2017

Junaid
3051048

Supervisor
Dr. Marshall Keyster

[Isolation and characterization of plant growth promoting endophytic bacteria from *Eriocephalus africanus* roots]

A thesis submitted in partial fulfillment of the requirements for the degree of Magister Scientiae in the department of biotechnology, University of the Western Cape

University of the Western Cape

Private Bag X17, Bellville 7535, South Africa

Telephone: +27-21- 959 2255/959 2762

Fax: +27-21- 959 1268/2266

Email: 3051048@uwc.zc.za

UNIVERSITY of the WESTERN CAPE

FACULTY OF NATURAL SCIENCE

GENERAL PLAGIARISM DECLARATION

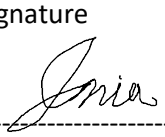
Name: Junaid Mia

Student number: 3051048

UNIVERSITY of the WESTERN CAPE

1. I hereby declare that I know what plagiarism entails, namely to use another's work and to present it as my own without attributing the sources in the correct way. (Refer to University Calendar part 1 for definition)
2. I know that plagiarism is a punishable offence because it constitutes theft.
3. I understand the plagiarism policy of the Faculty of Natural Science of the University of the Western Cape.
4. I know what the consequences will be if I plagiarise in any of the assignments for my course.
5. I declare therefore that all work presented by me for every aspect of my course, will be my own, and where I have made use of another's work, I will attribute the source in the correct way.

Signature



Date

8 December 2017

http://etd.uwc.ac.za/

Contents

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

ACKNOWLEDGEMENTS

I would first like to thank the almighty for blessing with all thesis opportunities and guiding me thought them.

I would like to thank my supervisor, **Dr. Marshall keyster** and the University of the Western Cape for giving me the opportunity to work on this projected. I would also like to thank Dr. keyster for creating a great working environment and his guidance throughout the projected. I want to thank the National Research Foundation for funding this project.

I also wish to thank my father, **Rafiq Mia** and mother **Shahnaaz Mia**, for their patience and support during this project. I want to thank them for their words of encouragement and guidance. I would like to say thank you to my annoying "scumbag" of a brother, **Ayyub Mia** for just being there to talk to and hang out with.

UNIVERSITY of the
WESTERN CAPE

I would like to thank the Environmental Biotechnology Laboratory (EBL) for their support and making the work environment enjoyable. Thank you to **Arun Gokul** and **Ruomou Wu** for all their help in the laboratory as well as the endophyte team **Alex Jason Siebritz**, **Brandon Istain** and **Tasheeq Ismail**. A thank you to my lab partners **Fahiem Carelse** and **Lee-Ann Tina Niekerk** for their contribution thought this project. Thank you to the EBL interns **Robynne Silver** and **Simira Lamberty** for their assistance in the lab.

I would like to thank my friends **Jihaad Pienaar**, **Reza Ahmed**, **Abu bakr Hartley, Josh Friedman**, **Ziyaad Valley** and **Toufeeq Allie** for all the relaxing gaming session. Last but not least I would like to thank **Robin Elize Hercules** for all support and encouragement she gave me throughout the project.

http://etd.uwc.ac.za/

List of Abrevations

IAA	Indole Acetic Acid
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
LB	Luria broth
PGPB	Plant Growth Promoting Bacteria
R2A	Reasoner's 2A agar
CAS	Chrome azurol S

http://etd.uwc.ac.za/

List of Figures

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

List of Tables

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

ABSTRACT

Endophytic bacteria are known to have an endosymbiotic relationship with plants and provide them with many beneficial properties. These bacteria stimulate plant hormones, provide protection from pathogens and increase nutrient availability in the environment. In this study some of these potential growth factors were tested.

Endophytic bacteria have the potential to be of great value for the increase of crop production. They offer a variety of processes that aid in plant growth promotion in an eco-friendly manner. The use of endophytic bacteria provides a cheaper and cleaner approach compared to industrial made fertilizers. They also have potential uses in bioremediation to clean the environment polluted by industrial processes.

Endophytes were isolated and showed significant growth improvement. Each isolate displayed different morphologies. Isolates were tested for classical growth promotion mechanisms such as the ability to solubilize phosphate, Indole-3-acetic acid and siderophore production. Inductively Coupled Plasma Optical Emission Spectrometry was performed to measure the effect of the isolates on the plants nutrient profile.

The isolates were then tested again while the plants were under heavy metal stress to determine if they were still capable of growth promotion. The plants were then assayed for cell death using Evans blue and biomass was measured to determine the effect of vanadium stress. Inductively Coupled Plasma Optical Emission Spectrometry was performed again to assess the change in nutrient profile while under vanadium stress.

Keywords: Endophytes, Siderophore, Indole-3-acetic acid, Vanadium, Plant growth promotion

http://etd.uwc.ac.za/

Chapter 1: Literature review

1.1 Introduction

The current increase in global population is placing a strain on food security as the agriculture industry struggles to keep up with food demands (Glick, 2014). The need to increase food production has led to the use of fertilizers and pesticides which have negative effects on the environment. An environmentally friendly approach is needed in order preserve nature while maintaining high crop production to meet food demands.

UNIVERSITY of the
WESTERN CAPE

Epiphytic bacteria are bacteria which are found on the surface of different parts of plants such as the roots, stems, leaves and even in the seeds of the plant. Current studies show that epiphytic bacteria normally don't cause the plant any harm and live non-parasitically on the surface. Some of these bacteria are able to produce an auxin hormone which aids in the growth of plants and plays a role in the life cycle of the bacteria.

The word endophyte means "within plant" which comes from the Greek word "endon" meaning within and "phyton" meaning plant (Kobayashi & Palumbo, 2000). Endophytes are microorganisms that have the ability to colonize plant tissue intracellular or intercellular without causing disease symptoms in the host plant during its life cycle (Miliute *et al*., 2015).Endophytes can be extracted and isolated

http://etd.uwc.ac.za/

from surface sterilized plant tissue (Hallmann *et al*., 1997). Endophytes have been isolated from many plant species and tissue types. There have been isolations of both gram-positive and gram-negative bacterial endophytes from several tissue types in numerous plant species. Multiple endophyte species have also been identified from a single plant. The main entry point for endophytes is the roots but they may even enter through the flowers or stems as well (Kobayashi & Palumbo, 2000). Endophytes are able to colonize different parts of plants ranging from the stems and leaves, including the intercellular spaces of the cell walls and xylem vessels of plant roots. They are also found in tissues or flowers, fruits (de Melo Pereira *et al*., 2012) and seeds (Trognitz *et al*., 2014). Bacterial populations tend to be less in stems and leaves when compared to the roots of a plant (Zinniel *et al*., 2002). The close association between endophytes and host plant is mediated through the compounds produced by the endophyte and the role it plays in the host cells (Brader *et al*., 2014).

UNIVERSITY of the
WESTERN CAPE

The genus *Eriocephalus* is commonly known as wild rosemary and is a member of the Asteraceae family. The genus is endemic to Southern Africa, with the highest appearance of the species in the Western and Northern Cape provinces of South Africa .The species of *Eriocephalus* have a wide range of habitats ranging from coastal locations to plains, mountains, and desert areas (Njenga, 2005). *Eriocephalus africanus* is a small fast growing evergreen shrub, the genus is of economic importance as some of its members are used in cosmetics as well as in

http://etd.uwc.ac.za/

traditional medicines such as antidepressants, antioxidants, antiseptics and anti-inflammatories (Catarino *et al*., 2014).

Brassica napus plants are of important economic value as they are often used in cooking because of their valuable source of dietary fiber. Brassica vegetables contain minute amounts of fats and high amounts of vitamins and minerals. Brassica plants are the third most important sources of edible oils (Gupta & Pratap, 2007). *Brassica napus* oils are high in oleic acid concentrations ~60% and contain other oils such as linoleic acid and linolenic acid. These fatty acids are considered desirable for human nutrition and superior to other plant oils by nutritionist (Rakow & Raney, 2003). Brassica oils have the lowest saturated fatty acid concentration of ant vegetable oil (Adamska *et al*., 2003).

UNIVERSITY of the
WESTERN CAPE

Plant-associated bacteria play an important role in providing plants with defenses against pathogens and help plants grow through various mechanisms. They are able to influence growth both directly as well as indirectly. Bacteria may influence nutrient uptake by means of nitrogen fixation or phosphate solubilization and may produce plant hormones such as auxins to stimulate plant growth (Glick, 2012). Bacteria have been shown to increase the germination rate of plants as well as their growth rate (Souza et *et al*., 2015).

http://etd.uwc.ac.za/

Studies have shown that due to the activity of rhizodeposits and root exudates, the environment of root systems has an abundance of microbes (Hiltner, 1904; Smalla *et al*., 2006; Hartmann *et al*., 2008). Bacterial populations tend to be less in stems and leaves when compared to the roots of a plant (Zinniel *et al*., 2002). The close association between endophytes and the host plant is facilitated through the compounds produced by the endophyte and the role it plays in the hosts cells (Brader *et al*., 2014).

1.2 Plant Growth Promoting Bacteria

Plant growth promoting bacteria (PGPB) may be applied in agricultural production to improve crop yield and reduce the use of fertilizers (Compant *et al*., 2010). Multiple endophyte species have also been identified from a single plant. PGPB are able to enhance the growth of plants as well as protect them from pathogen and abiotic stresses through a wide range of mechanisms. Bacteria that form close association with plants such as endophytes have the potential to have more influence on plant growth promotion. Interactions between plants and microbes play a role in the health, productivity and the richness of soils (Souza *et al*., 2015).

Endophytic bacteria produce an array of secondary metabolites as well as hydrolytic enzymes (Brader *et al*., 2014). The compounds produced by endophytic bacteria also possess antibacterial as well as antifungal properties. The investigation of novel endophytic metabolites and their role in plants is an active

http://etd.uwc.ac.za/

field of research. The current focus of endophytes antimicrobial activity is on the impact on pathogenic bacteria.

Endophytes may influence growth directly or indirectly (Glick, 2012). Bacteria may increase nutrient uptake via nitrogen fixation or phosphate solubilization, they may also stimulate plant hormones such as auxins. They can indirectly influence growth by protecting the host from plant pathogens.

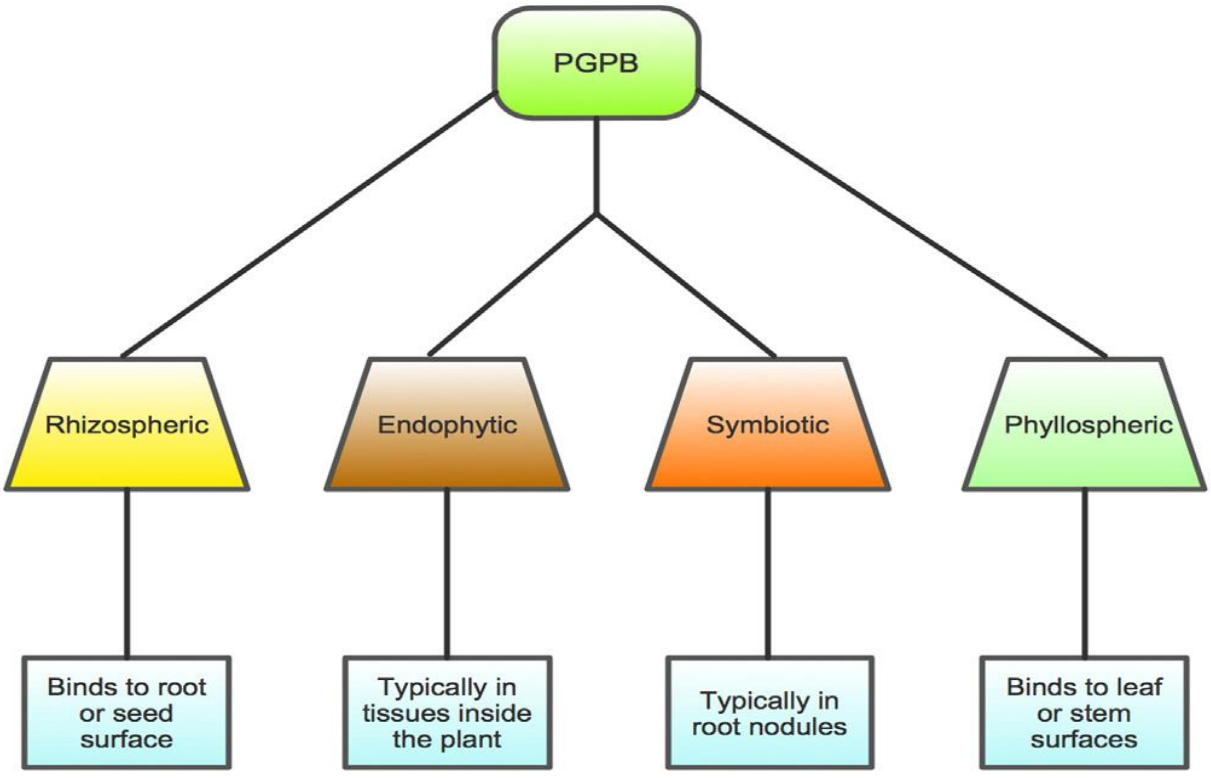


Figure 1: Diagram representing the various ways that plant growth promoting bacteria interact with plants. There are multiple modes of interactions between plants and bacteria, binding may occur at roots, stems or leaves (Glick, 2014).

http://etd.uwc.ac.za/

1.3 Nutrients and auxins

Phytohormones are plant hormones that play a role in plant growth development and stress response. Phytohormones are complex signaling networks that are able to regulate defense responses to abiotic as well as biotic stress (Schmelz *et al*., 2003). Auxins are plant hormones found in roots and stems and play a role in the growth and developments of plants; they were first described by Frits Warmolt Went in 1928.

