

Molecular selection and the taxonomy of rhizobacteria which suppress the growth of *Fusarium graminearum* on maize (*Zea mays* L.)

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DECLARATION

I, the undersigned, declare that the dissertation titled “Molecular selection and the taxonomy of rhizobacteria which suppress the growth of *Fusarium graminearum* on maize (*Zea mays* L.)”, which I herewith submit to the North-West University upon completion of the requirements set for the degree Master of Science in Biology, is my original work, all sources and citations used have been acknowledged. This work has not been submitted to any other university.

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DEDICATION

This work is dedicated to my beloved family, my mother Joana Annah Mokgakane, my sister Thabile Joyce Mokgakane, my nephew Lunathi Blessing Mnisi, and my brothers Bongane Mathews Mokgakane and Surprise Sandile Mokgakane.

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LIST OF ABBREVIATIONS

ACC:	1-aminocyclopropane-1-carboxylic acid
Al:	Aluminium
AMP:	Ampicillin
ANOVA:	Analysis of variance
C:	Chloramphenicol
Cd:	Cadmium
CFU:	Colony forming unit
Cu:	Copper
E:	Erythromycin
ESI-MS:	Electrospray ionization-mass spectrometry
Fe:	Iron
FTIR:	Fourier transform infrared
Ga:	Gallium
HCN:	Hydrogen cyanide
IAA:	Indole-3-acetic acid
OD:	Optical density
K:	Kanamycin
P:	Phosphorus
NH ₃ :	Ammonia
Pd:	Palladium
PGPR:	Plant growth-promoting rhizobacteria
TET:	Tetracycline
TLC:	Thin-layer chromatography
VA:	Vancomycin
Zn:	Zinc

ABSTRACT

The disease-causing agent *Fusarium graminearum* is considered a global cause of distress to the agricultural economy due to its undesirable effects and its ubiquity in the soil leading to infections in cereal grains. Therefore, healthier and effective inoculant alternatives are necessary to improve the currently applied practices. Plant growth-promoting rhizobacteria (PGPR) are some of the naturally occurring microbes that have a broad spectrum of activities that aid with plant growth and protection. This study investigated the cultural and molecular characteristics of novel *Bacillus* spp. that are commonly studied as PGPR that are known to attain antagonistic traits effective against maize plant pathogens such as *F. graminearum*. Forty-six (46) bacteria were successfully isolated from the rhizosphere of healthy maize crops in the North West Province, South Africa. Among these, only 3 strains, LBC4ME, LBC22HL and LBC35MW were found to be highly effective when tested for antifungal activities, particularly against the *F. graminearum* FG1 strain. These strains were further tested for plant growth-promoting (PGP) traits [Siderophores, indole-acetic-acid (IAA), phosphate solubilization, hydrogen cyanide (HCN), ammonia (NH₃) production, nitrogen fixation, hydrolytic enzyme production], antibiotic sensitivity using [Vancomycin (VA), chloramphenicol (C), kanamycin (K), tetracycline (TET), erythromycin (E) and ampicillin (AMP)], and biochemical traits. Molecular approaches (16S rDNA, rpoB, gyrB and tuf) of partial gene sequences were further used for the identification of the isolates and partial gene sequences (ItuD1, Bacc1, AS1, AF2) were used for the detection of lipopeptide biosurfactants biosynthesis. The results showed that LBC4ME, LBC22HL and LBC35MW strains exhibited a substantial fungal inhibition rate above 50% compared to the other isolated bacteria. The taxonomy of the obtained strains was identified as the Firmicutes phyla, genus *Bacillus* species LBC4ME (*Bacillus thuringiensis*), LBC22HL (*Bacillus velezensis*) and LBC35MW (*Bacillus thuringiensis*). The three (3) strains were also found to produce a broad range of antifungal activities through *in vitro* and *in vivo* tests. Antifungal efficiency validated by using pot experiments in the screen-house showed that the treatments containing individual antifungal bacterial cells and the treatments containing combined bacterial cells of the studied strains had a positive effect on disease severity (root length, shoot length, leaf rot, plant wilting) compared to the pathogen only and control treatments. The strains also showed effective antifungal inhibition zones against *F. graminearum* FG1 under different growth conditions [pH (2-10), temperature (4-45 °C), carbon (Glucose, D-fructose, sucrose, and D-galactose) and nitrogen sources (KNO₃, NH₄NO₃, (NH₄)₂SO₄, and urea)]. Viable production of antifungal lipopeptide

biosurfactants was displayed for surfactins, iturins, and fengycins molecular characteristics using chromatographic (Thin-layer chromatography (TLC)), spectroscopic analysis (Fourier transform infrared (FTIR) and the Electrospray ionization-mass spectrometry (ESI-MS)) analysis for the detection of surface-active lipopeptide molecules. The lipopeptide biosurfactants extracted from LBC4ME, LBC22HL and LBC35MW strains showed antifungal properties which demonstrated about 65-66% inhibition rate against *F. graminearum* FG1. The results obtained from this study have shown that the above-mentioned strains attained stable multipotent broad spectrum of antimicrobial activities and may be targeted and further used as potential biological control agents (BCAs) for *F. graminearum*, and thus, contribute to improving food safety and security.

Keywords: Agriculture, antagonism, bio-pesticides, mycotoxins, PGPR mechanisms, symbiosis, synergism

CHAPTER 1

1.1 GENERAL INTRODUCTION

The issue of phytopathogen *Fusarium* spp. mycotoxin contaminations in maize crops has been reported as a global and local concern, affecting environmental protection and sustainability, maintenance of economical global trade, and therefore, continuous attempts to control infection incidences are targeted (Alberts et al., 2016).

Maize (*Zea mays* L.) is one of the major cereal crops which is commercially grown in most developed and developing countries (Agbodjato et al., 2016). It is also used in subsistence or intensive commercial farming and in versatile applications as a principal ingredient for food (e.g. Maize meal), animal feed, agro-alimentary uses and raw material production (Rensburg et al., 2015). It is produced in some of the local provinces, namely, Mpumalanga, North West, Free State, and Kwazulu-Natal, plus, it ranks highly in demand in SA households as a staple food among other cereal grains (Beukes et al., 2017).

Its production may fluctuate from year-to-year depending on climate conditions (e.g. rainfall, floods, drought, etc.), production inputs (e.g. adapted cultivars, soil tillage, fertilization, insects and disease control) and financial resources (Rensburg et al., 2015). Reportedly, the cultivation of crops may be largely attacked by soil-borne pathogens through either a direct infection from the soil or through contamination from other sources (Ouda, 2014). One of the major practices commonly used to manage disease infections and soil-borne pathogens includes the application of synthetic pesticides, which in the long run have been reported to have devastating side effects on the environment and a potential health hazard (Guo et al., 2015).

