential for normal plant growth as it is required for transcription, the transport of proteins and for the proper function of enzymes such as cytochrome oxidase and plastocyanin (Yruela, 2005; Vinit-Dunand et al., 2002). This points to the fact that the increase in copper noted among plants treated with endophytes P2, P3, P4, P5 and P6 could have contributed to improved growth in

the host plant by leading to the increased synthesis and mobilization of proteins required for growth and the enhanced activity of the plants photosynthetic machinery through the enhanced synthesis of plastocyanin.

Potassium has been shown to be involved in a number of processes in plants including as an activator of multiple enzymatic reactions, in the process of ribosome-controlled protein synthesis and as a regulator of enzymes on a transcriptional and post-transcription level (Maathuis, 2009). This means that the increase noted among all of the isolates in terms of potassium content among the plants could contribute towards plant growth through increasing the efficiency of the plant's enzymatic reactions and through the enhanced mediation of protein synthesis at a ribosome level.

Calcium plays an important role in two biological processes that are integral to a plant's development. The first of these is as a secondary messenger at a cellular level and the second is the important function of maintaining the integrity and rigidity of the plant cell wall (Maathuis, 2009). Its importance to the strength of the plant cell wall shows that it is required for improved plant growth. All isolate treated plants were shown to have increased calcium levels, which could have contributed to the stronger, more stable stems noted in the experimental plants.

Magnesium plays a number of crucial roles in plants, the most important of which being its presence in the chlorophyll molecule as one of its key structural molecules (Maathuis, 2009). It is also involved in photosynthesis, functions as a number of enzyme co-factors and is able to stabilize nucleic acids (Maathuis, 2009). Magnesium could thus have been considered to improve the growth of the treated plants by allowing for the production of an increased number of chlorophyll molecules in the leaves, as well enhancing the photosynthetic activity of the plant (Maathuis, 2009). These improvements are situated around the leaves and could therefore be considered as one of the reasons why the endophyte treated plants had bigger leaves than in the control plants.

Manganese has two important roles in plants including functioning as an activator for a number of key enzymes throughout plant cells and as a catalyst for various enzymatic

reactions throughout the plant (Hansch & Mendel, 2009). This means that increased uptake in manganese noted in plants treated with endophytes P1, P2, P3, P5 and P6 could have allowed for these plants to increase the activity in certain enzymes related to nutrient absorption or growth which could have contributed to the increase in growth noted among these plants.

Zinc is required as a major component of a large number of proteins such as metalloenzymes and transcription factors throughout plant cells (Sadeghzadeh, 2013). It is also involved in quite a few RNA and DNA polymerases, enzymes relating to DNA splicing and was found as part of certain tRNA synthetases (Hansch & Mendel, 2009). This points to the possibility that the increased uptake of zinc noted in plants treated with endophytes P1, P2, P5 and P6 could have contributed to the increase in growth seen among these plants by contributing to the increase in activity and synthesis of these zinc containing proteins.

3.7.5 Phosphate solubilisation

All of the isolates tested were able to grow on pikovskaya agar; however, none of the isolates were shown to produce a halo around the colonies indicating that none of the isolates were able to solubilize phosphate. This does not coincide with phosphorous results seen in table 3; however, pikovskayas agar only tests for organisms that can solubilize phosphate and it cannot determine if the endophyte is able to cause an increase in the level of phosphorous through other mechanisms.

3.7.6 IAA production

Indole-3-acetic acid has been determined to contribute to a number of important growth promoting effects in plants such as improved stem, root and leaf growth and an increase in plant biomass (Flinn & Mei, 2010).

Although all strains were able to produce IAA to a certain extent but only isolate P3 was able to produce levels of IAA that were considered to be significant according to levels of IAA produced by other plant growth promoting bacteria. In fact, the level of IAA production shown to be at 45.53 µg/ml was higher than that seen in previous studies which found average IAA production to be between 13.96µg/ml and 22.88µg/ml among the isolates tested (Nain et al., 2012). Furthermore, although the amount of IAA produced by the other strains was low it

could still be utilized by the plant for certain processes. This was determined to be the case for the development of primary root growth where levels of IAA as low as 1 nM to 1 pM could promote plant growth (Glick & Patten, 2002). This means that even though strains P1, P2, P4, P5 and P6 are only able to produce small amounts of IAA they are still able to influence the growth of the plant to a certain extent through IAA production.

3.7.7 Siderophore

The results observed in the siderophore plate assay (Figure 6) indicates that all of the isolated endophytes were capable of siderophore production, which is supported by the results in table 3, where plants treated with strains P1, P2, P3, P5 and P6 had increased levels of iron compared to the control. The production of siderophores among all isolates presents other avenues by which these isolates can promote plant growth other than through the upregulation of iron in the plants.

The first of these mechanisms is through the protection of the host plant from a variety of different pathogens, by acting as an antagonist for the pathogen's growth inside of the host plant (Ferrando et al*.,* 2011). This comes about because the siderophores produced by fungal pathogens have a lower affinity for Fe^{3+} molecules than siderophores produced by most endophytic bacteria allowing for the endophytes to take up most of the Fe^{3+} molecules in the environment (de-Bashan & Bashan et al*.,* 2005). This has the effect of inhibiting the growth of pathogens trying to colonize the plant thereby protecting the plant from infection.

Siderophores may also act to prevent the plants ability to uptake Fe in cases where the roots are not able to use the bound Fe molecules, thereby preventing an adverse build-up of Fe that might influence the plants growth (Alexander & Zuberer, 1991). The inability of the roots to take up iron in this case could be due to sufficient levels of iron being present in the plant which if exceeded, could disturb the nutrient balance in the plant leading to a negative impact to the plant (Alatorre-Cobos et al*.,*2013; Alexander & Zuberer, 1991).

The combined effects of the different functions of siderophores are thought to be partly responsible for the improvement in the morphology of the isolate treated plants in this study.

3.7.8 ACC deaminase

As all isolates were found to possess ACC deaminase activity it is likely that this activity contributed towards the overall growth improvements of the plant. This could be contributed to three different factors arising from the effects on ACC deaminase on the levels of ethylene in each of the endophyte treated plants.

The first is due to the initial effect of ethylene on the seeds, which causes the seeds to begin germinating; however, if the level of ethylene is sustained it can inhibit the formation of the initial roots that develop after seed germination (Glick & Penrose, 2003). Thus, the role of ACC deaminase after the beginning of germination is to cleave any ACC present to prevent the levels of ethylene from staying at this elevated level. With the decrease in the levels in ethylene, the initial root growth can proceed uninhibited and can in fact lead to longer roots formation due to the extent of the reduction in ethylene (Glick & Penrose, 2003). The development of longer roots during the sapling phase could also act as an extra layer of protection for the developing plants thereby allowing them to survive the early stages of development (Glick & Penrose, 2001).

The second factor is due to the increase in root and stem length noted in plants after inoculation with endophytes with ACC deaminase (Glick & Penrose, 2001; Arshad et al*., 2003*). This enhanced root and stem growth comes about from the same mechanism of ACC degradation as mentioned above; however, this reduction in ethylene comes about later in the plant's life cycle as levels of stress related ethylene rise due to exposure to different biotic and abiotic factors (Glick & Penrose, 2001).

The third factor is dependent on the improved root size that is present due to the effects of the first two factors. The increased root size allows for enhanced nutrient and water uptake from the environment, which causes improved growth in the plant (Spaepen et al*.,* 2007). This coincides with what was seen in this study as it was found that all of the endophyte treated plants had longer and thicker roots than the control (Figure 3).

In conclusion, in this chapter, it was observed that all of the isolated endophytes were able to promote growth in *Brassica napus* when compared to the control non-endophyte treated plant, with isolates improving morphological characteristics, as well as nutrient acquisition

within the host plants. Isolate P4, however showed the least beneficial growth promotion effects among the isolated endophytes and as a result it was decided that only isolates P1, P2, P3, P5 and P6 would be used in the ensuing vanadium stress growth trials.

Chapter 4

Effect of endophytic bacteria on the abiotic stress tolerance capacity of *Brassica napus* under vanadium stress

4.1.1 16s DNA sequencing of isolated endophytic bacteria

A 16s rDNA sequence analysis was performed on the amplified PCR products of isolates P1, P2, P3, P5 and P6 obtained using the universal 16s primers E9F and U1510R as described in section 2.10.2. The sequencing results indicated that all of the endophytes were shown to be members of the *Rhodococcus* genus specifically of the species *Rhodococcus qingshengii*. As a result of this it was necessary to perform an Amplified ribosomal DNA restriction analysis of the 16s rDNA sequencing results in order to determine if the endophytes are in fact the same strain of *Rhodococcus qingshengii* or different strains of *Rhodococcus qingshengii.*

4.1.2 Amplified ribosomal DNA restriction analysis of 16s DNA sequences

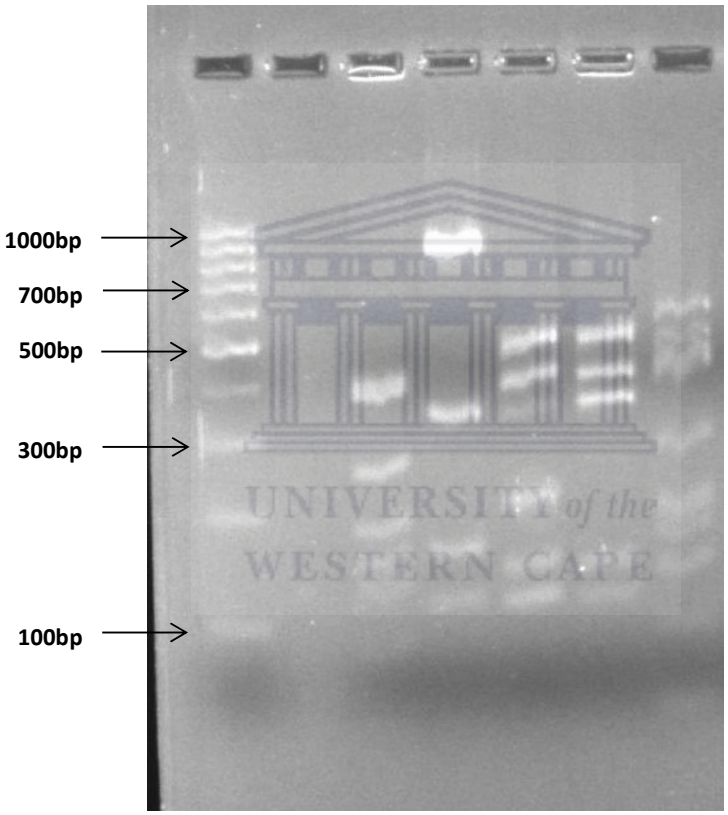

Figure 7: Showing a 3 % agarose gel of the Amplified ribosomal DNA restriction analysis of amplified PCR product: lane 1 – Gene ruler-100bp DNA ladder, lane 2 – control, lane 3 – P1, lane 4-P2, lane 5-P3, lane 6-P5, lane 7-P6.

The amplified ribosomal DNA restriction analysis of the digested 16s PCR products indicates that endophyte strains P1, P2, P3, P5 and P6 are 5 different strains of *Rhodococcus*

qingshengii, as indicated by the presence of unique banding patterns being seen among the digested DNA bands in each lane.

4.2 Comparative growth of plants

Figure 8.1: Showing growth of leaves in the 2nd, 3rd and 4th trifoliate of the control, P1 and P2 treatments under control and vanadium stressed conditions respectively. Plants were grown under non-stressed (water treated) and vanadium stressed conditions and grown for 46 days prior to harvest.

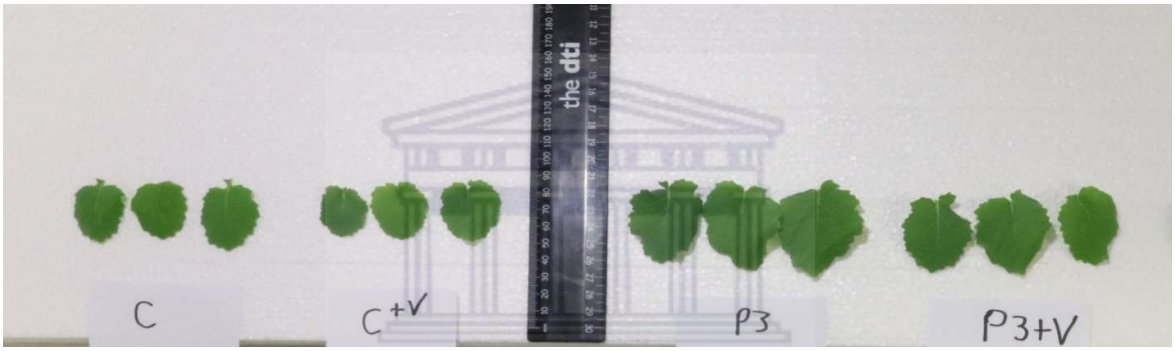

Figure 8.2: Showing growth of leaves in the 2nd, 3rd and 4th trifoliate of the control and P3 treatment under control and vanadium stressed conditions respectively. Plants were grown under non-stressed (water treated) and vanadium stressed conditions and grown for 46 days prior to harvest.

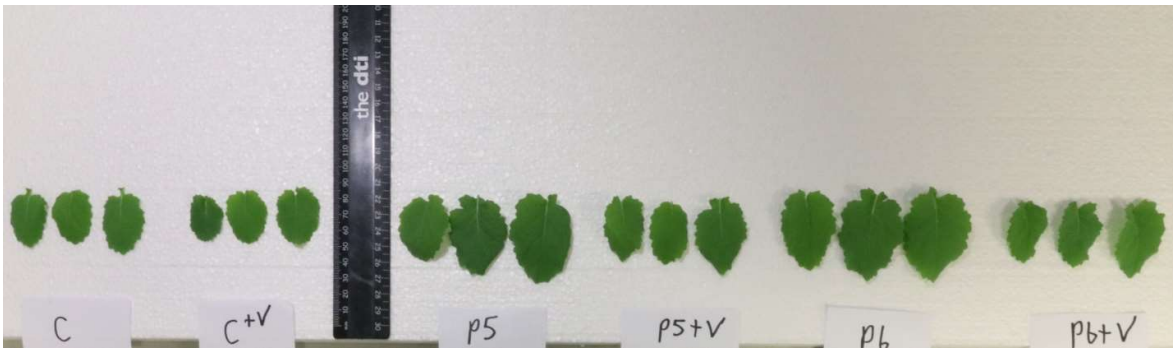

Figure 8.3: Showing growth of leaves in the 2nd, 3rd and 4th trifoliate control, P5 and P6 treatments under control and vanadium stressed conditions respectively. Plants were grown under non-stressed (water treated) and vanadium stressed conditions and grown for 46 days prior to harvest.

