Identification of *Pseudomonas* mechanisms contributing to maize (*Zea mays L.*) protection against *Fusarium graminearum*

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Dissertation accepted in fulfilment of the requirements for the degree *Masters of Science in Biology* at the North West University

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DECLARATION

I declare that, this work submitted for the degree of Masters in Microbiology at the North-West University, Mafikeng Campus, has not been submitted by me for a degree at this or any other University. This is my own work in design and execution, and that all material contained herein has been duly acknowledged.

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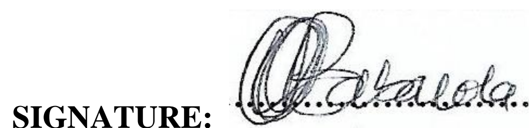
SIGNATURE: 

DATE: 05/03/2019

DEDICATION

This work is dedicated to God Almighty the giver of wisdom, knowledge, good health and life.

ACKNOWLEDGEMENTS

"I will praise you, Lord, with all my heart; I will tell of the wonderful things you have done." Psalms 9:1. Let me take this opportunity to thank my heavenly Father for making it possible for me to work tirelessly on this research project. His mercy has been abundant, His grace sufficient and His favour never ceased.

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Thank you all and may the Almighty God bless you!

OUTLINE OF DISSERTATION

This study consists of two major chapters submitted for publication in Accredited Journals. The Chapters contained therein are projected to be individual articles and describes the research work that has been performed to achieve the aim and objectives of this study.

Chapter 1 presents the general introduction of the study, aim, objectives and outline of the research.

Chapter 2 describes the literature review of the research.

Chapter 3 reports identification and screening of *Pseudomonas* species with in vitro anti- *F. graminearum* potential.

Chapter 4 describes the quantitative and qualitative screening of *Pseudomonas mediterranea* and *Pseudomonas putida* bio-protection mechanism against *F. graminearum* proliferation in maize.

Chapter 5 consists of the general conclusions from chapter 3 and 4 as well as future research prospects.

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LIST OF ABBREVIATIONS

Abbreviations	Full names
2,4 DAPG	2,4-diacetylphloroglucinol
ACCD	1-aminocyclopropane-1-carboxyl deaminase
AHC	Animal Health Center
bp	Base pairs
CAS	Chrome azurol S
CMC	Carboxylmethyl cellulose
CR	Crown root
DON	Deoxynivalenol
DSP	Desiccation protectant protein
EPS	Exopolysaccharide
FHB	Fusarium head blight
FSB	Fusarium seedling blight
GPX	Glutathione peroxidase
GRP	Glycine-rich RNA binding protein
HDTMA	Hexadecytrimethyl ammonium
HSP	Heat shock protein
IAA	Indole-3-acetic acid
ISR	Induced systematic resistance
LB	Luria Bertani agar
NWUAFM	North-West University Agricultural Farm

PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGPB	Plant growth promoting bacteria
PGPR	Plant growth promoting rhizobacteria
ROS	Reactive oxygen species
rpm	Revolutions per minute
Spp.	Species
TAE	Tris-Acetate-EDTA

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CHAPTER ONE

General abstract

Contamination of maize (Zea mays L.) with fumonisins in the field occurs when conditions favourable to the growth of Fusarium spp. such as high water activity by the time the plant reach near physiological maturity. The main source of the contamination is airborne conidia, mainly dispersed by wind, insects or rain. *Fusarium* spp. enters maize ear mainly via the silks or via ear wounds caused by birds or insects. This fungus is the most important ear and kernel rotting pathogen of maize. This fungus causes several infections in maize plants that cause significant quality and yield losses. The fungus also produces toxins that result in significant damage to the maize plant. In this study, three rhizospheric bacterial strains were isolated from two (Animal Health Center (AHC), North-West University, Mafikeng Campus, South Africa and (ii) North-West University Agricultural Farm, Molelwane (NWUAFM), South Africa) maize fields. Biochemical and morphological characteristics, nucleotide sequence analysis of the 16rDNA revealed that all the isolates belong to members of the genus *Pseudomonas.* The bacterial isolates were tested for their antagonistic activity against the growth of *F. graminearum* in-vitro. Various *Fusarium* growth inhibition traits (genes) including those for the production of the antibiotic phenazines (phz), pyoluteorine (phltb), pyrrolnitrin (prnD) and 2.4- diacetylphloroglucinol (2.4-DAPG). Moreover, all the *Pseudomonas* isolates were tested positive for the production of siderophore, indole-3-3acetic acid (IAA) and the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase. All isolates (*P. mediterranea, P. putida* and *P. fluorescens*) had the ability to produce siderophore with the highest production of 51.90% observed in *P. putida.* All isolates also produced indole-3-acetic acid, ACC deaminase activity and ammonia, while two isolates (*P. mediterranea* and *P. fluorescens*) solubilized phosphate and one (*P. mediterranea*) produced

hydrogen cyanide. *P. putida* was the most abundant and best performing isolate with the highest IAA production (9.57±0.66 µg/ml) and the highest ACC deaminase activity (0.87±0.12 µmol α-ketobutyrate mg protein^{-1} hour^{-1}). A gnotobiotic study was undertaken to test the antagonistic effect of *P. mediterranea* and *P. putida* against *F. graminearum* using three maize seed cultivars. The result indicated that the inoculation of maize seeds with the three *Pseudomonas spp.* significantly suppressed the growth of *F. graminearum* and also resulted in significant increase in important physiological parameters including root growth. The significance of this study is that it generated valuable baseline data and information on the biocontrol activities of *Pseudomonas mediterranea strain B5* and *Pseudomonas putida strain S6* against *Fusarium graminearum* infection. With further screening in the field trials, these *Pseudomonas* strains could be developed into agents for effective control of *F. graminearum* diseases in maize plants.

Keywords: *Fusarium graminearum, Pseudomonas*, growth inhibition, vermiculite, maize.

1.0 General Introduction

1.1 Background and rationale

Maize *(Zea mays L.)* is an important economic crop with an increasing area of cultivation in temperate climate regions due to the increasing demand for food and livestock feed (Shiferaw et al., 2013). Most maize contamination with fumonisins occurs in the field, near physiological maturity of the plants, when a high water activity in grain promotes *Fusarium* growth (Presello et al., 2008). Commercially produced seeds of maize (*Zea mays* L.) are almost universally treated with a fungicide prior to sale to protect the seeds from fungal infection after planting, or to deter the growth of seedborne fungal pathogens (Munkvold and O'Mara, 2002). Through numerous studies, it has been found that *Fusarium graminearum* is the main casual pathogen affecting small grain, cereals and maize (Adeniji and Babalola, 2018). *F. graminearum* produces deoxynivalenol (DON), which is a kind of mycotoxin that causes various toxic effects in humans and animals. This is confirmed by van Rensburg et al. (2016) who reported that infected maize has been commonly associated with human oesophageal cancer in South Africa. This fungal pathogen also causes seedling blight and root rot as well as head blight in most crops. However, not much attention has been given to the control of *Fusarium* seedling blight (FSB) as compared to that of *Fusarium* head blight (FHB) due to the high risk associated with mycotoxin contamination of grain in FHB.

To date, the main approach for controlling FSB are seed treatments with fungicides or biocontrol agents (Piotrowska-Seget et al., 2011). One of the most important biotic stresses affecting maize, wheat and sorghum grain in South Africa is caused by species belonging to the genus *Fusarium.* Among the *Fusarium* spp. the ones most commonly associated with three grain crop is *F. graminearum* (Beukes et al., 2017). Among the *Fusarium spp*. the ones most commonly associated with three grain crop is F. graminearum. Other *Fusarium* spp. affecting maize grain in South Africa include *F. moniliforme*, *F. proliferatum*, *F subglutinans*

and F. verticillioides, and with occurring less frequently (Boutigny et al., 2012). The discovery of fumonisins in South African maize grain by Bezuidenhout et al. (1988) sparked a significant interest in *Fusarium*-associated mycotoxins in the country and also worldwide. The growth and wellbeing of the plant also depends greatly on the rhizosphere. The rhizosphere microorganisms may include certain bacteria that may either be harmful to the plant or act as growth-promoters (Bouffaud et al., 2012). The utilization of a plant's own defence mechanism, which can be systemically activated upon exposure of plants to plant growth promoting bacteria (PGPB) strains or infection by the plant pathogen, is a fascinating area of research. This phenomenon is called induced systemic resistance (ISR). This mechanism is facilitated by PGPB organisms and activates through various defence compounds at the site of pathogen attack (Vanitha and Ramjegathesh, 2014). The mechanisms through which *Pseudomonas* spp. control plant diseases involve (i) competition for niches and nutrients, (ii) antibiosis, (iii) predation and (iv) induction of plant defence responses. Production of secondary metabolites like antibiotics, iron (Fe^{3+}) chelating siderophores and hydrogen cyanide is most often associated with fungal suppression by *fluorescent pseudomonas* (Lukkani and Reddy, 2014).

A number of disease-suppressive antibiotic compounds have been characterized, such as phenazines, pyrrole-type antibiotics, pyo-compounds and indole derivatives. The antibiotics pyoluteorin, pyrrolnitrin, phenazine-1-carboxylic acid (PCA) and 2,4-diacetylphloroglucinol (2,4-DAPG) are major determinants in biological control (Beukes et al., 2017). Some observations that also contribute to this mechanism of maize protection include indole-3-acetic acid (IAA) (Soussou et al., 2017). The involvement of phytohormone IAA in *Pseudomonas fluorescence*-mediated control of fusarium head blight disease of barley was first reported by Petti et al. (2012). IAA is the most abundant naturally occurring auxin with immense ability to regulate various aspects of plant development (Radhakrishnan and Lee,

2013). Iron chelating siderophores are known to have the ability to prevent proliferation of some phytohormones, and as a result to inhibit the growth of the phytopathogens (Glick, 2012). Very few studies have been done on the mechanism of action used by *Pseudomonas* spp. to protect maize from *F. graminearum* in South Africa. With the mechanism identified, maize production could be increased and the outbreak of fungal diseases on crops, grain cereals and maize by *F. graminearum* could be controlled with inexpensive methods.

1.2 Problem statement

F. graminearum, with a broad host range of pathogens, mostly infects crop plants, particularly wheat, maize, and barley, and results in severe loss of grain yield as well as quality reduction (Li et al., 2016). *F. graminearum* produces Deoxynivalenol, which is a kind of mycotoxin and it displays a wide range of toxic effects on animals and humans (Montibus et al., 2016). To humans and animals exposed to DON, the ingestion of contaminated food can induce toxic effects such as immunosuppression, neurotoxicity and teratogenicity (Savi et al., 2015). Some *Pseudomonas* strains possess mechanisms, which they use to protect plants from phytopathogens by suppressing the pathogen. These mechanisms through which *Pseudomonas* species control plant diseases involve production of secondary metabolites like antibiotics, Fe^{3+} chelating siderophores and hydrogen cyanide (Lukkani and Reddy, 2014). This research will investigate the mechanisms used by *Pseudomonas* in the protection of maize against *Fusarium graminearum*.

1.3 Research aims and objectives

1.3.1 Aim of the study

The aim of this study was to identify *Pseudomonas* strains as biocontrol agents against *F. graminearum*.

1.3.2 Objectives of the study

The objectives of this study were:

1. To isolate *Pseudomonas* from maize rhizosphere.
2. To identify *Pseudomonas* isolates with biocontrol potential for inhibition of *F. graminearum in vitro.*
3. To screen the *Pseudomonas* isolates for plant growth promoting traits.
4. Assay for the mechanisms of action for *Pseudomonas* protection.
5. To validate the biocontrol ability of *Pseudomonas* isolates under screen house conditions.

CHAPTER TWO

2.0 Literature review

2.1 *Fusarium graminearum*

Currently, commercial cultivars of maize (*Zea mays L.*) are vulnerable to *Fusarium* species infection, while the use of chemical pesticides are recalcitrant and environmentally undesirable (Chan et al., 2003). In parallel with developments in chemical control for increased effectiveness and in breeding for enhanced host-plant resistance, the feasibility of controlling *Fusarium* species has been successfully tested with selected microbial antagonists (Chan et al., 2003).

2.1.1 Fusarium head blight

Fusarium graminearum has a broad range of hosts, and infects crops such as barely, maize and wheat, and this causes severe loss of grains and reduction in quality of grains (Harris et al., 2015). The primary inoculum for the disease occurs when conidia (asexual spores) or ascospores (sexual spores) are dispersed on the heads of flowering cereal crop, then germinate, and invade the floral tissues of the host plant. The fungus enters through penetration of the epidermal cell walls directly with short infection hyphae and complex infection structures (Park and Yu, 2016). Damage caused by Fusarium infection has a huge negative economic impact throughout the world. One of these infection *is Fusarium head blight* of maize/wheat caused by two species of the fungus i.e. *F. graminearum* and *F. culmorum* (Jaillais et al., 2015). Fusarium head blight in wheat results from a complex of two species of pathogenic fungi, *F. culmorum and F. graminearum* (Jaillais et al., 2015). The main inoculum for the development of fusarium head blight are ascospores produced by Gibberella zeae on crop residues that remain on the soil surface after harvest and serve as sites for overwintering of the fungus (Khatibi et al., 2011). *Fusarium* consist of five main

species: *F. graminearum, F. culmorum, F. avenaceum, F. verticilloides* and *F. tritium* (Kuhnem et al., 2015). The most common of these species are *F. graminearum* and *F. culmorum*, which are also the most pathogenic, reducing the size of the grains (Ravensdale et al., 2014). Fusarium head blight is an attack on wheat by *F. graminearum*, which usually occurs in humid climates where the primary inoculum comes from either airborne ascospores or water-splashed conidia deposited directly in or among the spikelet of heads, usually during flowering (Wang et al., 2015a). Fusarium head blight is the most destructive crop diseases caused by *Fusarium graminearum* and is globally distributed (Sella et al., 2013).

2.1.2 Fusarium root rot

Fusarium root rot disease symptoms are manifested by a reddish-brown-black lesions on the tap root and hypocotyl, which is often accompanied by foliar chlorosis, vascular discolouration and wilt (Foroud et al., 2014). Being the common inhabitants of plant root ecosystems, fusaria and, particularly *Fusarium graminearum spp.* have been regularly studied for their interactions with the rhizobiome, motivated mainly by the importance of these organisms as soil-borne plant pathogens and the need to develop effective control mechanisms (Sandoval-Denis et al., 2018). Numerous fungal species are known to infect maize roots and eventually cause rot in maize plants in South Africa (Hugo, 2015). In Canada, root rot is a serious disease, and yet there has not been any available effective root rot management (Chang et al., 2015). Eight *Fusaraim* spp. have been associated with soybean roots and the species include *Fusarium oxysporum, F. graminearum, F. solani, F. avenaceun, F. tricinotum, F. sporotrichiodes, F. equiseti, F. poae* (Zhang et al., 2013). Members of the *F. solani* spp. complex (FSSC) are known to infect roots of soybean (Costa et al., 2016). Recent studies have confirmed that additional *Fusarium* spp. not belonging to FSSC can cause root rot and significant losses in soybean and these species include *F. graminearum*. Although

able to experimentally infect soybean pods, all other cited studies describe *F. graminearum* as a cause of damping-off or of crown and root rots of soybean (Sella et al., 2014).

2.2 The Genus *Pseudomonas*

Pseudomonas is the most important genus amongst other bacteria in the order Pseudomonadales and family Pseudomonadaceae. A group of bacteria among the genus *Pseudomonas*, which produces yellow-green fluorescent water-soluble pigments, are termed as fluorescent Pseudomonads (Tilak and Manoharachary, 2016). Fluorescent pseudomonads spp. are non-symbiotic rhizobacteria and a lot of attention has been given to them (Lee and Lee, 2015). Several studies describe the use of fluorescent pseudomonads as effective biocontrol agents against plant disease (Lukkani and Reddy, 2014). There is evidence to show that, inoculation of plants with the specific fluorescent *Pseudomonads* spp. strains results in increment of crop yield significantly (Kumar et al., 2012). This increase is associated with plant growth promotion and protection against pathogenic microorganism (Munees, 2014). Competition and antibiotic production by the species reduce the density and the harmful effect of pathogenic microorganisms (Shaikh et al., 2016). As biological control agents, fluorescent pseudomonads are important agriculturally and economically due to their production of secondary metabolites (Troppens et al., 2013).

2.2.1 *Pseudomonas* strains with biocontrol activities

Amongst the diverse range of fluorescent pseudomonads, specific strains that belong to *P. fluorescens*, *P. putida, P. aeruginosa* and *P. chlororapis*, have immense potential to be exploited as means of biocontrol agents because of their inherent capacity for the production of an array of metabolites and enzymes which mediate both biological control of pathogens and plant growth promotion in a wide variety of economically important agricultural crops (Subashri et al., 2016). This is confirmed by Mishra and Arora (2018) who reported that fluorescent pseudomonads are unique due to their ability to suppress a wide variety of

phytopathogens and that these rhizosphere bacteria are endowed with a state of art biocontrol machinery and hence used for the development of bioinoculants. Strano et al. (2017) also reported that strains such as *P. mediterranea* have been proposed as biological control agents, and that some of the strains have also been patented.

2.2.2 *Pseudomonas putida* and *Pseudomonas mediterranea*

P. putida and *P. mediterranea* are ubiquitous in the environment, including in water and soil (Selezska et al., 2012), and several *P. putida* and *P. mediterranea* strains that inhabit rhizosphere niches have been found to show excellent plant growth-promoting properties and to display effective biological control against various phytopathogens (Park et al., 2012). *Pseudomonas putida* and *P. mediterranea* are biofilm-forming gram-negative proteobacterium. Their biofilm components include a mannose-rich polysaccharide, *Psl*, a glucose-rich polysaccharide, *Pel*, and a mannose-derived biopolymer, alginate (Navarro et al., 2014). These strains are said to be non phytopathogenic and non necogenic because of their ability to produce plant growth promoting traits that activate biocontrol activity against the pathogens in plant. This is confirmed by a study conducted by Roquigny et al. (2017) who reported that in fact the ability of *Pseudomonas* strains to produce PGP traits and also posses biocontrol activity, does indeed make them non phytopathogenic and non necogenic strain.

2.3 Mechanisms of plant growth promotion by rhizobacteria

Soils consist of a diverse pool of microorganisms which includes bacteria, fungi, protozoa and algae (Glick, 2012). However, bacteria remain the most common and predominant microorganisms in most soils. Different conditions including temperature, moisture, presence of salt and other chemicals, influence the amount and type of bacteria found in most soils. These conditions also include the number and type of plants that inhabit the soils. Bacteria are known to use the same mechanism, whether they are free living, form symbiotic relationships with plants or are cyanobacteria, the mechanism used for plant growth

promotion is the same (Munees, 2014). There are two types of mechanisms used by PGPR, indirect and direct mechanisms. Direct mechanisms are those in which PGPB will either facilitate resource acquisition or modulate plant hormones (Ahemad and Saghir, 2012). In indirect mechanisms, PGPR will decrease the inhibitory effects of various pathogenic agents on development and plant growth, thus acting as biocontrol agents (Munees, 2014).

2.4 Direct plant growth promotion

2.4.1 Siderophore

Production of siderophore has been implicated in both direct and indirect enhancement of plant growth by plant growth promoting rhizobacteria. Iron is an essential micronutrient for almost all organisms in the biosphere (Sujatha and Ammani, 2013). Bacteria acquire iron by the secretion of low molecular mass iron chelators called siderophores, which have high association constants for compelling iron (Munees, 2014). Iron is not readily assimilated by either bacteria or plants because ferric ion, which is the predominant form in nature, is only sparingly soluble, so that the amount of iron available for assimilation by living organisms is extremely low (Gupta et al., 2015). Microorganisms have evolved specialized mechanisms for the assimilation of iron, and these mechanisms include the production of low molecular weight iron-chelating compounds known as siderophores (Arora et al., 2013). Some bacterial strains use the siderophores that they produce as biocontrol agents and these siderophores from PGPB can prevent some phytopathogens from acquiring sufficient amounts of iron ,thereby limiting their ability to proliferate (Glick, 2012).

