DEPARTMENT OF BIOTECHNOLOGY

Identification of candidate plant growth promoting endophytes from *Echium plantagineum* roots

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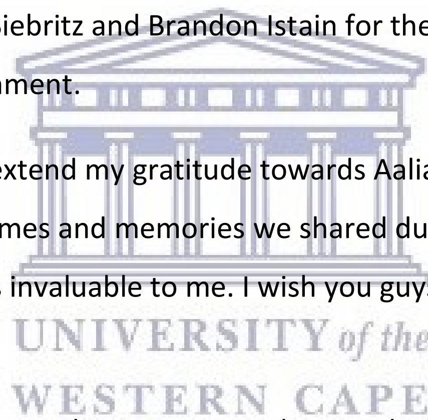

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List of Abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid
ACCD	1-aminocyclopropane-1-carboxylic deaminase
IAA	Indole-3-acetic acid
PGPE	Plant growth promoting endophyte
PGPB	Plant growth promoting bacteria
R2A agar	Reasoner's 2a agar

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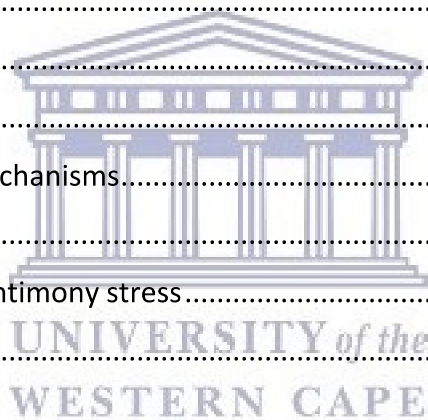
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// Abstract

The yearly increase of global population will result in a greater demand for crop production, but with the climates changes and a lack of available agricultural land it will become increasingly more difficult to provide sufficient crops to feed everyone adequately. Application of the PGPE has proven over the past researches to be able enhance growth of plants via various growth promoting mechanisms. To identify suitable growth promoting bacteria candidate, *E*. *plantagineum* plant was used to isolate endophytes from the root after surface sterilization. The isolates bacteria were used to inoculate *Brassica napus* L seeds. The effects of isolate's ability to promote growth were evaluated based on the certain growth parameters after 42 days in the green house. Isolate CP5 produced highest results in all growth parameter. Isolates CP5 was selected as potential candidate as significant improvement was shown by this isolate. This isolate was tested for the ability to produce ACC deaminase, solubilize phosphate, synthesize IAA and siderophore production. Furthermore isolate CP5 growth promotion abilities was tested on *Brassica napus* L under antimony stress.

Key words: *Brasscia napus* L, plant growth promoting endophytic bacteria, endophytes, *Echium plantagineum*, *ACC deaminase*, *Indole-3-acetic acid*, *phosphate solubilization*, *siderophore production*, *antimony*

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Identification of candidate plant growth promoting endophytes from *Echium plantagineum* roots

Chapter 1

Literature review

1.1 Introduction

Crop production can greatly influence food security of a region, this is because most of staple foods consumed are plant based. With each passing year the demands of consumers has become progressively difficult to maintain. This can be credited to the annual increase of global population which apply more pressure on the agricultural sector to produce sufficient crops. According to Global Food Security Index (GFSI) developed by Economist Intelligence Unit, South Africa as a whole is ranked first on Africa continent in terms of food security however studies suggest that there are regions in South Africa which still experience malnutrition (Labadarios *et al*. 2011; Battersby. 2012). Food security refers to a state in which the population have sufficient access to food in order meet their daily dietary requirements for a healthy life (FAO). This is determined by affordability, availability, quality and safety. Recently a new category was introduction, natural resources and resilience which seeks to explore a nation's risk of plant based resources and exposure to climate changes (GSFI). This category emphasizes the importance of initiatives of creating sustainable agricultural strategies but also to overcome effects of climate changes over the years causing flooding, drought and salinity. This is because plant based food greatly contributes to food security of a nation. However with development of residential and industrial sectors, less land are available for farming which ultimately will affects the crop yield which can be harvested (Wang, 2005; Gamalero *et al*. 2009). Additionally with the presence of emerging and remerging plant pathogen this can further constrict crop production (Miller *et al*. 2009). Should this inhibitory trend progresses, food security will be compromised as food resources required to feed the population will be severely limited and this can cause a wide range of social to economical conflicts. Therefore by

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increasing the yield of crop production by utilizing some form of feasible plant growth promoting application can improve current food security. Plant growth promotion can also increase the production of crops which are of low profitability due to unfavorable growth condition required, therefore ultimately reducing the dependence for imported planted based food.

Presently most strategies which aimed at improving the overall growth of crop involves the usage of chemical fertilizers, herbicides and pesticides which can provide satisfactory results, however these applications are not sustainable as numerous studies has shown that prolonged use of these chemical fertilizer and reagents will cause detrimental damage to the environment and personal health which is why these options are only viable as a short term solutions (Leach and Mumford, 2008). Therefore an efficient long term sustainable and environmentally friendly strategy is required in order to providing sufficient food for the global population. With the recent advancements on agricultural biotechnology, much attention has been diverted towards these plant growth promoting endophytic bacteria (Glick. 2014; Rashid *et al*.. 2012; Sheng *et al*.. 2008).

In this study the endophytic bacteria was extracted from a peculiar plant *Echium plantagineum* or better known as Salvation Jane or Paterson's curse, this plant originated from Mediterranean region and Western Europe but in the modern day it can be found throughout Eastern and Western regions of South Africa. These plants produce violet, red or blue color flowers which at first may seem harmless however can cause serious poisoning to livestock with a simple digestive system, this is because *E. plantagineum* produces high levels of pyrrolizidine alkaloids (Colegate *et al*. 2005). Despite being poisonous to livestock the plant proves to be very resilient, able to grow and proliferate in drought, often polluted environments with minimal nutrients available. The resiliency might be of the plant, but many studies shows that plant microbe interaction might play a role in the plant ability to tolerate and grow in hostile environments. The endophytic bacteria extracted from the *Echium plantagineum* plant may have plant growth promoting properties which can be utilized to improve the overall growth of agriculturally important plants by means of plant microbe interaction similarly to the *Echium plantagineum* plant.

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Figure 1.1 Parow industrial district, GPS coordinate (-33.928374; 18.609779). ***Echium plantagineum*** **plant with distinctive violet flower, thriving in dry, sandy and polluted soil near roadside of metal processing and logistic sites.**

1.2 Plant growth promoting endophytic bacteria

Endophytic bacteria reside within the internal tissues of a plant and are able to promote growth without eliciting any sign of infection or plant disease, thus these bacteria can be extracted from surface sterilized plant tissue. Plant growth promoting endophytic (PGPE) bacteria can stimulate growth promoting through growth promoting mechanisms available to be used by the isolates, these growth promoting mechanisms ranges from superior resource acquisition (nitrogen; phosphorus, other macro and micro elements) (Zehr *et al*.. 2003; Tilak *et al*.. 2005; Krey *et al*. 2013), stimulation of growth by modulation of phytohormone (Glick. 2012), sideophores production (Machua and Milagres. 2003) and auxin (Cassan *et al*. 2009) or indirectly increase the resilience of plant allow the plant to be more resistant towards stress therefore able to tolerate more stress induced from unfavourable environmental condition or plant related pathogenic infections (Tilak *et al*, 2005; Wang *et al*, 2009).

Plant growth promoting endophytic bacteria resides and proliferates within the plant's internal tissue, however many of the plant growth promoting bacteria which resides on

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the rhizosphere may also have the ability to enter and colonize internal tissue of plant (Sessitsch *et al*. 2004; Compant *et al*. 2005, 2008; Hallman and Berg. 2007;). Several recent studies confirm that the plants host a diverse endophytic community and these some of the endophytic bacteria are derived from rhizosphere (Sessitsch *et al*. 2002; Compant *et al*. 2005, 2010; Hardoim *et al*. 2015). Endophytic bacteria represent a subgroup of rhizobacterial communities which have the ability to enter the endorhiza (interior of root) of the host plant once the rhizosphere is colonized (Gray and Smith, 2005; Hallmann and Berg, 2007). Endophytic bacteria are more likely to exhibiting plant growth promoting effect than their counterparts which exclusively colonize the rhizosphere (Chanway *et al*. 2000). Following colonization of rhizosphere and rhizoplane, some of these bacteria are able to enter plant inner tissue and start growing to a range of 10^5 - 10^7 CFU g^{-1}. This penetration process does not always involve in active mechanism, therefore all rhizobacteria can be considered to be endophytic at a stage (Hardoim *et al*. 2008).

Bacteria may passively enter internal root tissue through cracks, such as those produced at root emergency site or created by deleterious microorganisms, as well as at the root tips. Certain nodulating bacteria have specialized mechanisms which can actively penetrate root systems (Hardoim *et al*. 2008). For example the interaction of symbiotic plant rhizobia with the semi aquatic legumes *Sesbania* and *Azohizobium caulinodans* (Goormachtig *et al*.. 2004), migration to inner tissue can occur through fissures at lateral root base and intercellular crack. Other rhizobia bacteria colonization occurs in the interior of hairy root before thread develop, after thread development they penetrate root tissues and specialized organs are formed by plant, known as nodules (Garg and Geetanjali. 2007). Bacteria may also enter internal root tissue from bacteria colonies producing cell wall degrading enzyme (CWDE) such as endoglucanases (Reinhold-Hurek *et al*. 2006) and endopolygalacturonidases. Bacteria which possess CWDE genes are able to secrete related enzymes which allow them to pass through the endodermis, following entry into internal root tissue the endophytic bacteria are able to translocate to the cortex of the root system via passive or active mechanisms. Once the endophytic reaches the root cortical zone, a blockade of endodermis tissue will prevent further colonization inside endorhiza (James *et al*. 2002). This can be shown in non nodulating

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bacteria *Azoarcus* sp. BH72 and *Burkholderia* sp. PsJN that utilize CWDE for endophytic colonization (Compant *et al*. 2005). In addition bacteria may also passively enter through sections of endodermal tissue which were tore during growth of secondary roots originating from pericycle (Gregory. 2006).

In the environment under natural conditions, penetration of endodermis can occur from deleterious bacteria breaking the endodermis, this allows the endophytic bacteria to pass through endodermis cell layer. After passing through the endodermis barrier the xylem of host plant can be reached. This progression can be demonstrated by *Herbaspirillum seropedicae* z67 in rice (James *et al*. 2002) and *B*. *phytofirmans* strain in grapevine (Compant *et al*. 2005b, 2008a). Previously mentioned indirect methods and the active penetrations of endophytes are known to induce systemic resistance (Rosenblueth and Martínez-Romero. 2006). In turn the plant defence mechanisms may control the endophytic colonization (Iniguez *et al*. 2005). Dicotyledonous plants are known to use salicylic acid (SA) and ethylene signalling pathway to control colonization by endophytes in laboratory conditions (Iniguez *et al*. 2005). Monocotyledonous plant like rice are able to interfere with endophytic colonization of *Azoarcus spp* by addition of jasmonic acid (JA) but not ethylene (Miché *et al*. 2006), suggesting that JA may also be involved in control of endophytic colonization inside root system.

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1.3 Mechanism of plant growth promoting by endophytic bacteria

1.3.1 Direct methods

1.3.1.1 Nitrogen fixation

Nitrogen is one of the main nutrients needed for plant proliferation and yield. Despite that nitrogen accounts for 78% of the atmosphere, these atmospheric nitrogen cannot to be utilized by plants without being convert to a usable form via biological nitrogen fixation (BNF) process. This process involves the reduction of nitrogen into ammonia by nitrogen fixing micro organisms using a complex enzyme system known as nitrogenase. Currently BNF process accounts for more than 2/3 of global nitrogen fixed whilst the rest is synthesized by Haber – Bosch process (Rubio and Ludden. 2008).

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Nitrogen fixation is limited to prokaryotes (bacteria, cyanobacteria) and can be classified into symbiotic nitrogen fixing bacteria such as those of rhizobiaccae (rhizobia) which form symbiotic relation with leguminous plant (Ahemad and Khan. 2012d; Zahran. 2001), other non – leguminous plant and non symbiotic (free living, endophytic) nitrogen fixing bacteria such as *Azotobacter*, *Gluconoacetobacter diazotrophicus* (Franche *et al*. 2009; Bhattacharyya and Jha. 2012). It is commonly believed that nitrogen fixation within the non symbiosis bacteria only accounts for a small percentage of which the associated plant requires (Glick. 2012). Majority of nitrogen made available to the plant is contributed by symbiotic bacteria *rhizobium* sp and cyanobacteria however the application of these bacteria is restricted to certain plant species.

Over the recent years growth promoting endophytic bacteria as potential biofertilizers has gained much traction as a viable application to improve plants growth (Bhattacharjee *et al*. 2008; Akhtar and Siddiqui, 2010). Several reports have shown there are already applications of endophytic bacteria in non legume plants such as kallar grass and sugarcane. Endophytic bacteria such as *Gluconoacetobacter diazotrophicus* is used as inoculants in sugarcane, these bacteria have the ability to fix nitrogen around 150 Kg N ha $^{-1}$ year $^{-1}$ (Muthukumarasamy *et al*. 2005). Potential nitrogen fixing endophytic bacteria *Azoarcus* inoculated in roots of kallar, increasing the yield as much as 20–40 ton ha $^{-1}$ year $^{-1}$ without adding more of any nitrogen based chemical fertilizer in soils which contain high concentration of salt and alkaline (Hurek and Reinhold, 2003).

The process of nitrogen fixation occurs through nitrogenase enzyme complex. This complex consists of two part metalloenzyme (a) dinitrogenase reductase which is the iron protein (b) dinitrogenase which consists of a metal cofactor. The dinitrogenase reductase supplies electrons with high reduction energy in turn these electrons are used by dinitrogenase to reduce nitrogen N_2 into ammonia NH_3. Based on this module three nitrogen fixing system have been established, (1) Mo - nitrogenase (2) V - nitrogenase (3) Fe - nitrogenase these systems presences are varied in different bacteria.

The genes that is responsible for nitrogen fixation is named Nif gene, they can be found is both symbiotic and free living, endophytic bacteria. Nif genes contain structural

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genes, gene responsible for Fe protein activation, biosynthesis of iron molybdenum cofactor, electron donation and regulatory genes.

1.3.1.2 Phosphate solubilization

Phosphorus is widely abundant in soil it exists in both organic and inorganic forms, however the amount of phosphorus which is available for plant uptake is limited, this is because plants are only able to utilize soluble phosphorus in the form of monobasic (H_2PO_4) and diabasic (HPO_4) ions (Bhattacharyya and Jha, 2012). Insoluble phosphorus exists within the soil as inorganic minerals in form apatites iron phosphate; aluminium phosphate; calcium phosphate or as organic form such as phosphotriesters, phosphoric esters and inositol phosphate (Glick. 2012; Khan. 2007).

Phosphorus deficiency is one of the primary factors limiting the plant growth causing browning of leaves and impaired root and shoot development. In order to mend the phosphorus deficiency within soil, phosphorus rich fertilizer has being the primary solution, however only 0.1% of the phosphorus contained within the fertilizer is utilized (Scheffer., 1992) whilst the rest of phosphorus is seized when absorbed into the soil and forms a precipitation reaction with others ions such as iron, aluminium and calcium pre existing within the soil (Mckenzie and Roberts. 1990; Goldstein. 1986).

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Phosphorus solubilizing bacteria (PSB) are able to utilize these insoluble phosphorus forms via solubilisation and mineralization processes. Solubilisation of inorganic phosphorus occurs as result of reaction of low molecular weight organic acid such as gluconic and citric acid and keto – gluconic acids all of which can be synthesized by various growth promoting bacteria (Zaidi *et al*. 2009). These organic acids produced by bacteria contain hydroxyl and carboxyl groups which chelate the ions bound to the phosphate creating a soluble form of phosphorus and in turn the pH of rhizosphere is decreased (Kpomblekou and Tabatabai., 1994). Furthermore the growth promoting bacteria have shown to possess the ability to covert phosphorus within organic materials into usable inorganic phosphorus through the process of mineralization. This process requires the catalyst enzyme phosphatase, this enzyme hydrolyze the phosphoric esters contained within organic material (Glick. 2012). These two processes can occur co exist within single bacteria (Tao *et al*. 2008).

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Application of PSB can increase crop yield up to 70% as it can aid in the uptake of native phosphorus along with insoluble phosphorus (Mohammadi. 2012). Plants with adequate supply of phosphorus will generally show improved quality of products. This is because these macronutrients are responsible for stimulation root development, meristematic development and aids in promoting flower formation and fruit production. Previous studies has shown that most prominent PSB includes *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Microbacterium*, *Pseudomonas*, *Rhizobium* and *Serratia* (Bhattacharyya and Jha, 2012).

1.3.1.3 Siderophore formation

Iron is another macronutrient which is abundant in the soil environment. It is required by both plant and microorganisms, however iron exists in the soil under aerobic conditions as Fe^{3+} ferric ion. Most of the Fe^{3+} forms insoluble hydroxides and oxyhydroxides, thus the availability of iron which can be assimilation is relatively low and additionally iron is only partially soluble which further contributes to limited uptake of iron. Under low iron conditions bacteria synthesis low molecular weight iron chelators (200 - 2000 Da) known as siderophore (Schwyn and Neilands. 1987) in order to obtain sufficient iron. Siderophores can be classified into three main categories hydroxamates, catecholates and carboxylates these siderophores differ from each other in terms of their functional group. Bacterial siderophores have a high affinity for ferric ions and depending if the bacteria is gram positive or negative the transportation strategy employed will vary.

Gram negative bacteria such as *Escherichia spp* have outer membranes which contain TonB-dependent receptors which are able to recognize and bind to Iron(lll)-siderophore complexes (Krewulak and Vogel, 2008) after Iron(lll)-siderophore complex bind to the surface receptor, the complex requires an energy dependant transport structure in order to migrates across cell membrane, once across the membrane the complex will be bounded by high affinity periplasmic binding protein and migrates across the cytoplasmic membrane to cytoplasm via ATP-binding cassette transport system thereafter the complex is released into the periplasmic space where iron is release from the complex through a reduction of iron(lll)-siderophore complex (Noinaj *et al*. 2010),

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during this reduction process siderophore maybe destroyed or recycled (Rajkumar *et al*. 2010). On the other end of the spectrum gram positive bacteria does not possess any outer membrane receptor as these bacteria lack an outer surface membrane. Instead the iron(lll)-siderophore complexes migrate into the cell via periplasmic siderophore binding proteins which are stationed onto the cell due the absence of periplasmic space (Fukushima *et al*. 2013). Thereafter the iron(lll)-siderophore complexes migrate to the cytoplasm in the same manner as the gram negative bacteria utilizing ABC transport system (Braun and Hantke, 2011). Furthermore siderophore can be an effective method of bioremediation (use of microorganisms to remove or consume pollutants from particular site) as siderophore may have affinity or selectivity for other heavy metal other than iron and can form complexes with heavy metal such as Al, Cd, Ni, Pb and Zn, (Rajkumar *et al*. 2010; Schalk., 2011), thereby decreasing the stress plants experiences from heavy metal contamination.