Plants require nutrients for growth and development like all living things (Uchida, 2000). There are 16 essential elements for plants (boron, calcium, carbon, chlorine, copper, hydrogen iron, magnesium, manganese, molybdenum, nitrogen, oxygen, phosphorus, potassium, sulphur and zinc) to obtain from the environment. Plants have different optimum and minimum nutrient requirements. Plants that take up excessive amounts of essential nutrients experience poor growth as the elements become toxic at high levels.

UNIVERSITY of the
WESTERN CAPE

1.3.1 Phosphate

Phosphorus is a key macronutrient in plants and plays a major role in most metabolic process ranging from energy transfer and storage to photosynthesis (Saber *et al*. 2005). This nutrient also plays a role in respiration (Khan *et al*. 2010) and various other biological processes in plants (Gyaneshwar *et al*., 2002).

http://etd.uwc.ac.za/

Phosphorus is abundant in the environment, however, it is not in a form that plants are able to utilize through their root system making it a major limiting factor in plant growth. Mining phosphate for the use in fertilizers requires large amounts of energy and involves high costs as it must be transported to manufacturing sites and then to farms. The minerals used as fertilizers on landscapes are not eco-friendly as nor is it sustainable. Continuous use of phosphate fertilizers leads to accumulation of Cadmium (Cd) as well as other heavy metals that may be taken up by the crops (Sharma *et al*., 2013). Endophytes that are able to solubilize phosphate can eliminate the need for phosphate fertilizers which can be harmful to the environment when used in excess.

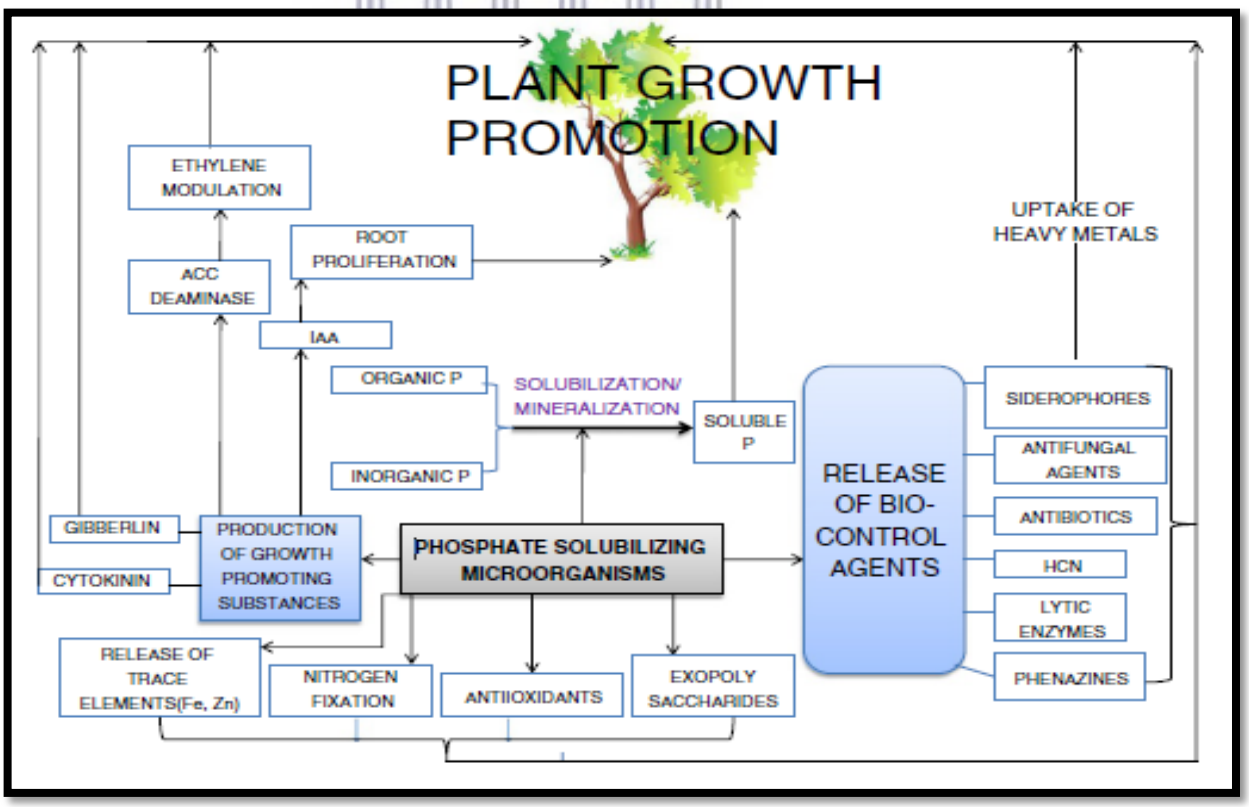


Figure 2: Mechanisms involved in plant growth promotion by phosphate solubilizing microorganisms. (Sharma *et al*., 2013)

http://etd.uwc.ac.za/

1.3.2 Indole-3-acetic acid

Indole-3-acetic acid (IAA) is a plant hormone and is one of the most common auxins found in plants (Lwin *et al*., 2012). Plant hormones influence and regulate a variety of cellular and physiological processes, ranging from cell division to fruit ripening. Auxins play a key role in the stimulation of the xylem and phloem, the development as well as root initiation.

IAA plays a role in the initiation of root elongation as well as increasing the number of root hairs and lateral roots, which aids in the uptake of various nutrients (Hsu , 2010). The rhizosphere has a rich supply of substrates for microorganisms to produce IAA as a secondary metabolite. A large amount of microorganisms produce IAA thought the metabolism of L-tryptophan.

UNIVERSITY of the
WESTERN CAPE

There are many microorganisms that produce IAA through the metabolism of L-tryptophan. There is a rich supply of substrates available to microorganisms in the rhizosphere to produce IAA as a secondary metabolite. IAA aids in the growth of longer roots and an increase in the number of root hairs, as well as lateral roots which play a role in nutrient uptake. The host plant's physiological process is disturbed by the bacteria's production of auxins and it is then used to benefit the bacteria (Hsu, 2010).

http://etd.uwc.ac.za/

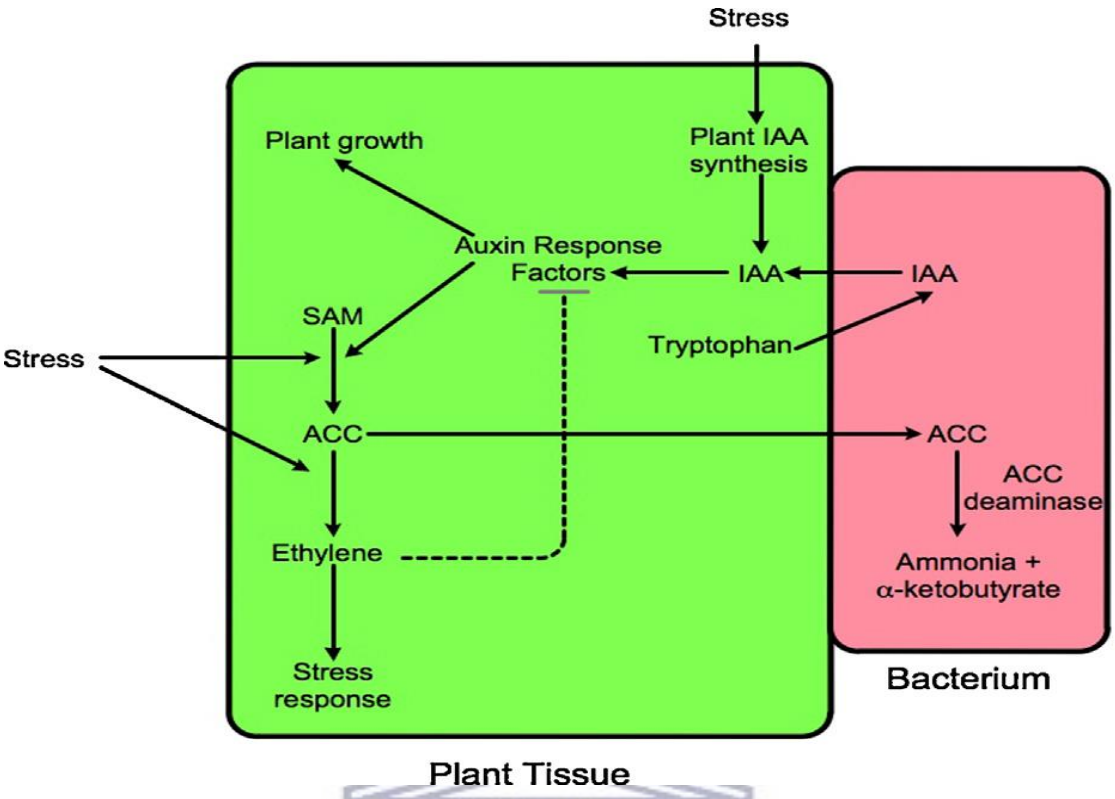


Figure 3: Diagram showing how bacteria and plant tissue interact. Endophytic bacteria that are able to produce ACC deaminase and synthesize IAA may facilitate plant growth promotion (Glick, 2014).

UNIVERSITY of the
WESTERN CAPE

1.3.3 Siderophores

One of the most important nutrients is iron as it is essential for many life forms. (Kiss & Farkas, 2008) The iron in the environment is mostly found in an insoluble form as Fe^{3+} and is not readily available to microorganisms. Bacteria therefore need a way to solubilize iron for uptake. A common mechanism used by bacteria is the production of siderophores. They are low-molecular iron chelators with high affinity for complexing iron. These siderophores also form complexes with other metals in the environment such as copper, zinc and cadmium.

http://etd.uwc.ac.za/

There are hundreds of different siderophores identified to date (Boukhalfa & Crumbliss, 2002). Their overall structures differ, however the functional groups are not diverse. The classification of siderophore are determined by metal binding sites which are either catechol (catecholate-), α-hydroxycarboxylic (hydroxycarboxylate) or hydroxamic acid (hydroxamate-) moieties sites.

Siderophores are able to play a role in the defence against phyto-pathogens. The siderophores are able to bind and reduce the available iron in the environment that the phyo-pathogens require to survive (Beneduzi *et al*., 2012). They have potential applications in medical, agricultural and environmental fields (Saha *et al*., 2016).

UNIVERSITY of the
WESTERN CAPE

1.4 Endophytic Bacteria Applications

Endophytes have been shown to have antimicrobial activity (Hui *et al*., 2013). They are also able to produce antibiotics Seo *et al*. (2010) have shown that some endophytic bacteria are viable bio-control agents that can be used against human and plant pathogens.

South Africa is a major supplier of vanadium (Moskalyk & Alfantazi, 2002) . It is thus expected that due to the mining of vanadium the surrounding areas will have increase levels. Vanadium at high concetraion just like other heavy metals poses a

http://etd.uwc.ac.za/

problem for plants and animals (Moskalyk & Alfantazi, 2002; Saco *et al*., 2013). Endophytic bacteria may be able to produce novel bioactive compounds, they are able to help the host plant cope with environmental stress and are able to detoxify and degrade heavy metals. They also have the potential to increase crop yields through germination increase and enhance plant growth rate.

1.4.1 Bioremediation

Bioremediation is a process that uses microorganisms to detoxify and reduce environmental contaminants (Farhadian *et al*., 2008). The process is a safe and economical method compared to the physiochemical methods commonly used (Bai *et al*., 2008). Plant associated bacteria are known for their crucial role in helping their host adapt to changes in the environment. Endophytic bacteria provide various growth promoting abilities as well as pathogen resistance as well as environmental stress tolerance.

UNIVERSITY of the
WESTERN CAPE

There are many industrial applications to heavy metals, however the wastewaters from these applications pollute the environment. The accumulation of toxic heavy metals in the environment has severe consequences in humans such as carcinogenesis, mental retardation and growth abnormalities (Zahoor & Rehman, 2009). Soils contaminated with heavy metals increase iron deficiency in plants and negatively affect their growth (Christian *et al*., 2008).

http://etd.uwc.ac.za/

Endophytic bacteria possess an advantage over epiphytic bacteria as they are partially protected from the high stress competitive environment of the soil (Guo *et al*., 2010). Endophytes have the potential to be a highly efficient biosorbent for heavy metal biosorption (Xiao *et al*., 2010).