2.4.2 Indole-3-acetic-acid (IAA)

PGPB benefit plants through several mechanisms that can act simultaneously during the different stages of the plant's cycle. These benefits include the production of phytohormones (e.g. indole-3-acetic acid) that promote plant growth (Moreira et al., 2016). IAA is a plant hormone involved in several mechanisms, such as promotion of cell elongation and cell

division, apical dominance, root development, differentiation of vascular tissue, ethylene biosynthesis and phototropism (Scagliola et al., 2016). IAA is the principal plant hormone classified into the family of indole derivatives of auxins (Nutaratat et al., 2016). About 80 % of bacteria from the rhizosphere are able to produce IAA , indicating a possible role in interaction with the plant (Naveed et al., 2015). Plant responses to IAA vary from one type of plant to another, where some plants are more sensitive to IAA than other plants; according to the particular tissue involved, for example, in roots versus shoots and as a function of the developmental stage of the plant (Glick, 2012).

2.4.3 2, 4-diacetylphloroglucinol (2,4-DAPG)

The antimicrobial metabolite 2,4-DAPG produced by *P. fluorescens* is a principal factor enabling this bacteria to control plant diseases caused by soil-borne pathogens (Yang and Cao, 2012). 2, 4-diacetylphloroglucinol is known as a polyketide, an antibiotic that is encoded by the eight-gene *phl*$^{+}$ cluster and has antimicrobial effects on phytopathogens (Moynihan et al., 2009). It has the ability to promote amino acid exudation from plant roots and induce systemic resistance in plants (Weller et al., 2011). However, despite its importance and the role it plays in a plant's wellbeing, 2,4-DAPG production is limited to a subset of *P. fluorescens* strains (Troppens et al., 2013). The term *phl*$^{+}$ *Pseudomonas* and 2,4-diacetylphloroglucinol producer, is used synonymously because detection of the gene correlates with the capacity to produce 2,4-DAPG (Kwak et al., 2012a).

2.4.4. 1-aminocyclopropane-1-carboxylate (ACC) deaminase

The enzyme 1-aminocyclopropane-1-carboxylate deaminase catalyzes the degradation of 1-aminocyclopropane-1-carobylic acid (ACC) and the enzyme has been detected in limited number of bacteria and plays a significant role in sustaining plant growth and development under biotic and abiotic stress conditions by reducing stress induced ethylene production in plants (Ali et al., 2013). Many PGPBs promote plant growth by expressing the enzyme 1-

aminocyclopropane-1-carboxylate (ACC) deaminase, which cleaves the immediate precursor of the plant hormone ethylene to produce α-ketobutyrate and ammonia (Win et al., 2018). .ACC deaminases significantly improve plant growth and tolerance to abiotic stresses by lowering stress-related ethylene levels in plants (Matsuoka et al., 2016). Many researchers have noted the positive effects of the ACC deaminase-producing bacteria in the rhizosphere in alleviating different stresses on plant growth and these bacteria includes Pseudomonas fluorescence and Pseudomonas putida (Jalili et al., 2009).

2.5 Indirect plant growth promotion

2.5.1 Pyrrolnitrin

Pyrrolnitrin was first described by Arima et al. (1964). Pyrrolnitrin is an inhibitor of fungal respiratory chain and thus a broad spectrum of antifungal metabolites produced by fluorescent and non-fluorescent strains of *Pseudomonas* (Sarker et al., 2014). A phenyl pyrrol derivative, pf *Prn,* has been developed as an agricultural fungicide (Dwivedi and Johri, 2003). The seven known antibiotics produced by Pf-5 as proposed by Yan et al. (2016) are pyrrolnitrin, hydrogen cyanide (HCN), 2,4-diacetylphloroglucinol (DAPG), pyoluteorin, orfamide A, rhizoxin analogy, and toxoflavin (Philmus et al., 2015).

2.5.2 Phenazines

Pseudomonas species secrete nitrogen containing heterocyclic antibiotics known as phenazines. Phenazines are bacterial, secondary metabolites that have long been recognized for their broad-spectrum antibiotic activity and have been widely used in the biological control of a range of fungal phytopathogens (Mavrodi et al., 2012). The role of phenazines as a biocontrol on numerous phytopathogens has been studied greatly (Jain and Pandey, 2016). Some phenazines derivatives such as phenazines-1-carboxylic (PCA), phanzine-1-carboxamide (PCN), 1-hydroxy phenazines, etc., possess antifungal activity. The following species of *Pseudomonas* encode for genes that produce phenazines derivatives; *P.*

aureofaciens 30-84, *P. fluorescens* 2-27 and *P. chlororephis* PCL 1391 (Fischer et al., 2013). The gene cluster *phzA-G* is responsible for phenazines biosynthesis in all phenazine-producing pseudomonads (Blankenfeldt, 2013). Phenazine-1-carboxylic acid (PCA) is produced by various pseudomonad strains, such as *Pseudomonas chlororaphis* 30-84, *Pseudomonas aeruginosa* GC-B26, *P. chlororaphis* PCL1391 and *P. fluorescens* 2-79 (Du et al., 2013).

2.5.3 Hydrogen cyanide (HCN)

Hydrogen cyanide is a volatile compound (Zhou et al., 2012). Volatile compounds are known to influence plant growth and development and in addition to this, bacterial volatiles have the ability to reduce growth of fungi (Groenhagen et al., 2013). This metabolite is produced from glycine under essentially microaerophilic conditions. In some *Pseudomonas* strains, the *hcnABC* genes encode for the HCN synthesis critical for HCN production (Fischer et al., 2013).

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CHAPTER THREE

In Vitro Identification and Screening of *Pseudomonas* Spp. with Biocontrol Potential for Inhibiting *F. Graminearum*

3.0 Abstract

Several diseases including Fusariosis cause significant yield losses in the production of maize worldwide. *Pseudomonas* spp. have the ability to inhibit growth of *F. graminearum* in maize. Rhizospheric *Pseudomonas* spp. were isolated from two maize fields in Mafikeng, South Africa. Three isolates showed physiological and morphological characteristics similar to that of the genus *Pseudomonas*. They revealed different degrees of high growth inhibition of *F. graminearum* by growing on 5% polyethylene glycol (PEG) 8000. Bacterial growth at different NaCl concentration and temperatures were evaluated and best optimum growth for all bacterial isolates was observed at 2% NaCl and temperature between 25°C and 30°C. Molecular identification of the three *Pseudomonas* isolates was done using 16S rDNA gene sequence analysis which gave the targeted sizes of 460bp for all isolates. These products were sequenced and computational analysis including BLAST search and phylogenetic analysis was performed to compare the isolates with other species in the GenBank library. Phylogenetic analysis revealed that all three isolates belong to the genus *Pseudomonas* with 99-100% similarity with the following accession numbers: MH666036, MH666037 and MH666038. *Fusarium* growth inhibition antibiotic primers with the following amplifications were observed: three isolates (*B5 and S6*) for *phl2*, two isolates (B5 and B9) for *pltB*, two isolates (B5 and B9) for *PRND,* two isolates (B5 abd B9) for *PHZ* together with the two plant growth promoting genes for ACC deaminase activity (B5, S6 and B9) and siderophore production (B5, S6 and B9) *Accd* and *Sid* were amplified by PCR. The amplification of the *Fusarium* growth inhibition and plant growth promoting primers indicates the presence of these genes in these bacteria. Hence, there is a need to develop techniques that assist in understanding the interactions that exist between the plant hosts and their resident microbes.

Keywords: *Pseudomonas, Fusarium graminearum,* amplification, molecular detection, plant growth promoting genes.

3.1 Introduction

Plant pathogens cause significant loss of agricultural yield and are important determinants of plant community structures and productivity (Dudenhöffer et al., 2016). Soil-borne plant pathogens particularly fungi and oomycetes are among the most harmful pathogenic microorganisms in agricultural environments resulting in major limitations to the production of food crops worldwide (Martini et al., 2015). The rhizosphere is a place of complex interactions between plants and microbes. Natural agricultural ecosystems depend directly on beneficial microorganisms that are present in the soil rhizosphere and help crops to reach higher productivities (Rosas, 2012). However, plant pathogens such as fungi often limit crop yields and cause large economic losses (Cordero et al., 2012). Fungi in the Genus Fusarium are ascomycetes which are characterised by their typical conidia and are often fusiform to sickle-shaped, characterized by an elongated apical cell and pedicellate basal cell. Several important *Fusarium* spp. including *F. graminearum, F. pseudo graminearum* and *F. avenaceum* were formerly classified in the genus *Gibberella* because of their teleomorph production (Foroud et al., 2014). The main causal agents in the temperate climates of some parts of the world are *Fusarium graminearum* (Müller et al., 2016). *Fusarium graminearum* is a necrotrophic fungal pathogen that causes Fusarium head blight (FHB) and crown rot (CR) on cereal crops growing in different regions of the world (Sella et al., 2014). *Fusarium graminearum* has a wide range of hosts including *gramineae (*wheat, barley, rice, maize, oat, etc.), cotton, kenaf, sweet potato and others (Zhao et al., 2010). For this reason, we concentrate on this particular species, as it is responsible for contamination of maize leading to loss of production of maize yield.

Rhizobacteria employ different strategies for competition with other microorganisms from soil including, production of antimicrobial compounds such as antibiotics or bacteriocins (Godino et al., 2016). A potential method for suppressing *F. graminearum* is plant growth

promoting rihzobacteria (PGPR) that have biocontrol activity against phytopathogens (Meyer et al., 2016). Many isolates of the genus *Pseudomonas* have these characteristics and are efficacious for improving plant growth and yields and for suppressing soil borne plant pathogens. The genus *Pseudomonas,* a gamma-proteobacterium, is a diverse group of microorganisms that occupy many different niches and exhibit versatile metabolic capacity (Goswami et al., 2015). *Pseudomonas aeruginosa, P. chlororaphis, P. fluorescens, P. stutzeri, P. pituda* etc. are some well-known non-pathogenic biocontrol agents showing strong plant growth-promoting activities. The suppression of phytopathogens and the beneficial effects of *Pseudomonas* can be caused by multiple factors such as: production of secondary metabolites and antibiotics which affect plant vigour, root weights, lengths and root branching (de Souza and Raaijmakers, 2003), antagonistic activity against other pathogenic bacteria, fungi, nematodes, oomycetes, plants, and viruses and trigger induced systemic resistance (ISR) (Meyer et al., 2016). The PGPB traits of *Pseudomonas* spp. have been widely studied. Nevertheless, little is known about their mechanism of action used to inhibit growth of *F, graminearum* in maize plants.

To eradicate the problem of Fusariosis in most plants, modern agro-biotechnological strategies are implemented for pathogen suppression in plants. These strategies include genetic engineering, germplasm screening, plant breeding etc., which have resulted in the development of hybrid products that are widely grown across different parts of the globe (Kant et al., 2012). However, *F. graminearum* may have become more aggressive or better adapted to the environment (Batemana et al., 2007), making the task of introducing new inhibition methods very difficult and strenuous. *Pseudomonas* bacteria have evolved several mechanisms to tackle the damages caused by *Fusarium graminearum* on plants. These mechanisms may include: modifications of phytohormones which play a major role in helping plants escape or survive biotic and abiotic stresses (Bell et al., 2014), alteration in

plant root morphology, accumulation of osmolytes, alteration of plant antioxidant defence mechanisms and the presence of *Fusarium* inhibitory genes which is a molecular mechanism (Mesterházy et al., 2015). Bacteria possessing these traits can be isolated and used to improve plant growth under the attack of Fusariosis. For successful application of this strategy, one should have a good knowledge of the ability and mechanisms used by these organisms to inhibit *Fusarium.* Many studies have revealed the successful isolation of *Pseudomonas* spp. from plants, but very few have been able to identify their mechanism of action in inhibiting growth of *F. graminearum* especially in maize plants. Identifying the genetic make-up of these bacteria as well as evidence of Fusariosis inhibitory genes may be of help in understanding the mechanism of action used by *Pseudomonas spp*. Knowledge gained from this study will go a long way in helping to select beneficial bacterial strains that can be used to improve plant growth under attack by *Fusarium*. Therefore, the objectives of this study were to:

1. Isolate, characterize and identify *Pseudomonas* from dry maize rhizosphere soil;
2. Determine the inhibition abilities of identified *Pseudomonas* isolates;
3. Evaluation of PEG, temperature, salinity and NaCl effects on bacterial growth;
4. Screen *Pseudomonas* spp. for the presence of inhibitory and plant growth promoting (PGP) genes.

3.2 Materials and methods

3.2.1 Sampling sites and sample collection

Six (6) rhizospheric soil samples were collected from two different maize fields, (i) behind Animal Health Center (AHC), North-West University, Mafikeng Campus, South Africa and (ii) North-West University Agricultural Farm, Molelwane (NWUAFM), South Africa. Soil samples were collected by carefully uprooting dry maize plants, and shaking the plants to remove soils loosely adhered to the plant roots. Soils tightly adhered to the roots were aseptically collected in sterile plastic bags and transported to the Microbial Biotechnology Laboratory in a cooler box. Collected samples were stored at -20°C for further analysis.

3.2.2 Isolation and selection of bacteria

Isolation of *Pseudomonas* isolates studies were carried out on King's B medium (KBM), malt agar (Alemu and Alemu, 2013). Bacterial isolation was carried out by suspending 1 g of each rhizosphere soil sample separately in 9 ml of sterile saline solution (0.85%) in 20 ml sterile test-tubes. The test-tubes were thoroughly shaken using a vortex machine and standard serial dilutions from 10^{-1} to 10^{-8} were made by transferring 1 ml of the soil suspension until the last test-tube. Aliquots of 200 µl from each dilution were spread using a glass rod on King's B medium (KBM), malt agar (pH 7.0±0.2) plates (these were performed in triplicate). For optimum growth, plates were incubated for 5 days at 25°C. Isolated bacteria with varying colour and shape were randomly selected and repeatedly streaked on freshly prepared P1852 *Pseudomonas* Agar plates (pH 7.0 +/- 0.2) supplemented with C8721 Cetrinix Supplement with 2 ml of 0.2N (930-65) sodium hydroxide which is a supplement recommended for selective isolation of *Pseudomonas* spp. This was done to obtain pure *Pseudomonas* cultures. Pure cultured bacterial strains were maintained on agar slants at 4°C.

3.2.3 Isolation and characterization of the fungal pathogens

Fusarium graminearum was kindly provided by Dr Claire Prigent Combaret (UMR CNRS 5557) Microbial Ecology of Lyon, University Lyon 1, France, and Prof Cristina Cruz, Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências da Universidade de Lisboa, Portugal, respectively, and they were maintained on potato dextrose agar (PDA Sigma-Aldrich P2182) plates (Adeniji and Babalola, 2018).

3.2.4 *In vitro* inhibition of *Fusarium graminearum* by *Pseudomonas*

The antifungal activity of rhizobacteria against *Fusarium graminearum* was assessed by dual culture method as described by Kumar et al. (2002). A disk (5 mm diameter) was cut out from a young culture of the fungal pathogen and placed in the middle of a Petri dish of potato dextrose agar (PDA). Ten (10) µl of a rhizobacteria suspension (approx. 10^8 CFU/ml) were spotted 2 cm on opposite sides of the infected agar block. The control plates contained monocultures of each pathogen. The plates were incubated at 26 ± 1°C and checked for zones of inhibition of mycelium growth after seven (7) days when the fungal mycelium had reached the edge of the plates. When the pathogen grew over the rhizobacteria, we concluded that rhizobacteria did not have antifungal activity. On the other hand, when fungal growth was inhibited by the rhizobacteria, the rhizobacteria antifungal activity (percentage of inhibition) was calculated by the following formula (Noumavo et al., 2015):

$$\%growth\ inhibition = \frac{r1 - r2}{r1} \times 100$$

Where, r1 = diameter of pathogen growth in monoculture (control); r2 = diameter of pathogen growth in dual culture. The test was done in three replicates.

3.3. Physiological and biochemical characterization

3.3.1 Hydrolysis of starch test

A loop full of each bacterial isolate was streaked on starch agar plates (Sigmon, 2008) in triplicate, plates were incubated at 25°C for 6 days. On the 6th day, iodine solution was added to each culture plate. After about a minute, excess iodine was carefully poured out from the culture plates. Controls consisted of un-inoculated starch agar plates. A yellow zone around the colony in a dark blue medium is considered positive for starch hydrolysis while the absence of a yellow zone is considered as negative. The test was done in three replicates.

3.3.2. Hydrolysis of casein test

The ability of selected isolates to hydrolyse casein was determined by spot-inoculating prepared skimmed milk agar plates with a loop full of each bacterial isolate. Un-inoculated skimmed milk agar plates served as the control for this experiment and all experiments were performed in triplicates. All plates (inoculated and control) were incubated at 25°C for 6 days. Observation of a clear zone around the bacterial growth on the agar culture plates indicated evidence of casein hydrolysis. The test was done in three replicates.

3.3.3 Effects of PEG 8000 on bacterial growth

Bacterial isolates were screened for growth ability in 250 ml conical flasks containing 50 ml sterile Luria Bertani (LB) Broth (pH 7.0±0.2) medium supplemented with 5% polyethylene glycol (PEG) 8000. Each flask was inoculated with 500 µl (optical density, OD = 0.2) of 5 day old bacterial cultures and incubated at 25°C under shaking conditions (150 rpm). Bacterial growth was determined at 6 days by measuring the OD of 2 ml of each culture at 600 nm using a UV spectrophotometer (Thermo Spectronic, Merck, SA). The controls for this experiment were PEG-free medium inoculated with bacteria. Growth of each bacterial isolate was represented graphically by plotting absorbance against time. Each was performed in triplicate. The test was done in three replicates.

3.3.4 Effects of temperature on bacterial growth

The effect of temp on the bacterial growth was assessed by growing10 µl (OD = 0.1) of each bacterial isolate in 10 ml of sterilized LB Broth medium containing 5% PEG-8000 and incubated at different temperatures (25°C, 30°C, 35°C, 40°C) under shaking conditions (150 rpm) for 6 days. The OD of each bacterial isolate was measured at 600 nm using a UV Spectrophotometer (Merck, SA). It was done in three replicates.

3.3.5 Effects of NaCl on bacterial growth

Tolerance to NaCl by bacteria was assessed according to the method of Ndeddy Aka and Babalola (2017) by inoculating 20 µl of each bacterial isolate in 20 ml sterilized LB broth medium containing varying concentrations of NaCl (0.2, 0.4, 0.6, 0.8, and 1.0%). Inoculated tubes were incubated for 6 days at 25°C and the OD was measured at 600 nm using a UV spectrophotometer (Thermo Spectronic, Merck chemicals, SA). It was done in three replicates.

3.3.6 Utilization of carbohydrate source

The selected bacterial strains were tested for their ability to utilize various carbon compounds as energy sources by the method recommended by Rico and Preston (2008). The carbon sources used were: D-galactose, D-glucose, D-xylose, sucrose, D-mannitol, lactose and fructose. The carbon sources were filter-sterilized using a micro-membrane filter (20 µm/cm) to ensure they were free from various contaminants. All inoculated tubes were incubated at 25°C for 5 days. Acid production by the isolates from the above carbon sources was also studied. The positive utilization (+) of a carbon source was considered when growth of an isolate on a tested carbon source was significantly better than the growth on the basal medium without a carbon source, while growth similar or less than growth on basal medium without a carbon source was considered as a negative utilization (-). It was done in three replicates.

3.3.7 Production of catalase

Luria-Bertani (LB) agar slants were inoculated with each bacterial isolate. Control for this experiment was an un-inoculated LB slant. All tubes were incubated at 25°C for 6 days, in triplicates. On the 5th day, a single colony from each inoculated tube was placed on a sterile glass slide and held at an angle while 2-3 drops of hydrogen peroxide (H_2O_2) was allowed to flow over the growth of each culture on the glass slide. Catalase production is indicated by the production of oxygen bubbles within one minute after addition of H_2O_2. It was done in three replicates.

3.3.8 Nitrate reduction

A loop full of spores from selected bacterial isolates was inoculated into 20 ml test-tubes containing 5 ml of sterilized nitrate broth and incubated at 25°C for 6 days. Controls were test-tubes containing broth without inoculation. On the 5th day of the incubation period, broths were tested for presence of nitrate by addition of two drops of reagent A (α-naphthylamine solution) and two drops of reagent B (sulfanilic acid solution) into 1 ml of broth or 1 ml of control. A change in colour of the broth to pink, orange or red indicated the presence of nitrate. Results were confirmed by the addition of a pinch of zinc dust after the reagents were added. A change in colour from pink, orange or red to the original colour of the broth confirms the result. It was done in three replicates.