Alternative sustainable practices are required as a better approach that favour sustainability (Agrawal et al., 2015). One of the current alternatives for synthetic applications in agriculture is plant growth-promoting rhizobacteria, which are listed as an economical, natural and sustainable disease control alternative practice (Pii et al., 2015). The application of PGPR in maize cultivation was previously shown to aid in the control of phytopathogen proliferation (Moussa et al., 2013). PGPR are an introduced system that encompasses a large taxonomic diversity of naturally occurring microbes that have a broad spectrum of studied mechanisms discovered to enable healthy plant growth and protection (Courty et al., 2015). Providentially, they have previously been tested and applied as an effective and friendly disease control alternative (Szentes et al., 2013; Franco-Correa & Chavarro-Anzola, 2016).

PGPR are natural soil bacteria mostly found to be present in the rhizosphere and are known to have a symbiotic or non-symbiotic relationship when they inhabit the roots of their desired host plants (Santiago et al., 2015). Their metabolic pathways produce substances which can inhibit pathogens from host plants and in turn promote plant growth, while the host plant provides the rhizobacteria with food from the roots as exudates (Kurabachew et al., 2013). Hence, there is a growing interest in obtaining PGPR with optimum antagonistic activity in terms of their direct and indirect mechanisms of action (Balan et al., 2017).

The commonly studied PGPR includes the genus *Bacillus* species, which are Gram-positive, rod-shaped, sporulating PGPR known to interact with several plants including maize crops (Ghodsolavi et al., 2013). They exhibit several plant growth-promoting (PGP) traits through numerous direct and indirect mechanisms that are described to be host specific and are found to positively assist host plants to survive challenging environmental conditions (Passari et al., 2016). These PGPR are commonly applied as BCAs and due to possible development of microbial resistance which may occur during the introduction or after application of BCAs product, also there is a constant need for novel agents and this requires proper screening, selection and characterization of the strains (Georget et al., 2015).

In previous studies, several *Bacillus* spp. have been reported to be successful BCAs against *F. graminearum* under both lab and screen-house conditions (Kaki et al., 2013; Shi et al., 2014). This study focuses on obtaining rhizobacteria from the local environment where maize is cultivated, screen them for biocontrol PGPR traits against a maize crop pathogen *F. graminearum*, and use molecular techniques to classify and validate the identity of the strains, and then further test the strains' biocontrol effectiveness in laboratory and pot experiments before declaring them as reliable PGPR strains which can suppress *F. graminearum* on maize. This study is designed to contribute and add to the existing information based on the overview of PGPR as biocontrol agents and their efficacy through direct and indirect applications against fungal plant pathogens.

1.2 Problem statement

The prevalence of *F. graminearum* is frequently reported as a major plant pathogen which is problematic in most cereal crop infections of maize. This leads to concerns affecting primary food production, health security, food safety, quality and security.

1.3 Aim of the study

This study aimed to investigate molecular selection and the taxonomy of rhizobacteria which suppress the growth of *Fusarium graminearum* on maize (*Zea mays* L.).

1.4 The objectives of the study

The aim of this study was achieved through the following objectives:

- To collect, screen and select potent bacterial strains which can inhibit the mycelium growth of *F. graminearum* *in vitro*
- To characterize and identify the taxonomy of the bacterial strains
- To conduct a screen-house assessment (*in vivo*) of the bacterial strains to assess their biological control capacity against *F. graminearum*

CHAPTER 2

LITERATURE REVIEW

2.1 Plant growth-promoting rhizobacteria

PGPR are a diverse community of free-living soil bacteria present in the rhizosphere of preferred plants, which is the zone near plant roots where plant secretions occur (Courty et al., 2015). They are commonly found to be in higher density compared to other microbial groups in the soil, and this is due to their symbiotic relationship with the plant caused by the higher concentration of nutrients (e.g. amino acids, organic acids and sugars) that are secreted by plants through the root exudates (Cordero et al., 2012; Perez-Montano et al., 2014).

Most PGPR are known to grow rapidly and they use a broad range of mechanisms when attached to a host plant, which involves several direct PGPR mechanisms which can influence growth on the plant cells by secretion of substances (e.g. organic acids, siderophores and phytohormones) to aid in nutrients, water and iron uptake (Rincon-Florez et al., 2013). Indirect mechanisms involve processes influencing growth outside the plant cells by secretion of secondary metabolites (e.g. Hydrogen cyanide and antibiotics), colonization sites /nutrient competition, and pathogen cell wall degrading enzymes (Kundan et al., 2015).

PGPR in the rhizosphere are confirmed to aid in the host plant's growth promotion, plus, support the plant under different environmental conditions, and reduce the proliferation of soil-borne pathogens at various stages of the plant's growth (Rayees et al., 2014). The plant-microbe relationship is considered to be an inter-dependent and complex interaction because it is affected by other influences in the rhizosphere such as abiotic and biotic factors (Oberson et al., 2013). PGPR are grouped as extracellular (ePGPR) when they are found on the rhizoplane, around the plant roots or in between the spaces of root cells and intracellular (iPGPR) when found inside the root cells, depending on their location on the plant (Gupta et al., 2015).

The PGPR community is diverse and includes both Gram-negative and Gram-positive genera including *Bacillus*, *Rhizobium*, *Sinorhizobium*, *Pseudomonas*, *Serratia*, *Streptomyces*, *Klebsiella*, *Azospirillum*, *Cupravidius*, *Enterobacter*, *Azotobacter*, *Alcaligenes*, *Burkholderia*, *Arthrobacter*, and *Bradyrhizobium* species (Prashar et al., 2014; Goswami et al., 2016). Due to the high population of soil bacteria in the rhizosphere, the bacteria are classified as PGPR when

they can express certain valuable traits proficient to support plant growth, protection and health through their numerous beneficial mechanisms (Chaitanya & Meenu, 2015).

There are many rhizobacteria associated with the soil and plants, some are considered deleterious by causing harm to the plant (e.g. phytotoxicity and causing an infection) or neutral (e.g. allelopathy, free attachment, enzyme release and nutrient flux) (Moustaine et al., 2017). It has also been observed that not all strains in a particular genus are considered PGPR due to the differences in their metabolism or genetic makeup, and thus, the taxonomic identification and species-specific traits are necessary to be investigated to clarify proper classification and application (Prasad et al., 2015).

2.2 Mechanisms of PGPR

Most PGPR have shown great potential as an ecological niche in the rhizosphere throughout the years. Their metabolic activities are beneficial for plant growth (Moustaine et al., 2017). Both the symbiotic and non-symbiotic rhizobacteria are found to utilize several PGPR mechanisms, however, their mechanisms may function differently depending on their genera, species, strains, soil pH, nutrient availability/supply and other environmental stimuli (Baysal & Tor, 2014). This means no two PGPR species in the same genus may have or use the same metabolic activities to influence the host plant (Gull, 2016).

2.2.1 Direct mechanisms

2.2.1.1 Phytohormone production

Plant sensitivity in response to PGPR activities prompts the secretion of plant growth regulators or plant hormones in very low concentrations that control growth, as well as mediate processes such as hormonal sensing, root system development, plant yield, cell expansion/extension/division, signalling, nutrient uptake, and more (Vacheron et al., 2013). The genera commonly reported to be involved in the production of phytohormones includes *Mycobacterium*, *Enterobacter*, *Bacillus*, *Burkholderia*, *Alcaligenes*, *Pseudomonas*, *BreviBacillus*, *Serratia*, *Klebsiella*, *Bradyrhizobium*, *Azotobacter* and *Rhizobium* species (Franco-Correa & Chavarro-Anzola, 2016). Most PGPR can produce different types of hormones which are involved in plant growth promotion and protection at different stages and locations of the plant (Figueiredo et al., 2016).