Reports have shown the acquisition of iron through bacteria via the bacterial siderophore under low iron conditions of commercially grown oats (Crowley and Kraemer, 2007). In addition siderophore produced by *Azotobacter vinelandii* such as azotochelin and azotobactin have the ability to chelate heavy metals beside iron such as molybdenum and vanadium (Wichard *et al*. 2009). Furthermore bacterial siderophore has shown to be an efficient solution for mobilization of metals from waste material accumulated from mines or metal contaminated soil (Rajkumar *et al*. 2010; 2012)

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Another bacterium which is capable of producing siderophore *Pseudomonas fluorescens* C7 produced Fe – pyoverdine complex can be used by *Arabidopsis thaliana* plants, resulting in increased levels of iron within plant tissue and facilitate plant growth (Vansuyt *et al*. 2007)

1.3.1.4 Indole – 3 – acetic acid (IAA)

Indole - 3 - acetic acid is a plant auxin, this auxin is produced approximately by 80% of bacteria those free living within the soil and in symbiosis to the plants. IAA affects plant development in multiple ways such as cell division and differentiation, root and xylem extension and stimulation of seed and tuber germination (Woodward and Bartel, 2005; Teale *et al*. 2006). In addition the IAA produced by bacteria is able to increase root

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surface area and length therefore increasing plant access to nutrients, furthermore IAA have shown to loosen the plant cell wall which in turn is able to facilitate root exudates which provide additional nutrients to support bacterial growth (Glick, 2012).

Exogenous IAA produced by bacteria can affect the plant development process differently, this depends on the amount of IAA produced which is available to the plant and the sensitivity of plant tissue towards changes in IAA concentration. The optimal concentration range for a given plant can be narrow, for example studies have shown morphological changes to *Arabidopsis* plant root system without affecting the plant growth when inoculated with *Pseudomonas thivervalensis*, an IAA producing strain at a concentration of 105 CFU/ml^{-1}, however inhibition of plant growth is shown when using concentration of 106 CFU/ml^{-1} and above (Persello - Cartieaux *et al*. 2001), however other factors such as metabolites may be contributed to the inhibitory effects of highly concentrated inoculants.

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IAA synthesis level can be altered by the amino acid tryptophan, tryptophan is direct precursor to IAA which involved in modulation of IAA. Tryptophan can be synthesized from rhizosphere from decomposed roots and bacterial cells or from root exudates. Biosynthesis of tryptophan begins at metabolic node of chorismate in a five step reaction process regulated by *trp* gene. Tryptophan is involved many pathway for the synthesis of IAA with indole - 3 - acetamide pathway and indole - 3 - pyruvate pathway more commonly deployed by bacteria, other pathways include conversion of trpytophan into indole - 3 - acetic aldehyde, conversion of tryptophan into indole - 3 - acetonitrile and tryptophan independent pathway (Davis. 2010; Ahemad and Kibret. 2014).

One of the major auxin pathway is Indole - 3 - pyruvate (IPA), it can also be found in plant pathogens (*P. agglomerans*) and PGPB such as *Azospirillum*, *Bacillus*, *Bradyrhizobium*. This pathway is a 3 step process, transanimation of tryptophan into IPA by enzyme aminotransferase. Aminotransferase activity has been shown in different IAA producing bacteria such as *Azospirillum brasilense*. Indole - 3 – acetaldehyde (IAAld) is produced following decarboxylation of IPA, IAA is produced as a result of oxidation of IAAld this reaction is catalyzed by dehydrogenase. The carboxylation step is catalyzed by

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key enzyme indole - 3 - pyruvate decarboxylase which is encoded by ipdC gene. The ipdC gene is important gene for this pathway to occurs, previous studies which inactivate this gene result in impaired IAA production.

The other pathway commonly used by microbial is indole - 3 - acetamide (IAM) pathway. This pathway can be found in many pathogenic strains. This pathway is a two step process, IAM is produced after tryptophan reaction with tryptophan monooxygenase (transcribed from iaaM gene). From here IAM is hydrolyzed to IAA and ammonia by IAM hydrolase which is encoded by iaaH gene. These gene have being characterized from previous plant pathogens strains (*Agrobacterium tumefaciens*, *P*. *savastanoi*, *Pseudomonas syringae*, *Pantoea agglomerans*) as well as symbiotic nitrogen fixing bacteria species (*Rhizobium*, *Bradyrhizobium*). Previous studies have shown that application of IAA producing bacteria where auxin balance in plant are altered plays in role of nodule genesis and other processes such as cell division, differentiation and vascular bundle formation. All these processes are needed for plant nodule formation. However biosynthesis of IAA phytostimulation alone is not enough to elicit growth promotion (Dobbelaere *et al*. 2003). In additive hypothesis suggests that unison of growth promoting mechanisms for example, phosphate solubilization, dinitrogen fixation and ACC deaminase together with IAA biosynthesis are responsible overall observed growth promotion and yield increase (Bashan and Holguin, 1997).

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In the study conducted by Glick *et al*. (1998), PGPB bind to the surface of root and seed of the plant, these growth promoting bacteria can also be found on leaves and flowers or migrate into the plant's internal tissue becoming endophytic bacteria. Plants release organic molecules from the root, these molecules are absorbed by PGPB which in turn synthesize and produce IAA. The IAA produced by bacteria along with endogenous IAA can either stimulate growth or induce the synthesis of plant enzyme ACC synthase which catalyze the formation of ACC. In this case the production of IAA stimulates the synthesis ACC synthase causing an influx of ACC thereby ultimately increase the production of ethylene. Some of the plant ACC is released from seed, roots (Penrose *et al*. 2001) is absorbed by ACC deaminase producing PGPB, the absorbed ACC is cleaved by ACC deaminase produced by bacteria (Penrose and Glick, 2003). Therefore this reduction of ethylene generated from IAA released from bacteria and pre existing within

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plant accomplished by ACC deaminase producing PGPB in turn can help to reduced fraction of the ethylene level in plant after various abiotic or biotic stresses.

1.3.1.5 1-Aminocyclopropane-1-carboxylate deaminase (ACCD) and ethylene in plants

Plant growth can be obtained via various mechanisms employed by PGPB, however one of the most notable mechanism which bacteria possesses to induce plant growth is the ability to utilize ACC deaminase (Glick, 2012). ACC deanimase is an enzyme which has the ability to cleave ACC which is direct precursor to ethylene. ACC deaminase cleaves ACC and converts it into ammonia and α-ketobutyrate (Honma and Shimomura, 1978), resulting in the overall decrease of ethylene levels within plants.

Ethylene is a hormone which is produced endogenously by almost all plants. This hormone under regulated concentration is essential to the growth and development of plants in various ways such as promoting root and root hair formation, ripening of fruit, induce seed germination and stimulate the synthesis of other plant (Khalid *et al*. 2006). Ethylene is also synthesized in high concentration when plants experience unfavourable conditions such as extreme temperatures, water logging, high salinity and drought, exposure to organic and inorganic compounds, radiation, various pathogenic bacteria and fungi infection. High levels of endogenous of ethylene affect the plant negatively inducing defoliation and other cellular processes which may result in growth inhibition (Saleem *et al*. 2007; Bhattacharyya and Jha, 2012).

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The effects of ethylene synthesized during stress can be demonstrated on a model where ethylene is produced in two intervals (Pierik *et al*. 2006; Van Loon *et al*. 2006; Glick *et al*. 2007). The first interval of ethylene is a small peak which consumes all existing amount of 1 – aminocyclopropane – 1 – carboxylate in the plant under stress. This triggers the transcription of genes which encodes for plant protective proteins. The second larger peak of ethylene occur after the synthesis of addition ACC by the plant as a response to stress this occurs several days later, the second peak of ethylene will impact the plant negatively as it induces senescence, chlorosis and leaf abscission. In this model high level ethylene is produced in response to stress, therefore treatments which can reduce the second larger peak of stress ethylene will in turn reduce the damages caused by stress ethylene.

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ACC deaminase producing PGPB can facilitating growth of the plant by alleviating the negative effects caused by stress ethylene. Ethylene production starts with ACC synthase catalyzing S-adenosylmethionine (SAM) into 1-aminocyclopropane-1-carboxylic acid (ACC) and 5'-methylthioadenosine (MTA), the ACC synthesized will be catalyzed into ethylene by ACC oxidase and the MTA will be converted into L-methionine. As mentioned previously ACCD cleaves ACC into ammonia and α-ketobutyrate, thus eliminating ACC the direct precursor to ethylene thereby ultimately lowering ethylene concentration within plants.

ACCD activity is triggered under the presence of ACC, ACCD activity is a multipart process which occurs very slowly, furthermore ACCD is only active within the first few hours following exposure to ACC and this activity is gradually decreased over time (Walsh *et al*. 1981; Jacobson *et al*. 1994). The activation of the enzyme ACCD is dependent on availability of ACC, this induction of ACCD enzyme can be observed when ACCD producing bacteria which was grown on enriched media was switched to minimal media enriched with ACC as the only source of nitrogen.

Several ACC deaminase producing bacterial strains have already been identified, some of these bacteria include *Acinetobacter*, *Achromobacter*, *Agrobacterium*, *Alcaligenes*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Ralstonia*, *Serratia* and *Rhizobium* (Shaharoona *et al*. 2007a,b; Nadeem *et al*. 2007; Zahir *et al*. 2008; Zahir *et al*. 2009; Kang *et al*. 2010). ACCD activity varies amongst the bacterial strains at different environmental conditions and in turn may provide an effective phytoremediation solution for host plant to proliferate and survive at these adverse environmental sites (Glick., 2005). Previous studies have shown the potential ACC deaminase producing bacteria, these bacteria was inoculated onto Indian mustard seeds (*B*. *napus*), canola (*B*. *napus*) and tomato (*L*. *esculentum*) was able to relieve stresses induced by the accumulation of inorganic contaminates such as zinc, nickel and lead within the soil. In addition root and shoot growth of Indian mustard and rapeseed seedlings was observed when grown under the presence of cadmium chloride (Belimov *et al*. 2005). Furthermore these ACCD producing bacteria can also improve the root system by changing the structure of the root system and the plant uptake matrix thereby allowing the roots to cover more area of soil and the plant root uptake system. The improvement

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of the roots system in turn will ultimately increase the uptake potential of heavy metal or other inorganic contaminates.

1.3 .2 Indirect method

1.3.2.1 Systemic resistance

Growth promoting endophytes can also promote plants to develop systemic resistance, becoming more resilient in adverse conditions and develop efficient systemic resistance towards pathogenic infections. Systemic resistance can be divided into induced systemic resistance (ISR) and systemic acquired resistance (SAR). SAR is developed when the plant has activated its defensive mechanism in response to pathogenic infection or other abiotic stresses, producing plant disease symptoms such as a localized lesion of brown, rotting of plant tissues (Van Loon *et al*. 1998). ISR differ from SAR as induction of plant growth promoting bacteria does not cause any external sign or infection or plant disease like symptoms. Plants which possess ISR are term primed and able to react more rapidly with more aggression to pathogenic infection by activating defensive mechanisms. ISR mechanism includes (1) promotion of developmental growth (2) physiological reduction plant disease symptoms (3) microbial antagonism endosphere and rhizosphere (4) biochemical resistance induction of cell wall reinforcement, phytoalexins and pathogenic related proteins (Berg, 2010). ISR does not target specific pathogen but act as a biocontrol which covers a variety of pathogenic diseases which may occur. Most of reported PGPB related ISR involves rhizobacterium, however endophytic bacteria also has the ability to activate ISR within plants. Inoculation of endophytic PGPB will activate ISR of plant resulting strengthening plant cell wall and change plant host physiological and metabolic response, providing a enhanced synthesis of plant defensive chemical compounds when facing pathogenic infection or other abiotic factors (Ramamoorthy *et al*. 2001 Nowak and Shulaev. 2003). For example following inoculation of endophyte *P. fluorescens* WCS417r onto tomato plant, a thickening of outer layer of cortical cell wall was observed when after colonization of hypodermal cells was observed (Duijff *et al*. 1997). Furthermore after inoculation of *Burkholderia phytofirmans* PsJN onto grapevine, the interaction resulted in the release of phenolic compound and reinforcement of cell wall in exodermis and cortical cell

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layers (Compant *et al*. 2005), biochemical changes also occurs include the accumulation of pathogenic related proteins such as chitinases and peroxidise (Park and Kloepper, 2000; Ramamoorthy *et al*. 2001; Jeun *et al*. 2004). Not all endophytic PGPB produce pathogen related proteins following infection, instead increase the build up enyzmes such as phenylalanine ammonia lyase, phytoalexins, polyphenol oxidase, and chalcone synthase (Chen *et al*. 2000; Ongena *et al*. 2000) ISR can be activated using two pathways, one pathway is dependent on the molecule salicylic acid (SA) and other is the SA independent pathway, which involve jasmonate and ethylene signalling (Verhagen *et al*. 2004; Pettersson and Baath, 2004). In addition other bacterial molecules can also induce ISR such as 2,4-diacetylphloroglucinol, flagella, siderophores, cyclic lipopeptides, lipopolysaccharides (LPS), and volatiles organic compounds (VOC) (Lugtenberg and Kamilova., 2009).

1.4 Heavy metal pollution

Heavy metal pollution has become a major environmental issue in recent times due to the rapid development of urbanization and industrialization. Heavy metals contamination can negatively affect the environment which in turn can have adverse consequence to agricultural important plants, disrupting normal metabolic processes, decrease or biomass and yield, furthermore heavy metal pollution is not only limited to the environment but can also cause adverse effects on microorganisms, animals and human health (Kidd *et al*. 2012). Plants have defence mechanisms in place to alleviate the effects of heavy metal toxicity.

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One of the major concerns regarding heavy metal released into the environment is that they are not degradable and without properly removal applications they remain within the soil almost indefinitely. Some heavy metals are also water soluble and are easily dissolved into solution. Currently applications that have been implemented for the removal of heavy metals include removal of contaminated sites, addition of chemicals which renders the metals in to a state which cannot be absorbed by plants or by soil washing. However these clean-up methods are very expensive and only feasible for small scale sites which are severely polluted, requiring a quick complete removal.

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Furthermore applications such as soil washing can render the soil infertile and not usable for biological processes (Pulford and Watson. 2003). This requires a change of strategy to a more sustainable, environmental friendly and effective method. In this context the uses of PGPE has since become a promising alternative strategy to decrease the amount of heavy metals within the soil, but also to enhance the growth of agriculturally important plants which are affected by heavy metal contamination.

Plant growth promoting endophytes can be the perfect method of remediating contaminated soils without adversely affecting the environment. Growth promoting endophytes are known to be able alleviate plants from the effects of biotic and abiotic stress such as drought, pathogenic infections and toxic materials, endohytes can also help to restore the natural ecological balance of the soil (Ryan *et al*. 2008). Through the increase of heavy metal particles released into the environment, PGPE has developed various mechanisms to cope with the heavy metal by reducing the toxicity (Rajkumar *et al*. 2013). By colonizing the plant, PGPE can restore soil fertility and promote growth of the host plant through regulation of auxin and phytohormones, nutrient availability and suppressing of plant pathogens (Doty. 2008; Aravind *et al*. 2010). In addition plant growth promoting endophytes which have resistance to certain heavy metals can decrease toxicity of metal and in turn reduced the bioavailability of the metals within soils (Rajkumar *et al*. 2009; Weyens *et al*. 2009; Ma *et al*. 2011).

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Objective

The concept of endophytic bacteria promoting plant growth has been well documented. However, not much research has been done on the potential of endophytic originating from weed plants to be used as an application to provide nutrients and improve growth of agriculturally important plants. The objective of this research is to isolate a potential of PGPE candidate from *Echium plantagineum* plant which can improve plant growth. Other objectives are to determine the growth promoting mechanisms which are available to be utilized by the candidate endophytic bacteria. Lastly to determine the isolate's ability to grow when exposed to heavy metal contamination, as well as to promote growth to plants which are under heavy metal stress.

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Chapter 2

Methods and material

2.1 Reagents and suppliers

Reagents	Supplier
Acetone	Merck Milliore
ACC	Sigma Alrich
Ammonium chloride	Sigma Alrich
Ammonium sulphate	Sigma Alrich
Bacto agar	Sigma Alrich
Borric acid	Sigma Alrich
Calcium chloride	Sigma Alrich
Calcium phosphate	Sigma Alrich
CAS dye	Sigma Alrich
Casamino acid	Sigma Alrich
Copper sulphate	Sigma Alrich
Disodium phosphate	Sigma Alrich
Ethanol absolute	Kimix
Evans Blue	Sigma Alrich
Ferric chloride	Sigma Alrich
Glucose	Sigma Alrich
Glycerol	Merek Millipore
Hexadecyltrimethylammonium bromide (H.D.T.M.A)	Sigma Alrich
Hydrochloric acid	Merek Millipore
Iron chloride	Sigma Alrich

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Iron sulphate	Sigma Alrich
Luria broth (LB)	Sigma Alrich
Magnesium sulphate	Sigma Alrich
Manganese sulphate	Sigma Alrich
Mannitol	Sigma Alrich
Nitric acid 70%	Sigma Alrich
Perchloric acid	Sigma Alrich
1,4-Piperazinediethanesulfonic acid dipotassium salt (PIPES)	Sigma Alrich
Potassium antimonyl tartrate	Sigma Alrich
Potassium chloride	Sigma Alrich
Potassium hydroxide	Merek Millipore
Potassium phosphate dibasic	Sigma Alrich
Potassium phosphate monobasic	Sigma Alrich
R2A agar	Sigma Alrich
Sodium chloride	Sigma Alrich
Sodium gluconate	Sigma Alrich
Sodium molybdate dihydrate	Sigma Alrich
Sodium perchlorate	Sigma Alrich
Tryptic soy broth	Merek Millipore
Tryptophan	Sigma Alrich
Yeast extract	Sigma Alrich
Zinc sulphate	Sigma Alrich

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2.1 Plant extraction and surface sterilization

The *E*. *plantagineum* plant was extracted from uncontrolled environment located in the Parow industrial sector, by the roadside of metal processing and logistic sites, GPS coordinate (-33.928374; 18.609779). Only the root of the plants was collected for further processing. The root was washed with water to remove excess soil before surface sterilization. Following washing, the root was surface disinfected by submerging the root in 70% ethanol for 3 minutes, 2.5% sodium perchlorate for 5 minutes, 70% ethanol for 1 minute and washed 4 times using sterilized distilled water. In order to confirm the root surface was disinfected, aliquot of sterilized water of the last wash was plated onto R2A agar and incubated at 28°C for 5 days, after 5 days the plate was inspected for any microorganism growth, the absence of microbial growth confirms the root surface was disinfected (Hallmann *et al*. 1997)

2.2 Isolation of endophytic bacteria

The roots was excised into smaller fragments and pulverized with 0.9% NaCl using sterilized mortar and pestle, after pulverizing the root samples were incubated in 15ml of sterilized water at28^{o}C for 4 hours to allow release the endophytic bacteria from the root tissue. Following incubation a serial dilution was performed (10^{-2} - 10^{-7}) using water containing root samples was plated in triplicate onto separate R2A agar plates containing per litre (yeast extract 0.5g; Proteose peptone 0.5g; Casein hydrolysate 0.5g; Glucose 0.5g; Starch 0.5g; Di-potassium phosphate 0.3g; Magnesium sulphate 0.024g; Sodium pyruvate 0.3g; Agar 15.0g; pH 7.2 ± 0.2) at 25°C and was subjected to incubation at 28°C for 5 days. After 5 days of incubation total of 7 morphologically different bacterial colonies (shape, size, color) appeared on the plates and was selected to be subcultured onto new R2A agar for pure colonies and subjected growth trial. The single colonies were stored in 15% glycerol at -80°C for subsequent analysis.