Non-stressed plants treated with endophytes (P1, P2, P3, P5, and P6) showed definite improvements in growth compared to the control, with visibly larger and darker leaves in each of the endophyte treatments. This is indicative of the overall growth improvement that was noted in the endophyte treated plants in comparison to the control treatment. Vanadium stressed plants showed a similar trend to their non-stressed counterparts, with the endophyte treated vanadium stressed leaves being noticeably larger and healthier than the control, with the control having an overall larger portion of the leaves displaying yellowing than compared to that found in the endophyte treated plants.

4.3 Biomass of leaves for non-stressed and vanadium stressed plants

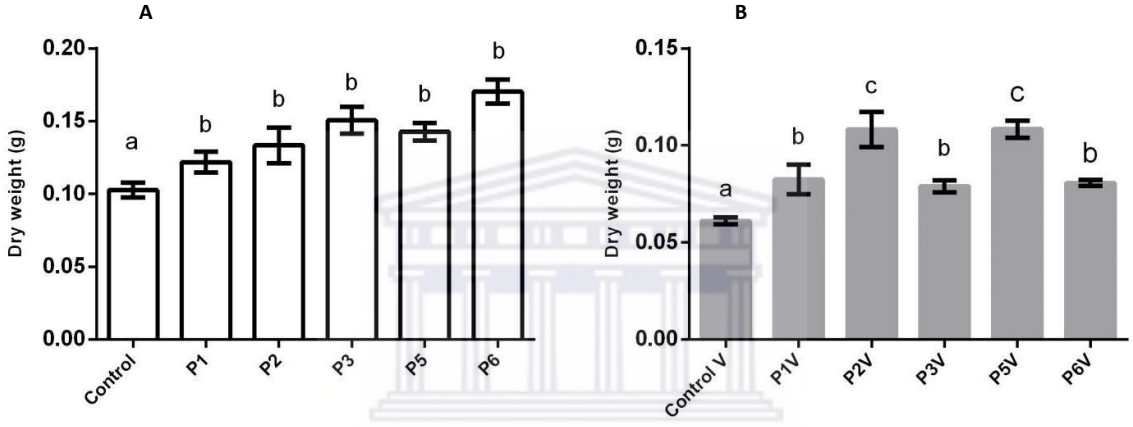

Figure 9: Biomass of leaves of *Brassica napus* plants treated with different endophyte strains (P1, P2, P3, P5 and P6) while under non-stressed (A) and vanadium stressed (B) conditions. The biomass was taken after 46 days of growth. The different letters above each column indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=3).

Brassica napus treated with endophytic bacteria under non-stressed conditions showed increases in biomass over the corresponding control with plants treated with isolates P1, P2, P3, P5 and P6 showing increases in biomass of 19%, 30%, 47%, 39% and 66% respectively over the control (Figure 9 A). A similar trend was seen in the vanadium stressed plants with each of the endophyte treatments showing an increase in leaf biomass over the vanadium stressed control, with isolates P1, P2, P3, P5 and P6 showing increases in biomass of 35%, 77%, 29%, 76% and 32% respectively (Figure 9 B). The vanadium stressed plants; however, displayed an

overall decrease in leaf biomass among all of the treatments in comparison to the corresponding non-stressed treatments, with the vanadium control showing a 40% decrease over the non-stressed control and P1, P2, P3, P5 and P6 showing a 32%, 19%, 48%, 25% and 53% decrease respectively (Figure 9).

4.4 Inductively coupled optical emission spectrometry

Table 6.1: Showing comparative nutrient levels measured in the leaves of control and experimental plants (mg/g) under non-stressed conditions using ICP-OES.

Element	Control	P1	P2	P3	P5	P6
K	5.355^{a}	6.007^{b}	5.941^{b}	6.592^{b}	6.429^{b}	5.498^{c}
Ca	2.074^{a}	2.228^{b}	2.772^{c}	2.056^{a}	1.718^{d}	2.004^{a}
Mg	0.517^{a}	0.592^{b}	0.740^{c}	0.577^{c}	0.484^{d}	0.508^{a}
P	0.496^{a}	0.494^{a}	0.874^{b}	0.504^{a}	0.475^{a}	0.461^{a}
Cu	0.0007^{a}	0.0006^{a}	0.0010^{b}	0.0006^{a}	0.0008^{a}	0.0004^{c}
Mn	0.0044^{a}	0.0045^{a}	0.0037^{a}	0.0026^{b}	0.0021^{c}	0.0027^{b}
Fe	0.0079^{a}	0.0087^{a}	0.0090^{a}	0.0153^{b}	0.0099^{a}	0.0058^{c}
Zn	0.012^{a}	0.014^{b}	0.011^{a}	0.011^{a}	0.008^{c}	0.009^{d}
V	0.0002^{a}	0.0002^{a}	0.0003^{b}	0.0004^{c}	0.0005^{b}	0.0004^{c}

The different letters above each number indicates statistically significant differences between means at P< 0.05 (Tukey-Kramer test), with statistical differences being compared per row. The colour scale indicates increased or decreased uptake in nutrients compared to the control, with green indicating an increase in nutrients and red indicating a decrease in nutrient content. The macronutrients and micronutrients were indicated with different coloured symbols, with the blue symbols indicating the macronutrients tested for, the green symbols indicating the micronutrients tested for and red indicating vanadium.

Table 6.2: Showing comparative nutrient levels measured in the leaves of control and experimental plants (mg/g) under vanadium stressed conditions using ICP-OES.

Element	Control V	P1V	P2V	P3V	P5V	P6V
K	6.4176^{a}	8.6121^{b}	6.6325^{a}	7.0022^{a}	6.5744^{a}	7.5561^{c}
Ca	2.7220^{a}	2.5776^{a}	2.4862^{a}	2.6245^{a}	2.1082^{b}	2.5130^{a}
Mg	0.5617^{a}	0.7055^{b}	0.5343^{b}	0.6187^{a}	0.5552^{a}	0.5523^{a}
P	0.6177^{a}	0.8487^{b}	0.4791^{c}	0.5994^{a}	0.6001^{a}	0.4994^{c}
Cu	0.0010^{a}	0.0016^{b}	0.0008^{a}	0.0011^{a}	0.0009^{a}	0.0026^{c}
Mn	0.0075^{a}	0.0077^{a}	0.0056^{b}	0.0085^{c}	0.0085^{c}	0.0059^{b}
Fe	0.0450^{a}	0.0162^{b}	0.0076^{c}	0.0107^{c}	0.0100^{c}	0.0077^{c}
Zn	0.0208^{a}	0.0195^{a}	0.0164^{b}	0.0263^{c}	0.0238^{c}	0.0160^{b}
V	0.0010^{a}	0.0012^{b}	0.0011^{b}	0.0008^{c}	0.0006^{c}	0.0007^{c}

The different letters above each number indicates statistically significant differences between means at P< 0.05 (Tukey-Kramer test), with statistical differences being compared per row. The colour scale indicates increased or decreased uptake in nutrients compared to the control, with green indicating an increase in nutrients and red indicating a decrease in nutrient content. The macronutrients and micronutrients were indicated with different coloured symbols, with the blue symbols indicating the macronutrients tested for, the green symbols indicating the micronutrients tested for and red indicating vanadium.

Table 6.3: Showing comparative nutrient levels measured in the roots of control and experimental plants (mg/g) under non-stressed conditions using ICP-OES.

Element	Control	P1	P2	P3	P5	P6
K	0.1935^{a}	0.2970^{b}	0.1817^{a}	0.3179^{c}	0.2215^{a}	0.2038^{a}
Ca	1.2536^{a}	1.4246^{b}	1.8041^{c}	2.6719^{d}	1.4359^{b}	2.8996^{e}
Mg	0.2488^{a}	0.3282^{b}	0.2706^{c}	0.4590^{d}	0.2765^{c}	0.3430^{b}
P	0.2343^{a}	0.3697^{b}	0.2757^{a}	0.4148^{c}	0.2887^{a}	0.2449^{a}
Cu	0.0013^{a}	0.0016^{a}	0.0019^{a}	0.0030^{b}	0.0019^{a}	0.0030^{b}
Mn	0.0040^{a}	0.0049^{b}	0.0088^{c}	0.0117^{d}	0.0085^{c}	0.0105^{d}
Fe	0.0442^{a}	0.0600^{b}	0.0850^{c}	0.1352^{d}	0.0784^{c}	0.1705^{e}
Zn	0.0083^{a}	0.0109^{b}	0.0118^{b}	0.0178^{c}	0.0129^{b}	0.0165^{c}
V	0.0003^{a}	0.0006^{b}	0.0005^{b}	0.0004^{a}	0.0004^{a}	0.0006^{b}

The different letters above each number indicates statistically significant differences between means at P< 0.05 (Tukey-Kramer test), with statistical differences being compared per row. The colour scale indicates increased or decreased uptake in nutrients compared to the control, with green indicating an increase in nutrients and red indicating a decrease in nutrient content. The macronutrients and micronutrients were indicated with different coloured symbols, with the blue symbols indicating the macronutrients tested for, the green symbols indicating the micronutrients tested for and red indicating vanadium.

Table 6.4: Showing comparative nutrient levels measured in the roots of control and experimental plants (mg/g) under vanadium stressed conditions using ICP-OES

Element	Control V	P1	P2	P3	P5	P6
K	0.2096^{a}	0.2426^{b}	0.2193^{a}	0.3358^{c}	0.3802^{c}	0.2330^{a}
Ca	2.4251^{a}	1.5217^{b}	1.1487^{c}	1.7201^{d}	2.2010^{a}	1.3513^{e}
Mg	0.2509^{a}	0.2631^{a}	0.2267^{b}	0.3603^{c}	0.4103^{d}	0.2526^{a}
P	0.2529^{a}	0.2938^{b}	0.2670^{c}	0.3874^{d}	0.4590^{e}	0.2997^{b}
Cu	0.0033^{a}	0.0019^{b}	0.0022^{c}	0.0022^{c}	0.0030^{a}	0.0018^{b}
Mn	0.0036^{a}	0.0055^{b}	0.0045^{c}	0.0086^{d}	0.0080^{d}	0.0066^{e}
Fe	0.0456^{a}	0.0508^{b}	0.0504^{b}	0.0665^{c}	0.0658^{c}	0.0615^{c}
Zn	0.0089^{a}	0.0123^{b}	0.0090^{c}	0.0172^{d}	0.0206^{d}	0.0125^{b}
V	0.0228^{a}	0.0135^{b}	0.0118^{b}	0.0122^{b}	0.0119^{b}	0.0134^{b}

The different letters above each number indicates statistically significant differences between means at P< 0.05 (Tukey-Kramer test), with statistical differences being compared per row. The colour scale indicates increased or decreased uptake in nutrients compared to the control, with green indicating an increase in nutrients and red indicating a decrease in nutrient content. The macronutrients and micronutrients were indicated with different coloured symbols, with the blue symbols indicating the macronutrients tested for, the green symbols indicating the micronutrients tested for and red indicating vanadium.

Table 6.5: Showing comparative translocation of nutrients from the roots to the leaves of experimental plants (mg/g) under vanadium stressed conditions using ICP-OES.

Element	Control V	P1V	P2V	P3V	P5V	P6V
K	3061%^{a}	3550%^{b}	3024%^{a}	2085%^{c}	1729%^{d}	3243%^{e}
Ca	112%^{a}	169%^{b}	216%^{c}	153%^{b}	96%^{a}	186%
Mg	224%^{a}	268%^{b}	236%^{a}	172%^{c}	135%^{d}	219%
P	244%^{a}	289%^{b}	179%^{c}	155%^{c}	131%^{d}	167%
Cu	31%^{a}	83%^{b}	36%^{a}	49%^{a}	29%^{a}	143%
Mn	209%^{a}	142%^{b}	124%^{b}	100%^{c}	107%^{c}	89%
Fe	99%^{a}	32%^{b}	15%^{c}	16%^{c}	15%^{c}	13%
Zn	235%^{a}	159%^{b}	181%^{c}	153%^{b}	116%^{d}	127%
V	4%^{a}	9%^{b}	10%^{b}	7%^{c}	5%^{d}	5%

The different letters above each number indicates statistically significant differences between means at P< 0.05 (Tukey-Kramer test), with statistical differences being compared per row. The colour scale indicates increased or decreased uptake in nutrients compared to the control, with green indicating an increase in translocation and red indicating a decrease in translocation. The macronutrients and micronutrients were indicated with different coloured symbols, with the blue symbols indicating the macronutrients tested for, the green symbols indicating the micronutrients tested for and red indicating vanadium.

The nutrient profile of the leaves and roots of endophyte treated plants and non-inoculated plants under non-stressed and vanadium stress conditions was determined through ICP-OES analysis.

Plants treated with endophyte P1 showed increased uptake in K, Ca, Mg and Fe by 12%, 7%, 14% and 15% respectively when compared to the control (Table 6.1). Plants treated with endophyte P2 showed increased uptake in K, Ca, Mg, P and Cu by 11%, 34%, 43%, 76% and 30% respectively when compared to the control (Table 6.1). Plants treated with endophyte P3 showed increased uptake in K, Mg and Fe by 23%, 12% and 94% respectively when compared to the control (Table 6.1). Plants treated with endophyte P5 showed an increased uptake of 20% in K when compared to the control (Table 6.1). Plants treated with endophyte P6 showed an increased uptake of 3% in K when compared to the control (Table 6.1).

Plants treated with endophyte P1 showed increased uptake in K, Mg, P and Cu by respectively when compared to the control (Table 6.2). Plants treated with endophyte P3 showed increased uptake in Mn and Zn by 13% and 26% respectively when compared to the control (Table 6.2). Plants treated with endophyte P5 showed increased uptake of 20% in K when compared to the control (Table 6.2). Plants treated with endophyte P6 showed an increased uptake of 3% in K when compared to the control (Table 6.2).