3.4. Molecular characterization of bacterial isolates

3.4.1 DNA extraction

For extraction of genomic DNA, bacteria were grown in 20 ml of LB Broth medium in Eppendorf tubes. The cultures were grown with constant agitation (150 rpm) at a temperature of 25°C for 7 days for optimum growth. Bacteria were harvested by centrifuging at maximum speed (4000rpm) for 10 min. Supernatants were discarded and cells were collected and re-suspended in sterile distilled water. The total DNA from each bacterium was extracted

using a DNA extraction kit (ZR soil microbe DNA MiniPrepTM kit (Zymo Research, USA) according to the manufacturer's instructions.

The concentration in ng/µl and purity of the extracted DNA was measured using a NanoDrop Spectrophotometer (Table 3.1). Amplification of a 460 bp fragment of the 16S rDNA gene was performed by polymerase chain reaction (PCR) using the universal 16S rDNA primers for bacteria B5 and B9 (Mulet et al., 2009) to confirm the identity of bacterial isolates. Details of primers are shown in Table 3.2. Amplifications were performed in a final volume of 25 µl consisting of 12.5 µl of 2x PCR Master mix (0.05 U/µl *Taq* DNA polymerase, 4 mM MgCl2 and 0.4 mM dNTPs (Fermentas)), 1 µl of genomic DNA template, 0.5 µl of each of the forward and reverse primers and 11 µl of nuclease-free water. The PCR mixture was subjected to 30 cycles in a C1000 thermal cycler (BioRad) with the following conditions: initial denaturation step at 95°C for 5 min; denaturation at 94°C for 30 s; annealing temperature of 55°C for 30 s; extension of 72°C for 1 min and final extension of 72°C for 10 min.

Table 3.1. Nanodrop readings of DNA concentrations of *Pseudomonas* isolates

Isolate	DNA Quantity (ng/µL)
S6	221.12
B9	109.27
B5	126.22

3.4.2 Polymerase chain reaction (PCR) amplification of biocontrol and plant growth promoting (PGP) genes in bacterial isolates

3.4.2.1 PCR amplification of biocontrol and plant growth promoting genes

Primers were tested for varying annealing temperatures using a gradient PCR machine from 45 to 60°C. Reaction volumes were 25 µl which consisted of 12.5 µl of 2x PCR Master-mix (0.05 U/µl *Taq* DNA polymerase, 4 mM $MgCl_2$ and 0.4 mM dNTPs (Fermentas)), 1 µl of

genomic DNA template (10 ng/ reaction mixture), 0.5 µl of each of the forward and reverse primers and 11 µl of nuclease-free water. The PCR started with a 95°C hot start for 10 min. The cycles consisted of 94°C denaturation temperature which lasted for 30 s/ cycle, a 48-65°C annealing temperature for 30 s, 72°C elongation step for 1 min, a final elongation step of 72°C for 10 min and a holding period at 4°C for infinity.

3.4.2.2 *Pseudomonas* protection mechanism of action

The sequence of primers phl2a, phl2b; PRND1, PRND2; PHZ1, PHZ2; PltBf, PltBr and the conditions used in PCR to amplify conserved genes *phl*D, *plt*B, *prn*D and *ph*Z involved in the synthesis of antibiotics 2,4-DAPG, pyoluteorin, pyrrolnitrin and phenazine respectively are described in table 3.2 (Hassan et al., 2011). Amplifications were performed in a final volume of 25 µl consisting of 12.5 µl of 2x PCR Master mix (0.05 U/µl *Taq* DNA polymerase, 4 mM MgCl2 and 0.4 mM dNTPs (Fermentas)), 1 µl of genomic DNA template, 0.5 µl of each of the forward and reverse primers and 11 µl of nuclease-free water. The PCR mixture was subjected to 30 cycles in a C1000 thermal cycler (BioRad). A quantity of 10 µL of each PCR product was separated on 1.2% agarose gel in 1× TBE buffer (90 mM of Tris-borate, 2 mM of EDTA) at 80 V for 60 min.

3.4.2.3 PCR amplification of PGP Genes

Siderophore (*Sid*) and ACC deaminase (*accd)* genes which are associated with plant growth promotion in each bacterial isolate were screened by PCR amplification. Table 3.2 shows all the oligonucleotide sequences used as primers in this study. The PCR for siderophore and ACC deaminase genes were performed as follows: 2 µl (about 10 ng) of each DNA extract was amplified with 12.5 µl of 2x PCR Master mix (0.05 U of Taq DNA polymerase4 mM MgCl2 and 0.4 mM dNTPs (Fermentas)), 1 µM of each primer and 8.5 µl of nuclease-free water, in a 25 µl reaction 54 mixture in a C1000 thermal cycler (BioRad) with the following PCR conditions: 30 cycles of denaturation at 94°C for 1 min, annealing temperatures of 55°C

for sid and 57°C accd for 45 s, extension of 72°C for 1 min and a final extension step of 72°C for 10 min.

Table 3.2 Oligonucleotide primers for PCR amplification of 16S, antibiotics and PGP genes

Gene	Primer	Forward Sequence	Temp	Reference
16S rDNA	*Ps for*	GGTCTGAGAGGATGATCAGT	55°	Mulet et al. (2009)
	Ps rev	TTAGCTCCACCTCGCGGC		
2,4-DAPG	*Phl2a*	GAGGACGTCGAAGACCACCA	67°	Sherathia et al. (2014)
	Phl2b	ACCGCAGCATCGTGTATGAG		
Pyoluteorin	*PltBf*	CGCAGCATGGACCCCCAGC	58°	Hassan et al. (2011)
	PltBr	GTGCCCGATATTGGTCTTGACC		
Pyrrolnitrin	*PRND1*	GGGGCGGGCCGTGGTGATGGA	68°	de Souza and Raaijmakers (2003)
	PRND2	YCCCGCSGCCTGYCTGGTCTG		
Phenazine	*PHZ1*	GGCGACATGGTCAACGG	56°	Raaijmakers et al. (1997)
	PHZ2	CGGCTGGCGGCGTATTC		
ACC	*accd1*	GTGACCCACCTGAATGTA	48°	Raddadi et al. (2008)
Deaminase	*accd2*	AAACGAGATGATTTACTTGG		
Siderophore	*Sid1*	GAGAATGGATTACAGAGGAT	58°	Raddadi et al. (2008)
	Sid2	TTATGAACGAACAGCCACTT		

3.4.3 Agarose gel electrophoresis

Genomic DNA and all PCR products were checked in 1% (w/v) agarose gel made by dissolving 1.5 g of agarose (Bi0-Rad, SA) in 150 ml of 1X Tris-acetate-ethylenediaminetetraacetate (TAE, pH 8). This mixture was heated in the microwave for 3 min after which it was allowed to cool. After solidification, 1 µl/ 10ml of ethidium bromide (EtBr) was added, combs were removed and the gel was placed in the electrophoresis tank containing 1X TAE buffer (40 nM Tris, 20 mM acetic acid and 100 mM EDTA, pH 8.0). DNA samples were prepared by mixing 7 µl of DNA template and 3 µl of 6x DNA loading

dye (Fermentas) while both PCR products and DNA ladders (1 kb or 100 bp used to estimate the size of bands for the genes) consisted of 5 µl volume. Samples were carefully loaded into the preformed wells in the gel. The gel was allowed to run for 60 min at 80 V. Results were visualized and photographed using a ChemDocTM MP System (Bio-Rad Laboratories, Hercules, CA, USA).

3.4.4 DNA purification, sequencing and phylogenetic analysis

Purification and sequencing of PCR products were performed by Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa using PRISMTM Ready Reaction Dye Terminator Cycle Sequencing Kit. The analysis of sequences and construction of phylogenetic tree were performed according to the methods described by Aremu and Babalola (2015). Analysis of chromatograms resulting from sequencing reaction for assurance and good quality sequence were performed using ChromasLite version 2.3.3 software (Technelysium Chromas). The chromatograms obtained were edited with BioEdit Sequence Alignment Editor (Hall) and consensus sequences were generated. The consensus sequences obtained were Blast in the NCBI database (www.ncbi.nlm.nih.gov) using the Basic Alignment Search Tool (BLASTN) for homology for identification of the bacteria. Sequences obtained were deposited in GenBank.

Analysis of phylogeny based on 16S rDNA gene was used to further characterize the bacterial isolates. Partial 16S rDNA sequences obtained were used to search for the reference nucleotide sequences in the NCBI GenBank database using BlastN algorithm (Altschul et al., 1997). MAFFT (version 7.0) was used to align multiple nucleotide sequences (Katoh, 2013) and the tree was drawn using MEGA 7 (Kumar et al., 2016). Techniques used in drawing the tree include: The Neighbour-Joining (NJ) with cluster-based algorithm used to calculate pairwise distance between and group sequences that are most similar to each other and the Maximum Likelihood used to compare a set of data against set of evolution models in order

to select the best model for the variation pattern of the sequences (Harrison and Langdale, 2005).

3.5 Data analysis

All quantitative data obtained from this study were statistically analysed by one way analysis of variance (ANOVA) using the Statistical Analysis Software (SAS), Version 9.4 (SAS, 2014). Significant mean differences were compared using New Duncan's Multiple Range Test (DMRT).

3..6 Results and discussion

3.6.1 Characterization and isolation of *Pseudomonas*

Three *Pseudomonas* spp. were selected from the rhizospheric soil samples based on their colony morphology and their biochemical properties. Isolate S6 was obtained from behind the AHC while the other two isolates coded as B5 and B9 were obtained from NWUAFM. All these isolates showed rapid growth under favourable conditions and after 7 days of incubation (25±1ºC) colonies were observed to be cream yellow/light brown, shiny and in some instances, the colour of the plate changed from light yellow to lime (Table 3.3). The biochemical properties showed that all tested bacterial isolates were positive for catalase activity, nitrate reduction test, starch hydrolysis and the utilization of glucose as a carbon source. However, only two bacterial isolates (B5 and B9) tested positive for hydrolysed casein. All isolates utilized D-galactose, D-xylose, sucrose and D-mannitol. The results obtained also showed that all the isolates utilized fructose except isolate S6, while this isolate was the only one that utilized lactose among all tested bacterial isolates. Table 3.3 shows detailed results on the biochemical and carbon utilization of bacterial isolates used in the study.

Based on the morphological, biochemical and physiological tests conducted on the bacterial isolates, it was established that isolates taken from dry maize rhizosphere belong to the *Pseudomonas* genus. The tested bacterial isolates were compared with the description of the cultural, biochemical and physiological properties of other *Pseudomonas* that fall in the same genus, hence the conclusion of the groups (Frasson et al., 2017). Similar studies have proven the predominance of *Pseudomonas* genus isolated from different hosts (Mulet et al., 2010). Nübling et al. (2016) in particular, isolated *Pseudomonas* spp. from vegetables, specifically lettuce, and he reported that commonly detected species on minimally processed vegetables are *P. chicorii*, *P. fluorescens, P. putida, and P. maltophila.*

Table 3.3: Morphological properties of isolated rhizospheric *Pseudomonas*

Isolate codes	**Isolate identification name**	**Growth colony nature**	**Texture of colony**	**Colour of plate**	**Formation of pigment**
S6	*Pseudomonas putida*	Fast and smooth	Bubbles on colony	Creamy yellow	Yes
B5	*Pseudomonas mediterranea*	Fast and smooth	Very sticky and firm	Light brown	Yes
B9	*Pseudomonas fluorescens*	Fast and smooth	Sticky and slimy	Greenish lime	Yes

Table 3.4: Physiological and biochemical properties of bacterial isolates

Biochemical and Physiological properties	Bacterial Isolates		
	S6	B5	B9
Inhibition ability	+	+	+
Nitrate reduction	+	+	+
Catalase activity	+	+	+
Starch hydrolysis	+	+	+
Casein hydrolysis	-	+	+
Carbohydrates utilization			
D-galactose	+	+	+
D-glucose	+	+	+
D-xylose	+	+	+
D-mannitol	+	+	+
Sucrose	+	+	+
Fructose	-	+	+
Lactose	+	-	-

Note: S and B stand for bacterial isolates

3.6.2 *In vitro* inhibition of *Fusarium graminearum* by *Pseudomonas*

In-vitro fungal growth inhibition assay using *Pseudomonas spp.* as antagonists yielded a high percentage of growth inhibition (Figure 3.1). The growth inhibition assay was observed over a period of 7 days. After 7 days of incubation *Pseudomonas putida* had the highest inhibition growth percentage of 87.50%, followed by *Pseudomonas mediterranea* with 81.25% and *Pseudomonas fluorescens* with 75.00%. One might have expected *Pseudomonas fluorescens* to be the highest but in this case it was not and this might be due to the limitations of using

biochemical characteristics to determine inhibition growth amongst isolates. *Pseudomonad*s that showed an inhibition halo around their streaks in the screening plates were selected to perform single antagonism assays.

Figure 3.1: Agar plate Pseudomonads inhibition of *Fusarium graminearum*.

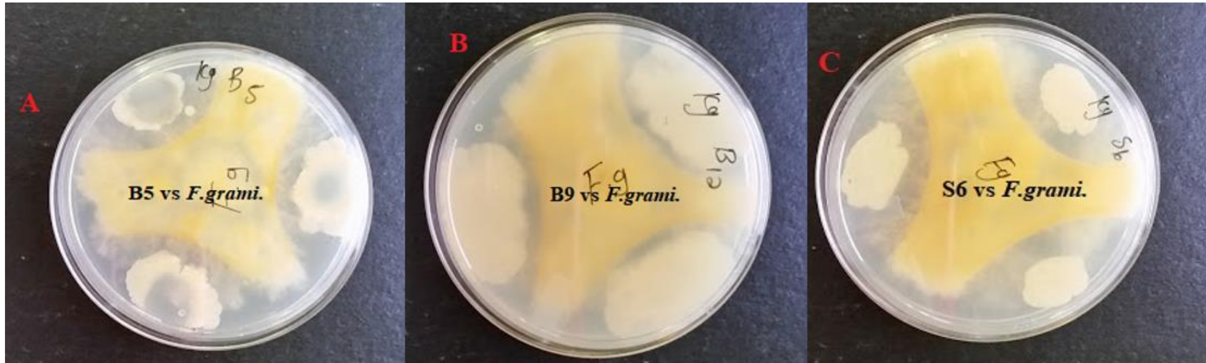

Figure 3.1: **A)** Inhibition zones of *F. graminearum* by *Pseudomonas mediterranea*. **B)** Inhibition zones of *F. graminearum* by *Pseudomonas fluorescens.* **C)** Inhibition zones of *F. graminearum* by *Pseudomonas putida.*

3.6.3 Effect of PEG 8000 on bacterial growth

For bacteria to survive abiotic stresses on the environment, high tolerance to PEG is required because tolerance to PEG medium is directly related to the ability of bacteria to survive and grow in environments with low water availability and high salt percentages. From this study, the effect of PEG on each bacterial growth was determined on LB medium amended with 5% PEG. The growth pattern of all bacterial isolates on medium supplemented with PEG in comparison with their controls is shown in Figure 3.2. From the results, growth varied among the isolates and bacterial growth decreased in PEG supplemented medium for all the isolates compared to their respective controls. In the control medium, maximum growths of 1.26±0.05, 1.04±0.8 and 1.57±0.06 were reached by isolates B5, B9 and S6 respectively. On the medium that was supplemented with PEG the maximum growth were 1.09±0.15,

0.8±0.15 and 1.43±0.07 for isolate B5, B9 and S6 respectively. Isolate S6 showed the highest optimum growth on both mediums.

All bacterial isolates tested showed significant growth in the presence of PEG. This indicates their potential to be used as bio-inoculants in alleviating growth of *Fusarium* even in biotic stressed environments. Different bacteria species of *Pseudomonas* have been reported by Chandra et al. (2018) to have the ability to inhibit *Fusarium* even in PEG supplemented medium. The result from this study is in agreement with that of Ali et al. (2013) who reported that *Pseudomonas* isolates also showed outstanding growth in PEG supplemented medium.

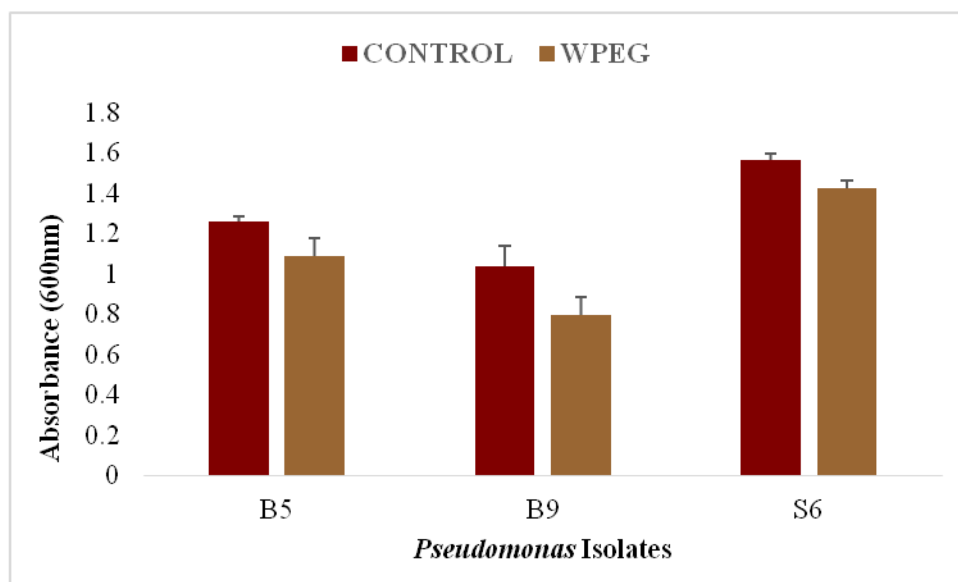

Figure 3.2: Effect of PEG 8000 on bacterial growth. WPEG = With PEG 8000

3.6.4 Effects of temperature on bacterial growth

The activity and growth of bacteria in soil depends on the soil temperature, which affects cellular enzymes. However, extremely high temperatures cause denaturation of protein structures while very low temperatures (closer to freezing point) cause the inactivation of enzymes and decrease in cellular metabolism (Akond et al., 2016). Most *Pseudomonas* spp. thrive better at temperatures that range between 25 and 30°C. In this study, three spp. of *Pseudomonas* isolates were grown under different temperatures. It was observed that all

tested bacterial isolates grew at various temperatures, although there were variations in the growth pattern of each isolate. For isolate B5 an optimum growth of 0.58±0.07 was observed at 25°C while B9 and S6 showed a high optimum growth of 0.36±0.13 and 0.29±0.03 at 25°C. Isolate S6 had the highest optimum growth of 0.66±0.07 at 30°C while growth optimums for B5 and B9 was reported as 0.34±0.07 and 0.22±0.07 respectively.

Results obtained from this study showed that isolates B5 and B9 would thrive best at room temperature while at 35°C they obtained 0.32±0.07 and 0.17±0.05 respectively, favouring growth of isolate S6 with the highest growth of 0.43±0.05. In this study, significant decrease in growth was observed for all isolates at 40°C with isolate B5 obtaining 0.22±0.04, B9: 0.17±0.03 and S6: 0.328±0.06. Most *Pseudomonas* spp. such at *P.* putida (Schmitz et al., 2015) are aerobic, indicating that oxygen is required for their growth. The effect of temperature on the growth of the tested bacterial strains showed that these bacteria can survive at varying temperature ranges. It indicates their potential to survive in temperate, harsh or hot climate conditions. The results obtained were similar to those obtained by Ndeddy Aka and Babalola (2017), that showed a decrease in the growth of bacterial isolates when the temperature was raised to 40°C. According to them, the decrease in growth at this temperature could be due to a decrease in metabolic activity of the bacterial isolates caused by the high temperature increase. These results also agree with Shakibaie et al. (2015), who reported that most mesophilic bacteria represent their highest attachment capacity at optimum cultivation temperature and the number of attached cells decreases with alteration from related optimum temperature. They were catalase and oxidase positive, showing growth at either of the extreme temperatures, 4°C and 40°C, and displayed oxidative utilization of glucose. Based on these typical characteristics, they are identified as *Pseudomonas* spp.