2.2.1.2 The 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase

ACC deaminase production is common for both Gram-negative and Gram-positive PGPR, to which, 1-aminocyclopropane-1-carboxylic acid (ACC) is an immediate ethylene precursor to α -ketobutyrate, which is essential for PGPR nutrition as a source of carbon (Ali et al., 2015). ACC deaminase by most PGPR can be utilized to avert ACC from the ethylene biosynthesis after PGPR producing ACC deaminase are attached to the host plant seed (Reddy, 2014). ACC deaminase regulates root and shoot elongation, plant flowering, defoliation, fruit ripening, seed germination and promotes nodulation in the genus *Bradyrhizobium* and *Bacillus* species (Glick, 2014).

ACC deaminase production enables root growth under normal and extreme environmental conditions by increasing the plant's stress tolerance through reduced ethylene production, during stressful conditions (Perez-Montano et al., 2014). However, ethylene is observed to be destructive at high concentrations leading to plant defoliation, reduced growth and reduced yield (Nandal & Hood, 2013).

2.2.1.3 Absciscic acid (ABA)

PGPR with ABA hormone production assist plants during biotic and abiotic stress by encouraging stomatal closure, whereby, water evaporation from the stomata is reduced and this enhances drought tolerance (Georget et al., 2015). For the plant to grasp into new sources of water, the ABA regulator promotes primary root elongation and also reduces lateral root formation (Sharif & Ryu, 2017). A study done by Salomon et al. (2014) showed that under water stress conditions, PGPR ABA production was increased in the plant inoculated with *Pseudomonas fluorescens* by 40% and *Bacillus licheniformis* by 70%.

2.2.1.4 Auxins or Indole-3-acetic acid (IAA)

Auxins (e.g. Indole acetic acid) control processes concerning plant development such as cell growth, plant weight/size, leaf surface area, stem elongation, branch growth, and root morphology variation, apical meristem divisions, and they are predominantly known to influence long and short term plant reactions as well as aiding in nutrient uptake (Gull, 2016). Synthesis of IAA is carried out through two main pathways among different PGPR, namely, L-tryptophan-dependent and L-tryptophan independent routes whereby, L-tryptophan-dependent is found to be the common pathway for most PGPR which further separate into three

pathways being the indole-3-pyruvic acid (IPyA), indole-3-acetamide (IAM), and tryptamine pathways (Goswami et al., 2016).

Approximately 80% of PGPR are commonly found to produce auxins (Sharif & Ryu, 2017). A study by Bailly et al. (2014) reported that indole production is a necessity for auxin signalling activation for the effective production of auxins by PGPR occurring in plant-microbe associations.

2.2.1.5 Cytokinins

Cytokinins are hormones produced mostly by PGPR genera *Pseudomonas*, *Paenibacillus*, *Bacillus*, *Pantoea*, and *Rhizobium* species (Bishnoi, 2015). The biosynthesis of these hormones is reported to be produced through two pathways, namely, direct pathway by developing N⁶-isopentenyl adenosine monophosphate (i6 AMP) from adenosine monophosphate (AMP) and dimethylallyl diphosphate (DMAPP), then development from zeatin-type compounds from the side-chain hydroxylation, whereas the indirect pathway involves tRNA having cis-zeatin releasing cytokinins via the turnover process (Salomon et al., 2014).

The presence of PGPR cytokinins on the plant increases root hair proliferation, stomatal opening, cell division, chlorophyll production, nutrition signalling, shoot and leaf elongation, and cell differentiation (Goswami et al., 2016). However, cytokinins could also decrease the lateral and primary form of root growth through ethylene activation (Sharif & Ryu, 2017). Studies by Liu et al. (2013) have shown that the upper part of the plant (aerial parts) treated with PGPR (e.g. *Bacillus* spp. and *Pseudomonas* spp.) cytokinins (zeatin) resulted in improved cytokinins biosynthesis.

2.2.1.6 Gibberellic acids (GAs)

GAs are an organised collection of phytohormones consisting of 136 molecules structured differently, which are made up of 19-20 carbon atoms (Bakthavatchalu et al., 2012). These hormones are pronounced regulators capable of translocating from the bottom part of the plant (roots) to the upper part (aerial) (Figueiredo et al., 2016). GAs mediate the upper plant's structural-developmental processes such as flowering, stem tissue morphological changes, fruit set, and seed germination (Glick, 2014). Wong et al. (2015) stated that the production of GAs by PGPR is most effective and notable on the aerial part of the plant when the same PGPR also produces auxins which are more likely to complement and enhance nutrient supply through root stimulation and promote growth.

2.2.1.7 Biological nitrogen fixation

This involves the production of PGPR organic substances which tend to act as primary nutrient suppliers (e.g. Biofertilizers) by contributing to nutrient mobility from the environment to the plant (Owen et al., 2015). The provision of scarce nutrients such as nitrogen (N) in the rhizosphere by PGPR is a direct effect for boosting the plant's growth. A large amount of N is essential for plant development and productivity, and it is vital for root hair formation, root organogenesis, and the growth of lateral roots (Courty et al., 2015). There are two groups of N-fixing PGPR, the first group is the symbiotic N₂ fixing rhizobacteria which involves root-associated PGPR with specificity traits that are proficient in nodule production with leguminous plants (e.g. *Rhizobium* spp.) (Oberson et al., 2013).

The second group involves free-living PGPR that does not have specificity traits and is made up of organisms called diazotrophic PGPR (e.g. *Bacillus* and *Pseudomonas* spp.) and they do not pierce the plant's tissues (Ahemad & Khan, 2012). Instead, they reside near the plant's roots where they convert atmospheric nitrogen (N₂) into simple forms that the plants can use and in exchange, the host plant provides the PGPR with carbon (C) fixed from the atmospheric carbon dioxide (CO₂) during photosynthesis (Nandal & Hooda, 2013).

The key enzyme necessary to fix the triple bonded N₂ gas into two ammonia (NH₃) molecules is the nitrogenase complex (Walley, 2013). Different PGPR genera have different N fixing systems when viewed structurally, and molybdenum nitrogenase attained in most diazotrophs seems to be the most commonly occurring activity for biological N fixation (Bishnoi, 2015). Some studies have observed that a plant treated with N fixing PGPR in an experiment with N-limited conditions, had major increases in plant gene expression for high-affinity transport systems (HATSs) (Kundan et al., 2015; Lakshmanan, 2015).

These HATSs are capable of taking up ammonium (NH₄), nitrate ions and or inorganic N limited in the root cells, therefore, this active process occurring in the root cell wall leads to available N for the plant (Pii et al., 2015). It has been observed that N deficiency in agricultural soils leads to reduced plant yields, therefore N fixing PGPR are considered potential alternatives to synthetic fertilizers in the agricultural space as bio-fertilizers and are also considered environmentally and economically friendly since they are largely distributed in nature (Prashar et al., 2014).

