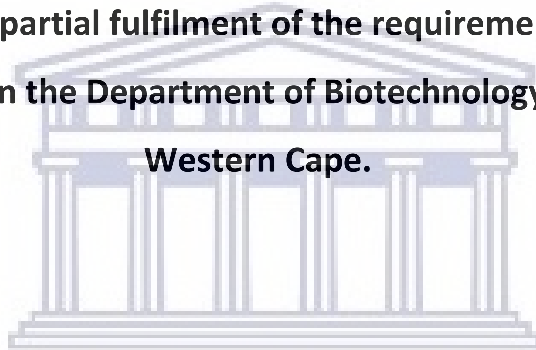
Isolation and characterisation of leaf endophytic bacteria from weed plants for enhancing salinity stress tolerance in *Brassica napus*

Tashreeq Ismail

A thesis submitted in partial fulfilment of the requirements for the degree of Magister Scientiae in the Department of Biotechnology, University of the Western Cape.



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KEYWORDS

1-aminocyclopropane-1-carboxylate (ACC) deaminase

Antioxidant enzymes

Brassica napus

Bi-lateral sequencing

Biomass

Cell death

Endophytes

Food security

Indole-3-acetic acid

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

Lipid peroxidation

Oxidative stress

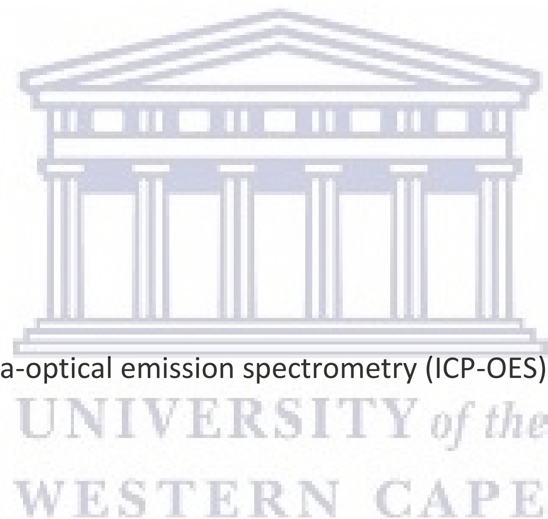
Phosphate solubilisation

Plant growth promoting bacteria (PGPB)

Reactive oxygen species (ROS)

Salt stress

Siderophore production



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ABSTRACT

Isolation and characterisation of leaf endophytic bacteria from weed plants for enhancing salinity stress tolerance in *Brassica napus*

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Masters Thesis, Department of Biotechnology, University of the Western Cape

In an ever changing environment, plants are constantly challenged by various abiotic stresses such as salinity, which limits global crop production. This directly affects food availability for the global population, which is projected to increase to 9.5 billion by 2050, which in turn places great pressure on natural resources and food security. These environmental adversities induce the accumulation of reactive oxygen species (ROS) hydrogen peroxide, hydroxyl and superoxide radicals which cause severe oxidative damage to plants. The equilibrium between the production and detoxification of ROS is then dependent on the modulation of enzymatic and non-enzymatic antioxidants to achieve plant homeostasis. Plant growth-promoting bacteria (endophytes) are an attractive biological approach that has been shown to possess several benefits to the host plant such as growth promotion, nutrient acquisition of environmental resources and plant-microbe interactions under diverse environmental conditions such as salinity. In this study, we investigated the effect of endophytic bacteria as a bio-inoculant to promote *Brassica napus* plant growth under saline and non-saline conditions by observational changes in plant physiology and biochemical responses of antioxidant enzymes ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD). Furthermore, we investigated the ability of these endophytes to aid a plant during nutrient acquisition of essential macro and micro elements required for plant growth and development via inductively coupled plasma optical emission spectrometry (ICP-OES). Moreover, we focused on characterisation based on plant growth promoting mechanisms indole-3-acetic acid, phosphate solubilisation, siderophore production and bi-lateral sequencing.

The results showed that plant growth promoting bacteria (PGPB) increased seedling emergence, plant growth and plant biomass compared to the control. Additionally, PGPB increased the acquisition of essential macro and micro elements compared to the control.

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The results showed that salt stress significantly decreased seedling emergence, plant growth, plant biomass and chlorophyll content. Additionally, salt stress increased lipid peroxidation, cell death and ROS accumulation. Furthermore, salt stress decreased macro and micro nutrient availability within the soil compared to the control. When salt stress plants were supplemented with plant growth promoting bacteria, the adverse effects observed in the salt treatment was reversed albeit not to the level of the control. Under saline conditions, PGPB increased seedling emergence, plant growth, plant biomass and chlorophyll content compared to salt-induced plants without PGPB. Additionally, under saline conditions, PGPB decreased ROS accumulation and increased antioxidant activity to counter the deleterious effects caused by salt stress. Furthermore, PGPB increased the acquisition of essential macro and micro elements compared to salt-induced plants without PGPB. The endophytes utilised in this study displayed the ability to produce indole-3-acetic acid and siderophore production. However, no endophyte displayed the ability to solubilise phosphate. Molecular identification methods showed that the endophytes utilised in this study are *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435.

Based on the results in this study we suggest that plant growth promoting bacteria (endophytes) could be utilized as a bio-inoculant to promote *Brassica napus* plant growth under saline and non-saline conditions. Furthermore, endophytes could aid a plant during nutrient acquisition under saline and non-saline conditions. Moreover, endophytes could induce plant-microbe interactions during environmental stress. This is evident by improved seedling emergence, plant growth and plant biomass combined with reduced ROS production and increased antioxidant activity. While a substantial amount of work has to be done in order to validate plant growth-promoting bacteria specifically endophytic bacteria, evidence suggests that endophytes could be utilised in agricultural practise.

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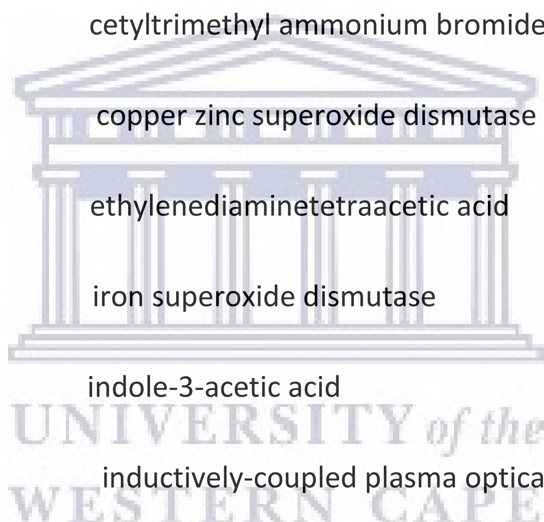
LIST OF ABBREVIATIONS

ACC	1-aminocyclopropane-1-carboxylate
ANOVA	analysis of variation
APX	ascorbate peroxidase
BLAST	basic local alignment search tool
CAT	catalase
CAS	chrome azurol s
CTAB	cetyltrimethyl ammonium bromide
Cu/Zn SOD	copper zinc superoxide dismutase
EDTA	ethylenediaminetetraacetic acid
Fe SOD	iron superoxide dismutase
IAA	indole-3-acetic acid
ICP-OES	inductively-coupled plasma optical emission spectrometry
KCN	potassium cyanide
KI	potassium iodine
LB	luria-bertani
MDA	malondialdehyde
Mn SOD	manganese superoxide dismutase
NBT	nitro blue tetrazolium chloride
NCBI	National Center for Biotechnology Information



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PGPB	plant growth-promoting bacteria
PvP	polyvinypyrrolidone
R_2A	reasoner's 2A
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
TBA	thiobarbituric acid
TCA	trichloroacetic acid
YEM	yeast mannitol

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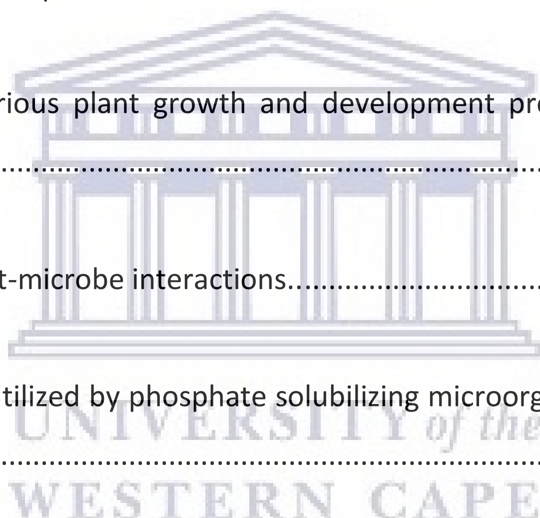
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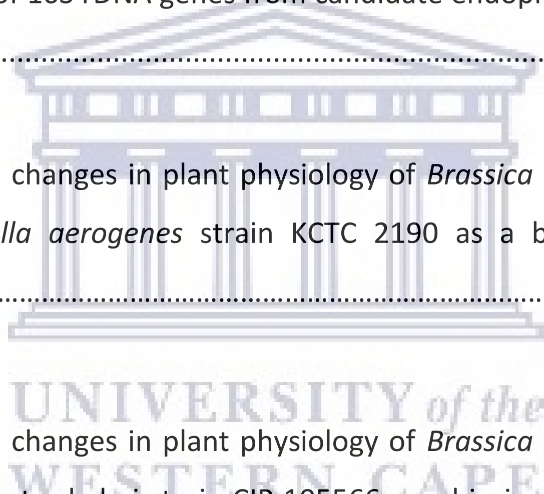
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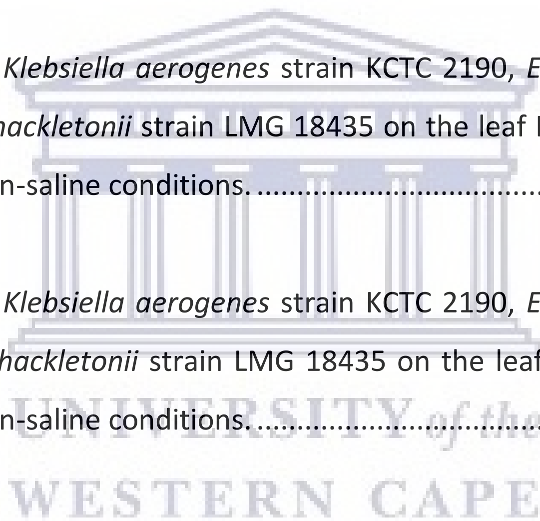
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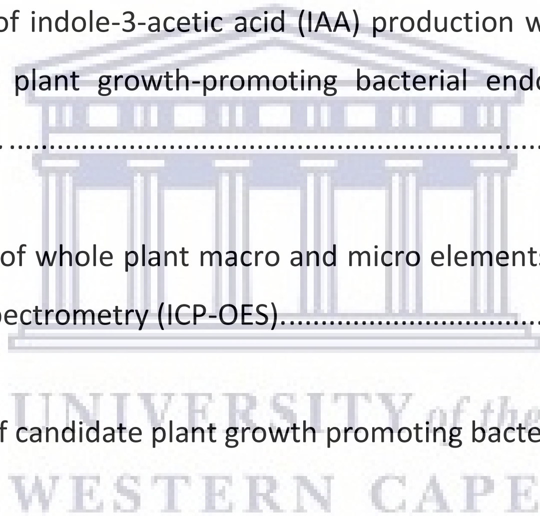

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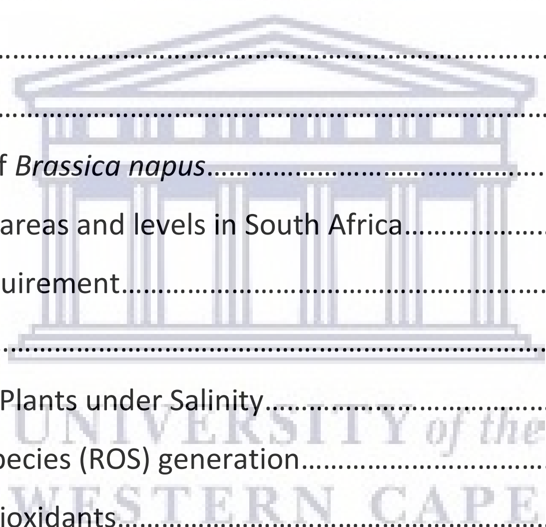

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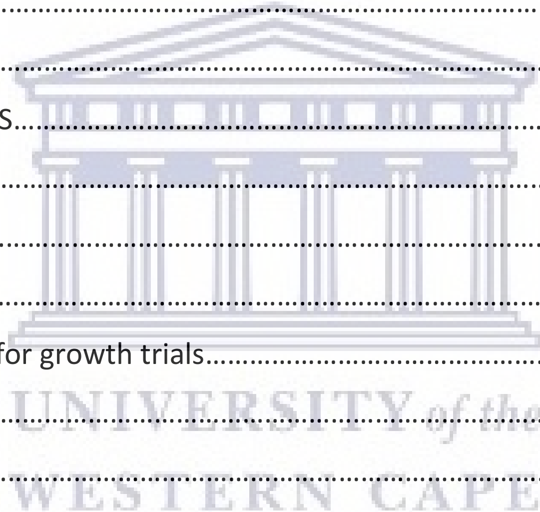

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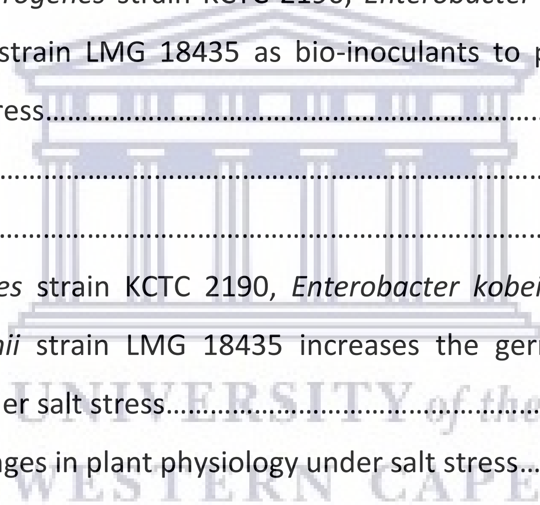
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CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Food security has recently received accumulative worldwide attention as the impact of population growth and climate change places new as well as additional stress on food systems (Stats SA, 2019). Recent studies have shown that approximately 821 million people in the world were affected by starvation in 2017 (FAO, IFAD, UNICEF, WFP and WHO, 2018). Additionally, the Global Hunger Index (GHI) indicated that 52 countries out of 119 countries had GHI scores that were rated as extremely alarming, alarming or serious in the same year (IFPRI, 2017). Essentially, more and more countries are acknowledging the need to attain food security within their domain (Stats SA, 2019). The right to food is embodied within Section 27(1) (b) of the South African Constitution which states that "everyone has the right to sufficient food and water" (Stats SA, 2019). Food security exists when people within a particular domain have economic, social and physical access to nutritional, safe and sufficient food at all times (Stats SA, 2019). Food security consists of four aspects namely; food stability, food utilisation, food accessibility and food availability (Stats SA, 2019). Therefore studies involving food security have become a fundamental aspect towards reducing food insecurity within South Africa.

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Food insecurity is a significant factor that still affects many South Africans even after fifteen years of democracy (du Toit, 2011). A study conducted by Stats SA, indicated that South Africa's population increased to 49 million people in 2009 with an annual population growth rate of 1.7% (du Toit, 2011). National food indicators revealed that South Africa had the ability to meet the needs of the growing population however; food insecurity still exists at a household level, especially within rural areas (du Toit, 2011). A study conducted by Demetre et al (2004) suggests that 14 million South Africans are facing food insecurity and 1.5 million children under the age of six suffers from stunted growth due to malnutrition (du Toit, 2011). This information has been supported by Machete et al (2004) that confirmed the majority of poor people affected by food insecurity are found within rural areas of South Africa. In 2004, the FOA released a report emphasizing the role that agriculture may play in addressing food insecurity within South Africa and the world (du Toit, 2011).

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The role that agriculture may play in achieving food security has not been fully addressed in literature. Thus, there is a need for further studies to determine the role that agriculture may play in achieving food security by investigating the impact of abiotic factors and population growth on agriculture. This review aims to provide a fundamental understanding of plant growth-promoting bacteria (i.e. endophytes) and the role they may play in agriculture.

1.2 Brassica napus

The Brassicaceae family (formerly known as Cruciferae) comprises of 375 genera and 3200 species of plants (Fang *et al*, 2017). The *Brassica* genus contains a 100 different species including *Brassica napus* or more commonly known as canola, rapeseed and oilseed rape (Fang *et al*, 2017). *Brassica napus* was first cultivated by ancient civilisations in the Mediterranean and Asia (Downey, 2016). During the 13th century canola oil was primarily used for lamps (Downey, 2016). *B. napus* was first commercially grown in Australia during 1969 (Downey, 2016).

B. napus was first deemed inedible for either animal or human consumption because canola seeds contain glucosinolates and erucic acid which is toxic to humans and other organisms (Downey, 2016). In the year 1970, high quality varieties of canola seeds containing significantly low levels of erucic acid and glucosinolates were achieved through very intensive breeding programs that occurred within several countries, including Australia (Downey, 2016). These varieties that meet the specific safety standard on the level of glucosinolates and erucic acid were termed canola (Downey, 2016).

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Figure 1.1: ***Brassica napus* (canola).** A canola plant has a stem that is between 75 cm – 175 cm in height, up to six large waxy blue-green leaves and small, yellow flower (Department of agriculture, forestry and fisheries, 2015).

1.2.1 Commercial uses of *Brassica napus*

Canola has been established as one of the world's most economically important plants and has become the third most important source of edible oil (El-Beltagi and Mohamed, 2010). During rapeseed oil production, a high protein animal feed is produced as a by-product that can be used during cattle feeding (Saeidnia and Gohari, 2012). The meal contains low levels of glucosinolates that is responsible for metabolism disruption in pigs and cattle (Saeidnia and Gohari, 2012). A study reported that a rapeseed oil diet contains 326 mg more plant sterols compared to an olive oil diet (Saeidnia and Gohari, 2012). The results showed that rapeseed oil decreased cholesterol absorption by 11% and increased excretion of sterols, bile acids and cholesterol by 51%, 32% and 9% respectively (Saeidnia and Gohari, 2012). Additionally, rapeseed oil can be used to produce margarine, biofuel, lubrication oil, soaps and plastics (Saeidnia and Gohari, 2012).

1.2.2 Canola production areas and levels in South Africa

Canola is said to be a relatively new crop in South Africa because it was introduced as early as 1994 (Department of agriculture, forestry and fisheries, 2015). Canola is mainly grown as a winter crop in the Western Cape and in smaller quantities in North West, Limpopo, KwaZulu-Natal, Eastern Cape, Free State and Northern Cape (Department of agriculture, forestry and fisheries, 2015). Although canola grain yields of 1516 kg ha^{-1} were considered as high under South African settings, an average yield of 2000 kg ha^{-1} and 3443

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kg ha^{-1} were recorded under USA and Canadian settings respectively (Cronjé, 2005). Each year for the past six years South Africa has imported more than 50% of its canola oil and 70% of its oilcake requirements from other countries (Department of agriculture, forestry and fisheries, 2015). This creates a big problem as the production of canola in South Africa is lower than the demand and favourable prices could be attained (Department of agriculture, forestry and fisheries, 2015).

1.2.3 Canola climatic requirement

Canola can be produced in cool weather environments with an optimal temperature of 21°C for growth and production (Department of agriculture, forestry and fisheries, 2015). Soil temperatures can range between 15°C - 20°C for optimal germination rates (Department of agriculture, forestry and fisheries, 2015). Even though 0°C is considered as the lowest growth temperature for canola, germination is significantly hindered by low temperatures (Department of agriculture, forestry and fisheries, 2015). Temperatures below 4˚C can cause damage during the flowering and seedling phase of canola (Department of agriculture, forestry and fisheries, 2015). Temperatures greater than 30°C can be harmful to the pollination of the flowers and will shorten the seed and pod growth phase to such an extent that both quality and yield are reduced (Department of agriculture, forestry and fisheries, 2015). The quality and quantity of canola seeds are always limited by the amount of water available to the crop, predominantly during seed maturation (Department of agriculture, forestry and fisheries, 2015). Canola will not produce high yields unless there is an adequate amount of sulphur, phosphorus and nitrogen available to the plant (Department of agriculture, forestry and fisheries, 2015).

1.3 Salinity

Soil salinity is one of the most dangerous abiotic factors affecting both ancient and modern civilisations (Rasool *et al*., 2013). Soil salinity limits the production of over 6% of the world's land and 20% of irrigated land which in turn negatively affects global crop production (Rasool *et al*., 2013). It is predicted that by the twenty-first century, 50% of agricultural land would be lost due to an increase in saline land (Hasanuzzaman *et al*., 2012). This directly affects the amount of food available for the global population, which is projected to increase to 9.5 billion by 2050 (Singh, 2015). Salt stress leads to a series of morphological,

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physiological, biochemical and molecular changes that adversely affect plant growth and development (Wang et al., 2001).

1.3.1 Oxidative Stress in Plants under Salinity

In plants, salinity reduces CO_2 availability and prevents carbon fixation, exposing chloroplasts to large amounts of excitation energy which increases ROS generation (Hasanuzzaman *et al*., 2012). Additionally, salinity reduces stomatal conductance to prevent water loss which reduces CO_2 concentration in the Calvin cycle (Hasanuzzaman *et al*., 2012). This reaction causes the reduction of oxidized NADP+ and increases O_2, resulting in the formation of the superoxide radical (O_2^-) (Hasanuzzaman *et al*., 2012). Furthermore, salt stress causes Na^+ ion and Cl^- ion toxicity to disrupt photosynthetic electric transport which increases O_2 (Hasanuzzaman *et al*., 2012). This reduces CO_2 in the Calvin cycle and induces photorespiration which leads to the formation of more hydrogen peroxide (Hasanuzzaman *et al*., 2012).

1.3.2 Reactive Oxygen Species (ROS) generation

ROS consist of a group of ions, reactive molecules and free radicals derived from oxygen (Kharusi *et al*., 2019). There are three reactive oxygen species namely; hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and superoxide radical (O_2^-) (Labrada *et al*., 2019). ROS plays two divergent roles within plants; at low concentrations ROS act as signalling molecules for the activation of defense mechanisms under stress whereas at high concentrations ROS cause significant cellular damage such as lipid peroxidation, protein oxidation, nucleic acid damage, enzyme inhibition and programmed cell death (PCD), which ultimately leads to cell death (Ahanger *et al*., 2017). Therefore the equilibrium between the production of ROS and the detoxification of ROS is tightly regulated by enzymatic and non-enzymatic mechanisms (Figure 1.2) (Caverzan *et al*., 2016).

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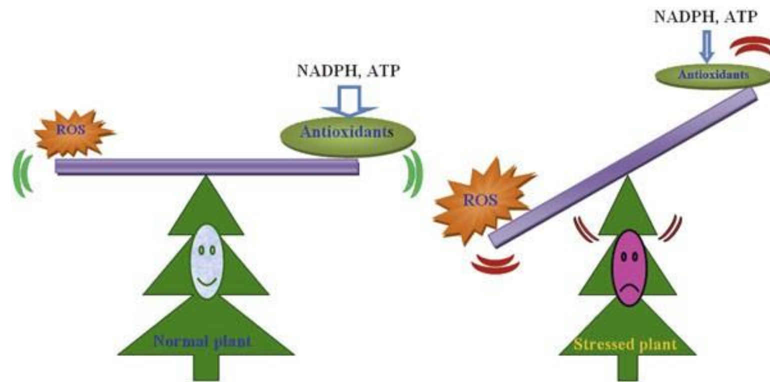

Figure 1.2: The equilibrium and imbalance between Reactive Oxygen Species (ROS) and antioxidants (Hasanuzzaman *et al*., 2012)**.** An equilibrium exist between ROS and antioxidant production during regular plant homeostasis, however this status is hampered during the overproduction of ROS molecules which leads to plant stress. Energy support plays an important role in the equilibrium between ROS and antioxidants.

1.3.3 Non-enzymatic antioxidants

Non-enzymatic antioxidants can be found in all cellular subunits, directly acting in the detoxification of Reactive Oxygen Species (ROS), radicals and reducing substrates for antioxidant enzymes (Varjovi *et al*., 2015). There are two major non-enzymatic antioxidants namely; ascorbate (AsA) and glutathione (GSH), among others (Figure 1.3) (Varjovi *et al*., 2015).

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1.3.3.1 Ascorbate

Ascorbate (AsA) or more commonly known as vitamin C, is an essential metabolite for plants (Racchi, 2013). AsA plays an important role as a cofactor for enzymes involved in antioxidant regeneration, phytohormone biosynthesis and photosynthesis (Ahanger *et al*., 2017). AsA protects plant metabolic processes against toxic radicals derived from oxygen such as H_2O_2, O_2^- and OH^- (Caverzan *et al*., 2016). During free radical scavenging, two AsA molecules are used by ascorbate peroxidase (APX) to reduce H_2O_2 into water via the ascorbate-glutathione cycle (Racchi, 2013). AsA can protect plant membranes either directly by scavenging toxic radicals or indirectly by zeaxanthin synthesis via the xanthophyll cycle (Ahanger *et al*., 2017).

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1.3.3.2 Glutathione

Glutathione (GSH) is a thiol containing tripeptide that can be found within various cell components such as the mitochondria, chloroplast and cytosol (Gondim *et al*., 2013). GSH has the ability to react non-enzymatically with H_2O_2, OH^- and O_2^- , therefore functioning as a direct free radical scavenger (Ahanger *et al*., 2017). Under salt stress, GSH directly protects a plant by regenerating water-soluble antioxidants like ascorbate via the AsA-GSH cycle and indirectly protects membranes by maintaining zeaxanthin and α-tocopherol in a reduced state (Hasanuzzaman *et al*., 2012). These functions results in the oxidation of GSH to form glutathione disulphide (GSSH) (Varjovi *et al*., 2015). The ratio between GSH and GSSH is controlled by the enzyme glutathione reductase (GR), which uses NAPDH to reduce GSSH resulting in two GSH (Gondim *et al*., 2013).

1.3.4 Enzymatic antioxidants

Enzymatic antioxidants play an important role in maintaining cell homeostasis when plants experiences stress (Varjovi *et al*., 2015). There are several enzymatic antioxidants namely; superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and ascorbate peroxidase (APX, EC 1.1.11.1) among others (Figure 1.3) (Varjovi *et al*., 2015).

1.3.4.1 Superoxide dismutase

Superoxide dismutase (SOD) is an enzyme that constitutes the frontline of defence against reactive oxygen species (ROS) as they possess the ability to reduce O_2^- into H_2O_2 and O_2 (Figure 1.4) (Rasool *et al*., 2013). SODs are classified according to their metal cofactor namely; FeSOD, MnSOD and Cu/ZnSOD (Hasanuzzaman *et al*., 2012). An increase in SOD activity reduces oxidative stress (Ahanger *et al*., 2017).

1.3.4.2 Catalase

Catalase (CAT) is a tetrameric heme-containing enzyme that is mainly responsible for scavenging H_2O_2 (Rasool et al., 2013). Catalase has the ability to reduce H_2O_2 into H_2O and O_2 (Figure 1.4) (Caverzan *et al*., 2016). One CAT molecule can reduce six million H_2O_2 molecules into H_2O and O_2 in one minute (Hasanuzzaman *et al*., 2012).

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1.3.4.3 Ascorbate peroxidase

The first step in the AsA-GSH cycle is the scavenging of H_2O_2 by the enzyme ascorbate peroxidase (APX) (Caverzan *et al.*, 2016). APX is a heme-containing enzyme that uses ascorbate as a substrate to reduce H_2O_2 into water while simultaneously producing monodehydroascorbate (MDHA) (Figure 1.4) (Racchi, 2013).