2.3 Inoculum preparation

100ml of LB was prepared and autoclaved at 121°C for 20 minutes, after LB was cooled 15ml of LB was transferred to 50ml conical tube and was inoculated with selected

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isolated bacteria. The inoculated LB was incubated at $28^{o}C$ overnight in the orbital shaker incubator at 200 rpm.

2.3 Seed preparation

Brassica napus L seeds were surface sterilized using 70% ethanol for 3 minutes followed by 2.5% sodium perchlorate for 5 minutes and rinsed with water 3 times. The surface disinfected seeds were treated with bacteria isolates by transferring the seeds into disc filter paper and soaked with inoculated LB for 20 minutes to allow bacteria to colonize the seeds, for control seeds filter paper was soaked for 20 minutes with uninoculated sterilized LB, all samples were performed in triplicates.

2.4 Germination/ growth trials

Germination was performed during the summer period (Mar - Apr) in the greenhouse set at $25^{o}C$ with natural lighting. Treated *Brasscia napus* L seeds and control seeds were transferred into cups contain a ratio of 2:1 of sand to soil. All the treatment and control were replicated 5 times therefore 4 seeds per cup and 5 cups were used per isolate. After the seeds were planted 5ml of the respective inoculated LB for control sterilized uninoculated LB was used and 100ml of sterilized water was added to cup. The plants received 100ml of sterilized water every two days. After 42 days the germinated plants were extracted the length of roots, shoots and dry weight were recorded. Three isolates (CP1, CP5, CP7) was selected based on their ability to promoted plant growth Isolate CP3 was not used for treatment as it failed to grow in liquid media.

2.5 Vigor index

Vigor index (VI) = (average shoot length + average root length) x germination percentage (Abdul Baki and Anderson, 1973).

2.6 Macro element quantification

The root and shoot samples for ICP analysis were grounded into fine powder using liquid nitrogen. 200 mg of grounded root and shoot sample was digested in 1.5ml of 62% HNO_3 and heated at $80^{o}C$ for 1 hour. After incubation the digested samples are diluted

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into (1:10) v/v 2% HNO_3 solution. The samples were analyzed for Ca, Mg, K and P by axially viewed inductively coupled plasma optical emission spectrometry (ICP-OES, Varian Inc). Same procedure was followed for ICP-OES analysis of plants grown in the presence of antimony.

2.7 1-Aminocyclopropane-1-carboxylic acid (ACC) metabolism assay

5.5ml of tryptic soy broth was used to make an overnight culture of the endophytic isolate, the culture was incubated for 48 hours at 30 ^{0}C in a rotary incubator at 225 rpm. After 48 hours of incubation, the culture was subjected to a 10 times dilution using 0.1 M of magnesium sulphate. 120 ul of modified DF media containing per litre (KH_2PO_4 4g; Na_2HPO_4 6g; $MgSO_4$ 0.2g; $FeSO_4$ 0.004; H_3BO_3 0.00001g; $ZnSO_4$ 0.00004g; $CuSO_4$ 0.00005g; Na_2MoO_4 0.00001g; $MnSO_4$ 0.00001g pH 6.8 ± 0.2) without nitrogen source was added to all the lanes utilized, 15 ul of 0.1 M of magnesium sulphate was added to first 4 lanes. 15 ul of ammonium sulphate was added to second 4 lanes and last 4 lanes 3mM of filter sterilized ACC was added. 15 ul of isolate was inoculated into each well. For control wells 15 ul of 0.1 M magnesium sulphate were added instead of isolate, O.D was measured at 550 nm after 0, 24, 48, 72 and 96 hours using Polarstar Omega system. The O.D value from this assay can be used to determine the isolate ability to utilize ACC as the only nitrogen source. The isolate was grown in two different nitrogen supplements ammonium sulphate and 1-aminocyclopropane-1-carboxylic acid (ACC) and a mineral nutrient supplement magnesium sulphate. The OD of the isolate supplemented with ACC as only nitrogen source were compared to the OD of isolate supplemented with ammonium sulphate and magnesium sulphate to determine the growth rate of the isolate supplemented with ACC. This assay was conducted according to the method described by Jacobson *et al*. (1994).

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2.8 Indole-3 acetic acid production

To determine the amount of IAA produced by the endophytic bacteria, the isolate underwent a colormetric assay using Salkowski method (0.5M ferric chloride; 35% perchloric acid) (Salkowski. 1885). Bacterial culture was incubated in YEM broth containing per litre (mannitol 5g; yeast extract; 0.5g; $MgSO_4$ 0.2g; NaCl 0.1g; K_2HPO_4

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0.5g; sodium gluconate 5g; 16.6% $CaCl_2$ 1ml pH 6.8 ± 0.2) supplemented with 100mg/L^{-1} tryptophan and without tryptophan for 24 hours. Following incubation the bacterial broth culture was centrifuged at 13 000 RPM for 7 minutes, 1 ml of the supernatant was mixed 2 ml of Salkowski reagent. Optical density was measured at 530 nm absorbance after 30 minutes of incubation in the dark. IAA concentration was determined using a standard curve.

2.9 Siderophore production using CAS agar media

Siderophore production by endophytic isolate was done using a modified method described by Schwyn and Neilands (1987). The media was prepared using four solutions. Solution 1 was prepared by dissolving 60.5 mg of CAS dye into 50ml of distilled deionized water. The CAS solution was mixed with 10ml of iron solution (1mM $FeCl_3.6H2O$; 10mM HCl) under stirring, this will create a dark purple solution which was added slowly to 40ml of H.D.T.M.A (1.82 mg/ml $^{-1}$). This solution was autoclaved and cooled to 50°C. Solution 2 was prepared by dissolving 30.24g of PIPES in 750ml distilled deionized water containing 0.3g KH_2PO_4, 0.5g NaCl and 1.0g NH_4Cl. The pH was adjusted to 6.8 with 50% KOH and brought to a final volume of 800ml. 15g of agar was added to the solution and autoclaved then cooled 50°C. Solution 3 was prepared by dissolving 2g glucose; 2g mannitol; 493mg $MgSO_4$; 11mg $CaCl_2$; 1.17mg $MnSO_4.H_2O$; 1.4mg H_3BO_3; 0.04mg $CuSO_4.5H2O$; 1.2mg $ZnSO_4.7H_2O$ and 1.0mg $Na_2MoO_2.2H_2O$. This solution was autoclaved and cooled to 50°C. After all the solutions were cooled to 50°C, Solution 2 was added to solution 3 after the addition of solution 4 consisting of 30ml of filter sterilized 10% (w/v) casamino acid. Solution 1 was added last and was carefully mixed without creating bubbles. The overnight plate culture of candidate isolate was spot inoculated onto the CAS agar media and was incubated at 30°C for 48 hours. Development of halo zone and color change around the inoculated isolate indicates siderophore activity.

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2.10 Phosphate solubilization

The endophytic bacteria was screened for the ability to solubilize phosphate, the screening process of the isolate utilizes the Pikovskaya agar, containing per litre (0.5 yeast extract, 10 $Ca_3(PO_4)_2$, 0.5 $(NH_4)_2SO_4$, 0.2 KCl, 0.1 $MgSO_4$. $7H_2O$, 0.0001 $MnSO_4.H_2O$, 0.0001 $FeSO_4$. $7H_2O$ and 15g agar pH 6.8 ± 0.2). Pikovskaya agar was poured into sterile petri plates and the isolate was spot inoculated. Following 3 - 5 days of incubation at 28 ^oC, isolates which created clear halo zone around spot which was inoculated considered as positive for phosphate solubilization.

2.11. Effect of antimony on isolate growth

Isolate was tested using LB broth supplement with 0.5 mg/l, 5 mg/L, 10 mg/L and 30 mg/L of antimony. The each concentration was inoculated with overnight culture of the isolate CP5 with optical density of 0.1 at 600nm. Following inoculation the samples were incubated at 32°C under shaking conditions until the growth reached plateau. The optical density was measured at 600nm using spectrometer (Thermo Scientific Multiskan Go)

2. 12 Effect of antimony on growth of Brasscia napus L

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Seeds of Brasscia napus L were surface sterilized using the same procedure as described in section 2.3. Following seed preparation, seeds were planted into cups contain sand and soil with a ratio of 2:1. After the seeds were planted 5ml of the inoculated LB was added for seed inoculation and for control sterilized uninoculated LB was used. *Brasscia napus* seeds were treated every second day with 100ml of sterilized 10uM of antimony solution and sterilized water was used for control per cup. The seeds were grown in the greenhouse set at 25°C with natural lighting. All the treatment and control were replicated 5 times therefore 4 seeds per cup and 5 cups were used per experiment. After 4 weeks of growth the plants dry weight were recorded.

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Chapter 3

Selection of candidate growth promoting endophytic bacteria

3.1 Results

3.1.1 Germination analysis

The growth percentage is recorded at the end of 42 days in greenhouse. Most of the Garnet seeds which were sown with bacterial isolates did not show any significant improvement in germination percentage or compared to control with exception to isolate CP5. Only isolates which showed germination above 30 percent were CP5, CP6 and CP7, where CP5 showed most significant improvement with 50 percent germination rate compared to uninoculated control where it is only 10% germinated (fig. 1).

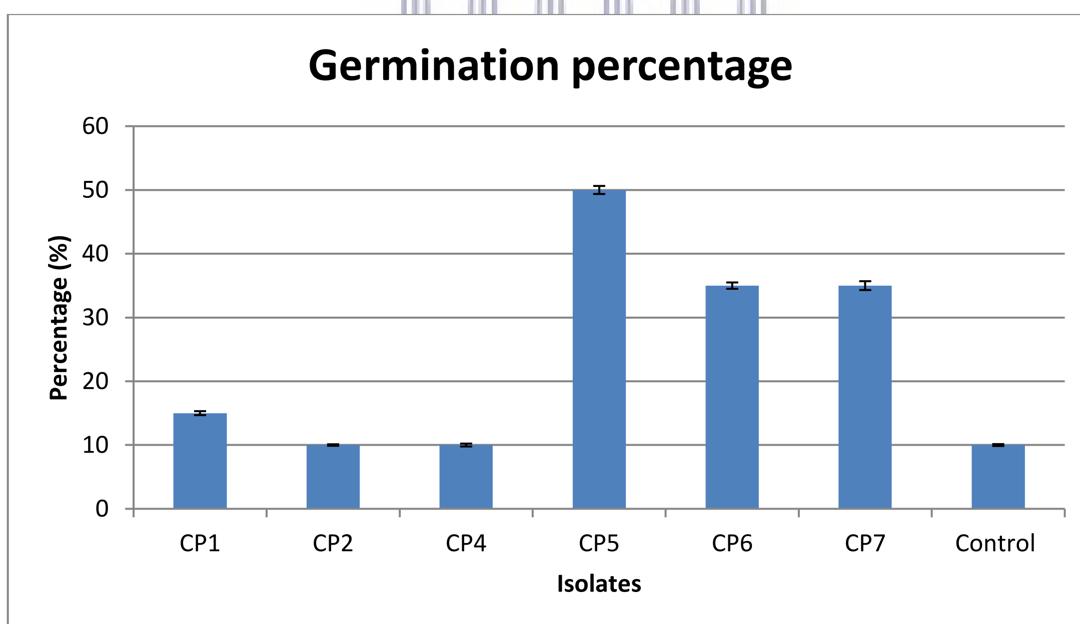

Figure 1: Germination percentage of *Brasscia napus* L after 42 days with and without treatment. Error bars represents (±SE) of the mean of 5 replicates per isolate. Means with different letters represents significant difference (P<0.05).

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3.1.2 Roots and shoot length

Among the selected isolates (CP1, CP5, CP7). Plants which were treated with isolate CP5 had the highest shoot length compared to control whilst plants treated with isolate CP7 had adverse effects on shoot length as CP7 caused a decrease in shoot length compared to control (**figure. 2**). Similarly plants which had the highest length in shoots also showed highest length in root growth, plants which was treated isolate CP5 showed the hightest length in root growth compared to control and plant which was treated isolate CP7 which caused a decrease in shoot length also caused a decrease in root length compared to control (**figure. 4**).

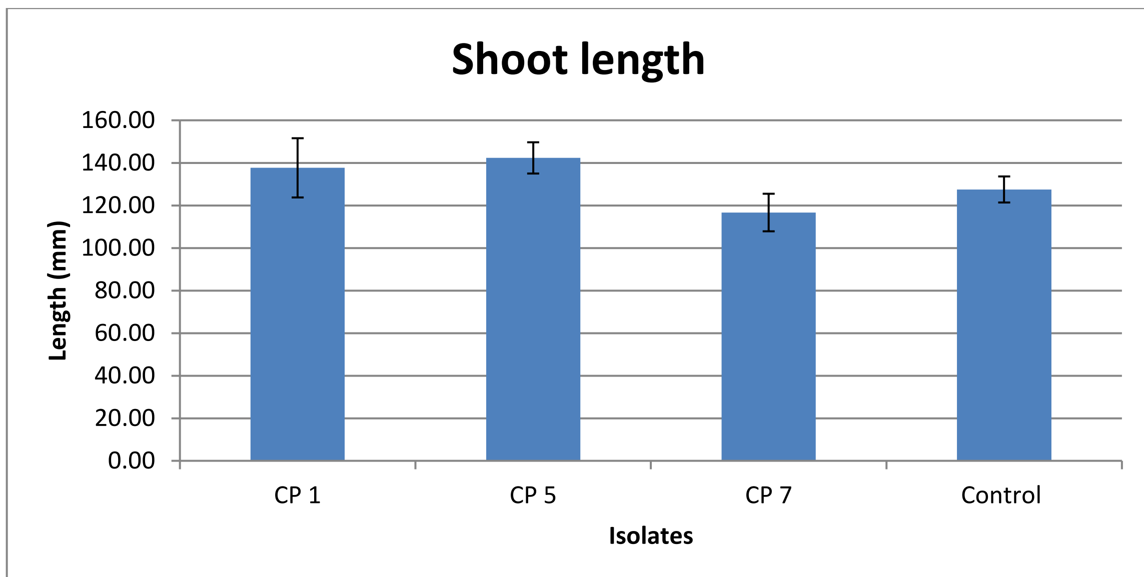

Figure 2.1. Average shoot length of *Brassica napus* L with and without inoculation. Error bars represents (±SE) of the mean of 3 replicates. Means with different letters represents significant difference (P<0.05).

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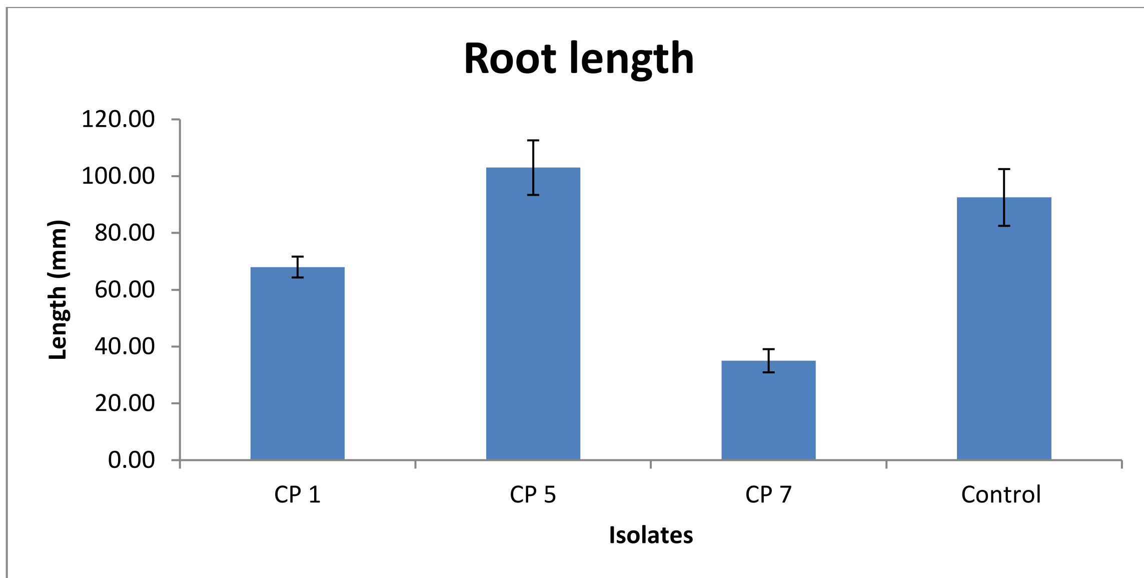

Figure 2.2. Average root length of *Brassica napus* L with and without inoculation. Error bars represents (±SE) of the mean of 3 replicates. Means with different letters represents significant difference (P<0.05).

Figure 4: *Brasscia napus* L seedlings after 42 days within greenhouse.

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3.1.3 Dry weights

The maximum increase in dry weight of both root and shoot was observed with plants inoculated with isolate CP5 compared to other isolates and uninoculated control (**figure 4, 5**). 2 Isolates (CP1 and CP7) had decreased the dry weight of shoots compared to control, where CP7 produce the lowest dry weight of shoot.