2.2.1.8 Phosphate solubilization

It is well recognized that phosphorus (P) comes after N as a limiting nutrient for plant absorption, regardless of its abundance in the soil, it undergoes precipitation due to its reaction with iron (Fe), aluminium (Al), or calcium (Ca) and consequently resulting in an unavailable form for plant usage (Vacheron et al., 2013). The monobasic (H_2PO_4^-) and dibasic (HPO_4^{2-}) ion phosphates are known to be accessible and suitable forms for plant absorption (Jha & Saraf, 2015). Organic P is mineralized by phosphate-solubilizing PGPR in the soil through the solubilization of complex-structured phosphates (e.g. Rock phosphate, aluminium phosphate and tri-calcium phosphate) (Lakshmanan, 2015). Consequently, helping the plant with phosphate availability by turning inorganic P into organic forms (Patel & Desai, 2015). Phosphate-solubilizing PGPR use the sugars from the root exudates as sources for energy to solubilize the insoluble phosphate by using primary mechanisms that involve the production of organic acids such as tartaric, oxalic, citric, malic, acetic, succinic, 2-ketogluconic and gluconic acids (Grobela et al., 2015). The secreted acids by the phosphate-solubilizing PGPR act as good chelators of proton efflux and acidify the rhizosphere which then becomes complementary to the discharge of phosphates from the mineral sources (Vacheron et al., 2013; Figueiredo et al., 2016). Phosphate-solubilizing PGPR are also collectively called phytase-producing bacteria (PPB), when they make soluble phosphate available to plants through phytase (myo-inositol hexakisphosphate phosphohydrolase) action, which is an enzyme mostly found to be active in the genera *Pseudomonas*, *Enterobacter*, *Rhizobium*, *Bacillus*, and *Klebsiella* spp. (Kundan et al., 2015; Owen et al., 2015).

Phosphonoacetate hydrolase, D- α -glycerophosphatase, C-P lyase, and phosphatase are other enzymes involved in the mineralization of organic P compounds (Munees & Mulugeta, 2014). From the rhizosphere's culturable population, an estimation of approximately 20-40% is found to be phosphate-solubilizing PGPR (Gupta et al., 2014). It is also shown by some studies that P deficiency in agricultural soils is dealt with by applying phosphatic fertilizers which are costly because a minute amount of them are used up by the plants while the rest becomes insoluble and complex in the soil, and consequently, they become undesirable (Ali et al., 2015; Franco-Correa & Chavarro-Anzola, 2016).

2.2.2 Indirect mechanisms

Indirect mechanisms involve microbial antagonism of PGPR which contribute to plant growth by controlling and assisting plants in terms of biological control and stress tolerance under stressed conditions (Prasad et al., 2015). This includes:

2.2.2.1 Microbial antagonism

This mechanism involves the inhibition of soil-borne pathogens through the PGPR production of antibiotics, antifungal metabolites, surface-active compounds (e.g. Biosurfactants), toxins, competition for colonization sites, nutrition and minerals (Sharma et al., 2014). Most Gram-negative and Gram-positive PGPR species are capable of producing volatile and non-volatile organic compounds, and the genera most commonly found active for this mechanism are *Pseudomonas*, *Bacillus*, *Serratia*, *Streptomyces* and *Stenotrophomonas* species (Nandal & Hooda, 2013).

2.2.2.2 Hydrogen cyanide production

Hydrogen cyanide (HCN) is a secondary metabolite that is phytotoxic and acts as an agent to constrain particular enzymes during metabolic reactions (Rayees et al., 2014). This compound is generated during the stationary phase of growth and occurs as free cyanide including non-dissociated HCN and cyanide anion in the metabolite solution (Jung & Park, 2015). Therefore, the cyanide ion is metabolized into smaller compounds and respired as HCN (Chaitanya & Meenu, 2015). PGPR producing HCN compete with other microorganisms through the utilisation of energy supply and electron transportation which also disturbs proper functioning of enzymes and recovery of cells, and consequently, leading to cell disruption and death of the opponent (Farag et al., 2013).

2.2.2.3 Production of volatile and non-volatile organic compounds

PGPR's volatile and non-volatile compounds are fungistatic substances when secreted in the soil environment at different concentrations and are also called volatile and non-volatile inhibitors (Ali et al., 2016). They are also initiators in terms of plant defence response and resistance (Saraf et al., 2014). They are organic and inorganic; examples of these inhibitors include allyl alcohol, trimethylamine, ammonia, benzaldehyde, acrylic acid, etc., and their production and effectiveness tend to depend on different conditions such as availability of nutrients/minerals, pH, environmental conditions, temperature, and the producing organism (Mariutto & Ongena, 2015). Some PGPR volatile compound production includes alkaloids,

which are reactive constituents produced in low concentrations and other volatile compounds include phenols, which are known to be involved in plant protection against pathogens (Ali et al., 2015).

2.2.2.4 Production of lytic enzymes

Most PGPR are shown to produce cell wall degrading enzymes such as lipases, phosphatases, dehydrogenase, glucanases, proteases, cellulases and chitinases which are capable of directly interfering with plant pathogenic attacks (Ouda, 2014). The proteases enzymes are known to expose the chitin microfibrils and the inner glucan layers by binding and opening the outer mannoprotein contained on the components of a fungal cell wall (Munees & Mulugeta, 2014). The cellulolytic enzymes are known to break down β -1.4 glycoside bonds and cellulose commonly present the cell walls in most phytopathogens and trigger elicited biotic defence in the host plant, also, the secretion and effectiveness of these lytic enzymes are identified to be dependant on several soil conditions such as soil pH and availability of nutrients in the soil (Reddy, 2014).

2.2.2.5 Antibiotic production

There are several antibiotic production mechanisms which are observed to damage the pathogen's cell walls through the production of surfactants. Some PGPR production of antibiotic products includes biosurfactants which are known to be surface-active compounds released through secondary metabolic pathways by most bacteria (Saraf et al., 2014). These products are described to be heterogeneous low molecular weight organic compounds and their toxins are produced in minute concentrations but effective enough to suppress soil-borne disease-causing agents and other deleterious competitive microorganisms (Ouda, 2014). An example of a well-studied genus is *Bacillus* spp., which produces multiple antibiotics (e.g. antifungal antibiotic lipopeptide biosurfactants) including cyclic lipopeptides (CLPs) fengycins, surfactins, and iturins which are currently known as the three main groups commonly found to be produced by most *Bacillus* species (Dunlap et al., 2013). These groups occur or coexist as a non-ribosomally synthesized family of closely related isoforms which are known to have different fatty acid side chains, about 7-10 amino acid hydrophilic head substitutions and peptide ring structures and lengths which are effectively proven to control most plant pathogens and some are useful in industrial applications as surface-active compounds (Prashar et al., 2014).