1.4.2 Application of endophytic bacteria in crops

Endophytic bacteria are able to increase crop germination rates (Lugtenberg *et al*., 2002). They also provide crops with improved nutrient uptake by mobilizing nutrients that would otherwise be unavailable to the host plant (Egamberdiyeva, 2007). The endophytic bacteria also provide the crops with protection from plant pathogens.

UNIVERSITY of the
WESTERN CAPE

Endophytes are able improves crop yield as well as the amount of resources that need to be used, this will aid in the crop production for food and feed crops as well as being of economic value. Crops such as canola, maize, soybean and other crops have been successfully inoculated with plant growth promoting bacteria in the laboratory as well as in field trials as shown by Glick *et al*. (1997) and Sharma *et al*. (2013). Egamberdiyeva (2007) showed that plants untreated with plant growth promoting bacteria had poor nutrient uptake when compared to treated plants.

http://etd.uwc.ac.za/

1.5 Justification

The current global population increase is threatening food security and as it stands food is already needed in poorer region around the world. Climate change is expected to add 5 – 170 million more people to be at risk of hunger by 2080 (Schmidhuber & Tubiello, 2007). There is a need for a method to crease crop production without cause damage that may be irreversible to the environment. The experiment is designed to isolate endophytic bacteria that may be used in agriculture industry to improve crop yield as well as decrease environmental pollution.

Agriculture doesn't not only offer production food, it also plays a role in economic income thought imports and exports. Brazil is a heavy importer of the nitrogenous fertilizers, the cost of nitrogen fertilizer is high. Brazil saves approximately US$ 7 billion per year by using microorganisms that are capable of nitrogen fixation (Hungria *et al*., 2013). South Africa is a net importer of fertilizers and thus using endophytic bacteria can reduce the cost of farming significantly. Endophytes offer a natural and environmentally friendly approach to increase crop production and protect the environment.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Chapter 2: Materials and Methods

2.1 Surface Sterilization of *Eriocephalus africanus* roots

A weed sample was harvested from Bellville area (-33.936453, 18.628520) and the roots were surface sterilized using 2% sodium perchlorate, 70% ethanol and autoclaved distilled water washes to get rid of any contaminating epiphytes. The samples were then plated the using pour plate technique after the last wash to check for successful surface sterilization using Reasoner's 2A agar (R2A). The plates were incubated at 30°C for 10 days.

2.2 Endophyte Extraction

The endophytes were extracted from the roots of *Eriocephalus africanus* with 1% sodium chloride using a mortar and pestle and incubated in 1 % sodium chloride water to allow for endophytic growth. A 10-fold serial dilution was performed where each dilution was plated on R2A agar. The plates were incubated at 30°C for 14 days. Endophytic colonies were purified by selecting isolated colonies and performing streak plating technique on R2A agar. The plates were then incubated at 30°C for 7days.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

2.3 Plant Growth

The isolates were grown in LB broths overnight. *Brassica napus* seeds were surface sterilized and imbibed in LB broths containing purified endophyte isolates, while the control only contained LB broth. Seeds were planted in a soil mixture of 2½:1 soil to silica sand and then pre-treated with water. The planted seeds were covered in 2 ml of endophyte culture. Plants were watered with 100 ml of water twice a week. Seeds were monitored for germination percentage and physiological growth differences over a 6 week period.

2.4 Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES)

An amount of 200 mg was weighed of each growth sample and then each sample was transferred to an eppendorf tube. The sample was then mixed with 6 % Nitric acid .Tubes were then wrapped in parafilm and placed in a heat block for 3 hours at 90°C. Using a syringe, 9 ml of 2 % nitric acid was taken up and 1 ml of sample. The sample was then filtered into a Greiner tube using a 0.45 um filter. ICP-OES analyses were done on the samples.

UNIVERSITY of the
WESTERN CAPE

2.5 Phosphate Solubilization plate assay

Single colony isolates were spot plated on phosphate plates made up of Yeast extract 0.05%, Dextrose 1%, Calcium phosphate 0.5%, Ammonium sulphate 0.05%, Potassium chloride 0.200%, Magnesium sulphate 0.01 %, Manganese sulphate 0.00001%, Ferrous sulphate 0.00001% and Agar 1.5%. *Escherichia coli (*Krx) was

http://etd.uwc.ac.za/

used as a control.The plates were then incubated at 30°C for 7 days and observed for the formation of halos around the isolates.

2.5 Indole Acetic Acid assay

Indole acetic acid (IAA) production was measured by a colorimetric test, using Van Urk Salkowski method (Yeast extract 0.1%, Mannitol 1%, Dipotassium phosphate 0.05%, Magnesium sulphate 0.02%, Sodium chloride 0.01%) with 0.1% tryptophan for 5 days at 30°C. The culture was centrifuged at 13000 rpm for 10 min and the supernatant was mixed in a 2:1 ratio with Salkowski reagent (0.25 M $FeCl_3$ and 35% perchloric acid) and incubated for 30 minutes. A standard curve was made by using Yeast Extract Manitol (YEM) and a known concentration of IAA ranging from 0-100 ug/ml. E.coli (krx) was used as a positive control and no microbe was used as the negative control. The optical density (OD) was read at 530 nm.

UNIVERSITY of the
WESTERN CAPE

2.6 Siderophore plate assay

Siderophore activity was measure using chrome azurol S (CAS) media as described by Alexander and Zuberer (1991). Isolates were spot inoculated on CAS plates and incubated at 37°C for 7 days. Plates were observed for formation of zone clearing around the colonies.

http://etd.uwc.ac.za/

2.7 Plant growth trial with vanadium

The isolates were grown in LB broths overnight. *Brassica napus* seeds were surface sterilized and imbibed in LB broths containing purified endophyte isolates, the control only contained LB broth. Seeds were planted in a soil mixture of 2.5:1 soil to silica sand and were pre-treated with water for the control and 350 µm sodium metavanadate for the experimental. The planted seeds were covered in 2ml of endophyte culture and the control seeds were given 2ml of LB. Plants were treated twice a week with 100 ml of 350 µm sodium metavanadate while the control was treated with 100 ml of water. Seeds were monitored for germination percentage and physiological growth differences over a 6 week period.

2.8 Cell death assay (Evans blue)

A modified version of the method described by Sanevas *et al*. (2007) was used to test for cell death. A 0.25% (w/v) Evans blue solution was prepared. A 1 cm^2 block was cut from a fresh leaf and transferred to an eppendorf tubes and 1 ml of Evans blue solution was added to the tubes. The roots were assayed by cutting 2 cm from the tip of the root and transferred to the tubes and 1 ml of Evans blue solution was added. The samples were then incubated for 1 hour at room temperature. The tubes were then rinsed to remove the Evans blue solution. The samples were then incubated overnight in water. The water was then decanted and 1 ml of a 1% (w/v) SDS solution was added to the tubes. The samples were then crushed and incubated at 65°C on a heating block for 1 hour. The samples were centrifuged to

UNIVERSITY of the WESTERN CAPE
http://etd.uwc.ac.za/

obtain the supernatant. The supernatants were then transferred to a microtitre plate and read on a spectrophotometer at 600nm.

2.9 Dry weight (Biomass) analysis

Once the plants had grown from the vanadium growth trial, they were removed from their pots. Three leaves from each plant were removed by cutting at the base of the leaf. The roots were removed by cutting them at the interface between the root and stem. The plant material was then placed in separate foil packets, holes were made in the packets to allow moisture to escape. The samples were then dried overnight in an incubator at 80°C. Once the samples were dry they were weighed and the values were recorded.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Chapter 3: Isolation of growth promoting Endophytic bacteria

3.1 Obtaining isolates

Endophytic bacteria have been cultured from different parts of plants (Nair & Padmavathy, 2014). The population present in the plants vary as the region, climate conditions and age of the plant all play a role in the condition needed for the bacteria to thrive. The population also differs within the same plant species due to these variations in environmental conditions. Bacterial populations tend to be less in stems and leaves when compared to the roots of a plant (Zinniel *et al*., 2002). The close association between endophytes and the host plant is mediated through the compounds produced by the endophytes and the role they play in the host cells (Brader *et al*., 2014). The extraction from the root of the plant yielded 3 endophytes of different morphologies.

UNIVERSITY of the
WESTERN CAPE

3.1.1 Results and Discussion

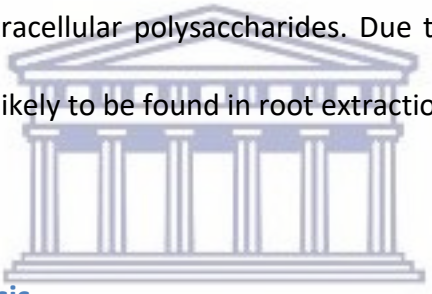
Figure 4: Purified endophyte isolates. Endophytic bacteria isolates were identified and labeled namely Wo, X and Ya

Each isolate displayed different morphologies which provide evidence that there exists a diverse population of endophytes interacting with plants. The Wo strains

http://etd.uwc.ac.za/

displayed an orange color with white pigmentation around the edges of the colony. The isolate displayed a dry flake like texture. The X strain was a lighter orange in color and was small with a round flat shape.

The Ya strain had a Mucoid texture and is the only isolate that exhibited this morphology which may play a role in the increase in germination as well as its interaction with the plant. A study done by Danhorn and Fugue in (2007) showed that mucoid strains' production of extracellular polysaccharides can increase biofilm formation and promote colonization of plants. A study done by in Bloemberg and Lugtenberg (2001) showed that mucoid strains adhere better to the roots because of the extracellular polysaccharides. Due to their longer adhesion, mucoid strains are more likely to be found in root extractions.


UNIVERSITY of the
WESTERN CAPE

3.2 Seed germination analysis

Bacteria have been shown to have an effect on the germination rate and increase plant growth rate (Souza *et al.*, 2015). Seedling emergences has also been shown to improve. Increasing endophyte resources can provide an increase in novel and effective bioactive compounds that cannot be chemically synthesized. The increase of seed germination leads to improved crop yield as fewer harvests are required and less resource used such as fertilizers.

http://etd.uwc.ac.za/

3.2.1Results and Discussion

Table1: Germination increase of isolates over 15 days. Treatment with endophytic strains yielded various increases in seed germination.

Strain	Day 4	Day 7	Day 11	Day 15
Control	50%[a]	67%[b]	67%[b]	67%[b]
Ya	100%[a]	100%[a]	100%[a]	100%[a]
Wo	83%[a]	83%[a]	100%[b]	100%[b]
X	67%[a]	67%[a]	83%[b]	83%[b]

Different letters indicate significant differences between means at P< 0.05 (anova) per row. Values are means ± S.E (N=3).

UNIVERSITY of the WESTERN CAPE

In this study, endophytes showed significant increase to seed germination after 4 days when compared to the control. The, Ya and Wo strains showed significant increases in germination with Ya having complete germination after 4 days and Wo achieving complete germination after 11 days. The X strain showed increased germination however it didn't achieve a perfect germination like the other two strains. The Ya strain increased initial seed germination by 100% while the Wo strain increased it by 66%. The X strain had an increase of 34% the lowest of the 3 isolates. The YA and Wo strain both had an overall increase of 49% while the X stain had a 24% increase to seed germination. The increase in germination percentages and the difference in times between isolates may be due to the endophyte isolates providing the seeds with different growth promoting mechanisms. The isolates may also be using the same mechanism but produce them at different concentrations.

http://etd.uwc.ac.za/

Plant endophytes are able to secrete growth hormones such as auxins and cytokins which help promote the germination as well as the growth of the plant (Paguia & Valentino 2016). They are also able to regulate nutrient uptake allowing for enhanced plant growth. IAA is also able to affect seed germination by affecting the activity of enzymes for example, in germinating pea seeds, the activity of glyoxalase I was regulated by IAA, resulting in higher rates of cell growth and development (Miransari & Smith, 2014). IAA was detected as early as the second day of germination in *Phaseolus vulgaris* (Bailek *et al*., 1992). Increases in some nutrients such as zinc play a role in increased seed germination, thus endophytic bacteria that are able to increase the uptake of these specific hormones or nutrients enhance the germination of seeds.

UNIVERSITY of the
WESTERN CAPE

Increases in seed germination provide an increase in yield of crops which increases profits. The increase in germination also allows for early harvests which saves farmers time and allows them to meet production demands sooner. All experimental strains showed better germination when compared to the control sample thus they each have one or more plant growth promotion mechanism such as nutrient regulation or hormone production.

http://etd.uwc.ac.za/

3.3 *B. napus* growth trial

The ever growing human population is creating a demand for an increase in food production, it is predicted that the demand will increase for another four decades (Godfray *et al*., 2010). Climate change poses a threat as it affects the optimal growing condition for crops. Growth promoting endophytic bacteria may be the solution to the high demand of food production. Plant growth promoting inoculates have been shown to have a variety of effects on plants ranging from increased germination to increased crop size (Souza *et al*., 2015).

3.3.1 Results and Discussion

Figure 5: Plant growth Physiology of isolates. Endophytic bacteria treated strains showed significant growth improvements. Isolates represent by letter as seen isolation.