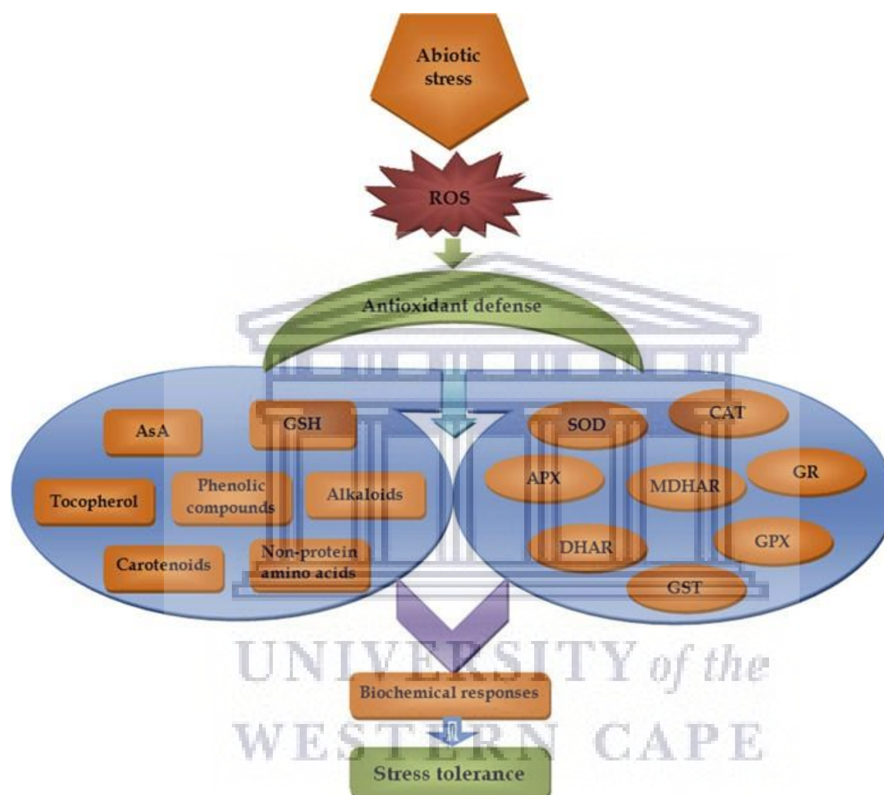

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Figure 1.3: Enzymatic and non-enzymatic mechanisms involved in the antioxidant system. (Hasanuzzaman *et al.*, 2012). Abiotic stress leads to significant ROS production levels that have adverse effects on plant growth and development. This phenomenon causes the activation of a plants natural antioxidant defense response which consists of enzymatic and non-enzymatic mechanisms. These mechanisms are responsible for biochemical response stimulus which provides stress alleviation or reduction, leading to plant stress tolerance.

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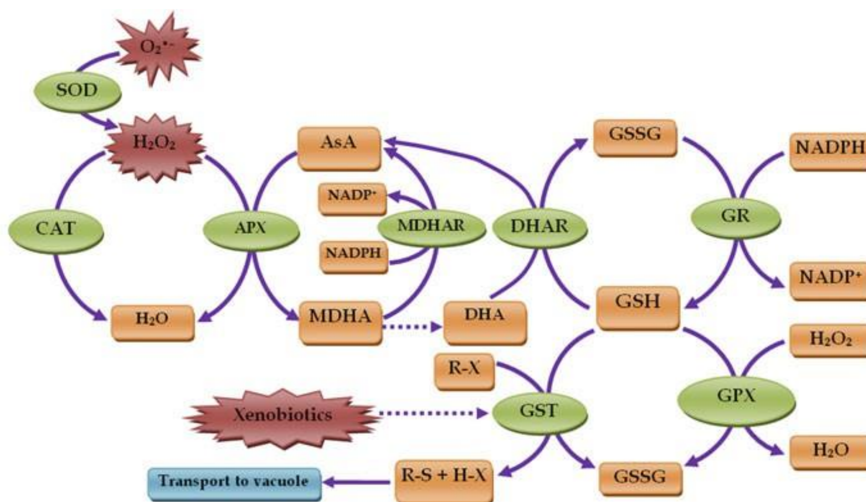

Figure 1.4: Reactive oxygen species (ROS) detoxification via various antioxidant enzymes. (Hasanuzzaman *et al.*, 2012). SOD reduces O_2^- into H_2O_2 and O_2, CAT reduces H_2O_2 into H_2O and O_2 and APX uses ascorbate as a substrate to reduce H_2O_2 into water.

1.4 Plant Growth-Promoting Bacteria (PGPB)

Soil contains a range of microorganisms including actinomycetes, algae, bacteria, fungi and protozoa, with bacteria being the most abundant (Ji *et al.*, 2013). Bacteria utilize a wide variety of substances for cell proliferation (Glick, 2014). Various soil conditions such as moisture, temperature and the presence of chemicals affect the type of bacteria found within soil (Basu *et al.*, 2017).

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Most bacteria are typically found within close proximity of plant roots because of the presence of various nutrients such as amino acids, organic acids and sugars (Glick, 2014). Bacteria utilize these nutrients and form interactions between plants which may either be beneficial, harmful or neutral (Figure 1) (Glick, 2005). These interactions may vary as the condition of soil changes (Glick, 2014).

Bacteria that positively influence plant growth and development are defined as plant growth-promoting bacteria (PGPB) (Kong and Glick, 2017). These bacteria facilitate plant growth mainly in suboptimal soil conditions (Kong and Glick, 2017). While a variety of soil bacteria are considered to be PGPB, not all bacterial strains of a specific genus and species are identical (Glick, 2005) Therefore, specific strains may promote plant growth whereas other strains may not have any physiological effect on a plant (Glick, 2014). *Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Burkholderia, Enterobacter, Klebsiella,*

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Pseudomonas and *Serratia* have been identified as plant growth-promoting bacteria (Ji *et al*., 2013).

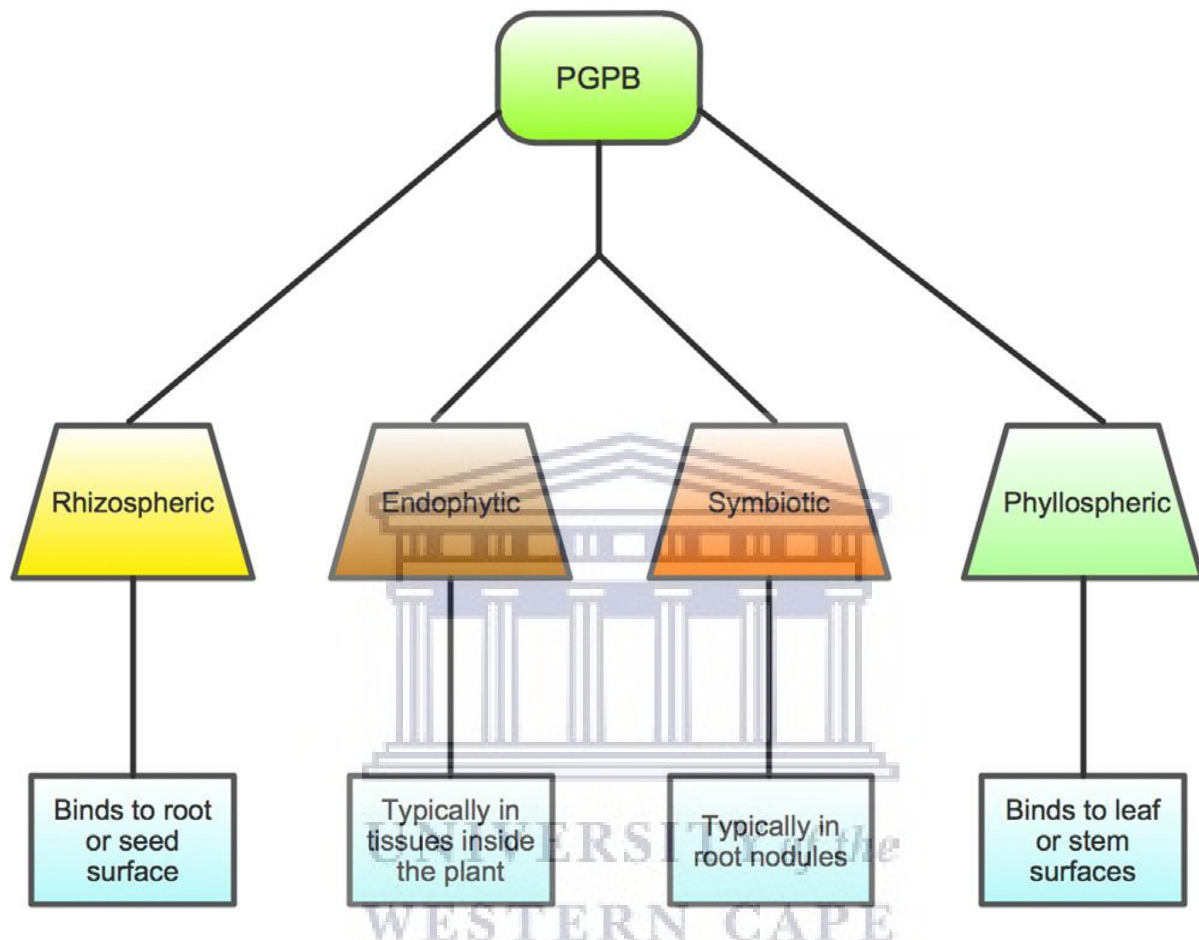

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Figure 1.5: Depicting various plant-microbe interactions. Plant growth-promoting bacteria (i.e. endophytes) reside within plant tissue (Glick, 2014).

1.4.1 Endophytes

Endophytes are defined as microorganisms that colonize intercellular and intracellular spaces of healthy plant tissue without causing any symptoms of disease (Miliute *et al*., 2015; Nair and Padmavathy, 2014; Yaish *et al*., 2015). As there are about 300 000 plant species that occur on earth with each plant, host to either one or more endophytes, the possibility to discover novel endophytes are considerable (Khan *et al*., 2016; Rosenblueth and Martínez-Romero 2006; Ryan *et al*., 2008).

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Endophytes have been isolated from every plant species that has been studied to date (Santoyo *et al*., 2016). It is stated that an endophytic-free plant is rarely found in nature and if such a plant exist, it would be less capable of coping with pathogens and more vulnerable to environmental stress (Santoyo *et al*., 2016). Endophytes have a distinct advantage compared to rhizobacteria because residing within plant tissue allows endophytes to always be in contact with plant cells (Rosenblueth and Martínez-Romero 2006; Santoyo *et al*., 2016). Endophytes colonise the roots through wounds and cracks caused by lateral root development or nematode and microbial activity (Compant *et al*., 2010; Gamalero *et al*., 2017). After colonisation within plant tissue, bacterial endophytes proliferate and invade xylem cells and vascular tissue (Gamalero *et al*., 2017). At this point, bacterial endophytes may spread towards the leaves and shoots of plants without causing harm (Compant *et al*., 2010; Gamalero *et al*., 2017). In this regard, endophytic bacteria colonize a biological niche that surpasses that of numerous phytopathogens, making them more effective as biocontrol intermediaries than rhizospheric bacteria (Compant *et al*., 2010). Within their capacity, endophytes are able to enhance plant growth, protect plants under adverse conditions and improve seedling emergence (Ryan *et al*., 2016).

Direct growth promotion occurs when endophytes aid the plant in the acquisition of environmental resources such as iron, phosphorus and nitrogen or modulate ethylene, cytokinin or auxin plant hormones (Glick, 2014). Indirect growth promotion occurs when endophytes prevent or limit plant damage caused by pathogens (Glick, 2014).

1.4.2 Direct growth promoting mechanisms

1.4.2.1 Indole-3-acetic acid (IAA)

Phytohormones are plant molecules that control numerous cellular processes such as fruit ripening, the formation of flowers and response to stress (Basu *et al*., 2017; Gamalero and Glick, 2011). They play a significant role in a plant's life cycle as they are responsible for cell differentiation (Basu *et al*., 2017). There are five classes of plant hormones that occur in nature namely abscisic acid, auxin, ethylene and gibberellin (Gamalero and Glick, 2011). Auxin is an important hormone required for plant growth and development (Tabatabaei *et al*., 2016). It affects the primary nutrition and development of plants by causing changes in the cell cycle which leads to xylem development and root elongation (Glick, 2012).

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Indole-3-acetic acid (IAA) is a natural plant hormone that belongs to the auxin class (Spaepen *et al*., 2007; Tabatabaei *et al*., 2016). It is primarily produced in seeds, stems and young leaves by decarboxylation and transamination tryptophan reactions (Sachdev *et al*., 2009). In plants, IAA controls physiological processes such as response to light and gravity, tissue differentiation, cell enlargement and division (Leveau and Lindow, 2004). Certain bacteria have the ability to contribute to the plant's auxin pool by producing IAA (Leveau and Lindow, 2004).

Acinetobacter, Azospirillum, Azotobacter, Bacillus and *Pseudomonas* are some of the various strains shown to produce IAA (Khan *et al*., 2016). Bacteria synthesize IAA through various pathways, with a single bacterial strain capable of utilizing one or more pathways (Gamalero and Glick, 2011; Khan *et al*., 2016). IAA is a metabolite derived from tryptophan-dependent and independent pathways such as indole-3-acetonitrile, indole-3-acetamide and indole-3-pyruvic acid (Mohite, 2013; Spaepen *et al*., 2007).

Microbial IAA producers may aid a plant by facilitating the production of longer roots, root hairs and lateral roots which increases the acquisition of nutrients (Basu *et al*., 2016; Mohite, 2013). They possess the ability to stimulate cell elongation through the modification of certain cell conditions such as increasing the osmotic content, water permeability and cell wall synthesis (Mohite, 2013). Hence, microbial IAA production plays a pivotal role in facilitating plant-bacteria interactions (Basu *et al*., 2017; Spaepen *et al*., 2007).

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1.4.2.2 1-Aminocyclopropane-1-Carboxylate (ACC) Deaminase

Ethylene is an important plant hormone that modulates plant growth and development and plays a vital role in response to plant stress (Kong and Glick, 2017; Nascimento *et al*., 2016). Ethylene affects various aspects of plant growth including the fruits, flowers, leaves, stems and roots of a plant (Figure 1.6) (Glick, 2014; Nascimento *et al*., 2016). Factors such as biological stress, plant hormones, nutrition, gravity, light and temperature can affect ethylene synthesis within a particular plant (Glick, 2014). One model proposed that stress ethylene is synthesized through two peaks (Glick, 2014; Nascimento *et al*., 2016).

The first ethylene peak utilizes 1-aminocyclopropane-1-carboxylate (ACC) which is the immediate precursor of ethylene in plant stress (Kong and Glick, 2017; Santoyo *et al*., 2016).

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ACC is responsible for initiating transcriptional genes encoding plant proteins capable of combating plant stress (Glick, 2014; Nascimento *et al*., 2016). The second ethylene peak occurs when a plant synthesizes additional ACC, initiating processes such as leaf abscission, chlorosis and senescence (Glick, 2014; Nascimento *et al*., 2016). Thus, any mechanism utilized by plants to lower ethylene levels should simultaneously decrease plant damage (Nascimento *et al*., 2016).

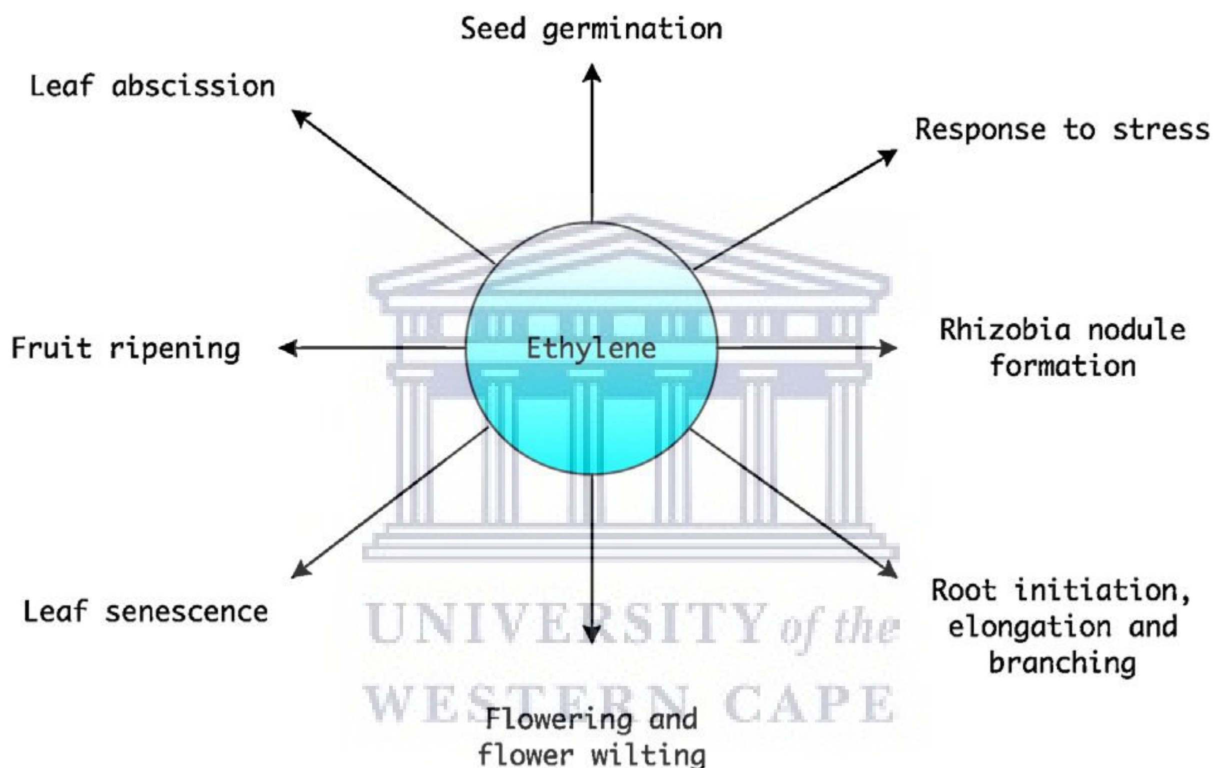

Figure 1.6: Depicting various plant growth and development processes affected by the plant hormone ethylene. Plant growth-promoting bacteria (PGPB) aid the plant by reducing high ethylene levels that cause chlorosis, leaf abscission and senescence (Glick, 2014).

Plant growth-promoting bacteria (PGPB) that possess the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase can promote plant growth by lowering ethylene levels (Khan *et al*., 2016; Kong *et al*., 2015). The enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase catalyzes the conversion of ACC into ketobutyrate and ammonia (figure 3, page 8) (Heydarian *et al*., 2016; Santoyo *et al*., 2016). ACC secreted from plant roots and seeds are metabolized by bacteria that possess the enzyme ACC deaminase (Khan *et al*., 2016). This stimulates plant ACC efflux, decreasing the concentration of ethylene and ACC and

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ultimately increasing root growth and development (Khan *et al*., 2016). PGPB that possess the enzyme ACC deaminase may be found on leaves, flowers or within internal tissue such as endophytes (Glick, 2014). Therefore, plants that grow in close association with PGPB that contains the enzyme ACC deaminase have longer shoots and roots and are more resistant to stress (Glick, 2014).

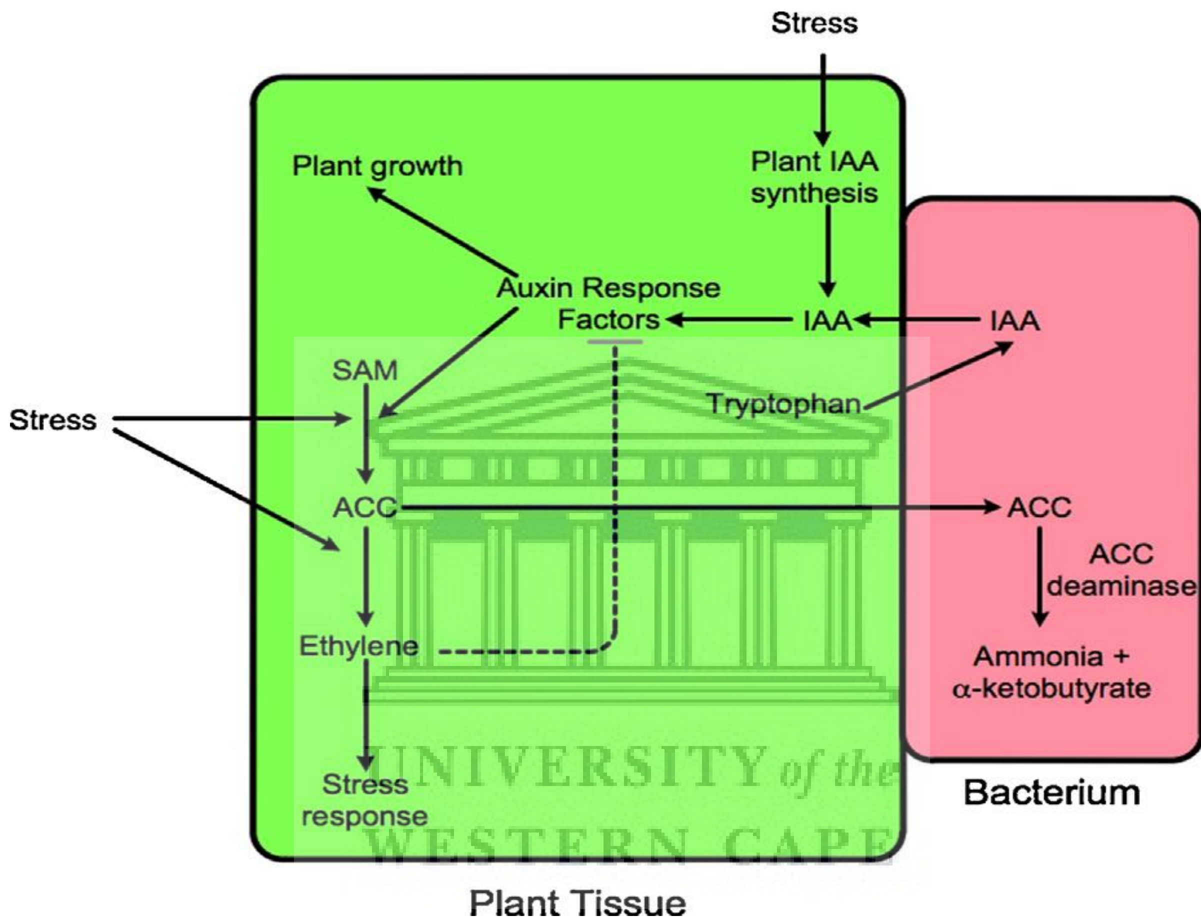

Figure 1.7: Depicting plant-microbe interactions. The enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase and the plant hormone indole-3-acetic acid (IAA) facilitate growth promotion by cleaving ACC into ammonia and ketobutyrate (Glick, 2014).

1.4.2.3 Phosphate solubilization

Phosphorus is an essential macro element required for plant growth and development (Brigido *et al*., 2017). It plays an important role in metabolic processes such as energy transfer, macromolecular biosynthesis, photosynthesis, respiration and signal transduction (Sharma *et al*., 2013). Phosphorus is abundant in soil and can be found in organic and

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inorganic forms, with the latter being more predominantly found (Gamalero and Glick, 2011).

Phosphorus deficiency hinders plant growth and development which ultimately decreases the quantity and quality of plants (Kong and Glick, 2011). Phosphorus deficiency is alleviated through the application of fertilizers; however this practice is expensive and reduces soil fertility (Khan *et al*., 2010). Due to the constant application of fertilizers having an adverse effect on crop sustainability and soil safety, agricultural communities are desperate to find alternative methods to achieve crop quantity and crop quality (Khan *et al*., 2010; Sharma *et al*., 2013). Therefore great emphasis is being placed on microorganisms capable of solubilizing phosphorus (Khan *et al*., 2010).

Microorganisms are important components of soil-plant interactions that affect plant growth and development (Glick, 2014). Fungi, bacteria and actinomycetes are some of the microorganisms that are involved in phosphate solubilisation (Khan *et al*., 2010). Plant growth-promoting bacteria (PGPB) are able to synthesize various organic compounds such as citric acid and gluconic acid that enable phosphate solubilisation (Basu *et al*., 2017). Another mechanism in which PGPB solubilizes phosphate occurs through the synthesis of phosphatases that hydrolyse phosphoric esters (Basu *et al*., 2017). Thus, insoluble phosphates are converted to a soluble form when PGPB secrete organic acids that lower soil pH (Figure 1.8) (Basu *et al*., 2017; Sharma *et al*., 2013). Therefore the use of endophytic inoculants that possess the ability to solubilize phosphate is considered as an environmentally friendly approach that could be utilized in crop productivity (Kong and Glick, 2011; Khan *et al*., 2010).

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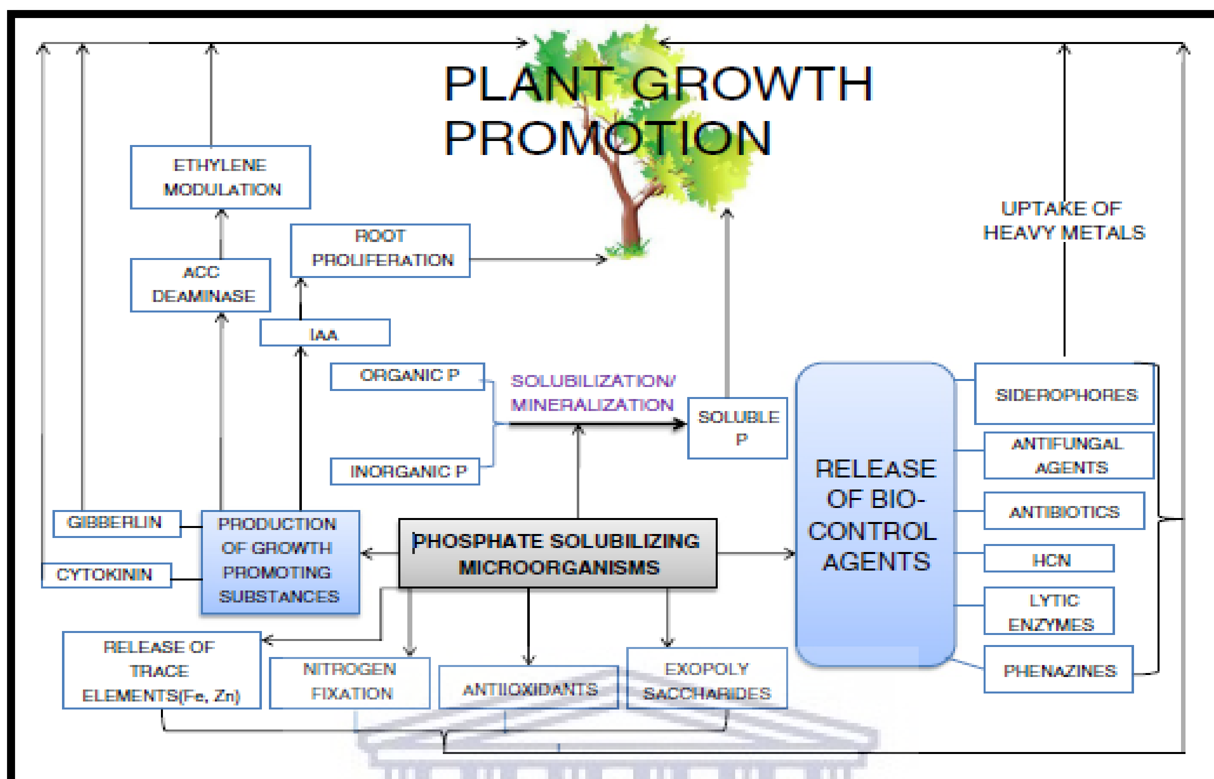

Figure 1.8: Mechanisms utilized by phosphate solubilizing microorganisms for plant growth promotion. Plant growth-promoting bacteria facilitate plant growth by converting insoluble phosphate into a bioavailable form that can be utilized by plants (Sharma *et al.,* 2013).

1.4.2.4 Siderophore production

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Iron is one of the most abundant elements found on earth and is an essential component of various biological processes such as chlorophyll biosynthesis, nitrogen fixation, photosynthesis and respiration (Basu *et al.*, 2017). Iron deficiency is caused by the low bioavailability of iron as it predominantly exists as insoluble ferric oxide (Fe(III)) in nature (Gamalero and Glick, 2011). Plant growth-promoting bacteria have the ability to convert Fe(III) into soluble ferric ion ($Fe2^{+}$) through the production of siderophores (Basu *et al.*, 2017; Gamalero and Glick, 2011).

Siderophores are low molecular weight molecules that have a high affinity for iron (Brigido *et al.*, 2017). They can be classified into three groups namely carboxylates, catecholates and hydroxamates (Gamalero and Glick, 2011). These classifications are based on three properties; ligand binding ability, functional group and structure (Basu *et al.*, 2017). Bacteria that secrete siderophores promote plant growth by reducing pathogens or facilitating the

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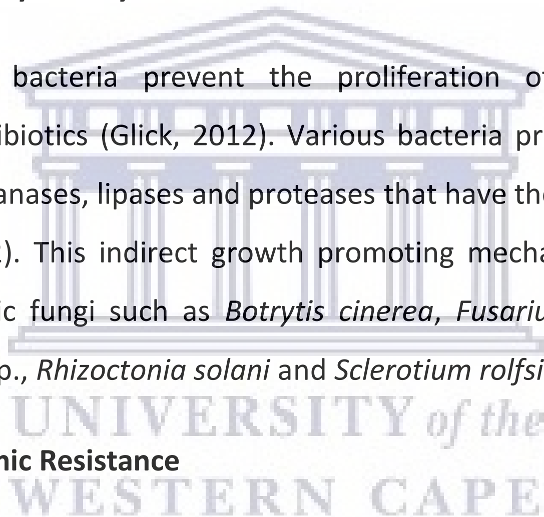
acquisition of iron (Basu *et al*., 2017; Gamalero and Glick, 2011). In addition, they are also able to eliminate microorganisms within their ecological niche by colonizing plant tissue (Basu *et al*., 2017).