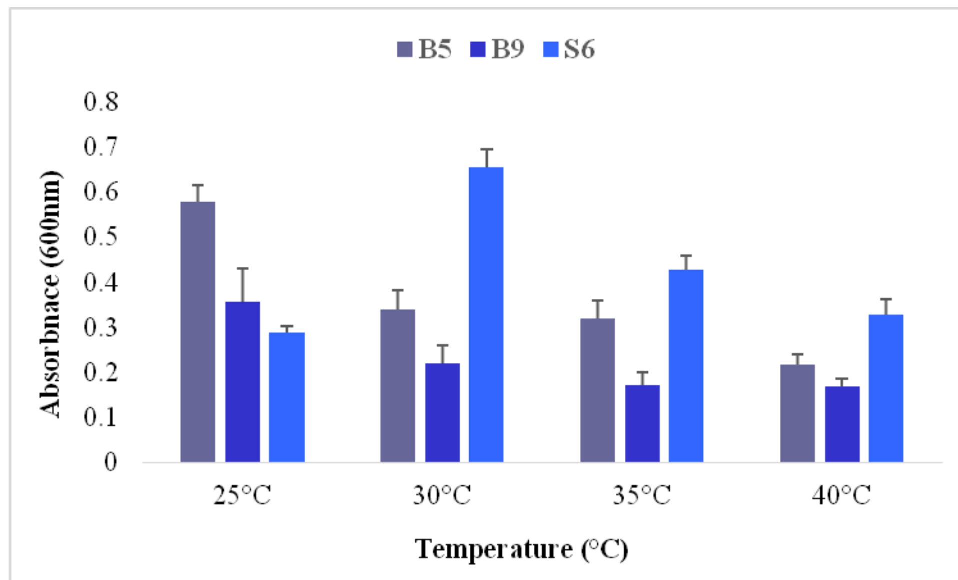

Figure 3.3: Effect of temperature on bacterial growth

3.6.5 Effects of sodium chloride (NaCl) on bacterial growth

Bacterial isolates B5, B9 and S6 were tested for their potential to withstand salinity stress by growing at different concentrations of sodium chloride (NaCl) 0, 2, 4, 6, 8, 10. Figure 3.4 showed that maximum growths of 0.56±0.06, 0.26±0.07 and 0.89±0.20 were observed for isolate B5, B9 and S6 respectively 0% NaCl. Optimum growth for all isolates was at 2% and results showed that the isolates grew to 0.78±0.05, 0.39±0.05 and 1.24±0.16 respectively. It was observed that S6 had the highest optimum growth at 2% with 1.24±0.16 while B5 and B9 reached growth of 0.78±0.05 and 0.39±0.05 respectively. On the other hand, the isolates decreased their growth at 4% to 0.75±0.17 by B5, 0.30±0.06 by B9 and 0.84±0.23 by S6. At 6% the isolates were declining in growth to 0.63±0.08, 0.19±0.06 and 0.72±0.10 by isolate B5, B9 and S6 respectively. The growth of the isolates kept declining as the concentration of NaCl increased with isolate B9 obtaining the lowest optimum of 0.16±0.05 at 8% amongst all tested isolates, while B5 obtained 0.43±0.10 and 0.62±0.03 for S6. No isolates showed growth at the highest NaCl concentration. At 10% B5 reached a growth of 0.38±0.12, B9 reached 0.19±0.07 and S6 reached 0.53±0.10. From the results obtained, all tested isolates

were able to grow at all the NaCl concentrations used in this study. This confirms their ability to withstand various salinity stress conditions and suggests their applicability for salt tolerance improvement in plant growth and survival. Results obtained also indicate that these bacteria strains are halotolerant because of their ability to withstand various levels of salinity stress. High salt concentration also affects osmotic pressure and causes protein denaturation, therefore halophilic bacteria possess specific enzymes in their active configuration that are only activated upon exposure to high salt concentrations (Akond et al., 2016). To confirm this study, Egamberdieva et al. (2016) reported that studies have indicated that the salt-tolerant *Pseudomonas extremorientalis* strain TSAU20, capable of growing with 4 % (684 mM) NaCl, is both an excellent root colonizer and antagonist towards a plant-pathogenic fungus. For example, accumulation of excess sodium plant cell walls rapidly lead to osmotic stress and cell death (Shrivastava and Kumar, 2014).

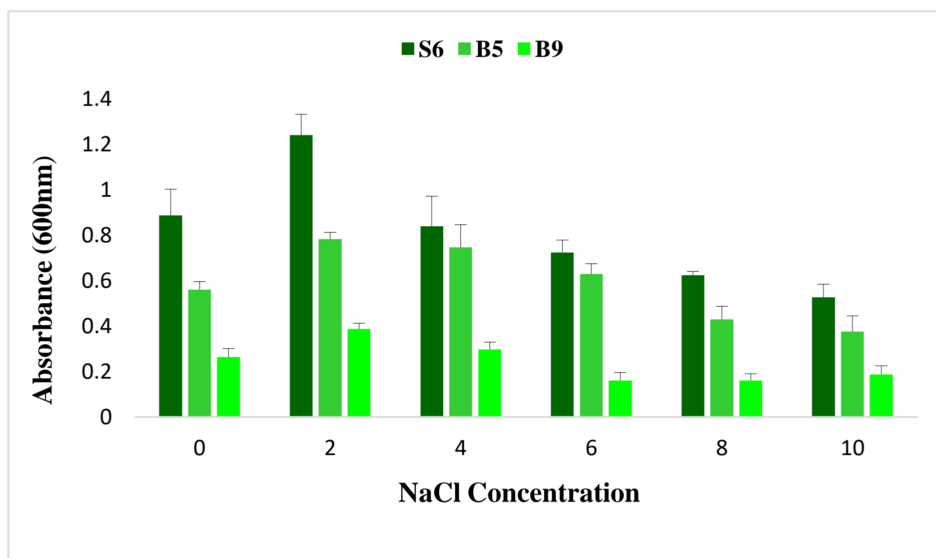

Figure 3.4: Effect of NaCl concentration on Bacterial growth

3.6.6 Molecular characterization of bacterial isolates

Extraction of DNA using Zymo (ZR) soil microbe DNA MiniPrep kit yielded high quality DNA according to the NanoDrop readings (Table 3.1). PCR amplification of the 16S rDNA

genes was successfully performed using Psfor and Psrev universal primers as shown in Table 3.2. The molecular marker (GeneRulerTM DNA Ladder) showing fragments of 1kb can be seen in the first Lanes in Figure 3.5. No non-specific amplification was observed. Bacterial identification was confirmed by computational analysis. The identification of *Pseudomonas* isolates based on genus level was performed by partial sequences of their 16S rDNA gene. Partial 16S rDNA nucleotide sequences from all bacterial isolates in this study were matched with similar sequences in the NCBI website using BLASTn program. The BLASTn homology search of NCBI for 16S rDNA gene sequences confirmed that all isolates were *Pseudomonas spp.* Isolates B9 had 100% similarity index with *Pseudomonas fluorescens* while isolates B5 and S6 showed 99% similarity to *Pseudomonas mediterranea* and *Pseudomonas putida* respectively. The gene sequences of all isolates were submitted to GenBank (NCBI) with the following accession numbers: MH666038 (B9), MH666037 (B5) and MH666036 (S6) (Table 3.5).

The three selected *Pseudomonas* isolates used in this study were subjected to sequencing and phylogenetic analysis. As shown in Figure 3.10, the evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model. The tree with the highest log likelihood (-3388.1425) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach and selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 18 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 607 positions in the final dataset. Neighbour Joining revealed the percentage of evolutionary relationships with

the inhibition *Pseudomonas* isolates according to the degree of differences among the sequences. Evolutionary analyses were performed in MEGA7 (Kumar et al., 2016).

Bacterial isolate B9 possessed the highest similarity index of 100% with *Pseudomonas fluorescens.* The other isolates (B5 and S6) also possessed very high similarity index of 99% with E-value = 0. These *Fusarium graminearum* inhibiting *Pseudomonas* isolates expressed a high similarity value above the 70% borderline degree of relatedness as suggested by Brenner et al. (2015). Furthermore, the similarities expressed by these isolates with the reference taxa belonging to different species, is due to the high similarity value exhibited in the values of DNA association which fall below the 70% threshold values (Stackebrandt and Goebel, 1994). According to Konstantinidis and Stackebrandt (2013), this shows a very high genetic relatedness that is progressively more reliable, as they cannot be wiped out overnight. In all, the high-level branching in the phylogenetic tree is in harmony with the traditional systematic divisions that classifies organisms belonging to the same family and genus into different species. The molecular identification of bacterial isolates is very important because it identifies organisms up to the species level and provides information about the organisms, such as the compounds they produce and whether they are novel or not (Adegboye and Babalola, 2012).

The analysis of 16S rDNA gene sequences has proven to be a very important method of phylogenetically characterizing microorganisms as it helps to explain the evolutionary relationship between organisms (Thenmozhi and Kannabiran, 2010). The phylogenetic relationship of the bacterial isolates used in this study was first estimated using a Blast search in the GenBank database and closest related strains were picked for pairwise sequence comparison, hence the construction of the phylogenetic tree. Most of the closest strains to the bacterial isolates in the present study have been associated with one stress tolerance or another. An example is the study by Goswami et al. (2016) which revealed the mechanism of

action of PGPR belonging to genera *Acinetobacter*, *Agrobacterium*, *Arthobacter*, *Azotobacter*, *Azospirillum*, *Bacillus, Burkholderia*, *Bradyrhizobium*, *Frankia, Pseudomonas, Rhizobium*, *Serratia* and *Thiobacillus*.

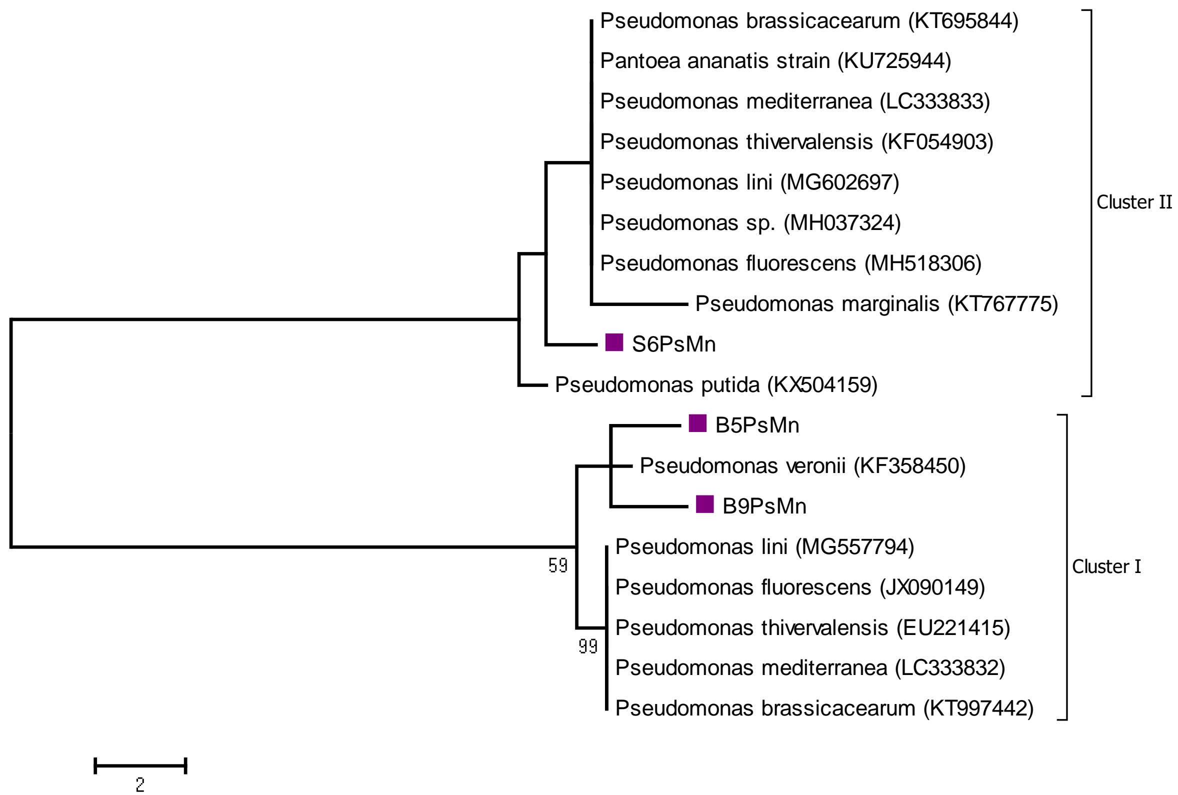

Figure 3.5: Neighbour-joining tree of the isolated pseudomonas isolates and representative species of pseudomonas bacteria based on partial 16S rDNA gene sequences. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. Only values greater than 50% are shown. The scale bar indicates 2 substitutions per nucleotide position.

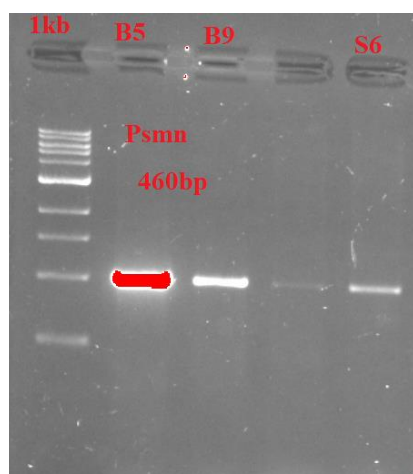

Figure 3.6 The agarose gel showing amplified DNA sequence for the *Pseudomonas* isolates (B5, B9 and S6) a at 989 bp. Lane 1= 1Kb molecular weight marker.

Table 3.5: Partial 16S rDNA sequence alignment results of the *Pseudomonas* isolates from the NCBI blast search.

Isolate	Accession no	Blast ID	Similarity (%)	E-value
S6	MH666036	*Pseudomonas putida*	99	0
B5	MH666037	*Pseudomonas mediterranea*	99	0
B9	MH666038	*Pseudomonas fluorescens*	100	0

The BLAST query grouped the *pseudomonas* isolates into different *Pseudomonas* spp.*, which* is closely related with the results obtained from the biochemical tests performed on the various isolates.

3.6.7 PCR amplification of genes coding for biocontrol traits

Bacteria have been found to possess certain genes which enhance their tolerance to abiotic and biotic stresses. This study used primer-specific PCR amplifications on total genomic DNA fractions that targeted certain genes responsible for inhibition of *Fusarium* in bacteria. The antibiotic biosynthetic potential was studied by PCR detection of the corresponding genes. The *phz* gene, involved in phenazines production, was detected in isolate B5 and B9

with a band size 429 bp (Figure 3.6) and no amplification was observed for isolate S6. These two isolates were the only isolates that detected the *phltB* which is a gene involved with the production of pyoluteorin, B5 and B9 produced a band size of 379 bp (Figure 3.6) while no amplification was observed for isolate S6. As shown in Figure 3.7, the PCR amplification of the *prnD* gene, required for pyrrolnitrin synthesis, was detected in all isolates (B5, B9 and S6) at an expected band size of 800 bp. Equally, the *phl2* gene encoding the synthesis of 2, 4-DAPG was also detected in the DNA fragments of isolates B5, B9 and S6 with a band size of 389 bp (Figure 3.7). Figure 3.8 shows the results for the amplification of the *sid* gene. The *sid* gene which encodes for siderophore production was detected in all isolates (B5, B9 and S6) with a band size of 452 bp. The ACC deaminase (*accd*) gene was amplified by PCR for all three isolates (Figure 3.9) with the reference primers as shown in Table 3.2. The isolates yielded an expected band size of 400 bp.

The ability of some of the tested isolates to amplify at the target product sizes of pathogen inhibition and PGP genes used in this study could indicate the presence of these genes in their genome or DNA chromosomes present in the nuclear regions of various bacterial cell walls. The present study is in harmony with the study of Raddadi et al. (2008) who reported a positive PCR for all bacterial strains tested for the *accd* gene coding for ACC deaminase enzyme. Ali et al. (2013) also reported that the *Pseudomonas* isolate SorgP4 amplified the *acds* gene. Bacteria possessing ACC deaminase gene can help lower the ethylene levels in plants and also assist in the protection of plants against certain environmental stresses such as drought, flooding, phytopathogens and heavy metals (Tiwari et al., 2016) which induce the synthesis of ethylene. The presence of the a*ccd* gene in the isolates (B5, B9 and S6) in this study could be of great importance in field application under stress conditions. The isolates amplified at 400 bp for *accd* gene. The presence of siderophore gene in bacterial isolates can enhance biocontrol of phytopathogenic fungi due to the competition for iron by plants and

improve the availability of iron to plants. This is confirmed by a study of Ferreira et al. (2018) who reported that the production of the secreted siderophores by the *P. mediterranea* strains and their application in environmental technologies are promising, eco-friendly and safe enough to be implemented both in metal removal and metal recovery processes. From the results obtained in the present study, all the isolates used in this study possessed one or more PGP genes. This clearly indicates their potential for possible use as bio- inoculants, not only to inhibit growth of *F. graminearum* in plants but also to serve as biofertilizers and biocontrol agents to facilitate plant growth.

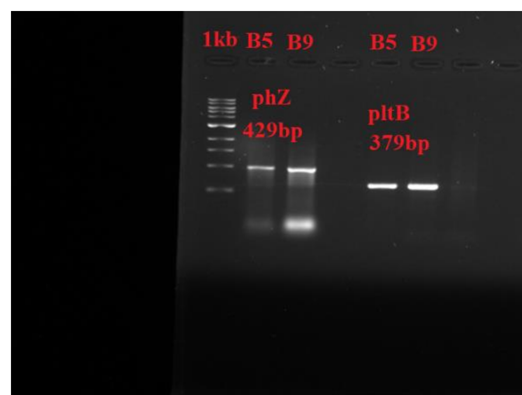

Figure 3.7: Agarose gel showing amplified DNA sequences of *phz* at 429 bp for isolate B5 and B9; and *phltB* sequences for B5 and B9 at 379 bp. Lane 1= 1Kb molecular weight marker.

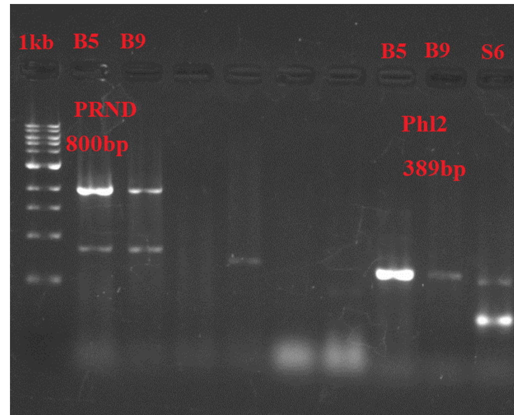

Figure 3.8: Agarose gel showing amplified DNA fragments of all isolates for *prnd* at 800 bp and for *phl2* at 389 bp. Lane 1= 1Kb molecular weight marker.

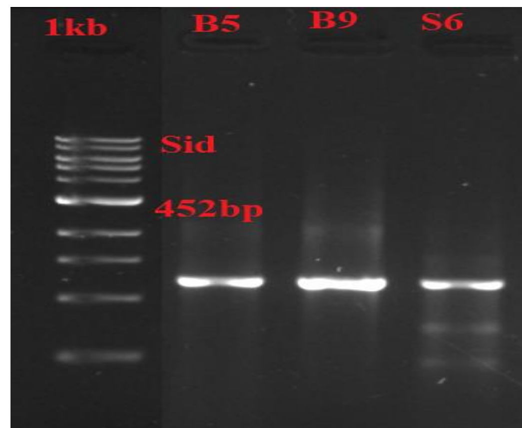

Figure 3.9: Agarose gel showing amplified DNA fragments of all isolates for *sid* at 452 bp. Lane 1= 1Kb molecular weight marker.

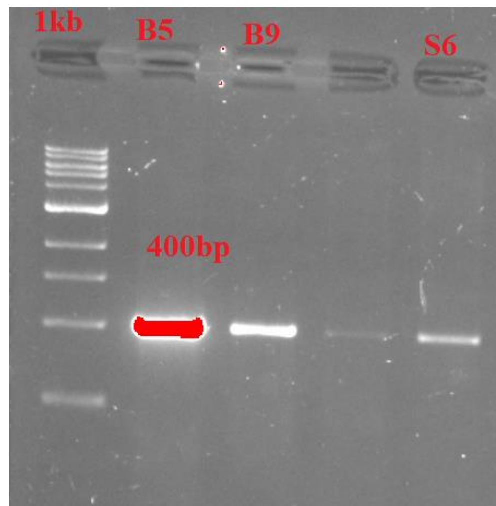

Figure 3.10: Agarose gel showing amplified DNA fragments of all isolates for *accd* at 400 bp. Lane 1= 1Kb molecular weight marker.

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CHAPTER FOUR

Quantitative and Qualitative Determination of the Modes of Action of *Pseudomonas* spp. Against Proliferation of *Fusarium graminearum* in Maize (Zea mays L.)