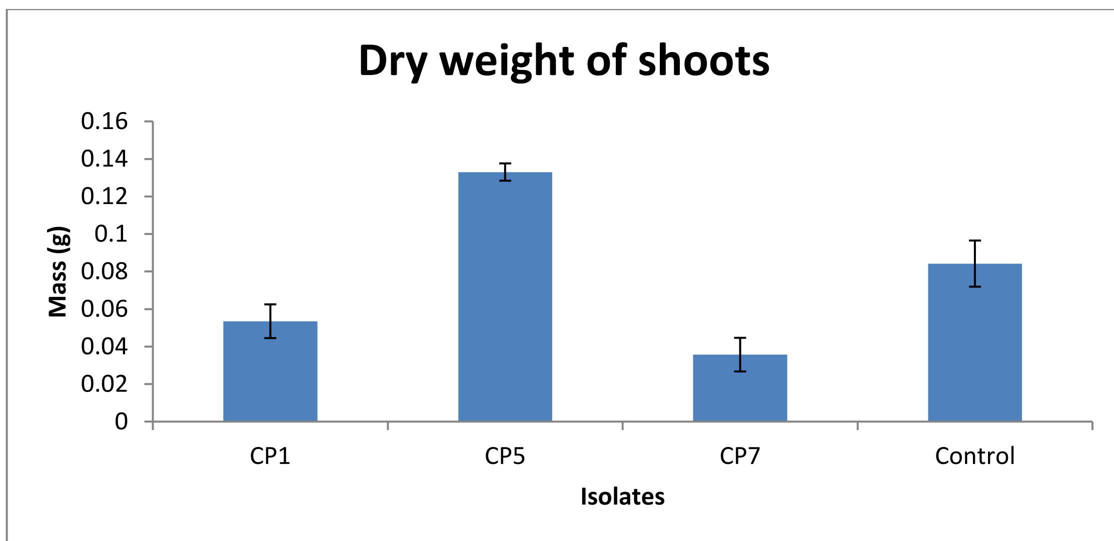

Figure 4: Dry weight of *Brassica napus* L plants shoots after 42 days. Error bars represents (±SE) of the mean of 3 replicates. Means with different letters represents significant difference (P<0.05).

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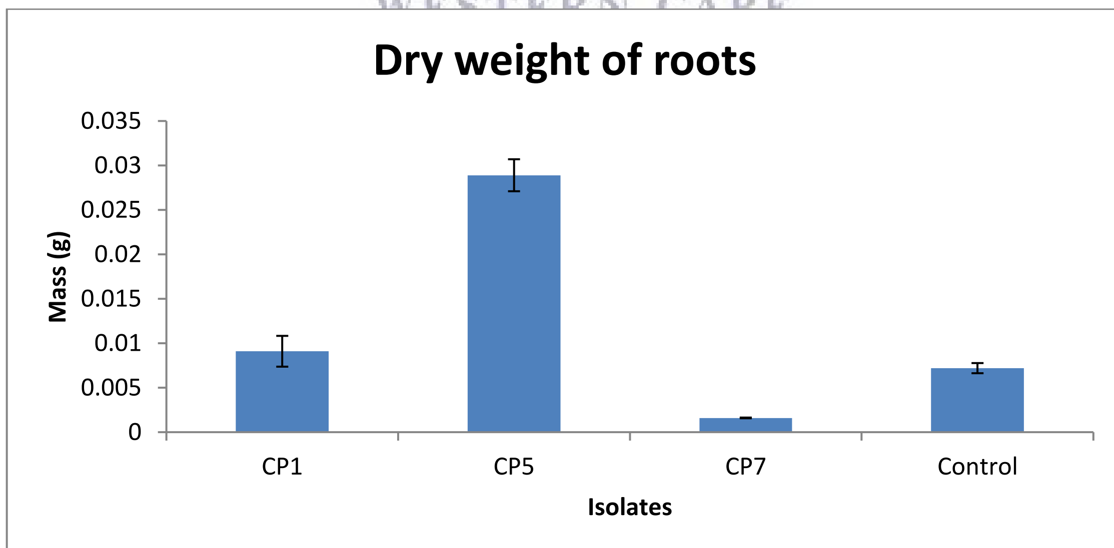

Figure 5: Dry weight of *Brassica napus* L plants roots after 42 days. Error bars represents (±SE) of the mean of 3 replicates. Means with different letters represents significant difference (P<0.05).

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3.1.4 Vigor index

Isolates	roots (mm)	shoots (mm)	Germination (%)	VI
CP 1	68	137.67	15	3085.05
CP 2	95	144	10	2390
CP 4	137.5	162.5	10	3000
CP 5	103	142.33	50	12266.5
CP 6	120	153.33	35	9566.55
CP 7	58	116.67	35	6113.45
Control	92.5	127.5	10	2200

Table 1: Ability of plant growth promoting endophytic strains on growth promotion of *Brassica napus* L seedling

3.1.5 Quantification of macro elements

Isolates which showed the greatest increased of plant dry weight also accumulated the most nutrients essential for growth. Plants which were treated with CP5 showed the highest uptake of all macro elements Ca, P, K, and Mg in both shoots and roots compared to other 2 isolates CP1, CP7 (**figure 6, 7**), This higher accumulation of macro nutrient observed from isolate CP5 could correlate of increased dry weight and biomass of the treated plant.

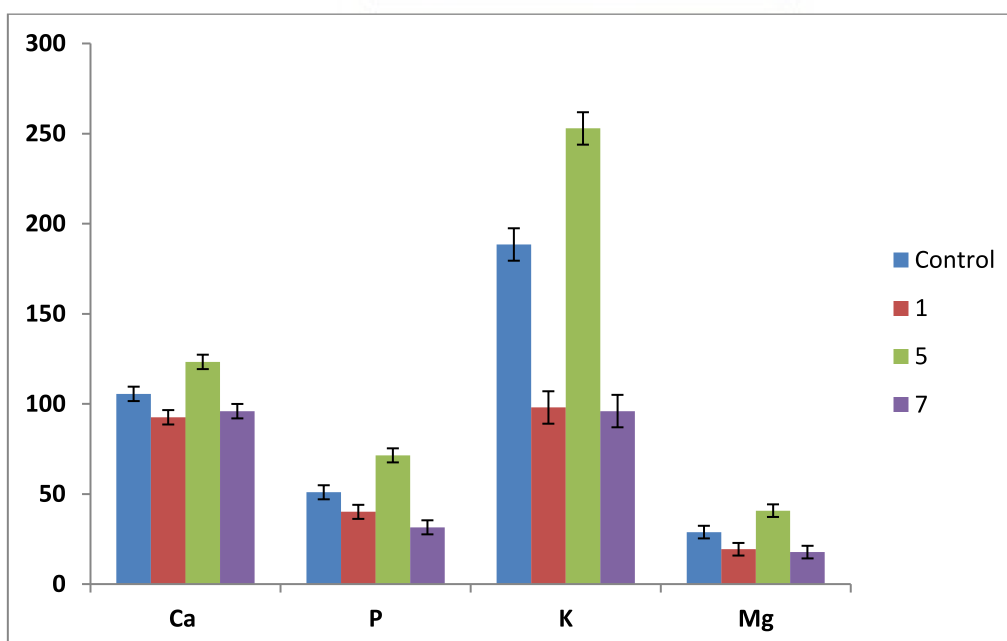

Figure 6: ICP result showing nutrient accumulation (part per million) within the shoots of *Brassica Napus* L treated with bacterial isolate CP 1, 5, 7 and control.

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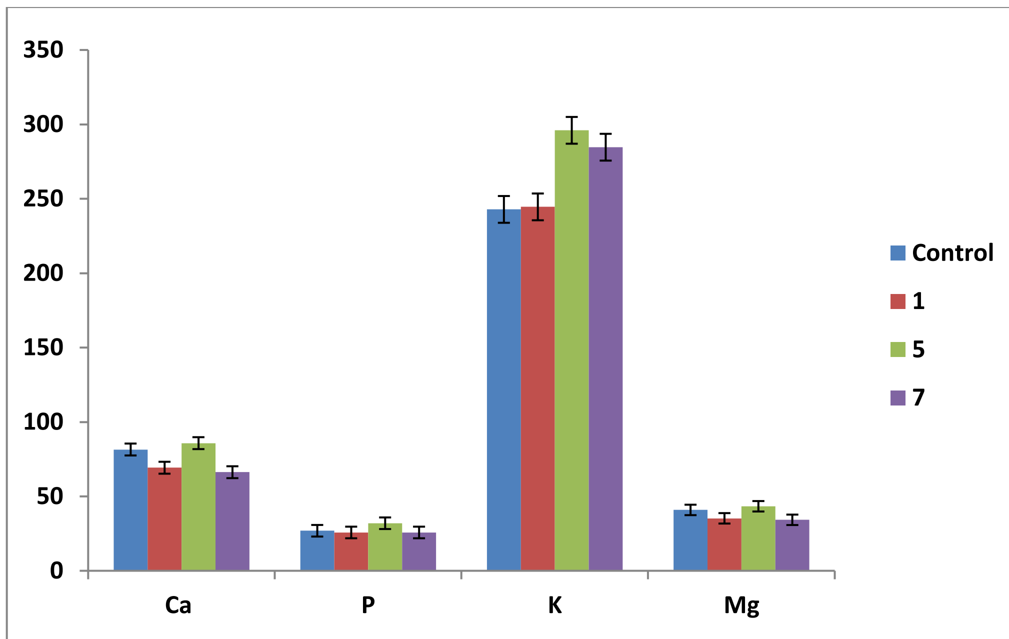
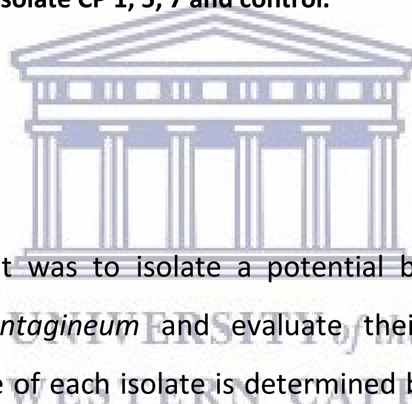


Figure 7: ICP result showing nutrient accumulation (part per million) within the roots of *Brassica Napus* L treated with bacterial isolate CP 1, 5, 7 and control.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3.2 Discussion

The aim of the experiment was to isolate a potential bacteria candidate from the internal tissues of *E. plantagineum* and evaluate their plant growth promoting capability. The performance of each isolate is determined by growth parameters which were based on length of the roots and shoots, dry weight of shoots and roots and vigor index.

The greenhouse experiment was set up to observe the effects of inoculation with endophytic bacteria isolates. Treatment with CP1, CP5 and CP1 showed an overall increase in germination percentage, vigor index (VI) and dry weight with exception to isolate CP7, compared to control. Amongst these potential growth promoting bacteria it is evident that CP5 showed the most potential, based on the growth parameters, plants which was treated with CP5 isolate produced the highest values in terms of VI, dry weight and germination percentage.

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3.2.1 Colonization

The *Brassica napus* L Garnet is a spring rapeseed, which is commonly extracted for its oil content, however this particular cultivar germination percentage under axenic condition is lower compared to other cultivars such as agarmax, from the results its show that germination percentage of control was only at 10% (**figure. 1**). The improvement of germination from inoculating CP5 isolate can be due its ability to colonize the seeds better in comparison to other isolates. Several traits which bacteria possess will allow better colonization. Bacteria which can influence greater germination will already interact and colonization during the seed stage therefore requires different mechanisms compared to seedling or budding stage. Germination of the seeds will subsequently release compounds in different quantities and composition therefore the bacterial population which can be supported will differ (Roberts *et al*. 2009). Another trait which benefits the colonization of seed is the release of phosphofructokinase which is encoded by the pfkA gene (Roberts *et al*. 1999). Previous studies also shown that bacteria are able to utilize adhesion factors such as fimbriae, calcium binding proteins and efflux pump which may also contribute to bacteria ability to colonize seed (Espinosa-Urgel *et al*. 2000; Molina *et al*. 2005; 2006). After the seed has been germinated, new root system will emerge from the seed which will induce root colonization by bacteria caused by chemotaxis induced motility and further migrate into the plant (Bacilio-Jiménez *et al*. 2003), studies has demonstrated that certain mutant of pseudomonas strains which contain non function flagella or twitching motility has decreased the ability of bacteria to colonize the root (Camacho Carvajal, 2001). Following colonizing roots bacteria may migrate further into the plant via passive and direct mechanisms. Bacteria may passively enter internal tissue through cracks, such as those produced at root emerging site or created by other microorganisms to gain entry or bacteria can actively gain access to internal tissue by possessing the ability to secrete cell wall degrading enzymes such as endoglucanases (Reinhold-Hurek *et al*. 2006), cellulase and pectinase (Ebeltagy *et al*. 2000; James *et al*. 2002). Aside from the bacterial traits at times the plant may possess certain genes which such as those involved in ethylene signalling pathway or kinase shr5 which may play a part in plant and bacteria interaction resulting whether or not the bacteria is able colonize plant's

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internal tissue. (Vinagre *et al*. 2006; Cavalcante *et al*. 2007; Liu *et al*. 2012). Even though all the seeds were subjected to liquid media containing the isolates and allowed time colonize, the ability of these isolates to colonize the seeds will differ because certain bacteria traits will give an advantage in terms of colonizing whilst those that lack these traits might not colonize as effectively. Another factor to include which can be linked to affect the seed germination percentage is the seed. A good quality seed must be free from pathogenic infection or plant like disease and will produce high germination percentage with high index vigor (Dent *et al*. 2004).

Once seed start to germinate organic compounds are released from the emerging root system which can be used by bacteria as energy source to proliferate, this environment will be able to support a higher bacterial population as a result of more nutrient available compared to the low nutrient environment around the seed (Baker and Cook. 1974). Successful colonization within the seed internal tissue will prove to be advantageous to endophytic bacteria, as these bacteria inhabiting the seed will be well adapted to the environment and can proliferating at higher rate within plant tissue. This can be due to less competition in terms of nutrient acquisition and space with other microorganism originating from the external environment (Kaga *et al*. 2009; Hardoim *et al*. 2012). Inoculating the seed with Isolate CP5 has showed the highest germination percentage among other treated plants, this may be good indication of the isolate CP5 ability to initially colonize seed and furthermore gain entry into the internal tissue before germination occurs or during germination where the cracks and fissures created by emerging roots.

3.2.2 IAA stimulation of plant growth

Another possible method which bacteria utilize to promote growth and germination of treated plants was the production of the auxin indole-3-acetic acid (IAA). This commonly occurring auxin is produced the bacteria as a result of L-tryptophan metabolism (Johri *et al*.. 2003) which is capable of increasing germination percentage (Tokala *et al*. 2002; Ali B and Hasnain. 2007; Glick. 2012). L-tryptophan can be found as one the compound released as roots exudates (Khamna *et al*. 2010) the seeds used for the this experiment was all same specie and was all grown in the same environmental conditions therefore

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the concentration of L-tryptophan level released by the plant should be uniform across all treated and control plants. Since the amount of L-tryptophan released was uniform the ability to promote germination will depend on the efficiency of L-tryptophan metabolism to produce IAA. Many studies has being conducted showing the positive effects of IAA. Gagne-Bourgue *et al*, (2013) showed that switchgrass seedling inoculated with *Bacillus* and *Microbacterium* strains stimulated increased plant growth compared to the non treated plant, both of these strains produced IAA. El-Tarabily *et al*, (2005) showed that streptomyces spp which has the ability to produce IAA was able to improve the overall growth and germination of tomato plant and another study showed that inoculation of bean seeds with IAA producing *Klebsiella* strain was able to significantly increase the adventitious root length of seedlings (Chaiharn and Lumyong. 2011). In the currently study Brassica Napus L treated with CP5 exhibited the highest root and shoot elongation compare to all other isolates and control (**figure 2, 3**), this result correlates with previous studies which used auxin producing plant growth promoting bacteria (PGPB) to treat *Brassica* spp resulting in the increase of shoot length and number of branches per plant (Asghar *et al*. 2004). Root elongation of *Brassica Napus* L was also observed after inoculation with IAA producing PGPB (Sheng and Xia. 2006).

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However plants are sensitive to the changes of the concentration of IAA, additional IAA produced by bacteria can also have adverse effects on the plant. The optimal IAA concentration level range for a given plant can be narrow and the effects of the auxin are based on the concentration released, studies have shown that inoculation of *Pseudomonas thivervalensis*, a strain which produced IAA at a concentration of 105 CFU/ml^{-1} promoted growth of *Arabidopsis* plant, however inhibition of plant growth was shown when concentration of IAA was at 106 CFU/ml^{-1} and above was reported (Persello-Cartieaux *et al*. 2001), Sarwar and Kremer (1995) compared the auxin production level of a growth promoting and inhibiting bacteria, the growth inhibiting bacteria released high concentration of IAA thus decreased root length of *Convolvulus arvensis*, similar results was observed in another study using *Lactuca sativa* L (Baranzani and Friedman. 1999). Another study used IAA producing *Bacillus* strains to promoted growth of corn found that strains which showed growth promotion did not produce high level of IAA (Araujo and Guerreiro. 2010).

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IAA concentration could be one of the potential factors which influenced the differentiation in germination percentage and the variation in shoots and roots length of certain treated plants. In the present study isolate CP7 has 35% germination percentage which is one of the better performing isolate in terms of germination, however when observing the overall length of the plant treated with isolate CP7 it produced lowest overall length, this could be due to the IAA concentration released by bacteria was within the optimal range for germination however the concentration of IAA released did not promote any significant growth, similar results was observed for plants inoculated with CP5 was able to achieve 50% germination percentage but did not achieve highest overall length (**figure. 1,2,3**).