Siderophores participate in bioremediation by binding to various metals such as arsenic, cadmium, chromium, copper, gallium, lead, magnesium, manganese, plutonium, radionuclides and zinc thereby alleviating the stress imposed on plants within heavy metal-contaminated soil (Gamalero and Glick, 2011). Therefore, siderophores play a significant role in plant growth and protection (Gamalero and Glick, 2011).

1.4.3 Indirect growth promoting mechanisms

1.4.3.1 Antibiotics and lytic enzymes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Plant growth-promoting bacteria prevent the proliferation of plant pathogens by synthesizing different antibiotics (Glick, 2012). Various bacteria produce enzymes such as cellulases, chitinases, glucanases, lipases and proteases that have the ability to lyse cell walls of pathogens (Glick, 2012). This indirect growth promoting mechanism has been utilized against various pathogenic fungi such as *Botrytis cinerea*, *Fusarium oxysporum, Pythium ultimum, Phytophthora* spp., *Rhizoctonia solani* and *Sclerotium rolfsii* (Glick, 2012).

1.4.3.2 Induced Systemic Resistance

Plant growth-promoting bacteria (PGPB) have the ability to induce a specific plant mechanism known as induced systemic resistance (ISR) (Glick, 2012). ISR occurs when a plant is exposed to pathogens (Glick, 2012). Plants that possess ISR have the ability to counteract the adverse effects of pathogens by inducing defensive mechanisms (Glick, 2012). Plant hormones such as ethylene and jasmonate play an important role in ISR as they are responsible for stimulating a plant's defence response to various pathogens (Glick, 2012). There are various ISR signals such as $\beta\beta$-glucans, chitin, cyclic lipopeptide surfactants, pyoverdineand salicylic acid (Glick, 2012).

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1.5 Justification

During the last decade, the global population has increased at an alarming rate and is predicted to reach 9.5 billion by the year 2050 (Basu *et al*., 2017). Global climate change is consequently going to affect agriculture because of the increase in surface temperature that will directly reduce soil moisture (Glick, 2012). Environmental stress such as limited nutrient availability and salinity, limits plant growth and productivity (Miliute *et al*., 2015). Subsequently, agricultural food production needs to significantly improve in order to feed the growing population (Glick, 2012). Current agricultural practice includes the use of chemical fertilizers; however, these agricultural methods have harmful effects on soil ecology and human health (Glick, 2012; Glick, 2014; Miliute *et al*., 2015). Thus, new environmentally friendly approaches have to be utilized in order to maintain sustainable agricultural production and to overcome threats that reduce the quality and quantity of crop productivity (Basu *et al*., 2017; Glick, 2014; Miliute *et al*., 2015). Therefore, the application of endophytic bacteria as a bio-inoculant could be utilized in agriculture to address the problem (Glick, 2012; Glick, 2014).

Aims and Objectives

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The aim of this study was to isolate candidate plant growth-promoting endophytes from weed leaves, utilize these candidate plant growth-promoting endophytes as a bio-inoculant to promote *Brassica napus* plant growth under salt stress and characterise these candidate plant growth-promoting endophytes. The association between plants and bacteria has been studied for many decades; however, the mechanisms utilized by plant growth-promoting bacteria (PGPB) are not well understood, making it difficult to reproducibly improve plant growth in an applied setting. Furthermore, to our knowledge, the use of plant growth-promoting bacteria (i.e. endophytes) as a biological approach to achieve *Brassica napus* plant growth promotion under saline conditions has not been well documented throughout literature. Therefore, this study aims to achieve *Brassica napus* plant growth promotion under axenic and saline conditions. The first objective of this study was to increase the germination rate of *Brassica napus*. Secondly, was to analyse observational changes in plant physiology such as root length, shoot length and plant biomass. Thirdly, was to characterise these bacteria based on their plant growth promoting mechanisms (IAA, Siderophore

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production, phosphate solubilisation) and identify these bacteria via bi-lateral sequencing. Fourthly, was to identify the overproduction of ROS (H_2O_2, O_2^- and OH^-) and analyse the antioxidant response (APX, SOD and CAT) to salt stress. Furthermore, we investigated cell death, lipid peroxidation and chlorophyll content. Lastly, we quantified micro and macro elements (Cu, Fe, Mn, Zn, Ca, K, P and Mg) via inductively coupled plasma optical emission spectrometry (ICP-OES). Therefore, this study aims to provide a fundamental understanding of Plant Growth Promoting Bacteria (PGPB) and the role they may play in agriculture.

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CHAPTER 2

MATERIALS AND METHODS

Table 2.1: List of chemicals/reagents and suppliers

Chemical/reagent	Supplier
Acetic acid	Sigma-Aldrich
Agar	Sigma-Aldrich
Ammonium chloride	Sigma-Aldrich
Ammonium sulphate	Sigma-Aldrich
Boric acid	Sigma-Aldrich
Calcium chloride	Sigma-Aldrich
Calcium phosphate	Sigma-Aldrich
Casamino acid	Sigma-Aldrich
Cetyltrimethyl ammonium bromide	Sigma-Aldrich
Copper (II) sulphate pentahydrate	Sigma-Aldrich
Chloroform	Sigma-Aldrich
Dextrose	Sigma-Aldrich

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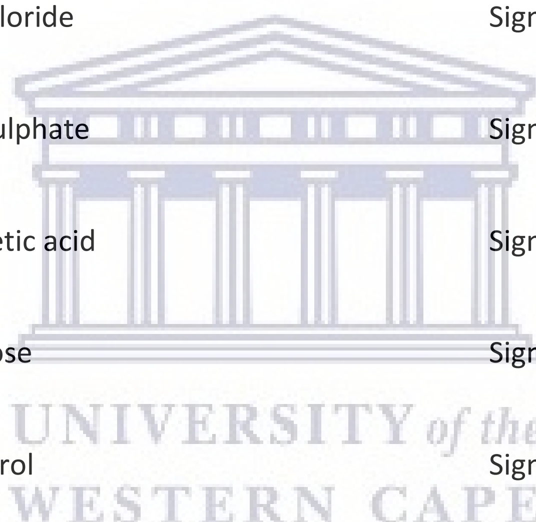
Di-potassium hydrogen phosphate	Sigma-Aldrich
Di-potassium phosphate	Sigma-Aldrich
2,2’,2”,2”’-(Ethane-1.2-diyldinitrilo)tetraacetic acid	Sigma-Aldrich
Ethanol	Sigma-Aldrich
Evans Blue	Sigma-Aldrich
Ferric chloride	Sigma-Aldrich
Ferrous sulphate	Sigma-Aldrich
Glacial acetic acid	Sigma-Aldrich
Glucose	Sigma-Aldrich
Glycerol	Sigma-Aldrich
Hexadecyl trimethyl ammonium	Sigma-Aldrich
Hydrochloric acid	Sigma-Aldrich
Hydrogen peroxide	Sigma-Aldrich
Iron (III) chloride hexahydrate	Sigma-Aldrich
Isoamyl alcohol	Sigma-Aldrich
Luria-Bertani (LB) broth	Sigma-Aldrich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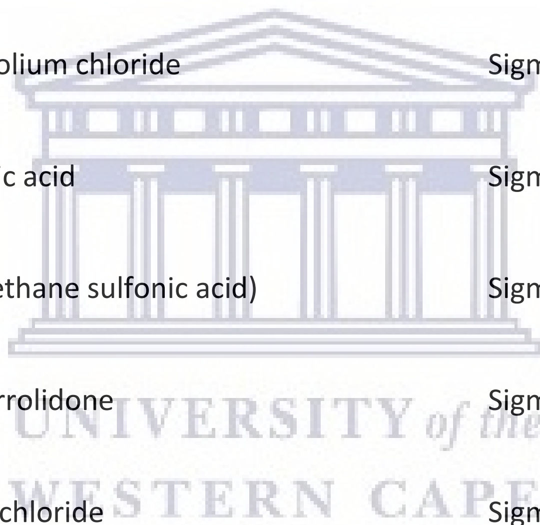
Magnesium sulphate	Sigma-Aldrich
Magnesium sulphate heptahydrate	Sigma-Aldrich
Manganese sulphate	Sigma-Aldrich
Mannitol	Sigma-Aldrich
β-Mercaptoethanol	Sigma-Aldrich
65% nitric acid	Sigma-Aldrich
Nitro blue tetrazolium chloride	Sigma-Aldrich
Perchloric acid	Sigma-Aldrich
Piperazine-N,N’-bis(2-ethane sulfonic acid)	Sigma-Aldrich
Polyvinylpyrrolidone	Sigma-Aldrich
Potassium chloride	Sigma-Aldrich
Potassium cyanide	Sigma-Aldrich
Potassium dihydrogen phosphate	Sigma-Aldrich
Potassium iodine	Sigma-Aldrich
Potassium phosphate	Sigma-Aldrich
Reasoner’s agar	Sigma-Aldrich



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Riboflavin	Sigma-Aldrich
Sodium chloride	Sigma-Aldrich
Sodium dodecyl sulfate	Bio-Rad
Sodium hypochlorite	Sigma-Aldrich
Sodium molybdate dihydrate	Sigma-Aldrich
Thiobarbituric acid	Sigma-Aldrich
Trichloroacetic acid	Sigma-Aldrich
Tryptophan	Sigma-Aldrich
Yeast extract	Sigma-Aldrich
Zinc sulphate heptahydrate	Sigma-Aldrich

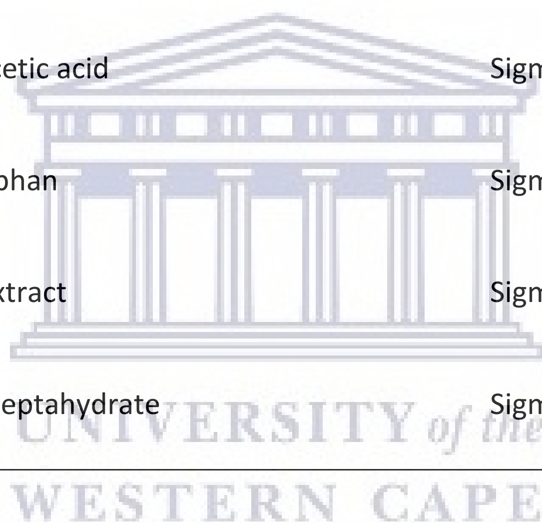
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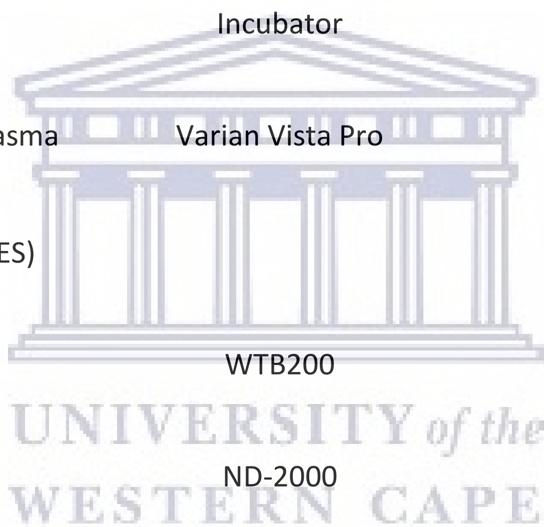
Table 2.2: List of equipment

Instrument	Model	Company
Centrifuge	5415D	Eppendorf
Enduro™ GDS Gel Documentation System	GDS-1302	Labnet International
Heating Block	ABHZ	FMH Instruments
Incubator	Quincy Lab Economy Digital Incubator	ProLab Scientific
Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES)	Varian Vista Pro	Varian
Mass Balance	WTB200	Radwag
NanoDrop™	ND-2000	ThermoFisher Scientific™
Polymerase Chain Reaction (PCR) Thermal Cycler	T100	Bio-Rad
Spectrophotometer	Ultraspec 2	Biochrom
Spectrophotometer Plate Reader	POLARstar	Omega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2.1 Sample collection

Weed plant samples were extracted from arid soil conditions (-33°56’6’’, 18°37’41’’) at the University of the Western Cape, Cape Town, Western Cape, South Africa. Each sample was washed with dH_2O to remove any excess soil before removing the leaves of each sample for subsequent analysis.

2.2 Surface sterilization

Surface sterilization was performed to remove any epiphytic bacteria by sequentially using 100% ethanol, 2% sodium hypochlorite, 70% ethanol and dH_2O. The final wash was spread plated onto Reasoner’s 2A (R_2A) agar and incubated at 30°C for 6 days. The absence of microbial growth following the 6 day incubation was an indication that surface sterilization was successful.

2.3 Endophytic extraction

Leaf samples were crushed in a 0.9% NaCl solution using a sterile mortar and pestle. Thereafter, leaf samples were incubated at room temperature for 3 hours. Following incubation, a 10-fold serial dilution (10^{-2}-10^{-6}) was performed and each dilution was spread plated onto Reasoner’s 2A (R_2A) agar which was incubated at 30°C for 6 days. Colonies were subcultured onto R_2A agar and incubated at 30°C for 4 days. Single colonies were inoculated into Luria-Bertani (LB) broth and incubated at 37°C in an orbital shaker until an optical density (OD_{600nm}) of 0.5. Each isolate was stored in 20% glycerol for subsequent analysis.

2.4. Inoculum preparation for growth trials

Each isolate was inoculated into Luria-Bertani (LB) broth and incubated at 37°C in an orbital shaker at 225 rpm for 48 hours. This methodology was also followed for the inoculum preparation for growth trials under saline conditions.

2.4.1 Seed preparation

Brassica napus L (AV-Garnet) seeds were surface sterilized using 100% ethanol, 0.35% sodium hypochlorite, 70% ethanol and dH_2O. The final wash was spread plated onto Reasoner’s 2A (R_2A) agar and incubated at 30°C for 6 days. The absence of microbial growth

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following the 6 day incubation was an indication that surface sterilization was successful. The surface sterilized seeds were treated with each bacterial inoculum and incubated at 37°C in an orbital shaker at 225 rpm for 60 minutes. Control *Brassica napus* L (AV-Garnet) seeds were treated with sterile Luria-Bertani (LB) broth. This methodology was also followed for the inoculum preparation for growth trials under saline conditions.

2.5 Growth trials

Growth trials were conducted to determine the effect of each bacterial inoculum on *Brassica napus* L (AV-Garnet) by observational changes in plant physiology. Growth trials were conducted in a greenhouse set at 21°C with natural lighting. *Brassica napus* L (AV-Garnet) seeds were planted in cups containing a sterile 1:2 soil to silica sand ratio. Each cup contained three *Brassica napus* L (AV-Garnet) seeds treated with 2 ml of each inoculum which was replicated in triplicate. Each plant was treated with 100 ml of water every Tuesday and Friday for 42 days. Root and leaf dry weight was measured by allowing the respective plant material to dry out in an oven set at 80°C for 48 hours. Supplementary plant material was harvested using liquid nitrogen for the quantification of micro and macro elements via inductively coupled plasma optical emission spectrometry (ICP-OES) analysis.

2.6 Indole-3-acetic acid (IAA) production

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Identification of indole-3-acetic acid (IAA) producing micro-organisms was conducted using a colorimetric estimation of IAA by (Gordon and Weber, 1951). Each isolate was inoculated into Yeast Mannitol (YEM) broth (1 g yeast extract, 10 g mannitol, 0.5 g dipotassium phosphate, 0.2 g magnesium sulphate, 0.1 g sodium chloride, pH 7.0) supplemented with and without 0.1% tryptophan which was incubated at 37°C in an orbital shaker at 225 rpm for 96 hours. A 2 ml inoculum volume was centrifuged at 13, 200 x *g* for 10 minutes and 1ml of supernatant was mixed with 2 ml of Salkowski reagent (0.5 M ferric chloride and 35% perchloric acid). The mixture was incubated in the dark at room temperature for 30 minutes before the optical density was spectrophotometrically measured at 530 nm. IAA concentration was determined from a standard curve with a known IAA concentration ranging from 0 µg/ml – 1000 µg/ml. The *Escherichia coli* strain KRX was used as a control.

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2.7 Phosphate solubilisation

Identification of phosphate solubilizing micro-organisms was conducted using Pikovskayas agar by (Pikovskayas, 1948). Pikovskayas agar was prepared with 0.500 g yeast extract, 10.000 g dextrose, 5.000 g calcium phosphate, 0.500 g ammonium sulphate, 0.200 g potassium chloride, 0.100 g magnesium sulphate, 0.0001 g manganese sulphate, 0.0001 g ferrous sulphate and 15.000 g agar. Isolates were spot inoculated onto Pikovskayas agar and incubated at 30°C for 5 days. The *Escherichia coli* strain KRX was used as a control.

2.8 Siderophore production

Identification of siderophore producing micro-organisms was conducted using Chrome Azurol S (CAS) agar by (Alexander and Zuberer, 1991). All glassware was washed with 3 M hydrochloric acid. CAS agar was prepared from four solutions which were sterilized separately before mixing. Solution one was prepared by mixing 10 ml of 1 mM iron(III) chloride hexahydrate with a 50 ml CAS solution. The mixture was added to a 40 ml hexadecyltrimethylammonium aqueous solution. Solution one was sterilized at 121°C for 20 minutes and cooled to 50°C. Solution two was prepared by dissolving 30.24 g of piperazine-N,N'-bis(2-ethanesulfonic acid) into 800 ml of a salt solution (0.3 g potassium dihydrogen phosphate, 0.5 g sodium chloride and 1 g ammonium chloride, pH 6.8). Solution two was sterilized at 121°C for 20 minutes and cooled to 50°C after adding 15 g of agar. Solution 3 was prepared by dissolving 2 g glucose, 2 g mannitol, 493 mg magnesium sulfate heptahydrate, 11 mg calcium chloride, 1.17 mg manganese(II) sulfate monohydrate, 1.4 mg boric acid, 0.04 mg copper(II) sulfate pentahydrate, 1.2 mg zinc sulphate heptahydrate and 1 mg sodium molybdate dehydrate into 70 ml dH_2O. Solution three was sterilized at 121°C for 20 minutes and cooled to 50°C. Solution four was prepared by filter sterilizing 30ml of 10% (w/v) casamino acid. Solution two, three and four were mixed before solution one was added. Isolates were spot inoculated onto Chrome Azurol S (CAS) agar and incubated at 30°C for 5 days. The *Escherichia coli* strain KRX was used as a control.

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2.9 Quantification of micro and macro elements via inductively coupled plasma optical emission spectrometry (ICP-OES)

A 200 mg plant sample was digested with 1 ml of 65% nitric acid at 90°C for 3 hours on a heating block. Following digestion, a filter sterilized 10-fold dilution was performed using 2% nitric acid. Micro and macro elements, Cu, Fe, Mn, Zn, Ca, K, P and Mg were subsequently analysed thereafter. This methodology was also followed for the quantification of micro and macro elements via inductively coupled plasma optical emission spectrometry (ICP-OES) under saline conditions.

2.10 Inoculum preparation for genomic DNA extraction

Each isolate was inoculated into Luria-Bertani (LB) broth and incubated at 37°C in an orbital shaker at 225 rpm for 48 hours.

2.10.1 Genomic DNA extraction

The Cetyltrimethyl Ammonium Bromide (CTAB) method was used to extract genomic DNA. A 2 ml inoculum volume was centrifuged at 13, 200 x *g* for 5 minutes in order to pellet the DNA. Following centrifugation, the pellet was re-suspended in 300 µl TE buffer and 50 µl 10% sodium dodecyl sulfate was added before incubation in a 65°C hot water bath for 15 minutes. Thereafter, 500 µl 1.5X cetyltrimethyl ammonium bromide and 5 µl 2-mercaptoethanol was added before incubation in a 65°C hot water bath for 60 minutes. An equal volume of chloroform:isoamyl alcohol (24:1) was added before centrifugation at 13, 200 x *g* for 15 minutes. The aqueous phase was transferred to a sterile eppendorf tube containing 50 µl 10% cetyltrimethyl ammonium bromide and an equal volume of chloroform:isoamyl alcohol (24:1) which was subsequently centrifuged at 13, 200 x *g* for 15 minutes. The aqueous phase was transferred to a sterile eppendorf tube before DNA precipitation. DNA was precipitated with a 0.6 volume of ice cold absolute ethanol for 60 minutes before centrifugation at 13, 200 x *g* for 2 minutes. DNA was purified twice with 70% ice cold ethanol before re-suspension in 10 µl TE buffer for long term storage.

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2.11 Nucleic acid analysis

Nucleic acid analysis was performed using the NanoDrop 2000 Spectrophotometer (Thermo Scientific) at absorbance peaks of 280 nm, 260 nm and 230 nm respectively. A 2 µl TE buffer volume was used as a blank and 2 µl of genomic DNA was subsequently analysed thereafter.

2.12 Amplification of 16S rDNA via polymerase chain reaction (PCR)

The 16S rRNA gene of each isolate was amplified with the universal bacterial 16S rDNA primers E9F (5'-GAGTTTGATCCTGGCTCAG-3') and U1510R (5'-GGTTACCTTGTTACGACTT-3'). The 50 µl reaction mixture contained 1X One *Taq* Standard Reaction Buffer, 200 µM dNTPs, 0.2 µM forward and reverse primer, 1.25 U/µl One *Taq* DNA polymerase and 100 ng of DNA. DNA samples were amplified on a Thermal Cycler (T100, BioRad). The following PCR parameters were used to amplify the 16S rDNA genes; initial denaturation at 94°C for 30 seconds, 30 cycles of denaturation at 94°C for 30 seconds, primer annealing at 55°C for 60 seconds and extension at 68°C for 1 minute/kb. Final extension occurred at 68°C for 5 minutes.

2.13 Agarose gel electrophoresis

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Successful amplification of the 16S rDNA genes was determined via agarose gel electrophoresis. A 1 % (w/v) agarose gel was prepared with 1X TAE buffer (40 mM Tris-base, 20 mM acetic acid, 1 mM EDTA, pH 8.4). Electrophoresis was conducted within 1X TAE buffer for 90 minutes at 80 V. Agarose gels were visualized using the Enduro^{TM} GDS Gel Documentation System via UV Illumination at 302 nm.

2.14 16S rDNA sequencing

Successful 16S rDNA gene amplicons were sequenced at the Central Analytical Facility (Stellenbosch University) via Bi-lateral sequencing. The raw data sequences were edited using the program Bioedit Sequence Alignment Editor Version 7.2.6. The sequences were then analysed using the National Center for Biotechnology Information (NCBI) database. The Basic Local Alignment Search Tool (BLAST) was used to conduct a nucleotide BLAST on the respective sequences to determine the identification of each endophyte.

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2.15 Growth trials under saline conditions

Growth trials were conducted to determine the effect of each bacterial inoculum on *Brassica napus* L (AV-Garnet) by observational changes in plant physiology under saline conditions. Growth trials were conducted in a greenhouse set at 21°C with natural lighting. *Brassica napus* L (AV-Garnet) seeds were planted in cups containing a sterile (autoclaved) 1:2 soil to silica sand ratio. Each cup contained three *Brassica napus* L (AV-Garnet) seeds treated with 2 ml of each inoculum which was replicated in triplicate. The experimental design consisted of the following treatments: Control: Plants without salt stress and inoculum, Saline experimental: Plants with salt stress, Endophyte experimental: Plants with inoculum and Endophyte experimental under saline conditions: Plants with inoculum under salt stress. The soil of the saline experimental and the endophyte experimental under saline conditions was pre-treated with 100 ml of 50 mM NaCl before planting the seeds. The control plant and the endophyte experimental was treated with a 100 ml of water and the remaining two experimental design plants were treated with a 100 ml of 100 mM NaCl solution every Tuesday and Friday for 42 days. Leaf dry weight was measured by allowing the respective plant material to dry out in an oven set at 80°C for 48 hours. Supplementary plant material was harvested using liquid nitrogen for the quantification of micro and macro elements via inductively coupled plasma optical emission spectrometry (ICP-OES) analysis, antioxidant enzyme determination, reactive oxygen species determination, lipid peroxidation and chlorophyll content.

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2.16 Evans Blue Assay (cell death)

Cell viability was determined using a modified method of (Sanevas *et al*., 2007). A 1 cm^3 square fresh leaf material was inserted into an Eppendorf tube containing 1 ml of a 0.25% (w/v) Evans blue solution. The samples were incubated at room temperature for 1 hour in the Evans blue solution. Following the incubation period, the samples were rinsed with dH_2O to remove the Evans blue solution. The samples were then incubated overnight in deionised water at room temperature. The water was decanted and 1ml of a 1% (w/v) sodium dodecyl sulphate (SDS) solution was added to the samples. The samples were then crushed in the SDS solution and incubated at 65° C on a heating block for 1 hour. Following the incubation period, the samples were centrifuged at 13, 200 x *g* for 10 minutes to pellet

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the plant material and obtain the supernatant. The supernatants were aliquoted into a 96 well microtitre plate and spectrophotometrically measured at 600 nm.

2.17 Protein extraction

A 100 mg of leaf material was weighed into three different Eppendorf tubes for each sample. A 0.5 ml protein extraction buffer containing [0.004 M phosphate buffer, 1 mM EDTA and 5% (w/v) PvP] was added to one of the three tubes. The homogenate in the tube was then further mixed using a vortex. The samples were centrifuged at 13, 200 x *g* for 5 minutes to pellet the plant material and obtain the supernatant. The supernatant was transferred to the second Eppendorf tube containing a 100 mg of leaf material. The previous steps were then repeated for the second and third Eppendorf tube. The supernatant was removed from the third tube and inserted into a clean Eppendorf tube. The protein concentration of each sample was quantified using a Bradford Assay and thereafter stored at -20° C.

2.18 A kinetic spectrophotometric assay to determine catalase activity

The catalase activity was determined using a modified method of (Aebi, 1984). The principle of the assay hinged on the decrease in absorbance due to the dissociation of H_2O_2. A reaction mixture containing 200 mM K_2HPO_4 (pH 7.0), 1 mM EDTA, 100 mM H_2O_2 and 20 µl protein extract was added and made up to a volume of 1 ml using dH_2O. The absorbance of the reaction mixture was spectrophotometrically measured at 240 nm. The extinction coefficient 39.4 $mM^{-1}.cm^{-1}$ was used to calculate the catalase activity.

2.19 A spectrophotometric assay to determine total superoxide dismutase activity

The total amount of superoxide dismutase activity was determined using a method by (Sigh *et al.*, 2007). A reaction mixture containing 20 mM phosphate, 0.1 mM Nitrotrezolium blue chloride (NBT), 0.005 mM riboflavin, 10 mM methionine, 0.1 mM (EDTA) and 20 µl protein extract was aliquoted into a 96 well microtitre plate and made up to a volume of 210 µl using dH_2O. The plate was then incubated on a light box at room temperature for 20 minutes. Following the incubation period, the samples were spectrophotometrically

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measured at 560 nm. One unit of SOD represents the amount that inhibited NBT photoreduction by 50%.

2.20 A kinetic spectrophotometric assay to determine total ascorbate peroxidase activity

The total amount of ascorbate peroxidase activity was determined using a method by (Sigh *et al*., 2007). An Eppendorf tube containing 20 µl of protein extract and 2 mM ascorbic acid was incubated at room temperature for 5 minutes. A reaction mixture containing 71.43 mM K_2HPO_4, 0.36 mM ascorbate and 0.714 mM H_2O_2 was made up to a volume of 1 ml using dH_2O. It should be noted that 0.714 mM H_2O_2 was added last to initiate a kinetic reaction before the samples were spectrophotometrically measured at 290 nm. The extinction coefficient 2.8 $mM^{-1}.cm^{-1}$ was used to calculate the total amount of ascorbate peroxidase activity.

2.21 A spectrophotometric assay to determine hydroxyl ion concentration

Hydroxyl ion concentration was determined using a method by (Halliwell *et al*., 1988). A 50 mg plant sample quantity was homogenised in 1 ml of a 10 mM phosphate buffer (pH 7.4) containing 15 mM 2-Deoxy-D-Ribose. Thereafter, the samples were incubated at 37° C for 2 hours. A reaction mixture containing 3 ml of a 0.5% (w/v) TBA made up in 5 mM sodium hydroxide (2ml), 1ml glacial acetic acid and 700 µl of sample was heated at 100° C for 30 minutes. Thereafter, the samples were cooled on ice for 5 minutes before the samples were centrifuged at 13, 200 x *g* for 5 minutes to pellet the plant material and obtain the supernatant. The supernatants were aliquoted into a 96 well microtitre plate and spectrophotometrically measured at 532 nm and 600 nm respectively. The extinction coefficient 155 $mM^{-1}.cm^{-1}$ was used to calculate the hydroxyl ion concentration.