4.0 Abstract

Identification and characterisation of *Pseudomonas* for PGPB characteristics is necessary as it helps to recognize and use certain species for growth promotion and suppression of pathogens in plants. The *Pseudomonas* isolates P. *fluorescens strain B9, P. mediterranea strain B5 and P. putida strain S6* were screened for the presence of PGP characteristics as well as the amounts produced. Through biochemical assays, it was observed that all isolates produced siderophore with the highest production of 51.90% found in *P. putida.* All isolates produced indole-3-acetic acid, ACC deaminase activity and ammonia, while two produced solubilized phosphate and one produced hydrogen cyanide. *P. putida* was the most abundant and best performing isolate with the highest IAA (9.57±0.66 µg/ml) and the highest ACC deaminase activity (0.87±0.12 µmol α-ketobutyrate mg protein^{-1} hour^{-1}). A greenhouse experiment was performed to evaluate the effect of two isolates *P. mediterranea* and *P. putida* on growth inhibition of *F. graminearum* in maize using two different genotypes of maize seeds and two inoculation methods. Results showed that the greatest increase in physiological parameters (Chlorophyll index, number of root and leaves, leaf area, root length, dried leaf weight, dried root weight, dried shoot weight and dried plant biomass weight) were obtained when the two bacteria were c-inoculated on maize seeds such as in chlorophyll (9,86±0,74 and 8,64±1,29 for treatment Sp+F+B1+B2 and Sb+F+B1+B2 respectively) and dry root weight (16,69±3,46 and 15,47±1,23 for treatment Sp+F+B1+B2 and Sb+F+B1+B2 respectively). Increases in measurements were also observed in individually inoculated plants, highest number of leaves (12±0,00 for treatment Sd+F+B2 followed by treatment Sp+F+B1 and Sp+F+B2 both with 11,67±0,58), compared to the control plants. Higher growth was also observed in plants whose seeds were coated with vermiculite than the plants whose seeds were inoculated without coating. The ability of these isolates to produce PGP characteristics as well as *Fusarium* growth inhibition shows that they

can be effectively used to promote plant growth in plants in either greenhouse or field conditions.

Keywords: Maize seed *FRANCE DK315*, Maize seed *PR37Y15*, greenhouse, plant growth promotion, vermiculite, *Pseudomonas putida*, *Pseudomonas mediterranea*.

4.1 Introduction

Maize (*Zea mays* L.) is one of the most important crops worldwide and is used by many countries as a major source of food. However, several diseases infect this important crop causing severe damage and yield loss (Kwak et al., 2012b). These diseases include Fusarium head blight (FHB) and stalk rot. *Fusarium graminearum* (previously also named *Gibberella zeae*), which can cause gibberella stalk rot, is thought to possess the highest pathogenicity and aggressiveness among the species responsible for stalk rot (Zhang et al., 2016). FHB is caused by *Fusarium graminearum* and FHB is a major fungal disease of multiple cereal crops including wheat, barley, maize, oat, rye and triticale. FHB causes significant yield losses, reduces grain quality and contaminates the grain with fungal mycotoxins (Machado et al., 2018). Depending on the nature of plant spp. Involved, different interactions occur between bacteria, fungi and plant, which can affect the plant negatively or positively (Kumar et al., 2012).

Microbial activity in rhizosphere soil affects plant health and growth of many rhizobacteria have the potential to inhibit growth of *F. graminearum* in maize by producing certain plant growth promoting rhizobacteria (PBPR) traits. The majority of the major group of PGPR belong to members of the genera of PGPR belongs to genera *Acinetobacter*, *Azospirillum*, *Burkholderia*, *Agrobacterium*, *Arthobacter*, *Pseudomonas*, *Azotobacter*, *Bradyrhizobium*, *Rhizobium*, *Frankia*, *Serratia*, *Thiobacillus* and *Bacillus* (Singh et al., 2015). Bacteria are important soil components, able to form mutualistic and beneficial associations with most plants. Symbiotic bacteria are capable of conferring stress tolerance to a wide variety of their plant hosts through phytohormonal modifications, production of exopolysaccharides, accumulation of osmolytes and acting as a defence against reactive oxygen species. These bacteria are also able to synthesize antibiotic substances, fix atmospheric nitrogen, produce soluble iron compounds (siderophore), and solubilize inorganic phosphates. In addition, they

serve as plant growth regulators by producing the phytohormones indole-acetic acid (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC), cytokines and gibberellins (GA). These outstanding properties of PGPR facilitate the efficient stimulation of plant growth during unfavourable environmental conditions like plant attack by *Fusarium*. Several studies have revealed the successful application of isolated PGPB on *Fusarium* growth inhibition improvement in plants (Machado et al., 2018) and most of these studies have concentrated on certain groups of bacteria species such as *Bacillus* and *Pseudomonas***.**

The use of *Pseudomonas* spp. to inhibit growth of *Fusarium graminearum* in plants has received a lot of attention over the years but only little is fully understood about the mechanism of action used by these species. *Pseudomonas*, found mostly in soils, are widely known for their antibiotic production and their outstanding ability to survive in unfavourable environments (Singh et al., 2015). Their ability to produce certain PGP properties has also been identified, but with little information on the extent of these properties produced (Ali et al., 2013). Hence, this study was conducted to:

1. Screen *Pseudomonas* spp. with the potential to inhibit growth of *Fusarium graminearum* by the production of plant growth promoting (PGP) elements;
2. Determine the amount of certain PGP elements (IAA, ACC deaminase, siderophore) produced by the *Pseudomonas* bacteria;
3. Evaluate the effect of *Pseudomonas* inoculation on the growth of two varieties of maize (*Zea mays* L.) seeds; and
4. Determine the effect of inoculation method on growth inhibition of *F. graminearum* in maize.

4.2 Materials and methods

4.2.1. Phosphate solubilisation and Hydrogen activity

To evaluate the ability of *Pseudomonas* isolates to solubilize phosphate (appendix page 134), 10 µl of freshly prepared culture were spot inoculated on Pikovskaya's agar plates containing 2% tri-calcium phosphate. Inoculated plates were incubated at 30°C for 72 h, plates were observed for the appearance of a clear zone around the *Pseudomonas* colonies (Browne et al., 2009). The test was done in three replicates. Hydrogen cyanide activity was determined according to the protocol of Bakker and Schippers (1987). Bacterial cultures were separately streaked on Luria Bertani (LB) agar amended with 0.4% (w/v) of glycine. A Whatman no. 1 filter paper soaked in 0.5% (w/v) picric acid in 2% (w/v) sodium carbonate was placed on the lid of the Petri dish. Thereafter, plates were properly sealed with parafilm and incubated for seven days. The change in colour of the filter paper from yellow to deep orange indicates a positive result. The test was done in three replicates.

4.2.2 Indole-3-acetic acid production

For qualitative determination of indole-3-acetic acid production by *Pseudomonas* isolates the method of Ghirardi et al. (2012) was employed. Freshly prepared bacterial cultures (20 µl) were inoculated in LB broth (20 ml) amended with 5 mmol tryptophan and incubated at 27°C for 4 days. After incubation, 1 ml of bacterial culture was transferred into sterile Eppendorf tubes and centrifuged at 5,000 g for 15 min. The supernatant was collected in a 15 ml centrifuge tube and 2-3 drops of orthophosphoric acid was added alongside 4 ml of Salkowsky reagent (50 ml of 35% perchloric acid in 1 ml of 0.5 M FeCl3). The contents in the tubes were incubated at room temperature under dark conditions for 20 min, the development of a pink colour indicated IAA production. The absorbance of the pink color was read using a UV spectrophotometer (Thermo Spectronic, Merck chemical, SA) at 530 nm. The amount of IAA produced by each bacterial isolate was determined by the generation

of a standard curve (appendix page 135). Standards were made in LB broth at 0,5,10, 20, 50 and 100 μg/l including a control consisting of LB broth only, 2 ml of Salkowsky reagent was added to 1 ml of each standard and incubated at room temperature for 20 min. Absorbance was read at 530 nm using a UV spectrophotometer (Thermo Spectronic, Merck chemical, SA). The test was done in three replicates.

4.2.3 Siderophore, Ammonia and ACC deaminase production

The production of siderophore by bacterial isolates was assayed according to a protocol described by Schwyn and Neilands (1987) using an indicator dye, chrome azurol S (CAS). Briefly, 60.5 mg of CAS was dissolved in 50 ml of distilled water and mixed with 10 ml of iron (III) solution (1 mM $FeCl_3$. $6H_20$ in 10 mM HCl). The mixture was slowly added while constantly stirring with a magnetic stirrer to 72.9 mg of hexadecyltrimethylammonium (HDTMA) bromide dissolved in 40 ml distilled water and then autoclaved at 121°C for 15 min. The final mixture (100 ml) was added while stirring to 900 ml of sterilized LB broth adjusted to pH 6.8 and poured into petri plates. Upon solidification, freshly prepared bacterial cultures were spot inoculated on the petri plates and incubated at 25°C for 7 days. A yellowish- orange halo around the bacterial colonies was considered a positive result for siderophore production (appendix page 133). The amount of siderophore produced by each bacterial isolate was estimated following the protocol of Alexander and Zuberer (1991) using a modified CAS assay solution. Hexadecyltrimethylammonium (HDTMA, 21.9 mg) was dissolved in 25 ml of distilled water with constant stirring under low heat. In a 50 ml flask, 1.5 ml of 1 mM FeCl3. 6H20 in 10 mM HCl was added to 7.5 ml of 2 mM CAS. This solution was slowly added to the HDTMA solution and the resultant mixture transferred to a 100 ml flask. A buffer solution was prepared by dissolving 9.76 g of 2-(N-morpholino)ethanesulfonic acid (MES) in 50 ml distilled water and the pH adjusted to 5.6 with 50% KOH. This buffer solution was then added to the flask containing the dye solution

while distilled water was added to get a final volume of 100 ml. A shuttle assay solution was prepared by adding 87.3 mg of 5-sulfosalicyclic acid to the above solution before use. All three isolates to be tested for siderophore were inoculated in 5 ml sterilized LB medium without added Fe and incubated at 25°C for five (5) days. Bacterial cells were pelleted by centrifugation at 3000 g for 10 min and the supernatant was collected in tubes. The concentration of siderophore in the supernatant was obtained by mixing 100 µl of CAS assay solution with 100 µl of supernatant and allowing to equilibrate for 3-4 h, the absorbance of the 200 µl mixture was measured at 630 nm using a UV spectrophotometer (Thermo Spectronic, Merck chemicals, SA). The test was done in three replicates. The percentage siderophore produced was calculated by the equation:

$$\% \text{ Siderophore units} = \frac{Ar - As}{Ar} \times 100$$

Where Ar = Absorbance of reference at 630 nm (CAS reagent),

As = Absorbance of sample at 630 nm

The production of ammonia by *Pseudomonas* isolates was tested by inoculating 10 µl (0.2 OD) of freshly prepared *Pseudomonas* cultures into test tubes containing 10 ml of peptone water. The inoculated test tubes were incubated at 25°C for 48-72 h after which 1 ml of Nessler's reagent was added to each test tube, and any colour changes were observed. A change in the colour of the media to yellow or brown specifies a positive result for ammonia production. The experiment was done in triplicate (Laslo et al., 2012). The test was done in three replicates. Three *Pseudomonas* bacterial isolates used in this study were screened for ACC deaminase activity based on their ability to utilize ACC as sole nitrogen source. All bacteria were first grown on 5 ml of Tryptone-Soy broth (TSB, rich medium) and incubated at room temperature for 48 h. Bacterial cells were harvested by centrifugation at 5000 g for 5 min, washed twice with sterile 0.1 M Tris-HCl (pH 7.5) and resuspended in 1 ml of 0.1 M Tris-HCl (pH 7.5). Washed bacterial cells were spot inoculated on petri plates containing

modified Dworkin and Foster salts minimal medium (Dworkin and Foster, 1958). Minimal salts medium was composed of 2 g glucose, 2 g gluconic acid, 2 g citric acid, 4 g KH_2PO_4, 6 g Na_2HPO_4, 0.2 g $MgSO_4.7H_2O$ and 10 ml micro nutrient solution (200 mg $CaCl_2$, 200 mg $FeSO_4.7H_2O$, 15 mg H_3BO_3, 20 mg $ZnSO_4.7H_2O$, 10 mg Na_2MoO_4, 10 mg KI, 10 mg NaBr, 10 mg $MnCl_2$, 5 mg $COCl_2$, 5 mg $CuCl_2$, 2 mg $AlCl_3$, 2 mg $NiSO_4$ and 1000 ml distilled H_2O) in 990 ml distilled H_2O amended with 3 mM ACC as a sole nitrogen source. Negative control for this experiment was Petri plates containing only DF minimal salts medium without ACC while the positive control consisted of plates containing DF minimal salts medium + 0.2% (w/v) $(NH_4)_2SO_4$. Inoculated plates were incubated at 30°C for 5 days. The growth of bacterial isolates on DF minimal plates containing ACC was used to compare those of the positive and negative controls. Petri plates were selected based on bacterial growth by utilizing ACC as sole source of nitrogen. The experiment was repeated three times. Alpha-ketobutyrate concentration in each sample was determined by comparing with a standard curve (appendix pp 120) generated as follows: alpha-ketobutyrate solutions (500 µl) of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 mM were mixed each with 400 µl of 0.56 N HCl and 150 µl of 2,4-DNP solution. One (1) ml of 2 N NaOH was then added and mixed. Absorbance was measured at 540 nm and the values obtained for the absorbance against concentration (mM) were used to generate a standard curve. The test was done in three replicates.

4.3. Greenhouse experiments

Greenhouse experiments were conducted to: (i) evaluate the effect of bacterial inoculation on the growth of maize plant when the maize seeds have been inoculated with *Fusarium graminearum* and (ii) evaluate the effect of the method of inoculation on growth inhibition of *Fusarium graminearum* in maize. Maize, known to be very susceptible to *Fusarium*, was chosen based on its high nutritional and economic importance in South Africa. Knowledge

gained from this will help to find suitable means of improving maize growth and yield, despite the ravaging attack by this pathogen (Adeniji and Babalola, 2018).

4.3.1. Preparation of Pseudomonas inoculum

The two *Pseudomonas* isolates, *Pseudomonas putida* (MH666036) and *Pseudomonas mediterranea* (MH666037) were chosen and used in this experiment based on their outstanding ability to r inhibit the growth of *Fusarium graminearum in-vitro* as well as their plant growth promoting potentials. The inoculants were prepared by growing the bacterial strains in 250 ml conical flasks containing 100 ml of sterilized LB broth. Inoculated flasks were incubated at 25°C under constant shaking (120 rpm) for 7 days. Pellets were collected by centrifugation at 10,000 rpm for 20 min and washed twice with sterile distilled water. Pelleted cells were resuspended in 0.01M phosphate buffer at pH 7 and adjusted to an absorbance of 1.2 at 600 nm with a UV spectrophotometer (Thermospectronic, Merck, SA) (Ndeddy Aka and Babalola, 2017).

4.3.2 Soil collection and pot experiment preparation

Soil for the trial was collected from behind the Animal Health Centre of the North-West University, Mafikeng Campus at 27°S Latitude, 27°E Longitude and elevation of 1282.7 m. The soil was collected at the surface 0-20 cm depth, oven dried at 70°C for 48 h, passed through a 2 mm sieve and autoclaved at 121°C for 15 min. Sterilized soil was allowed to cool for 2 days after which 10 kg of soil was aseptically transferred into plastic pots.

4.3.3 Seed viability test

Seed germination tests were conducted to evaluate the effect of bacterial inoculation on the germination of the test seeds. Prior to the test, maize seeds of the variety *France DK315* and *PR37Y15* obtained from the North-West University were washed first with tap water, then soaked in 2% sodium hypochlorite ($NaClO_2$) solution for 15 min and severally rinsed with

sterile distilled water to remove the remains of the disinfectant (Madhaiyan et al., 2007). Thereafter, 4 clean Petri plates (replicated three times) were prepared by placing two filter papers at the bottom of each plate and subsequently 10 ml of each bacterial suspension or 10 ml of sterile distilled water (in the case of the control) was pipetted in each Petri plate. Sterile seeds were immersed in 10 ml of bacterial suspension for 5 h in a rotary shaker at 150 rpm after which 20 seeds were placed in each petri plate and incubated for 10 days at 25°C. Germinated seeds in each petri plate were counted and five seedlings per plate were randomly selected for growth parameter measurements (shoot length, root length and dry seedling weight). The test was done in three replicates. Percentage germination and vigor index were estimated according to the method of Ghorbanpour et al. (2013) as follows:

$$\text{Germination rate (\%)} = \frac{n}{N} \times 100$$

Where n is the number of germinated seeds after 7 days and N is the total number of seeds

Vigor index = % germination x total length of seedling (shoot length + root length)

4.3.4 Preparation of maize seeds for greenhouse experiment

Maize seeds of varieties *France DK315* and *PR37Y15* were firstly immersed in 70% ethanol for 15 min and washed three times with sterile distilled water. Thereafter, the seeds were soaked in 2% sodium hypochlorite ($NaClO_2$) solution for 10 min, and then thoroughly rinsed twice with sterile distilled water.

4.3.5 Seed inoculation with bacterial isolates

In this study, two methods of inoculation were employed to enable the bacterial isolates to adhere to the surface sterilized maize seeds. This was to determine the mode of inoculation on growth inhibition of *F. graminearum* in maize plants. Firstly, surface sterilized maize seeds were inoculated by direct immersion in bacterial cultures (1.5 OD_{600}/ ml) for 12 h. Following immersion, seeds were resuspended in 1% carboxylmethyl cellulose (CMC,

binder) in a 500 ml conical flask and finely ground and sterilized vermiculite was spread all over the seeds until they were completely coated. Both the directly inoculated and coated seeds were left to dry overnight in a sterile laminar flow chamber prior to being sown in the greenhouse. The test was done in three replicates.

4.3.6 Seed inoculation with Fusarium graminearum

Preparation of *Fusarium graminearum* was described by Adeniji and Babalola (2018) *Fusarium graminearum* isolate (F) was used as inoculum. *Fusarium graminearum* was kindly provided by Dr Claire Prigent Combaret (UMR CNRS 5557) Microbial Ecology of Lyon, University Lyon 1, France, and Prof Cristina Cruz, Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências da Universidade de Lisboa, Portugal, respectively, and they were maintained on potato dextrose agar (PDA Sigma-Aldrich P2182) plates One hundred and twenty (120) sterile pre-germinated maize seeds were inoculated in 10^7 spores ml^{-1} of *F. graminearum*. The overnight pre-germinated (120) bacterized seeds above were also air dried and eighty grains were aseptically removed and submerged in the spore suspension of each fungal pathogen (10^7 spores ml^{-1}) (Munkvold and O'Mara, 2002).

4.3.7 Greenhouse evaluation of bacteria for inhibition of Fusarium graminearum

In the factorial greenhouse experiment, a total of sixty (60) pots (23-cm diameter) were used, representing five (5) treatment combinations based on three (3) experimental factors (seed treatments and bacterial types) and maize varieties, with three replicates each. The three experimental factors used in the present study include:

1. Two seed treatments (inoculation method): directly inoculated seeds and vermiculite coated seeds as described in section 4.2.3.5 above

2. Four types of seed inoculation: without bacteria isolate (control),with *F. graminearum* only, with *P. mediterranea and F. graminearum*, with *P. putida and F. graminearum,* and combination of both bacterial isolates with the fungi, and

3. Varieties of maize: *PR37Y15* and *France DK315*

All experimental pots containing treatments were arranged in a completely randomized design (CRD). Six (6) seeds were sown per plastic pot containing 10 kg of sterilized soil at a depth of 5 cm. Ten days after germination, each pot was thinned and only five (5) seedlings were left in each pot. Pots not inoculated with either bacteria or fungi: were watered with 1L of water every day for ten (10) weeks. Pots inoculated with *F. graminearum* only: fungal suspension of 50 ml (1.5 OD per ml) was added 2cm away from the root zone in each pot, eleven (11) days after germination and this routine was repeated every two (2) weeks for ten (10) weeks. Pots co-inoculated with *F. graminearum* and *Pseudomonas* spp: fungal suspension of 50 ml (1.5 OD per ml) was added 2cm away from the root zone in each pot and bacterial suspension of 50 ml (1.5 OD per ml) was added near the plant root zone in each pot and this was done every 2 weeks until the end of the experiment (10 weeks). After every two (2) weeks above ground data (chlorophyll content index, shoot length, number of plant roots and leaves and leaf area) were collected and one (1) plant from each pot would be carefully uprooted from the pots, this routine was repeated for ten (10) weeks. After uprooting, plants were washed with distilled water to remove adhering soil and root lengths were measured for each plant. Plant shoot and roots were packaged in aluminium foil papers and dried in an oven at 68°C for three days. Dry root and shoot weights were collected using a weighing balance. Thereafter, plant samples were stored in polyethylene bags at -4°C for further analysis. The greenhouse experiments were conducted twice and data were analysed together. The twenty (20) treatment combinations used in the present study are presented below as follows:

1. Sd	6. Sp	11. Sd+F+B1+V	16. Sp+F+B1+V
2. Sd+V	7. Sp+V	12. Sd+F+B2	17. Sp+F+B2
3. Sd+F	8. Sp+F	13. Sd+F+B2+V	18. Sp+F+B2+V

4. Sd+F+V 9. Sp+F+V 14. Sd+F+B1+B2 19. Sd+F+B1+B2

5. Sd++F+B1 10. Sp+F+B1 15. Sd+F+B1+B2+V 20.Sp+F+B1+B2+V

where: C= Control Maize seed, F= *Fusarium graminearum,* Sd= Maize seed *France DK315*, Sp= Maize seed *PR37Y15,* V= vermiculite coated, B1 = bacteria B5 (*P. mediterranea*), B2 = bacteria S6 (*P. putida*).