The *Bacillus* spp. genus produces multiple extracellular metabolites which are known to be active in the biological control of several plant pathogens (e.g. *Rhizoctonia*, *Botrytis* and *Fusarium* spp.) (Reddy, 2014). The mechanism of antibiosis is a microbe-microbe interaction whereby there is a conflict between the microorganisms involving secretions of deleterious diffusion of chemical complex molecules between them that result in the death of the other (e.g. Bacterial-fungal interaction (BFI)) (Walley, 2013).

Other antibiotic production mechanisms have been observed to inhibit the pathogen's zoospores formation, transport systems, cellular respiration (e.g. Fusaric acid and hydrogen cyanide), cell wall synthesis (e.g. Butyric acid and penicillin) (Jha & Saraf, 2015). According to Patel et al. (2015), the effectiveness of the produced antibiotic depends on the sensitivity or resistance of the other microbe and most PGPR antibiotic-producing strains are shown to produce more than one antibiotic and are evaluated as good BCAs and antagonists for pathogen control.

2.2.2.6 Systemic response induction

Acquired systematic resistance (ASR) and induced systematic resistance (ISR) are the defence responses of plants to chemical or microorganism's attack (Prasad et al., 2015). The ISR responses are activated by non-pathogenic PGPR molecules called elicitors whereas ASR is activated by a pathogenic attack resulting in resistance to the unaffected plant parts (Perez-Montano et al., 2014). During ISR, some PGPR are shown to have an interaction with the host plant leading to an activation of the pathogenic defence initiated from the roots spreading to the shoot and this relies on jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) signals from the host plant (Glick, 2014).

The different signal pathways for ASR are induced through SA whereas ISR through ET and JA; and they also overlap with other signalling molecules, such as homoserine lactones, cyclic lipopeptides, siderophores, and lipopolysaccharides (Ghodsilavi et al., 2013). Several pathogen-related proteins have been reported to be induced by both accumulation of JA and ET molecules together, sometimes ET enhancing JA response or SA suppressing JA responses, or one molecule interfering with another molecule's defence response (occurring vice versa) (Beneduzi et al., 2012).

2.2.2.7 Siderophore production

Iron (Fe) is essential for plant growth due to its assistance and involvement in numerous metabolic reactions such as photosynthesis or respiration for plant development and health (Radzki et al., 2013). In aerobic environments, this micronutrient is primarily found as the ferric ion (Fe^{3+}) and forms insoluble oxyhydroxides and hydroxides in the soil which is in an unavailable form for plant absorption (Baysal & Tör, 2014). Therefore, obtaining it in a suitable form is a necessity for both rhizobacteria and plants, and thus, some PGPR can manage to attain Fe by releasing siderophores which are Fe chelators with low-molecular-mass known for creating stable complexes with Fe and further making the metal soluble in water (Munees & Mulugeta, 2014).

These siderophores can also make stable complexes with other heavy metals (e.g. Copper (Cu), Zinc (Zn), Aluminium (Al), Cadmium (Cd), Palladium (Pd), and Gallium (Ga)) (Radzki et al., 2013; Chaitanya & Meenu, 2015). The siderophore-ferric complex is formed when siderophores are released by some PGPR at the surface of the bacterial cell and bind with Fe limiting-dependent receptors, then the released Fe^{3+} becomes active ferrous iron (Fe^{2+}) in the cytoplasm (Sharifi & Ryu, 2017). The siderophores are useful to the plants as a source of Fe (Pii et al., 2015). Siderophore production by some PGPR is shown to prevent the proliferation of phytopathogens in the roots due to the competition for iron absorption, consequently depriving other microorganisms of Fe intake, and therefore, making it difficult to survive (Tokpah et al., 2016).

2.2.2.8 Quorum sensing (QS)

QS is the exchange of cell-to-cell (bacteria-bacteria) communication, in the rhizosphere, this communication takes place among PGPR populations (Vacheron et al., 2013). During this communication, there is a release, detection and production of chemical signalling molecules called auto-inducers (Salomon et al., 2014). These molecules increase in concentrations due to the cell density functions and involve the regulation of gene expressing coordinates based on the cell population density (Baysal & Tör, 2014). This phenomenon permits a synchronized population behaviour & controls gene expression through collective communication and is only allowed by several species of PGPR present in the rhizosphere with the same traits instead of just one bacterium (Jung & Park, 2015).

Most auto-inducers are observed to operate as universal signals for inter-species communication while others as signals for intra-species (within species) communication (Saraf et al., 2014). Also, QS helps in the regulation of information including PGPR competence (e.g. Antibiotic production) and plant-microbe interactions (Mariutto & Ongena, 2015).

2.3 *Fusarium graminearum*

F. graminearum Schwabe [teleomorph *Gibberella zeae* (Schweinitz) Petch] is an ascomycete soil-borne fungal pathogen which is the causative agent of *Fusarium* head blight (FHB), seedling blight, scab of maize, ear, root and stalk rot on the cereal grain maize (Zhang et al., 2013). This pathogen produces a variety of virulent mycotoxins, including trichothecenes (TRIs), fumonisins (FUS), deoxynivalenol (DON), nivalenol (NIV) and zearalenones (ZEA) known to be hazardous to the health of animals and humans through the infected host (Beukes et al., 2017).

Different lineages of *Fusarium* spp. cause infections on their preferred host plant under a wide range of environmental conditions (Shi et al., 2014). Consequent to an initial infection, they may cause continuous infections that can occur at any life stage growth in a host's development and making it a major threat in the agricultural and industrial production of cereal grains including maize (Lee et al., 2015). The primary reproduction of the *Fusarium* spp. takes place when its conidia (asexual spores) and ascospores (sexual spores) are spread on the heads of a flowering plant, through germination, and further invades the floral tissues of the infected plant, the fungal infection occurs directly through the penetration of the epidermal cell walls with short infectious hyphae and complex infectious structures, and thus, leading to the presence of virulent contaminating mycotoxigenic properties (Zhang et al., 2013).

CHAPTER 3

Biocontrol screening, characterization and assessment of *Bacillus* spp. with potent antagonistic traits against *F. graminearum* on maize (*Zea mays* L.)