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2.22 A spectrophotometric assay to determine superoxide content

Superoxide content was determined using a modified method of (Russo *et al*., 2008). Eight 1 cm^3 squares of fresh leaf material was inserted into an Eppendorf tube containing 10mM KCN (to inhibit Cu/Zn SODs), 10 mM H_20_2 (to inhibit Mn and Cu/Zn SODs), 2% SDS (to inhibit Mn and Fe SODs) and 80 µM NBT made up to a volume of 800 µl using a 50 mM potassium phosphate (pH 7.) solution. The samples were incubated at room temperature for 20 minutes. Following the incubation period, the samples were centrifuged at 13, 200 x *g* for 5 minutes to pellet the plant material and obtain the supernatant. The supernatants were aliquoted into a 96 well microtitre plate and spectrophotometrically measured at 600 nm. The extinction coefficient 12.8 $mM^{-1}.cm^{-1}$ was used to calculate the superoxide content.

2.23 A spectrophotometric assay to determine hydrogen peroxide content

Hydrogen peroxide content was determined using a modified method of (Valikova *et al*., 2000). A (0 nm, 5 000 nm, 10 000 nm, 15 000 nm, 20 000 nm and 25 000 nm) standard was prepared by diluting H_20_2 using dH_2O. The standards were then aliquoted in triplicate into a 96 well microtitre plate. A reaction mixture containing 20 mM dipotassium hydrogenphosphate (K_2HPO_4) and 500 mM potassium iodine (KI) was added to the standard and to 50 µl protein extract. The samples were incubated at room temperature for 20 minutes on a shaker before being spectrophotometrically measured at 390 nm.

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2.24 Determination of lipid peroxidation by quantifying MDA

Lipid peroxidation was determined using a modified method of (Zhang *et al*., 2007). A 100 mg of leaf material was weighed into Eppendorf tubes containing 5 volumes of 6% (w/v) trichloroacetic acid (TCA). The samples were centrifuged at 13, 200 x *g* for 10 minutes to pellet the plant material and obtain the supernatant. A 200 µl volume of supernatant was aliquoted into an Eppendorf tube containing 300 µl of a 0.5% (w/v) thiobarbituric acid (TBA). The samples were heated at 90° C for 20 minutes and thereafter placed on ice for 10 minutes. Following the incubation period, the samples were spectrophotometrically measured at 532 nm and 600 nm respectively. The absorbance at 600 nm was subtracted from the absorbance at 532 nm to correct for non-specific turbidity. The extinction coefficient 155 $mM^{-1}.cm^{-1}$ was used to calculate MDA content.

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2.25 Determination of chlorophyll A and B

Chlorophyll content was determined using a modified method of (Oancea *et al*., 2005). A 100 mg of leaf material was weighed into an Eppendorf tube containing 10 volumes of 100% (w/v) acetone. The samples were spectrophotometrically measured at 662 nm and 644 nm respectively. All optical readings were used in a calculation to determine the different chlorophyll species concentration.

2.26 Statistical analysis

Statistical analysis was performed using One-way ANOVA followed by Sidak's multiple comparisons test using GraphPad Prism version 6.0, GraphPad Software, La Jolla California USA.

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CHAPTER 3

UTILIZATION OF ENDOPHYTIC BACTERIA AS A BIO-INOCULANT TO PROMOTE *BRASSICA NAPUS* PLANT GROWTH

3.1 Introduction

Brassica napus more commonly known as rapeseed or canola, is a yellow flowering member of the Brassicacae family (Fang *et al*., 2017). Canola has been established as one of the world's most economically important plants and has become the third most important source of edible oil (El-Beltagi and Mohamed, 2010). Canola seeds contain low levels of erucic acid and glucosinolates that plays an important role in human health (Saeidnia and Gohari, 2012). Additionally, canola can also be used to produce margarine, biofuel, lubrication oil, soaps and plastics (Saeidnia and Gohari, 2011).

Canola is a relatively new crop in South Africa mainly grown as a winter crop in the Western Cape (Department of agriculture, forestry and fisheries, 2015). Although canola grain yields of 1516 kg ha^{-1} were considered as high under South African conditions, an average yield of 2000 kg ha^{-1} and 3443 kg ha^{-1} were recorded under USA and Canadian conditions respectively (Cronjé, 2005). Canola is also highly susceptible to pests and diseases such as slugs and stem rot among others (Fang *et al*., 2017).

Current agricultural practice includes the use of chemical fertilizers to address these problems; however, these agricultural methods have harmful effects on soil ecology and human health (Glick, 2012; Glick, 2014, Miliute *et al*., 2015). Therefore, in order to achieve an increase in agricultural productivity, various sustainable and environmentally friendly strategies need to be undertaken (Gamalero and Glick, 2011). To this extent, the use of plant growth-promoting bacteria (i.e. endophytes) is an attractive biological approach that could be utilized in agriculture to address these problems (Gamalero and Glick, 2011).

In this chapter nine candidate plant growth-promoting bacterial endophytes were utilized as bio-inoculants to promote *Brassica napus* plant growth. Based on observational changes in plant physiology, isolates that elicit beneficial effects on *Brassica napus* such as an increase

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in germination rate, root and shoot structure as well as plant biomass, were further analysed for possible plant growth promoting characteristics such as indole-3-acetic acid, phosphate solubilisation and siderophore production. Additionally, we investigated their ability to aid *Brassica napus* in nutrient acquisition via inductively coupled plasma optical emission spectrometry (ICP-OES). Because there are about 300 000 plant species that occur on earth with each plant, host to either one or more endophytes, the possibility to discover novel endophytes are considerable; therefore we identified these isolates using molecular techniques.

3.2 Results

3.2.1 Candidate plant growth promoting bacteria increases the germination percentage of *Brassica napus*

Brassica napus were grown and treated as described in section 2.5. The germination percentage of *Brassica napus* was calculated after 10 days as germinating seedlings may take 4 - 10 days to emerge. The results indicate that all isolates increased germination percentage when compared to the control (Table 3.1). Isolates E2 and E6 reached a 100% germination rate after 6 days. Isolates E1, E2, E6 and E7 reached a 100% germination rate after 10 days. Evidence suggests that utilization of endophytic bio-inoculum increases seedling emergence.

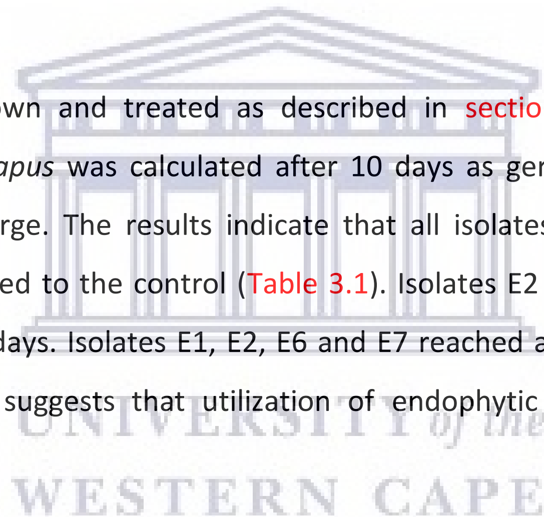
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Table 3.1: Germination percentage of *Brassica napus* after 10 days.

	4 days	5 days	6 days	10 days
Control	11%	56%	67%	67%
E1	11%	67%	89%	100%
E2	0%	78%	100%	100%
E3	0%	44%	78%	89%
E4	22%	44%	56%	89%
E5	11%	56%	67%	78%
E6	0%	56%	100%	100%
E7	33%	78%	78%	100%
E8	0%	44%	78%	89%
E9	0%	78%	78%	78%

3.2.2 Observational changes in plant physiology

Candidate plant growth promoting bacteria differentially influenced *Brassica napus* physiology (root and shoot structure) as observed in (Figure 3.1), (Figure 3.2) and (Figure 3.3) respectively. Inoculums E1-E6 displayed an adverse effect on root growth compared to the control; however, inoculums E7-E9 augmented root growth compared to the control. All inoculums (E1-E9) displayed a beneficial effect on shoot structure compared to the control. Inoculum E6 displayed the least degree of plant growth. Overall, inoculums E7-E9 displayed an advantageous effect on root, shoot and leaf structure when compared to the control (Figure 3.3). Based on the effect of isolate E7, E8 and E9 on *Brassica napus*, subsequent analysis of these isolates were conducted.

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Figure 3.1: Observational changes in plant physiology of *Brassica napus* as per the effect of utilizing endophytic strains E1, E2 and E3 as bio-inoculants. Overall, E3 displayed the most growth promotion compared to E1 and E2.

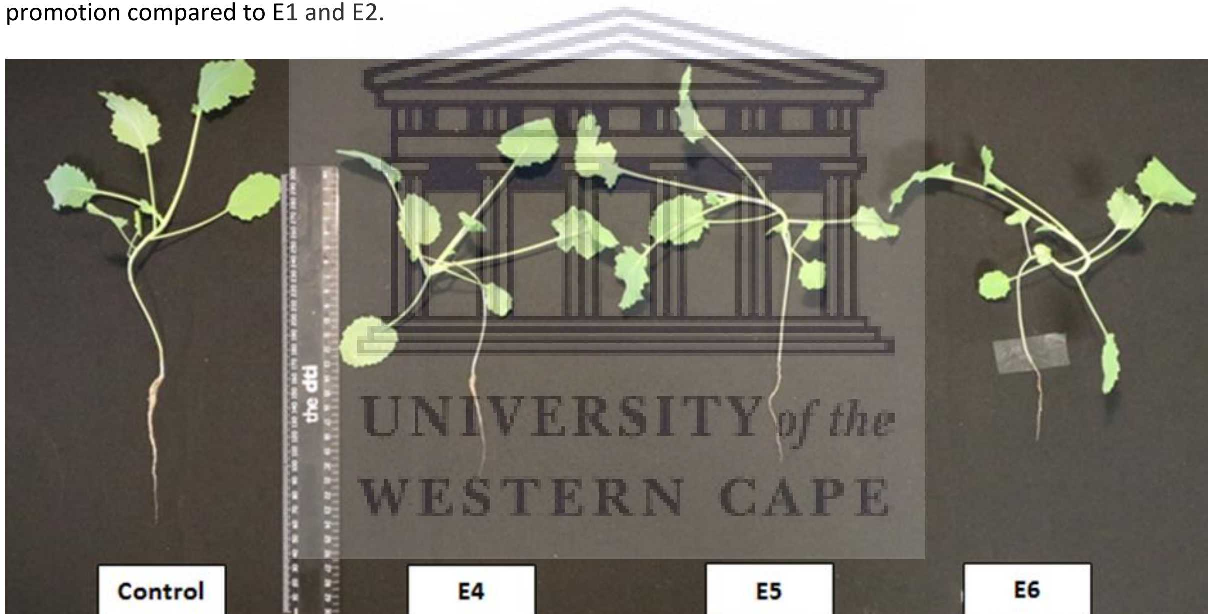

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Figure 3.2: Observational changes in plant physiology of *Brassica napus* as per the effect of utilizing endophytic strains E4, E5 and E6 as bio-inoculants. Overall, E6 displayed the least growth promotion compared to E4 and E5.

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Figure 3.3: Observational changes in plant physiology of *Brassica napus* as per the effect of utilizing endophytic strains E7, E8 and E9 as bio-inoculants. E7, E8 and E9 displayed a significant increase in growth promotion compared to the control.

3.2.3 Candidate plant growth promoting bacteria increases root dry weight of *Brassica napus*

Inoculum E7, E8 and E9 increased root dry weight of *Brassica napus*. Figure 3.4 shows that utilisation of inoculum E7-E9 displayed an increase in root dry weight compared to the control which could be correlated to the change in plant physiology during growth trials (Figure 3.3). Inoculum E7-E9 displayed a 182%, 383% and 990% increase in root dry weight compared to that of the control. Inoculum E7-E9 displayed a statistical difference which is represented by the letters b, c and d (Figure 3.4).

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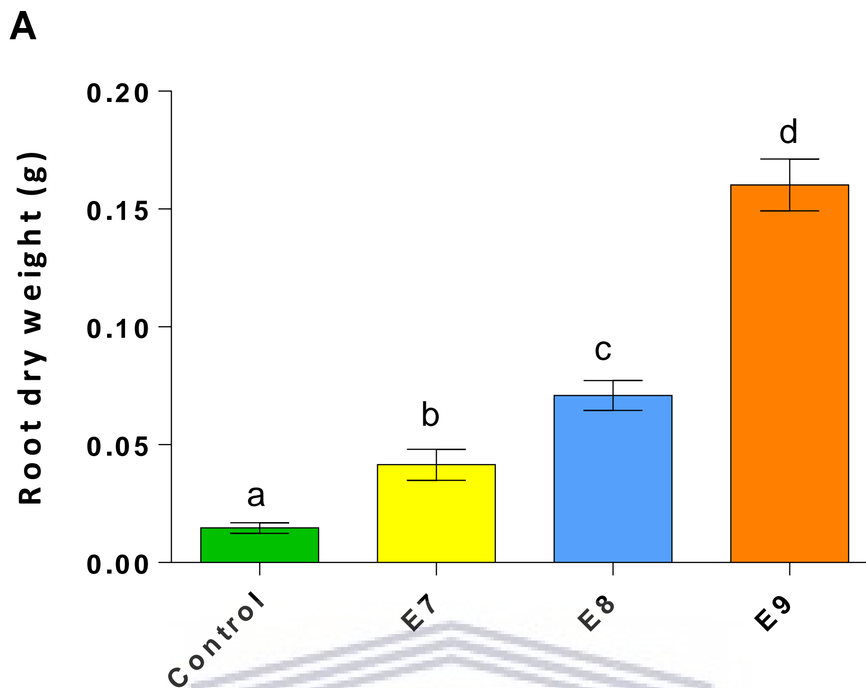

Figure 3.4: Effect of endophytic strains E7, E8 and E9 on the root dry weight of *Brassica napus*. Statistical analysis was performed using one-way ANOVA, where significance was represented by a P< 0.05. Different letters indicate significant differences between means at P< 0.05. Values are means ± S.E (n=3).

3.2.4 Candidate plant growth promoting bacteria increases leaf dry weight of *Brassica napus*

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Inoculum E7, E8 and E9 increased leaf dry weight of *Brassica napus*. Figure 3.5 shows that utilisation of inoculum E7-E9 displayed an increase in leaf dry weight compared to the control which could be correlated to the change in plant physiology during growth trials (Figure 3.3). Inoculum E7-E9 displayed a 14%, 52% and 91% increase in leaf dry weight compared to that of the control. Inoculum E7-E9 displayed a statistical difference which is represented by the letters b and c (Figure 3.5).

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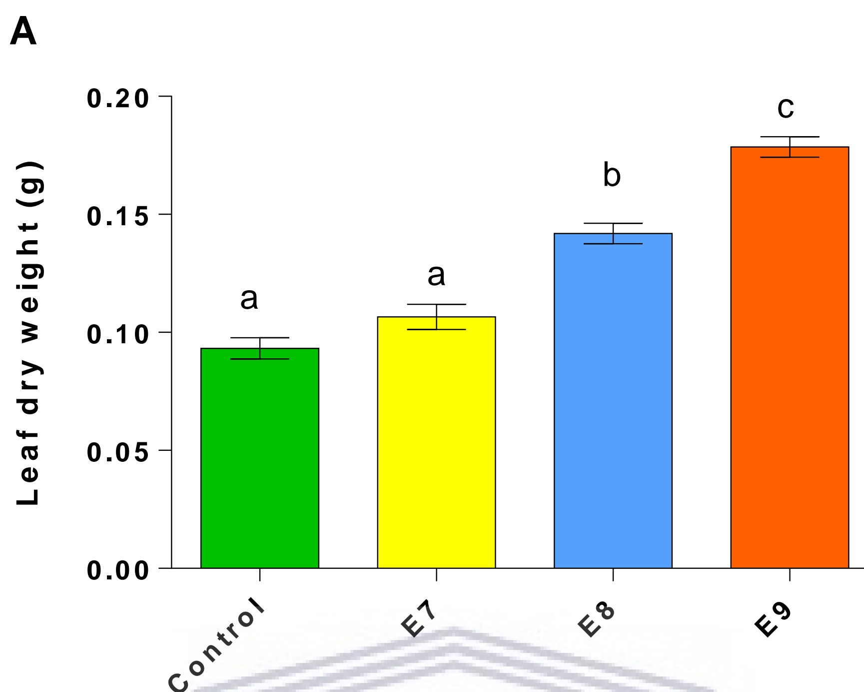

Figure 3.5: Effect of endophytic strains E7, E8 and E9 on the leaf dry weight of *Brassica napus*. Statistical analysis was performed using one-way ANOVA, where significance was represented by a P< 0.05. Different letters indicate significant differences between means at P< 0.05. Values are means ± S.E (n=3).

3.2.5 Candidate plant growth promoting bacteria produces indole-3-acetic acid

Candidate plant growth promoting bacterial isolates E7, E8 and E9 displayed the ability to produce indole-3-acetic acid. Isolate E7, E8 and E9 produced 69.1 µg/ml, 110.0 µg/ml and 348.3 µg/ml of IAA. The *Escherichia coli* strain KRX produced 39.8 µg/ml of IAA. The amount of IAA produced by isolate E7, E8 and E9 correlates to the increase in root length which is represented in (Figure 3.3).

Table 3.2: Identification of indole-3-acetic acid (IAA) production within the presence of L-tryptophan by candidate plant growth-promoting bacterial endophytes E7, E8 and E9 isolated from weed leaves. Table 3.2 represents indole-3-acetic acid production that was conducted in triplicate. Different letters indicate significant differences between means at p < 0.05 as per row. Values are means ± S.E (n=3).

Endophyte	Control	E7	E8	E9
Concentration µg/ml	39.8[a]	69.1[b]	110.0[c]	348.3[d]

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3.2.6 **Phosphate solubilisation analysis**

The following equation was used to calculate phosphate solubilisation. Phosphorus solubilisation index (PSI) = A/B; where A is the total diameter of the halo and B is the colony diameter. No isolate possessed the ability to solubilise phosphate when compared to the control. An isolate that produces a phosphorus solubilisation index (PSI) ≥ 2 is regarded as phosphate solubilising bacteria. The *Escherichia coli* strain KRX produced a phosphorus solubilising index (PSI) of 1.25.

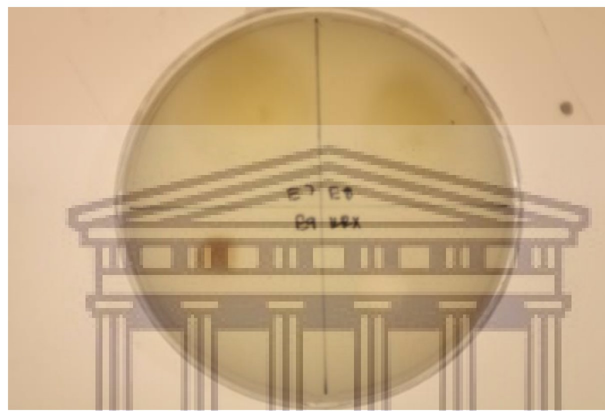

Figure 3.6: Identification of phosphate solubilisation on Pikovskayas agar by candidate plant growth-promoting endophytic bacteria E7, E8 and E9 isolated from weed leaves. Figure 3.6 is a representative phosphate solubilising plate assay that was conducted in triplicate.

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3.2.7 **Candidate plant growth promoting bacteria produces siderophore**

Candidate plant growth promoting bacterial isolates E7, E8 and E9 displayed the ability to produce siderophore. The following equation was used to calculate the percentage of siderophore production. Percentage yield of siderophore production (%YS) = [(Halo diameter – colony diameter)/colony diameter) x 100]. Isolate E7, E8 and E9 produced 67%, 12.5% and 88% siderophore production. The *Escherichia coli* strain KRX produced 38% siderophore production.

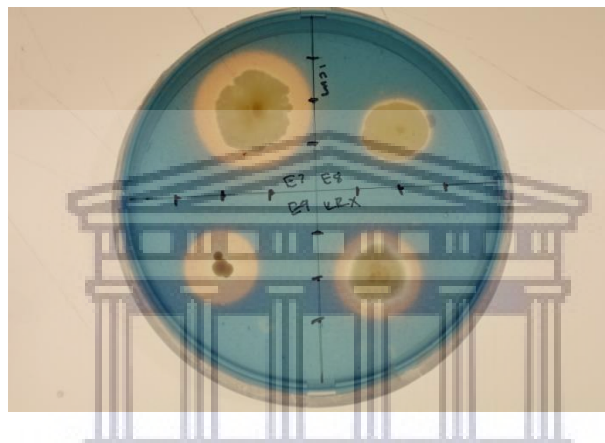

Figure 3.7: Identification of siderophore production on Chrome Azurol S (CAS) agar by candidate plant growth-promoting endophytic bacteria E7, E8 and E9 isolated from weed leaves. Figure 3.7 is representative siderophore producing plate assay that was conducted in triplicate.

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3.2.8 Candidate plant growth promoting bacteria increase micro and macro element acquisition

In Table 3.3, yellow represents the midpoint, red represents a decrease and green represents an increase. Candidate endophytic bacteria E7, E8 and E9 augmented K, Ca, Mg, P, Zn, Mn and Fe compared to un-inoculated *Brassica napus*. Cu decreased in all samples when compared to un-inoculated *Brassica napus*. Isolates E7, E8 and E9 augmented potassium by 7.735 ppm, 71.018 ppm and 92.376 ppm respectively. Isolates E7, E8 and E9 augmented calcium by 33.427 ppm, 38.153 ppm and 49.745 ppm respectively. Isolates E7, E8 and E9 augmented magnesium by 5.421 ppm, 6.919 ppm and 9.098 ppm respectively. Isolates E7, E8 and E9 augmented phosphate by 3.283 ppm, 2.791 ppm and 5.416 ppm respectively. Isolates E7, E8 and E9 reduced copper by 0.01 ppm, 0.007 ppm and 0.001 ppm respectively. Isolates E7, E8 and E9 augmented zinc by 0.065 ppm, 0.105 ppm and 0.281 ppm respectively. Isolates E7, E8 and E9 augmented manganese by 0.117 ppm, 0.139 ppm and 0.225 ppm respectively. Isolates E7 and E9 augmented iron by 0.145 ppm and 0.209 ppm however isolate E8 reduced iron by 0.031 ppm.

Table 3.3: Quantification of whole plant macro and micro elements via inductively coupled plasma optical emission spectrometry (ICP-OES). Table 3.3 represents the quantification of whole plant micro (blue) and macro (purple) elements (part per million) that was conducted in triplicate. Different letters indicate significant differences between means at $p \leq 0.05$ as per row. Values are means ± S.E (n=3).

Element	Control	E7	E8	E9
K	490.109[a]	497.844[a]	561.127[b]	582.485[b]
Ca	250.106[a]	283.533[b]	288.259[b]	299.851[b]
Mg	41.688[a]	47.109[a]	48.607[a]	50.786[a]
P	43.225[a]	46.508[a]	46.016[a]	48.641[a]
Cu	0.114[a]	0.104[a]	0.107[a]	0.113[a]
Zn	1.330[a]	1.395[b]	1.435[b]	1.611[c]
Mn	0.848[a]	0.965[b]	0.987[b]	1.073[c]
Fe	0.459[a]	0.604[b]	0.428[a]	0.668[b]

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3.2.9 **16S rDNA amplicon analysis**

Lane 1 represents the Quick-Load® 1kb DNA ladder I NEB. Lane 2 represents the negative control that was used in the PCR amplification. Lane 3 represents the candidate bacterial strain E7. Lane 4 represents the candidate bacterial strain E8. Lane 5 represents the candidate bacterial strain E9. Bacterial strains E7, E8 and E9 were amplified and gel electrophoresed to resolve the DNA fragments on the basis of their molecular weight compared to the Quick-Load® 1kb DNA ladder I NEB.

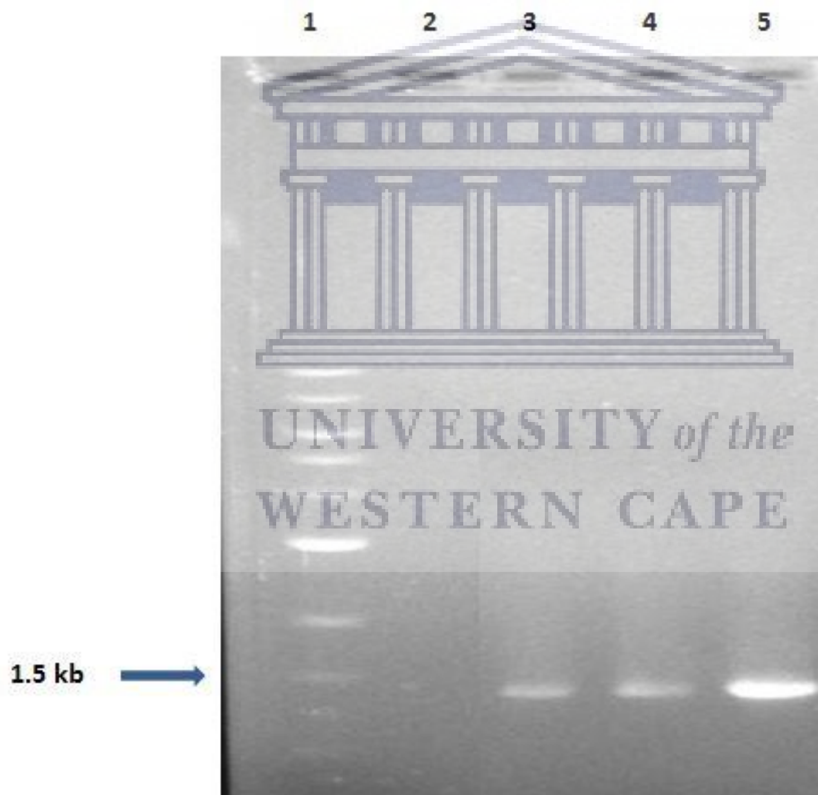

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Figure 3.8: Amplification of 16S rDNA genes from candidate endophytic bacterial strains E7, E8 and E9. Universal primers E9F and U1510R were used to amplify 16S rDNA genes isolated from endophytic bacterial strains E7, E8 and E9.

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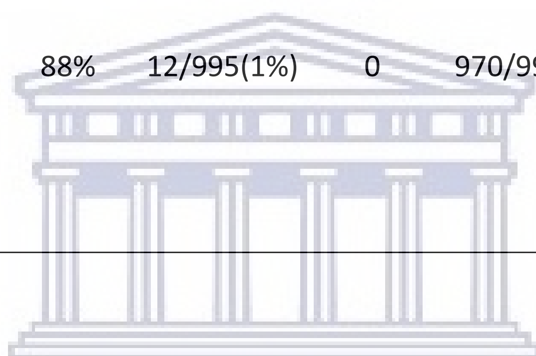
3.2.10 **16S rDNA amplicon identification**

Table 3.4: Identification of candidate plant growth promoting bacteria (endophytes). Table 3.4 represents the top two identification names for isolates E7, E8 and E9 when using the National Center for Biotechnology Information (NCBI) database and the Basic Local Alignment Search Tool (BLAST).

Endophyte	Description	Query cover	Gaps	E-value	Identity	Accession
E7	*Klebsiella aerogenes* strain KCTC 2190	84%	28/866(3%)	0	828/866(96%)	NR_102493.2
E8	*Enterobacter kobei* strain CIP 105566	96%	14/1114(1%)	0	1096/1114(98%)	NR_028993.1
E9	*Bacillus shackletonii* strain LMG 18435	88%	12/995(1%)	0	970/995(97%)	NR_025373.1



3.3 Discussion

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In this chapter, we utilized candidate plant growth-promoting endophytes as a bio-inoculant to promote *Brassica napus* plant growth and characterise these isolates based on their plant growth promoting mechanisms, nutrient acquisition capabilities and molecular identity. The results displayed observational changes in plant morphology when utilizing each isolate as a bio-inoculant during the growth trials conducted in the greenhouse. Utilizing inoculum E1-E6 displayed adverse effects on *Brassica napus* whereas inoculum E7-E9 displayed plant growth promotion when compared to the control. This evidence suggests that the ability of inoculum E7, E8 and E9 to promote plant growth could be due to the plant growth-promoting characteristics such as indole-3-acetic acid production and siderophore production of these particular isolates. Furthermore, these isolates possess the ability to aid a plant in nutrient acquisition of micro and macro elements, which are essential for plant growth, development and survival. The molecular results in this study indicate that candidate plant growth promoting bacterial endophytes responsible for *Brassica napus*

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plant growth promotion are E7 - *Klebsiella aerogenes* strain KCTC 2190, E8 - *Enterobacter aerogenes* strain ATCC 13048 and E9 - *Bacillus shackletonii* strain LMG 18435.

3.3.1 Candidate plant growth promoting bacterial endophytes improves *Brassica napus* germination rate

Brassica napus is an important oil rapeseed due to its economic and nutritional value. However, germination rates of this particular cultivar produces low yield due to its high susceptibility. Table 3.1 depicts the low germination percentage of canola under axenic conditions. Therefore increasing the germination percentage of this particular cultivar is of utmost importance as it plays a role in global food security.