Plants treated with endophyte P1 showed increased uptake in K, Ca, Mg, P, Mn, Fe and Zn by 53%, 14%, 32% and 32%, 22%, 36%, 32% respectively when compared to the control (Table 6.3). Plants treated with endophyte P2 showed increased uptake in Ca, Mg, Mn, Fe and Zn by 44%, 9%, 119%, 92% and 43% respectively when compared to the control (Table 6.3). Plants treated with endophyte P3 showed increased uptake in K, Ca, Mg, P, Cu, Mn, Fe and Zn by 64%, 113%, 85%, 77%, 126%, 191%, 206% and 115% respectively when compared to the control (Table 6.3). Plants treated with endophyte P5 showed an increased uptake in Ca, Mg, Mn, Fe, Zn by 15%, 85%, 113%, 77% and 56% when compared to the control (Table 6.3). Plants treated with endophyte P6 showed an increased uptake in Ca, Mg, Cu, Mn, Fe, Zn by 131%, 38%, 126%, 161%, 286% and 99% when compared to the control (Table 6.3).

Plants treated with endophyte P1 showed increased uptake in K, P, Mn, Fe and Zn by 16%, 16%, 51%, 11% and 39% respectively when compared to the control (Table 6.4). Plants treated with endophyte P2 showed increased uptake in P, Mn, Fe and Zn by 6%, 25%, 10%, and 2% respectively when compared to the control (Table 6.4). Plants treated with endophyte P3 showed increased uptake in K, Mg, P, Mn, Fe and Zn by 64%, 85%, 77%, 191%, 206% and 115% respectively when compared to the control (Table 6.4). Plants treated with endophyte P5 showed increased uptake in K, Mg, P, Mn, Fe and Zn by 14%, 11%, 23%, 113%, 77% and 56% when compared to the control (Table 6.4). Plants treated with endophyte P6 showed increased uptake in P, Mn, Fe and Zn by 5%, 161%, 286% and 99% when compared to the control (Table 6.4).

4.5 Levels of cell death in *Brassica napus* under non-stressed and vanadium stressed conditions

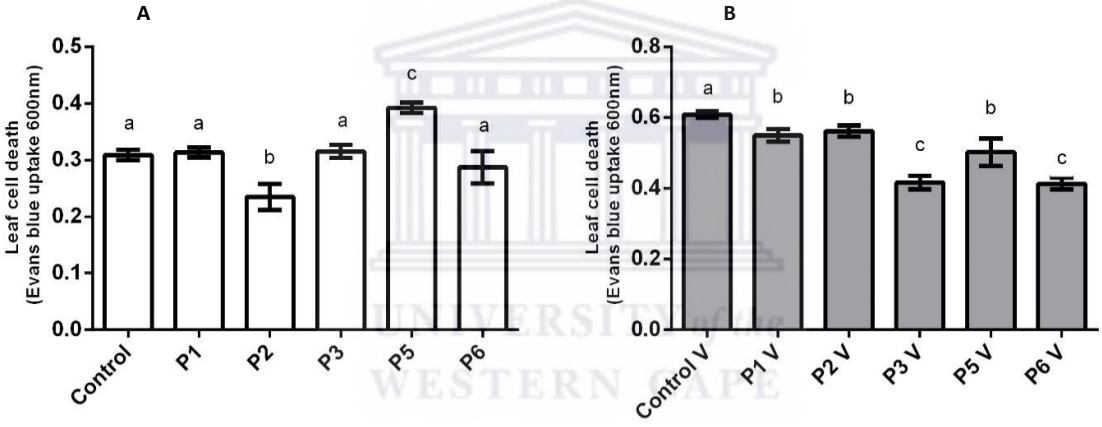

Figure 10: The effect of non-stressed (A) and vanadium stressed (B) conditions on cell death in *Brassica napus* treated with endophytic bacteria (P1, P2, P3, P5, and P6). The levels of cell death in the non-stressed and vanadium stressed plants determined after 46 days of growth using the Evans blue assay. The different letters above each column indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=3).

Levels of cell death in plants treated with isolates P1, P3 and P6 under non-stressed conditions had levels of cell death that were statistically similar to the control (Figure 10 A). Plants treated with isolate P2 under non-stressed conditions showed a 24% decrease in cell death; however, not all of the isolates showed beneficial effects on cell death with isolate P5

displaying a 27% increase in cell death over the control (Figure 10 A). In the vanadium stressed growth trials all of the endophyte treatments showed an ameliorating effect on cell death in comparison to the vanadium stressed control with P1, P2 and P5 having statistically similar levels of reduction in cell death and isolate P3 and P6 showing a decrease of 32% and 31% respectively in comparison to the control (Figure 10 B). The results further indicate that although the endophyte treatments showed beneficial effects under vanadium stress when compared to the control, there was still an overall increase seen in cell death in the vanadium stressed treatments compared to the non-stressed treatments, with the vanadium stressed control, P1, P2, P3, P5 and P6 showing increases of 95%, 87%, 143%, 31%, 31% and 31% respectively.

4.6 Superoxide content in *Brassica napus* under non-stressed and vanadium stressed conditions

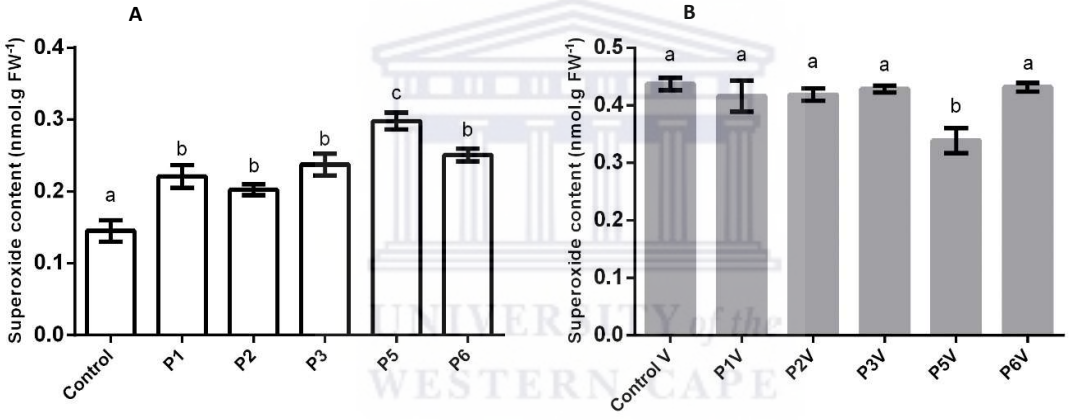

Figure 11: The effect of non-stressed (A) and vanadium stressed (B) conditions on levels of superoxide in *Brassica napus* treated with endophytic bacteria (P1, P2, P3, P5, and P6). The levels of superoxide in non-stressed and vanadium stressed plants determined after 46 days of growth. The different letters above each column indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=3).

Levels of superoxide in under non-stressed conditions (Figure 11 A) in plants treated with endophytes P1, P2, P3, P5 and P6 showed an increase of 52%, 39%, 64%, 105% and 73% respectively when compared to the control. This trend is not seen in the vanadium stressed treatments (Figure 11 B) as it can be seen that other than endophyte P5, which showed a 23% decrease over the vanadium stressed control, all of the remaining endophytes had levels of

superoxide that was statistically similar to the vanadium stressed control. When comparing the vanadium stressed treatments to the control, it was noted that all of the treatments showed increases in superoxide over their non-stressed counterparts with control showing a 201% increase over the non-stressed control and endophytes P1, P2, P3, P5 and P6 showing increases of 120%, 141%, 79%, 33% and 71% respectively.

4.7 Hydrogen peroxide content in *Brassica napus* under non-stressed and vanadium stressed conditions

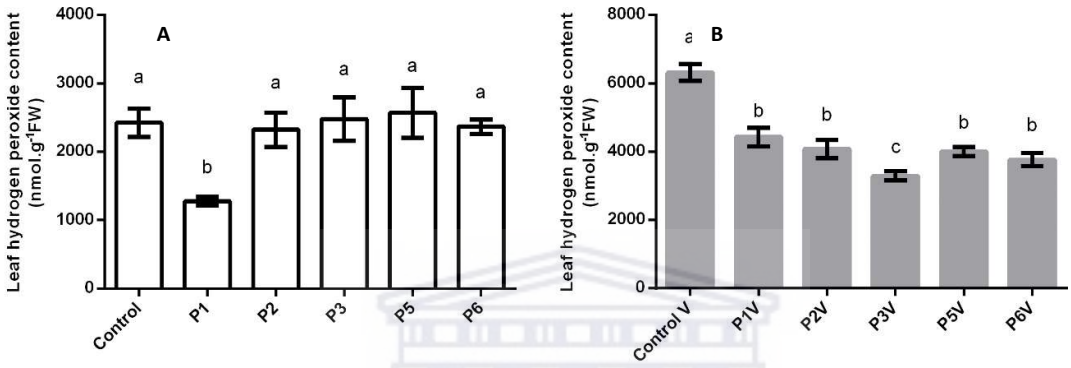

Figure 12: The effect of non-stressed (A) and vanadium stressed (B) conditions on hydrogen peroxide levels in *Brassica napus* treated with endophytic bacteria (P1, P2, P3, P5, and P6). The levels of hydrogen peroxide in non-stressed and vanadium stressed plants were determined after 46 days of growth. The different letters above each column indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=3).

Plants under non-stressed conditions that were treated with endophyte P1 showed a beneficial decrease in H_2O_2 of 47% when compared to the non-stressed control, whereas the other endophyte treatments showed a level of hydrogen peroxide that was statistically similar to the non-stressed control (Figure 12 A). In the vanadium stressed trials (Figure 12 B) all of the endophyte treatments showed significant decreases in levels of hydrogen peroxide over the vanadium stressed control, with P1, P2, P5 and P6 all showing statistically similar decreases of between 30% and 46%, with P3 showing the largest decrease of 48% compared to the vanadium stressed control. It was also determined that vanadium increased the levels of hydrogen peroxide in all treatments, with the control, P1, P2, P3, P5 and P6 showing increases of 161%, 129%, 52%, 33%, 154% and 44% respectively.

4.8 Hydroxyl ion concentration in *Brassica napus* under non-stressed and vanadium stressed conditions

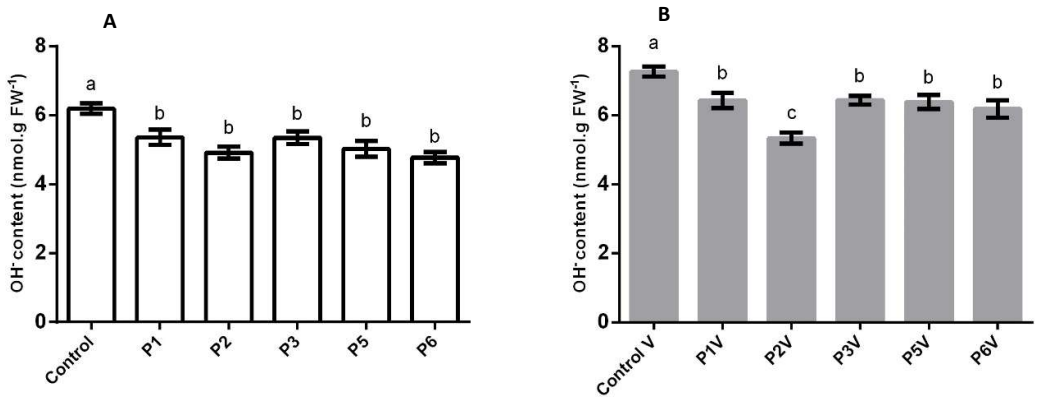

Figure 13: The effect of non-stressed (A) and vanadium stressed (B) conditions on the levels of Hydroxyl radical (•OH) in *Brassica napus* treated with endophytic bacteria (P1, P2, P3, P5, and P6). The levels of Hydroxyl ion in non-stressed and vanadium stressed plants were determined after 46 days of growth. The different letters above each column indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=3).

The levels of hydroxyl radical under non-stressed conditions (Figure 13 A) showed a significant decrease in all plants treated with endophytes with P1, P2, P3, P5 and P6 showing decreases of 13%, 21%, 14%, 19% and 23% in hydroxyl radical concentration compared to the control, respectively. A similar trend was found in their vanadium stressed counterparts with endophytes P1, P2, P3, P5 and P6 showing decreases of 10%, 25%, 25%, 10% and 13% compared to the vanadium stressed control. Treatment with vanadium was also determined to increase the hydroxyl ion concentration in all treatments compared to the non-stressed treatments, with treatments showing an increase of 15%, 20%, 9%, 20%, 27% and 29% for the control, P1, P2, P3, P5 and P6 respectively (Figure 13).

4.9 Levels of lipid peroxidation in *Brassica napus* under non-stressed and vanadium stressed conditions

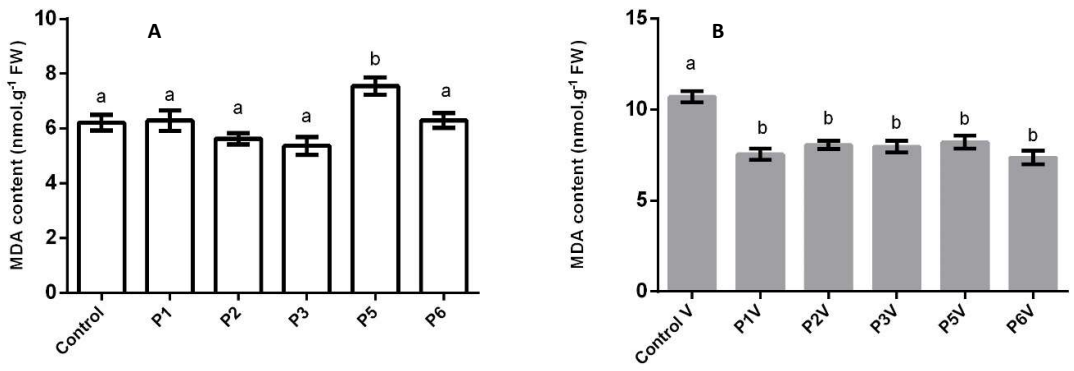

Figure 14: The effect of non-stressed (A) and vanadium stressed (B) conditions on the levels of lipid peroxidation in *Brassica napus* treated with endophytic bacteria (P1, P2, P3, P5, and P6). The levels of lipid peroxidation in non-stressed and vanadium stressed plants were determined after 46 days of growth. The different letters above each column indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=3).