4.4 Data analysis

All experimental data obtained from this study were analysed by One-way analysis of variance ANOVA using the Statistical analysis software (SAS), version 9.4 (SAS, 2014). For each treatment, generated data were presented as arithmetic means ± standard deviation/standard error. Significantly, different means were separated using New Duncan Multiple Range Test (DMRT) at 5% level of significance.

4.5 Results and discussion

4.5.1 Growth inhibition of *F. graminearum* by *Pseudomonas*

Plants are continuously exposed to abiotic and biotic stresses such as attack by pathogen, salinity and cold (Egamberdieva et al., 2016). *Fusarium graminearum,* being one of the most serious environmental problems affecting the growth and development of maize plants and subsequently agricultural yields and food supply, has gained research attention over the years. The ravaging effects of *Fusarium graminearum* on maize plant can be reduced by the action of certain PGPB such as *Pseudomonas* spp. These bacteria are capable of tolerating and surviving under harsh environments through the regulation of phytohormones, production of ACC deaminase activity, accumulation of osmolytes, production of volatile compounds and antioxidant defence. In this greenhouse assay, *P. putida* (MH666036) and *P. mediterranea* (MH666037), showed impressive inhibition of *F. graminearum* with 87.50% and 81.25% respectively.

4.5.2 Characterization of bacteria for PGP traits

All tested isolates produced multiple plant growth promoting characteristics. The results of the qualitative plant growth promoting tests conducted are shown in Table 4.1. Results revealed that all three isolates tested were positive for ammonia, ACC deaminase activity, indole-3-acetic acid and siderophore. Isolate B5 and B9 tested positive for phosphate solubilisation by showing clear zones around colonies on plates while isolate S6 was positive for hydrogen cyanide activity.

Table 4.1: Qualitative plant growth promoting properties of *Pseudomonas* isolates.

PGP TRAITS	B5	B9	S6
Ammonia Production	+	+	+
Indole-3-acetic acid	+	+	+
Siderophore Production	+	+	+
ACC deaminase activity	+	+	+
Phosphate solubilisation	+	+	-
Hydrogen Cyanide	+	-	-

Note B and S stands for bacterial isolates

4.5.3 Ammonia production

The role of bacteria in the control of phytopathogens in plants is another area that has gained attention in recent times. Some bacterial species are able to indirectly increase plant growth by inhibiting the growth of pathogens (Dobbelaere et al., 2003). The methods used by bacteria to inhibit pathogenic growth may include the secretion of volatile compounds like ammonia and other antifungal enzymes, the production of HCN, competitive secretion and the production of siderophores (Brimecombe et al., 2000). In the present study, all tested bacterial isolates produced ammonia. Plant growth promoting bacteria produce ammonia as a secondary metabolite, playing a major role in antagonistic effects (Compant et al., 2005) (Table 4.1).

4.5.4 Indole-3-acetic acid production in *Pseudomonas* isolates

Plants developmental processes are regulated by the production of phytohormones in their various parts. The phytohormone, indole-3-acetic acid, plays a major role in plant development and its supply is capable of supporting its host during stress conditions like drought and pathogenic attacks (Sathya et al., 2017). It also improves seedling growth, and

cell differentiation, as well as enhancing both elongation and development of lateral roots in plants (Sathya et al., 2017). The results of IAA production by the tested bacterial isolates are shown in Table 4.1 and Figure 4.1. Figure 4.1 shows results of *Pseudomonas* isolates with L-tryptophan and without L-tryptophan (control). In the control medium (WxTRY) isolate S6 *(P. putida)* showed the highest IAA production of 7.08±0.77 µg/ml, followed by B5 *(P. mediterranea)* with 4.87±1.31 µg/ml and the lowest reading of IAA production was obtained by B9 *(P. fluorescens*) with 3.36±0.34 µg/ml. In a medium containing tryptophan (WTRYP) B9 *(P. fluorescens)* was also the lowest with 4.36±0.43 µg/ml, followed by B5 *(P. mediterranea)* which was higher with 7.18±1.25 µg/ml and S6 *(P. putida)* with the highest IAA production of 9.57±0.66 µg/ml. Several rhizospheric bacteria have been documented for their ability to produce IAA as well as their different biosynthetic pathways of IAA production (Duca et al., 2018). Results from the present study revealed that all tested bacterial isolates produced indole-3-acetic acid. However, the amount of this metabolite produced was significantly different in each isolate.

The increased amount of IAA produced by these bacteria in the medium used was due to the presence of L-tryptophan, as corroborated by Idris et al. (2007) who revealed that the secretion of IAA can be increased by the addition of L-tryptophan in medium. In this regard, inoculating maize plants with IAA producing bacteria can improve the growth and development of the maize plants under the attack of *Fusarium graminearum*. The IAA result of this study is in agreement with previous reports of IAA production by bacteria. Studies have shown that the endophytic IAA producing *Pseudomonas spp. UW4* improved seed germination, growth and root elongation in plants (Duca et al., 2018). Marathe et al. (2017) suggested that the IAA produced by the plants is proposed to act as a stimulator of cell elongation and proliferation, and enhancement of mineral and nutrient uptake from the soil by the host plant. The production of phytohormones such as IAA is another commonly reported

mechanism of plant growth promotion (Patten and Glick, 2002). In this study, all tested isolates produced IAA. IAA promotes root development and nutrient uptake, making it a very important mechanism of PGP. This was also confirmed by Kamble and Galerao (2015) who reported that beside plant growth, IAA also affects the central metabolism of bacteria as studied in case the of *Sinorhizobium meliloti.* Alternative bacteria for IAA production are *Arthrobacter*, *Bacillus*, *Klebsiella, Streptomyces atrovirens* and *Paenibacillus* species.

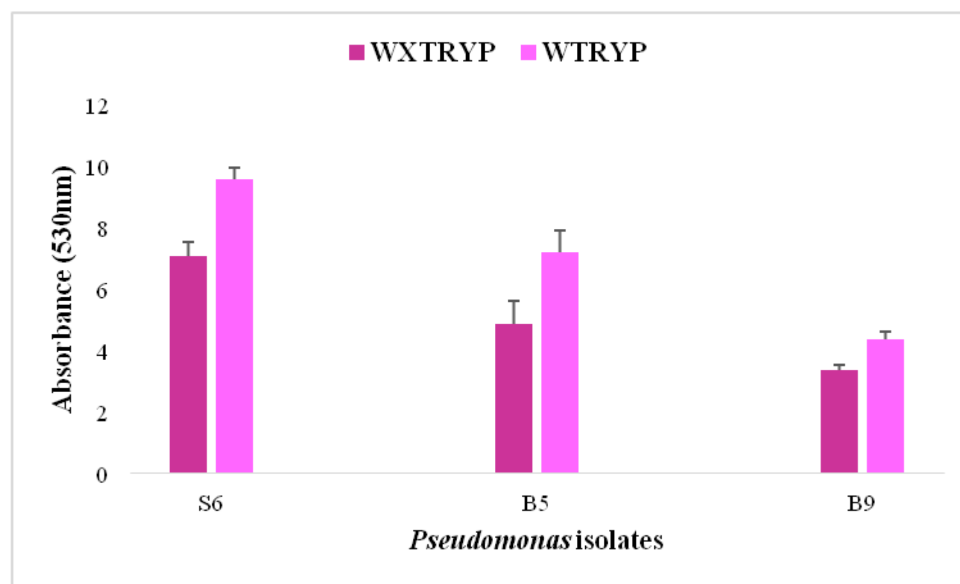

Figure 4.1: Percentage of Indole-3-Acetic Acid (IAA) production by *Pseudomonas i*solates. WXTRY= Medium without L-tryptophan (control), WTRYP= = Medium with L-tryptophan.

4.5.5 Siderophore and hydrogen cyanide (HCN) Production

Both qualitative and quantitative siderophore production was detected on all bacterial isolates tested (Table 4.1 and Figure 4.2). In the quantitative siderophore tests, statistically different values of percentage siderophore production were obtained for the tested bacteria. The maximum percentage was produced by *P. putida* (51.90±1.80*)* followed by *P. mediterranea strain B5* (47.84±0.81) and the lowest production of siderophore was *P. fluorescens strain B9* (41.33±1.19). Data obtained for siderophore production by bacterial isolates are shown in Figure 4.1. The production of low molecular weight metal chelators (siderophore) by the

tested bacteria isolates offers them a competitive advantage for their use as biocontrol agents and to contribute to disease suppression in plants due to insufficient supply of essential minerals in environment (Laslo et al., 2012). A stimulated biosynthesis may cause these tested bacterial isolates to directly secrete antimicrobial compounds. In antagonism effect development, siderophore production and antifungal effects play major roles, although antifungal effects encompass other features (Selvakumar et al., 2011). Results obtained from the present study concur with the work of Laslo et al. (2012) who reported that 36.2% of tested isolates produced siderophore. Quan et al. (2010) detected different types of siderophores in *Pseudomonas* spp. Among all tested bacterial isolates for HCN activity, only *P. mediterranea* produced HCN, indicating its potential use as a biocontrol agent.

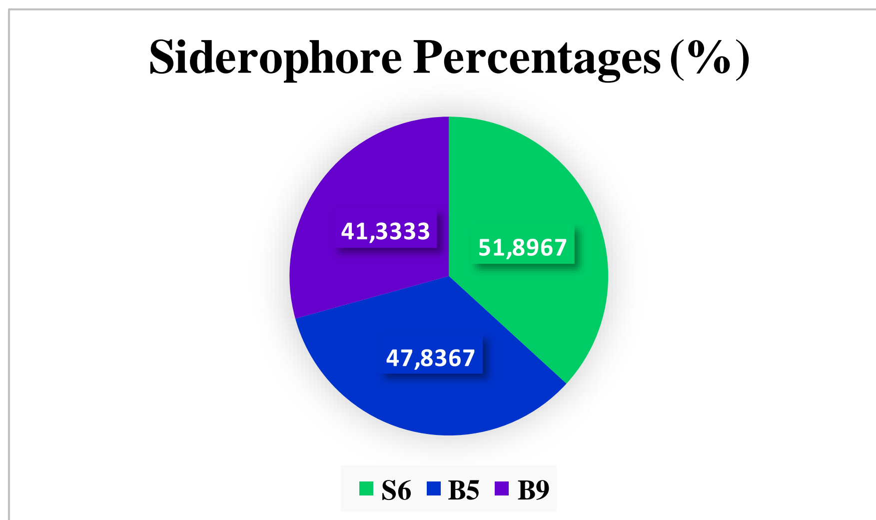

Figure 4.2: Percentages of siderophore production by *Pseudomonas* isolates.

4.5.6 ACC deaminase activity (ACC) of *Pseudomonas* isolates

The introduction of growth inhibition of *Fusarium graminearum* ACC deaminase producing bacteria to *Fusarium graminearum* infested soils helps to improve inhibition of its growth in plants by lowering the production of ethylene. Several studies have reported the production of ACC deaminase activity in *Pseudomonas* spp. (Glick, 2012; Rashid et al., 2004). *Pseudomonas* spp. are capable of surviving in *Fusarium graminearum* infected environments

by adhering to the roots of developing seedlings or on seed coats of plants, causing the deamination of ACC (the immediate precursor of ethylene in plants) by ACC deaminase, which decreases the level of plant ethylene and consequently enhances plant growth and development. The mechanism of action of ACC deaminase producing bacteria in the improvement of both abiotic and biotic stresses is by the reduction of ethylene levels through the activity of the enzyme ACC deaminase which breaks down ACC into α-ketobutyrate and ammonia instead of ethylene (Arshad et al., 2008). In the present study, *Pseudomonas* spp. were screened for ACC deaminase activity and all tested bacteria were positive (Table 4.1), though the levels of ACC deaminase activity produced varied among *Pseudomonas* isolates (Figure 4.3). Two types of medium were prepared for this test. One medium did not contain ACC (WxACC) and was considered as the control and the other medium contained ACC (WACC). In both mediums isolate S6 *(P. putida)* had the highest production of ACC. In WxACC S6 obtained 0.60±0.16 µmol/min and in WACC 0.87±0.12 µmol/min of ACC deaminase activity production.

B5 *(P. mediterranea)* produces 0.47±0.07 µmol/min and 0.61±0.35 µmol/min in the WxACC and WACC mediums respectively. Isolate B9 *(P. fluorescens)* had the lowest production of ACC deaminase in both mediums. In WxACC it only produced 0.23±0.66 µmol/min and in WACC 0.5±0.16 µmol/min levels of ACC was produced. In the preceding chapter, PCR amplification of ACC gene revealed that the tested isolates amplified the *accd* gene primer when run on agarose gel. *Pseudomonas* spp. producing ACC deaminase activity are known to improve the growth of a wide range of plants under stressful conditions like attack from pathogens, salinity, heavy metals and flooding (Belimov et al., 2009). They also play major roles in plant nodulation processes in different leguminous plant spp. (Wang et al., 2015b).

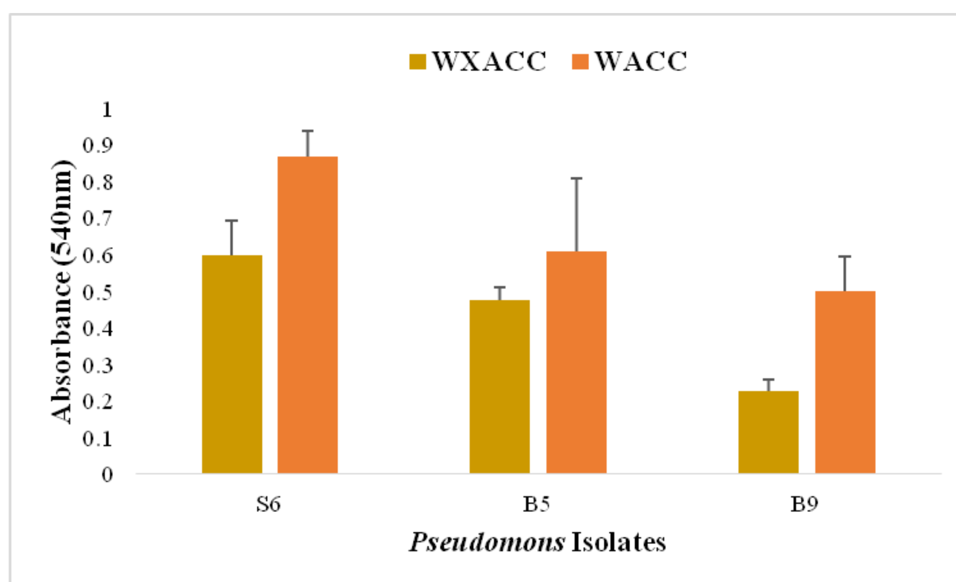

Figure 4.3: Percentages of ACC Deaminase Activity (ACC) production by *Pseudomonas* isolates. WxACC = Medium without ACC deaminase (control), WACC = Medium with ACC deaminase.

4.5.7 Phosphate solubilisation in *Pseudomonas* isolates

Solubilisation of phosphate is an important mechanism of plant growth promotion (Richardson, 2001). Bacteria are capable of increasing the availability of phosphorus (P) to plants through mechanisms such as the secretion of phosphatase to free P bound in organic matter and the production of organic acids/ chelating substances that helps to decrease rhizosphere pH (Chen et al., 2006; Rashid et al., 2004). From the results in Table 4.1, it can be seen that isolates B5 and B9 out of the three tested bacterial isolates solubilized phosphate by showing clear zones around colonies (*P.putida* and *P.flourescens* respectively). Several bacterial species of *Pseudomonas* and *Bacillus* have also been reported to solubilize inorganic phosphates. A similar effect has also been demonstrated on wheat by *Micromonospora aurantiaca*, *Streptomyces griseus, and Streptomyces sp*. (Sathya et al., 2017), under P-deficient soil. Almoneafy et al. (2012) reported the solubilisation of phosphate in three strains of *Subtillus* D29, Am1 and H8. Rodríguez and Fraga (1999) also reported phosphate

solubilisation in *Pseudomonas striata* and *Bacillus polymyxa*. In this regard, the result from the present study conforms to other previous studies on phosphate solubilisation.

4.5.8 Seed germination test

The results of the seed germination tests by *Pseudomonas putida* (MH666036) and *Pseudomonas mediterranea* (MH666037) are shown in Table 4.2. The least germination and vigor index of 42.09±2.8 and 273.82±59.66 respectively were obtained by B1, followed by Up with a germination percentage and vigor index of 42.17±2.87 and 505.35±37.95 respectively. In the same manner, maximum shoot and root lengths of 6.42±0.14 and 6.55±0.09 cm respectively, were observed in G1, followed by E1 which obtained maximum shoot and root length 6.12±0.47 and 6.51±1.10 cm respectively.

On the other hand the least shoot and root length of 2.8±0.58 and 3.56±0.59 cm respectively was seen in B2, followed by the least shoot and root length of 3.02±0.41 and 3.45±0.56 respectively which was observed in B1. The least shoot and root length were obtained by maize seeds that were not inoculated with any *Pseudomonas* spp. These are maize seeds that had been inoculated with *Fusarium graminearum* and vermiculite only. Based on the seed germination test, treatment G1 gave significantly higher ($P<0.05$) shoot length (6.42±0.14), root length (6.55±0.09), germination percentage (85.31±5.00) and vigor index (1106.50±51.70) compared to the other treatments and the control. However, the root length from treatment G1 was not significantly different from that of E1, H1, F1, Up, A2 and Ud which also had very high root lengths (6.51±1.10, 6.50±0.70, 6.29±0.21, 6.20±0.43 6.04±0.53 and 6.00±0.22) respectively. The least shoot length (3.02±0.412 and 2.8±0.58), root length (3.45±0.56 and 3.56±0.59) germination percentage (42.09±2.81 and 43.5±0.77) and vigor index (273.82±59.66 and 277.59±28.43) was obtained from treatments B1 and B2 respectively, which were not significantly different ($P > 0.05$) from each other. PGPR capable of colonizing both the surface and inner parts of plant roots play essential roles that directly

or indirectly influence plant growth and development (Gerhardt et al., 2009). In this study, maize seed treatment with the two selected bacterial strains *P. putida* and *P. mediterranea* significantly improved the emergence and growth of the seedlings. Different mechanisms have been proposed for the promotion of plant growth by PGPR, which include the indirect enhancement of seed germination and vigor index by reduction in the incidence of seed mycoflora that can negatively affect plant growth (Begum et al., 2003). In a study by Duarah et al. (2011) , amylase activity was increased during rice and legume germination after treatment with PGPR. Starch was hydrolysed by amylase to metabolisable sugars to provide the roots and shoots of germinating seeds with the energy to grow.

Table 4.2: Seed germination test.