In this study, we have shown that utilising endophytic bacterial strains E1-E9 increased the germination percentage of *Brassica napus*. Table 3.1 depicts the effect of each endophytic strain on the germination rate of *Brassica napus*. An overall increase in germination percentage was observed by endophytic strains E1-E9. The increase in germination percentage could be due to colonisation between seed and microbe. Various factors influence colonisation such as inoculum survival, spermosphere multiplication, root surface attachment and root colonisation (Nelson, 2004). Root colonisation and endophytic competent development forms the fundamental basis for plant growth-promoting bacteria to elicit their positive capabilities (Nelson, 2004).

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Endophytes are able to colonise plant tissue via passive penetration that occurs at root cracks and tips created by root emergence sites or deleterious micro-organisms. Flagella, lipopolysachharides, pili and twitching motility has been proven to affect plant-microbe colonisation (Bohm *et al*., 2007). Another factor that may aid penetration is the production of bacterial cell wall degrading enzymes (CWDEs) (Lodewyckx *et al*., 2002). Transport systems and metabolic pathways involved in recognising and catabolising polysaccharides allow endophytes to penetrate plant tissue (Fouts *et al*., 2008). Passive and active mechanisms have been shown to translocate endophytes within plant tissue enabling endophytic progression to the root cortex. At this point, the endodermic barrier may prevent colonisation as only certain bacteria are able to pass (Gregory, 2006). Endophytes possess cell wall degrading enzymes that provide access to continue colonisation (James *et al*., 2002). These reports have been observed by Herbaspillum seropedicae Z67 within rice,

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B. phytofirmans strain PsJN within grapevine and various other endophytic bacteria (Compant *et al*., 2005; James *et al*., 2002). Evidence suggests that endophytic bacterial strains E1-E9 possess these prerequisites for colonisation and this may be observed by the germination percentage rate of each strain on the *Brassica napus*.

3.3.2 Candidate plant growth promoting bacterial endophytes improves *Brassica napus* plant growth and plant biomass

In this study, we have shown that utilising bacterial endophytic strains E7, E8 and E9 as a bio-inoculant significantly improves *Brassica napus* plant growth and plant biomass compared to the control (Figure 3.3), (Figure 3.4) and (Figure 3.5). An increase in *Brassica napus* plant morphology (roots and shoot structure) can be seen in (Figure 3.3) which directly correlates to the increase in plant biomass seen in (Figure 3.4) and (Figure 3.5) respectively. A study conducted by Ji et al (2018) reported that PGPB increased plant height, root length and the dry weight of *Panax ginseng*. Evidence suggests that plant growth promoting bacteria (endophytes), specifically in this study, i.e. isolates E7, E8 and E9 improved root elongation, shoot elongation, root biomass and shoot biomass (Figure 3.3), (Figure 3.4) and (Figure 3.5). Therefore, one can conclude that these isolates elicit positive effects on *Brassica napus*.

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3.3.3 Indole-3-acetic acid (IAA) production

In this study, we have shown that isolate E7, E8 and E9 possess the ability to produce indole-3-acetic acid (Table 3.2). IAA has the ability to affect plant nutrition and development by initiating variations within the cell cycle which may lead to root elongation and xylem development (Glick, 2012).

In this study endophytic bacterial strains were isolated from weed leaves which are known for IAA production by means of transamination and decarboxylation tryptophan reactions. Plants inoculated with endophytic bacterial strains E7-E9 displayed an increase in germination rate compared to un-inoculated plants (Table 3.1). Plants inoculated with endophytic bacterial strains E7-E9 displayed increased root length, root hairs and lateral roots after the 42 day greenhouse experiment (Figure 3.3). An increase in root length facilitated the acquisition of essential nutrients which can be displayed by root and leaf

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biomass (Figure 3.4) and (Figure 3.5). The quantification of micro and macro elements via ICP-OES displayed an increase in potassium, calcium, magnesium, phosphorus, zinc and manganese compared to un-inoculated plants (Table 3.3). Indole-3-acetic acid production was measured following the colorimetric assay by Gordon and Weber. In this assay, isolates were grown in medium containing mannitol at 37°C which resulted in high IAA concentrations (Table 3.2).

These results can be supplemented by the following statements and findings. Sachdev et al (2009), Spaepen et al (2007) and Tabatabaei et al (2016) stated that IAA is predominantly produced in stems, young leaves and seeds by means of transamination and decarboxylation tryptophan reactions. This phenomenon could account for the high IAA production displayed in (Table 3.2), as isolates E7, E8 and E9 were extracted from young weed leaves. In 2009, Fatima et al showed that IAA and plant growth-promoting bacteria increase germination rates as well as root and shoot growth (Table 3.1) (Mohite, 2013). Basu et al (2016) and Mohite (2013) stated that micro-organisms that possess the ability to produce IAA can support plant growth and development by inducing the production of longer lateral roots and root hairs (Figure 3.3) which promotes nutrient acquisition (Table 3.3). Patten and Glick utilised a *P. putida* strain that produced high IAA concentrations to increase canola root growth by 50% (Figure 3.3) (Patten and Glick, 2002). Factors such as carbon source, pH, and temperature affects IAA production (Mohite, 2013). In 2005, Shilts et al reported the production of IAA concentrations in medium containing galactose and mannitol (Mohite, 2013). In 2012, Sudha et al reported that the optimum temperature for IAA production was 37°C for *Bacillus* spp. and Rhizobium spp. In another report, root elongation was observed in *Sesbania aculeata* by *Azotobacter* spp., *Brassica campestris* by *Bacillus* spp., *Vigna radiata* by *Pseudomonas putida* and in *Pennisetum americanum* by *Azospirillum brasilense* (Ghosh *et al.,* 2003; Patten and Glick, 2002). Bacillus, Enterobacter, Xanthomonas, Pseudomonas, Alcaligenes, Azotobacter, Acetobacter and Agrobacterium are some of the various genera that has been shown to produce high concentrations of IAA (Khan *et al*., 2016). Therefore, the correlation between the findings in this study and literature provides enough evidence to state that isolates E7, E8 and E9 possibly promoted *Brassica napus* plant growth via indole-3-acetic acid production.

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3.3.4 **Phosphate solubilisation**

In this study, we have shown that isolates E7, E8 and E9 doesn't possess the ability to solubilise phosphate (Figure 3.6). Phosphorus is an important macro element prerequisite for plant growth and development (Brigido *et al*., 2017). Phosphorus is copious in soil and can occur in inorganic and organic forms with the inorganic form largely established in soil (Gamalero and Glick, 2011). Actinomycetes, bacteria and fungi are some of the microorganisms that are able to solubilise phosphate for plant uptake (Khan *et al*., 2010).

Identification of phosphate solubilising micro-organisms was conducted on Pikovskayas agar. A clearing halo formation around the colony is an indication that micro-organisms are able to mineralise or solubilise phosphate. According to (Figure 3.6), endophytic bacterial strains E7, E8 and E9 displayed no ability to solubilise or mineralise phosphate. This evidence is further supplemented by (Table 3.3) which shows that E7, E8 and E9 showed no significant increase in phosphorus compared to the control.

3.3.5 **Siderophore production**

In this study, we have shown that isolates E7, E8 and E9 possess the ability to produce siderophores (Figure 3.7). Siderophores are low molecular weight molecules that have a high affinity for iron (Brigido *et al*., 2017). Iron is one of the most abundant elements found on earth and is an essential component of various biological processes such as chlorophyll biosynthesis, nitrogen fixation, photosynthesis and respiration (Basu *et al*., 2017). Bacteria that secrete siderophores promote plant growth by reducing pathogens or facilitating the acquisition of iron (Basu *et al*., 2017; Gamalero and Glick, 2011).

Identification of siderophore-producing micro-organisms was conducted on Chrome Azurol S (CAS) agar. An orange halo around the colony is an indication that micro-organisms are able to produce siderophores. No sign of chlorosis were observed in plants inoculated with endophytic bacterial strain E7 and E9 which could be due to their ability to produce siderophores. Isolates E7, E8 and E9 were able to produce 67%, 12.5% and 88% siderophores respectively. This evidence is supplemented by (Table 3.3) which shows that iron increased in isolate E7 and E9.

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These results can be supplemented by the following statements and findings. Plants inoculated with siderophore producing bacteria (SPB) facilitate the acquisition of iron through numerous mechanisms such as ligand exchange reactions, siderophore-Fe complexes and chelate degradation (Rajkumar *et al*., 2009). Previous studies reported that SPB inoculum simultaneously promotes plant growth (Figure 3.3) and increases iron uptake in plants (Table 3.3) (Rajkumar *et al*., 2009). Studies conducted by Crowley and Kraemer showed that SPB supplied iron to oats under limited iron availability (Rajkumar *et al*., 2009). Sharma et al evaluated the ability of a Pseudomons strain GRP3 to increase iron uptake on Vigna radiate over a period of 45 days (Rajkumar *et al*., 2009). They concluded that signs of chlorosis decreased and iron accumulation increased (Rajkumar *et al*., 2009). Therefore, the correlation between the findings in this study and literature provides enough evidence to state that isolates E7, E8 and E9 possibly promoted *Brassica napus* plant growth via siderophore production.

3.3.6 **Nutrient acquisition**

In this study, we have shown that isolates E7, E8 and E9 possess the ability to aid a plant in nutrient acquisition. Plant growth and development depend on a number of mineral elements that needs to be utilised from their surrounding environment (Gruzak, 2001). These minerals may serve as ionized species to provide charge balance in cellular compartments, osmotic solutes needed to maintain proper water potential, cofactors in enzymatic reactions and structural components in macromolecules (Gruzak, 2001). In this study, various micro and macro elements such as copper, zinc, manganese, iron, potassium, calcium, magnesium and phosphorus were quantified via inductively coupled plasma optical emission spectrometry. This analysis was conducted to determine an increase or decrease in mineral elements as a result of the inoculum.

In this study, the regulation of essential nutrients and the effect that endophytic bacterial strains have on this regulatory process can be seen by either an increase or decrease of micro and macro elements (Table 3.3).

Isolates E7, E8 and E9 increased the level of potassium (K) available to *Brassica napus* and displayed a statistical difference compared to the control and E7 (Table 3.3). Potassium (K) is a macronutrient that plays an important role in plant growth and development (Tripathi *et*

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al., 2014). Potassium is linked to many physiological processes which help improve transportation, assimilates, water relations, enzyme activation and photosynthesis (Tripathi *et al*., 2014). A study conducted by Helal and Mengel (1979) and Leigh and Wyn Jones (1986) showed that a lack of K^+ ions in plants inhibits protein synthesis (Tripathi *et al*., 2014). Pfluger and Mengel (1972) reported that K is responsible for the activation of NADPH during photosynthesis (Tripathi *et al*., 2014). Chrispeels et al (1999) stated that K plays an important role in the plant metabolic system which involves transportation of phytohormones, nutrients, metabolites and water through xylem and phloem (Tripathi *et al*., 2014). It has been shown that stomata conductance is dependent upon K^+ ions because of their movement between guard cells, and therefore minimises abiotic stress (Tripathi *et al*., 2014).

Isolates E7, E8 and E9 increased the level of calcium (Ca) available to *Brassica napus* and displayed a statistical difference compared to the control (Table 3.3). Ca is one of the most essential elements required in the form of calcium ions (Ca^{2+}) that is involved in many cellular processes in all living organisms (Tripathi *et al*., 2014). It is required for cell elongation, division, growth and many other biological functions (Tripathi *et al*., 2014). During plant growth and development, Ca is involved in processes such as enzyme activation, water movement and salt balance induction and stomata conductance (Tripathi *et al*., 2014). Ca increases nutrient acquisition, plant tissue resistance, cell wall strength and root development (Tripathi *et al*., 2014). Hepler (2005) observed that Ca controls various processes during plant growth and development and that Ca deficiency causes black spots and yellow coloration on leaves (Tripathi *et al*., 2014). Several studies revealed that Ca protects plants from abiotic and biotic stress via Ca^{2+}/H^+ antiporter and Ca^{2+}-ATPase signalling channels (Tripathi *et al*., 2014). Furthermore, Li et al (2003) observed that the HSP gene expressed Ca signalling pathways during salt stress in tobacco (Tripathi *et al*., 2014).

Isolates E7, E8 and E9 increased the level of magnesium (Mg) available to *Brassica napus* but displayed no statistical difference compared to the control (Table 3.3). Magnesium (Mg) is a common element that plays a pivotal role in photosynthesis as it is the central atom for chlorophyll. Therefore Mg deficiency degrades chlorophyll content causing leaves to become yellow which is commonly known as chlorosis (Tripathi *et al*., 2014). A study revealed that an appropriate level of Mg increased antioxidant enzyme and molecule

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activity in mulberry, bean, maize and pepper (Tripathi *et al*., 2014). Comparatively, Tewari et al (2006) showed that Mg deficiency increased oxidative stress in mulberry plants by enhanced ROS generation that triggered redox changes in cellular metabolism (Tripathi *et al*., 2014). A study conducted by Ding et al (2008) revealed that low levels of Mg in rice was negatively associated with Malondialdehyde (MDA) and antioxidative enzymes however, the exogenous application of Mg increased photosynthetic rates, chlorophyll content, yield, shoot biomass, SOD, CAT and APX activity (Tripathi *et al*., 2014). Chen et al (2012) reported that Mg alleviated abiotic stress in rice and Jones and Huber (2007) stated that adequate Mg levels can control various harmful plant diseases such as root rot caused by *Rhizoctonia solani* in bean plants, leaf spot diseases caused by *Botrytis* spp. in castor bean and early blight diseases caused by *Alternaria solani* in potato (Tripathi *et al*., 2014).

Isolates E7, E8 and E9 increased the level phosphorus (P) available to *Brassica napus* but displayed no statistical difference compared to the control (Table 3.3). Phosphorus (P) is found in all plant cellular membranes in the form of phosphate where it plays essential roles in ATP, RNA and DNA, therefore, it is an essential component for plant growth and development (Tripathi *et al*., 2014). P is a required component for photosynthetic processes that convert solar energy into chemical energy (Tripathi *et al*., 2014). It increases the rapid growth of plant root systems needed to extract nutrients from the soil (Tripathi *et al*., 2014). A study conducted by Kavanova et al (2006) reported that P deficiency caused a decline in cell elongation and cell division in the leaves of grass (Tripathi *et al*., 2014). Comparatively, Postma and Lynch (2011) reported that optimal P levels increased maize growth (Tripathi *et al*., 2014).

Isolates E7, E8 and E9 decreased the level of copper (Cu) available to *Brassica napus* but displayed no statistical difference compared to the control (Table 3.3). Copper (Cu) is a transitional element that regulates multiple biochemical reactions in plants (Tripathi *et al*., 2015). Cu acts as an important enzyme cofactor and is involved in auxin regulation, protein synthesis, phenol metabolism, lignifications, photosynthesis and respiration (Tripathi *et al*., 2015). Copper proteins localised in peroxisomes, chloroplasts and the cytoplasm act as scavengers of ROS molecules (Tripathi *et al*., 2015). Cu deficiency causes improper growth rate, chlorosis of young leaves, leaf curling and plant development (Tripathi *et al*., 2015). High levels of Cu can cause the inhibition of root and shoot growth, stunting, chlorosis and

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necrosis (Tripathi *et al*., 2015). Therefore, proper regulation, assimilation and acquisition of Cu are important for plant growth and development (Tripathi *et al*., 2015).

Isolates E7, E8 and E9 increased the level of zinc (Zn) available to *Brassica napus* and displayed a statistical difference compared to the control (Table 3.3). Zinc (Zn) is another transitional element that is either a structural constituent or regulatory cofactor for various enzymes and proteins (Tripathi *et al*., 2015). Zn is responsible for several plant processes such as promoting the metabolism of auxins, proteins, carbohydrates and pollen formation (Tripathi *et al*., 2015). Zn regulates cell membranes and elicits defense mechanisms against any harmful pathogens (Tripathi *et al*., 2015). The presence of Zn in CAT and SOD act as cofactors to protect plants during oxidative stress (Tripathi *et al*., 2015). A study conducted by Cakmak (2000) confirmed that Zn plays an important role in various antioxidant enzymes such as glutathione reductase during oxidative stress (Tripathi *et al*., 2015). Zinc deficiency causes necrosis, leaf curling, leaf shortening and size reduction (Tripathi *et al*., 2015).

Isolates E7, E8 and E9 increased the level of manganese (Mn) available to *Brassica napus* and displayed a statistical difference compared to the control (Table 3.3). Manganese (Mn) is a microelement that occurs in many oxidative states such as 0, II, III, IV, VI but preferably found as II, III and IV in biological systems (Tripathi *et al*., 2015). The bioavailability of Mn is affected by various soil conditions (Tripathi *et al*., 2015). It is responsible for the biosynthesis of fatty acids, proteins, lipids and ATP (Tripathi *et al*., 2015). Furthermore, manganese is also responsible for the bioactivation of Mn-CAT and Mn-SOD enzymes therefore Mn plays an important role in stress response by serving as a cofactor for various antioxidant enzymes (Tripathi *et al*., 2015). However, over expression of Mn levels leads to ROS generation, particularly OH^-, resulting in oxidative stress (Tripathi *et al*., 2015). Comparatively, Mn deficiency causes chlorosis, necrosis, delayed maturity and premature leaf fall (Tripathi *et al*., 2015).

Isolates E7 and E9 increased the level of iron (Fe) available to *Brassica napus;* however, isolate E8 decreased the level of iron (Fe) available to *Brassica napus* (Table 3.3). Isolate E8 displayed no statistical difference in iron compared to the control (Table 3.3). Iron (Fe) is a microelement required by a plant in trace amounts for optimal growth and productivity (Tripathi *et al*., 2015). Iron can be found in various cellular components such as vacuoles,

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mitochondria and chloroplast (Tripathi *et al*., 2015). Iron is involved in chlorophyll biosynthesis, photosynthesis, DNA biosynthesis, gene regulation, oxygen transport and cellular respiration (Tripathi *et al*., 2015). The most prominent sign of Fe deficiency in plants is chlorosis in young leaves which is caused by a decrease in chlorophyll biosynthesis (Tripathi *et al*., 2015). Several reports stated that excessive Fe concentrations led to enhanced oxidative stress and the production of ROS (Tripathi *et al*., 2015).

This evidence suggests that endophytic bacterial strains could possibly play an integral role in the acquisition of essential nutrients required by the plant for growth and development. This facilitative mechanism could be due to plant growth promoting characteristics indole-3-acetic acid and siderophore production. These statements can be substantiated by previously mentioned studies that have been documented throughout this study.

3.3.7 **Molecular identification of bacterial endophytes**

In this study, we have shown that isolates E7, E8 and E9 promote *Brassica napus* plant growth and development through direct mechanisms such as indole-3-acetic acid and siderophore production. These isolates also displayed the ability to aid a plant in nutrient acquisition. Therefore, determining the identification of these isolates was of utmost importance to agricultural studies.

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In this study, genomic DNA was extracted from isolates E7, E8 and E9 using the Cetyltrimethyl Ammonium Bromide (CTAB) method. The DNA extracts were analysed using the NanoDrop 2000 Spectrophotometer before subsequent analysis. The 16S rDNA gene of each isolate was amplified using the universal bacterial 16S rDNA primers E9F (5'-GAGTTTGATCCTGGCTCAG-3') and U1510R (5'-GGTTACCTTGTTACGACTT-3'). Successful amplification of the 16S rDNA genes was determined via agarose gel electrophoresis. Agarose gels were visualized using the Enduro^{TM} GDS Gel Documentation System via UV Illumination at 302nm (Figure 3.8). Successful 16S rDNA gene amplicons were sequenced at the central analytical facility via Bi-lateral sequencing. The raw data sequences were edited using the program Bioedit Sequence Alignment Editor Version 7.2.6. The sequences were then analysed using the National Center for Biotechnology Information (NCBI) database. The Basic Local Alignment Search Tool (BLAST) was used to conduct a nucleotide BLAST on the respective sequences to determine the identification of each endophyte. As depicted by

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(Table 3.4), isolate E7 was identified as *Klebsiella aerogenes* strain KCTC 2190, E8 was identified as *Enterobacter kobei* strain CIP 105566 and E9 was identified as *Bacillus shackletonii* strain LMG 18435.

Klebsiella aerogenes is a Gram-negative bacterium that belongs to the family *Enterobacteriaceae* (Iyer *et al*., 2017). This bacterium can be isolated from various habitats such as water, air, soil and plants (Iyer *et al*., 2017). *Klebsiella* is capable of existing as free endophytic bacteria that colonize plant tissue (Hazen *et al*., 2014). Gang et al (2018) stated that *Klebsiella* is a plant growth promoting bacterial strain that is capable of promoting plant growth through various direct and indirect mechanisms such as phosphate solubilization, auxin production, nitrogen fixation and abiotic stress protection. Additionally, Liu et al (2018) stated that *Klebsiella* promotes plant growth by fixing nitrogen, producing siderophores, gibberellic acid, indole-3-acetic acid, 1-aminocyclopropane-1-carboxylate deaminase and solubilizing phosphate. A study conducted by Liu et al (2018) showed that *Klebsiella* mediates resistance against *Heterodera glycines* and promotes soybean growth. Furthermore, Kuan et al (2016) showed that *Klebsiella* enhanced nitrogen remobilization, nitrogen fixation and vegetative growth of maize plants under greenhouse conditions.

Enterobacter kobei is a Gram-negative, facultative anaerobic rod that belongs to the family *Enterobacteriaceae* (Jha *et al*., 2011). *Enterobacter* spp. are known to possess various PGP characteristics such as nitrogen fixation, soil phosphorous solubilisation, antibiotic production, siderophore production, chitinase production, ACC deaminase and enhanced soil porosity (Jha *et al*., 2011). *Enterobacter* contributes to plant growth and development through three functions; lessening or preventing plant diseases, facilitating nutrient acquisition from the soil to the plant and synthesizing certain compounds for plant growth and development (Jha *et al*., 2011). Kavita et al (2008) reported that *Enterobacter* inoculum increased mung bean plant growth under heavy metal toxicity. A study conducted by Deepa et al (2010) showed that *Enterobacter* can solubilize phosphate (60.1-79.5 mg/ml/day) after ten days of incubation; produce IAA (23.8-104.8 mg/ml/day) after 48 hours of incubation, HCN and siderophores. Furthermore, *Enterobacter* increased cowpea root and shoot length compared to the uninoculated control (Jha *et al*., 2011).

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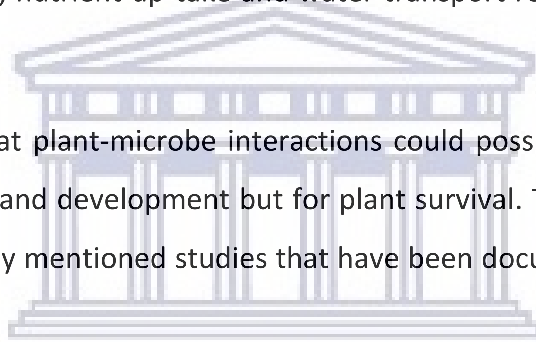
Bacillus shackletonii is a Gram-positive, facultative anaerobic rod that exhibits a wide range of characteristics that allow them to thrive in various environments such as airborne dust, marine sediments, water, hot springs and soil (Liu *et al*., 2015). *Bacillus* spp. can secrete several metabolites that trigger plant growth and prevents pathogenic infections (Radhakrishnan *et al*., 2017). During drought, salinity and heavy metal toxicity, *Bacillus* secretes siderophores and exopoysaccharides which inhibits the movement of toxic ions, adjust ionic balance and water transport in plants (Radhakrishnan *et al*., 2017). Additionally, *Bacillus* regulates intracellular phytohormone metabolism and increases plant stress response through the synthesis of IAA, gibberellic acid and ACC deaminase (Radhakrishnan *et al*., 2017). Furthermore, *Bacillus* spp. are capable of mitigating the detrimental effects of abiotic and biotic stress via *Bacillus* induced physiological changes, including antioxidant defence system activation, nutrient up-take and water transport regulation (Radhakrishnan *et al*., 2017).



This evidence suggests that plant-microbe interactions could possibly play an integral role not only for plant growth and development but for plant survival. These statements can be substantiated by previously mentioned studies that have been documented throughout this study.

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Chapter three described the effect of endophytic bacteria on *Brassica napus* germination percentage, plant physiology and biomass. Isolates E7, E8 and E9 increased the germination percentage, plant physiology and biomass of *Brassica napus*. Isolates E7, E8 and E9 displayed the ability to produce indole-3-acetic acid and siderophore production however the ability to solubilise phosphate was not displayed. Furthermore, isolates E7, E8 and E9 displayed the ability to aid *Brassica napus* during nutrient acquisition. The molecular results identified isolates E7, E8 and E9 to be *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 respectively.

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CHAPTER 4

Utilization of *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 as bio-inoculants to promote *Brassica napus* plant growth under salt stress

4.1 Introduction

In an ever changing environment, plants are continuously faced with numerous abiotic stress factors such as salinity which causes a significant loss in crop yield (Labrada *et al*., 2019). Soil salinity is one of the most dangerous abiotic stress factors that limits the production of over 6% of the world's land and 20% of irrigated land (15% of total cultivated areas) and negatively influences crop production worldwide (Abbas *et al*., 2019; Hasanuzzaman *et al*., 2012; Rasool *et al*., 2013). Furthermore, increased salinity of agricultural land is expected to have deleterious global repercussions, resulting in up to 50% land loss by the twenty-first century (Abbas *et al*., 2019; Hasanuzzaman *et al*., 2012; Rasool *et al*., 2013). This directly affects food availability for the global population, which is projected to increase to 9.5 billion by 2050 (Abbas *et al*., 2019; Singh, 2015).

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Some of the harmful effects of salinity have been associated with an increase in chloride (Cl^-) and sodium (Na^+) ions, which provide essential conditions for plant survival by impeding various plant mechanisms (Labrada *et al*., 2019). Both Na^+ and Cl^- cause numerous physiological disorders in plants however, Cl^-, is the most dangerous (Kharusi *et al*., 2019). Plant growth water use efficiency and transpiration has been associated with relative salt tolerance due to the build-up of Cl^- (Labrada *et al*., 2019). Salinity affects upper plant parts, root growth and physiology which essentially impact their function in nutrient uptake (Hasanuzzaman *et al*., 2012).

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A key sign of salt stress at the molecular level is the enhanced production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and superoxide radical (O_2^-) (Gondim *et al*., 2013). ROS are extremely volatile in nature because they are capable of interacting with numerous cellular molecules and metabolites, which can lead to irreversible metabolic dysfunction and death (Gondim *et al*., 2013; Kharusi *et al*., 2019; Labrada *et al*., 2019). Plants have developed enzymatic and non-enzymatic scavenging pathways or detoxification systems capable of countering the damaging effects of ROS such as ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD), glutathione (GSH), and ascorbate (AsA) (Rasool *et al*., 2013). In plant cells, specific ROS-producing and scavenging systems are found in different organelles and the ROS-scavenging pathways from different cellular subunits are coordinated (Hasanuzzaman *et al*., 2012). Recent studies have shown that moderately low levels of ROS act as signalling molecules that induce abiotic stress tolerance by modulating the expression of defense genes (Hasanuzzaman *et al*., 2012). Furthermore, several studies have shown that plants with increased levels of antioxidants, exhibited greater resistance to various environmental stress factors (Hasanuzzaman *et al*., 2012).

Studies have shown that plant growth promoting bacteria (PGPB) (endophytes) are an attractive biological approach that could be utilized to alleviate or reduce salt stress (Abbas *et al*., 2019; Glick, 2014; Santoyo *et al*., 2016). These bacteria provide tolerance to host plants during abiotic stress such as salinity and belong to diverse genera such as *Microbacterium*, *Pantoea*, *Achromobacter*, *Rhizobium*, *Pseudomonas*, *Bacillus*, *Paenibacillus*, *Enterobacter*, *Burkholderia*, *Methylobacterium*, *Azospirillum*, and *Variovorax* (Abbas *et al*., 2019; Ji *et al*., 2013). Endophytes elicit several benefits to their host plant such as growth promotion, protection from pathogens and plant-microbe interactions under diverse environmental conditions (Santoyo *et al*., 2016).

In this chapter we utilised *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 as bio-inoculants to promote *Brassica napus* plant growth under salt stress. We investigated the ability of these strains to reduce or alleviate salt stress in *Brassica napus* by evaluating antioxidant enzyme response during the overproduction of ROS molecules such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and superoxide radical (O_2^-). Additionally, we investigated cell death, lipid

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peroxidation and chlorophyll content. Furthermore, we investigated their ability to aid a plant during nutrient acquisition under salt stress. Previous studies have shown plant growth promotion by utilising *Klebsiella, Enterobacter and Bacillus* spp., however to our knowledge, this is the first study utilising *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 as bio-inoculants to promote *Brassica napus* plant growth under salt stress.