The levels of lipid peroxidation in plants under non-stressed conditions (Figure 14 A) treated with endophytes P1, P2, P3 and P6 showed statistically similar levels of lipid peroxidation compared to the control; however, plants treated with endophyte P5 showed an increase of 22% compared to the non-stressed control. Exposure to vanadium stress was found to increase lipid peroxidation in all treatments with the control having an increase of 72% compared to the non-stressed control and plants treated with endophytes P1, P2, P3, P5 and P6 showing increases of 20%, 43%, 48%, 9% and 17% compared to their non-stressed counterparts respectively (Figure 14). Endophyte treated plants showed significantly reduced levels of lipid peroxidation compared to the control, with endophytes P1, P2, P3, P5 and P6 showing decreases of 30%, 25%, 26%, 23% and 31% respectively.

4.10 Levels of Superoxide dismutase activity in *Brassica napus* under non-stressed and vanadium stressed conditions

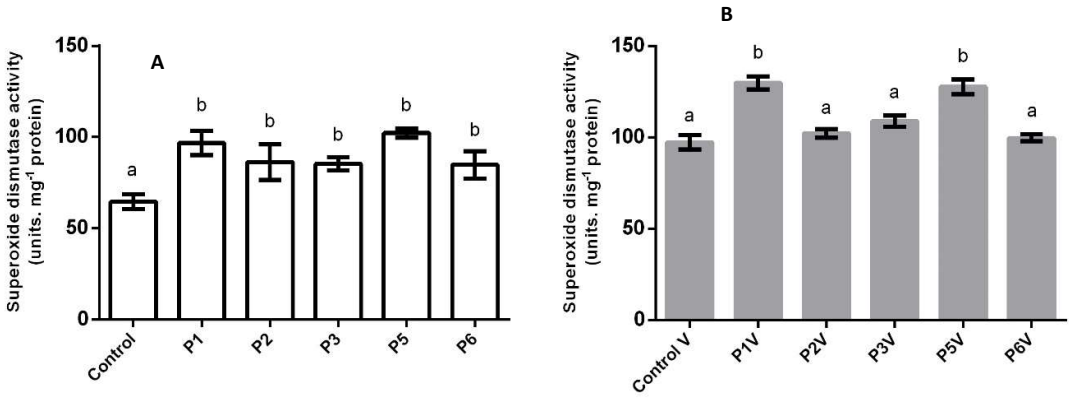

Figure 15: The effect of non-stressed (A) and vanadium stressed (B) conditions on the levels of Superoxide dismutase (SOD) in *Brassica napus* treated with endophytic bacteria (P1, P2, P3, P5, and P6). The levels of Superoxide dismutase in non-stressed and vanadium stressed plants were determined after 46 days of growth. The different letters above each column indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=3).

The levels Superoxide dismutase activity under non-stressed conditions (Figure 15 A) showed a significant increase in all plants treated with endophytes, with endophytes P1, P2, P3, P5 and P6 showing increases of 50%, 34%, 52%, 38% and 51% respectively. Vanadium stress was found to have upregulated the activity of SOD in all of the treatments with the control, P2, P3 and P6 having statistically similar levels of SOD and endophytes P1 and P5 displaying an increase in SOD activity of 33% and 31% respectively compared to the vanadium stressed control (Figure 15 B). The level of SOD activity in the vanadium stressed treatments was found to be significantly higher than the non-stressed controls with the control, P1, P2, P3, P5 and P6 showing levels of SOD that were 51%, 34%, 18%, 28%, 25% and 18% higher compared to their non-stressed counterparts (Figure 15).

4.11 Levels of catalase activity in *Brassica napus* under non-stressed and vanadium stressed conditions

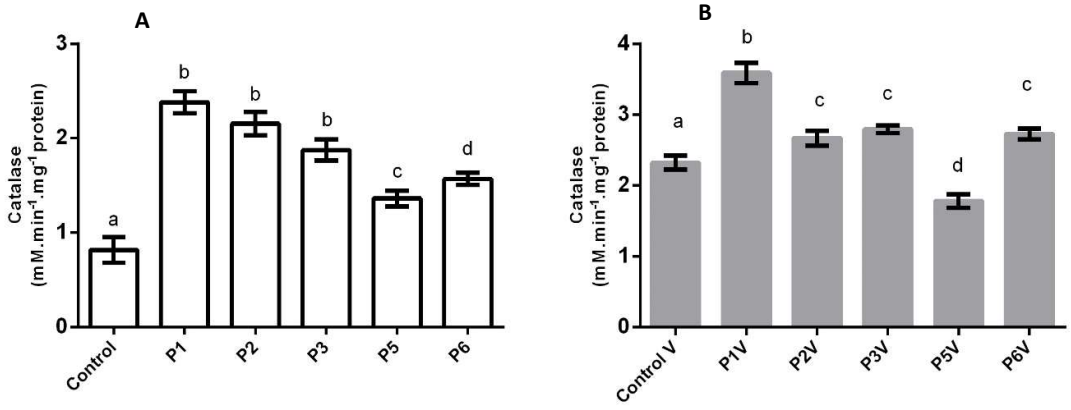

Figure 16: The effect of non-stressed (A) and vanadium stressed (B) conditions on the levels of Catalase in *Brassica napus* treated with endophytic bacteria (P1, P2, P3, P5, and P6). The levels of Catalase in non-stressed and vanadium stressed plants were determined after 46 days of growth. The different letters above each column indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=3).

The levels of catalase activity under non-stressed conditions showed a significant increase in all plants treated with endophytes, with endophytes P1, P2, P3, P5 and P6 showing increases of 191%, 164%, 129%, 67%, 234% respectively (Figure 16 A). Vanadium stress was found to have upregulated the activity of catalase in endophyte treatments P1, P2, P3 and P6 with these treatments showing levels of activity that was 48%, 10%, 15% and 12% higher than the activity of the vanadium stressed control (Figure 16 B). It was also found that endophyte P5 downregulated catalase activity by 27% compared to the vanadium stressed control (Figure 16 B). The level of catalase activity in the vanadium stressed treatments was found to be significantly higher than the non-stressed treatments with the control, P1, P2, P3, P5 and P6 showing levels of catalase that were 197%, 51%, 24%, 49%, 31% and 74% higher compared to the non-stressed treatments respectively (Figure 16).

4.12 Levels of Ascorbate peroxidase activity in *Brassica napus* under non-stressed and vanadium stressed conditions

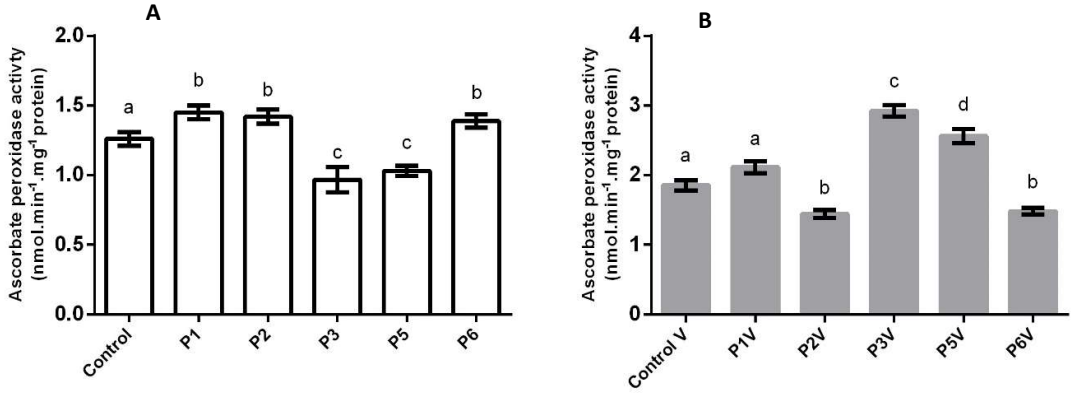

Figure 17: The effect of non-stressed (A) and vanadium stressed (B) conditions on the levels of Ascorbate peroxidase activity (APX) in *Brassica napus* treated with endophytic bacteria (P1, P2, P3, P5, and P6). The levels of Ascorbate peroxidase in non-stressed and vanadium stressed plants were determined after 46 days of growth. The different letters above each column indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=3).

The levels of ascorbate peroxidase activity under non-stressed conditions showed a significant increase in plants treated with endophytes, with endophytes P1, P2, and P6 showing increases of 15%, 13% and 18% respectively (Figure 17 A). Those plants under non-stressed conditions that were treated with endophytes P3 and P5 had significantly lower levels of ascorbate peroxidase activity with P3 and P5, showing decreases of 23% and 18% (Figure 17 B). Vanadium stress was found to have upregulated the activity of ascorbate peroxidase in endophyte treatments P3 and P5 with these treatments showing levels of activity that was 58% and 38% higher than the activity of the vanadium stressed control respectively (Figure 17 B). It was also found that endophyte P2 and P6 downregulated ascorbate peroxidase by 22% and 20% compared to the vanadium stressed control respectively (Figure 17 B). The level of ascorbate peroxidase in the vanadium stressed treatments was found to be significantly higher than the non-stressed treatments with the control, P1, P2, P3, P5 and P6 showing levels of catalase that were 47%, 46%, 2%, 203%, 148% and 7% higher compared to the non-stressed treatments respectively (Figure 17).

4.13 Discussion

The work reported here aimed to investigate the effects of vanadium stress on the morphology and biomass of *Brassica napus* that was treated with endophytes (P1, P2, P3, P5 and P6) and a control. As this chapter is not focused on determining the growth promoting effects of the endophytic bacteria, information on the germination and stem biomass have not been recorded as the focus has shifted more towards the internal biochemical changes that occurred in the leaves of *Brassica napus* under prolonged exposure to vanadium. In order to more accurately determine what changes occurred to the plants, experiments were carried out in order to determine the cell viability (cell death) and levels of lipid peroxidation. It was also necessary to determine how the oxidative state of the plants was affected as result of changes brought about by exposure to vanadium stress. As such experiments were carried out to see what levels of O_2^-, H_2O_2 and hydroxyl radical (•OH). With the change in levels of ROS within the plants it was also deemed necessary to ascertain the status of antioxidant enzymes which play a role in the detoxification of these reactive oxygen species, including SOD, Catalase and APX.

4.13.1 16s DNA sequencing of isolated endophytic bacteria

The 16s rDNA sequencing results indicated that all of isolated endophytes belonged to the species *Rhodococcus qinshengii* and upon subsequent amplified ribosomal DNA restriction digest analysis (ARDRA) of the amplified PCR products it was determined that the endophytes are all unique strains of *Rhodococcus qinshengii* (Figure 7). Rhodococcus are gram positive, aerobic, non-motile, mesophiles, that are found ubiquitously throughout the environment (Warhurst & Fewson, 1993).They possess a wide range of metabolic activities and can produce a multitude of secondary metabolic products, which makes them economically important as sources for the development of new and improved version of important prodcuts (van der Geize & Dijkhuizen, 2004; Xu et al., 2007). They have also been extracted from plant tissues before and have been shown to possess plant growth promoting abilities (Hong et al., 2016).

4.13.2 Comparative growth of plants

The addition of endophytes (P1, P2, P3, P5 and P6) to *Brassica napus* under both non-stressed and vanadium stressed conditions (Figure 8.1, 8.2 and 8.3) was found to have an overall

beneficial effect on the size and morphology of *Brassica napus* leaves, with leaves appearing greener and larger than the control. Vanadium stressed plants were also shown to have a marked decrease in leaf growth compared to their non-stressed counterparts, with signs of leaf rolling and yellowing in leaves; however, it was noted that those plants treated with endophytic bacteria (P1, P2, P3, P5, and P6) had visibly larger and healthier looking leaves than in the vanadium stressed control. These results indicate that endophyte P1, P2, P3, P5 and P6 are able to curtail the negative morphological effects caused by vanadium stress.

A similar result to that seen in the vanadium stressed control was found by Saco et al. (2013) who noted that exposure to vanadium in *Phaseoulus vulgaris* at concentrations at or above 240µM caused noticeably shorter and more rounded leaves, that displayed a markedly reduced surface area. The change in leaf morphology was attributed to vanadium causing a decrease in the number of chloroplasts present in the cells, thereby leading to a possible decrease in the photosynthetic activity in the leaves (Saco et al., 2013). This development was also noted in the leaf morphology of *Brassica juncea L.* grown in soil amended with vanadium. In the study it was seen that leaves displayed yellowing and withering symptoms, with the intensity of the symptoms showing a positive correlation to the concentration of vanadium, with the most severe symptoms noted from plants grown in the soil treated with the highest concentration of vanadium (Tian et al., 2015). This indicates that the adverse effects that vanadium has on plant morphology, significantly reduces plant growth and therefore the comparatively healthier and bigger leaves noted in the endophyte treated plants under vanadium stress are likely to be due to mechanisms employed by the endophytes themselves. This was supported by a study on maize under chromium stress conducted by Islam et al. (2016) who noted that exposure to chromium severely stunted the growth of maize leaves by 54% when compared to the stress-free control; however, the addition of the endophyte *Proteus mirabilis* strain *T2Cr* to maize under chromium stress, was able to reduce the extent of the damage to 32% when compared to the control. A possible reason for the improvement in morphology in endophyte treated plants could be due to endophytes upregulating the host plants genes for antioxidant synthesis, resulting in an increase in antioxidant activity allowing for the enhanced scavenging of harmful stress induced ROS (Lata et al., 2018; Hamilton et al., 2012).

The results seen in the non-stressed endophyte treated leaves coincided with morphological results seen in a study by JI et al. (2014) using rice cultivars. In the study it was found that rice plants treated with the endophyte Bacillus subtilis strain CB-R05 possessed significantly larger and healthier looking leaves, which was thought to be due to the endophytes ability to produce IAA, solubilize phosphate and produce siderophores. This coincides with what was seen in this study, as it should be noted that all of the endophytes possess the ability to produce IAA, are capable of siderophore production and were found to upregulate the uptake of nutrients in their host plant. The combination of these three factors are all thought to be contributing factors to the increase in plant size noted among plants treated with these endophytes.

4.13.3 Biomass of leaves for non-stressed and vanadium stressed plants

Vanadium stress (Figure 9 B) was found to cause a decrease in biomass compared to the corresponding non-stressed plants (Figure 9 A), furthermore it was found that treatment with endophytic bacteria increased leaf biomass in both treatment types compared to the control plants.

The result seen in this study, was supported by similar results seen in a study by Vachirapatama et al. (2011) that noted that leaves of Chinese green mustard treated with 80mg/L of NH_4VO_3 exhibited significantly reduced biomass in comparison to non-treated controls, indicating that exposure to vanadium stress significantly impacts the growth potential in most plants. These findings were supported by the results seen by Kaplan et al. (1990) who noted a decrease of up to 40% in the biomass of leaves in Soybean when treated with 6mg/L $VOSO_4$.