3.2.3 ACC production

One of the commonly plant hormone associated with plant growth and development is ethylene, this plant hormone regulates many physiological development of the plant such as root and shoot growth, germination and flowering (Arshad and Frankenberger. 2002). Ethylene is synthesized endogenously by almost all plants, under normal condition ethylene is produced at according to the plant requirements. Ethylene can also be synthesized when the plant experiences stress caused by salinity, drought, heavy metals, phytopathogens and wound caused by insects (Hilt and Bessis. 2015; Roberts and Tucker. 2013; Habben *et al*. 2014), as a result of these stresses the synthesis of endogenous ethylene will significantly increase and this increase of ethylene level often found to cause various growth inhibition and premature senescence (Ali *et al*. 2012; Bhattacharyya and Jha. 2012). In the present study the seedlings were grown within a green house environment however these seedlings are still vulnerable to stress caused by insects, decreased nutrient uptake and receiving insufficient sunlight. Upon the induction of stress this result in the accumulation of 1-aminocyclopropane-1-carboxylate (ACC) which lead to increased ethylene production, ACC is a direct precursor to ethylene and much of the plant ethylene synthesized is influenced by endogenous ACC concentration (Mckeon *et al*. 1982). Effects of ethylene stress can be decreased by isolates which are able produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Abeles *et al*. 2012; Chen *et al*. 2013). ACC deaminase can reduce the concentration of ethylene by converting ACC into ammonia and α-ketobutyrate thus

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decreasing the ethylene level within plant (Honma and Shimomura. 1978; Siddikee *et al*. 2012). Previous studies has shown the effects of inoculation of seed or root with ACC deaminase producing bacteria resulted in the plant root elongation, promotion of shoot growth, improved nodulation and nutrients uptake such as nitrogen, phosphorus and potassium (Shaharoona *et al*. 2008; Nadeem *et al*. 2009; Glick. 2012). It is evident that inoculation with ACC deaminase producing bacteria can promote plant growth, however these ACC deaminase producing bacteria may also be the causation of low germination percentage obtained among the treated plants.

Ethylene previously discussed to cause adverse effects to plants at higher concentrations however this hormone is required for germination of seeds (Matilla. 2000; Linkies and Leubner-Metzger. 2012). In current study isolates CP1, CP7 did not show any major increase in germination percentage over the control plant in the overall length of the plant, this can be due to bacterial isolates which are able to produce ACC deaminase promoted elongation of the plant root and shoot however in turn lowering the overall ethylene concentration thus reducing germination capability of the seeds. The elongation   Ethylene concentration within the plant can also be elevated through interaction of IAA produced endogenously by plant or by bacteria, IAA as previously discussed can stimulate root and shoot growth and elongation, furthermore IAA can also stimulate the transcription of ACC synthase, this plant enzyme ACC synthase catalyzes the production of ACC and therefore IAA can stimulate the production ethylene (Penrose *et al*, 2001). It can be expected that bacteria isolates which is able to synthesize IAA will improve germination but at the same time also cause deleterious effects on the plant due the elevated ethylene concentration, however previous research has also shown that as the concentration of ethylene increase within the plant this create a feedback which causes the inhibition of IAA signal transduction and this will limit the amount of IAA which are able to stimulate ACC synthase transcription (Glick *et al*. 2007; Stearns *et al*. 2012). Previous research has shown that the ethylene level within the plant were much lower from bacteria which release IAA together along with ACC deaminase compare to the bacteria which solely produce IAA, this is because the presence of ACC deaminase will cause the decrease of endogenous ethylene concentration of the plant therefore the overall inhibitory feedback of IAA signal

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transduction is also decreased so that the IAA produced can still promote growth and stimulate ACC synthase transcription (Potuschak *et al*. 2003; Glick. 2014). Previously discussed interaction between phytohormones and auxins operating in unison or individually can influence the germination, root and shoot growth in the present study, isolate CP7 which achieved 35% germination however the treated plants showed a significant decrease in root and shoot length and dry weight compare to control (**figure 4, 5**). Plants treated with isolate CP1 produced the higher overall plant length (root + shoot) compared to control, however CP1 treated root and shoot dry weight did not shown any significant improvement at accumulation of biomass (**figure 4, 5**), whereas plants treated with isolate CP5 produced the significant root and shoot length improve, furthermore seeds treated with CP5 also showed the highest VI and produced the highest germination percentage and the overall biomass of the *Brassica Napus* L (**figure 1, 4, 5**). This may suggest that isolate CP5 was able release higher amount of ACC deaminase and IAA compared to the other isolates and this in turn improved plant performance in all growth parameters, this finding correlates with study done by Rashid *et al* (2012) where growth promotion was evaluated on Canola plants which was treated with various endophytic bacteria isolated from tomato plant (*Solanum lycopersicum*), the results showed that *Agrobacterium* strains which produced low levels of ACC deaminase and auxins did not shown any major growth, whereas *Pseudomonas* spp. strains which showed high level of ACC deanimase level and moderate level of IAA had the greatest growth promotion effect on the canola plant.

3.2.4 Nutrient uptake

Much of the growth promotion effects of bacterial isolates can be shown by the increase of biomass of the plant. The increase of plant biomass can be credited to the acquisition of nutrients necessary for the growth of plants. Many previous research has being conducted which has shown that the plant growth promoting bacteria are able to facilitate the acquisition nutrients, these nutrients include phosphorus, nitrogen, iron and other important resources such as calcium, potassium and magnesium which the plants can utilize for growth (James. 2000; Subramanian *et al*. 2014; Rajkumar and Freitas. 2009). Much of the increase of dry weight observed from plants treated with isolate CP5 can be credited to superior nutrient uptake mechanisms which bacteria are

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able utilize, such as phosphate solubilisation or nitrogen fixation and sideophore production.

Many researches has shown the plant growth promoting effects of bacteria which are capable of phosphate solubilisation, nitrogen fixation or other nutrient uptake mechanisms. For example Dias *et al*, 2009 showed that treating strawberry *Fragaria ananassa* with IAA producing and phosphate solubilising endophytic bacteria was able to promote the growth of roots and shoots of the plant. Previously research conducted by Dey *et al* (2004) has shown that peanut *Arachis hypogaea* L inoculated with *Pseudomonas* spp. which is capable of producing IAA and siderophore in additional also possess the ability to solubilise tri calcium phosphate caused a significant increase pod, stem yield and nodule dry weight, similar results was shown by Marques *et al*. (2010) *Zea mays* was treated with *Ralstonia* which was able to produce IAA, ammonia and siderophore production, this resulted in the greater increase of biomass, root and shoot elongation and higher nutrient accumulation of *Zea mays* over other isolates and control. Furthermore Hurek and Reinhold-Hurek, (2003) observed the effects of nitrogen fixing bacteria Azoarcus which was present within the roots of kallar grass, the presence of the nitrogen fixing bacteria cause the increase of kallar grass yield by 20 - 40t ha^{-1}/ $year^{-1}$, these results correlates with experiment done by Marroquí *et al*. (2001) utilizing *Rhizobium tropici* which was constructed with a deletion of a gene which encode for glycogen synthase, this deletion was to further facilitate synthesis of ATP as nitrogen fixation require large amount of ATP, bean plants treated with the mutant *Rhizobium tropici* showed a drastic increase in number of nodules developed and dry weight compared to bean plants treated the wild type strain. Correlation these studies to current experiment, these studies suggests that beside the interaction between phytohormone and auxins, isolates CP5 may possess superior nutrient acquisition capabilities including phosphorus solubilisation, nitrogen fixation or siderophore production, this can be substantiated by ICP analysis conducted which showed the accumulation of macro-elements was much higher in roots and shoots of the plant that was inoculated with isolate CP5 compared to plants that was inoculated with CP1, CP7 and control (**Table. 2**). The macro elements analyzed Ca, Mg, K and P all has an important role to play in the development of plants. Nutrients such as calcium is

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required which plays an important role in structural development of cell wall and membrane such as elongation of plant cells, fortify and stabilize cell wall by forming calcium pectate and aid in heat resistance by inducing heat shock proteins (Tuna *et al*. 2007).

Much of the photosynthesis occurring within plants involves magnesium, this nutrient is required for the formation chlorophyll, generation of ATP in chloroplast, photosynthetic carbon dioxide fixation, oxidation within plant tissue and facilitates the synthesis of wide range organic compounds and proteins required for functioning and growth of plants (Cakmak and Kirkby. 2008; Waraich *et al*. 2011). Many regulatory functions of the plant which involves the regulation of exchange of carbon dioxide, protein synthesis, oxygen and water, generation of ATP through photosynthesis, transport of sugars and nutrients like magnesium and calcium requires potassium, plants which experience potassium defiency usually have smaller growth pattern and yellowing of the leaves (Ashley *et al*. 2006; Mäkelä *et al*. 2012).

Phosphorus is essential macro nutrient required for cellular processes. Adenosine triphosphate synthesis from photosynthesis involves the use of P, therefore the amount P the plant is able to uptake will affect its growth, furthermore much of the DNA synthesis also requires phosphorus, however this nutrient one of most limited resource because it exists mostly as apatite which the plant cannot utilize (Thakur *et al*. 2014).

From the findings from previous conducted researches has shown that inoculating plants with endophytic bacteria which possess these previously mentioned plant growth promoting traits has substantial potential to increase the root and shoot elongation, dry weight and nutrient accumulation, Results from the present study clearly shows that isolates CP5 holds the most growth promoting potential, previous studies also suggests that isolates CP5 may possess superior nutrient acquisition capabilities such phosphorus solubilisation or nitrogen fixation and was able to releases higher level of ACC deaminase or IAA compared to other isolates as CP5 showed the highest value in all growth parameter, thus leaving isolate CP5 the perfect plant growth promoting endophytic bacteria candidate.

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Chapter 4

Plant growth promoting mechanisms

4.1 Introduction

The ability for the bacteria to promote growth is evident from the results obtained during growth trials. Bacteria can utilize various growth promoting mechanisms to achieve overall growth increase. Endophytic bacteria can achieve growth promotion by improve the acquisition and cycling of essential nutrients such as phosphorus or iron which otherwise are not readily available for uptake or low in abundance within the soil (Dias *et al*. 2009). Endophytic bacteria can also augment phytohormones and auxins yo benefit the plant in term of increasing shoots and roots growth increase. Phytohormones synthesized by PGPE can also help to alleviating the effects of environmental stress. In addition previous research has shown that residing within the inner tissue of the host plant can be advantageous to the bacteria in terms of improved protection from biotic and abiotic stresses exerted from the outside of the plant. Furthermore plants often experience greater increase of growth when inoculated with endophytic bacteria compare to bacteria which are limited to rhizoplane (Chanway *et al*. 2000). However, the mechanisms which the isolated bacteria were able to utilize in order promote growth of the *Brassica napus* plant still remains relatively unclear and not much research has been conducted to using weed endophytes as a means of bio-fertilizer.

The acquisition and cycling of nutrients can be achieved through the usage of bacteria capable of utilizing one or more plant growth promoting mechanisms. Phosphate is one of most limited nutrient as most of phosphorus present in soil is in an insoluble state therefore unable to be used by the plant. Certain endophytic bacteria can synthesize enzymes or acids sometimes in combination which are able to solubilize the organic and inorganic bound phosphorus, thereby rendering the phosphorus available for uptake by the plant. The quantity of iron which can be absorbed by plants in soil is generally very limited with exception to acidic soil, the limited usable iron can be accredited to its lack

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of solubility (Mercado-Blanco *et al*. 2007). Iron is essential for both plants and microorganisms growth, it is required in many metabolic processes. Inadequate amount of soluble iron will cause bacterial growth to decrease this will trigger the production of low molecular weight molecules termed as siderophore by bacteria. These siderophore have a strong affinity for Fe+ ions and forms an iron siderophore complex, the iron siderophore complexes is then transported to the plant through protein receptor which was synthesized following the development of siderophores. Furthermore because endophytic bacteria colonize the plant's inner tissue they are prone to develop siderophores in order to survive within an environment which greatly lacks bioavailable iron (Reinhold-Hurek. 2011).

Aside from the improved uptake of nutrients, the growth promoting endophytes can also elevate phytohormones and auxins produced. Ethylene in plants is required for numerous growth and developmental processes. However, when the plant experiences hostile environmental conditions ranging from flooding, drought, extreme cold or heat and high salinity this will trigger stress ethylene to be synthesized within plants. Ethylene produced at high concentration has being shown to cause symptoms such as plant necrosis, decoloration and growth inhibition, therefore any means to decrease the elevated ethylene level may prove to be effective at decrease the damage to the plant. Certain bacteria can synthesize enzyme ACC deaminase, this enzyme is able to cleave ACC into ketobutyrate and ammonia (Honma and Shimonmura. 1978), this trait of producing ACCD in turn can lower the overall level of stress ethylene because ACC is a direct precursor to ethylene (Hardoim *et al*. 2008; Pliego *et al*. 2011; Glick *et al*. 2007).

The synthesis of bacterial ACCD can improve the host plant's tolerance to biotic or abiotic factors (Wang *et al*. 2002), auxins such as IAA on the other hand can improve the stimulation of several developmental processes. These processes include the elongation of cell by modulation of the osmotic content of cell, increase water permeability and increase cell wall synthesis, improve root formation and amount of root hair and lateral roots (Datta and Basu. 2000). Majority of plant growth promoting bacteria can produce IAA, these bacteria accomplishes this through the utilization of an auxin precursor tryptophan (Spaepen *et al*. 2009), this also extends to the population of endophytic bacteria (Vandan *et al*. 2010; Shcherbakov *et al*. 2013). Given the immense potential of

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growth promoting bacteria to cause growth improvement and elevated tolerance towards hostile conditions this study will aim to investigate the growth promoting mechanisms available to the endophytic bacteria. In addition many studies have been conducted on growth promoting bacteria residing in the soil or rhizoplane, not much emphasis has being placed on the potential of growth promoting endophytic bacteria even less for endophytes residing within weeds. This study seeks to explore the plant growth promoting mechanisms available to the isolate CP5. This isolate was selected based on its overall performance to improve plant growth during the growth trials (**section. 3**).

4.2 Results

4.2.1 Phosphate solubilization

Phosphate solubilization was tested using Pikovsaya agar described in section 2.3. Bacteria can solubilise phosphate by releasing several compounds which in turn are able to release these phosphate ions which otherwise would be rendered insoluble. Within 5 days of incubation clear halo rings forms with a diameter of (15mm) (**figure 4.1**). The zone of clearance increased in size upon further incubation. Maximum zone of clearance was observed after 12 days of incubation with a diameter of 23mm (**figure 4.2**). During the incubation period the isolate also grew and expanded covering the clearing made from solubilizing phosphate. *Rhodococcus qingshengii* was used as a control due to lack of phosphate solubilization activity.

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Figure 4.1 ***Rhodococcus qingshengii*** **on Pikoskaya's agar.** P4, P5 and P6 are *R. qingshengii*. No zone of clearance was formed on the media.

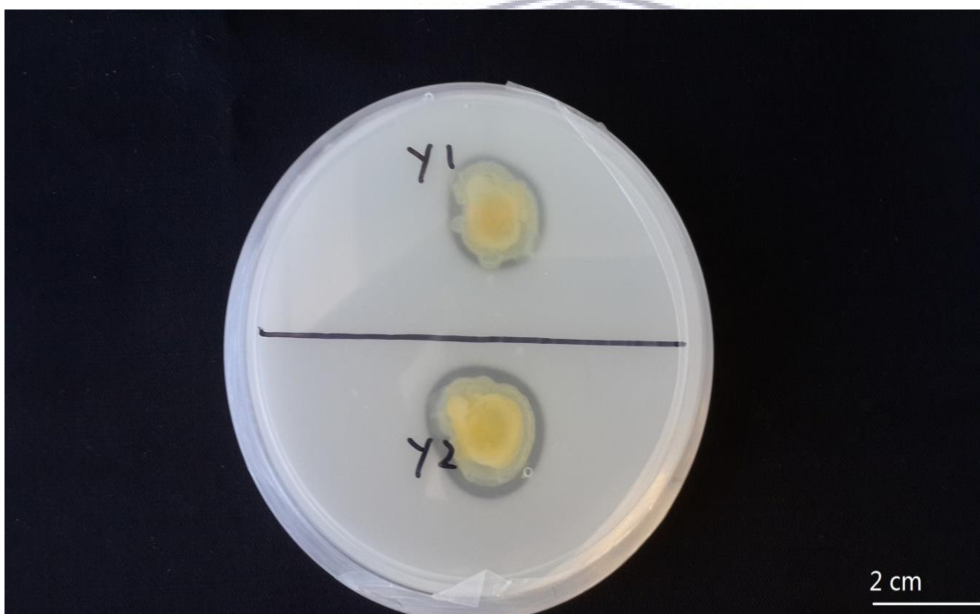

Figure 4.2 Phosphate solubilization by isolate CP5 on Pikoskaya's agar. Y1 and Y2 are both isolate CP5 (Y2) showed the maximum clear halo zone diameter 23 mm. Lighter colonies surrounded the darker central colony, this is due to the bacteria growing out from the initial spot of inoculation.

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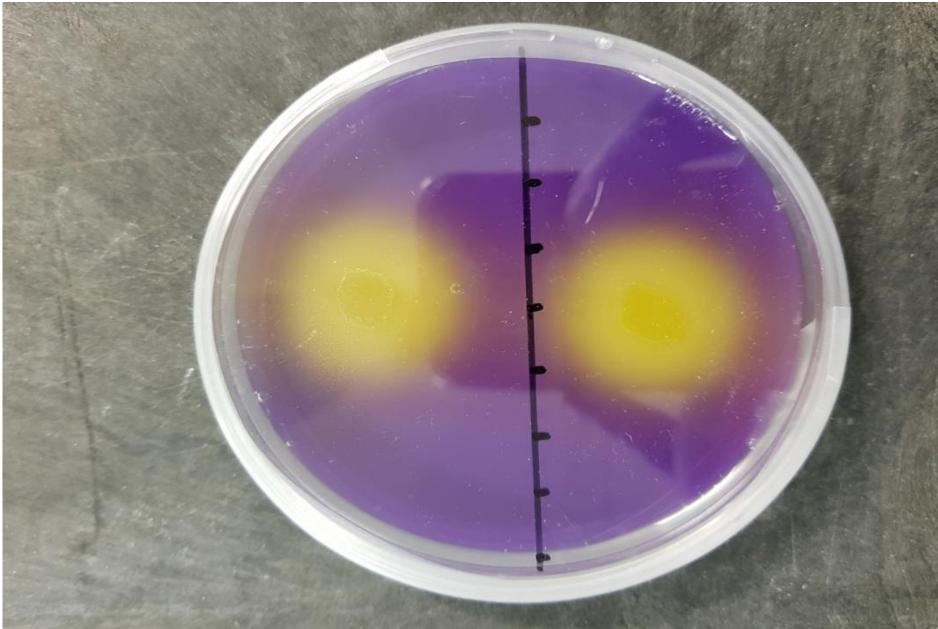

Figure 4.3 Production of organic acid using Pikoskaya agar supplemented with bromocresol purple. Color yellow indicates a decrease in pH from the release of acids.