4.2 Results

4.2.1 *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increases the germination percentage of *Brassica napus* under salt stress

Brassica napus were grown and treated as described in section 2.15. The germination percentage of *Brassica napus* was calculated after 10 days as germinating seedlings may take 4 - 10 days to emerge. The results show that *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increased germination percentage of *Brassica napus* under saline and non-saline conditions (Table 4.1). Under axenic conditions, *Brassica napus* displayed a 23% and 11% increase in germination rate on day 5 and 6 compared to saline conditions. Evidence suggests that salt stress inhibits seedling emergence. Candidates E7, E8 and E9 displayed a 33% increase in germination rate after 10 days compared to the control. Evidence suggests that utilization of endophytic bio-inoculum increases seedling emergence. Under saline conditions, these strains displayed a 44% increase in germination rate after 10 days compared to *Brassica napus* grown in saline conditions without endophytic inoculum. Evidence suggests that utilization of endophytic bio-inoculum increases seedling emergence under salt stress.

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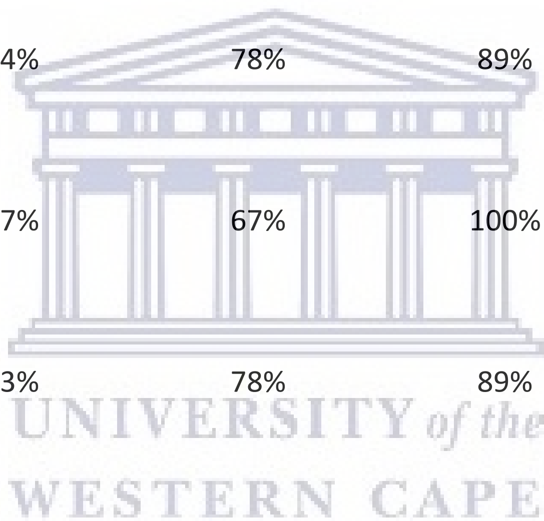
Table 4.1: Germination percentage of *Brassica napus* under saline conditions after 10 days.

	4 days	5 days	6 days	10 days
Control	11%	56%	67%	67%
Salt	11%	33%	56%	56%
***Klebsiella aerogenes* strain KCTC 2190**	0%	56%	100%	100%
***Klebsiella aerogenes* strain KCTC 2190 + salt**	0%	56%	78%	100%
***Enterobacter kobei* strain CIP 105566**	44%	78%	89%	100%
***Enterobacter kobei* strain CIP 105566 + salt**	67%	67%	100%	100%
***Bacillus shackletonii* strain LMG 18435**	33%	78%	89%	100%
***Bacillus shackletonii* strain LMG 18435 + salt**	22%	67%	100%	100%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4.2.2 Observational changes in plant physiology under salt stress

Klebsiella aerogenes strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increased *Brassica napus* plant growth under saline and non-saline conditions as observed in (Figure 4.1), (Figure 4.2) and (Figure 4.3) respectively. Under non-saline conditions, *Brassica napus* displayed an increase in plant growth compared to saline conditions. Under saline conditions, *Brassica napus* displayed plant growth inhibition compared to non-saline conditions. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increased *Brassica napus* plant growth compared to the control. Plants inoculated with *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 displayed salt stress alleviation compared to un-inoculated plants. These observations can be seen in (Figure 4.1), (Figure 4.2) and (Figure 4.3) respectively.

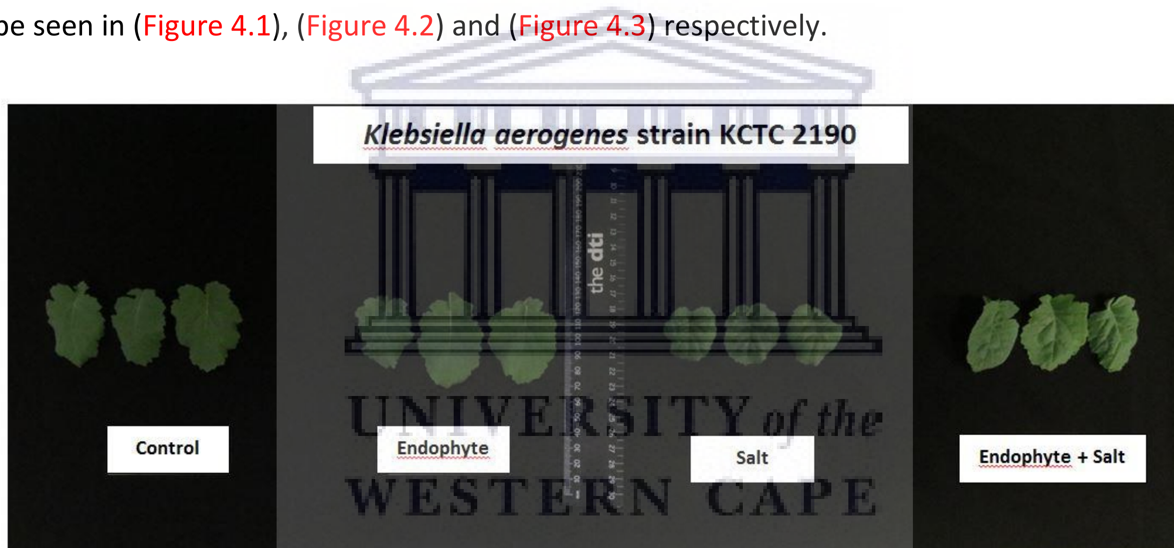

Figure 4.1: Observational changes in plant physiology of *Brassica napus* leaves as per the effect of utilizing *Klebsiella aerogenes* strain KCTC 2190 as a bio-inoculant under salt stress. *Klebsiella aerogenes* strain KCTC 2190 promotes *Brassica napus* plant growth under saline and non-saline conditions.

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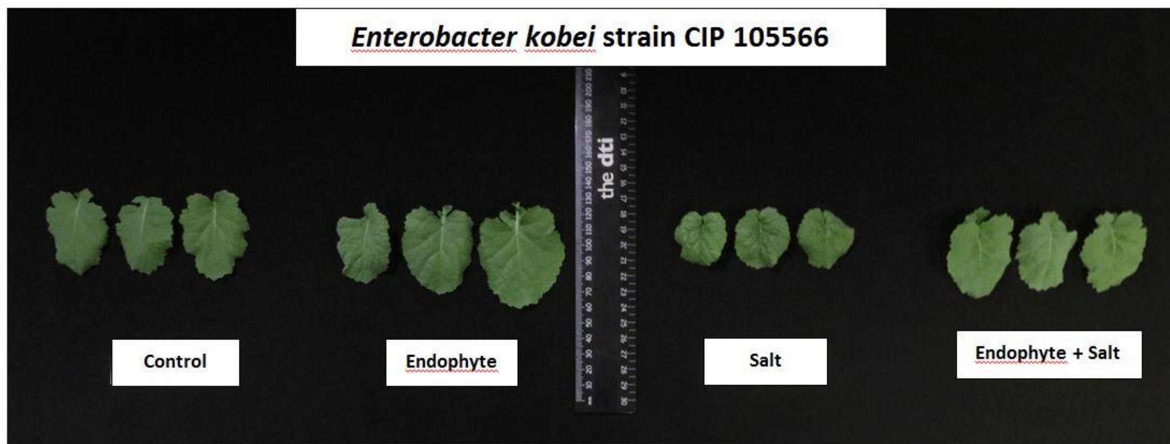

Figure 4.2: Observational changes in plant physiology of *Brassica napus* leaves as per the effect of utilizing *Enterobacter kobei* strain CIP 105566 as a bio-inoculant under salt stress. *Enterobacter kobei* strain CIP 105566 promotes *Brassica napus* plant growth under saline and non-saline conditions.

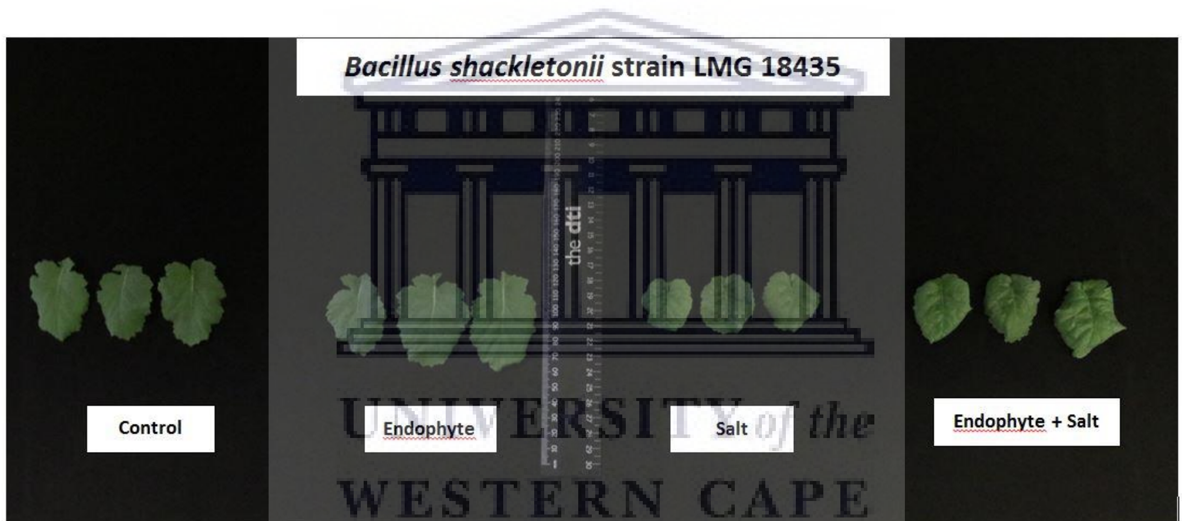

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Figure 4.3: Observational changes in plant physiology of *Brassica napus* leaves as per the effect of utilizing *Bacillus shackletonii* strain LMG 18435 as a bio-inoculant under salt stress. *Bacillus shackletonii* strain LMG 18435 promotes *Brassica napus* plant growth under saline and non-saline conditions.

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4.2.3 *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increases leaf dry weight of *Brassica napus* under saline and non-saline conditions

Klebsiella aerogenes strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increased *Brassica napus* leaf biomass under saline and non-saline conditions. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 compared to the axenic control. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increased *Brassica napus* leaf biomass by 5%, 36% and 20% compared to the axenic control. *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 displayed a statistical difference compared to the axenic control. The salt-induced control displayed *Brassica napus* leaf biomass inhibition compared to the axenic control. The salt-induced control reduced *Brassica napus* leaf biomass by 47% compared to the axenic control. The salt-induced control displayed a statistical difference compared to the axenic control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increased *Brassica napus* leaf biomass compared to the salt-induced control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increased *Brassica napus* leaf biomass by 49%, 53% and 30% compared to the salt-induced control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 displayed a statistical difference compared to the salt-induced control.

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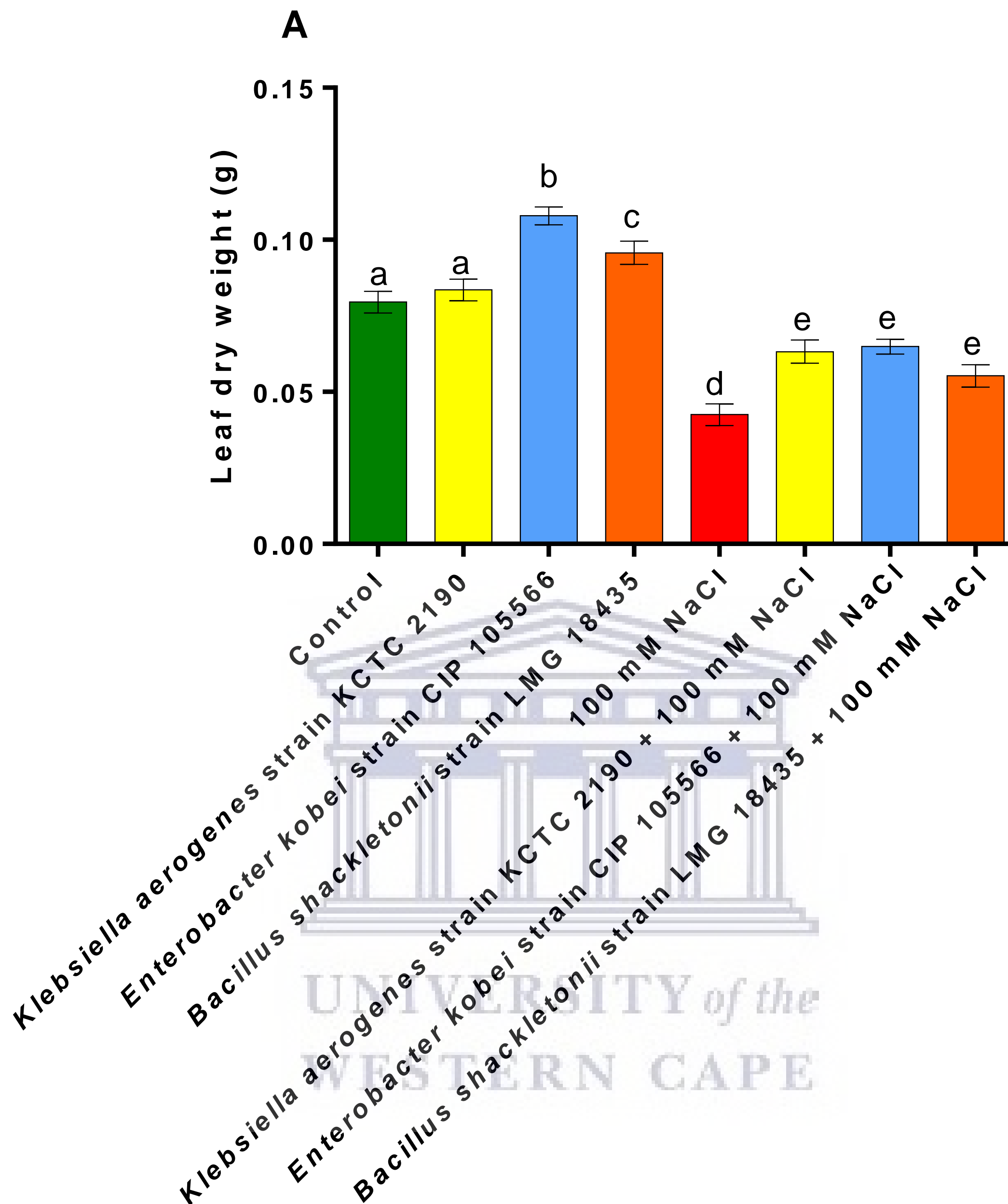

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Figure 4.4: The effect of *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 on the leaf dry weight of *Brassica napus* under saline and non-saline conditions. Statistical analysis was performed using one-way ANOVA, where significance was represented by a $P < 0.05$. Different letters indicate significant differences between means at $P < 0.05$. Values are means ± S.E (n=3).

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4.2.4 *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increases *Brassica napus* chlorophyll content under saline and non-saline conditions

Klebsiella aerogenes strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increased *Brassica napus* chlorophyll content under saline and non-saline conditions. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increased *Brassica napus* chlorophyll content compared to the axenic control. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increased *Brassica napus* chlorophyll content by 3%, 14% and 5% compared to the axenic control. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 displayed no significant statistical difference compared to the axenic control. The salt-induced control displayed *Brassica napus* chlorophyll content inhibition compared to the axenic control. The salt-induced control reduced *Brassica napus* chlorophyll content by 80% compared to the axenic control. The salt-induced control displayed a statistical difference compared to the axenic control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increased *Brassica napus* chlorophyll content compared to the salt-induced control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increased *Brassica napus* chlorophyll content by 251%, 354% and 249% compared to the salt-induced control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 displayed a statistical difference compared to the salt induced control.

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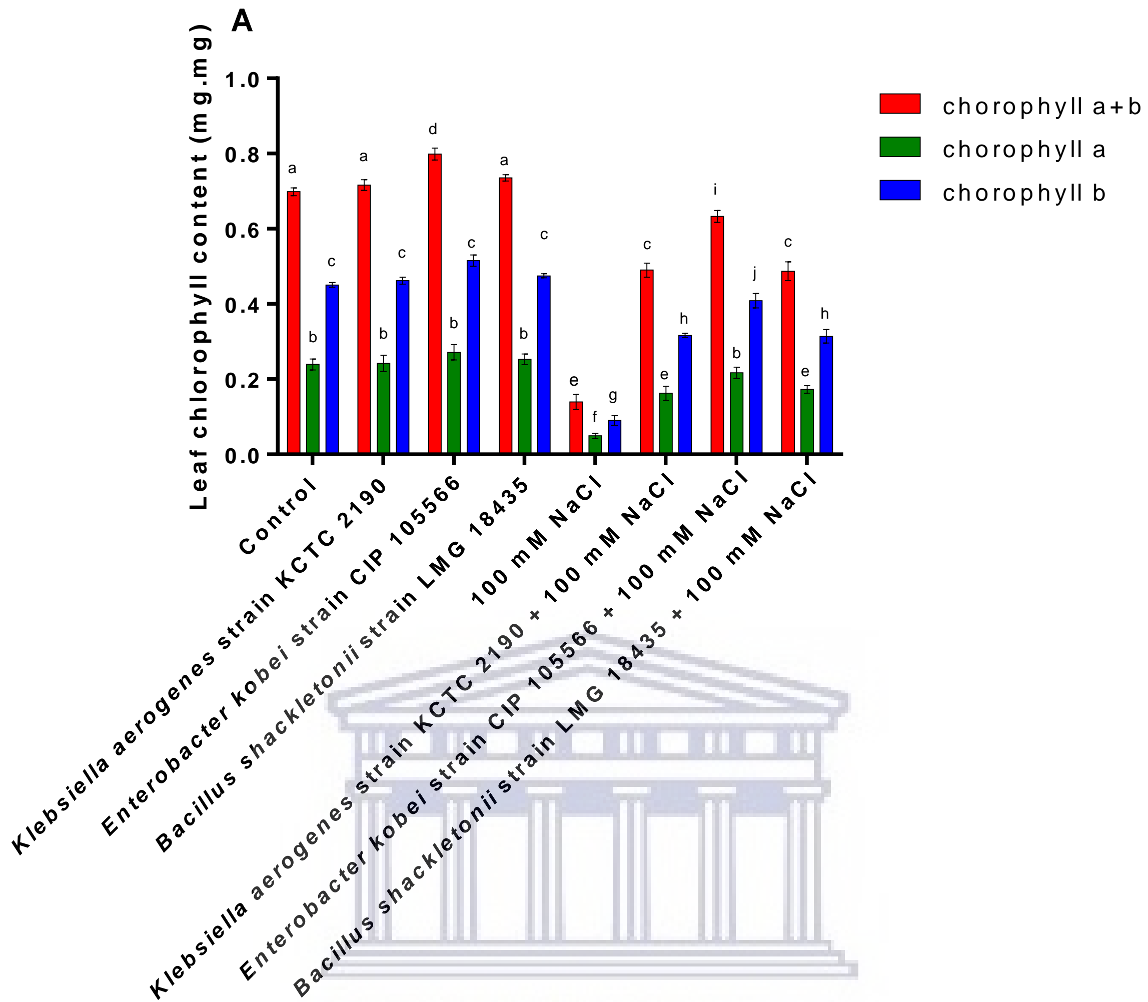

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Figure 4.5: The effect of *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 on *Brassica napus* chlorophyll content under saline and non-saline conditions. Statistical analysis was performed using one-way ANOVA, where significance was represented by a $P< 0.05$. Different letters indicate significant differences between means at $P< 0.05$. Values are means ± S.E (n=3).

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4.2.5 *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 reduces *Brassica napus* leaf lipid peroxidation under saline and non-saline conditions

Klebsiella aerogenes strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG reduced *Brassica napus* leaf lipid peroxidation under saline and non-saline conditions. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 reduced lipid peroxidation compared to the axenic control. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 reduced lipid peroxidation by 35%, 58% and 42% compared to the axenic control. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 displayed a statistical difference compared to the axenic control. The salt-induced control increased lipid peroxidation compared to the axenic control. The salt-induced control increased lipid peroxidation by 55% compared to the axenic control. The salt-induced control displayed a statistical difference compared to the axenic control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435, reduced lipid peroxidation compared to the salt-induced control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435, reduced lipid peroxidation by 49%, 54% and 36% compared to the salt-induced control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 displayed a statistical difference compared to the salt-induced control.

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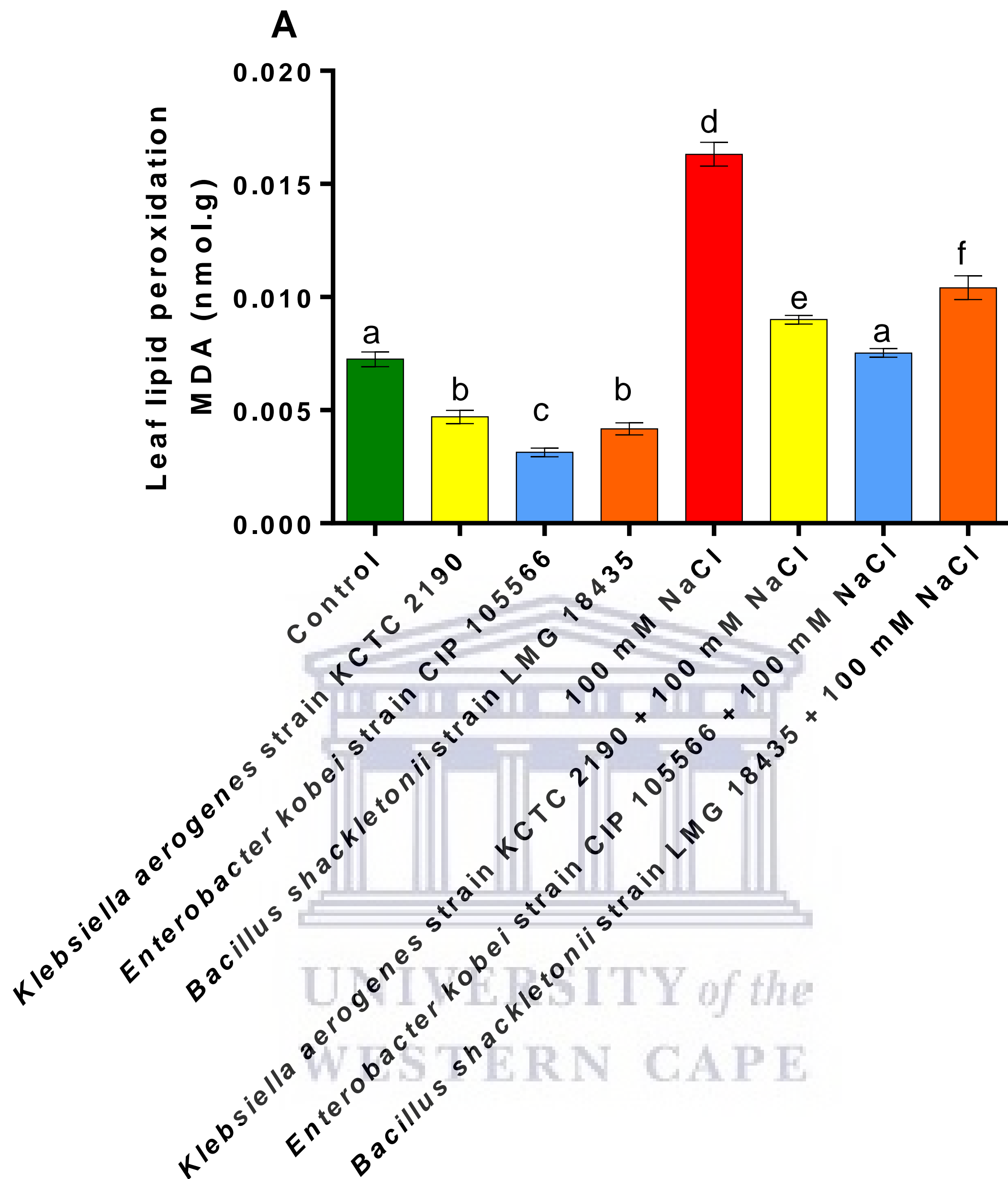

Figure 4.6: The effect of *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 on the leaf lipid peroxidation of *Brassica napus* under saline and non-saline conditions. Statistical analysis was performed using one-way ANOVA, where significance was represented by a P< 0.05. Different letters indicate significant differences between means at P< 0.05. Values are means ± S.E (n=3).

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4.2.6 *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 reduces *Brassica napus* leaf cell death under saline and non-saline conditions

Klebsiella aerogenes strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG reduced *Brassica napus* leaf lipid peroxidation under saline and non-saline conditions. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 reduced cell death compared to the axenic control. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 reduced cell death by 3%, 28% and 16% compared to the axenic control. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 displayed no statistical difference compared to the axenic control. The salt-induced control increased cell death compared to the axenic control. The salt-induced control increased cell death by 197% compared to the axenic control. The salt-induced control displayed a statistical difference compared to the axenic control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435, reduced cell death compared to the salt-induced control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435, reduced cell death by 50%, 53% and 26% compared to the salt-induced control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 displayed a statistical difference compared to the salt-induced control.

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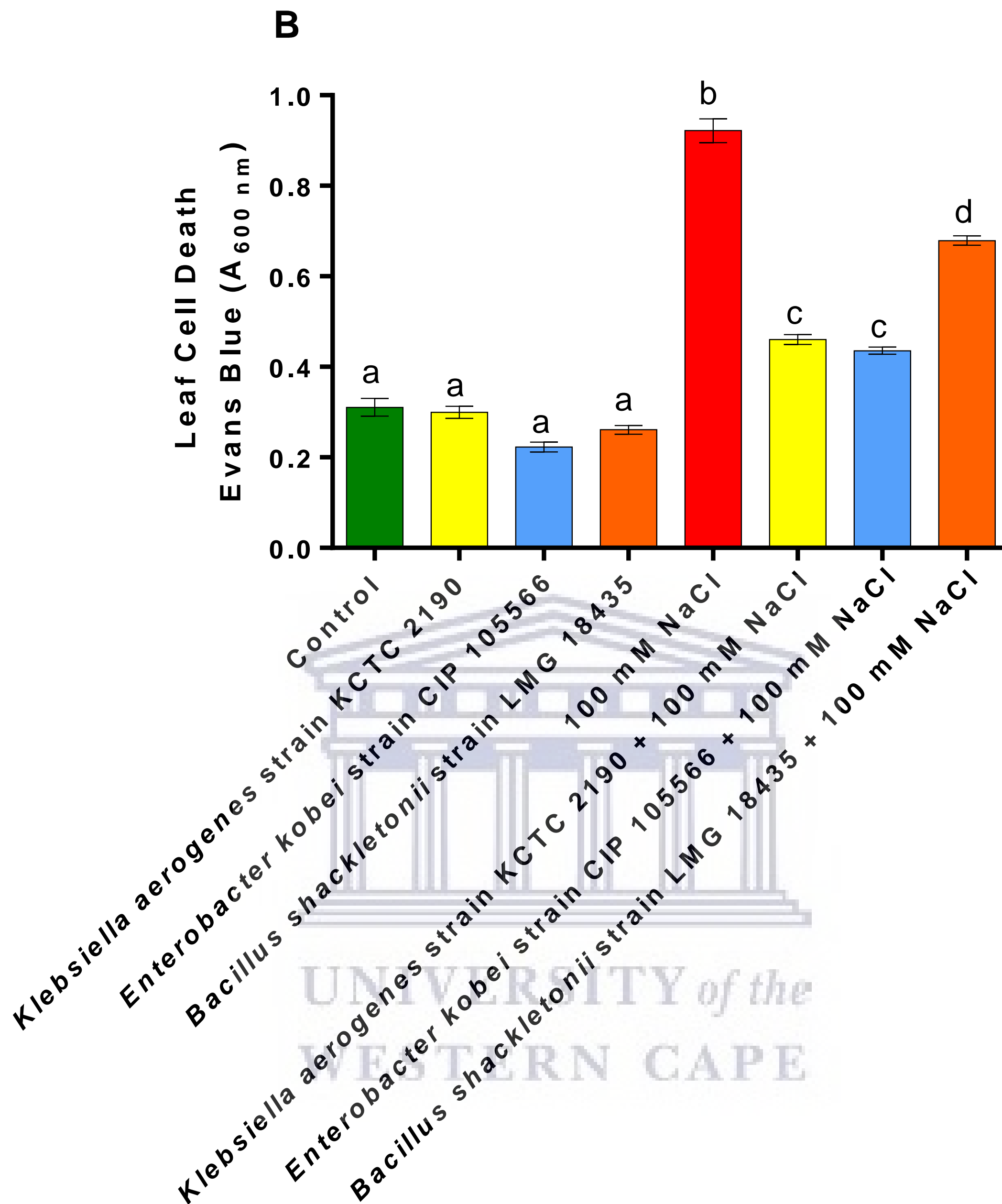

Figure 4.7: The effect of *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 on the leaf cell death of *Brassica napus* under saline and non-saline conditions. Statistical analysis was performed using one-way ANOVA, where significance was represented by a P< 0.05. Different letters indicate significant differences between means at P< 0.05. Values are means ± S.E (n=3).