This significant decrease in biomass could be as a result of a decrease in the plants ability to absorb nutrients through the plasma membrane leading to a decrease in overall plant growth. The mechanism behind this is thought to be due to the increase in the intracellular concentration of vanadium leading to a decrease in the activity of hydrogen-translocation ATPase found at the plasma membrane. This inhibitory effect of vanadium is therefore able to impede the ability of the plasma membrane to efficiently transfer nutrients into the cell resulting in a deficiency in crucial nutrients required for plant growth (Wuilloud et al., 2000).

The beneficial increase exhibited in the biomass of endophyte (P1, P2, P3, P5, P6) treated plants under vanadium stress indicates that these endophytes posess mechanisms with which to ameliorate the deleterious effects that vanadium stress can impose on plant growth. This result could be due to a number of different mechansims that endophytes P1-P6 were previously found to possess such as in the case of the ability to synthesise ACC deaminase (Table 5). It has been noted before that increased levels of ethylene produced when a plant is under stress can have an inhibitory effect on plant growth and development (Nadeem et al., 2006). As such, since vanadium puts significant stress on plants exposed to it, then it should also contribute towards an increase in ethylene stress levels that could impact the overall growth of the affected plants roots, which in turn would result in a decrease in the growth and total biomass of the plant. Therefore, if an endophyte such as those used in this study is able to cleave ACC, the precursor for ethylene production, prior to the plant being able to utilize it to produce more ethylene, then it should decrease the overall amount of stress ethylene produced when the host plants are under stress (Glick, 2015; Nadeem et al., 2006; Zhang et al., 2011). This would thereby result in an increase in the resistance of the enodphyte treated plants to the imposing stress they are exposed to. This hypothesis was supported by the results seen by Zhang et al. (2011) who found that inoculation of *Brassica napus* with two different ACC deaminase producing bacteria, resulted in a significant increase in the biomass of the host plants when compared to the control (Zhang et al., 2011).

A similar result was seen in a study by Sheng et al. (2008) in which *Brassica napus* exposed to 800 $mg.kg^{-1}$ of lead (Pb) showed a marked decrease in biomass when compared to the non-stressed control, however, when the plants were treated with two different endophyte strains in separate treatments, the decrease in biomass was significantly smaller than in the control under lead without endophyte treatment. The beneficial effects seen in this study was deemed to be due to one of two mechanisms that the endophytic bacteria could utilize, namely ACC deaminase synthesis and through siderophore production.

This could also be due the endophytic bacteria utilizing their own ROS scavenging systems to augment the antioxidative systems of their host plant in conjunction with upregulating the synthesis of antioxidants in the host plant (Zaets et al., 2010; Pavlo et al., 2011). This

mechanism is important to alleviating the loss of biomass caused in plants under heavy metal exposure, as it has been seen in multiple studies that plants under heavy metal stress usually have high ROS concentrations which can lead to the development of aberrant morphological states such as decreased biomass, leaf chlorosis, lipid peroxidation and eventually cell death if left untreated (Zaets & Kozyrovska, 2012; Zhang et al., 2007)

4.13.4 Nutrient profile of Brassica napus under non-stressed and vanadium stressed conditions

Plants require specific nutrients in certain ratios in order to grow and develop properly. These nutrients consist of the macronutrients and micronutrients, with the former being required in large quantities and the latter being required in trace amounts for proper plant development (Maathuis, 2009; Hansch & Mendel, 2009). In light of the changes in morphology and biomass noted in the control and endophyte treated plants under vanadium stressed conditions, it was determined to be necessary to establish what effect vanadium stress had on the nutrient profile in non-stressed plants in comparison to the vanadium stressed plants.

In the leaves of plants under non-stressed conditions, it was found that all of the endophytes were able to upregulate one or more of the nutrients in comparison with the control. Among endophyte treatments P2 had the largest effect on nutrient uptake, with these plants showing significantly improved levels of K, Ca, Mg, P and Cu (Table 6.1). Endophytes P1 and P3, were also able to promote the uptake of multiple nutrients with P1 improving uptake in K, Ca, Mg and Zn and P3 causing an increase in the concentration of K and Fe. Endophytes P5 and P6 were shown to be only able to improve the uptake of K; however, they also decreased the concentrations for certain nutrients, indicating a certain antagonistic effect to the uptake of these nutrients. It should be noted though that the downregulation of nutrients seen in these plants should not have negatively affected the plant as leaves for plants treated with endophytes P5 and P6 were significantly bigger, healthier and had a higher biomass in comparison to the control (Figure 8.3). The increases in nutrients noted for the above treatments should have contributed to the increase in leaf size (Figure 8) and biomass (Figure 9 A) seen in the endophyte treated plants.

Vanadium stress was found to improve the nutrient uptake in the leaves of almost all treatments, with the control, P1, P3, P5 and P6 showing significant overall increases in all of the macronutrients and micronutrients (Table 6.2) that was tested for in this study. Endophytes P2 and P3 both displayed decreases in nutrients under vanadium stress, with P3 having decreased concentrations of Fe and plants treated with endophyte P2 having experienced decreases in the concentration of Ca, Mg, P, Cu and Fe when compared to plants treated with endophyte P2 under non-stressed conditions (Table 6.1). It should also be noted that there was an extremely low level of translocation of vanadium from the roots to the leaves (Table 6.5) with the control, P1, P2, P3, P5 and P6 showing translocation of 4%, 9%, 10%, 7%, 5% and 5% respectively. This indicates that it is highly likely that *Brassica napus* sequesters most of the vanadium that enters the plant inside the roots, in order to prevent the accumulation of vanadium in the upper plant tissues. This low level of translocation coincides with what has been previously seen in soybean grown in soil treated with 500mg kg^{-1} V. In the study it was found that the percentage of translocation from roots to the leaves of soybeans grown under these conditions was 1.8%, with the concentration of vanadium being highest in the roots, slightly lower in the stems and significantly lower in the leaves (Yang et al., 2017). The reason for this was thought to be as a primary mechanism of vanadium tolerance in soybean, as the roots would continue to accumulate vanadium over time, while the concentration of vanadium in the leaves and shoots would remain at much lower levels (Yang et al., 2017). This is thought to be the case in this study as well, as the concentration of vanadium in the leaves of the stressed plants, though significantly higher than in the non-stressed leaves, was only six fold higher (Figure 5.2); however, this was not the case in the roots, where at its lowest, V concentration was 23.2-fold higher than in the non-stressed roots, but at its highest was 76-fold higher in the vanadium stressed control roots as opposed to the non-stressed root (Table 6.4).

The roots of the non-stressed endophyte treated plants exhibited the most significant impact that endophytes had on the nutrient profile of their host plants seen in this study, as it was seen that all of these treatments displayed significantly higher levels in almost all of the macronutrients and micronutrients when compared to the non-stressed root control (Table 6.3). This can be seen as one of the possible reasons why plants treated with endophytes P1,

P2, P3, P5 and P6 were larger and healthier, with longer and thicker roots compared to the endophyte free control, as was seen in the previous growth trial (Figure 3). In addition to this it can be assumed that the increase in overall nutrient uptake seen in the leaves in the second growth trial, is in part due to the increased concentration of nutrients seen in the endophyte roots as it allowed for an enhanced pool of nutrients for the leaves to draw from (Table 6.1).

Vanadium stress was found to significantly disrupt the nutrient uptake efficiency in all treatments in the roots, with vanadium being the only nutrient that showed a consistent increase in all treatments (Table 6.4). When comparing the endophyte treatments to the control, it was found that except for a slight decrease in the concentration of Mn, the control was the only treatment among the vanadium stressed root treatments, that had increased uptake for all of the remaining macronutrients and micronutrients. This appears to be a positive development; however, when taking into account that the same trend was found in the vanadium stressed leaves and the abnormal morphology and low biomass that was noted in the leaves, it can be inferred that the roots were likely to have experienced aberrant morphological and ultrastructural changes as well (Table 6.4).

The improvements found in the nutrient profile in the roots and the leaves of the non-stressed endophyte inoculated treatments were attributed to the effects of different plant growth promoting mechanisms utilized by the endophytes, including facilitating the improved uptake of nutrients from the environment through the release of compounds such as siderophores, the regulation of phytohormones such as IAA and ethylene and the general improvement of the size and health of the roots (Figure 3) of the plant allowing for increased absorption of nutrients from the environment (del Carmen Orozco-Mosqueda et al., 2016). A similar result to this was seen in lentil plants inoculated with *Providencia vermicola,* which resulted in the upregulation of K, P, Ca, Fe, Cu and Zn, when compared to the non-inoculated control, which was in part due to *P. vermicola* producing siderophores, solubilizing phosphate and due to the beneficial effects of bacterial IAA production (Islam et al., 2016). This shows that the results seen in the non-stressed endophyte treatments are attributable to the effect of endophytes on the host plant and although the mechanisms used in this study is slightly

different, they are still able to promote enhanced nutrient acquisition in plants under normal conditions.

It has also been shown previously that endophytes can help improve nutrient uptake in plants under heavy metal stress, by increasing the uptake of certain nutrients which are able to antagonize the uptake of the heavy metal by controlling the membrane bound transport channels, by limiting the effects of stress ethylene in the host plant and by promoting increased antioxidant activity in host (Rajkumar et al., 2009; Islam et al., 2016). This was supported by the results seen in a study conducted on chromium stress in maize plants in which it was found that inoculation with *Proteus mirabilis* strain *T2Cr* resulted in the increase in the concentration of K and Fe in comparison to the non-inoculated control. The increase in iron in this case was attributed to the utilization of bacterial siderophores; however, the mechanism behind the increase in phosphorous was not determined (Islam et al., 2016).

The upregulation and downregulation that has been found for many of the macronutrients and micronutrients among the different treatments in this study, highlights the need to elucidate the functions of the different nutrients and how they might respond under vanadium stress.

Potassium plays important roles in processes required for proper metabolism in the cell, with it being involved in transport across membranes and in initiating the activity of many different enzymes (Amtmann & Armengaud, 2009). The improvement of K in vanadium stressed plants could be as a result of K being recruited in large amounts due to its ability to minimize oxidative damage in plant cells by reducing the concentration of ROS produced during photosynthesis and by inhibiting the ROS generating NADPH oxidase (Shen et al., 2000). This coincides with the uptake of K being upregulated in the leaves of all vanadium treated samples (Figure 6.2) and in the roots of the control as well as plants treated with endophytes P2, P3, P5 and P6 (Figure 6.4).

Calcium is required for the formation and integrity of the cell membrane and is responsible for coordinating the crosslinking of membrane bound phospholipids (Maathuis, 2009). This is why the decrease in Ca content within the plant due to heavy metal stress can lead to

decrease in the structural integrity of the cell membrane (Maathuis, 2009). Calcium is also involved in many cellular signaling processes which depends on a sufficient concentration of Ca to work (Maathuis, 2009; Sanders et al., 1999). The decrease in calcium content was noted to have occurred in the roots of plants treated with endophytes P2 and P6 (Table 6.4) and the leaves of plants treated with endophyte P2 (Table 6.2).

Magnesium could help alleviate the effects of oxidative stress through the upregulation of antioxidant activity (Nazar et al., 2012). It is also used in the synthesis of chlorophyll a and therefore the downregulation or upregulation seen in Mg concentrations, might be due to a decrease in chlorophyll production under vanadium stressed conditions or conversely due to an increase in chlorophyll production under non-stressed conditions (Chen et al., 2010). Magnesium is also highly essential for the function of a number of important enzymes including ATPase, phosphatases, RNA polymerase, glutathione synthase and protein kinases (Shaul, 2002). This essentiality means that if there is a significant increase or decrease in the cellular concentration of magnesium, then it can disrupt the function of multiple key enzymes required for growth and development in the plant.

Phosphorous is highly important for the function and structure of membrane bound lipids, nucleic acids and serves as a key role in cellular energy transfer due to its pivotal role in ATP molecules (Amtmann & Armengaud, 2009). This means that if there is a deficiency in the required level of cellular phosphorous then it could disrupt the integrity of essential cellular membranes throughout the plant, such as the plasma membrane, which when coupled with the possible reduction in the activity of the plasma membrane bound, hydrogen-translocation ATPase, could have resulted in the disruption of nutrient uptake across the plasma membrane.

Copper is known to be important for cell wall synthesis, contributing to protection from oxidative stress and it is essential for proper photosynthesis activity (Hansch & Mendel, 2009). The increases noted in the levels of Cu and Zn noted amongst some of the vanadium stressed roots and leaves, could be due to their use in the synthesis of Cu/Zn SODs in order to counteract the increase in ROS induced by vanadium exposure (Imtiaz et al., 2015b; Yu and Rengel, 1999). (Wuilloud et al., 2000).

Zinc is required as a key requirement in the production of a large number of proteins, metalloenzymes and transcription factors (Sadeghzadeh, 2013). It also plays a role in maintaining the integrity of cellular membranes, to regulate gene expression required for elevated tolerance to abiotic and biotic stressors and is needed during protein synthesis (Marschner, 2011). The result of its deficiency would therefore lead to the disruption of cellular membrane integrity, as well as the proper function of genes required for the induction of tolerance to different stressors. This points to the possibility that the increased uptake of zinc noted in the leaves of plants treated with endophytes P2, P3 and P5 (Table 6.2) and the roots of plants treated with endophytes P1, P2, P3, P5 and P6 (Table 6.4), could have been as a result of the plant attempting to initiate increased transcription of the genes required for abiotic stress tolerance, in order to ameliorate the negative effects of vanadium exposure.

Manganese has two important cellular functions with the first being a catalytically active metal, such as in the case of Mn-SODs and the second is that it serves to activate certain enzymes within the cell (Hansch & Mendel, 2009) Manganese therefore can be seen as a means to promote tolerance towards vanadium stress, via the production of Mn-SOD in order to scavenge vanadium stress induced O_2^- and it could possibly activate the cellular antioxidant enzymes in order to further reduce the oxidative burden in the plant.

An increase in the level of Iron might be able to help reduce the effect that vanadium stress has on the plant by increasing plant growth and helping to maintain the structural integrity and number of chloroplasts in the cell under vanadium stress (Nada et al., 2007). If the level of iron is deficient, however then this can lead to damage to the chloroplasts thereby resulting in a strong chlorotic state in the leaves (Hell & Stephan, 2013). Iron also plays the role as an important co-factor in certain redox reactions, as a key component in iron-based plant specific proteins and is involved in the function of electron transport chains for photosynthesis (Hell & Stephan, 2013).