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4.2.2 Indole-3-acetic acid production (IAA)

IAA production was tested using the Salkowski reagent. Development of red to pink color was observed within 1 minute after adding Salkowski reagent, the color intensity was increased over 30 minutes of incubation. The concentration of IAA was compared using YEM media one with tryptophan and a second without tryptophan. Results from this study showed that IAA production was significantly higher in media supplemented with tryptophan compared to isolate grown in tryptophan free media. Following incubation isolates CP5, and control supplemented with tryptophan produced 35.86 ug/ml and 8.33 ug/ml respectively (**figure 4.3**). IAA was produced in very low quantities in tryptophan free media, isolates CP5 and control produced 2.4 ug/ml and 0.32 ug/ml respectively (**figure 4.4**)

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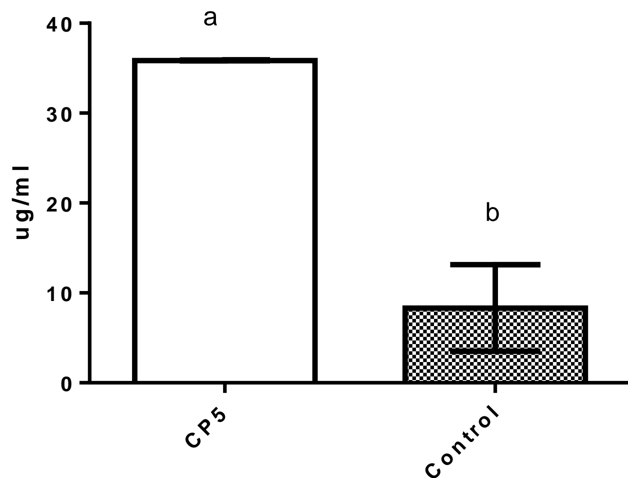

Figure 4.4 IAA production by isolate supplemented with tryptophan. Error bars represents (±SE) of the mean of 3 replicates. Means with different letters represents significant difference (P<0.05).

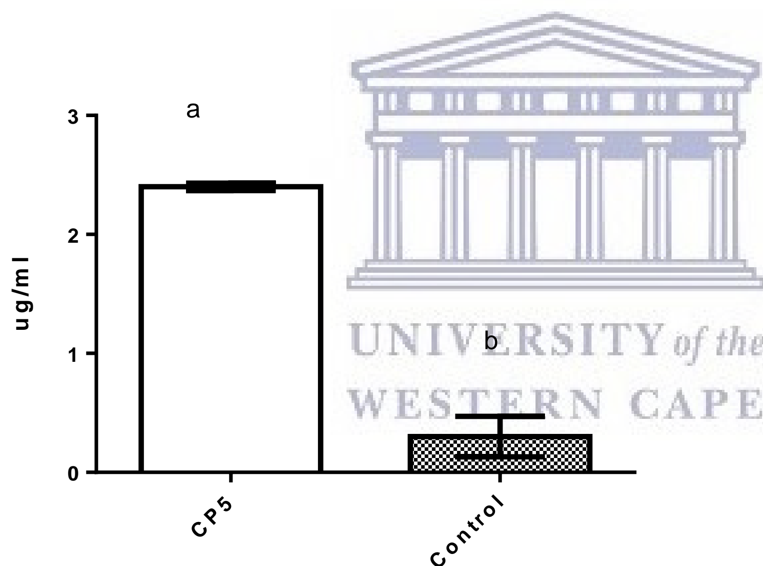

Figure 4.5 IAA production isolates with no tryptophan supplementation. Error bars represents (±SE) of the mean of 3 replicates. Means with different letters represents significant difference (P<0.05).

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4.2.3 ACC deaminase metabolism assay

ACC deaminase activity can be determined by the endophyte ability to utilize ACC as the nitrogen source. The results showed that the isolate CP5 tested positive for ACC deaminase activity, the highest optical density of 0.75 was reached at day 3. The

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bacteria performed best in terms of growth in media which contained higher concentration of magnesium sulphate with the highest recorded optical density of 0.95.

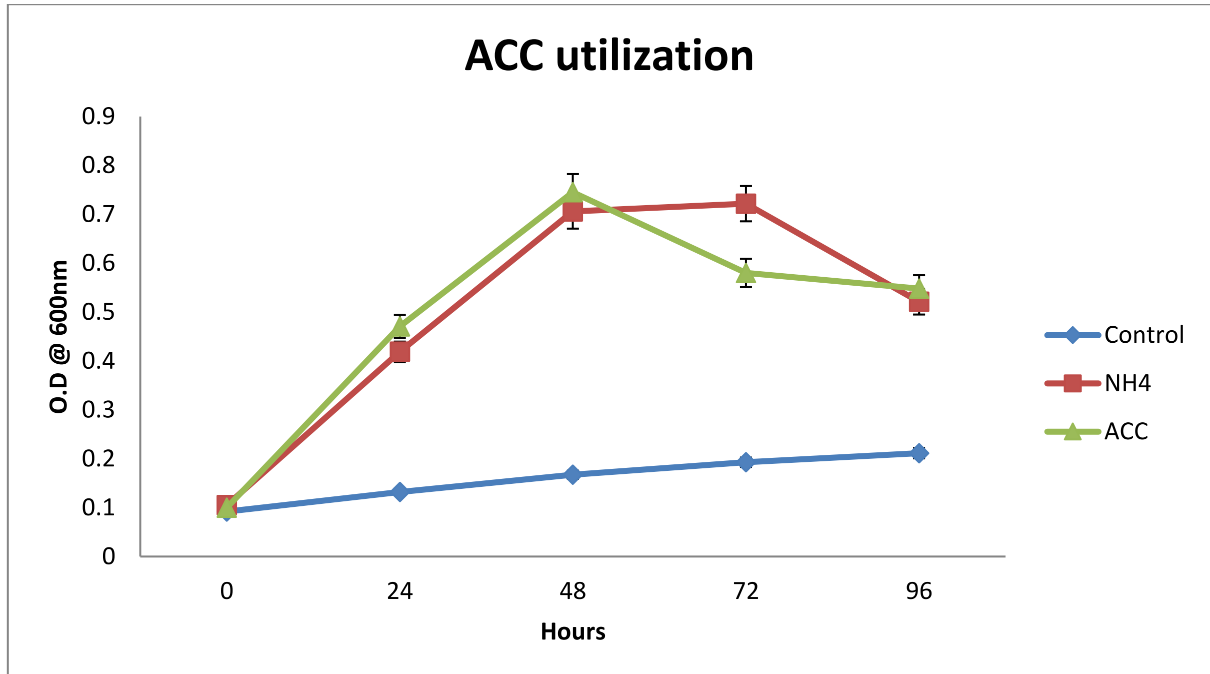

Figure 4.6 Utilization of ACC by isolate as the sole nitrogen source compared to ammonium sulphate. Error bars represents (±SE) of the mean of 3 replicates.

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4.2.4 CAS medium

Siderophore production can be determined by development of colored halo zone from the original blue color of the CAS agar. Isolate CP5 was able to growth on the CAS agar media however did not produce any colored halo zone around the inoculated colony when compared to the positive control, *Rhodococcus qingshengii* displaying a clear formation of colored halo around the colony (**figure 5.4**)

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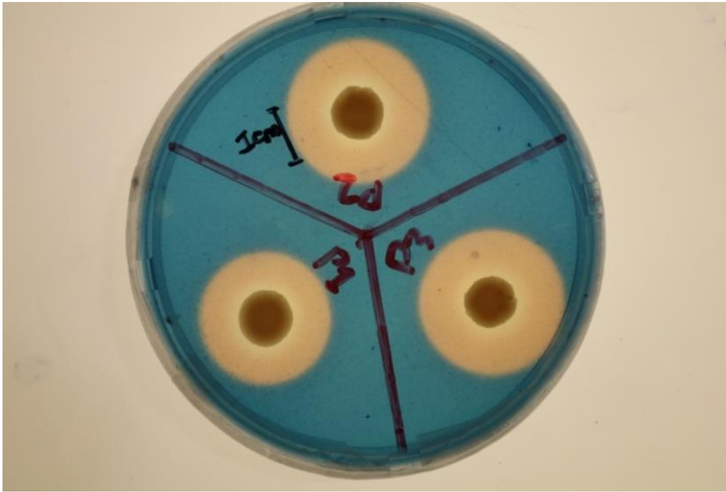

Figure 4.7 Siderophore activity using CAS blue agar plates. Clear formation of colored halo produced by *R. qingshengii*.

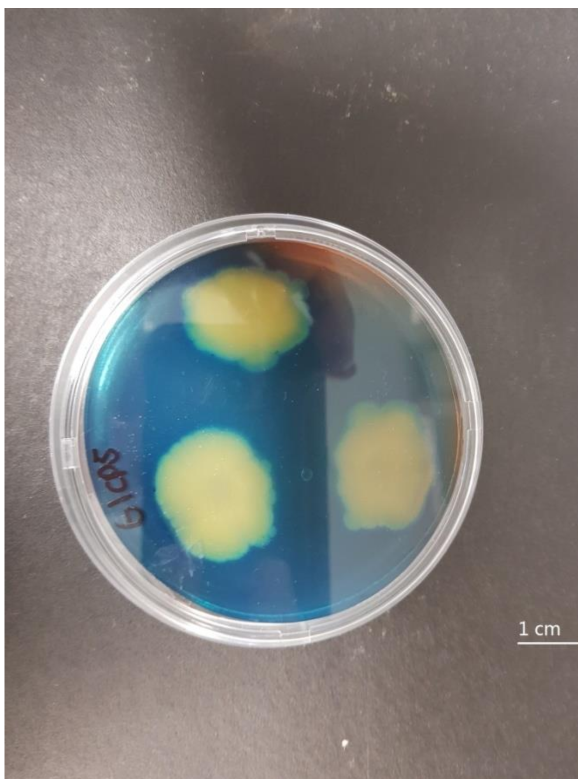

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Figure 4.8 Siderophore activity using CAS blue agar plates. CP5 did not produce any color change to the media.

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4.3 Discussion

4.3.1 Phosphate solubilization

Phosphorus is an important macronutrient required for numerous developmental processes and currently the best method of phosphate delivery is through chemical fertilizers. However, this method of delivery is rather inefficient as much of the phosphates present within the fertilizers are rapidly converted into insoluble forms, thus rendering majority of the phosphate unavailable for the plant to uptake. Therefore the conversion of these insoluble forms of phosphate into usable soluble phosphate by microorganisms can in turn influence the amount of bio-available phosphate (Richardson *et al*. 2001). The formation of clear halo zone from the data present in the study shows the isolate CP5 was able to solubilize phosphate. This can be attributed to the synthesis of substances which can solubilize phosphate or organophosphate during the period of growth, this efficiency of this process can vary depending on numerous factors such as the amount of nutrients available and the state of the bacteria physiologically and during the growth period (Reyes *et al*. 1999).

Many theories have been proposed as to explain the mechanism of phosphate solubilization and the most popular is the theory of acid synthesis. The acid theory suggests that a bacterium can solubilize phosphate through the synthesis of low molecular weight organic acids then followed by a decrease of pH in the medium (Goldstein. 1995; Puente *et al*. 2004; Kim *et al*. 1997). Organic acids produced can chelate ions bounded to the phosphates using the hydroxyl and carboxyl groups (Kpomblekou and Tabatabai. 1994). This theory coincide with other studies done which analyzed different PSB strains and various organic acids was detected such as fumaric, malic, citric, alpha keto butyric, oxalic, and gluconic acid (Lapeyrie *et al*. 1991; Fasim *et al*. 2002). In a fairly recent study reports of decrease of pH from 7.21 to Ph 4.24 using media containing $Ca_3(PO_4)_2$ as the phosphate source was observed (Zhu *et al*. 2011). In the present research, change of color from purple to yellow was observed in phosphate solubilizating medium with added bromocresol purple (**figure 4.3**), this change of color to yellow indicates a decrease in pH in the medium which correspond to the acid theory.

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Phosphate solubilization endophytic bacteria can be developed into an effective method of transforming insoluble phosphorus bound to ions into soluble usable source of phosphorus, therefore further research will be conducted in order to analyze the type of organic acids produced. Furthermore additional endophytic bacteria will be extracted from plants grows in regions which experience high environmental stress such as high salinity or heavy metal pollution. Extracting endophytic bacteria from these extreme environmental conditions is important in order to increase the chances of locating efficient phosphate solubilizing bacteria as previous studies suggests that phosphorus solubilizing performance can be affect by the environmental conditions, more so for the regions which experience stress (S´anchez-Porro *et al*. 2009; Yoon *et al*. 2001).

4.3.2 Indole-3-acetic acid production

The ability of IAA production can be a good indicator for screening growth promoting bacteria, this suggests that bacteria which produces IAA have a profound impact on the growth of the plant (Wahyudi *et al*. 2011). In the present study IAA produced by the isolate CP5 was significantly higher in the presence of tryptophan, this might be because tryptophan is a precursor for IAA production. The significant increase of IAA production from isolate CP5 from tryptophan supplementation corresponds with previous research which recorded an increase of IAA production from 1.47 ug/ml to 32.8 ug/ml when supplemented with 1 - 5 mg/ml of tryptophan (Ahmad *et al*. 2005). Furthermore other research also showed a higher production of IAA from bacteria using concentrations of tryptophan ranging from 0.05 to 0.25 mg/ml and the maximum IAA production was observed at 0.2 mg/ml (Bharucha *et al*. 2013). Beside the addition of tryptophan, IAA production can be influenced by factors such as temperature, duration of incubation, pH, availability of oxygen, carbon source and nitrogen source (Patten and Glick. 2002; Sarwar *et al*. 1992; Yuan *et al*. 2011; Santi *et al*. 2007). Bacterial IAA production is an important growth promoting trait as this auxin is involved in various plant developmental processes (Belimov *et al*. 2015; Spaepen *et al*. 2007).

The production of IAA by isolate CP5 could be the causation for the improved root elongation, lateral root formation, shoot growth and seed germination.

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However, it is important to note that more of this auxin released does not always result to growth promotion. Previous research has shown an inhibition of roots and shoots length of chickpeas seedlings when IAA was exogenously applied at a concentration of 1 uM compared to control, furthermore at 10um complete inhibition of the root system and inhibition shoot growth and lateral shoot development was observed (Malik *et al*. 2011). Another study showed similar results where canola seedlings root length was increased following inoculation with *Pseudomonas putida* which produces a low concentration of IAA, however when inoculated with high IAA producing mutant a 33% decrease of root growth was observed (Xie *et al*. 1996). The results obtained in the present research corresponds with previous studies conducted,

4.3.3 ACC deaminase activity

Reduction of high concentration of ethylene in plants by plant growth promoting endophytic bacteria through ACC deaminase activity is also an important trait to consider when screening for plant growth promoting candidate. Application of plant growth promoting microorganisms to increase the yield of agricultural important crops in regions which often experience stressed conditions is rapidly becoming an enticing biotechnological method as opposed to the use of pesticides or chemical fertilizers which can be detrimental to environment especially after long term usage (Saharan and Nehra. 2011). Inoculation of ACC deaminase producing bacteria can induce improved development of root system and in turn this can improve the shoot growth as well, furthermore inoculation of ACC deaminase producing bacteria can positively affect the development seedling of various crops (Belimov *et al*. 2002; Zahir *et al*. 2003; Glick *et al*. 2005).

In the present study isolate CP5 growth rate of the ACC supplemented media was very similar to the growth rate in the media containing ammonium sulphate, only until the 72 hour mark that the growth rate of isolate supplemented ACC started to decrease compared to ammonium sulphate (Figure 4.6). This assay shows the isolate CP5 was able to utilizing ACC as the sole nitrogen source, as growth was observed in the ACC supplemented media, thus confirming ACC deaminase activity. Canola plants which

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were inoculated with CP5 showed a significant increase of lateral roots, root length and shoot length compared to the uninoculated plants, the increased growth can be attributed to the ACC deaminase released by the isolate, The findings in this study are in agreement with other studies done demonstrating the improved growth of roots and shoots in plants treated with ACC deaminase producing bacteria (Mayak *et al*. 2004; Shaharoona *et al*. 2006). In addition the increase of roots and shoots of the inoculated plant can also be attributed to IAA released which forms a synergic interaction with ACC deaminase to stimulate root development (Glick. 2014; Noreen *et al*. 2012).

4.3.4 Siderophore production

Production of siderophores is a strategy or mechanism employed by bacteria to overcome the lack of usable iron present in the environment. Previous studies have shown that bacteria which are able to produce siderophores can greatly affect the uptake of iron by plants. Siderophore affinity for iron remain to be one of their most consistent feature, these low weight molecules can also have affinity for other heavy metals such as zinc and copper (Chincholkar *et al*. 2007; Dimkpa. 2009; Gururani *et al* 2012). Production of siderophores can be determined by a change of color on the CAS agar plate, the color change indicates the chelating of iron from the CAS media by bacterial siderophores produced (Schwyn and Neilands. 1987).

There are two distinct groups of siderophore, hydroxamate and catechol, each cause a different color reaction on the CAS plate. At neutral pH (pH of the CAS media), the formation of monohydroxamate and trihydroxamate complexes will develop a reddish orange and orange color respectively. Catechol complexes at neutral pH will develop a reddish purple color (Neiland. 1984; Payne. 1994). This can be observed from the *R. qingshengii* which was used as a positive control in the present experiment (Figure. 4.7), a clear development of orange halo formed around the colony, whereas with isolate CP5 no formation of colored halo was seen, which indicates the isolate does not produce siderophores. Furthermore literatures show that siderophores also have antagonistic effect capable of suppressing certain plant disease. This suppression of plant disease is based on siderophore mediated competition, this process reduces the

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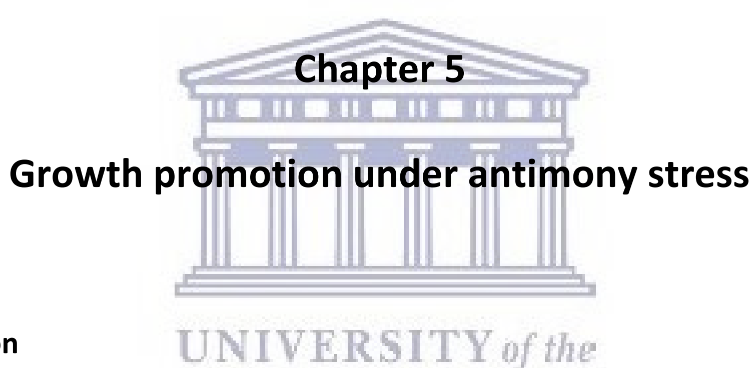
plant pathogens can occur in the rhizosphere or within plant inner tissue by directly competing for iron and this in turn will cause a decreased amount of iron available for pathogenic bacteria growth and spore germination (Gupta *et al*. 2001; Chandra *et al*. 2004). Therefore more research must be conducted in order to fully explore the capability of antagonistic effects of siderophores.