After 6 weeks of growth, endophyte isolates showed significant differences in growth compared to control samples. The Wo treated plants had the biggest leaves and most defined root structure while the Ya and X treated plants possessed firm stems. The Ya treated plants showed the most consistent growth results

http://etd.uwc.ac.za/

suggesting that it may be responsible for multiple growth promoting properties. The Wo treated plants increased plant overall size by 31%, the X treated plants showed an increase of 19% and the Ya had an increase of 13% in overall size.

Experimental plants all exhibited faster growth rates, stronger stems as well as more defined roots systems. The experimental samples also had large leaf surface area which aids in chlorophyll production. Light and nutrient availability play a direct role in the size of the leaves of plants (Jurik *et al*., 1982). The endophytes may increase nutrient uptake of elements such as magnesium and manganese which play key roles in chlorophyll production and photosynthesis. The increase in stem strength may also be due to nutrient uptake.

UNIVERSITY of the
WESTERN CAPE

The radius and length of roots can play a role in nutrient uptake by plants growing in soil (Silberbush & Barber, 1984).The increase in root length by the endophyte isolates and increase in nutrient uptake can be seen in the ICP analyses in table 2 and physiology in figure 5. The increase in root length may be due to increased production in IAA which is known to initiate root elongation. Pattern and Glick (2002) showed that bacterial IAA plays a major role in root development.

3.4 Inductively coupled plasma optical emission spectroscopy (ICP-OES)

Inductively coupled plasma optical emission spectroscopy (ICP-OES) was used to determine the nutrient profile of the growth trial plants. The ICP-OES results will

http://etd.uwc.ac.za/

give an indication of how the endophyte interacted with the uptake of important nutrients. Data obtained from ICP-OES analysis shows that each endophyte regulated nutrient uptake differently. Endophytes are able to aid in the uptake and availability of nutrients, improve stress tolerance and provide an enhanced defense against disease (Ryan *et al.*, 2008). The ability for endophytes to aid in growth promotion is linked to the plant growth promoting hormones it produces. Endophytes may also increase the available amount of nutrients in the environment such as nitrogen and phosphorus though its activity (Glick, 2012). All of the isolates regulated nutrient uptake in at least three elements when compared to the control sample.

3.3.1 Results and Discussion

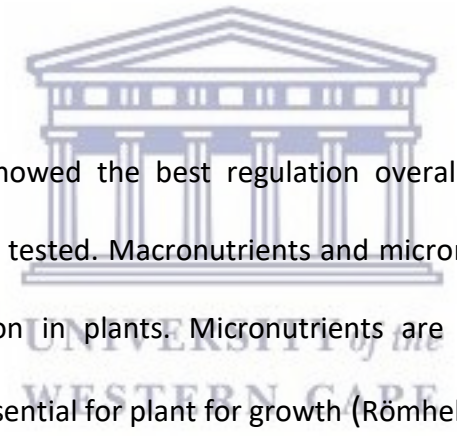
Table 2: Inductively coupled plasma optical emission spectrometry (ICP-OES) of plants treated with endophytes. Macronutrients represented in green and micronutrients in blue. Color scale from green (increase in uptake) to red (decrease in uptake).

Element	C	X	Ya	Wo
Mn	0,828 b	0,905 a	0,719 c	0,830 b
Fe	0,943 b	1,328 a	1,245 a	1,302 a
Cu	0,020 c	0,029 b	0,035 a	0,025 b
Zn	1,450 b	1,681 a	1,231 c	1,280 c
Mo	0,005 d	0,018 c	0,071 a	0,035 b
Ni	0,046 c	0,092 a	0,050 c	0,068 b
P	27,733 b	29,946 a	27,080 c	27,420 b
K	1084,640 c	1167,803 a	991,550 d	1132,487 b
Ca	152,666 b	208,233 a	191,805 a	195,925 a
Mg	55,996 b	58,616 a	61,385 a	57,507 a

Different letters indicate significant differences between means at P< 0.05 (anova) per row. Values are means ± S.E (N=3). Values are in mg/Kg.

http://etd.uwc.ac.za/

Elements such as Manganese (Mn) showed an increase of 9%, Zinc (Zn) with an increase of 16%, Calcium (Ca) was up by 36%, Iron (Fe) increased by 41% and Nickel (Ni) showed a 100% increase in plants treat with the X strain. The Ya treated plants showed an increase of Magnesium (Mg) by 11%, Ca 26%, Fe 32% and Copper (Cu) by 75%.The Wo treated plants showed increases of nutrient uptake for Potassium (K) by 4%, Fe 38% and Ca by 28%. All isolates show an increase uptake of Ca, Fe and Cu. The increase uptake of Fe and Cu may be caused by siderophore activity produced by the isolates.


UNIVERSITY of the
WESTERN CAPE

The X strain sample showed the best regulation overall, inducing a significant increase in all elements tested. Macronutrients and micronutrients play a key role in physiological function in plants. Micronutrients are only required in small amounts but are still essential for plant for growth (Römheld & Marschner, 1991).

Nickel (Ni) is a necessary element that aids in the functioning of the enzyme urease and improves seed germination (Jones & Jacobsen, 2005). The accumulation of urea due to a lack of nickel causes necrotic lesions on the tips of leaves. The increase in Ni for X and Wo strains might have contributed to its increased germination. The Ya strain must therefore possess another growth promoting mechanism for its increased germination as the amount of Ni is equivalent to the control.

http://etd.uwc.ac.za/

The element zinc (Zn) is involved with various metabolic activities by influencing the activities of carbonic anhydrase and hydrogenase. The deficiency in Zn leads to a plants growth being stunted, chlorosis and affects the quality of harvested crops (Hafeez *et al*., 2013). The increase in Zn in the X strain may have allowed it to increase metabolic activity which could have helped with the increase of other nutrient's uptake.

Calcium (Ca) is a multifunctional nutrient in the physiology of crop plants. It plays a role in the structure and permeability of membranes and cell wall strength as well as its thickness. A deficiency in Ca leads to weakened stems (Easterwood, 2002). All experimental samples showed higher amounts of calcium which may have improved the stems as they appeared to be stronger when compared to the control plants.

UNIVERSITY of the
WESTERN CAPE

Plants require Copper (Cu) as an essential micronutrient for normal growth and development. Cu acts as a structural element in regulatory proteins and participates in photosynthetic electron transport, mitochondrial respiration machinery, oxidative phosphorylation and iron mobilization (Yruela, 2005). All the endophyte treated plants showed significant increase in copper uptake and increased growth. The increase in growth rate may have been due to the plant now having more Cu available for normal growth.

http://etd.uwc.ac.za/

Potassium (K) is essential for many physiological processes, such as photosynthesis, translocation of photosynthates into sink organs, maintenance of turgescence, activation of enzymes, and reducing excess uptake of ions such as Sodium (Na) and Fe in saline and flooded soils (Cakmak, 2005).Potassium deficiency causes severe reduction in photosynthetic CO_2 fixation and impairment in partitioning and utilization of photosynthates. Potassium aids in growth of plants as well as helping with biotic and abiotic stress response (Ahmad & Maathuis, 2014). This is evident in the size of the plants inoculated with the Wo and X strain.

Manganese (Mn) plays a role in photosynthesis and controls multiple oxidation-reduction systems (Jones & Jacobsen, 2005). A deficiency in manganese can look similar to that of iron deficiency (interveinal chlorosis) namely yellow leaves with green veins. The increase of Mn in the X strain may have allowed the plant to grow faster and healthier. The leaves observed in plants treated with endophytes were larger and greener based on surface area.

UNIVERSITY of the
WESTERN CAPE

Magnesium (Mg) is an enzyme activator and its most important role is as the central atom in the chlorophyll molecule (Shaul, 2002). Iron (Fe) plays a role in numerous functions most notably with chlorophyll synthesis and in enzymes for electron transfer. The two major problems with iron as a free ion is its toxicity and insolubility (Hell & Stephan, 2003). The increase in Mg may have allowed the plants

http://etd.uwc.ac.za/

to absorb light better which is required for photosynthesis, thus improving plant growth. The increase in Fe observed in plants treated with endophytes may be due to siderophore production.

In 1953 D.Mulder published his “Les elements mineurs en culture fruitière” that shows us how nutrient uptake is influence by each other. The chart shows that Ca and P have an antagonistic nature to each other. The table shows that the control ratio for P:Ca is 1:5.5 and as Ca increases in the treated plants, the ratio changed to 1:7 in all isolates showing that P was being taken up less by the plant. This could be due to the antagonistic relationship between P and Ca. The same relationship is shown between P and Fe where the control ration was 1:31 and the experimental samples were about 1:22. The increase in Cu can lead to a decrease in Mn according to Mulders chart as they also have an antagonistic effect on each other. The control ration was 1:40 and as Cu increased in the experimental samples the ratio for X and Wo treated plants became 1:32 while Ya treated plants has a ration of 1:20 as it had the highest increase in Cu whilst X and Wo were statistically the same.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Chapter 4: Characterization of endophytic bacteria

4.1 Phosphate solubility

There are six essential macronutrients nutrients for plants, Mg, N, S, K, P and Ca (Vance *et al*., 2003). Due to its insolubility, phosphorus is a major limiting factor in plant growth, despite it being abundant in soil. It exists in a form that is unavailable to plants though root uptake. Phosphorus is a bio-critical element which is essential for plant nutrition, it plays a role in a majority of metabolic processes. Mining phosphate for the use in fertilizers is energy intensive. Excessive use of the fertilizer causes damage to the environment (Khan *et al*., 2010; Sharma *et al*., 2013). Endophytic bacteria offer an eco-friendly approach to supply plants with phosphorus without causing harm to the environment.

UNIVERSITY of the
WESTERN CAPE

4.1.1 Results and Discussion

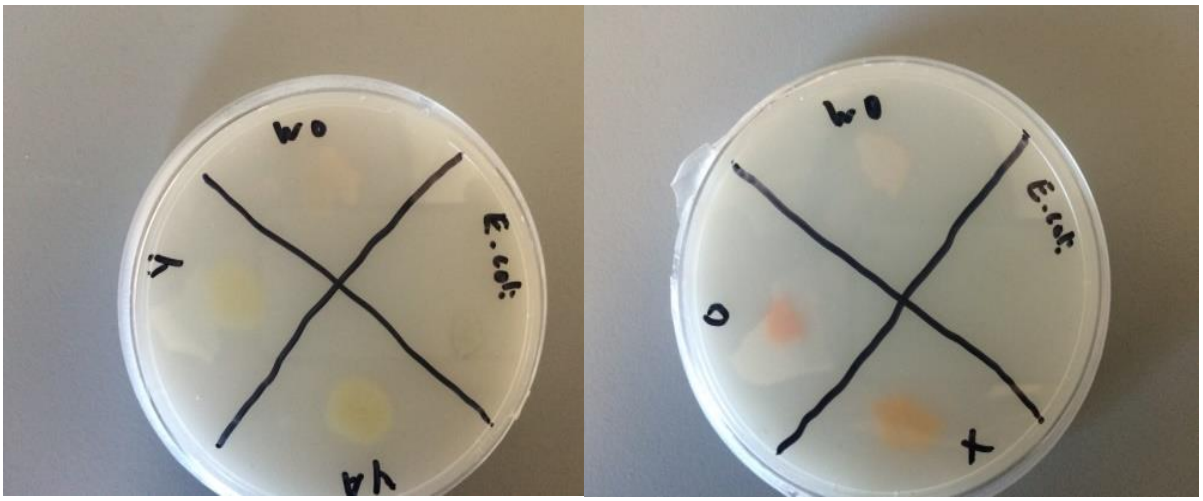


Figure 6: Phosphate solubility of endophyte isolates. Isolates were spot inoculated and observed for formation of zone clearings to identify phosphate solubization. O is a replicate of X and Y is a replicate of Ya.

http://etd.uwc.ac.za/

All isolates tested negative for phosphate solubilization as no halos were formed around the colonies on the phosphate plates, indicating that the endophytes are able to grow on the media but not solubilize phosphate. *E.coli* krx was used as a negative control and also produced no halo. This result is supported by ICP-OES analyses (table 2) which show that phosphate nutrient levels were similar in all experimental samples when compared to the control. Therefore, these endophytic bacteria likely did not play a role in the regulation of phosphate to benefit plant growth promotion.

4.2 Indole-3- Acetic Acid production

One of the most physiologically active auxins in plants is Indole acetic acid (IAA), a common product of the metabolism of L-tryptophan which is produced by many microorganisms such Plant Growth Promoting Rhizobacteria. Auxins are plant hormones that play a role in various plant growth mechanisms such as the improvement of root initiation (Lwin *et al*., 2012) and seed germination. . Esesami *et al*. (2015) showed that the production of IAA aids in microbe to plant interaction and protects the microbe from the plants natural defense mechanisms. All isolates were able to produce IAA in various quantities (table 3).

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

4.2.1 Results and Discussion

Table 3: Indole-3-acetic acid production of isolates. A 0-100 ug/ml standard was used to measure the amount of IAA produced by each isolate.