Treat.	S. Length (cm)	R. Length (cm)	Germ (%)	V. Index
A1	5.47±0.40^{a-b}	5.81±0.19^{ab}	47.02±2.68^{d-f}	519.17±45.70^{ef}
A2	5.79±0.73^{ab}	6.04±0.53^{a}	52.01±7.70^{de}	618.72±144.97^{de}
B1	3.02±0.41^{d}	3.45±0.56^{c}	42.09±2.81^{f}	273.82±59.66^{g}
B2	2.8±0.58^{d}	3.56±0.59c	43.5±0.77^{f}	277.59±28.43g
C1	5.48±0.83^{a-c}	5.17±0.77^{ab}	72.28±5.54^{bc}	767.87±37.14^{cd}
C2	4.74±0.12^{bc}	5.14±0.17^{ab}	53.80±3.09^{de}	532.11±42.60^{ef}
D1	5.44±1.10^{a-c}	5.61±0.91^{ab}	73.62±7.80^{bc}	802.84±67.51^{bc}
D2	4.71±0.40^{bc}	5.10±0.11^{ab}	55.40±5.05^{d}	541.92±27.81^{ef}
E1	6.12±0.47^{ab}	6.51±1.10^{a}	68.72±2.91^{c}	866.46±97.08^{bc}
E2	5.64±0.61^{a-c}	5.78±0.63^{ab}	53.77±5.58^{de}	616.56±104.80^{de}
F1	6.11±0.11^{ab}	6.29±0.21^{a}	72.25±2.59^{bc}	894.07±44.28^{bc}
F2	4.85±1.90^{a-c}	5.21±1.78^{ab}	45.58±4.68^{ef}	448.44±139.49^{ef}
G1	6.42±0.14^{a}	6.55±0.09^{a}	85.31±5.00^{a}	1106.50±51.70^{a}
G2	4.77±1.30^{bc}	5.143±1.28^{ab}	55.40±4.96^{d}	548.83±205.71^{ef}
H1	5.97±0.83^{ab}	6.50±0.70^{a}	77.16±2.66^{b}	965.20±150.50^{ab}
H2	4.12±0.82^{cd}	4.39±0.66b^{c}	50.47±4.72^{d-f}	427.72±69.21^{fg}
Ud	5.77±0.48^{ab}	6.00±0.22^{a}	45.^{34}±0.00^{ef}	533.65±30.58^{ef}
Up	5.80±0.61^{ab}	6.20±0.43^{a}	42.17±2.87^{f}	505.35±37.95^{ef}

Where: Up = un-inoculated seed PR37Y15, Ud = un-inoculated seed DK315; A1 = seed PR37Y15 + *F. graminearum,* A2 = seed DK315 + *F. graminearum;* B1 = seed PR37Y15 + *F. graminearum* + vermiculite, B2 = seed DK315 + *F. graminearum* + vermiculite; C1 =

seed PR37Y15 + *F. graminearum* + *P. putida,* C2 = seed DK315 + *F. graminearum* + *P. putida;* D1 = seed PR37Y15 + *F. graminearum* + *P. putida* + vermiculite, D2 = seed DK315 + *F. graminearum* + *P. putida* + vermiculite; E1 = seed PR37Y15 + *F. graminearum* + *P. mediterranea,* E2 = seed DK315 + *F. graminearum* + *P. mediterranea;* F1 = seed PR37Y15 + *F. graminearum* + *P. mediterranea* + vermiculite, F2 = seed DK315 + *F. graminearum* + *P. mediterranea* + vermiculite; G1 = seed PR37Y15 + *F. graminearum* + *P. putida* + *P. mediterranea,* G2 = seed DK315 + *F. graminearum* + *P. putida* + *P. mediterranea,* H1 = seed PR37Y15 + *F. graminearum* + *P. putida* + *P. mediterranea* + vermiculite, H2 = seed DK315 + *F. graminearum* + *P. putida* + *P. mediterranea* + vermiculite. All values are means of triplicate determinations ± S.E. Means followed by the same letters are not significantly different at $P \leq 0.05$ according to New Duncan's Multiple Range Test (DMRT).

4.5.8.1 Genotypic difference between maize seed *PR37Y15* and *FRANCE DK315*

Figure 4.4 shows the genotypic difference between maize seeds of *FRANCE DK315* and *PR37Y15*. The higher chlorophyll content index (CCI) obtained was by *PR37Y15* with 7.00±2.03 µ/g in comparison to CCI obtained by *FRANCE DK315* with 6.98±2.21 µ/g. *PR37Y15* had the higher number of leaves (11.74±1.53 leafs) while *FRANCE DK315* had the least number of leaves (11.40±1.5 leaves). , *PR37Y15* also had the higher number of roots (23.63±3.54 roots) while *FRANCE DK315* had the lower with (22.45±2.83 roots). In a similar manner, *PR37Y15* had longer root length (32.45±8.33 cm) compared to *FRANCE DK315* (32.37±9.54 cm). *FRANCE DK315* had the lower dried leaf weight (DRLW) of 12.77±5.28 g and *PR37Y15* obtained the highest DRLW with 14.71±4.87. *FRANCE DK315* had the lower dried root weight (DRRW) with 14.00±4.66 and *PR37Y15* had the higher DRRW with 17.62±4.29 g. *PR37Y15* obtained the higher dried shoot weight (DRSW) of 16.23±10.30 g and on the other hand *FRANCE DK315* obtained the lower DRSW with 15.42±9.92 g. The

lower total plant biomass was obtained (TPB) by *FRANCE DK315* was 42.65±4.63 g and the higher was obtained by *PR37Y15* with 48.61.45±6.21 g.

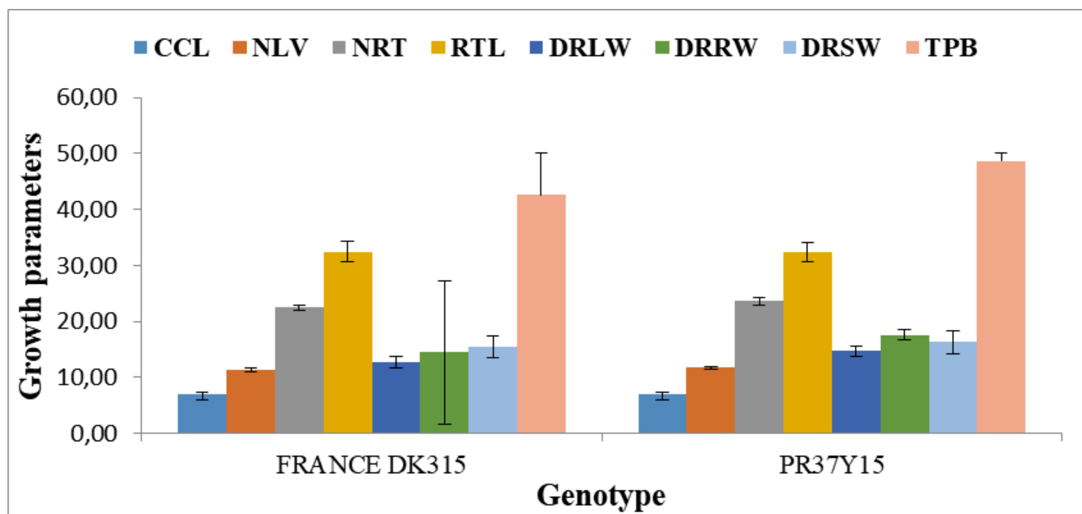

Figure 4.4: Genotypic deference of maize seeds. CCI = chlorophyll content index, NLV = number of leaves, NRT = number of roots, DRLW = dried leaf weight, DRRW = dried root weight, DRSW = dried shoot weight and TPB = total plant biomass.

4.5.9 Effect of *Pseudomonas* on growth of *F. graminearum* in maize

Results from the greenhouse study are shown in Table 4.3. The study showed that the plants inoculated with both bacterial isolates (B1 and B2) produced better levels of growth parameters. Treatment Sp+F+B1+B2 obtained significantly higher ($P<0.05$) chlorophyll content index (CCI) with the value of 9.86±0.74 µ/g, followed by Sd+F+B1+B2 with CCl value of 8.64±1.29 µ/g. The lower values of CCl production was seen in treatments Sd and Sp with 4.45±0.20 and 4.27±0.93 µ/g respectively. From the table, it can be observed that bacterial isolate B1 (*P. mediterranea*) performed better in general for CCl. Sp+F+B1+B2 gave significantly higher ($P<0.05$) number of roots (NRT) produced with 26±2.65 followed by Sd+F+B2 with 24±2.00 while the lowest NRT production was seen in treatment Sp and Sd with 19±1.00 and 20.36±1.42. Treatments Sp and Sd had the shortest root length (RTL) of 21.37±1.18 and 22.85±0.34 cm respectively and the longest RTL was observed in treatments

Sp+F+B1+B2 with 39.04±4.85 cm and Sd+F+B2 with 35.97±4.37 cm. The lower dried leaf weight (DRLW), dried root weight (DRRW), dried shoot weight (DRSW) and dried plant biomass weight (DRPW) was observed in treatment Sd which obtained 6.18±1.43g, 6.04±0.61g, 4.68±1.44g and 16.90±3.48g respectively. Sd was not significantly different from that of Sp which also had low DRLW, DRRW, DRSW and DRPW (6.69±0.9, 6.24±1.72, 5.39±0.89 and 18.32±3.57) respectively. The higher DRLW, DRRW, DRSW and DRPW was observed in Sd+F+B1+B2 (15.47±1.23, 16.39±2.15, 26.36±3.62 and 58.22±7.01) respectively. All results obtained showed that physiological parameters were enhanced by the inoculation with both bacterial isolates (B1 and B2), as well as with individual bacterial isolates (B1 or B2), as better growths were observed in plants that were inoculated than the ones that were not inoculated. This confirms the growth inhibition of *Fusarium graminearum* on maize plants by bacterial *(Pseudomonas*) inoculation.

Table 4.3 also shows that maize seeds that have not been inoculated by either *Pseudomonas* spp. or *F. graminearum* had lower values in all physiological growth parameters. Seeds that had been inoculated with *F. graminearum* only also had the lowest values of physiological growth parameters as compared to those that had been inoculated with *pseudomonas* spp. From the results, one can conclude that *Pseudomonas* spp. can inhibit growth of *F. graminearum* in maize plants and this was confirmed by the trend in the highest values of growth parameters shown by all treatments that were co-inoculated with both *Pseudomonas* spp.

T able 4.3: Effect of *Pseudomonas* spp. inoculation on growth inhibition parameters of *Fusarium graminearum* in maize plants.

Treatment	CCI (µ/g)	RTL (cm)	DRLW (g)	DRRW (g)	DRSW (g)	DRTPW (g)
Sd	4.45±0,20^{f}	24,37±1,18^{d}	6,18±1,43^{h}	6,04±0,61^{b}	4,68±1,44^{g}	16.90±3.48^{g}
Sd+F	5.35±0,54^{ef}	27,79±4,17^{cd}	11,23±2,94^{ef}	11,12±2,04^{b}	6,24±0,84^{e-f}	28.59±5.82^{d-f}
Sd+F+B1	6,60±1,16^{de}	35,04±8,59^{bc}	6,06±0,94^{h}	14,25±2,56^{b}	5,7±0,74^{fg}	26.01±4.24^{c-e}
Sd+F+B1+B2	8,64±1,29^{b}	30,4±1,47^{b-d}	15,47±1,23^{de}	16,39±2,15^{b}	26,36±3,62^{b}	58.22±7.01^{f}
Sd+F+B2	6,5±0,84^{de}	25,97±4,37^{cd}	10,21±1,45^{fg}	11,59±2,43^{b}	21,29±1,83^{c}	43.09±5.6^{b-e}
Sp	4,27±0,93^{f}	25,85±0,34^{cd}	6,69±0,96^{gh}	6,24±1,72^{b}	5,39±0,89^{fg}	18.32±3.57^{c-e}
Sp+F	5,26±0,45^{ef}	28,17±2,58^{bc}	13,06±1,13^{f}	15,28±8,03^{b}	5,69±0,55^{fg}	34.03±9.71^{c-d}
Sp+F+B1	6,68±1,26^{de}	30,2±4,20^{b-d}	11,43±0,16^{ef}	12,84±1,02^{b}	8,15±1,29^{de}	32.42±2.47^{c-e}
Sp+F+B1+B2	9,86±0,74^{ab}	30,04±4,85^{d}	13,69±3,46^{cd}	13,57±2,57^{b}	18,71±3,05^{c}	45.97±9.08^{ef}
Sp+F+B2	6,58±0,39^{de}	29,45±1,69^{bd}	14,32±3,80^{bc}	14,77±5,21^{b}	24,58±1,71^{b}	53.67±10.72^{de}

Legend: Sd= Maize seed *FRANCE DK315*, Sp= Maize seed *PR37Y15*, Treat= treatment, B1= bacteria isolate B5 *(P. mediterranea*), B2= bacteria isolate S6 (*P. putida), F= Fusarium graminearum.* CCI = chlorophyll content index, RTL = root length, DRLW = dried leaf weight, DRRW = dried root weight, DRSW = dried shoot weight and DRTPW = dried total plant biomass weight. All values are means of triplicate determinations ± S.E. Means followed by the same letters are not significantly different at $P \leq 0.05$ according to New Duncan's Multiple Range Test (DMRT).

The mean data on the effect of inoculation method on the growth of plants are presented in Table 4.4. The effect of the two inoculation methods used in the study showed that greater growth parameters were observed in plants whose seeds were bound with carboxylmethyl cellulose and coated with vermiculite than the directly inoculated plants. From the results, treatment Sp+F+B1+B2+V and Sd+F+B1+B2+V produced higher CCI value (μ/g) of 13.66±3.99 and 12.10±1.30 respectively as compared to Sp+F+B1+B2 and Sd+F+B1+B2 that produced 11.62±2.00 and 12.30±1.64 (μ/g) respectively when the two bacterial isolates were directly inoculated with both isolates. Based on the results Sp+F+B1+B2+V and Sd+F+B1+B2+V gave significantly higher ($P<0.05$) plant height (P.HEIG) with 177.36±27.36 and 175.08±11.49 respectively. For the control seeds, results showed that better CCI values were observed in plants whose seeds were immersed in 1% CMC and coated with vermiculite as CCI values of 10.87±1.84 and 10.67±1.93 μ/g respectively for treatment Sd+F+ V and Sp+F+ V were observed in them as compared to Sd+F and Sp+F that produced 6.87±0.84 and 7.10±1.23 μ/g respectively. The lower CCI values were obtained for plants whose seeds were only immersed in distilled water, treatments Sd and Sp which produced 6.10±0.81 and 5.78±.

Table 4.4: Effect of bacterial inoculation method on growth inhibition parameters of *Fusarium graminearum* in maize plants.

Treatment	CCI(µ/g)	P.HEIG (cm)	NLV	DRLW (g)	DRRW (g)	DRSW (g)
Sd	6.10±0.81^{ef}	175.22±19.64^{d}	22.64±1.50^{g}	13.72±4.18^{h}	9.59±5.18^{b}	17.71±5.58^{g}
Sd+F	6.87±0.84^{d-f}	187.16±15.98^{b-d}	11.83±1.47^{d-f}	11.41±3.59^{ef}	12.44±3.71^{b}	17.71±5.65^{e-g}
Sd+F+ V	10.87±1.84^{c-e}	157.16±18.98^{b-d}	14.83±1.77^{b-d}	17.41±8.58^{bc}	17.44±9.71^{b}	21.71±7.65^{d}
Sd+F+B1	7.3±0.36d^{e}	149.40±21.05^{bc}	10.67±1.63^{c-e}	10.28±6.04^{h}	8.35±6.27^{b}	15.96±4.26^{fg}
Sd+F+B1+ V	12.30±1.64^{b-d}	152.75±24.05^{b-d}	13.67±1.83^{ab}	14.29±8.54^{d-f}	11.04±9.27^{b}	17.96±7.26^{de}
Sd+F+B1+B2	11.10±1.27^{bc}	165.08±10.46^{b-d}	11.33±1.21^{f}	13.68±3.45^{b-d}	12.06±4.55^{b}	17.11±6.72^{b}
Sd+F+B1+B2+V	12.10±1.30^{ab}	175.08±11.49^{a}	113.33±1.31^{ab}	15.68±5.45^{a}	14.06±5.55^{b}	19.11±7.72^{a}
Sd+F+B2	8.33±1.03^{de}	165.78±4.78^{cd}	11.67±1.50^{b-e}	9.62±1.76^{fg}	5.78±2.84^{b}	16.37±10.27^{c}
Sd+F+B2+ V	11.46±1.13^{a-c}	175.56±6.78^{b-d}	18.67±1.57^{b-d}	10.62±6,22^{c-f}	8.78±4.84^{b}	23.37±12.27^{b}
Sp	5.78±0.66^{f}	174.11±20.27^{cd}	11.17±2.04^{g}	11.95±4.06^{gh}	10.13±5.66^{b}	13.96±6.08^{fg}
Sp+F	7.10±1.23^{ef}	152.19±27.66^{b-d}	11.17±1.72^{c-e}	11.04±8.17^{d-f}	7.03±5.23^{b}	15.39±7.67^{fg}
Sp+F+V	10.67±1.93^{ab}	182.19±28.66^{b-d}	14.17±1.98^{ab}	12.04±10.47^{b}	9.03±5.68^{b}	18.39±8.67^{d}
Sp+F+B1	8.00±0.77^{de}	160.00±9.80^{b-d}	11.67±0.81^{c-e}	11.96±3.09^{ef}	11.78±8.20^{b}	19.39±5.97^{d-f}
Sp+F+B1+V	10.32±0.97^{b-e}	170.99±11.64^{b-d}	14.67±0.95^{b-e}	12.93±5.03^{b-d}	12.78±8.94^{b}	21.39±7.97^{d}
Sp+F+B1+B2	11.62±2.00^{ab}	170.66±26.31^{b-d}	11.33±1.21^{ef}	11.88±2.57^{c-f}	8.60±4.16^{b}	19.27±7.56^{c}
Sp+F+B1+B2+V	13.66±3.99^{a}	177.36±27.36^{a}	12.33±1.45^{a}	12.80±4.57^{a}	11.60±5.16^{b}	25.27±9.56^{a}
Sp+F+B2	8.82±1.39^{de}	170.92±20.15^{b-d}	11.33±1.63^{c-e}	11.42±3.48^{b-e}	5.80±3.01^{b}	21.53±13.77^{b}
Sp+F+B2+V	9.87±2.39^{b-d}	182.85±24.15^{b-d}	14.33±1.98^{b-d}	11.69±4.46^{b-d}	7.80±4.01^{b}	29.53±14.67^{a}

Legend: Sd= Maize seed *FRANCE DK315*, Sp= Maize seed *PR37Y15*, V= vermiculite coated, Treat= treatment, B1= bacteria isolate B5 *(P. mediterranea)*, B2= bacteria isolate S6 (*P. putida), F= Fusarium graminearum.* CCI = chlorophyll content index, PHEIG = plant height, NLV = number of leaves, DRLW = dried leaf weight, DRRW = dried root weight and DRSW = dried shoot weight. All values are means of triplicate determinations ± S.E. Means followed by the same letters are not significantly different at $P \leq 0.05$ according to New Duncan's Multiple Range Test (DMRT).

The data on plant height, number of leaves per plant, number of roots per plant, leaf area, dry leaf weight, dry shoot and root weight also revealed that for all the treatments, plants whose seeds were immersed in 1% CMC and coated with vermiculite were better in terms of growth and all the parameters measured than the plants whose seeds were either directly inoculated with bacteria or distilled water. *Fusarium graminearum* is a serious environmental problem in agriculture as it causes severe loss in plant yield. The fungus invades plant vascular tissues and induces severe wilting of the foliage by blocking xylem transport and impeding the movement of water. Controlling the vascular wilt pathogen is difficult for several reasons; the pathogens live deep in the interior of their host plants, many vascular wilt pathogens are soil-borne and produce persistent resting structures that are able to survive for long periods of time in the absence of host plants (Boukerma et al., 2017).