ABSTRACT

The ascomycete fungal pathogen *F. graminearum* is regarded to be a threat to commercial crops. As a strategy to manage the infection of this pathogen, PGPR have been introduced as alternative potential biocontrol agents (BCAs) with multifunctional mechanisms which are of value to plant growth. These mechanisms may influence particular host plant growth directly or indirectly. This study was carried out to screen bacteria isolated from healthy maize rhizosphere for plant growth-promoting (PGP) tests (Phosphate solubilization, nitrogen fixation, IAA, siderophores, NH₃, and HCN production), hydrolytic enzymes' production (Cellulases and proteases), antibiotic sensitivity, and antifungal activity under *in vivo* and *in vitro* conditions. To obtain effective PGPR strains, 3 potent strains (LBC4ME (*B. thuringiensis*), LBC22HL (*B. velezensis*), and LBC35MW (*B. thuringiensis*)) were selected and identified through biochemical, physiological, and molecular techniques. To distinguish amongst the strains, partial 16S rDNA gene sequence and multilocus sequence analysis (MLSA) of 3 protein-coding housekeeping genes *rpoB*, *gyrB*, and *tuf* gene sequences were used to further identify the strains. A screen-house pot experiment was conducted to evaluate the biocontrol efficacy of these strains against the phytopathogen *F. graminearum* FG1 strain infection on the maize crop. The results showed that the strains were *Bacillus* spp. that belonged to the Firmicutes phyla which were able to exhibit several plant growth-promoting traits (Phosphate solubilization, nitrogen fixation, IAA, siderophores, NH₃, HCN production, production of hydrolytic enzymes) known to assist through the direct and indirect growth of the host plant and also showed effective inhibition rate (above 40%) against *F. graminearum* FG1 under laboratory and screen-house conditions. The strains were found to possess spores, plasmid DNA and also grew optimally and expressed effective antifungal activity when grown at pH 7, temperature 37 °C, in the presence of glucose and (NH₄)₂SO₄. These results show the possibility of using this *Bacillus* spp. strains as biopesticides in the treatment of plant diseases.

Keywords: Antifungal activity, plant diseases, plasmid DNA, protein-coding genes, taxonomy

3.1 Introduction

The soil-borne fungus *F. graminearum* is commonly known to be a plant pathogen and is the leading cause of numerous crop diseases such as FHB and rotting resulting in plant damage, defoliation and wilting in maize, thus, infection management is critical (Dunlap et al., 2013). PGPR are known to be beneficial in terms of disease management to their host plant through several direct and indirect mechanisms of action (e.g. Phosphate solubilization, HCN production, IAA production, nitrogen fixation, siderophore production, production of hydrolytic enzymes, etc.) (Georget et al., 2015).

The PGPR community is diverse, and sometimes species in the same genus might not contain similar plant growth-promoting traits, therefore, specific identification is necessary to discriminate between the bacteria (Alajlani et al., 2016). Among the many available PGPR, the *Bacillus* spp. is considered to be the most beneficial, due to their ability to form spores, these spores are vigorously resistant to extreme conditions and have a long shelf-life lasting up to three (3) years and are found to further form stable products (Liu et al., 2013).

This study is focused on the biological control which involves only the bacterial strains that can effectively prevent the growth of *F. graminearum* on maize crops. Screening of PGPR is recommended as a foundation to better select potent strains and since some PGPR are known to be host-specific, selecting native strains from the specific host is advisable over using exotic commercial inoculants caused by several factors including climatic and environmental adaptations which may influence rhizosphere colonization and the PGPR's functionality after application (Yamamoto et al., 2015). Identification of *Bacillus* spp. using conventional molecular techniques such as PCR through the 16S rDNA gene sequence intended for revealing bacterial phylogeny is important (Ali et al., 2014). However, their characterization has so far been considered a challenge due to this genus's high genetic similarities leading to difficulties through the discrimination of closely related species such as *B. thuringiensis* and *B. cereus*, etc. (Kaki et al., 2013; Rosselló-Móra & Amann, 2015).

Consequently, alternative species-specific identification genes are introduced to further elucidate among the related species, namely, the MLSA RNA polymerase β subunit (*rpoB*), TU elongation factor (*tuf*), and DNA gyrase β subunit (*gyrB*) genes which have been previously recommended to distinguish and identify bacterial taxons including well-conserved regions (Caamano-Antelo et al., 2015). Achieving a distinctive characterization and efficiency of the

genus *Bacillus* spp. is necessary, due to its potent application as PGPR, and thus, this work aimed to obtain native PGPR strains, screen and identify them for biological control traits against *F. graminearum* then further assess their biocontrol efficiency through *in vitro* tests using individual and consortium inoculant applications in pot treatments under screen-house conditions. Also to establish potential BCAs strains that can be used as antifungal agents against maize crops.

3.2 Materials and methods

3.2.1 Samples' area collection

This study complies with the North-West University ethics committee requirements and has received the ethics clearance number NWU-00364-19-S9. The collection sites were located in the Ngaka Modiri Molema District and Dr. Ruth Segomotsi Mompati districts. Soil samples were randomly collected from 3 locations (Mareetsane, Morwatshetlha & Vergelee) that had healthy maize plantations in the North West Province, SA, during June 2015 as shown in Figure 3.1. The temperature range in these areas in the summer is from 17-31 °C and 3-21 °C in the winter, and the annual rainfall is about 360 mm (Online source. [www. South African weather service.co.za](http://www.SouthAfricanweather.service.co.za)). The soil and its associated root materials were removed from the ground with a trowel and placed in sterile polythene bags, the samples were transported to the laboratory and placed in the refrigerator (4 °C) until further use.