Strain	C(-)	C(+)	Ya	Wo	X
Concentration (µg/ml)	0[d]	8[b]	68[a]	3[c]	9[b]

Different letters indicate significant differences between means at P< 0.05 (anova). Values are means ± S.E (N=3).

The Ya strain produced the most IAA at 68 µg/ml while the Wo and X strain produced significantly lower amounts. The increase in germination of the Ya treated plants can be related to the IAA produced. The Wo strain which had a similar germination rate did not produce as much IAA, indicating that this strain may possess another mechanism which aided in the increase of seed germination. The X strain produced more IAA than the Wo, however its germination was not as high.

UNIVERSITY of the
WESTERN CAPE

IAA stimulates the production of longer roots and increases the number of root hairs and lateral roots which contribute in nutrient uptake (Datta & Basu, 2000). IAA is a metabolite derived from tryptophan (Trp) by various Trp-dependent and Trp-independent pathways in bacteria and plants. There can be more than one pathway present in a bacterium (Pattern & Glick, 1996). The Wo and X treated plants in figure 5 show larger root systems when compared to the control which may be due to the IAA produced.

http://etd.uwc.ac.za/

A study done by Patil *et al*. (2011) showed endophytic bacteria producing 25 µg/ml of IAA. The Ya strain produced almost 3 times the amount showed in this study. In another study done by Mohite (2013) it was shown that high production of IAA is between 10-50 µg/ml for rhiziphere microorganism, the endophytic strain Ya out produced this by 18 µg/ml.

4.3 Siderophore activity

Siderophores (Greek for "iron carrier") are small molecular iron chelators that microbes produce in order to obtain iron in a soluble form (Behnsen & Raffatellu, 2016). Siderophores can be produced by animal or plant microbes, it is most common in plant rhizobacteria (Rajkumar *et al*., 2010). All isolates were able to produce siderophore activity of various levels.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

4.3.1 Results and Discussion

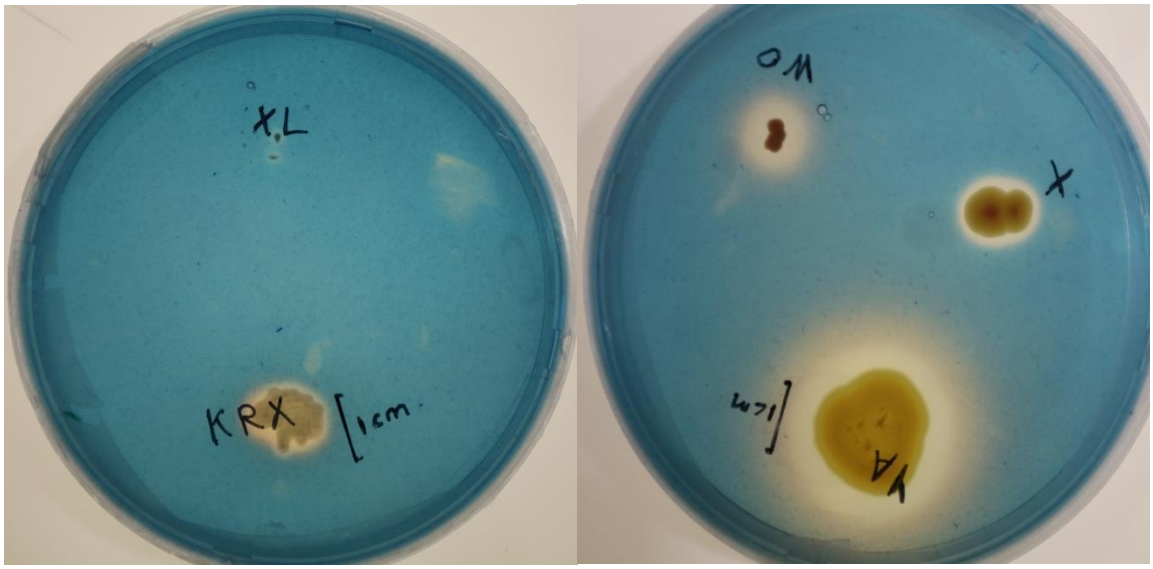


Figure 7: Siderophore activity of spot inoculated isolates on CAS media. Zone clearing indicates positive activity.

Siderophore activity was calculated as % = ((Zone clearing – colony size)/ colony size)*100.The Wo colony had a zone of clearing of 10 mm and a colony size of 4 mm producing an activity of 150% which was the highest observed of the 3 isolates. The Ya colony had a zone of clearing of 30 mm and a colony size of 16mm producing 88% activity while the X colony had a zone of clearing of 12 mm and a colony size of 9 mm producing 33% activity. The *E.coli* krx strain served as a positive control and had a zone clearing of 12 mm and a colony size of 10 mm producing an activity of 20%.The *E.coli* strain XL gold served as a negative control produced no zone clearing as expected.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

The confirmation of siderophore activity supports the increase of Fe and Cu uptake seen in ICP-OES analyses (table 2). The increase in uptake is statistically the same for Fe in all isolates while Cu is statistically the same in X and Wo. The Ya strain was statically the most up regulated for Cu, which may be due to it being more active in the plant than the other isolates.

Extreme environmental conditions stimulates optimum growth and siderophore production activity in bacteria (Rajkumar *et al*., 2010). The isolates having siderophore production may allow it to aid plants that are under heavy metal stress. This makes them ideal candidates for bioremediation as they will be able to regulate the metals in the environment. Siderophores can also play a role in pathogens defense as they limit the available Fe in the environment for pathogens (Beneduzi *et al*., 2012). The isolates have the potential to be used as a bio-control to protect plants from infections.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Chapter 5: Effect of Vanadium toxicity on endophytic bacteria

5.1 Seed germination under vanadium stress

High levels of heavy metals in soil negatively impact plant growth and seed germination (Sethy & Ghosh, 2013). Plants have adapted biochemical and genetic strategies through evolution to manage heavy metal stress. Seeds are highly sensitive to the environment and protective to external stresses. Once seeds start to develop they become sensitive to the stress from the environment (Li *et al*., 2005). Germination and seedling establishment are critical stages which affected both quality and quantity of crop yields(Tian *et al*., 2014)

UNIVERSITY of the
WESTERN CAPE

5.1.1 Results and Discussion

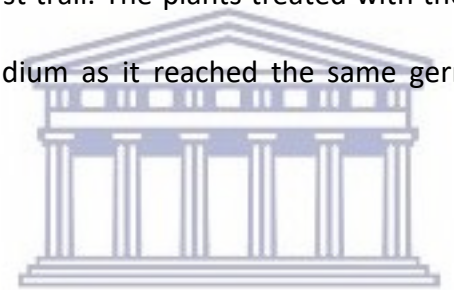
Table 4: Germination increase of isolates under vanadium stress. Endophytic strains show various increases in seed germination.

Strain	Day 4	Day 7	Day 11	Day 15
Control	50%[a]	67%[b]	67%[b]	67%[b]
V	50%[a]	78%[b]	78%[b]	78%[b]
Ya	50%[a]	78%[b]	83%[c]	83%[c]
Wo	67%[a]	78%[b]	83%[c]	83%[c]
X	67%[a]	83%[b]	83%[b]	83%[b]

Different letters indicate significant differences between means at P< 0.05 (anova) per row. Values are means ± S.E (N=3).

http://etd.uwc.ac.za/

The results obtained in table 1 show significant increases in seed germination for seeds treated with endophytic isolates however, in table 4 it shows that while under vanadium stress the germination increase was hindered. The control's germination remained the same in both tests, vanadium stress increased germination which may be due to a defensive mechanism. A study by Kaya *et al*. (2006) showed that plants under stress may increase germination however, they varied in time taken to germinate. Treatment with the isolates all yielded an increase in germination but the Ya and Wo isolates were unable to reach maximum germination as in the first trail. The plants treated with the X isolate displayed the most tolerance to vanadium as it reached the same germination as the normal growth trial.



UNIVERSITY of the
WESTERN CAPE

5.2 Alleviating Vanadium Stress in *B.Napus* using endophytic isolates

Vanadium is a trace element in soil, however elevated amounts of it is toxic to plants in the environment and can have an impact on the plants physiology and biomass. Heavy metals in high concentration can decrease seed germination and reduce biomass. Excess heavy metal in soils can reduce shoot and root elongation, as well as nutrient and productivity loss (Sethy & Ghosh, 2013). Bacteria have several mechanisms in which they can mobilize immobilize or transform metals. This helps them reduce the toxicity to tolerate the uptake of heavy metal ions. The metal can bind to extracellular material, thus preventing it from entering the cell (Rajkumar *et al*., 2010).

http://etd.uwc.ac.za/

5.2.1 Results and Discussion

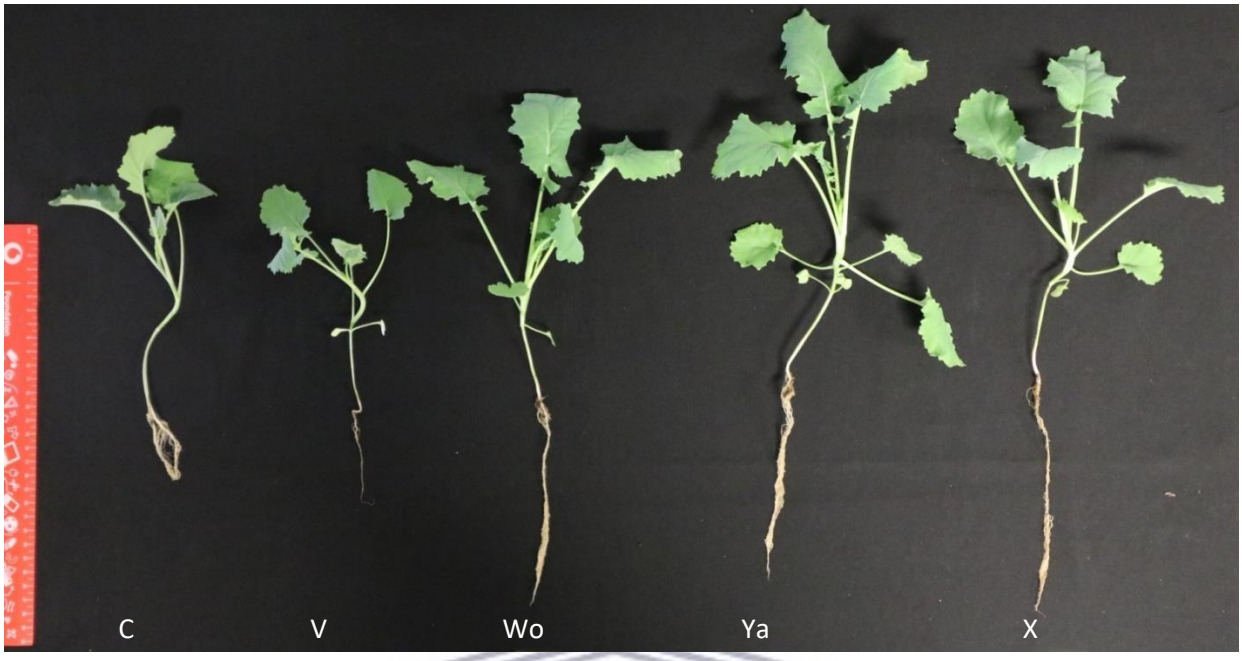


Figure 8: Plant growth Physiology of isolates under vanadium stress. Treatment with endophytic bacteria strains yielded significant growth improvements. Isolates represent by letter as seen isolation. C represents the control and V represents plants treated with vanadium.

UNIVERSITY of the
WESTERN CAPE

After 6 weeks of growth, plants treated with endophyte isolates displayed significant differences in growth compared to control and vanadium treated plants. Plants treated with vanadium showed a 12% decrease in overall plant size. The Ya treated plants showed the most significant increase of plant size of 60%. The X and Wo treated plants showed an increase of 50% and 41% respectively. A study by John *et al*. (2012) showed that heavy metal toxicity caused a decline in growth parameters in *Brassica juncea*. The leaves in the vanadium plants showed less development when compared to the control, the stem and roots were not well developed showing the negative effect that excess vanadium cause. The vanadium treatment showed no visible effect on the plants

http://etd.uwc.ac.za/

containing the endophyte isolates. The decrease in root development in the vanadium treated plants may impact its nutrient uptake.

The experimental samples had leaves with larger surface area while under vanadium stress when compared to both the control and the vanadium samples. The root structures of the experimental samples showed the same trend that was seen in the leaves. The increase in root development may be due to the production of IAA by the bacteria. The Ya sample showed a thicker root system while the X and Wo strains show longer root system which may be an attempt to escape the vanadium and reach more nutrients. The size of the leaves for the experimental samples was significantly larger than those of the control and vanadium treated plants. The leaves showed no sign of yellowing under vanadium stress. The Ya strain had the biggest leaves and most defined root structure overall.