This study shows that the isolate CP5 has the ability to solubilize phosphates, synthesize IAA and ACCD. Multiple plant growth promoting mechanisms present in the isolate CP5 could explain the vast improvement of seed germination percentage, roots and shoots elongation and biomass of the plant in the growth trials.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Chapter 5

Growth promotion under antimony stress

5.1 Introduction

Antimony (Sb) is a metalloid found in the Group 15 of the periodic table, this metalloid has an atomic mass of 121.76u. Sb is one of the most versatile metalloids in the world and has been widely incorporated in the industrial production of flame retardants, glassware additive, ceramic, paint, ammunition and battery plants. Sb also has medical application particularly with treatment of leishmaniasis (Carlin. 2000; Filella *et al*. 2002). Currently China produces around 14 thousand tons of Sb annually, ranking China to be the largest producers of this metal, with South Africa ranked within the top 5 of the Sb producer (USGS. 2016).

The wide range of applications requiring antimony causes a high demand for Sb. This creates an increase in Sb mining and smelter plants which results in an elevated level of Sb to be released into environment contaminating soils and waters, especially at regions which are nearby Sb mines and smelting area (Scheinost *et al*, 2006; He. 2007; Wang *et*

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al. 2011). Antimony pollution can occur both naturally or due to anthropogenic activities, however studies suggests human activity is the the main factor influencing the environmental Sb level, as majority of the Sb released into the environment is a result of mining, Sb processing plants, fuel combustion and waste incineration (Shotyk *et al*. 2005; Qi *et al*. 2008; Wilson *et al*. 2010). Antimony and related compounds react in a similar fashion to arsenic and are hazardous to humans and may cause numerous health issues from headaches, vomiting to more severe conditions such as kidney and liver damage furthermore Sb is also known to be carcinogenic. The other factor to consider is that heavy metals like Sb cannot be biologically degraded and which mean the metal remains in the soil. Most heavy metals including Sb are water soluble which can be dissolved into water which renders physical separation impossible (Hussein *et al*. 2004), and once soluble this creates a method for translocation to other areas such as arable land.

The extent of Sb pollution can also affect agriculturally important plants often accompany by decrease of plant growth. In addition Sb is readily absorbed by plant root from contaminated soil which subsequently can affect the consumers (Ainsworth *et al*. 1991). Previous studies have shown the growth inhibitory effects of Sb toxicity when radish (*Raphanus sativus*) was exposed to Sb induced sludge (Fjallborg and Dave. 2004). This growth inhibition of root and shoot of rice (*Oryza sativa*) was also observed when exposed to antimony potassium tartrate and potassium antimonite (He and Yang. 1999).

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The decrease of crop production can impose a great threat to the food security of a country, this facilitates the increase of dependence on importing food sources in order to sustain food demands. There are currently applications in place to remedy the effects of heavy metal contamination in soil, however these methods often include soil replacement, physio-chemical based extraction and in situ decontamination. These methods are very expensive and only effective in small scale regions which are extensively polluted and require a complete clean-up. In addition to the ineffectiveness and high running costs, these methods often creates waste and render the soil infertile for plant growth (Glass. 1999; Pulford and Watson. 2003). Many reports shows that growth promoting bacteria can influence the uptake of heavy metals in plants, this suggests that bacteria can be developed into an effective method of removing heavy

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metals such as antimony in the soil through bioremediation (Glick. 2003; Chen *et al*. 2005; Abou-Shanab *et al*.2007). This leads to the utilization of plant growth promoting endophytic bacteria as a potential strategy not only to removing heavy metals from contaminated areas but also to enhance the overall growth and improve upon the tolerance of desired plants experiencing heavy metal stress. The objective of this research was to determine the growth rate of the endophytic isolate in the presence of antimony. Another objective includes the determination of inoculating PGPE on Brassica napus L grown in the presence of antimony.

5.2 Results

5.2.1 Effect of antimony on PGPE growth

The growth of the isolate culture supplemented with antimony was incubated until the growth reached plateau, in this case it took 9 hours. Figure 5.1 shows isolate CP5 which was grown in media supplemented with antimony had a significant decrease of growth compared to the control. Lowest OD was observed following incubation 30 mg/L of Sb. At 0.5 mg/L concentration the OD of the PGPE decreased substantially, lower than the control at the third hour mark, followed by a gradual decrease upon further incubation. Maximum OD of PGPE culture was also observed at 0.5 mg/L. *E*. *coli* KRX strain was subjected to the same Sb concentrations, however the KRX growth was severely affect by the presence of antimony. Figure 5.2 shows OD values for KRX were lower compared to the endophytic isolate when grown in the presences of Sb. Maximum OD for KRX was observed at 0.5mg/L Sb concentration with lowest OD recorded at 30 mg/L.

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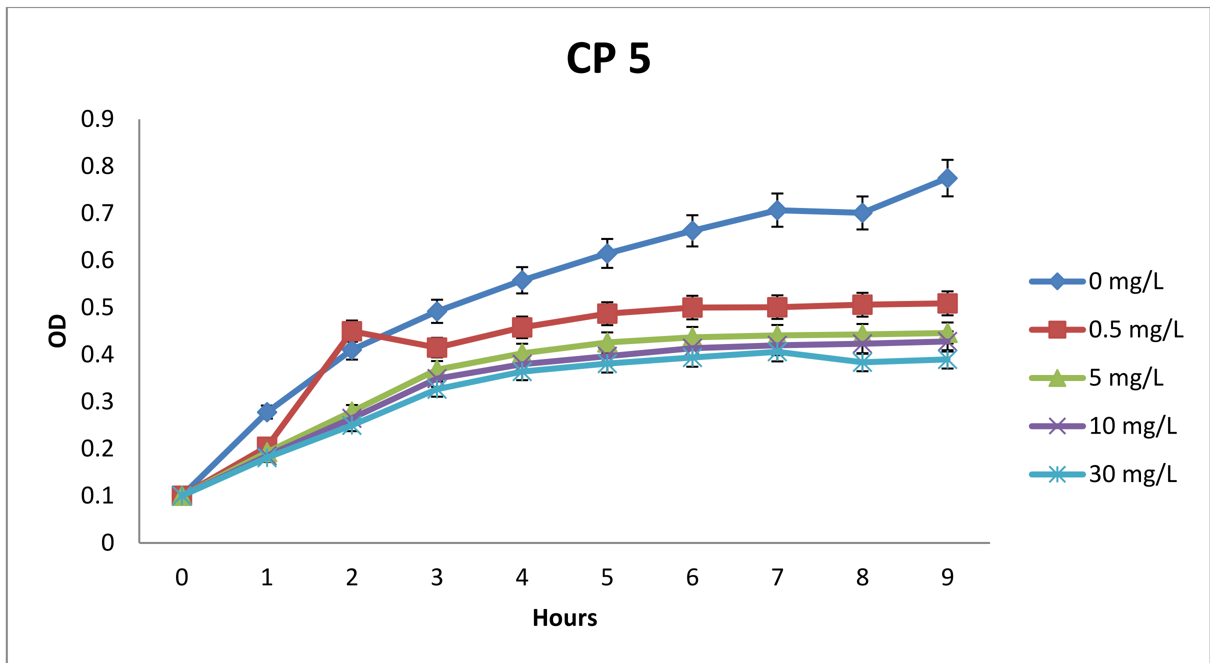

Figure 5.1 Antimony effect on endophytic isolate growth after 9 hours of incubation at 32^{0}C. Each value represents the mean of a triplicate study (±SE).

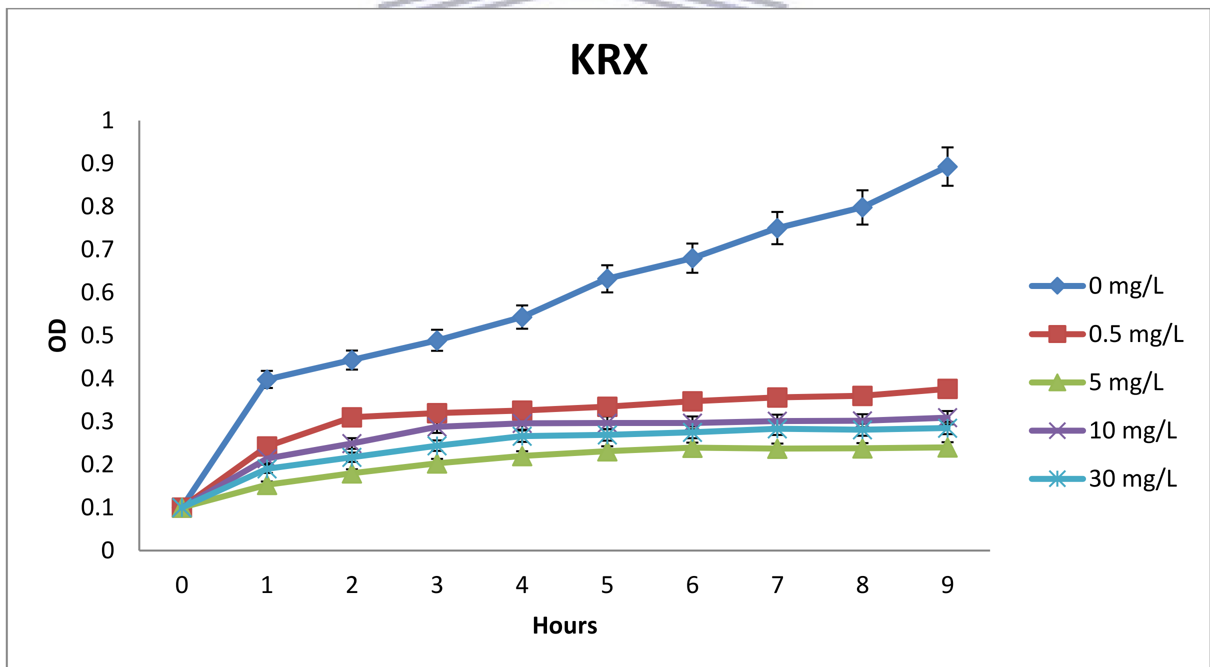

Figure 5.2 Antimony effect on KRX growth after 9 hours of incubation at 32^{0}C. Each value represents the mean of a triplicate study (±SE).

5.2.2 Effects of PGPE on the growth of Brassica napus treated with antimony

Brassica napus seedlings inoculated with and without PGPE were treated with 10 uM of potassium antimony tartrate solution for 4 weeks. Figure 5.3 shows that plants which

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were treated solely with Sb did not experienced significant growth inhibition in terms of shoots compare to the control and inoculated. However significant growth inhibition of roots was observed with plants treated with Sb compare to the control and inoculated. Plants which were inoculated with PGPE showed a maximum growth of the root system, PGPE was able to increase the biomass of roots even in the presence of antimony.

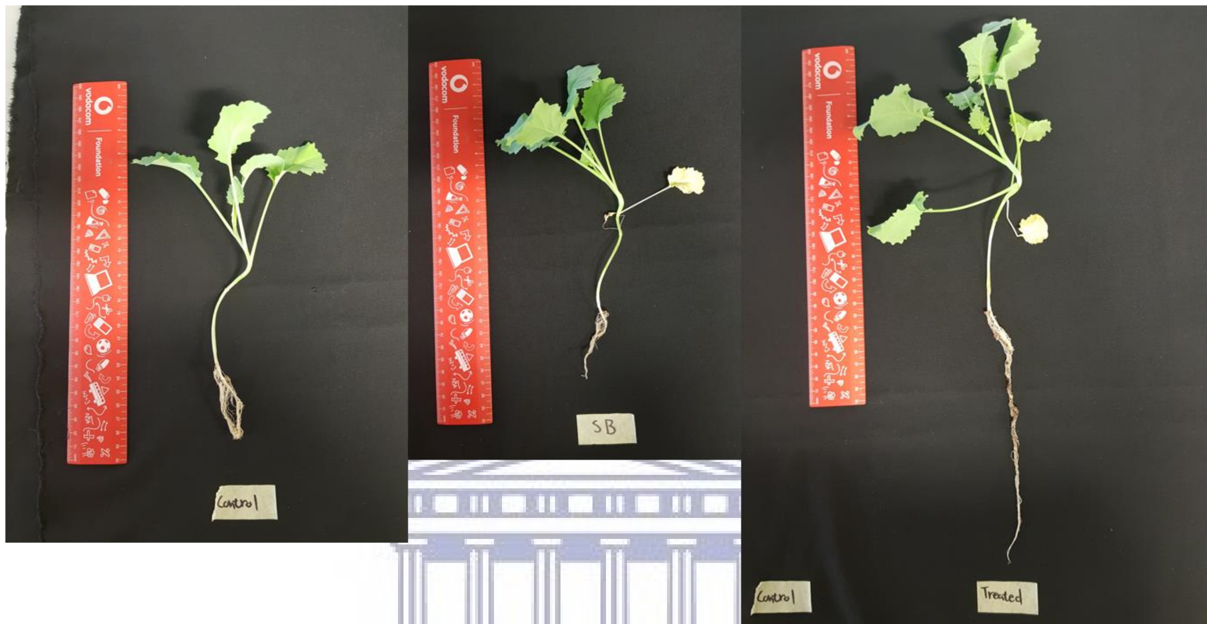

Figure 5.3 Effect of antimony on root and shoot growth of 4 weeks old Brassica napus L seedlings. Treated represent plant grown in Sb inoculated with isolate CP5. Sb represents plants grown in Sb without inoculation. Control plant was inoculated and was treated with only water.

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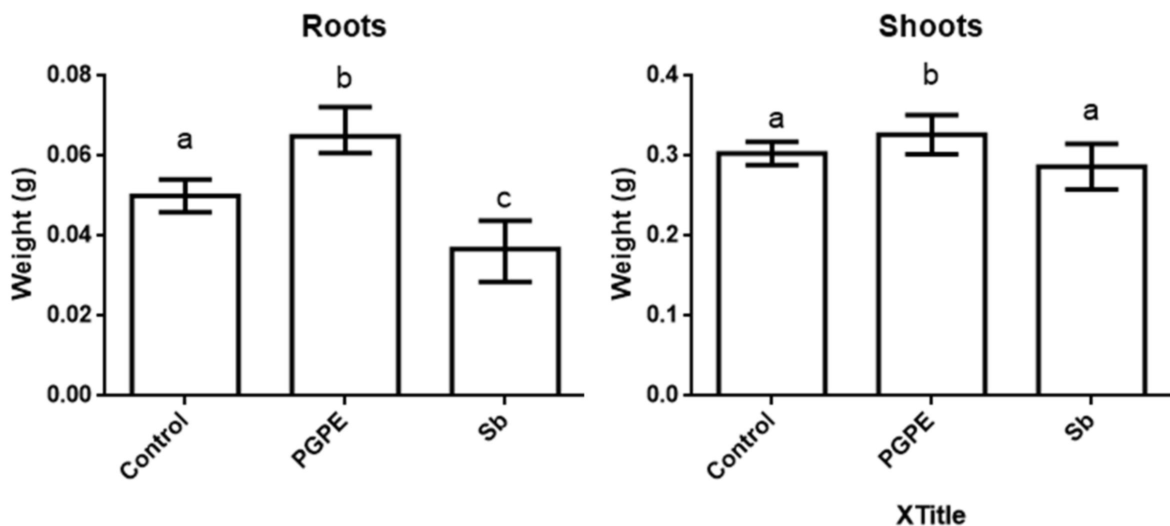

Figure 5.4 Effect of antimony on roots and shoots biomass. Mean values of each biomass with different letter are significantly different from each other ($P < 0.05$). Error bars represent (± SE) of the mean.

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ICP-OES was conducted to observe the effects of Sb on the macro element profile (Ca, K, P, Mg, Fe and Sb) of *Brassica napus* L. Nutrient accumulation within the shoots was mostly unaffected with K being the only element decreased. Inoculating the Sb affected plants with the PGPE bacteria showed an increase in accumulation of all macro nutrients and Sb within the roots and shoots. (**figure 5.4 and 5.5**).

Antimony seem to affect the Fe uptake the most in the plants, a significant decrease in Fe uptake in both roots and shoots was observed from the Sb treated plants compared to control. However, inoculation of PGPE onto Sb treated plants did show increase of iron in both roots and shoots compare to the uninoculated plants (**Figure 5.5**). Sb was present roots and shoots of the both inoculated and uninoculated treated plants (**Figure 5.6**). Sb accumulation was more prominent in the roots than the shoots. However inoculating plants with growth promoting endophytic seems to cause much more Sb to absorb in the root compare than the uninoculated plants. In addition small amount of Sb was also detected in the control plants which were not treated any Sb.

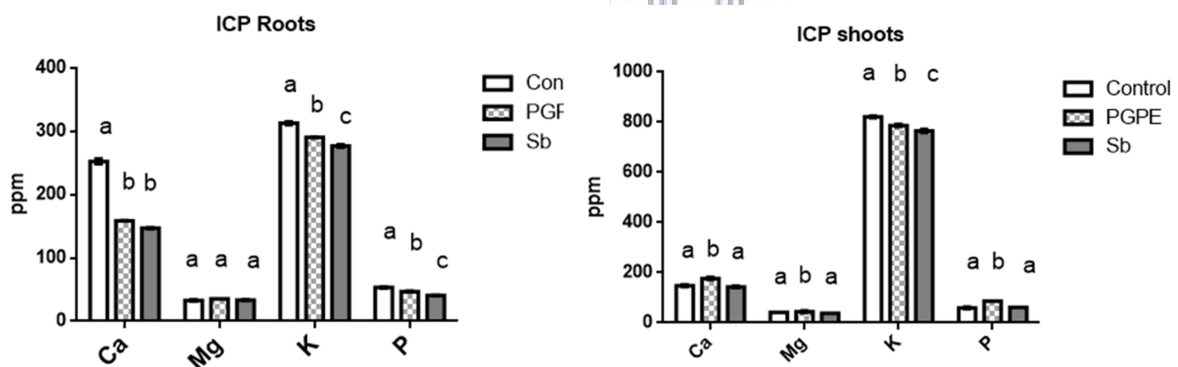

Figure 5.5 Influence of antimony on macro nutrient accumulation (part per million) within the roots and shoots of *Brassica Napus* L inoculated with and without PGPE. Mean values of each element with different letter are significantly different from each other (P< 0.05). Error bars represent (± SE) of the mean.