4.13.5 Levels of cell death in Brassica napus under non-stressed and vanadium stressed conditions

Plants exposed to abiotic stress, can experience increased levels of ROS, that can lead to DNA damage, lipid peroxidation, membrane instability and eventually cell death (Chaoui et al.,

1997). Cell death is intimately connected with increased concentrations of ROS when the plant is under stressful conditions and therefore with the possibility of increased ROS concentrations due to vanadium exposure, it was necessary to measure the levels of Cell death in *Brassica napus* both in non-inoculated and endophyte treatments (Karuppanapandian et al., 2011).

The levels of cell death in the plants treated with endophytes P1, P2, P3 and P6 were shown to have no statistical difference when compared to the non-stressed control indicating that treatment with these endophytes did not negatively affect the cell death of their host plants (Figure 10 A). It was also noted; however, that plants under non-stressed conditions treated with endophyte P5 had levels of cell death that were significantly higher than the control, indicating that it had a negative effect on cell viability in their host plants (Figure 10 A).

This increase in cell death could have come about as a result of the 105% increase in superoxide content over the control (Figure 10 A), as this was the most significant change in reactive oxygen species observed in the plants treated with endophyte P5. It should also be noted that plants treated with endophyte P5 displayed significantly higher levels of lipid peroxidation (Figure 14 A) than the control, which can be considered as one of the factors that contributed to the noted increase in cell death.

Among those plants treated with vanadium it was found that all of the endophyte treatments displayed levels of cell death that was significantly lower than the vanadium stressed control, with endophytes P3, P5 and P6 showing extensive decreases in cell death (Figure 10 B); however, even though the endophytes were able to increase the level of cell viability under vanadium stress, there was still a significant increase in cell death among all of the treatments in comparison to their non-stressed counterparts (Figure 10). The increased levels of cell death under heavy metal stress seen here, was similar in nature to the results found in chickpea seedlings treated with varying concentrations of vanadium. In the study it was found that chickpea seedlings treated with vanadium ranging from 50mg kg^{-1} to 200mg kg^{-1} experienced increasing levels of cell death in a directly proportional manner to the concentration of vanadium (Imtiaz et al., 2018). This observation helped to show that vanadium stress can induce an increase in plant cell death, which was attributed to the

increased ROS accumulation noted in the seedlings due to exposure to vanadium (Imtiaz et al, 2018). This could help to explain why endophyte treated plants under vanadium stress had lower levels of cell death compared to the control, as all of the endophyte treated plants had significantly lower concentrations of H_2O_2 (figure 11 B) and •OH (Figure 13 B) in comparison to the vanadium stressed control. These endophyte treatments also have also been found to have antioxidant activities that are much higher than the control and this could have contributed to the lowered cell death concentration, as it is known that the intervention of sufficiently high levels of antioxidant activity can help halt cell death (Apel and Hirt, 2004; Mittler et al., 2004; Manoharan et al., 2005; Karuppanapandian et al., 2011).

4.13.6 Superoxide content in Brassica napus under non-stressed and vanadium stressed conditions

The significant increases in superoxide levels noted among the endophyte treated plants under non-stressed conditions though unexpected, was determined to be non-detrimental to the host plants in the case of plants treated with endophytes P1, P2, P3 and P6 as their levels of lipid peroxidation (Figure 14 A) and cell death (Figure 10A) showed no significant increases compared to the control in each respective case. In the above cases it was thought to be that the increase in levels of superoxide, could be due to a recently discovered phenomenon in which certain heterotrophic bacteria were found to produce exogenous superoxide at levels that are far above those found intracellularly (Diaz et al., 2013). This would help to explain the increase in superoxide levels seen among non-stressed endophyte treated plants, as the exogenous superoxide that would usually be released into the environment would instead be released into the internal environment of the plant. This would also be help to explain the increase in SOD noted for all of the endophyte treatments under non-stressed conditions (Figure 15 A), as it would need to be upregulated in order to offset the adverse effects that the increased O_2^- would bring about.

This however did not appear to be the same for plants treated with endophyte P5 as it was noted that the levels of superoxide in the host plant of endophyte P5 had the highest superoxide levels among all of the treatments, furthermore it also had significantly increased levels of lipid peroxidation (Figure 14 A) and cell death (Figure 10 A). This could be explained by an observation made by White and Torres (2010) involving endophytic fungi present in tall

fescue plants. In the study it was found that endophytic fungi present in the apoplastic spaces between plant cells would release O_2^- onto the plasma membrane of the nearby cells to oxidize these membranes thereby causing leakage of intracellular nutrients into the apoplastic space (White & Torres, 2010). This mechanism, though likely to not be utilized by isolate P5 for nutrient acquisition, could still be inadvertently employed through the increase noted in O_2^-; however, the increase might have exceeded the threshold beyond which SOD, was no longer able to scavenge sufficient O_2^-, which resulted in the oxidation of the resulting membranes and the resulting increase in lipid peroxidation that was noted. The increase in lipid peroxidation could also have been the result of the action of the ROS hydroperoxyl (HO_2•), that is produced due to the protonation of O_2^- under aqueous conditions (Gill & Tuteja, 2010). The hydroperoxyl radical is highly reactive and can accept electrons from poly-unsaturated fatty acids thereby leading to the initiation of lipid peroxidation (Karuppanapandian et al., 2011). When taking into account the increase in O_2^- content along with possible conversion of a portion of this O_2^- in (HO_2•) it stands to reason that the increased lipid peroxidation could be due in some part to the effects of the hydroperoxyl radical.

It should also be noted that vanadium exposure was found to exacerbate the levels of superoxide in leaf tissue in *Brassica napus* in all of the treatment types with the control having statistically similar O_2^- levels to those found in endophyte treatments P1, P2, P3, P6 (Figure 11 A), whereas the treatment carried out with endophyte P5 showed significantly reduced levels of O_2^- compared to the vanadium stressed control. This shows that under vanadium stress endophyte P5 was able to successfully regulate the production or scavenging of O_2^-, to the extent that it was able to decrease the adverse effect that increased O_2^- concentrations would incur in the host plant. The increase in the concentration of O_2^- found in the stressed control was similar to the results found in a study conducted on *Brassica napus* under vanadium stress, which exhibited a 71% increase in O_2^- levels when compared to the control (Gokul et al., 2018). This indicates that the increase in O_2^- seen in this study can be attributed to the plant's exposure to vanadium stress.

4.13.7 Hydrogen peroxide content in Brassica napus under non-stressed and vanadium stressed conditions

Exposure to vanadium caused a significant rise in the levels of hydrogen peroxide in leaf tissue compared to the non-stressed treatments (Figure 12), with the vanadium stressed control exhibiting the highest concentration of H_2O_2 among all of the stressed treatments. This result was supported by the findings of Imtiaz et al. (2018) who found that chickpea treated with increasing concentrations of vanadium, led to a directly proportional rise in H_2O_2 concentrations with a maximum level of H_2O_2 found at the highest treatment of 200mg. kg^{-1} V (Imtiaz et al., 2018). This shows that the increase in H_2O_2 seen in this study can be attributed to the plants exposure to vanadium, indicating that any reduction in H_2O_2 seen in the vanadium stressed endophyte treatments, should be as a result of the endophytic bacteria playing a role in reducing the stress imposed by vanadium on *Brassica napus*. This was further supported by the results found in a study conducted on copper stress in the leaves of lentil plants, which found that lentils grown in soil amended with copper were shown to have increased levels of H_2O_2 in their leaves compared to the control; however, plants grown in soil amended with the same concentration of copper, exhibited significantly reduced levels of H_2O_2 when treated with the endophytic bacteria *Providencia vermicola* (Islam et al., 2016). This was attributed to the endophyte causing an increase in the levels of the antioxidant enzymes APX and Catalase, leading to the scavenging of excess stress induced H_2O_2, so that the deleterious effects of the increased ROS production could be mitigated (Islam et al., 2016). This coincides with what was seen in this study as it was found that endophytes P1, P3 and P5 upregulated APX activity (Figure 17 B) whereas endophytes P1, P2, P3 and P6 upregulated Catalase activity (Figure 16 B), indicating their involvement in the decrease in H_2O_2 seen in these treatments in comparison to the vanadium stressed control.

In the non-stressed treatments endophytes P2, P3, P5 and P6 displayed levels of H_2O_2 that was determined to be statistically similar to the non-stressed control. Endophytes P1 on the other hand displayed a significant downregulation in levels of H_2O_2. The similarity in H_2O_2 levels in the control to those seen in plants treated with endophyte P2, P3, P5 and P6 could be due to the upregulation seen in catalase activity for these treatments, as it can be inferred that the significantly higher levels of O_2^- seen in the non-stressed endophyte treatments

(Figure 11 A) would result in the increased synthesis of SOD, as was seen by the upregulation in SOD activity in the endophyte treated plants in comparison to the control (Figure 15 A). The consequence of the increase in SOD activity would naturally be the production of more H_2O_2, leading to an imbalance in the production-scavenging relationship maintained between O_2^-, H_2O_2 and SOD. The plant would therefore need to increase the synthesis of Catalase (P1, P2, P3, P5, P5) (Figure 16 A) and APX (P1, P2, P6) (Figure 17 A) in order to scavenge the newly formed H_2O_2 and restore the delicate balance between ROS and antioxidants, as can be inferred by the statistically similar levels of H_2O_2 seen in endophyte treatments P2, P3, P5 and P6. In addition to this the decrease in H_2O_2 seen in endophyte treatment P1, could be explained due to its relatively high activity of both Catalase and APX, along with the possibility that the larger percentage of H_2O_2 produced in the other treatments might have been localized in the peroxisomes, whereas a larger percentage of the H_2O_2 produced in the plants under the treatment of endophyte P1 may have been localized to the chloroplast and cytosol. This is of importance as catalase has a relatively low affinity for H_2O_2 due to it requiring two H_2O_2 molecules to operate, whereas APX has a much higher affinity for H_2O_2, as it can catalyse H_2O_2 when only one molecule is present (Asada, 2006). In addition to this, catalase is predominantly found in the peroxisomes and APX is found in the chloroplasts and cytosol, making it the most ubiquitous H_2O_2 scavenging antioxidant.

4.13.8 Hydroxyl radical concentration in Brassica napus under non-stressed and vanadium stressed conditions

The Hydroxyl radical is the most reactive of the reactive oxygen species with it being able to disrupt cellular macromolecules, induce lipid peroxidation, damage DNA and initiate the onset of cell death in plant tissues, making it highly toxic to plants especially when under biotic and abiotic stress (Hippeli & Elstner, 1997). In light of this determining its concentration in leaf tissue was determined to be highly important for this study, in order to determine what effect vanadium stress had on its production in *Brassica napus* treated with endophytes and without (control).

Vanadium stress was found to induce a definite increase in the concentration of •OH in all of the vanadium stressed treatments; however, it was noted that the endophyte treated plants displayed statistically lower levels of •OH compared to the vanadium stressed control (Figure

13 B). This trend was also seen in the non-stressed treatments, with endophytes displaying decreased levels of •OH compared to the non-stressed control (Figure 13 A). The increase in OH^- noted among all of the plants under vanadium stress is thought to be attributed to the increased conversion of H_2O_2 into •OH via the metal dependent Haber-Weiss/Fenton reaction. (Maksymiec, 2007). The reason for this hypothesis relies on the fact that the Haber-Weiss cycle is dependent on the reaction of reduced heavy metal ions with H_2O_2 in order to take place, resulting in the production of Fe^{3+} along with OH^- (Mittler et al., 2004). This in turn means that if there is an increase in the available pool of intracellular reduced metal ions such as Fe^{2+}, along with an increase in the concentration of intracellular H_2O_2, then this could lead to an upregulation in the production of •OH (Sharma et al., 2012). When this observation is taken into account along with the fact that superoxide is able to reduce Fe^{3+} into Fe^{2+}, it can therefore be established that a possible reason for the increase noted in the levels of •OH seen in this study was due to the upregulation in the production of O_2^- (Figure 11 B) and H_2O_2 (Figure 12 B) seen under vanadium stress.

The downregulation in the levels of hydroxyl radical seen in vanadium stressed plants treated with endophytes was attributed to combination of the upregulation of the hydrogen peroxide scavenging enzymes catalase (Figure 16 B) or ascorbate peroxidase (Figure 17 B). This assumption is made based on the consideration that the hydroxyl radical has been found to have no known enzymatic scavengers, meaning that endophytes are not able to directly reduce the levels of the hydroxyl radical in their respective host plants through any currently known mechanisms (Gill & Tuteja, 2010).

All of the non-stressed endophyte treatments displayed marked decreases in •OH compared to the control; however, the reason behind this could not be determined as all of the endophyte treatments displayed increased levels of O_2^- (Figure 11 A) and statistically similar levels of H_2O_2 (Figure 12 A) respectively when compared to the control, without having a similar level of •OH to the control. A possible reason behind why this occurred could be due to the release of siderophores by the endophytes in order to increase their uptake of iron. Siderophores are low molecular weight iron chelating compounds and is produced by bacteria when there is insufficient iron in the local environment to meet the needs of the endophyte

(Rajkumar et al., 2010). When this occurs then the siderophore is able to bind Fe^{3+} ions present from the greater surrounding environment for the eventual absorption and uptake by the endophyte (Rajkumar et al., 2010). In addition to this it is well known that in order for •OH to be produced from the Haber-Weiss cycle, Fe^{3+} needs to donate an electron to H_2O_2; however, if the siderophores in the environment can bind free Fe^{3+} ions inside the plant, then the endophyte should be able to directly lower the pool of Fe^{3+} inside the plant resulting in the decreased conversion of H_2O_2 to •OH (Gill & Tuteja, 2010; Karuppanapandian et al., 2011; Rajkumar et al., 2009). This particular mode of utilization of siderophores could possibly be used by endophytic bacteria under certain conditions, as it has been seen before that endophytes usually have to cope with low levels of available iron ions in the plant tissues within which they reside (Idris et al., 2004; Sessitsch et al., 2004).

4.13.9 Levels of lipid peroxidation in Brassica napus under non-stressed and vanadium stressed conditions

Levels of malondialdehyde (MDA), a secondary breakdown product produced as a result of lipid peroxidation and has been shown to be a reliable indicator of the extent of lipid peroxidation due to its stable nature and accuracy with which it can be quantified (Davey et al., 2005). It was therefore decided to carry out a TBARS assay in order to determine the levels of MDA in *Brassica napus* leaf tissue under vanadium stressed and non-stressed conditions to ascertain the effects each treatment had on lipid peroxidation in the plant tissue.