UNIVERSITY of the
WESTERN CAPE

The treated plants resistance to the stress of excess vanadium added to the environment might have been mitigated by the isolates producing siderophores. The siderophores may have acquired iron and produced biofilms to protect itself and the plant. The increase in root length caused by the isolates may have helped the plant obtain more nutrients thus allowing the plants growth not to be hindered.

http://etd.uwc.ac.za/

5.3 Cell death

Programmed cell death (PCD) is a physiological response to selectively eliminate damaged or deregulated cells (Pennell & Lamb, 1997). Plants under stress from the environment or pathogen infection will activate PCD as defensive mechanism (Lam *et al*., 2001). Cell death is a useful indicator to measure the amount of damage a stress caused. The Evans blue assay works on the basis that cells that are not damaged won't allow the Evans blue to be taken up.

5.3.1 Results and Discussion

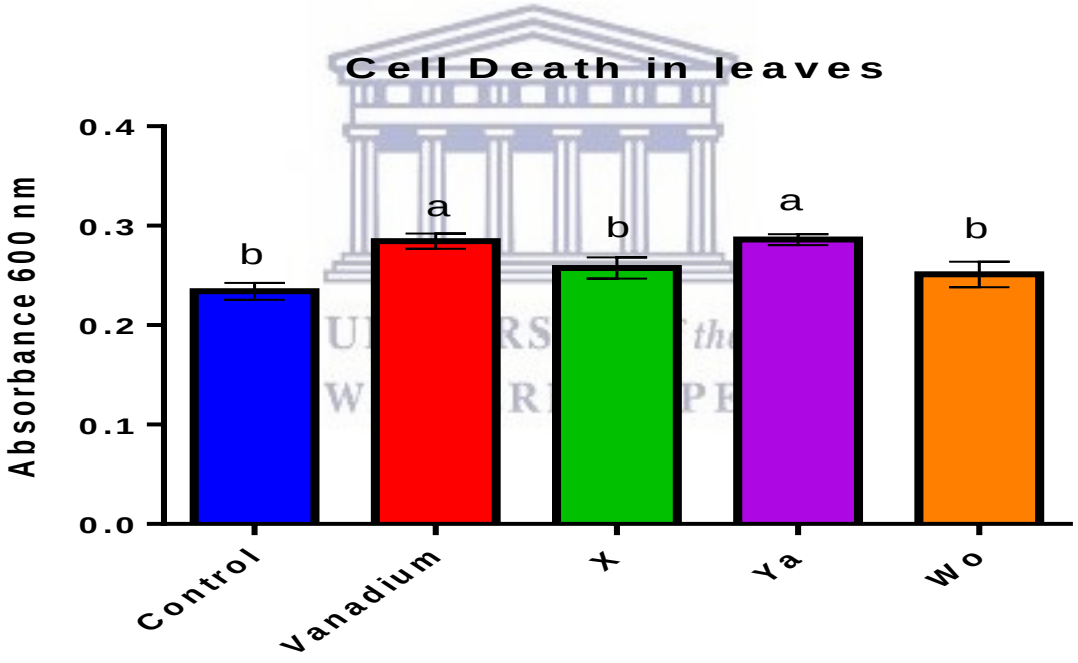


Figure 9: The effect of endophytic bacteria on cell death of vanadium treated *Brassica napus* leaves. Vanadium was applied to cultivars, the cell death of the leaves were determined. Different letters indicate significant differences between means at $P < 0.05$ (anova). Values are means ± S.E (N=3).

http://etd.uwc.ac.za/

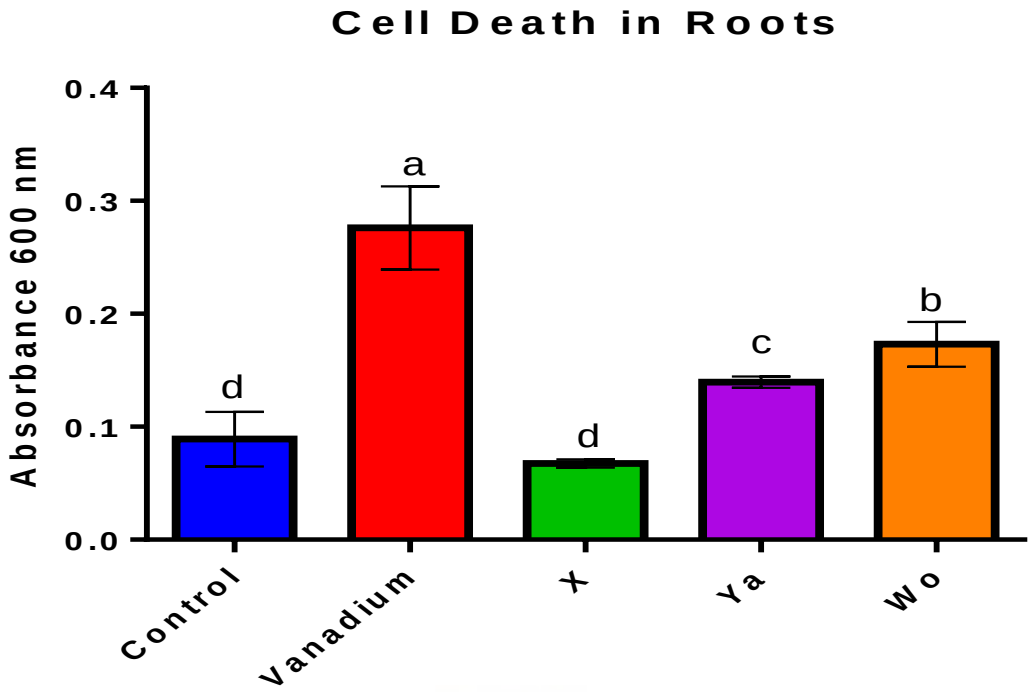


Figure 10: The effect of endophytic bacteria on cell death of vanadium treated *Brassica napus* roots. Vanadium was applied to cultivars, the cell death in the roots were determined. Different letters indicate significant differences between means at P< 0.05 (anova). Values are means ± S.E (N=3).

Cell death in the leaves where increase by 22% in vanadium plants and 20% in plants treated with Ya. Plants treated with X and Wo showed an increase in cell death of 9%. The cell death in the roots of vanadium plants where increased by 175%. The Ya and Wo samples showed an increase of 40% and 70% respectively. The plants treated with the X strain showed a decrease in cell death by 25%.

In figure 9 the vanadium treated plants and the Ya treated plants had the highest cell death in leaves while the control, X and Wo samples were statistically the same. The cell death in the leaves do not show drastic changes as it is not seen in figure 8 where all the leaves show no deterioration or yellowing. This suggests the damage may be limited to the roots as defense mechanisms.

http://etd.uwc.ac.za/

In figure 10 the vanadium treated plants showed the highest cell death in the roots and the effect can be seen in figure 8 as the roots are smaller in size and length. The control samples has significantly less cell death compared to the vanadium treated plants. The plants treated with endophyte isolates all showed significant decreases in cell death compared to the vanadium treated plant. The X strain was statistically the same as the control showing a high tolerance for vanadium while the Ya strain reduced the cell death by ~50%. The Wo strain showed the least tolerance of the three isolates, however it still had a high tolerance for vanadium.

The cell death in roots (figure 10) shows that the isolates provided a process to the plant to help mitigate the vanadium stress. It has been observed that Fe plays a role in the formation of biofilms in microorganisms (Glick *et al*., 2010).The isolates all produced siderophores which may have aided in biofilm formation that protected both the plant and microbes from the vanadium stress.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

5.4 Biomass

A study done by Gokul (2013) showed that vanadium stress decreased plant biomass. The decrease in biomass effects crop yield as more crops will be needed to meet supply demands. The dry weights of the plants were determined to see if the isolates were able to improve plant biomass under vanadium stress.

5.4.2 Results and Discussion

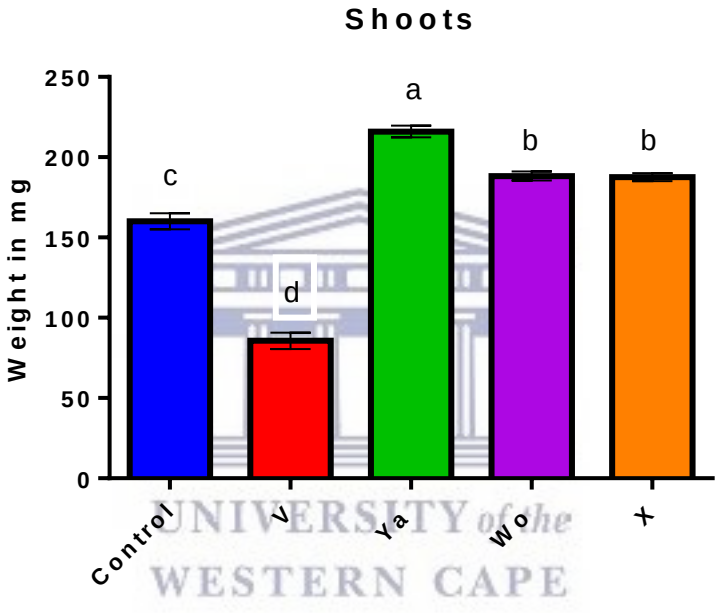


Figure 11: The effect of endophytic bacteria on dry weight of vanadium treated *Brassica napus* shoots. Vanadium was applied to cultivars, the dry weight in the shoots were determined. Different letters indicate significant differences between means at P< 0.05 (anova). Values are means ± S.E (N=3).

http://etd.uwc.ac.za/

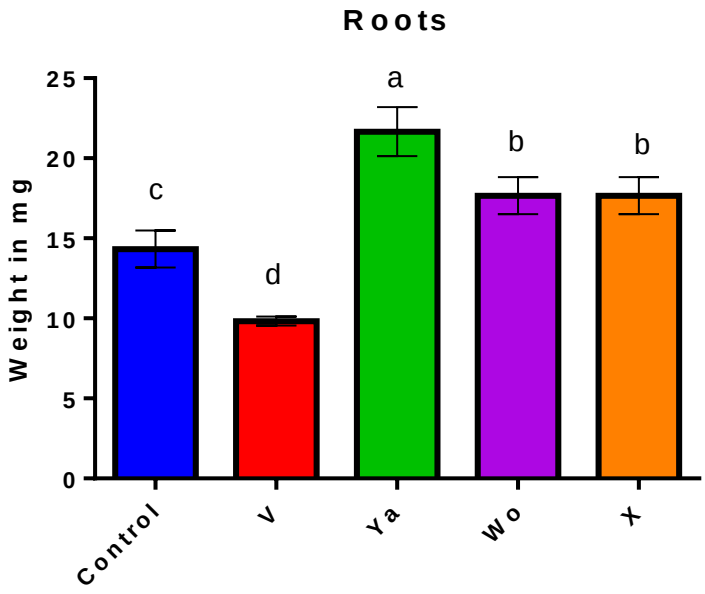


Figure 12: The effect of endophytic bacteria on dry weight of vanadium treated *Brassica napus* roots. Vanadium was applied to cultivars, the dry weight in the roots were determined. Different letters indicate significant differences between means at P< 0.05 (anova). Values are means ± S.E (N=3).

Dry weight of the vanadium treated plants shoots showed a decrease of 46% and the root a decrease of 29%. The Ya sample showed the biggest increase in both root (50%) and shoot (33%).The Wo and X samples showed very similar biomass increases. The shoots showed an increase of 19% while the roots biomass increased by 29%. John *et al*. (2012) showed that heavy metal stress decreased plant biomass and cause a decrease in root length in *Brassica juncea*.

The vanadium treated plants show that the increase in vanadium had a negative impact on biomass when compared to the control in both roots and shoots. The endophyte treated plants show that they were able to overcome the vanadium toxicity and improve biomass in both the roots and shoots when compared to both the control and vanadium treated plants.The trend in biomass is an expected result

UNIVERSITY of the WESTERN CAPE
http://etd.uwc.ac.za/

as the difference in plant size and root development could be seen in figure 8. The increase of biomass for isolates compared to the vanadium treated plant show that the microbes are able to help the plants growth heath despite the stress vanadium causes.

5.5 Inductively coupled plasma optical emission spectroscopy (ICP-OES)

Inductively coupled plasma optical emission spectroscopy (ICP-OES) was used to determine the nutrient profile of the growth trial plants and the uptake of vanadium. The ICP-OES results will give an indication of how the endophyte interacted with the uptake of important nutrients while the plant was under heavy metal stress of vanadium.Build-up of heavy metals in plant tissue leads to adverse effects on growth as well as an impaired nutrition uptake. Heavy metals can induce iron deficiency and the resulting low iron content affects chlorophyll biosynthesis and hinders chloroplast development (Parida *et al.*, 2003).

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

5.5.1 Results and Discussion

Table 5: Inductively coupled plasma optical emission spectrometry (ICP-OES) of cultivars treated with vanadium. Macronutrients represented in green and micronutrients in blue. Color scale from green (increase in uptake) to red (decrease in uptake).