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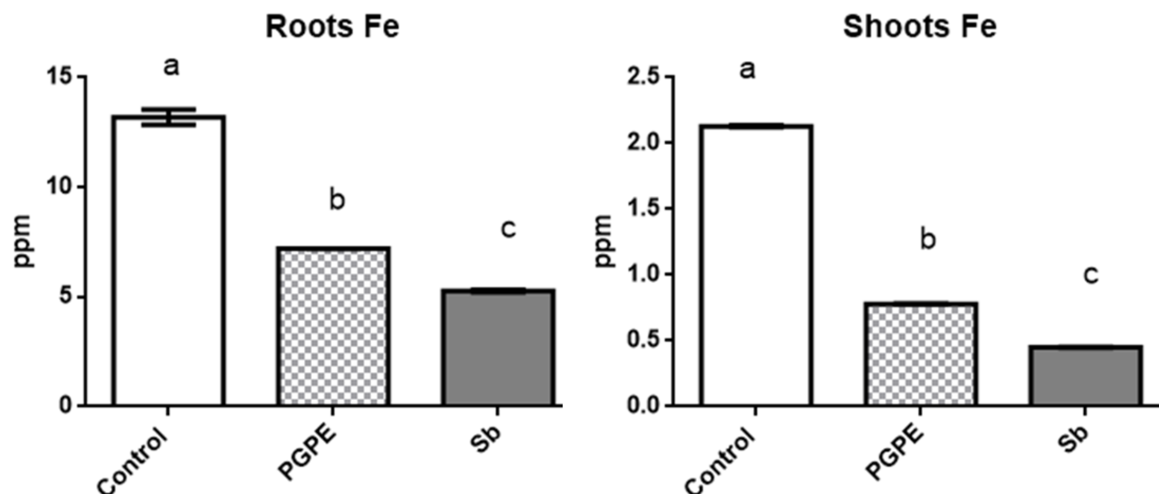

Figure 5.6 Influence of antimony on iron accumulation (part per million) within the roots and shoots of *Brassica Napus* L inoculated with and without PGPE. Mean values of each Fe with different letter are significantly different from each other (P< 0.05). Error bars represent (± SE) of the mean.

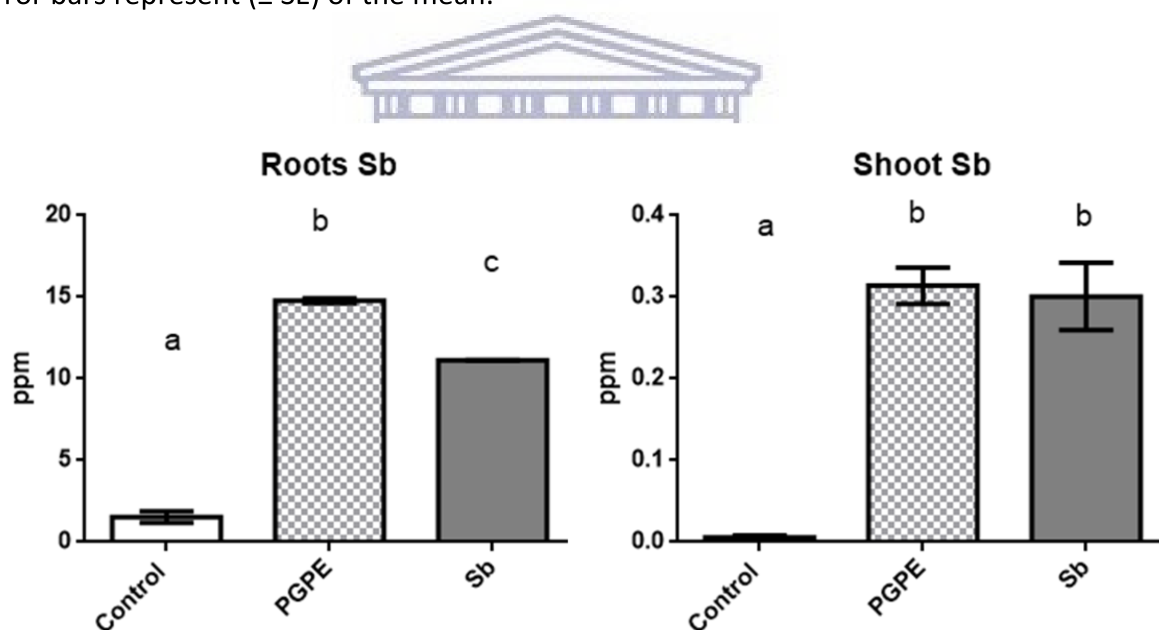

Figure 5.7 Antimony accumulation (part per million) within the roots and shoots of *Brassica Napus* L inoculated with and without PGPE. Mean values of each Sb with different letter are significantly different from each other (P< 0.05). Error bars represent (± SE) of the mean.

Evans Blue assay was conducted to show the extent of plant tissue damage from exposure to Sb. Roots and shoots which was treated solely with Sb showed the maximum absorbance at 0.23 OD and 0.28 OD respectively. Inoculation of endophytic

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bacteria seems to lower absorbance of both roots and shoots. Absorbance was much higher in the shoots than roots.

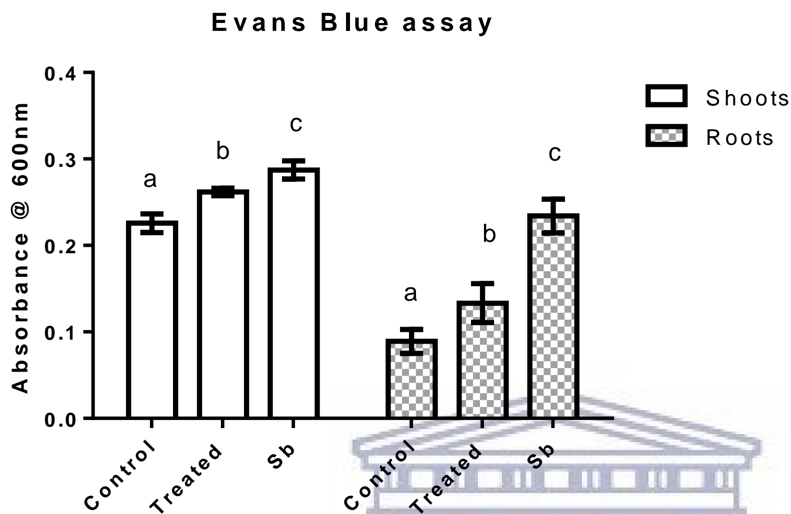

Figure 5.8 Antimony induced cell death on shoots and roots. Error bars represents (±SE) of the mean of 3 replicates. Means with different letters represents significant difference as per roots and shoots (P<0.05).

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5.3 Discussion

5.3.1 Effect of antimony on PGPE growth

Heavy metal contamination in the environment has since became more prominent in the recently years due to wide usage of these metals in the production of electronics, ammunition, batteries and paint. This is especially true in the case of antimony, as antimony is one of the most mined and used metal. Presently heavy metal toxicity poses a great threat to the environment. Many previous applications developed to remedy this problem are still inefficient and expensive. Due to the above mentioned short comings much emphasis has been placed on the interaction between growth promoting endophytic bacteria and its ability to alleviate and promote growth of the plants which experience heavy metal stress. To our knowledge not much research has being

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conducted to explore the potential of endophytic bacteria as a strategy to improve plant growth grown in regions which are contaminated with antimony in South Africa.

In order for PGPE to promote plant growth in high concentrations of heavy metals it would be highly advantageous for the PGPE to have a degree of tolerance toward the heavy metal first, this could in turn result in better colonization and proliferation once inoculated to plants. Present experiment was conducted to document the toxicity of Sb at different concentrations on the growth of PGPE isolate. Even though antimony did decrease the growth of endophytic bacteria at all concentrations, the tolerance toward antimony can be seen when compared to the growth of KRX in the presence of antimony. KRX is commonly used for protein expression and does not inherently have any heavy metal tolerance. In this study the maximum OD recorded for KRX was lower than the minimum OD recorded for the PGPE isolate (**Figure 5.2**), this shows that isolate CP5 was able to grow better at the highest Sb concentration than KRX growth at the lowest Sb concentration, thus confirming that endophytic isolate does have a degree of tolerance to antimony.

Studies suggest that PGPE tolerance to antimony can be accredited to the biosorption of Sb to the cell wall from viable and non viable cell, this is accomplished through metabolism independent pathways (Vijayaraghavan and Yun. 2008). Heavy metal ions are readily transported into cell surface through interaction between metals and functional groups present on the cell surface, these functional groups include hydroxyl, carbonyl, carboxyl, sulfhydryl, thioether, sulfonate, amine, amide and phosphonate (Ma *et al*. 2011). Other metal binding processes such as ion efflux, electrostatic exchange and microprecipitation can occur independently and interact to create a greater metal biosorption effect (Volesky and Schiewer. 1999). Tolerance to Sb can also be attributed to metabolism dependent pathways, Sb can be transported into viable bacterial cells and accumulate intracellularly within the cell membrane through cell metabolic cycle (Malik. 2004). Once the metal is inside the bacterial cells and depending on the bacteria, Sb can be precipitated, accumulated, sequestered within certain intracellular organelles or translocated to other specific structure like bacterial vacuoles (Ma *et al*. 2011).

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5.3.2 Plant growth promotion in antimony contaminated soil.

It is well documented that heavy metals can interfere with number of developmental processes of plants and microorganisms (Araujo and Monteiro. 2005; Atici *et al*. 2005) It is well documented that heavy metal toxicity causes the plants to experience oxidative stress from the direct release of reactive oxygen species and methylglyoxal which in turn upset the ionic homeostasis within the plant cells (Hossain *et al*. 2012; Sytar *et al*. 2013) which can lead to inhibition of functional enzymes and damage to cell structures (Jadia and Fulekar. 2009). Aside from oxidative stress heavy metals are known to cause the release of ethylene (Pennazio and Roggero. 1992). ACC leads to the formation of ethylene, high levels of hormone in high concentrations can adversely affect plant growth and inhibits root and shoot elongation, lateral root formation and root hair growth in heavy metal contaminated soils. Most of Antimony released into environment is mainly through anthropogenic sources and the extent of Sb pollution is not limited to industrial but also starting to affect urban and agricultural regions which are why it is important to understand. In addition the prevalence of antimony pollution can be seen even in South African soil, results from ICP showed that trace amounts Sb was present in plants with no Sb solution added. This indicates that the Sb was already present in the soil from other sources (**Figure 5.7**).

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The present study demonstrates the inhibitory effect of Sb toxicity, *Brassica napus* L seedlings grown in the presence of antimony all showed a degree of biomass reduction, with much of the biomass reduction occurring at the root system. Promotion or reduction of plant biomass can often reflect the growth of a plant. When plant growth is inhibited roots and shoots of the plant tend to shorter, thinner and have less lateral branching, this results in reduction of biomass. In contrast to when plants experience growth promotion, roots and shoots development of the plant is enhanced result in longer, more dense and more lateral branching of roots and shoots system, which ultimately leads to higher biomass of the plant.

In our study when the seedlings were inoculated with PGPE it seems to alleviate inhibitory effects of heavy metal toxicity mainly by improving the root biomass of *Brassica napus* L seedlings, as the shoots did not experience significant growth decrease

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(**Figure 5.3**). The tolerance toward Sb could be accredited ACC deaminase activity from the endophytic bacteria to hydrolyze ACC which in turn decrease the subsequent amount of Sb mediated ethylene, ACC deaminase as described in section 4.2 is an enzyme which can be produced by PGPE CP5 which cleaves ACC into α-ketobutyrate and ammonia which can be used as a nitrogen source. In our study seedling grown in Sb which was inoculated with endophytic isolate CP5 showed significant improvement of root growth over the uninoculated and control seedlings. The improvement of plant development can also be attributed to the release of auxin IAA by PGPE. Recently study suggests that IAA produced by PGPE might be pivotal to modulating the plant endophyte interaction and plant growth in the presence of heavy metal (Shin *et al*. 2012). IAA as describe in section 4.3 can stimulate primary root development when produced at low concentrations by bacteria, when produced in high concentration primary root development is inhibited, lateral and adventitious roots development is stimulated. This means that endophytic bacteria can help plants overcome heavy metal toxicity facilitate root development by modulating phytohormone homeostasis through ACC production (Patten and Glick. 2002). Other report has shown similar findings where IAA and ACC deaminase synthesized by endophytes was able to improve growth of S. nigrum plant under cadmium stress (Luo *et al*. 2011). Our findings in this research is in agreement with previous reports which showed ACC deaminase producing bacteria capable of enhancing plant growth by alleviate stresses created by heavy metal contamination (Zhang *et al*. 2011; Han *et al*. 2015).

Antimony toxicity can cause damage to plant cells. Results from Evan Blue assay revealed that the absorbance was the highest in both root and shoot of plants grown solely in the presence of Sb, this indicates that Sb caused the most damage to the plant cells (**Figure 5.8**). Antimony toxicity was most prominent in the roots, this can observed by the decrease of root growth. This decrease can be related to the high amount Sb accumulated particularly in the roots, which in turn results in root cell damage, therefore ultimately causing growth inhibition. Cell death occurred within shoots was higher compared to roots even thought accumulation of Sb was much higher in the shoots, this could be that the part of shoot which was extracted and subjected to the

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assay was much older compare to the roots, therefore the high amount of pre-existing dead cell was already present.

Isolate CP5 is also capable or solubilizing phosphates which can further attribute to improve plant root and shoot development through improving phosphate availability and changing availability of heavy metals (Wu *et al*. 2010; Gupta *et al*. 2014). Studies show that heavy metal tolerant endophytes are able to solubilize precipitated phosphates in heavy metal contaminated soil through acidification of soil, release of organic acids, chelation of phosphate ions and ion exchange or mineralization of organic phosphorus through the release of extracellular acid phosphatase, (Nautiyal *et al*. 2000; van der Hiejden *et al*. 2008). These phosphate solubilizing processes induce growth of plants which are experiencing heavy metal stresses by providing the plants with additional usable phosphates.

ICP-OES reveals the bioaccumulation of Sb and how Sb might affect the bioaccumulation of other macro elements (Ca, Mg, K, P and Fe) in the plant. Figure 5.4 shows that the uptake of Ca, K, P, Mg and Sb in the shoots was significantly higher compared to the roots, with Sb inhibited uptake of all macro nutrients. The inhibition of nutrients is more prominent in roots than the shoots, with only Mg remaining relatively unaffected in the roots. Figure 5.6 show that Sb concentration was much higher in the roots compare shoots, this could explain the major growth inhibition experienced by the roots, as higher concentration of Sb accumulates the toxicity exerted becomes more prominent in the roots resulting elevated oxidative and ethylene stress. Previous research indicates that root growth is more susceptible to heavy metal toxicity compare to shoot growth or seed germination (Araujo and Monteiro. 2005), this is in agreement with another study done on the effects of Pb, Cu and Ni on lettuce seeds (*Lactuca sativa*), the result shows that root inhibition was much prominent compared to shoot inhibition (Seneviratne *et al*. 2016). Present study supports this inhibitory trend.

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ICP also revealed that the uptake of Sb was greater in seedling roots inoculated with PGPE than the uninoculated (**Figure 5.7**). This could be attributed to the improved root formation from plant growth promoting activities of the endophyte, the improved root formation in turn can increase the uptake of Sb from the soil. Antimony did show some

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inference with the uptake of Ca, Mg, K and P mostly in roots of seedlings. The elevate uptake of P observed in both shoots and roots of the inoculated plant can be attributed the phosphate solubilization capabilities of the endophyte. Phosphate as described in section 3.2.4 is involved in many developmental processes such cell division and development of new tissue, phosphate also play a role in protein synthesis. Deficiency in phosphate often result in reduced growth, poorly developed root system, yellowing of the leaves and development of abnormal dark green or reddish-purple color. Beside Sb, nutrient availability can also be influenced by other of macro elements. In this study accumulation of K and Ca was much greater than Mg and P in both roots and shoots, according the Mulder's chart high concentrations K and Ca may also have antagonistic effect to other macro elements which ultimately the decrease the availability of P and Mg to the plant. Furthermore results from ICP shows that Sb significantly inhibits the uptake of iron, as concentration of Fe in Sb treated seedlings were significantly lower for roots and shoots. However currently there is no information available on the interaction between Sb and iron in plants. From this study we conclude that the PGPE CP5 does have a degree of heavy metal tolerance and is able to promote plant growth even under heavy metal stress. This makes PGPE an ideal solution to alleviate heavy metal toxicity in plant but also to enhance plant growth under stress.

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Identification of mechanisms of action which can be used in conjunction with other bacteria or fungi to facilitate a broader spectrum of resistance to plant against pathogens. Biotechnology can further improve on bacterial strains which contain target traits e.g stability and more suited for colonization by creating transgenic strain that can combine multiple mechanism of action.

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Conclusion and Future prospects

Plant growth promoting endophytic bacteria technology has the potential to become an integral part of agricultural practice. This microbial assisted plant growth promotion has only successfully implemented very few regions at a small scale. It is important to understand the plant and endophyte interaction, as the knowledge gained can allow for improvement in designing strategies which will increase efficiency of PGPE to promote growth and remediate heavy metal contaminated sites. The application of PGPB may decrease global dependence on chemical fertilizers. In future PGPB is expected to replace the majority of chemical fertilizer, pesticides and artificial growth regulators which have numerous environmental side effects to sustainable agriculture. Furthermore improvement of PGPE technology can in turn grant access to farmer in both developed and developing countries. In the current study results are divided into three sections.

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Section three seeks to extract and isolate endophytic bacteria from the *Echium plantagineum*. The extracted endophytic population are then subjected to plant growth trials using *Brasscia napus* L as the model plant in order to find the best plant growth promoting endophytic bacteria. The endophytic bacteria performance was evaluated based on index vigor, increase of biomass. Endophytic bacteria CP5 was selected as the best plant growth promoter as the isolate score the highest in index vigor and increase of biomass.

Section four seeks to explores the growth promoting mechanisms available to the isolate CP5. The plant growth promoting mechanisms tested were ACC deaminase activity, IAA production, phosphate solubilization and siderophore production. Each of these mechanisms is important but not always required to elicit plant growth. These mechanisms can be combined to work synergistically to facilitate plant growth promotion. CP5 was able to solubilize phosphate, produce ACC deaminase and produce IAA. However, endophytic bacteria CP5 did not synthesize any siderophores.

Section five shows the ability for of isolate CP5 to grow in the presence of Sb. Isolate was also subjected to growth trial similar to section 3, however in this growth trial the

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model plant was treated with Sb to demostrate the inhibitory effect of this heavy metal and how inoculating the plants experiencing Sb stress with CP5 may alliviate effect of Sb and at the same time to improve the plant growth.

Results from this thesis strongly suggest that isolate CP5 is a great potential candidate plant growth promoting and remediation applications. This isolate has multiple growth promoting mechanisms, tolerance toward heavy metal and promote growth of plants in heavy metal contamination regions.

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