Vanadium stress was found to increase the levels of lipid peroxidation among all of the treatments; however, it should be noted that the endophyte treated plants showed consistently lower levels of MDA compared to the vanadium stressed control (Figure 14 B). The increase in MDA noted in this study was mirrored by results seen in a study on wheat leaves under zinc and chromium stress, in which exposure to 0.1mM of each heavy metal caused an increase of 33% and 32% in lipid peroxidation for each treatment respectively (Panda et al, 2003). In the above study the increase in lipid peroxidation was attributed to an increase in ROS, in particular as a result of a significant increase in H_2O_2, which is similar to what was found in this study where the vanadium stressed control had levels of H_2O_2 that was 161% higher (Figure 12) than the non-stressed control, as well as a 201% increase in O_2^- (Figure 11) over the non-stressed control. The decrease in lipid peroxidation noted in

endophyte treated vanadium stressed plants compared to the vanadium stressed control, could therefore be due to the comparatively lower levels of H_2O_2 (Figure 12 B) and •OH (Figure 13 B) seen in these plants compared to the control.

Levels of lipid peroxidation in the non-stressed trial were found to be statistically similar to the control for treatments P1, P2, P3 and P6; however, those plants treated with endophyte P5 showed increased levels of lipid peroxidation. The statistically similar result seen in lipid peroxidation shows that these endophyte treatments did not negatively affect their host plants, indicating that the levels of ROS within these treatments were well within the range that could be considered "normal" levels for *Brassica napus* and should be in the range of normal signaling processes (Slesak et al., 2007). This hypothesis was supported by the statistically similar levels of cell death seen in these treatments when compared to the control (Figure 10 A). This, however was not the case in plants treated with endophyte P5 which displayed significantly higher levels of lipid peroxidation compared to all of the other non-stressed treatments. This was thought to be as a result of the significant increase noted in the levels of superoxide (Figure 11) in these plants, as it has been shown previously that O_2^- can contribute towards the onset of lipid peroxidation in plant tissues, through cellular membrane disruption and through its conversion into the highly toxic hydroperoxyl radical (White & Torres, 2010; Sharma et al., 2012).

4.13.10 Levels of Superoxide dismutase activity in Brassica napus under non-stressed and vanadium stressed conditions

Superoxide dismutase is an enzymatic antioxidant employed by plants to lower the levels of intracellular superoxide in order to prevent the plant from accumulating an excess of O_2^- which can cause the plant to enter into a state oxidative stress (Scandalios, 1993). Vanadium stress induced a significant increase in Superoxide dismutase activity in all stressed treatments in comparison with non-stressed treatments, with the control, P2, P3 and P6 having statistically similar levels of SOD activity and vanadium stressed plants inoculated with P1 and P5 displaying significantly higher levels of SOD activity than the rest of the treatments (Figure 15 B). The upregulation in SOD seen in all of the stressed treatments was thought to be brought about by the significant increase in levels of O_2^- that was induced as a result of exposure to vanadium (Figure 11 B). This upregulation in O_2^- would help to initiate the

increased synthesis of new SOD protein thereby leading to enhanced levels of SOD activity, which could allow the plant to more efficiently scavenge a larger percentage of the O_2^- produced due to vanadium stress (Verma & Dubey, 2003; Fatima & Ahmad, 2005). This observation is supported by the results seen in a study assessing the effects of vanadium in 3 different chickpea cultivars, where it was noted that chickpea plants that were exposed to vanadium stress, displayed a significant upregulation in SOD activity irrespective of the cultivar (Imtiaz et al., 2015a). The increase in SOD noted in plants treated with endophyte P5 led to a noticeable reduction in O_2^- in the host plant; however, this did not occur in plants treated with endophyte P1 (Figure 11 B). The reason for this is thought to be that plants treated with endophyte P1 experienced enhanced levels of O_2^- production throughout the 46-day growth period leading to an increase in SOD activity; however, this upregulation in SOD activity might not have been sustainable resulting in a gradual decrease in the level of SOD activity over time, eventually leading to a decline in the amount of O_2^- scavenged. This was supported by the results seen by Panda et al. (2003) who found that wheat plants exposed to varying levels of Zinc and chromium stress experienced a gradual decrease in SOD activity over time, with the level of SOD activity starting at a high level, decreasing gradually and then stabilizing at a lower concentration than the initial starting concentration (Panda et al., 2003). The increase in SOD seen in plants treated with P5 was comparable to the results seen in mustard plants under nickel stress, where it was found that exposure to nickel at toxic concentration caused a significant rise in the levels of SOD in all of the treatments tested in the experiments; however, those plants treated with an endophytic *Pseudomonas spp.* displayed the highest level of SOD among all of the treatments (Tak, 2015).

For the non-stressed treatments, those plants inoculated with endophytes showed a significant increase in the levels of SOD activity compared to the control; however, this was determined to be as a result of the need to scavenge the increased O_2^- found in plants under these treatments (Figure 11 A). This, however does not detract from the possibility that endophytes could have also contributed to the upregulation in SOD activity, due to the augmentation of the plants antioxidant machinery by mechanisms employed by the endophyte, as a means to offset the increased O_2^- levels that it induced in the host plant (Duponnois et al., 2006; Islam et al., 2016, White & Torres, 2010).

4.13.11 Levels of catalase activity in Brassica napus under non-stressed and vanadium stressed conditions

Inoculation with endophytes was found to significantly upregulate the activity of catalase in the non-stressed plant treatments (Figure 16 A). This could be due to the increased levels of O_2^- noted in the endophyte treated plants being dismutated, leading to an increase in the levels of H_2O_2 present in the intracellular environment of the plant. This would therefore require catalase to be upregulated in order to scavenge the newly formed H_2O_2, thereby redressing the imbalance in ROS and returning it back to a state at which the plant was no longer experiencing oxidative stress.

Exposure to vanadium stress was determined to increase catalase activity in plants treated with endophytes P1, P2, P3 and P6 in comparison to the vanadium stressed control; however, it was found that catalase activity in plants treated with endophyte P5 was significantly downregulated in comparison to the control (Figure 16 B). The decrease in catalase seen in vanadium stressed plants treated with endophyte P5 could be due to the fact that catalase is sensitive to levels of O_2^-, with increasing concentrations of O_2^- leading to the increased possibility of inactivation of the Catalase enzyme (Calmak, 2000). It could also be due to the downregulation in the enzyme synthesis activity as a result of vanadium inadvertently causing damage to the plants enzyme synthesis machinery (Feiereband & Engel, 1986; MacRae & Ferguson, 1985). The increase in catalase activity seen in the remaining endophyte treatments when compared to the control, was supported by a similar result seen in a study done on mustard seedlings. In the study it was found that treatment of mustard seedlings with 100mg kg^{-1}, 300mg kg^{-1} and 500mg kg^{-1} of nickel increased the levels of catalase activity in mustard seedlings; however, the catalase activity did not increase beyond the level of activity that was observed in the plants treated with 100mg kg^{-1} of nickel (Tak, 2015). In contrast to this, the mustard seedlings inoculated with an endophytic *Bacillus spp.* also exhibited increased catalase activity in all three treatments; however, the level of catalase activity was significantly higher than the control in the case of 300mg kg^{-1} and 500mg kg^{-1} treatments (Tak, 2015). The result seen in the above study supports what was seen in the endophyte treated vanadium stressed plants which shows that endophytic bacteria can improve the level of catalase activity when their host is under heavy metal stress.

4.13.12 Levels of Ascorbate peroxidase activity in Brassica napus under non-stressed and vanadium stressed conditions

Ascorbate peroxidase is an antioxidant enzyme that catalyses the conversion of H_2O_2 and ascorbic acid into water and dehydroascorbate, resulting in a reduction of the concentration of intracellular H_2O_2 thereby lowering the oxidative burden on the plant (Karuppanapandian et al., 2011).

Vanadium stress was found to induce three distinct changes in the activity of APX in the vanadium stressed plants with the control and in plants treated with endophytes P1 displaying statistically similar levels of increase in APX activity, treatments P3 and P5 having significantly highly levels of vanadium compared to the control and plants treated with endophytes P2 and P6 showed significant decrease in APX activity (Figure 17 B). The increase in APX activity was attributed to the plant upregulating APX in order to counteract the significant increase noted in H_2O_2 in the plants under vanadium stress (Figure 12 B). This is supported by a study on lead toxicity in *Triticum aestivum L.*, which noted a 50% increase in APX activity in plants that were treated with 1.5mM Pb when compared to the control (Lamhamdi et al., 2011). The upregulation of APX activity in plants treated with endophytes P3 and P5, significantly exceeded the levels seen in the control and is thought to be due in part to the plant upregulating APX activity and also due to certain endophytes having the capability to activate gene expression for antioxidant enzymes in host plants in order to resist the damage brought on by abiotic stress (Duponnois et al., 2006). This ability to initiate the upregulation of APX activity under heavy metal stress was also seen in *P. vermicola* which was shown to have increased APX activity in lentil plants when under copper stress when compared to the non-inoculated control under the same conditions (Islam et al., 2016). The decrease in APX activity noted in plants treated with endophyte P2 and P6, could possibly be attributed to the significant damage applied by the increased concentration of ROS on the structure of APX, leading to the disruption of its antioxidant capability (Martinez-Romero & Rosenbleuth, 2006). This decrease in APX activity has been seen previously in Sunflower plants treated with different concentrations of vanadium. In the study it was found that sunflower treated with 3.25mg L^{-1}, 7.5mg L^{-1} and 15mg L^{-1} all experienced significantly decreased APX activity compared to the non-stressed control (Abedini et al., 2017). This

indicates that the antioxidant diminishing effects of vanadium can occur even at the point vanadium is present at relatively lower concentrations, which supports the hypothesis that the significantly higher concentration of vanadium used in this study could have resulted in the downregulation in APX activity.

In the non-stressed conditions, it was found that endophytes P1, P2 and P6 significantly upregulated APX activity and plants treated with P3 and P5 had significantly reduced levels of APX activity when compared to the control (Figure 17). The upregulated activity noted here may be due to a possible increase in H_2O_2 created as a result of SOD scavenging the increased O_2^- noted among endophyte treated plants (Figure 11). The upregulation noted in APX activity (Figure 17) coincides with the upregulation noted in catalase activity (Figure 14) seen in non-stressed endophyte treated plants. This indicates that both of these enzymes were needed in order to scavenge the excess H_2O_2, which stands to reason since Catalase is localised to the peroxisomes and APX is localized to the chloroplast, mitochondria and cytosol (Dat et al., 2000), therefore it is likely that there was a sufficient concentration of H_2O_2 in the peroxisomes, as well as the chloroplasts, cytosol or mitochondria, which would have required the upregulation of both Catalase and APX to scavenge. In addition, when taking into account the different affinities that APX (µM) and catalase (mM) have for H_2O_2, it stands to reason that the level of APX activity could be upregulated even if a much lower concentration of H_2O_2, in comparison with total leaf H_2O_2, was present in the chloroplast or mitochondria in comparison (Gill & Tuteja, 2010).

In conclusion, in this chapter, it was determined that exposure to vanadium has a detrimental effect on the health of *Brassica napus L* plants, with it reducing plant leaf size, significantly altering the regulation and uptake of nutrients within the plant and leading to the increased production of harmful ROS and increasing the levels of certain antioxidants to counteract this rise in ROS. Treatments with endophytes P1, P2, P3, P5 and P6 was found to ameliorate the effects of vanadium stress on *Brassica napus* to various extents, with plants treated with these endophytes displaying enhanced biomass, increased leaf size, lower levels of certain ROS molecules and enhanced levels of antioxidants. This indicates that these endophytes could

play a role in increasing crop yield by promoting increased tolerance to abiotic stressors such as heavy metals in crop plants.

Chapter 5

Conclusion and future perspectives

Endophytic bacteria have been shown to possess a variety of different mechanisms for the promotion of plant growth, such as the production and regulation of plant hormones, the production of ACC deaminase, phosphate solubilization and overall nutrient upregulation. Endophytes may use any combination of these mechanisms to promote growth and therefore the endophytes isolated in this study were tested for the ability to use any of these growth promoting characteristics.

The results of the plant growth trials indicated that all of the isolates showed significantly improved growth compared to the control plants furthermore, P1 was found to have the best germination rate and isolate P6 was found to have the best effect on improving plant growth, in terms of increased root, stem and leaf size. Furthermore, the results seen from comparing the biomass correlates with those seen in the comparative growth results. This was further supported by the results of the ICP-OES analysis which indicated that all of the endophytes were involved in increasing the uptake of one or more nutrients in their respective plants. P1 was found to be the best nutrient regulator overall; however, certain isolates were better able to upregulate the intake of certain micronutrients in comparison to isolate P1. In the biochemical tests all of the isolates were shown to produce siderophores and ACC deaminase, while none of the isolates were capable of solubilizing phosphate and P3 was the only isolate capable of significant IAA production. When taking into account the overall plant growth promotion capabilities of all of the isolates, it was determined that although P4 could promote plant growth, its overall effect was not significant enough and was therefore excluded from the experiments that followed after the preliminary plant growth promotion experiments.

Once the endophytes were confirmed to possess growth promotion characteristics, this study then went further to determine what effect treatment with the chosen endophytes (P1, P2, P3, P5, and P6) would have on the physical and oxidative state of *Brassica napus* under the effects of vanadium stress. Vanadium stress was found to be detrimental to the health of endophyte free *Brassica napus* with it causing stunted leaf growth, decreased biomass, significantly enhanced lipid peroxidation and increased cell death among when compared to its non-stressed counterparts. When compared to the vanadium stressed control, it was

found that all of the endophyte treated plants showed varying degrees of enhanced tolerance to the detrimental effects caused by vanadium stress. This was observed through a significant increase in leaf size, enhanced biomass and significantly decreased levels of both lipid peroxidation and cell death when compared to the vanadium stressed control. It was also found that vanadium stress disrupted the normal uptake of nutrients in the plants, with the vanadium stressed control displaying upregulation of certain nutrients in both the leaf and root tissues; however, this upregulation was determined to be non-beneficial when taking into account the physiological changes (reduced size, decreased biomass, enhanced lipid peroxidation, increased cell death) noted in those plants under vanadium stress. In relation to this it was found that there was an increase in the uptake of vanadium in both the roots and leaves of these plants; however, the vast majority of vanadium uptake was localized to the roots, which indicated that the plant could have been protecting itself through this mechanism. This trend in nutrient upregulation was also noted in the vanadium stressed endophyte treated plants; however, it was found that the overall extent of nutrient upregulation was lower when compared to the control, which pointed to the possibility that the endophytes were able to protect their hosts from the effects of vanadium stress, as the change in nutrient uptake was linked to the increased uptake of vanadium by the plants. Exposure to vanadium was also determined to induce an increase in the levels of the harmful stress induced reactive oxygen species O_2^-, H_2O_2 and •OH among all of the treatments; however, it was found that the endophytes treated plants possessed lowered levels of these ROS compared to the vanadium stressed control. As a result of these differences noted in ROS among the vanadium stressed and non-stressed plants it was deemed necessary to ascertain what changes occurred in the antioxidant profile among the treatments. It was found that exposure to vanadium stress caused an increase in the activity of SOD in all of the vanadium stressed treatments, an increase in the activity of catalase for the control and plants treated with endophyte P1, P2, P3 and P6 and an increase in the activity of APX for the control and plants treated with endophytes P1, P2 and P6. Conversely vanadium caused a decrease in the activity of catalase in plants treated with endophytes P5 as well as a decrease in the activity of APX in plants treated with endophytes P2 and P6.