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4.2.7 *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 reduces *Brassica napus* leaf H_2O_2 content under saline and non-saline conditions

Klebsiella aerogenes strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG reduced *Brassica napus* leaf H_2O_2 content under saline and non-saline conditions. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 reduced H_2O_2 content compared to the axenic control. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 reduced H_2O_2 content by 34%, 50% and 36% compared to the axenic control. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 displayed a statistical difference compared to the axenic control. The salt-induced control increased H_2O_2 content compared to the axenic control. The salt-induced control increased H_2O_2 content by 87% and displayed a statistical difference compared to the axenic control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435, reduced H_2O_2 content compared to the salt-induced control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435, reduced H_2O_2 content by 28%, 43% and 17% compared to the salt-induced control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 displayed a statistical difference compared to the salt-induced control.

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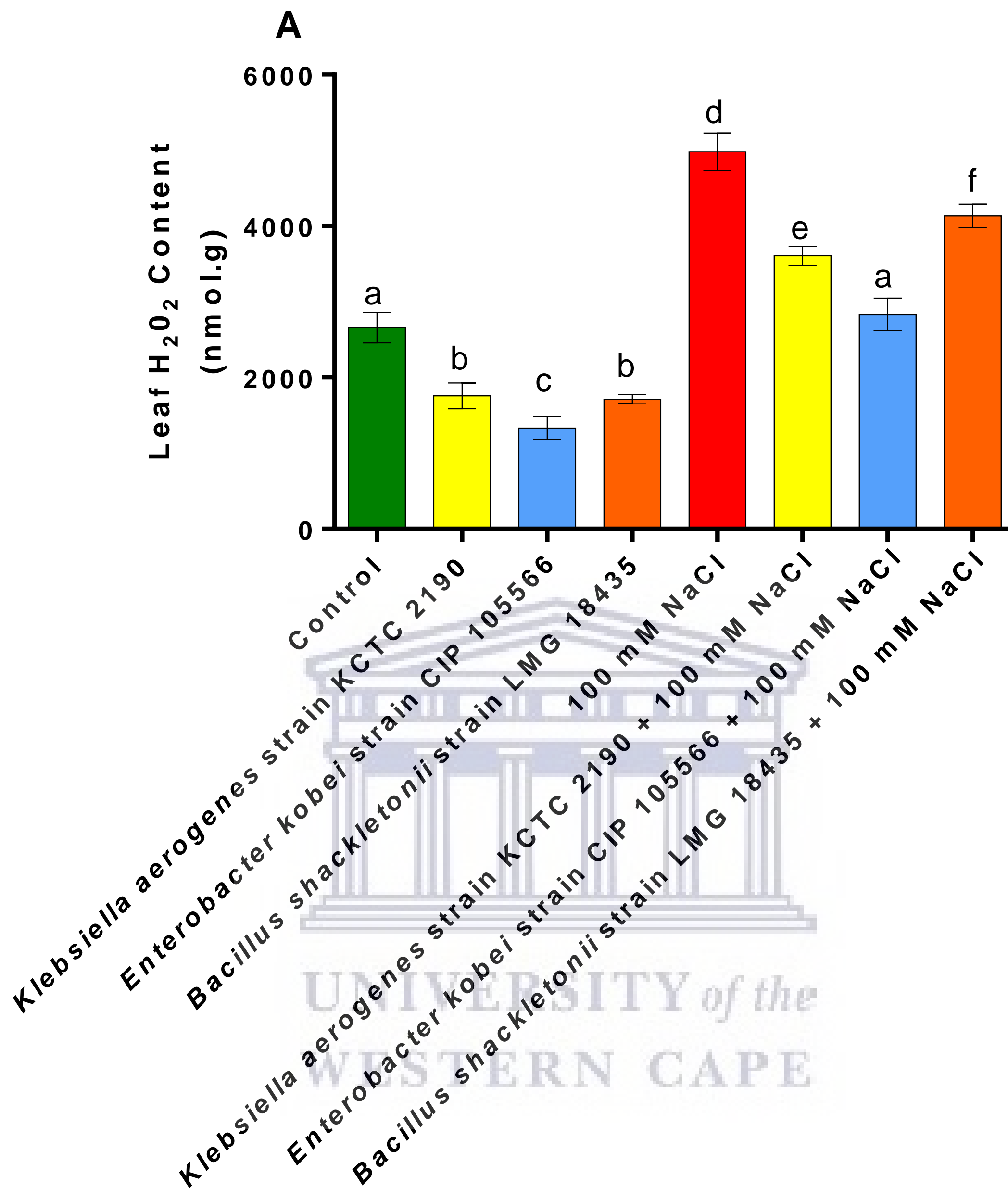

Figure 4.8: The effect of *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 on the leaf H_2O_2 content of *Brassica napus* under saline and non-saline conditions. Statistical analysis was performed using one-way ANOVA, where significance was represented by a P< 0.05. Different letters indicate significant differences between means at P< 0.05. Values are means ± S.E (n=3).

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4.2.8 ***Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 reduces *Brassica napus* leaf O_2^- content under saline and non-saline conditions**

Klebsiella aerogenes strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG reduced *Brassica napus* leaf O_2^- content under saline and non-saline conditions. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 reduced O_2^- content compared to the axenic control. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 reduced O_2^- content by 22%, 44% and 31% compared to the axenic control. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 displayed a statistical difference compared to the axenic control. The salt-induced control increased O_2^- content compared to the axenic control. The salt-induced control increased O_2^- content by 47% compared to the axenic control. The salt-induced control displayed a statistical difference compared to the axenic control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435, reduced O_2^- content compared to the salt-induced control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435, reduced O_2^- content by 20%, 30% and 14% compared to the salt-induced control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 displayed a statistical difference compared to the salt induced control.

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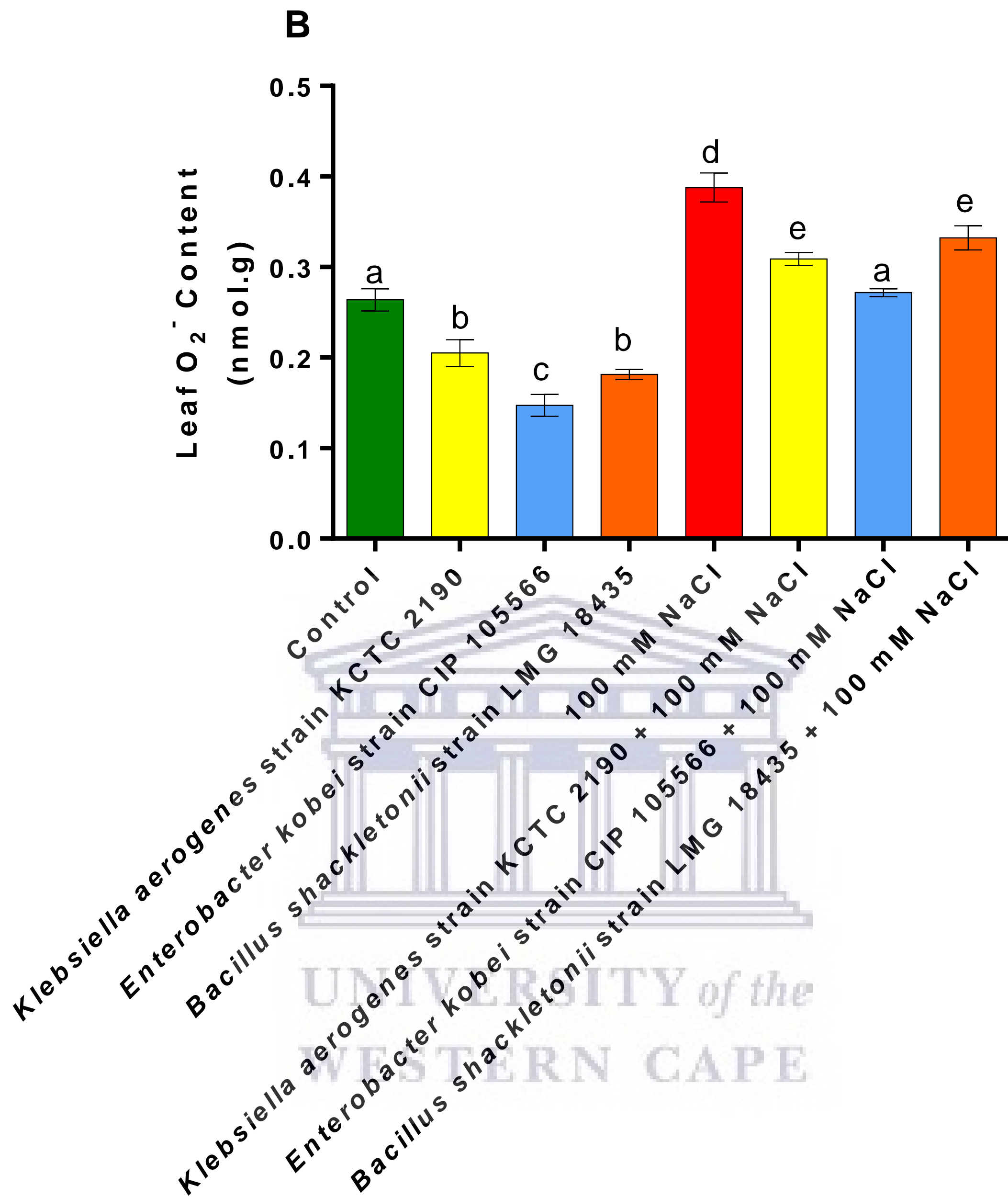

Figure 4.9: The effect of *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 on the leaf O_2^- content of *Brassica napus* under saline and non-saline conditions. Statistical analysis was performed using one-way ANOVA, where significance was represented by a $P < 0.05$. Different letters indicate significant differences between means at $P < 0.05$. Values are means ± S.E (n=3).

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4.2.9 ***Klebsiella aerogenes*** **strain KCTC 2190,** ***Enterobacter kobei*** **strain CIP 105566 and** ***Bacillus shackletonii*** **strain LMG 18435 reduces** ***Brassica napus*** **leaf OH^- content under saline and non-saline conditions**

Klebsiella aerogenes strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG reduced *Brassica napus* leaf OH^- content under saline and non-saline conditions. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 reduced OH^- content compared to the axenic control. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 reduced OH^- content by 14%, 27% and 18% compared to the axenic control. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 displayed a statistical difference compared to the axenic control. The salt-induced control increased OH^- content compared to the axenic control. The salt-induced control increased OH^- content by 70% compared to the axenic control. The salt-induced control displayed a statistical difference compared to the axenic control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435, reduced OH^- content compared to the salt-induced control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435, reduced OH^- content by 32%, 41% and 19% compared to the salt induced control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 displayed a statistical difference compared to the salt-induced control.

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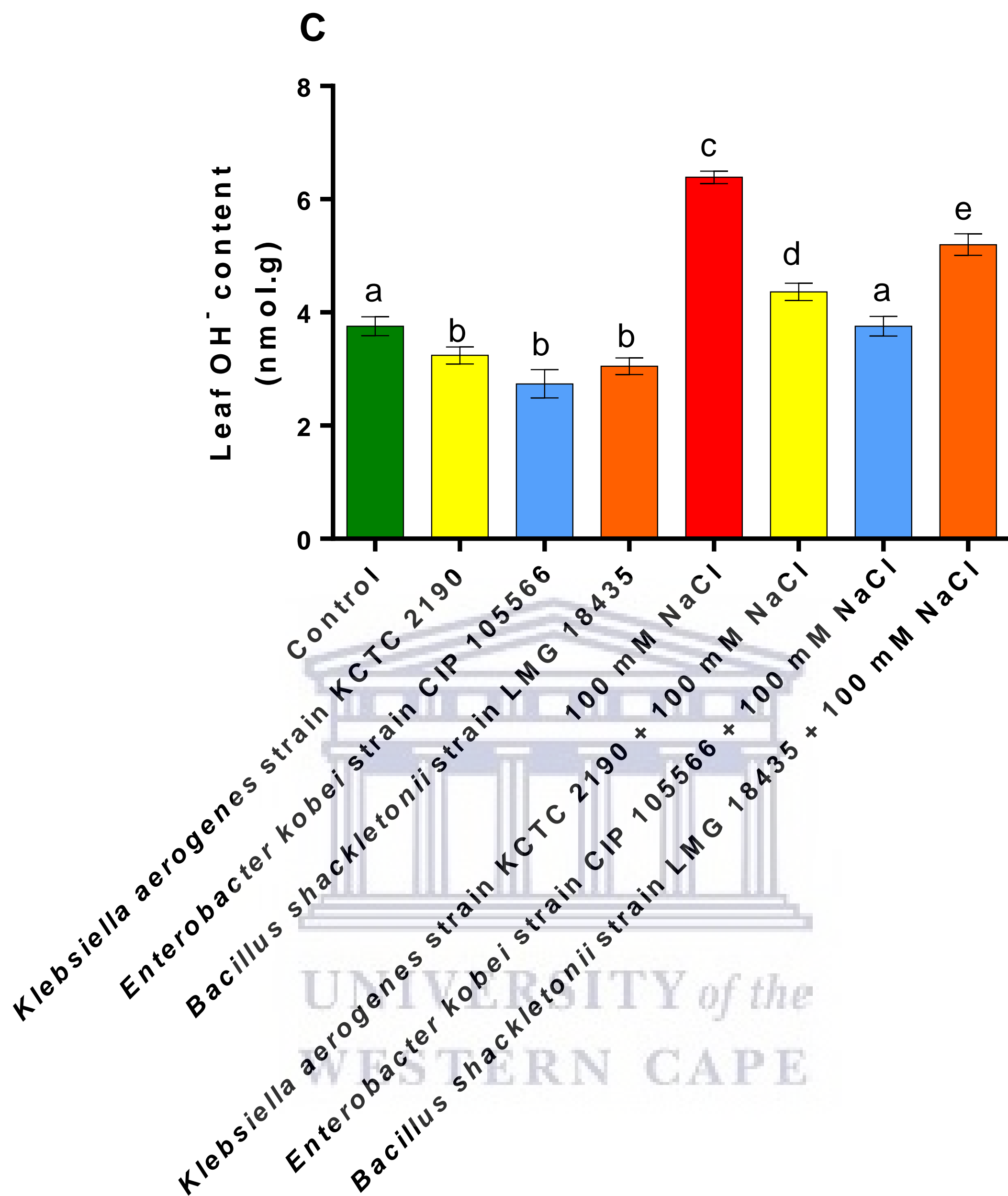

Figure 4.10: The effect of *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 on the leaf OH^- content of *Brassica napus* under saline and non-saline conditions. Statistical analysis was performed using one-way ANOVA, where significance was represented by a P< 0.05. Different letters indicate significant differences between means at P< 0.05. Values are means ± S.E (n=3).

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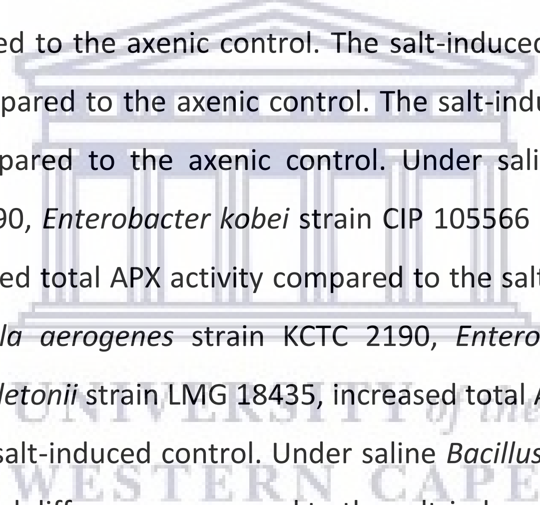
4.2.10 ***Klebsiella aerogenes*** **strain KCTC 2190,** ***Enterobacter kobei*** **strain CIP 105566 and** ***Bacillus shackletonii*** **strain LMG 18435 increases** ***Brassica napus*** **total leaf APX activity under saline and non-saline conditions**

Klebsiella aerogenes strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG increased *Brassica napus* total leaf APX activity under saline and non-saline conditions. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increased total APX activity compared to the axenic control. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increased total APX activity by 132%, 251% and 218% compared to the axenic control. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 displayed a statistical difference compared to the axenic control. The salt-induced control increased total APX activity compared to the axenic control. The salt-induced control increased total APX activity by 269% compared to the axenic control. The salt-induced control displayed a statistical difference compared to the axenic control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435, increased total APX activity compared to the salt induced control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435, increased total APX activity by 13%, 72% and 1% compared to the salt-induced control. Under saline *Bacillus shackletonii* strain LMG 18435 displayed a statistical difference compared to the salt-induced control.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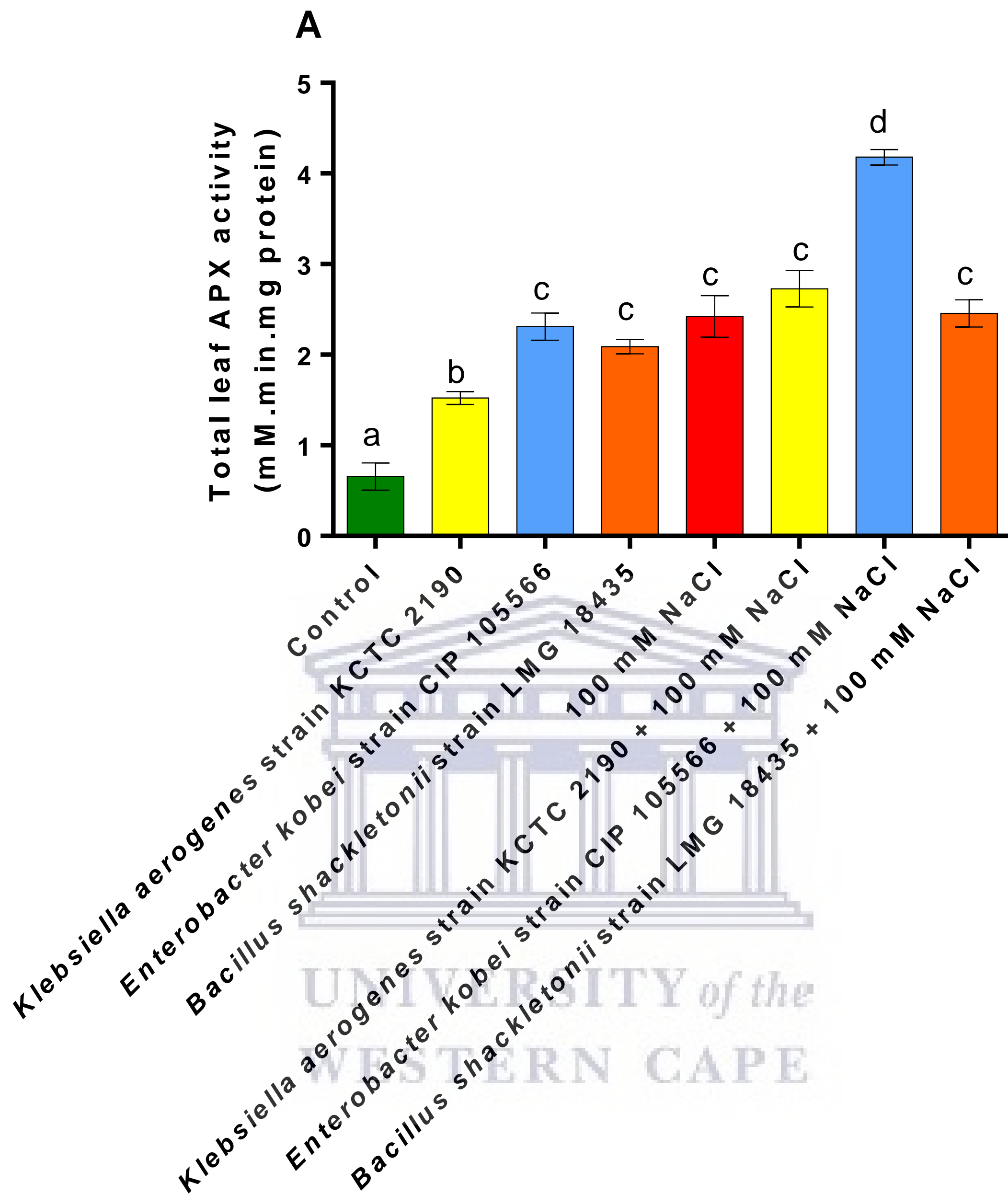

Figure 4.11: The effect of *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 on the total leaf APX activity of *Brassica napus* under saline and non-saline conditions. Statistical analysis was performed using one-way ANOVA, where significance was represented by a $P < 0.05$. Different letters indicate significant differences between means at $P < 0.05$. Values are means ± S.E (n=3).

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4.2.11 ***Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increases *Brassica napus* total leaf CAT activity under saline and non-saline conditions**

Klebsiella aerogenes strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG increased *Brassica napus* total leaf CAT activity under saline and non-saline conditions. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increased total CAT activity compared to the axenic control. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increased total CAT activity by 23%, 127% and 79% compared to the axenic control. *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 displayed a statistical difference compared to the axenic control. The salt-induced control increased total CAT activity compared to the axenic control. The salt-induced control increased total CAT activity by 138% compared to the axenic control. The salt-induced control displayed a statistical difference compared to the axenic control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435, increased total CAT activity compared to the salt-induced control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435, increased total CAT activity by 50%, 228% and 40% compared to the salt-induced control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 displayed a statistical difference compared to the salt-induced control.

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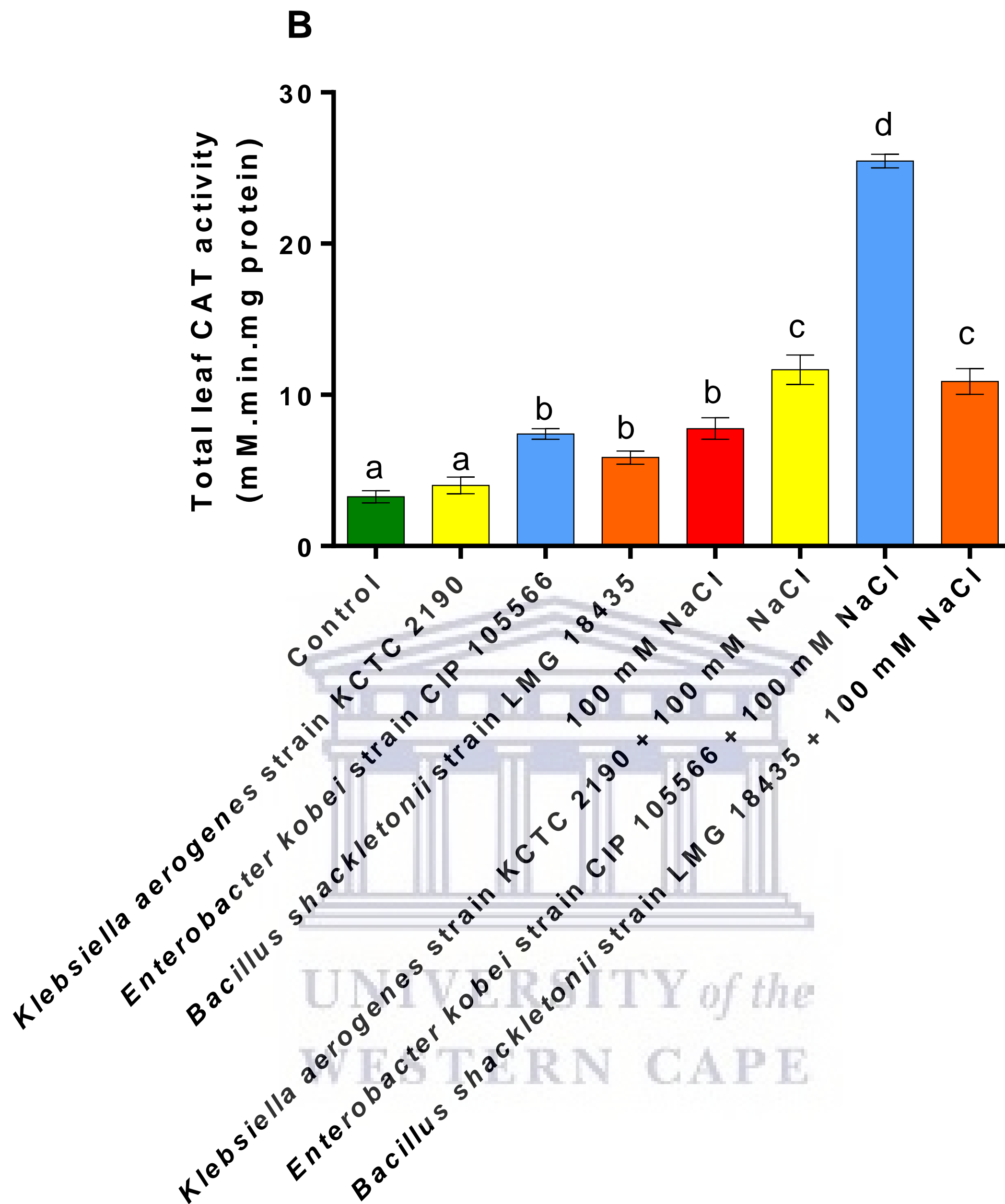

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Figure 4.12: The effect of *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 on the total leaf CAT activity of *Brassica napus* under saline and non-saline conditions.tatistical analysis was performed using one-way ANOVA, where significance was represented by a $P< 0.05$. Different letters indicate significant differences between means at $P< 0.05$. Values are means ± S.E (n=3).

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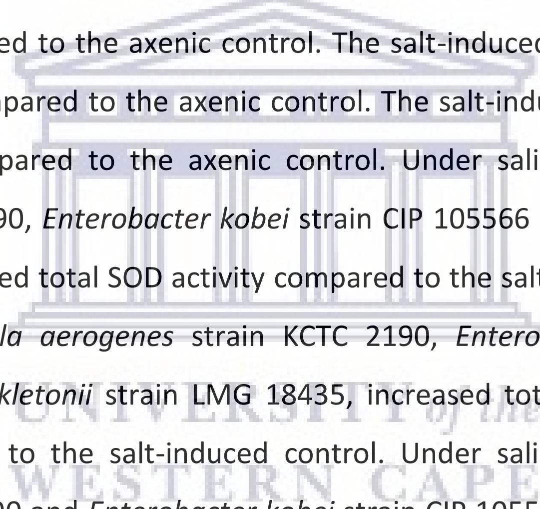
4.2.12 ***Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increases *Brassica napus* total leaf SOD activity under saline and non-saline conditions**

Klebsiella aerogenes strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG increased *Brassica napus* total leaf SOD activity under saline and non-saline conditions. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increased total SOD activity compared to the axenic control. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increased total SOD activity by 198%, 279% and 233% compared to the axenic control. *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 displayed a statistical difference compared to the axenic control. The salt-induced control increased total SOD activity compared to the axenic control. The salt-induced control increased total SOD activity by 290% compared to the axenic control. The salt-induced control displayed a statistical difference compared to the axenic control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435, increased total SOD activity compared to the salt-induced control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435, increased total SOD activity by 38%, 127% and 1% compared to the salt-induced control. Under saline conditions *Klebsiella aerogenes* strain KCTC 2190 and *Enterobacter kobei* strain CIP 105566 displayed a statistical difference compared to the salt-induced control.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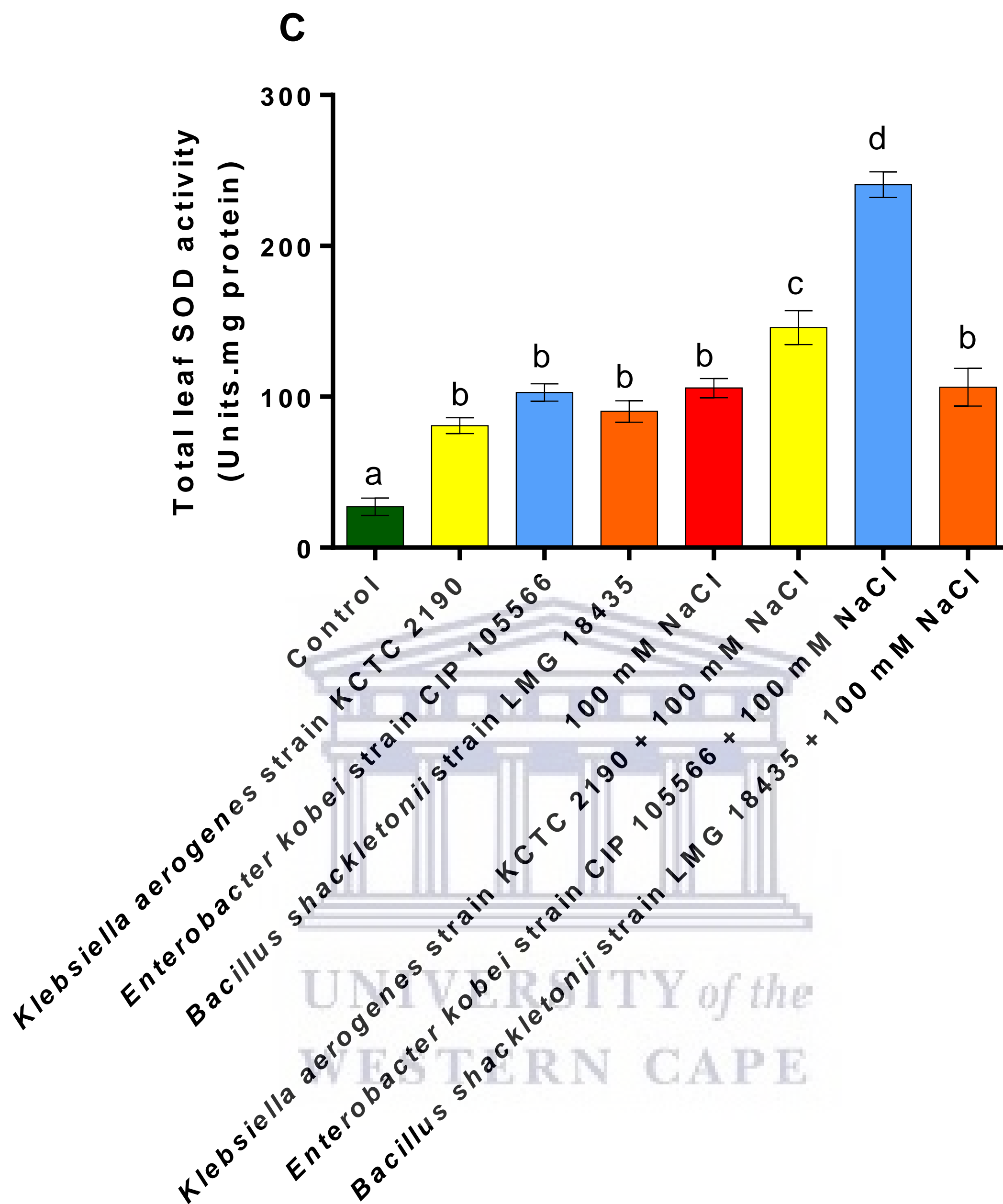

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Figure 4.13: The effect of *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 on the total SOD leaf activity of *Brassica napus* under saline and non-saline conditions. Statistical analysis was performed using one-way ANOVA, where significance was represented by a $P < 0.05$. Different letters indicate significant differences between means at $P < 0.05$. Values are means ± S.E (n=3).