Element	C	V	X	Wo	Ya
Mn	1,065 b	0,945 d	1,269 a	1,002 c	1,066 b
Fe	0,539 a	0,528 a	0,482 b	0,232 d	0,314 c
Cu	0,070 c	0,069 c	0,100 a	0,077 b	0,097 a
Zn	1,738 d	1,629 e	2,662 a	1,876 c	2,251 b
Mo	0,019 b	0,011 c	0,018 b	0,012 c	0,024 a
Ni	0,066 a	0,051 b	0,056 b	0,052 b	0,057 b
P	40,909 c	38,073 d	55,631 a	40,919 c	45,920 b
K	821,164 b	688,845 d	1020,820 a	740,406 c	813,508 b
Ca	165,459 b	144,070 d	170,629 a	150,888 c	171,950 a
Mg	42,205 b	35,731 d	45,074 a	35,259 d	37,328 c
V	0,013 e	0,045 b	0,058 a	0,037 c	0,027 d

Different letters indicate significant differences between means at P< 0.05 (anova) per row. Values are means ± S.E (N=3). Values are in mg/Kg.

UNIVERSITY of the
WESTERN CAPE

The nutrient profile in table 5 shows that vanadium has a negative impact on all nutrients in the plant. The control shows that there was a trace amount of vanadium in soil. The amount of vanadium increased in treated plants showing that the concentration added was taken up by the plants. Treatment with the Ya and Wo isolates showed a decrease in vanadium uptake suggesting a defensive mechanism to protect the plant, while treatment with the X isolate showed an increase in vanadium uptake suggesting it may use the heavy metal for various processes or absorbs it to protect its host. The increase in vanadium in plants

http://etd.uwc.ac.za/

treated with the X isolate didn't show any increase in cell death as seen in figure 9 and 10.

Vanadium treated plants showed a decrease in all nutrients with the exception of Cu when compared to the control. The uptake of micronutrients such as Mo was decreased by 44% and Ni by 23%. Macronutrients K, Ca and Mg all decreased by ~15% while P was down by 7%. The nutrient poor profile seen in table 4 caused by vanadium stress can be seen in the damaged root system (figure 8) as it hinders the plants ability to obtain nutrients. The cell death in the roots (figure 10) further supports this.

All isolates showed an increase in Cu and Zn and a decrease in Fe uptake. The Ya isolate increased the uptake by 39% in Cu, 30% Zn and showed a decrease by 42% in Fe. The Wo isolates increase uptake in Cu by 11%, Zn 8% and a decrease in Fe by 57%. The X isolate showed significant increases in micronutrients such as Zn (53%), Cu (42%) and macronutrients such as P (36%) and K (24%).

All isolates showed an improvement in nutrient profile when compared to the vanadium treated plants. The increase in the root system (figure 8) may have provided the plants with the increase in nutrients. This also suggests that the endophytes helped to mitigate the damage caused by excess vanadium.

http://etd.uwc.ac.za/

The X isolate had the best nutrient profile as can be seen in table 2 and table 4. This suggests that, despite not having the best results in classical plant growth promoting mechanisms such as IAA, phosphate solubilization or siderophore production when compared to the other isolates it plays a major role in the plants nutrient regulation.

There was a decrease in Fe in all isolate treated plants when compared to the control and vanadium treated plants despite the endophytes being able to produce siderophores. The X isolate had the lowest production of siderophore activity and saw the lowest decrease in Fe between the isolates while The Wo strain showed the highest production and the biggest decrease in Fe uptake. The Isolates may be using the Fe for itself while under vanadium stress to produce biofilms for protection. The isolates show an increase in Fe under normal conditions (table 2) while the reverse is seen under vanadium stress (table 5). The isolated siderophore activity (Figure 7) and the decrease in Fe seen, suggests that the higher the siderophore activity the higher the decrease in Fe. A similar trend can be seen in Cu however, it shows that the higher the siderophore activity the higher the increase in Cu.

Mulder chart (1953) showed that P and Fe have an antagonistic relationship. There is an increase in P (table 5) that was not seen in the first growth experiment (table 2). The ratio for the control and vanadium treatment was 1(Fe):75(P). The experimental samples have varying rations, X 1:114, Wo 1:177 and Ya 1:146. The

UNIVERSITY of the
WESTERN CAPE
http://etd.uwc.ac.za/

increase in P may be due to the isolates using Fe for itself thus having less effect on the on the antagonistic relationship. The ration between Ca and K was 1:5 for all samples with the expectation of the X isolate which was 1:6. The disturbance in the ration is due to the X isolate having an increase in Mn which was not seen the other samples.

http://etd.uwc.ac.za/

6. Conclusion and Future Work

There is a diverse endophytic bacteria population present in the roots of plants, each contributing a plant growth promoting mechanism ranging from nutrient regulation to hormone production. Endophytic bacteria may possess more than one mechanism to aid in plant – microbe interaction and improve plant growth. All isolates improved plant growth in both stand and heavy metal growth trials.

The Ya strain displayed IAA production which aided in growth promotion root elongation and seed germination. It also demonstrated high siderophore production activity. The Wo and X strains also possessed IAA production however, it was far lower than the Ya strain. The Wo strain produced the highest siderophore activity, the Ya and X were both able to produce siderophores as well. The X strain regulated multiple essential nutrients to improve plant growth. The other strains were able to improve a few nutrients however, it was not as significant as the X strain. All strains enabled a high tolerance for vanadium. The tolerance may be due to the production of siderophores and therefore should be tested against other heavy metals. All 3 strains are promising candidates for improved crop growth without the use of commercial fertilizers. The strains tolerance to vanadium stress makes them ideal candidates to be tested for bioremediation capability.

Future studies will involve characterization of endophytes with test such as gram stain, starch hydrolysis, motility and various other classical characterization tests.

UNIVERSITY of the
WESTERN CAPE
http://etd.uwc.ac.za/

Nitrogen fixation is another key test that may reveal a key growth promoting property as nitrogen in the same way that phosphorus is a key element that is limited in the environment. Mixed endophyte culture tests should be done to combine the properties of all 3 strains to test if they IAA producing and the nutrient regulator strain can work in combination. Another classical plant growth promoting trait to look into is 1-aminocyclopropane-1-carboxylate (ACC) deaminase. The isolates are to be sequences to identify them as novel or known microorganism. The endophytes can also be applied to other stresses such as salinity and drought.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

7. REFERENCES

Adamska, E., Cegielska-Taras, T., Kaczmarek, Z. and Szała, L., 2003. Multivariate approach to evaluating the fatty acid composition of seed oil in a doubled haploid population of winter oilseed rape (Brassica napus L.).*Journal of applied genetics*, *45*(4), pp.419-425.

Ahmad, I. and Maathuis, F.J., 2014. Cellular and tissue distribution of potassium: physiological relevance, mechanisms and regulation. *Journal of plant physiology*, 171(9), pp.708-714.

Alexander, D.B. and Zuberer, D.A., 1991. Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. *Biology and Fertility of soils*, *12*(1), pp.39-45.

Bai, H.J., Zhang, Z.M., Yang, G.E., Li, B.Z. (2008). Bioremediation of cadmium by growing Rhodobacter sphaeroides: kinetic characteristic and mechanism studies. *Bioresource Technology*. 99, pp.7716–7722.

Barber, S.A. and Silberbush, M., 1984. Plant root morphology and nutrient uptake. Roots, nutrient and water influx, and plant growth, (rootsnutrientan), pp.65-87.

Behnsen, J. and Raffatellu, M., 2016. Siderophores: more than stealing iron. *MBio*, *7*(6), pp.e01906-16.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Beneduzi, A., Ambrosini, A. and Passaglia, L.M., 2012. Plant growth-promoting rhizobacteria (PGPR): their potential as antagonists and biocontrol agents. *Genetics and molecular biology*, *35*(4), pp.1044-1051.

Bialek, K., Michalczuk, L. and Cohen, J.D., 1992. Auxin biosynthesis during seed germination in Phaseolus vulgaris. *Plant Physiology*, 100(1), pp.509-517.

Bloemberg, G.V. and Lugtenberg, B.J., 2001. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. *Current opinion in plant biology*, 4(4), pp.343-350.

Boukhalfa, H. and Crumbliss, A.L. (2002) Chemical aspects of siderophore mediated iron transport. Biometals 15, 325–339.

Brader, G., Compant, S., Mitter, B., Trognitz, F. and Sessitsch, A., 2014. Metabolic potential of endophytic bacteria. *Current opinion in biotechnology*, 27, pp.30-37.

UNIVERSITY of the
WESTERN CAPE

Cakmak, I., 2005. The role of potassium in alleviating detrimental effects of abiotic stresses in plants. *Journal of Plant Nutrition and Soil Science*, 168(4), pp.521-530.

Catarino, M.D., Sobral, A.J.F.N. and Cardoso, S.M., 2014. Novel phenolics in Eriocephalus genus. *Planta Medica*, 80(16), p.P1L130.

Christian, O.D., Aleš, S., Paulina, D., Andre, S., Wilhelm, B., Erika, K. (2008). Involvement of siderophores in the reduction of metal-induced inhibition of auxin synthesis in *Streptomyces spp*. *Chemosphere.* 74, pp.19–25.

http://etd.uwc.ac.za/

Compant, S., Clément, C. and Sessitsch, A., 2010. Plant growth-promoting bacteria in the rhizo-and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. *Soil Biology and Biochemistry*, 42(5), pp.669-678.

Danhorn, T. and Fuqua, C., 2007. Biofilm formation by plant-associated bacteria. Annu. Rev. Microbiol., 61, pp.401-422.

Datta, C. and Basu, P.S., 2000. Indole acetic acid production by a Rhizobium species from root nodules of a leguminous shrub, Cajanus cajan. *Microbiological research*, 155(2), pp.123-127.

De Melo Pereira G. V., Magalhaes K. T., Lorenzetii E. R., Souza T. P., Schwan R. F. (2012). A multiphasic approach for the identification of endophytic bacterial in strawberry fruit and their potential for plant growth promotion. *Microbial Ecology*, 63 (2), pp.405–417.

UNIVERSITY of the
WESTERN CAPE

Easterwood, G.W., 2002. Calcium's role in plant nutrition. *Fluid J*, *10*, pp.16-19.

Egamberdiyeva, D. (2007). The effect of plant growth promoting bacteria on growth and nutrient uptake of maize in two different soils. *Applied Soil Ecology*. 36(2-3), pp.184-189.

Ehmann, A., 1977. The Van Urk-Salkowski reagent—a sensitive and specific chromogenic reagent for silica gel thin-layer chromatographic detection and identification of indole derivatives. *Journal of Chromatography* A, 132(2), pp.267-276.

http://etd.uwc.ac.za/

Etesami, H., Alikhani, H.A. and Hosseini, H.M., 2015. Indole-3-acetic acid (IAA) production trait, a useful screening to select endophytic and rhizosphere competent bacteria for rice growth promoting agents. *MethodsX*, 2, pp.72-78.

Farhadian, M., Vachelard, C., Duchez, D., Larroche, C. (2008). In situ bioremediation of monoaromatic pollutants in groundwater: a review. *Bioresource Technology*. 99, pp.5296–5308.

Glick B. R. (2014). Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Microbiological Research*, 169 (1), pp.30–39.

Glick, B.R., 2012. Plant growth-promoting bacteria: mechanisms and applications. *Scientifica*, 2012.

Glick, R., Gilmour, C., Tremblay, J., Satanower, S., Avidan, O., Déziel, E., Greenberg, E.P., Poole, K. and Banin, E., 2010. Increase in rhamnolipid synthesis under iron-limiting conditions influences surface motility and biofilm formation in Pseudomonas aeruginosa. *Journal of bacteriology*, *192*(12), pp.2973-2980.

Godfray, H.C.J., Beddington, J.R., Crute, I.R., Haddad, L., Lawrence, D., Muir, J.F., Pretty, J., Robinson, S., Thomas, S.M. and Toulmin, C., 2010. Food security: the challenge of feeding 9 billion people. *science*, *327*(5967), pp.812-818.

Guo, H., Luo, S., Chen, L., Xiao, X., Xi, Q., Wei, W., Zeng, G., Liu, C., Wan, Y., Chen, J. and He, Y. (2010). Bioremediation of heavy metals by growing hyperaccumulaor endophytic bacterium Bacillus sp. L14. *Bioresource Technology*. 101(22), pp.8599-8605.

http://etd.uwc.ac.za/

Gupta, S.K. and Pratap, A., 2007. History, origin, and evolution. *Advances in Botanical Research*, 45, pp.1-20.

Gyaneshwar, P., Kumar, G.N., Parekh, L.J. and Poole, P.S., 2002. Role of soil microorganisms in improving P nutrition of plants. In *Food Security in Nutrient-Stressed Environments: Exploiting Plants' Genetic Capabilities* (pp. 133-143). Springer Netherlands.

Hafeez, B., Khanif, Y.M. and Saleem, M., 2013. Role of zinc in plant nutrition-a review. *American journal of experimental Agriculture*, 3(2), p.374.