This shows the potential of these endophytes to be used to promote crop plant growth; however, future studies should be done before it could be used in that capacity. This would include conducting field trials to determine the extent to which the endophytes can promote plant growth in the field in a non-static environment. Combination studies combining two or more of the endophytes should also be carried out to determine if endophytes with different growth promoting mechanisms could act in a synergistic manner to improve plant growth to an extent that is above that of either endophyte on its own. It would also include determining to what extent translocation of nutrients from the soil to the roots and from the roots to the leaves occurs, under different soil conditions, with the soil being either nutrient poor or nutrient dense. It will also be necessary to perform indirect mutagenesis on the endophytes to attempt to obtain endophytes with improved siderophore production and then testing whether these mutants can help protect their hosts under low iron conditions. In addition to this it will be essential to perform whole genome sequencing on the normal and mutant endophytes in order determined what changes have occurred in the genomes of the mutants resulting in the increase in siderophore activity.

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Appendix

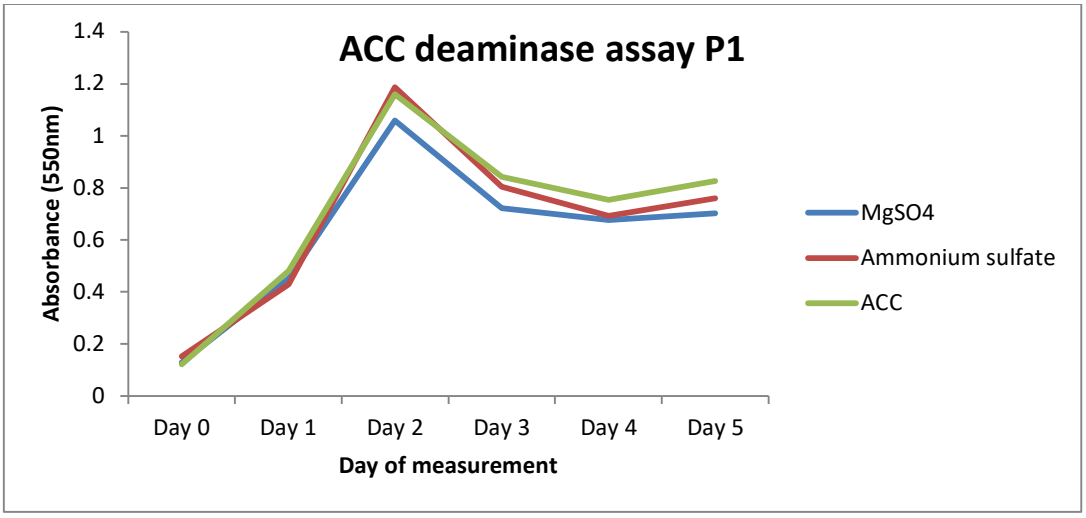

Figure 18: Level of ACC deaminase activity of isolated endophyte P1 as indicated by growth on ACC media compared to growth on magnesium sulphate and ammonium sulphate containing media

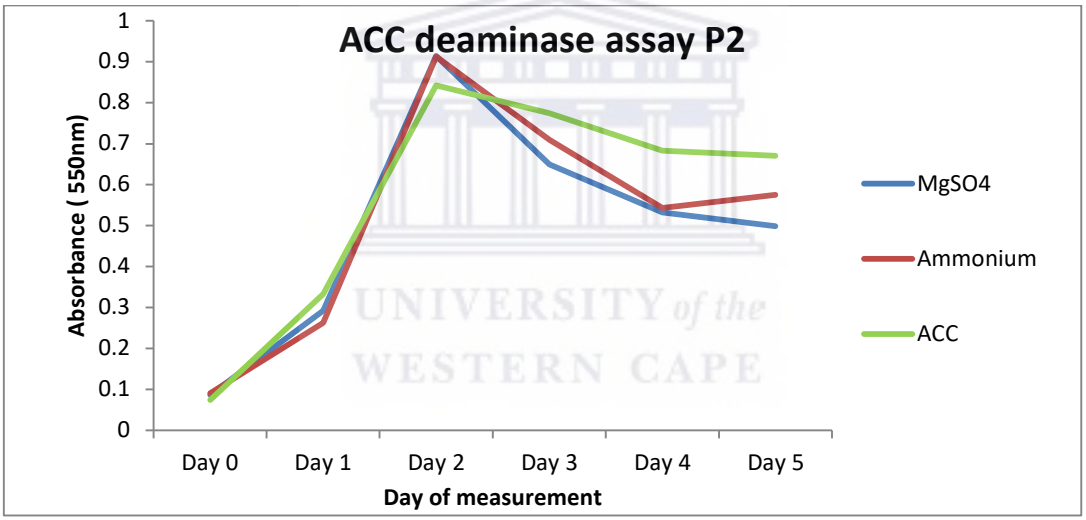

Figure 19: Level of ACC deaminase activity of isolated endophyte P2 as indicated by growth on ACC medium compared to growth on magnesium sulphate and ammonium sulphate

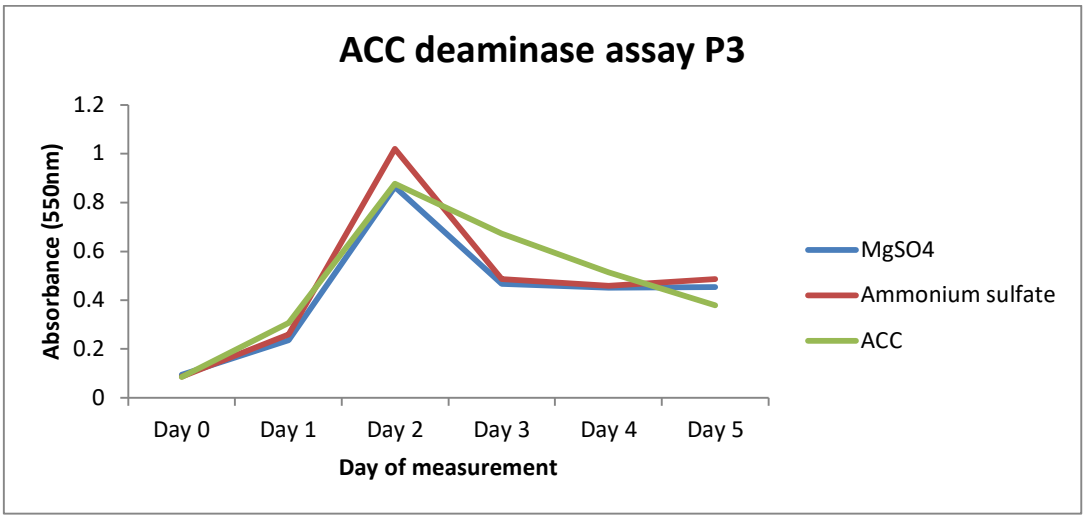

Figure 20: Level of ACC deaminase activity of isolated endophyte P3 as indicated by growth on ACC media compared to growth on magnesium sulphate and ammonium sulphate containing media

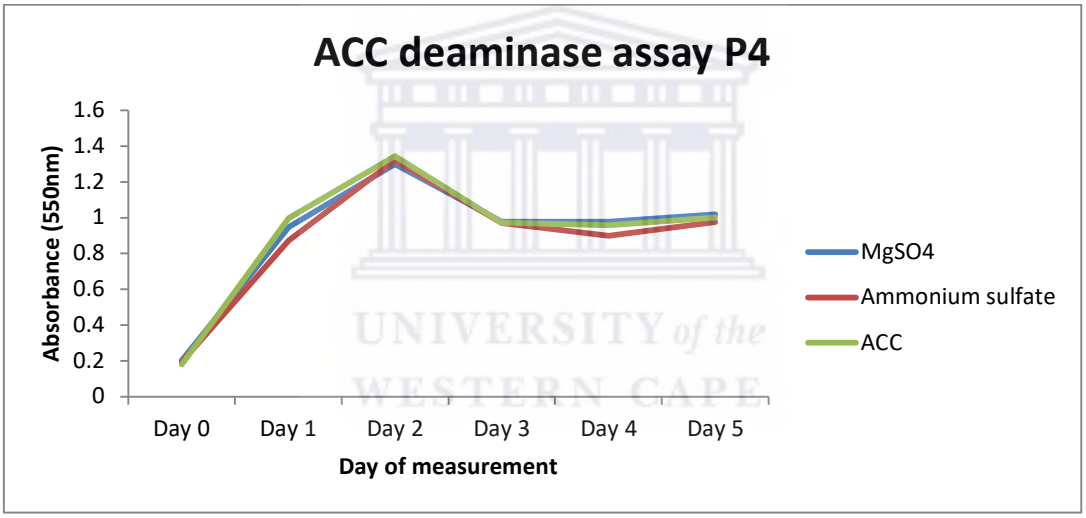

Figure 21: Level of ACC deaminase activity of isolated endophyte P4 as indicated by growth on ACC media compared to growth on magnesium sulphate and ammonium sulphate containing media

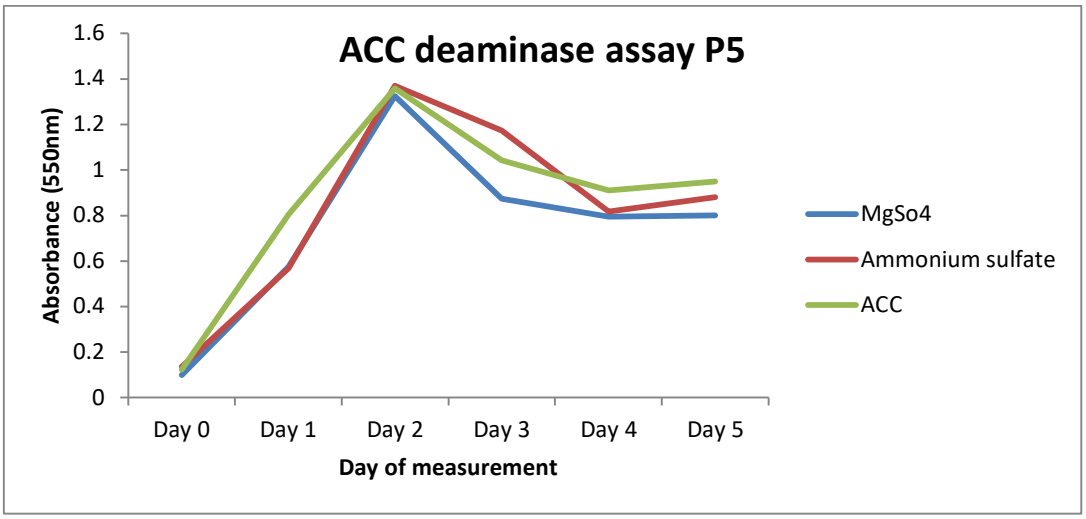

Figure 22: Level of ACC deaminase activity of isolated endophyte P5 as indicated by growth on ACC media compared to growth on magnesium sulphate and ammonium sulphate containing media

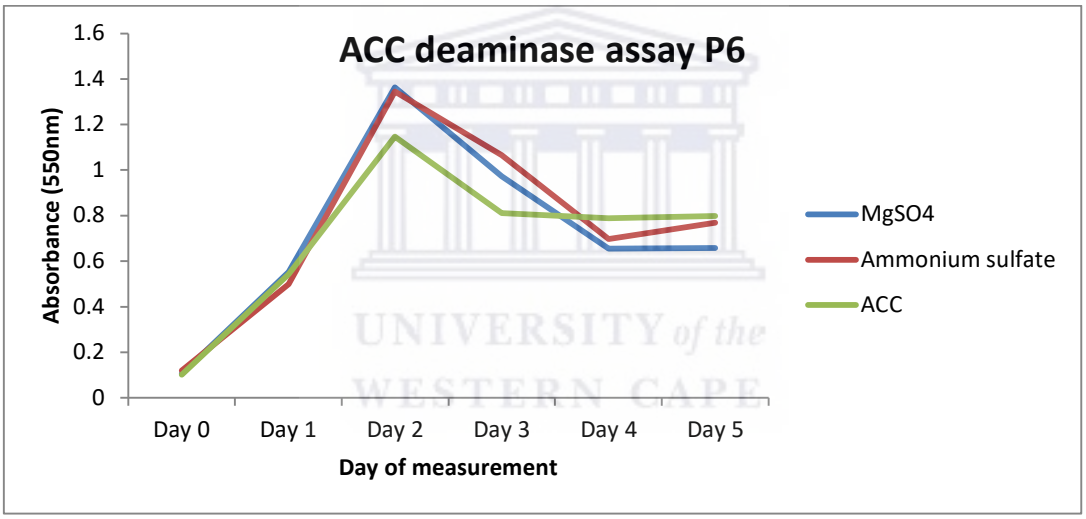

Figure 23: Level of ACC deaminase activity of isolated endophyte P6 as indicated by growth on ACC media compared to growth on magnesium sulphate and ammonium sulphate containing media

Table 7: Showing the 16s rDNA sequencing results for endophytes P1, P2, P3, P5 and P6

Endophyte	Description	Identity
P1	Rhodococcus qingshengii strain JCM 15477	99%
P2	Rhodococcus qingshengii strain JCM 15477	99%
P3	Rhodococcus qingshengii strain JCM 15477	99%
P5	Rhodococcus qingshengii strain JCM 15477	99%
P6	Rhodococcus qingshengii strain JCM 15477	98%