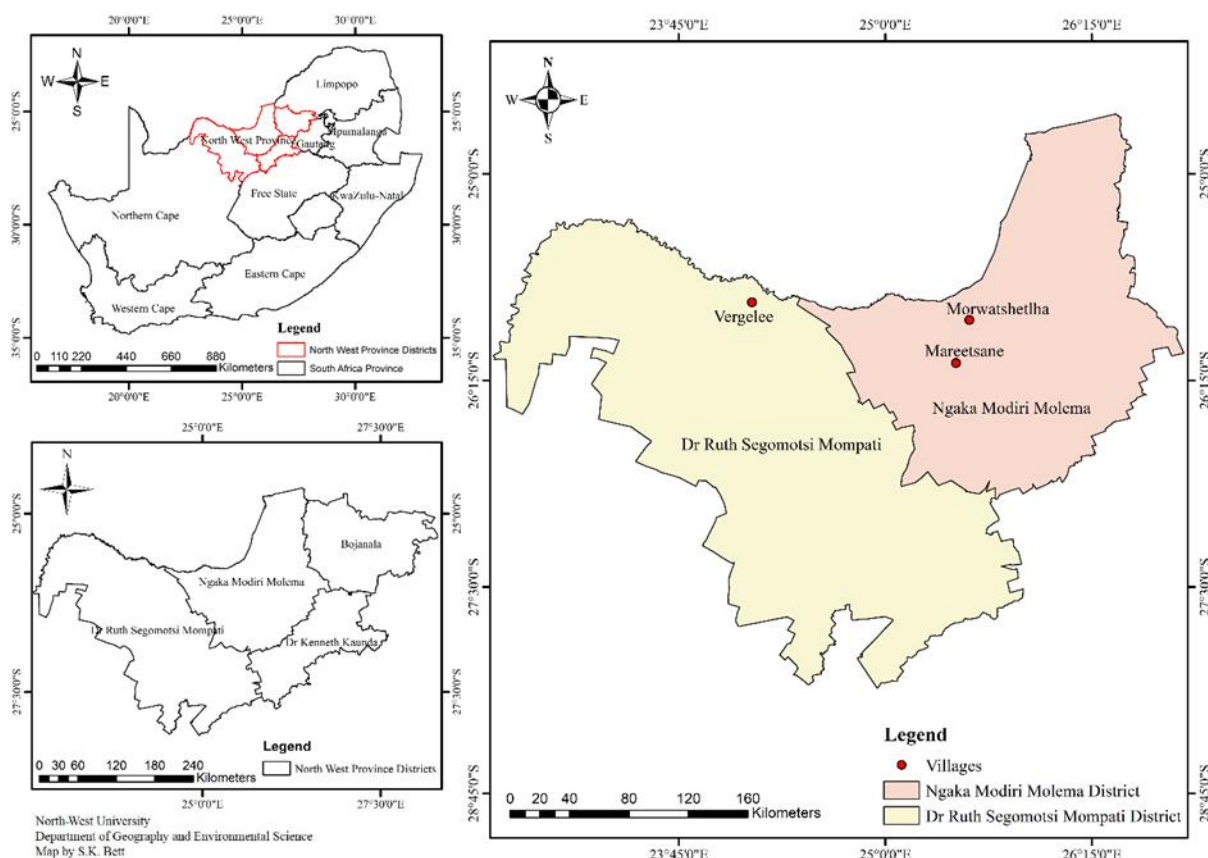


Figure 3. 1: Map showing the samples' area collection locations (highlighted with red dots), North West province, SA.

3.2.2 Bacterial isolation

The isolation of rhizobacteria was carried out using the spread plate technique. Ten (10 g) grams of the soil samples were transferred into a 100-mL Erlenmeyer flask containing 90 mL of phosphate-buffered saline (Per 100 mL distilled water: 1.2 g Na_2HPO_4 , 0.18 g NaH_2PO_4) at pH 7 (6 mol/L of HCl and 2 mol/L of NaOH were used to balance the pH)) and placed on a rotary shaker (200 rpm) at 25 °C for 30 min, then left to settle down the large particles for 10 min. Then 1 mL of the bacterial phosphate buffer solution (PBS) was sequentially diluted from the 10^{-2} to 10^{-6} dilutions, about 80 μL of the 10^{-2} to 10^{-6} dilutions were spread plated in triplicates on Petri dish plates containing Tryptic Soy Broth (TSB) solidified with 1.5% nutrient agar (NA) (MERCK). After 48 h of incubation at 25 °C, plates containing different colonies were selected from each replicate and the bacterial colonies were counted. The colonies were selected from the Petri plates based on their different morphology, shape, height and colour. Strains were sub-cultured again on a Tryptic Soy Agar (TSA) and the purified strains were separated as working cultures in 1 mL slants containing TSA, and overlaid with sterile mineral

oil which was then stored at 4 °C and others were stored as master stocks in TSB medium containing 10% glycerol at -80 °C (Guiñazú et al., 2013; Fan et al., 2015).

3.2.3 *In vitro* screening for fungal antagonistic activity

Fungal pathogen and cultural conditions: A Pathogenic *F. graminearum* FG1 strain culture was kindly provided by Dr. Claire Prigent-Combaret from the UMR-CNRS 5557 Ecologie Microbienne, France. The strain was sub-cultured by inoculation on a Potato Dextrose Agar (PDA) Petri dish then incubated at 25 °C for 120 h. The sub-cultured fungal plates were kept at 4 °C until further use. All the microbial growth mentioned in the tests were estimated by CFU/mL determination and by measuring the absorbance of the culture at OD_{600 nm} by using a spectrophotometer: Spectronic 20 D+, USA. About 6 mol/L HCl and 2 mol/L NaOH were used to balance the pH of the medium on all the conducted experiments, except the ones specified differently. The medium was autoclaved for 15 min at 121 °C for sterilization.

3.2.3.1 Antifungal assay

The agar diffusion method was used to test bacterial strains for antifungal activity. The bacteria were grown for 24 h at 25 °C in TSB medium. The fungus *F. graminearum* FG1 strain was grown in a Mueller Hinton agar (MHA) medium (1000/mL distilled water: 30 g beef infusion, 17.5 g casein acid hydrolysate, 1.5 g starch, 20 g agar, pH 7.2) for 96 h at 25 °C. In order to obtain a diluted spore suspension of the fungus at 10⁵ CFU/mL, the mycelium spores were scraped from the plate and suspended in 10 mL of sterile normal saline solution (NSS) for about one (1h). Wells of 8 mm (one well per plate) in diameter were punched using a sterile cork borer into the MHA solid media, then 0.1 mL of diluted spore suspensions were streaked on the punched plates with a cotton swab. The wells were filled with 200 µL (2 x 10⁷ CFU/mL) of the TSB bacterial culture. An MHA plate streaked with the spore suspension without a bacterial culture was used as a control plate. The plates were incubated for 120 h at 25 °C and the antifungal activity was evaluated by measuring the size of the zone of inhibition against the fungal growth (Ahmad et al., 2008).

The inhibition rate in a percentage formula was calculated according to Ahmad et al. (2008):

$$IR\% = \frac{R-r}{R} \times 100$$

Whereby: R = Diameter of control fungal mycelium growth

r = Diameter of fungal mycelium in presence of inoculated bacteria

3.2.3.2 Optimum conditions for maximum growth and antagonistic activity

The bacterial strains showing antifungal activity were further tested for optimum growth and antagonism of *F. graminearum* FG1 under different growth conditions. Bacterial strains were grown for 48 h in a TSB medium to test the effect of different temperature ranges (4 °C, 25 °C, 37 °C, and 45 °C) against fungal growth. To test the effect of various hydrogen ion concentration (pH) against fungal growth, strains were inoculated in TSB medium with different pH ranges (2, 4, 7, and 10), then grown for 48 h at 25 °C. To test the effect of different carbon and nitrogen sources, strains were grown in a Modified Mineral-Based Medium (MMM) (1000/mL distilled water: 1 g KH₂PO₄, 1.25 g Na₂HPO₄·2H₂O, 1 g (NH₄)₂SO₄, 0.5 g MgSO₄·7H₂O, 0.05 g CaCl₂, 0.05 g FeSO₄) supplemented with 1 mL trace element salt stock solution (1000/mL distilled water: 40 µg FeSO₄, 40 µg MnSO₄, 20 µg ZnSO₄, 5 µg CuSO₄, 4 µg CoCl₂, 5 µg Na₂MoO₄, 0.5 µg CaCl₂, 136 µg KH₂PO₄, 1 mg NaCl) with carbon sources using 2 g of 1000/mL: Glucose, D-fructose, sucrose, and D-galactose and for different nitrogen sources using 2 g of 1000/mL: KNO₃, NH₄NO₃, (NH₄)₂SO₄, and urea then inoculated for 48 h at 25 °C. The bacterial growth of the cultures was measured for absorbance with an optical density at (OD_{600 nm} = 0.5) after incubation and the concentration of the fungal mycelium spores was at 10⁵ CFU/mL. The antagonistic activity of the bacterial strains was tested through the dual culture method on MHA medium plates using the cultures grown from different pH, temperatures, nitrogen and carbon sources and the zone of inhibition was recorded after incubation for 72 h at 25 °C (Zhou et al., 2016).