Hallmann, J., Quadt-Hallmann, A., Mahaffee, W.F. and Kloepper, J.W., 1997. Bacterial endophytes in agricultural crops. *Canadian Journal of Microbiology*, *43*(10), pp.895-914.

UNIVERSITY of the
WESTERN CAPE

Hartmann, A., Rothballer, M. and Schmid, M., 2008. Lorenz Hiltner, a pioneer in rhizosphere microbial ecology and soil bacteriology research. *Plant and Soil*, *312*(1-2), pp.7-14.

Hell, R. and Stephan, U.W., 2003. Iron uptake, trafficking and homeostasis in plants. *Planta*, 216(4), pp.541-551.

HILTNER, L.T., 1904. Ober neuter erfahrungen und probleme auf dem gebiete der bodenbakteriologie unter besonderer berucksichtigung der grundungung und brache. *Arbeiten der Deutshen Landwirschafthchen Gesellschaft*, *98*, pp.59-78.

http://etd.uwc.ac.za/

Hsu, S.Y., 2010. *IAA production by Streptomyces scabies and its role in plant microbe interaction* (Doctoral dissertation, Cornell University)

Hui, S., Yan, H., Qing, X., Renyuan, Y. and Yongqiang, T. (2013). Isolation, characterization, and antimicrobial activity of endophytic bacteria from Polygonum cuspidatum. *African Journal of Microbiology Research*. 7(16), pp.1496-1504.

John, R., Ahmad, P., Gadgil, K. and Sharma, S., 2012. Heavy metal toxicity: Effect on plant growth, biochemical parameters and metal accumulation by Brassica juncea L. *International Journal of Plant Production*, *3*(3), pp.65-76.

John, R., Ahmad, P., Gadgil, K. and Sharma, S., 2012. Heavy metal toxicity: Effect on plant growth, biochemical parameters and metal accumulation by Brassica juncea L. *International Journal of Plant Production*, *3*(3), pp.65-76.

UNIVERSITY of the
WESTERN CAPE

Jones, C. and Jacobsen, J., 2005. Plant nutrition and soil fertility. Nutrient management module, (2), p.11.

Jurik, T.W., Chabot, J.F. and Chabot, B.F., 1982. Effects of light and nutrients on leaf size, CO2 exchange, and anatomy in wild strawberry (Fragaria virginiana). *Plant Physiology*, 70(4), pp.1044-1048.

Kaya, M.D., Okçu, G., Atak, M., Çıkılı, Y. and Kolsarıcı, Ö., 2006. Seed treatments to overcome salt and drought stress during germination in sunflower (Helianthus annuus L.). *European journal of agronomy*, *24*(4), pp.291-295.

http://etd.uwc.ac.za/

Khan, M.S., Zaidi, A., Ahemad, M., Oves, M. and Wani, P.A., 2010. Plant growth promotion by phosphate solubilizing fungi–current perspective. *Archives of Agronomy and Soil Science,* 56(1), pp.73-98.

Kiss, T. and Farkas, E. (1998) Metal-binding ability of desferrioxamine B. J. Inclusion Phenom. Mol. Recognit. Chem. 32, 385–403.

Kobayashi, D.Y. and Palumbo, J.D., 2000. Bacterial endophytes and their effects on plants and uses in agriculture*. Microbial endophytes*, 19, pp.199-233.

Kouas, S., Labidi, N., Debez, A. and Abdelly, C., 2005. Effect of P on nodule formation and N fixation in bean*. Agronomy for sustainable development*, 25(3), pp.389-393.

Lam, E., Kato, N. and Lawton, M., 2001. Programmed cell death, mitochondria and the plant hypersensitive response. *Nature*, *411*(6839), pp.848-853.

UNIVERSITY of the
WESTERN CAPE

Li, W., Khan, M.A., Yamaguchi, S. and Kamiya, Y., 2005. Effects of heavy metals on seed germination and early seedling growth of Arabidopsis thaliana. *Plant growth regulation*, *46*(1), pp.45-50.

Logan, B.E., Hamelers, B., Rozendal, R., Schröder, U., Keller, J., Freguia, S., Aelterman, P., Verstraete, W. and Rabaey, K., 2006. Microbial fuel cells: methodology and technology. *Environmental science & technology*, 40(17), pp.5181-5192.

Lugtenberg, B., Chin-A-Woeng, T., Bloemberg, G. (2002). Microbe– plant interactons: principles and mechanisms. *Antonie van Leeuwenhoek* . 81, 373–383.

http://etd.uwc.ac.za/

Lwin, K., Myint, M., Tar, T. and Aung, W. (2012). Isolation of Plant Hormone (Indole-3-Acetic Acid - IAA) Producing Rhizobacteria and Study on Their Effects on Maize Seedling. *Engineering Journal*, 16(5), pp.137-144.

Miliute, I., Buzaite, O., Baniulis, D. and Stanys, V., 2015. Bacterial endophytes in agricultural crops and their role in stress tolerance: a review. *Zemdirbyste-Agriculture*, 102(4), pp.465-478.

Miransari, M. and Smith, D.L., 2014. Plant hormones and seed germination. *Environmental and Experimental Botany*, 99, pp.110-121.

Moskalyk, R.R. and Alfantazi, A.M., 2002. Nickel laterite processing and electrowinning practice. *Minerals Engineering*, *15*(8), pp.593-605.

UNIVERSITY of the
WESTERN CAPE

Mulder, D., 1953. Les Elements Mineurs en Culture Fruitière, 1o Convegno Nazionale de Frutticoltura. *Montana de Saint Vincent*, pp.188-198.

Nair, D.N. and Padmavathy, S., 2014. Impact of endophytic microorganisms on plants, environment and humans. *The Scientific World Journal*, *2014*.

Njenga, E.W., 2005. The chemotaxonomy, phylogeny and biological activity of the genus *Eriocephalus L*. (Asteraceae) (Doctoral dissertation, Faculty of Health Sciences, University of the Witwatersrand).

http://etd.uwc.ac.za/

Paguia, E.F. and Valentino, M.J.G., 2016. Seed germination promoting activity of fungal endophytes in Rice (Oryza sativa L.) seeds. *Asian Journal of Plant Science and Research*, 6(4), pp.37-39.

Parida, B.K., Chhibba, I.M. and Nayyar, V.K., 2003. Influence of nickel-contaminated soils on fenugreek (*Trigonella corniculata L.*) growth and mineral composition. *Scientia horticulturae*, *98*(2), pp.113-119.

Patil, N.B., Gajbhiye, M., Ahiwale, S.S., Gunjal, A.B. and Kapadnis, B.P., 2011. Optimization of Indole 3-acetic acid (IAA) production by Acetobacter diazotrophicus L1 isolated from Sugarcane. *International journal of environmental sciences*, 2(1), p.295.

Patten, C.L. and Glick, B.R., 1996. Bacterial biosynthesis of indole-3-acetic acid. *Canadian Journal of Microbiology*, 42(3), pp.207-220.

UNIVERSITY of the
WESTERN CAPE

Patten, C.L. and Glick, B.R., 2002. Role of Pseudomonas putida indoleacetic acid in development of the host plant root system. *Applied and environmental microbiology*, *68*(8), pp.3795-3801.

Pennell, R.I. and Lamb, C., 1997. Programmed cell death in plants. *The Plant Cell*, *9*(7), p.1157.

Pratap, A. and Gupta, S.K., 2007. Unusual floral morphology in advanced generations of intergeneric hybrids between Brassica napus and Eruca sativa.

http://etd.uwc.ac.za/

Rajkumar, M., Ae, N., Prasad, M.N.V. and Freitas, H., 2010. Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. *Trends in biotechnology*, *28*(3), pp.142-149.

Rakow, G. and Raney, J.P., 2003, July. Present status and future perspectives of breeding for seed quality in Brassica oilseed crops. In *Proc. 11th Int. Rape Seed Congress, Copenhagen, Denmark* (pp. 181-185).

Römheld, V. and Marschner, H., 1991. Function of micronutrients in plants. *Micronutrients in agriculture*, (micronutrientsi2), pp.297-328.

Saber, K., Nahla, L., Ahmed, D., Chedly, A. (2005): Effect of P on nodule formation and N fixation in bean. *Agron. Sustain. Dev.* , 25 , 389–393.

Saco, D., Martin, S. & San Jose, P. 2013. Vanadium distribution in roots and leaves of *Phaseolus vulgaris*: morphological and ultrastructural effects. *Biol Plantarum*, 57(1):128-132.

Saha, M., Sarkar, S., Sarkar, B., Sharma, B.K., Bhattacharjee, S. and Tribedi, P., 2016. Microbial siderophores and their potential applications: a review. *Environmental Science and Pollution Research*, *23*(5), pp.3984-3999.

Sanevas, N., Sunohara, Y. and Matsumoto, H., 2007. Characterization of reactive oxygen species-involved oxidative damage in Hapalosiphon species crude extract-treated wheat and onion roots. *Weed biology and management*, *7*(3), pp.172-177.

http://etd.uwc.ac.za/

Schmelz, E.A., Engelberth, J., Alborn, H.T., O'donnell, P., Sammons, M., Toshima, H. and Tumlinson, J.H., 2003. Simultaneous analysis of phytohormones, phytotoxins, and volatile organic compounds in plants. *Proceedings of the National Academy of Sciences*, *100*(18), pp.10552-10557.

Schmidhuber, J. and Tubiello, F.N., 2007. Global food security under climate change. *Proceedings of the National Academy of Sciences*, *104*(50), pp.19703-19708.

Seo W.T., Lim W.J., Kim E.J., Yun H.D., Lee Y.H., Cho K.M. (2010). Endophytic Bacterial Diversity in the Young Radish and Their Antimicrobial Activity against Pathogens. *Journal of the Korean Society for Applied Biological Chemistry*. 53, pp.493-503.

Sethy, S.K. and Ghosh, S., 2013. Effect of heavy metals on germination of seeds. *Journal of natural science, biology, and medicine*, *4*(2), p.272.

UNIVERSITY of the
WESTERN CAPE

Sharma, S., Sayyed, R., Trivedi, M. and Gobi, T. (2013). Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. *SpringerPlus*. 2(1), pp587

Shaul, O., 2002. Magnesium transport and function in plants: the tip of the iceberg. *Biometals*, 15(3), pp.307-321.

Silberbush, M. and Barber, S.A., 1984. Phosphorus and potassium uptake of field-grown soybean cultivars predicted by a simulation model. *Soil Science Society of America Journal*, *48*(3), pp.592-596.

http://etd.uwc.ac.za/

Smalla, K., Sessitsch, A. and Hartmann, A., 2006. The Rhizosphere:'soil compartment influenced by the root'. *FEMS microbiology ecology*, *56*(2), pp.165-165.

Souza, R.D., Ambrosini, A. and Passaglia, L.M., 2015. Plant growth-promoting bacteria as inoculants in agricultural soils. *Genetics and molecular biology*, *38*(4), pp.401-419.

Tian, Y., Guan, B., Zhou, D., Yu, J., Li, G. and Lou, Y., 2014. Responses of seed germination, seedling growth, and seed yield traits to seed pretreatment in maize (Zea mays L.). *The Scientific World Journal*, *2014*.

Trognitz F., Piller K., Nagel M., Borner A., Bacher C.-F., Rechlik M., Mayrhofer H., Sessitsch A. (2014). Isolation and characterization of endophytes isolated from seeds of different plants and the application to increase juvenile development. *Tagung der Vereinigung der Pflanzenzüchter und Saatgutkaufleute Osterreichs*, 65, pp.25–28.

UNIVERSITY of the
WESTERN CAPE

Uchida, R., 2000. Essential nutrients for plant growth: nutrient functions and deficiency symptoms. *Plant nutrient management in Hawaii's soils*, pp.31-55.

Vance, C.P., Uhde-Stone, C. and Allan, D.L., 2003. Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. *New phytologist*, *157*(3), pp.423-447.

Xiao, X., Luo, S.L., Zeng, G.M., Wei, W.Z., Wan, Y., Chen, L., Guo, H.J., Cao, Z., Yang, L.X., Chen, J.L., Xi, Q. (2010). Biosorption of cadmium by endophytic fungus (EF) Microsphaeropsis sp. LSE10 isolated from cadmium hyperaccumulator Solanum nigrum L. *Bioresource Technology*. 101, pp.1668–1674.

http://etd.uwc.ac.za/

Yruela, I., 2005. Copper in plants. *Brazilian Journal of Plant Physiology*, 17(1), pp.145-156.

Zahoor, A and Rehman, A. (2009). "Isolation Of Cr(VI) Reducing Bacteria From Industrial Effluents And Their Potential Use In Bioremediation Of Chromium Containing Wastewater". *Journal of Environmental Sciences.* 21(6), pp.814-820.

Zinniel, D., Lambrecht, P., Harris, N., Feng, Z., Kuczmarski, D., Higley, P., Ishimaru, C., Arunakumari, A., Barletta, R. and Vidaver, A., 2002. Isolation and Characterization of Endophytic Colonizing Bacteria from Agronomic Crops and Prairie Plants. *Applied and Environmental Microbiology*, 68(5), pp.2198-2208.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/