In this study, the growth and survival of maize plants were affected because *F. graminearum* was introduced to almost all plants. However, better growth was observed in bacterial inoculated maize plants than the un-inoculated plants as better survival, dry leaf, dry root and shoot weight, number of leaves, number of roots and chlorophyll content were observed. Over the years, PGPB have been used mostly to promote plant growth because of their ability to stimulate plant growth through certain mechanisms such as the production of plant growth regulators and fixation of nitrogen (Lucy et al., 2004). Studies have demonstrated other beneficial effects of PGPB on plants including their ability to inhibit growth of *F. graminearum* in maize plants (Wang et al., 2014; Yang and Cao, 2012). Although this hemi-biotrophic fungus can spend most of its life cycle in maize, it can also exist as a facultative saprophyte and infect seeds and/or lateral roots from plant residues or soils (Arias et al., 2016). There are no efficient chemical treatments that exist to cure infected plants. Management with chemical fungicides causes serious environmental problems and they are toxic to non-target organisms as well (Hardoim et al., 2008). One approach to prevent

contamination with fungal toxins is by developing and using cultivars more resistant to diseases. However, to date, neither commercial maize hybrids nor inbred lines have achieved the desired resistance levels. Moreover, increasing concern about food safety urges scientists to search for environmentally friendly alternatives for the management of fungal diseases. The use of microorganisms with antagonistic activities against *Fusarium graminearum* may be a promising approach for resolving the issue since it is safe for both humans and ecosystems. *Pseudomonas* is a genus of bacteria that can produce antagonistic compounds, such as phenazine-type antibiotics, hydrogen cyanide, pyoluteorin, hydrolytic enzymes, siderophores, etc. Similar to this study, the antifungal properties against *Fusarium* spp. have been identified in *Pseudomonas*, and the siderophore-mediated competition for iron with soil-borne pathogens has been suggested to be a key mechanism to restrict the proliferation and root colonization of *Fusarium oxysporum* (Yu et al., 2017). It has also been reported that the siderophore produced by *Pseudomonas fluorescens* induced systemic resistance against *Fusarium* wilt of radish (Chandra et al., 2018).

In the preceding study, *P. mediterranea strain B5* and *P. putida strain S6* showed the presence of glutathione peroxidase, indicating their capability of inhibiting growth of *F. graminearum* by the avoidance of oxidative damage. This could also be the reason behind the greener leaves of the inoculated plants over the un-inoculated ones. We also observed that the severity of *F. graminearum* was more pronounced on the un-inoculated plants than the inoculated plants as the un-inoculated plants showed more signs of wilting than the inoculated plants. Damage to plant proteins often results from stress exposure, therefore it is necessary to maintain proteins in their functional forms to enable plants to survive under stressful conditions (Wang et al., 2015b).

Plant proteins like the Heat-shock proteins (HSP), Malic-enzyme proteins (ME), glycine-rich RNA binding proteins (GRP) and desiccation protectant proteins are often synthesized

during biotic and abiotic stress conditions and are recognized as mechanisms of stress tolerance in plants (Wahid et al., 2007). They play major roles in translocation, protein folding, degradation and assembly in several cellular processes. They can also assist in stabilizing and refolding of proteins under different conditions of stress (Wang, 2007). From the preceding study on *F. graminearum* growth inhibition genes, *P. putida* and *P. mediterranea* were observed to possess the proteins glycine-rich binding protein, and Malic-enzyme and this could have also contributed to their better survival under the attack of *F. graminearum*. The growth of plants depends highly on differentiation, enlargement and cell division. In addition, *F. graminearum* affects the physiological, morphological, ecological and genetic processes of plant growth. Studies have described the roles of PGPB in modifying plant metabolism under biotic and abiotic stress conditions by mechanisms including indole-3-acetic acid production, ACC deaminase activity, nitrogen fixation and antioxidant production (Dimkpa et al., 2009). PGPB are also capable of producing compatible solutes (glycine-betaine and proline) that assist in the processes of osmoregulation (Dimkpa et al., 2009). In the present study, better growth inhibition of *F. graminearum* was observed in bacterial inoculated plants than un-inoculated plants. This could be because of production of IAA, ACC deaminase and glycine-rich protein by these bacterial isolates.

Besides the inoculation of plants with single strains of PGPB, co-inoculation or combination of two strains also inhibited growth of *F. graminearum* in plants to an even greater extent (Wang, 2007). From the results obtained in the present study as shown in Table 4.3, better inhibition was observed in the co-inoculation of seed *FRANCE DK315+F.graminearum+P. mediterranea strain B5 +P. putida* strain S6 (Sd+F+B1+B2) and seed PR37Y15+*F.graminearum+P. mediterranea* strain B5 + *P. putida* stain S6 (Sp+F+B1+B2) in maize plants as better dry shoot and root weight, chlorophyll content and numbers of roots

and leaves were observed in the plants. Moreover, wilting of leaves was observed to be lower in the co-inoculated plants than those inoculated with either *P. mediterranea* or *P. putida*, which were much better than the control.

The results from this study are in agreement with the study of Bergottini et al. (2015) who reported that native bio-inoculants had a highly significant positive effect in growth of yerba mate seedlings in soil. All native strains produced a significant increase on the shoot and root dry weight in comparison to un-inoculated controls. According to Wang et al. (2012), darker green leaves, lighter wilting symptoms, relative electrical conductance, increased leaf proline and chlorophyll content and intension of root recovery were observed in BBS treated plants. In a similar study, exopolysaccharide producing bacterial strains *Alcaligenes faecalis*, *Proteus penneri* and *Pseudomonas aeruginosa* exhibited better growth inhibition in maize compared to individual PGPB strains (Bell et al., 2014).

The effect of inoculation method on the growth inhibition of *F. graminearum* of *P. mediterranea strain B5* and *P. putida* strain B9 in maize seeds are shown in Table 4.4. Results obtained revealed that plants whose seeds were co-inoculated with *P. mediterranea* and *P. putida* and coated with vermiculite showed better increase in growth parameter measurements compared to the plants whose seeds were directly co-inoculated with the bacterial isolates.

Similarly, better growth was observed when seeds were un-inoculated but coated with vermiculite compared to the plants with seeds merely immersed in distilled water. In all, seed inoculation with the combination of the two bacterial strains was more effective than inoculation with individual bacterial strains. The better results obtained from the growth parameter measurements of the vermiculite coated seeds as compared to the un-coated seeds of both inoculated and un-inoculated seeds on the effect of inoculation method used in this study could have been due to the presence of 1% Carboxylmethyl cellulose (CMC), as this

adhesive may have facilitated the better binding of the bacterial isolates to the seeds. Carboxymethyl cellulose is an adhesive that has also been used in drug and food industries (Ibarra et al., 2016). It plays major roles in binding inoculants to seeds and also protects the seeds from desiccation as well as providing nourishment for the inoculated plants. In addition, the coating of the seeds with vermiculite may have helped to protect the seeds from possible insect and pathogen attacks. From the results obtained in the present study, it is encouraged that for efficient growth inhibition of *F. graminearum*, inoculated seeds should be bound as well as coated with good binding and coating agents as this will reduce pest attacks, pathogen attacks, preserve seeds for longer periods and enhance growth of all plants.

4.6 References

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CHAPTER FIVE

5.1 General conclusion and future research prospects

The high demand for maize by consumers worldwide brings maize fields under a lot of strain. This in turn puts maize producers and suppliers under a lot of pressure to supply and produce maize. Because of pathogens such as *Fusarium*, many agricultural farmers have resorted to synthetic pesticides. Global increases in food production achieved in recent decades have required large (15–20 times) increases in the use of synthetic pesticides to control pests, pathogens and weeds (Oerke, 2006) but the increasing use of synthetic pesticides is no longer sustainable. Synthetic pesticides have proven to cause economic challenges, as they are expensive to manufacture. For this reason, the problem needs a careful revision and one should clarify several aspects that were not consider to have a serious importance. Climate change increases the chances of disease epidemics in many crop producing areas, which is also an argument for significantly increasing resistance to pathogens (Mesterházy et al., 2015).

The search for alternative solutions for agriculture has prompted researchers to take a second look at the range of microorganisms long known to provide benefits to agricultural production and is driving rapid growth in markets for bio pesticides and PGP microorganisms (O'Callaghan, 2016). PGPB facilitate plant growth, helps them to resist and inhibit growth of pathogens such as *F. graminearum*, and could thereby play a major role in solving the problem of global food insecurity. *F. graminearum* growth inhibition by PGPB can be enhanced through a variety of mechanisms ranging from phytohormonal modifications, ACC deaminase activity, to alteration in root morphology and molecular techniques (Hardoim et al., 2008). Several bacteria have been isolated and used to inhibit growth of *Fusarium* in a variety of crops, but with only little knowledge of the mechanism of action used by *Pseudomonas* to inhibit growth of *F. graminearum* in maize plants. This therefore made it

necessary to isolate, characterize and identify *Pseudomonas* spp. from dry maize plant with the intention of evaluating their PGP characteristics as well as their ability to inhibit growth of *F. graminearum* in maize plants. In this study, *Pseudomonas* has been identified as a bacterium that has the ability to protect maize from *F. graminearum*. IAA production was performed in a rich media and this was to allow the *Pseudomonas* to grow in favourable conditions. Minimal medium would have compromised the growth of the *Pseudomonad strains.*

From the present study, three *Pseudomonas* strains were successfully isolated and characterized from maize rhizospheric soils using culture dependent techniques. The results obtained showed that the bacterial isolates from this study showed tolerance to 5% polyethylene glycol (PEG) 8000. The three isolates could also survive and inhibit growth of *F. graminearum* even in low water supply conditions. Results showed that concentration of PEG affected growth of bacteria in PEG containing medium, as the growth in medium without PEG was higher with the maximum of *Pseudomonas putida* at 1.57±0.06. Bacterial growth at different NaCl concentration and temperatures were evaluated and best optimum growth for all bacterial isolates was observed at 2% NaCl and between 25°C and 30°C. Malik et al. (2008) reported that only 1% of soil bacteria can be cultured using culture-dependent techniques. With the use of culture-independent techniques such as temperature gradient gel electrophoresis (TGGE), more new strains of *Pseudomonas* spp. have been isolated. All tested bacterial isolates in this study showed tolerance to different concentrations of PEG, temperatures and saline conditions indicating their potential for possible use as *F. graminearum* growth alleviators in plants. However, there is need for further clarification on the precise mechanisms employed by the bacteria to inhibit growth of *F. graminearum* and salinity stresses.

Furthermore, molecular techniques such as Scanning electron microscopy (SEM) could be used to further characterize the bacterial strains. There is also the need to evaluate the effect of bacterial combinations, such as bacteria from different genera or even from other possible pathogens that can share a beneficial and mutualistic relationship with these bacterial strains, with the hope that they will be able to inhibit growth of *F. graminearum* in maize. *Fusarium* growth inhibition was amplified with the following antibiotic primers phl2, pltB, PRND, PHZ together with the two plant growth promoting genes for ACC deaminase activity and siderophore production (*accd* and *Sid*). Similar results were also reflected in in chapter 3, were all the *Pseudomonas* strains presented the ability to produce siderophore in vitro and also in chapter 4, were the presence of gene coding for siderophore production was detected in all three *Pseudomonas* strains. However, no sequencing analysis was performed to match them with available *F. graminearum* growth inhibiting genes on the GenBank database, hence the need for further investigation. Also, new technologies such as real time-polymerase chain reaction (RT-PCR) could be used for better results and also to quantify the levels at which these genes were expressed. The products obtained from *Fusarium* growth inhibition gene expression could also be used in the engineering of new inhibitory bacteria as well as in the construction of biosensors. Most *Pseudomonas* bacterial strains exhibited multiple PGP properties, both qualitatively and quantitatively.

Through biochemical assays, it was observed that all isolates produced siderophore, indole-3-acetic acid, ACC deaminase activity and ammonia, while two isolates solubilized phosphate and one produced hydrogen cyanide. However, only bacterial isolates *P. mediterranea* and *P.putida* were chosen for *F. graminearum* growth inhibition studies in the greenhouse due to their outstanding ability to grow both on the highest concentrations of PEG 8000 and their ability to produce high amounts of ACC deaminase, siderophore and indole-3-acetic acid. The effects of individual and combined inoculation of these *Pseudomonas* strains on

inhibition of *F. graminearum* growth in maize plants were tested by a series of experiments. The inoculation of bacteria improved seed germination of maize as compared to un-inoculated seeds. Maximum germinated percentage was seen in seeds treated with a combination of the two bacterial strains (*P. mediterranea strain B5* and *P. putida strain S6*) while the lowest percent germination was seen in the un-inoculated seeds.

A greenhouse experiment evaluated the effect of inoculation of *P. mediterranea strain B5* and *P. putida strain S6* on growth inhibition of *F. graminearum* in maize. Results obtained revealed that the combination of both bacteria strains produced better results compared to inoculation with individual strains, and that co-inoculated plants showed significant increase in chlorophyll content, shoot, number of roots, number of leaves and leaf area per plant, and in shoot and root dry weights. For individual inoculations, maximum growth parameters were observed in plants inoculated with *P. putida* compared to plants inoculated with *P. mediterranea*, while there was a significant decrease in the growth of un-inoculated plants. The effect of inoculation method on the growth inhibition of *F.* graminearum by these bacterial isolates on maize revealed that plants whose seeds were co-inoculated with *P. mediterranea* and *P. putida* and coated with vermiculite gave better increase in the measured growth parameters compared to the plants whose seeds were directly inoculated with the bacterial isolates. Similarly, better growth was also observed in those whose seeds were un-inoculated but coated with vermiculite compared to the plants whose seeds were merely immersed in distilled water. Based on the results obtained from the greenhouse study, it is therefore recommended that *Pseudomonas* spp. are most preferred for inhibiting growth of *F. graminearum*. Maize seeds should be inoculated with the two bacterial isolates with subsequent suspension in 1% CMC solution of vermiculite coating, which is necessary to ensure proper adherence of bacteria to the seeds. Growth inhibition of *F. graminearum* by

Pseudomonas bacteria is an emerging technology that is cost effective and efficient to help solve the problem of low crop productivity and yield.

Results of this study have proven that the inoculation of growth inhibiting *P. mediterranea* and *P. putida* increased plant growth as well as reducing the attack of *F. graminearum* on the tested plants. However, further studies need to be undertaken to observe if these mechanisms can work on all crops and not on maize alone and whether or not these bacterial spp. can inhibit growth of other *Fusarium* spp. in various crops. Furthermore, in spite of the recent progress in growth inhibition of *F. graminearum*, co-inoculation in the inhibition growth of *F. graminearum* in plants still needs more attention therefore more research work is required in this area to give new insights for better agricultural productivity. Even though the study used specific gene primers, they cannot fully confirm whether the gene is responsible for the inhibition of Fusarium graminearum growth.

Hence, our recommendations for future research works are as follows:

1. To assess the root colonization of the maize plants by the bacterial strains *P. mediterranea* and *P. putida*.

2. To test the effects of inoculation of growth inhibition of *F. graminearum* bacterial *P. mediterranea* and *P. putida* under field conditions.

3. To study their effects on different plants and various *Fusarium* spp.
4. Functional study of genes such as deletion and knockout methods to confirm if specific genes are the mechanisms responsible for Fusarium inhibition.

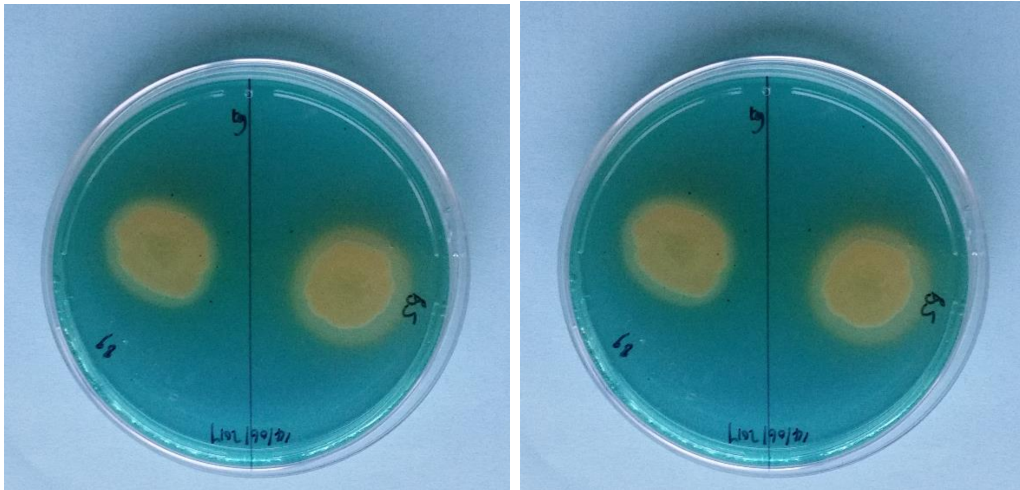
5.2 References

Mesterházy A., Lehoczki-Krsjak S., Varga M., Szabó-Hevér Á., Tóth B., Lemmens M. (2015) Breeding for FHB Resistance via Fusarium Damaged Kernels and Deoxynivalenol Accumulation as Well as Inoculation Methods in Winter Wheat. *Biocontrol Science and Technology* 452:1166-1195.

O'Callaghan M. (2016) Microbial inoculation of seed for improved crop performance: issues and opportunities. *Applied Microbiology and Biotechnology:*1-18.

Oerke E.-C. (2006) Crop losses to pests. T*he Journal of Agricultural Science:* 144:31-43.

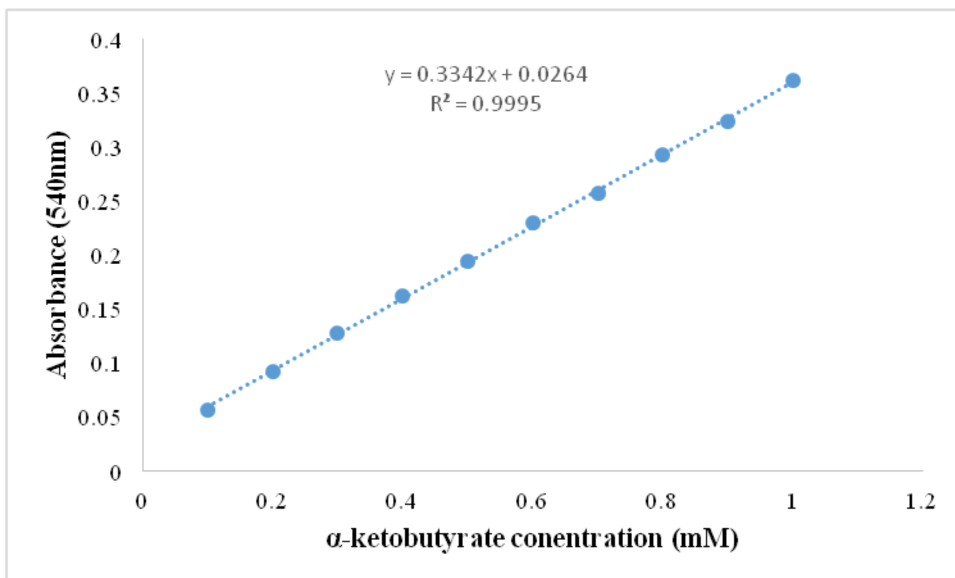
APPENDIX



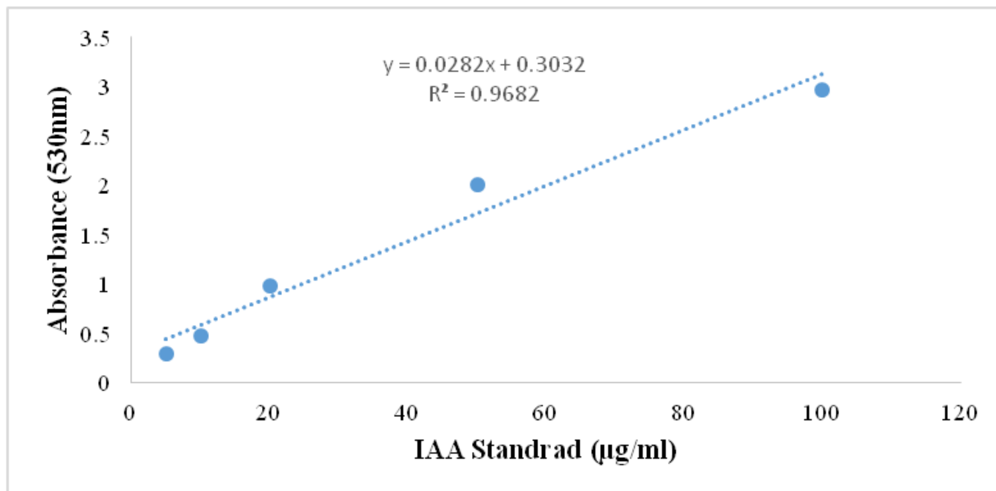
1: Image showing positive siderophore production by *Pseudomonas* isolates

2: Image of phosphate solubilisation assay by *Pseudomonas* isolates

3: Greenhouse evaluation of growth inhibition of *F. graminearum* by *Pseudomonas* in maize plants



4: Concentration of α-ketobutyrate produced by *Pseudomonas* isolates



5: Standard Curve for IAA production by *Pseudomonas* isolates