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4.2.13 *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 increases micro and macro elements under saline soil conditions

In Table 4, yellow represents the midpoint, red represents a decrease and green represents an increase. *Klebsiella aerogenes* strain KCTC 2190 augmented K, Ca, Mg, Zn, Mn and Fe by 2.4029 ppm, 8.7251 ppm, 0.9382 ppm, 0.0133 ppm, 0.2240 ppm and 0.9027 ppm respectively compared to 100 mM NaCl induced soil. *Klebsiella aerogenes* strain KCTC 2190 reduced P and Cu by 0.5713 ppm and 0.0064 ppm compared to 100 mM NaCl induced soil. *Enterobacter kobei* strain CIP 105566 augmented K, Ca, Mg, P, Zn, Mn and Fe by 4.6965 ppm, 35.882 ppm, 1.1897 ppm, 0.2527 ppm, 0.0315 ppm, 0.0752 ppm and 1.2128 ppm respectively compared to 100 mM NaCl induced soil. *Enterobacter kobei* strain CIP 105566 reduced Cu by 0.0034 ppm. *Bacillus shackletonii* strain LMG 18435 augmented K, Ca, Mg, P, Mn and Fe by 1.4592 ppm, 13.1264 ppm, 0.2911 ppm, 0.7982 ppm, 0.0737 ppm, 2.0393 ppm respectively compared to 100 mM NaCl induced soil. *Bacillus shackletonii* strain LMG 18435 reduced Cu and Zn by 0.008 ppm and 0.0161 ppm respectively compared to 100 mM NaCl induced soil.

Table 4.2: Quantification of saline soil macro and micro elements via inductively coupled plasma optical emission spectrometry (ICP-OES). Table 4.2 represents the quantification of saline soil micro (blue) and macro (purple) elements (part per million) that was conducted in triplicate. Different letters indicate significant differences between means at $p < 0.05$ as per row. Values are means ± S.E (n=3).

Element	100 mM NaCl	*Klebsiella aerogenes*	*Enterobacter aerogenes*	*Bacillus shackletonii*
K	5.0004[a]	7.4033[b]	9.6969[c]	6.4596[b]
Ca	65.4920[a]	74.2171[b]	101.3740[c]	78.6184[b]
Mg	4.7131[a]	5.6514[a]	5.9028[a]	5.0043[a]
P	4.6630[a]	4.0917[a]	4.9157[a]	5.4612[a]
Cu	0.0623[a]	0.0559[a]	0.0588[a]	0.0543[a]
Zn	0.4454[a]	0.4587[a]	0.4770[a]	0.4292[a]
Mn	0.4265[a]	0.6505[a]	0.5017[a]	0.5002[a]
Fe	3.2566[a]	4.1593[a]	4.4695[a]	5.2960[a]

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4.3 Discussion

In this chapter, we utilised *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 as a bio-inoculant to promote *Brassica napus* plant growth under saline and non-saline conditions by investigating their ability to modulate ROS production and antioxidant response. Furthermore, we investigated their ability to aid a plant during nutrient acquisition under saline and non-saline conditions. The results displayed observational changes in plant morphology when utilizing *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 as a bio-inoculant during growth trials conducted in the greenhouse. Utilizing *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 promoted *Brassica napus* plant growth when compared to the axenic control. Salt stress inhibited *Brassica napus* plant growth when compared to the axenic control. However, plants supplemented with *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 displayed *Brassica napus* plant growth promotion under saline conditions when compared to the salt-induced control. The biochemical results in this study indicates that *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 reduced ROS production and increased antioxidant response. Furthermore, *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 increased macro and micro nutrients under saline and non-saline conditions, which are required by a plant for growth and development.

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4.3.1 *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 improves *Brassica napus* germination rate under saline and non-saline conditions

In this chapter we have again shown that *Brassica napus* produces a low yield due to its high susceptibility (Table 4.1). However, *Brassica napus* supplemented with *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 augmented germination percentage when compared to the axenic control (Table 4.1). The low germination percentage of *Brassica napus* under axenic conditions were even further reduced when plants were exposed to (100 mM NaCl) salt stress (Table 4.1). Numan et al (2018) stated that high levels of salinity have harmful effects on different stages of a plants life cycle such as seed germination. However, *Brassica napus* supplemented with *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 under salt stress showed augmented germination percentage compared to the salt-induced control (Table 4.1). A study conducted by Hussein and Joo (2018) showed that seeds coated with PGPB, increased seed germination and growth during salt stress.

The increase in germination percentage could be due to colonisation between seed and microbe which was explained in section 3.3.1. Furthermore, Ilangumaran and Smith (2017) explained that PGPB secretes exopolysaccharides (EPS) responsible for attachment to soil particles forming an enclosed matrix of microcolonies which provides protection against environmental fluctuations, water and nutrient retention. Egamberdieva et al (2019) stated that EPS improves plant yield by acting as a seed priming agent which enhances germination. Furthermore, Sandhya and Ali (2015) stated that EPS acts as a physical barrier around the roots and support plant growth during high salt stress (Ilangumaran and Smith, 2017). A study conducted by Yang et al (2016) showed that quinoa seeds inoculated with *Enterobacter* sp. and *Bacillus* sp. improved germination under 400 mM NaCl conditions (Ilangumaran and Smith, 2017). Evidence suggests that *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 could possibly possess the prerequisites for colonisation mentioned in section 3.3.1 and the ability to secrete EPS. This may be observed by the germination percentage rate of each strain on *Brassica napus* under saline and non-saline conditions.

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4.3.2 *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 improves *Brassica napus* plant growth and plant biomass under saline and non-saline conditions

In this chapter we have shown that *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 increased *Brassica napus* plant growth under saline and non-saline conditions (Figure 4.1), (Figure 4.2) and (Figure 4.3). Induced exposure to salt stress (100 mM NaCl) caused plant growth inhibition and leaf curling when compared to the axenic control (Figure 4.1), (Figure 4.2) and (Figure 4.3). Previous reports showed that salt stress affected the growth of soybean, wheat, pepper and sorghum (Khan *et al*., 2019). However, *Brassica napus* supplemented with *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 under salt stress augmented plant growth compared to the salt-induced control (Figure 4.1), (Figure 4.2) and (Figure 4.3). Researchers reported that bacteria capable of producing IAA, significantly enhanced plant growth under salt stress (Khan *et al*., 2019). This statement correlate to the findings within this study as the isolates used in this study displayed the ability to produce IAA. Khan et al (2019) stated that PGPB inoculum contains a sufficient amount of growth regulators that may influence future root growth, cell elongation, tissue differentiation, plant growth and development. A similar trend to the trend observed during plant growth and development can be seen when evaluating the effect of *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 on *Brassica napus* plant biomass under saline and non-saline conditions (Figure 4.4). Furthermore, *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 displayed the ability to increase macro and micro nutrient availability within saline and non-saline soil (Table 4.2) and (Table 4.3) which could aid the plant during growth and development (Figure 4.1), (Figure 4.2) and (Figure 4.3). Khan et al (2019) stated that IAA producing bacteria increased root length which offered plants better access to nutrients available in the soil. The ability of *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 to promote *Brassica napus* plant growth and development could be due to their ability to produce IAA (Table 3.2).

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These results can be supplemented by the following statements and findings. Studies have shown that phytohormones produced by PGPB plays an essential role in the modulation of plant physiology under salt stress (Egamberdieva *et al*., 2019). Glick (2012) stated that PGPB may aid plants to overcome abiotic stress by providing the plant with IAA that directly stimulates plant growth even in the presence of other inhibitory compounds. Furthermore, Glick (2012) described how IAA promotes plant growth when the amino acid tryptophan is excluded by plant roots and then taken up by PGPB where it is converted into IAA. The IAA produced by PGPB is then secreted, taken up by plant cells and together with the plants IAA, induces a auxin signal transduction pathway which includes various auxin response factors. This causes plant cells to grow and proliferate simultaneously as some of the IAA promotes gene transcription encoding the enzyme ACC synthase. Egamberdieva et al (2019) observed that plants inoculated with PGPB increased mineral uptake, plant protection and enhanced plant growth under saline conditions. A study conducted by Yao et al (2010) showed that PGPB modulated IAA synthesis in plant tissue increased the growth parameters of cotton under saline conditions. Some of the best known IAA producing PGPB under salt stress is *Azotobacter*, *Arthrobacter*, *Azospirillum*, *Bacillus*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Rahnella* and *Stenotrophomonas* (Egamberdieva *et al*., 2019).

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4.3.3 *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 improves *Brassica napus* chlorophyll metabolism under saline and non-saline conditions

Chlorophylls are unique photosynthetic pigments that capture solar energy and convert it into chemical energy that is used to build essential carbohydrate molecules used as a food source for plant growth and development (Pareek *et al*., 2017).

In this study we have shown that *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 increased *Brassica napus* chlorophyll metabolism under saline and non-saline conditions (Figure 4.5). Induced exposure to salt stress (100 mM NaCl) caused the reduction of chlorophyll a, chlorophyll b and total chlorophyll content when compared to the axenic control (Figure 4.5). When plants are exposed to salt stress it stimulates an enzyme called chlorophyllase which degrades protein pigments, resulting in photoinhibition (Heidari, 2012). The reduction in

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chlorophyll content due to salt stress has been detected in *Sesbania sesban* (Abd_Allah *et al.*, 2016), *Ephedra alata* (Alqarawi *et al.*, 2014) and *C. arietinum* (Rasool *et al.*, 2013). However, *Brassica napus* supplemented with *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 under salt stress augmented chlorophyll metabolism compared to the salt-induced control (Figure 4.5). Salt stress has been shown to cause stunted plant growth due to the de novo synthesis of proteins and chlorophyll components (El-Tayeb 2005), while *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 interactions increased chlorophyll synthesis through enhanced metabolism and photoassimilates (Abd_Allah *et al.*, 2017). Stefan et al (2013) stated that PGPB has the capability to improve crop resilience under salt stress by regulating growth promoting pathways resulting in proper growth. The results in this study correlates with the results of Stefan et al (2013) and Mohamed and Gomaa (2012) who reported improved runner bean and *Raphanus sativus* plant growth due to PGPB inoculation which caused an increase in chlorophyll synthesis (Abd_Allah *et al.*, 2017). Furthermore, they suggested that an increase in magnesium can aid pigment synthesis and might be one of the key reasons for the increase in chlorophyll content (Abd_Allah *et al.*, 2017). A study conducted by Dawwam et al (2013) reported that changes in N, P and K induced by PGPB stimulate pigment synthesis in potato plants. Similarly, in this study we observed an increase in magnesium, phosphate and calcium induced by PGPB within saline soil which can be seen in (Table 4.3). The protective mechanisms displayed by *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 against the deleterious effects of salt stress might contribute to an enhanced photosynthetic efficiency rate which will maintain the function of the pigment protein complex (Rasool *et al.*, 2013).

Evidence suggests that *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 could possibly alleviate the toxicity of salt stress on photosynthetic machinery through the stimulation of pigment synthesis and their related components.

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4.3.4 *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 reduces *Brassica napus* lipid peroxidation and cell death under saline and non-saline conditions

In this study we have shown that *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 reduced *Brassica napus* lipid peroxidation and cell death under saline and non-saline conditions (Figure 4.6) and (Figure 4.7). Induced exposure to salt stress (100 mM NaCl) affected the integrity of the membrane structure, causing an increase in lipid peroxidation which in turn led to an increase in cell death when compared to the axenic control which can be seen in (Figure 4.6) and (Figure 4.7) respectively. This correlates to the findings of Ahmad et al (2012) who reported membrane damage and membrane dysfunction caused by salt stress. However, *Brassica napus* supplemented with *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 under salt stress reduced lipid peroxidation which in turn led to decreased cell death compared to the salt-induced control which can be seen in (Figure 4.6) and (Figure 4.7) respectively. This correlates to the findings of Han et al (2014) who reported that *Bacillus subtilis* inoculum reduced lipid peroxidation of white clover. Several studies have documented the induced effect of salinity on membrane damage due to the overproduction of ROS which led to lipid peroxidation (Abd_Allah *et al.*, 2017). For example, salt induced riverhemp, basil and mustard displayed increased levels of ROS and lipid peroxidation which inhibited plant growth (Abd_Allah *et al.*, 2017). This correlates to the findings in this study which showed that induced exposure to (100 mM NaCl) salt stress increased the ROS production which can be seen in (Figure 4.8), (Figure 4.9) and (Figure 4.10) and lipid peroxidation (Figure 4.6) which inhibited plant growth (Figure 4.1), (Figure 4.2) and (Figure 4.3). Alqarawi et al (2014) stated that salt stress affects unsaturated poly fatty acid composition which leads to membrane dysfunction.

Evidence suggest that *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 could possibly regulate membrane function by maintaining the optimal ratio between saturated poly fatty acids and unsaturated poly fatty acids and by the reduction of ROS generation.

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4.3.5 *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 reduces *Brassica napus* ROS production under saline and non-saline conditions

Salt stress has severe effects on plant growth and development due to the overproduction of reactive oxygen species (H_2O_2, OH^- and O_2^-) (Numan *et al*., 2018). The overproduction of ROS exceeds the scavenging ability of a plants natural defense system resulting in oxidative stress (Abbas *et al*., 2017). Oxidative stress damages cellular components resulting in their dysfunction and ultimately cell death (Numan *et al*., 2018). Under these conditions plants start the production of enzymatic antioxidants such as APX, CAT and SOD to scavenge the overproduction of ROS (Rasool *et al*., 2012).

In this study we have shown that *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 reduced the production of ROS molecules (H_2O_2, OH^- and O_2^-) under saline and non-saline conditions (Figure 4.8), (Figure 4.9) and (Figure 4.10) respectively. Induced exposure to salt stress (100 mM NaCl) caused a significant increase in ROS production compared to the axenic control (Figure 4.8), (Figure 4.9) and (Figure 4.10) resulting in plant growth inhibition which can be seen in (Figure 4.1), (Figure 4.2) and (Figure 4.3) respectively. Similarly in a study conducted by Khan et al (2019), the authors reported a significant increase in ROS production when soybean seedlings were exposed to salt stress. Furthermore, they reported a decrease in ROS production when soybean seedlings were inoculated with PGPB. In another study by Hahm et al (2017), the authors reported that PGPB alleviated salt stress in pepper by reducing ROS production through increased APX and CAT activity. The results in this study displayed similarities to that of khan et al (2019) and Hahm et al (2017) when, *Brassica napus* supplemented with *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 under salt stress reduced ROS production compared to the salt-induced control (Figure 4.8), (Figure 4.9), (Figure 4.10), resulting in plant growth promotion which can be seen in (Figure 4.1), (Figure 4.2) and (Figure 4.3) respectively. Similar results were reported where PGPB reduced ROS production by enhanced antioxidant activity of various ROS scavenging enzymes under increasing salt stress in okra, potato and lettuce (Khan *et al*., 2019).

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The ameliorative role of *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 against salt stress in *Brassica napus* might be due to the reduction of ROS production and upregulation of antioxidant capacity.

4.3.6 *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 increases *Brassica napus* antioxidant response under saline and non-saline conditions

Antioxidants are compounds produced by plants to counteract oxidative stress caused by an imbalance of ROS (Racchi, 2013). To reduce the damaging effects of ROS, plants regulate enzymatic antioxidant defenses such as ascorbate peroxidase, catalase and superoxide dismutase (Varjovi *et al*., 2015). Previous studies suggested that PGPB possess the ability to promote plant growth by enhancing plant tolerance towards salt stress by improving the plant antioxidant potential of APX, CAT and SOD (Numan *et al*., 2018). Ascorbate peroxidase is an enzyme that plays an important role in catalyzing the conversion of H_2O_2 into H_2O while using ascorbate as an electron donor (Caverzan *et al*., 2016). Catalase plays a crucial role during stress by eliminating H_2O_2 (which is a signalling molecule that rapidly diffuses through membranes) to prevent membrane and organelle damage (Abd_Allah *et al*., 2017). Superoxide dismutase is a key antioxidant enzyme involved in the scavenging of O_2^- and H_2O_2 for the reduction of the Haber-Weiss reaction and the formation of OH^- (Abd_Allah *et al*., 2016).

In this study we have shown that *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 increased the antioxidant activity of APX, CAT and SOD under saline and non-saline conditions (Figure 4.11), (Figure 4.12), (Figure 4.13). Induced exposure to salt stress (100 mM NaCl) caused a significant increase in antioxidant activity compared to the axenic control (Figure 4.11), (Figure 4.12), (Figure 4.13) which indicates that a plant is in oxidative stress. Hahm et al (2017) stated that plants inoculated with PGPB decreased the negative effects of oxidative stress by producing an increase in antioxidative enzymes. An increase in SOD, CAT and APX acticvity in response to salt stress has been reported in riverhemp by Abd_Allah et al (2016) and chickpea Rasool et al (2013). In this study, *Brassica napus* supplemented with *Klebsiella*

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aerogenes strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 under saline conditions increased the antioxidant activity of APX, CAT and SOD compared to the salt-induced control (Figure 4.11), (Figure 4.12), (Figure 4.13), which displays a reduction in oxidative stress by producing an increase in antioxidant activity and plant growth (Figure 4.1), (Figure 4.2), (Figure 4.3). This correlates to the statement made by Hahm et al (2017) that although salt stress induced higher levels of antioxidant activity, PGPB inoculum further induced antioxidant activity, which led to accelerated ROS detoxification. Therefore the up-regulation of APX, CAT and SOD in this study could account for the plant protection from free radical induced membrane dysfunction (Abd_Allah *et al*., 2017). Furthermore, the increased activity of CAT in *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 inoculated plants could modulate plant growth by protecting sensitive organelles such as chloroplasts, where important metabolic processes occur (Abd_Allah *et al*., 2017). Hahm et al (2017) stated that PGPB such as *Enterobacter clocae*, *Pseudomonas pseudoalcaligenes* and *Bacillus sp*., increased CAT and APX activity in Jatropha leaves in response to salt stress.

These findings suggest that *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 could possibly play an important role in plant stress tolerance by modulating physiological and biochemical processes.

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4.3.7 *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 improves *Brassica napus* nutrient acquisition within saline and non-saline soil

Soil salinity has detrimental effects on plant growth and development, as the roots cannot access water from salt accumulated areas (Abbas *et al*., 2019). The presence of salt ions in the soil reduces a plant's ability to access essential minerals and nutrients required for plant growth (Gondim *et al*., 2013). This affects important periods of a plant's life cycle such as seed germination, chlorophyll metabolism and plant growth (Rasool *et al*., 2013). The application of PGPB to soil can promote plant growth by improving the bioavailability of essential minerals and nutrients (Numan *et al*., 2018).

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In this study we have shown that *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 increased the acquisition of essential macro and micro nutrients within saline and non-saline soil (Table 4.2) and (Table 4.3). A study conducted by Bharti et al (2016) showed that PGPB inoculum improved the acquisition of important minerals by compartmentalizing toxic ions. Similarly, in this study we have shown that *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 improved the acquisition of essential macro and micro elements which could be due to their ability to compartmentalize toxic ions. A study conducted by Sigh and Jha (2016) revealed that Na^+ ion accumulation in plants is harmful to plant growth and disrupts calcium and potassium mobility within plant cells. In this study, induced exposure to salt stress (100 mM NaCl) caused the reduction of essential elements (Table 4.3) when compared to the axenic control (Table 4.2). However, soil supplemented with *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 under salt stress augmented nutrient availability compared to the salt-induced control (Table 4.3). Labrada et al (2019) stated that an increase in Na^+ ions induces osmotic stress which leads to oxidative damage which affects the uptake of potassium, changing the K/Na ratio. However, Abd_Allah et al (2017) reported that chickpea inoculated with *Bacillus subtilis* displayed an increase in the K/Na ratio, thereby inhibiting Na^+ ion accumulation, which might have an impact on the expression of transport coding genes. Furthermore, Abd_Allah et al (2017) reported a significant increase in magnesium, potassium, phosphorus and nitrogen, in chickpea plants inoculated with *Bacillus subtilis* inoculum which resulted in plant growth promotion. These findings can be correlated to the findings in this study, as *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 augmented macro and micro elements under saline (Table 4.3) and non-saline soil conditions (Table 4.2) which resulted in *Brassica napus* growth promotion under saline and non-saline conditions which can be seen in (Figure 4.1), (Figure 4.2) and (Figure 4.3) respectively. Additionally, Khan et al (2019) explained that changes in soil pH, increases nutrient accessibility due to organic acid secretion and chelation via siderophore production. This leads to the maintenance of osmolyte accumulation and water homeostasis and stimulates carbohydrate transport and metabolism which prevents photoinhibition and promotes plant growth during salt stress

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(Khan *et al*., 2019). Furthermore, Hahm et al (2017) stated that siderophore-producing bacteria can alter the acquisition of various metals, including Cu, Zn and Fe. These findings can be correlated to the findings in this study, as *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 displayed the ability to produce siderophores.

Evidence suggests that *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter aerogenes* strain ATCC 13048 and *Bacillus shackletonii* strain LMG 18435 could possibly aid *Brassica napus* during nutrient acquisition under saline and non-saline soil conditions via siderophore production.

Chapter four describes the effect of *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 on *Brassica napus* germination percentage, plant physiology and biomass, lipid peroxidation and cell death, chlorophyll metabolism, ROS production, antioxidant activity and nutrient regulation. Under non-saline conditions, *Brassica napus* displayed reduced germination percentage, plant physiology and biomass, chlorophyll metabolism, antioxidant activity and nutrient regulation. Additionally, *Brassica napus* displayed increased ROS production, lipid peroxidation and cell death. However, *Brassica napus* supplemented with *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 displayed augmented germination percentage, plant physiology and biomass, chlorophyll metabolism, antioxidant activity and nutrient regulation. Additionally, *Brassica napus* supplemented with *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 showed reduced ROS production, lipid peroxidation and cell death. Similarly, salt stress significantly reduced germination percentage, plant physiology and biomass, chlorophyll metabolism, antioxidant activity and nutrient regulation. Furthermore, salt stress displayed increased ROS production, lipid peroxidation and cell death. However, *Brassica napus* supplemented with *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 under salt stress augmented germination percentage, plant physiology and biomass, chlorophyll metabolism, antioxidant activity and nutrient regulation. Additionally, *Brassica napus* supplemented with *Klebsiella aerogenes* strain KCTC

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2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 under salt stress reduced ROS production, lipid peroxidation and cell death.

CONCLUSION AND FUTURE WORK

Throughout literature, salinity resulted in the depletion of a large number of global food production and as a result these global changes has led to alarmist projections that seem to argue for additional strategies by which food supply can be guaranteed. These considerations have aroused strong interest in studying plant abiotic stress responses. This study describes the regulatory effect of plant growth promoting bacteria (endophytes) on *Brassica napus* plant growth under saline and non-saline conditions by evaluating changes in plant physiology and biochemical response. Furthermore, the characterisation and identification of these microbes is of utmost important to agricultural studies.

Chapter three described the effect of endophytic bacteria on *Brassica napus* germination percentage, plant physiology and biomass. Isolates E7, E8 and E9 increased the germination percentage, plant physiology and biomass of *Brassica napus*. Isolates E7, E8 and E9 displayed the ability to produce indole-3-acetic acid and siderophore production however the ability to solubilise phosphate was not displayed. Furthermore, isolates E7, E8 and E9 displayed the ability to aid *Brassica napus* during nutrient acquisition. The molecular results identified isolates E7, E8 and E9 to be *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 respectively.

Chapter four describes the effect of *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 on *Brassica napus* germination percentage, plant physiology and biomass, lipid peroxidation and cell death, chlorophyll metabolism, ROS production, antioxidant activity and nutrient regulation. Under non-saline conditions, *Brassica napus* displayed reduced germination percentage, plant physiology and biomass, chlorophyll metabolism, antioxidant activity and nutrient regulation. Additionally, *Brassica napus* displayed increased ROS production, lipid peroxidation and ultimately cell death. However, *Brassica napus* supplemented with *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 augmented germination percentage, plant physiology and

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biomass, chlorophyll metabolism, antioxidant activity and nutrient regulation. Additionally, *Brassica napus* supplemented with *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 showed reduced ROS production, lipid peroxidation and cell death. Similarly, salt stress significantly reduced germination percentage, plant physiology and biomass, chlorophyll metabolism, antioxidant activity and nutrient regulation. Furthermore, salt stress displayed increased ROS production, lipid peroxidation and cell death. However, *Brassica napus* supplemented with *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 under salt stress showed augmented germination percentage, plant physiology and biomass, chlorophyll metabolism, antioxidant activity and nutrient regulation. Additionally, *Brassica napus* supplemented with *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 under salt stress showed reduced ROS production, lipid peroxidation and ultimately cell death.

Evidence suggests that the positive effects of *Klebsiella aerogenes* strain KCTC 2190, *Enterobacter kobei* strain CIP 105566 and *Bacillus shackletonii* strain LMG 18435 could be due to their ability to produce indole-3-acetic acid, siderophore and enhance nutrient acquisition. Future research in plant-microbe interactions should explore the application of bio-inoculants to various crops. The application of a mutli-strain bacterial consortium over a single inoculum could potentially be a more effective approach for reducing the harmful effects of plant stress. While a substantial amount of work has to be done in order to validate plant growth promoting bacteria, specifically endophytic bacteria becoming the basis in agricultural practise, evidence suggests that the world is on the verge of a paradigm shift in plant agriculture.

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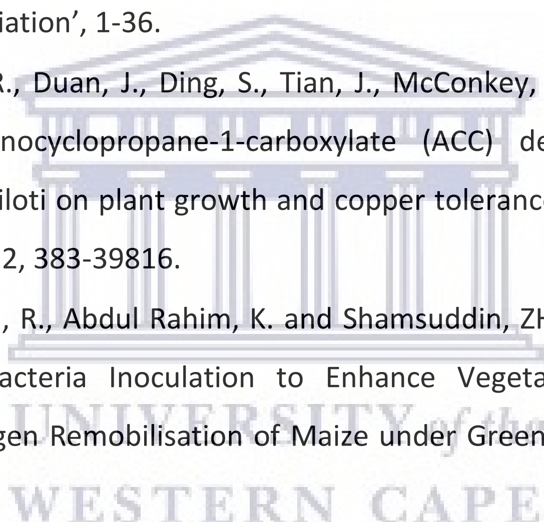
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