3.2.4 Preliminary identification analysis

The bacterial strains were preliminarily identified by a selection of their cultural conditions, morphological, physiological, and biochemical characterization based on standard identification methods. All the microbial growth mentioned in the tests were estimated by CFU/mL determination and by measuring the absorbance of the culture at OD_{600 nm} by using a spectrophotometer: Spectronic 20 D+, USA. About 6 mol/L of HCL and of 2 mol/L NaOH were used to balance the pH of the medium on all the conducted experiments, except the ones specified differently. The medium was autoclaved for 15 min at 121 °C for sterilization.

All experiments were repeated 3 times. Tests include:

3.2.4.1 API®50CH ID strips kit test

The catalase and casein tests were carried out according to Bergey's manual of systematic bacteriology (Bergey & Holt, 2012).

This experiment was carried in order to identify the bacterial strains through the utilisation of carbon using the following as main ingredients in the tests strips: Glycerol (GLY), Erythritol (ERY), D-arabinose (DARA), L-arabinose (LARA), D-ribose (RIB), D-xylose (DXYL), L-xylose (LXYL), D-xylose (ADO), Methyl-beta-D-xylopyranoside (MDX), D-galactose (GAL), D-glucose (GLU), D-fructose (FRU), D-mannose (MNE), L-sorbose (SBE), L-rhamnose (RHA), Dulcitol (DUL), Inositol (INO), D-mannitol (MAN), D-sorbitol (SOR), Methyl-alpha-D-mannopyranoside (MDM), Methyl-alpha-D-glucopyranoside (MDG), N-acetylglucosamine (NAG), Amygdalin (AMY), Aburitin (ARB), Esculin ferric citrate (ESC), Salicin (SAL), D-cellobiose (CEL), D-maltose (MAL), D-lactose (Bovine origin) (LAC), Melibiose (MEL), D-saccharose (sucrose) (SAC), D-trehalose (TRE), Inulin (INU), D-Melezitose (MLZ), D-raffinose (RAF), Amidon (starch) (AMD), Glycogen (GLYG), Xylitol (XLT), Gentiobiose (GEN), D-turanose (TUR), D-lyxose (LYX), D-tagatose (TAG), D-fucose (DFUC), L-fucose (LFUC), D-arabitol (DARL), L-arabitol (LARL), Potassium gluconate (GNT), Potassium 2-ketogluconate (2KG) and Potassium 5-ketogluconate (5KG). Which were carried out using the API®50CH ID strips kit (Biomérieux, USA) according to the manufacturer's guidelines for screening and identification of the strains from the APILAB software.

3.2.4.2 Gram staining

This experiment was carried out according to Kumari and Dhingra (2013). Bacterial strains grown on NA for 24 h at 25 °C were stained with Gram reagents using the standard procedure to determine their Gram reactions.

3.2.4.3 Oxidase test

Oxidase test was done to identify strains with the ability to produce cytochrome c oxidases. The bacterial strains were grown in a Nutrient Broth (NB) for 24 h at 25 °C. The bacterial suspensions were applied on the commercial oxidase strips and observed for a purple colour change after 1 min. A control test was done using commercial oxidase strips streaked with NB without bacterial inoculum (Kumari & Dhingra, 2013).

3.2.4.4 Nitrate reduction test

The strains were tested for the ability to reduce nitrate to nitrite. Nitrate broth with Durham tube was prepared in a screw-cap tube. The tubes were then inoculated with the strains and incubated at 30 °C for 48 h. Nitrate reagents A and B were sequentially added to each tube, the appearance of red or pink colour was a positive reaction for the presence of nitrite in the tube and no colour change indicates a negative reaction. The development of an orange colour after the addition of reagent C was also observed for a negative reaction to confirm the non-existence of nitrate in the tube (Kumari & Dhingra, 2013).

3.2.4.5 Spore production

The strains were tested for the production of spores. The strains were grown on TSB medium in 200 mL Erlenmeyer flask for 24 h at 25 °C in a rotary shaker (200 rpm) (Model HZ-300, Thomas Scientific, USA). After incubation, the growth was measured by checking the optical density (OD_{600 nm}). The cultures were further heated to 80 °C for 10 min for bacterial cell removal, 0.1 mL of bacterial solution was sequentially diluted from 10⁻² to 10⁻⁶ dilutions then spread plated onto TSA plates and incubated for 72 h at 25 °C to test for bacterial growth. Control tests were conducted as TSA plates without the spore solution (Zalila-Kolsi et al., 2016).

3.2.4.6 Protease production

Testing strains for the ability to reduce litmus milk (reduction of casein). The cultures were grown on Skim Milk Agar (SMA) medium (Per 100 mL distilled water: 10 g skim milk powder, 0.5 g peptone, 1.5 g agar, pH 7) for 24 h at 25 °C. The litmus milk was observed for the appearance of clear zones around culture growth, control tests were conducted as SMA plates without bacterial inoculation (Kumari & Dhingra, 2013).

3.2.4.7 Cellulase production

Testing strains for the ability to produce cellulases. The Czapek-mineral salt agar medium (100/mL distilled water: 0.2 g NaNO₃, 0.1 g KH₂PO₄, 0.05 g MgSO₄, 0.5 g KCl, 0.2 g peptone, 2 g agar, pH 7) was inoculated with strain cultures and incubated for 24 h at 25 °C. After incubation, the plates were further flooded with hexadecyltrimethylammonium bromide and observed for the formation of empty zones around the bacterial growth. A control test was Czapek-mineral salt agar plates inoculated with distilled water without bacterial inoculations (Kumari & Dhingra, 2013).

3.2.5 Production of antimicrobial metabolites

3.2.5.1 Qualitative and quantitative estimation of IAA production

An assay for IAA production was performed to detect the ability of the strains to produce IAA. Quantitative IAA analysis was carried out at different tryptophan concentrations (0, 100 and 200 mg/mL). Bacterial strains were grown in TSB supplemented with tryptophan for 48 h at 25 °C. After incubation, cultures were further centrifuged (Benchtop centrifuge, model ECH-TYP, USA) for 30 min at 3000 (rpm), about 2 drops of orthophosphoric acid and 4 mL of Salkowski reagent (50/mL: 1 mL 0.5 M FeCl₃ solution, 35% perchloric acid) was added to 2 mL of the liquid supernatant. Colour change to pink indicated positive results for IAA production and no colour change indicated negative results for IAA production. Negative control was performed using TSB and tryptophan medium without bacterial inoculations. Further readings were done by checking for optical density (OD_{530 nm}) (spectrophotometer: Spectronic 20 D+, USA) (Ahmad et al., 2008).

3.2.5.2 NH₃ production

The ability of strains to produce NH₃ was tested. The bacterial cultures were grown in TSB for 24 h at 25 °C. After incubation, 100 µL of bacterial cultures were inoculated in 10 mL peptone water (Sigma, USA) and were grown in 200 mL Erlenmeyer flask for 48 h at 28 °C. Then Nessler's reagent (0.5 mL) was added to each flask and observed the colour development from brown to yellow which indicated positive results for NH₃ production, and no colour change indicated negative tests (Ghyselinck et al., 2013).

3.2.5.3 HCN production

The ability of strains to produce HCN was determined. A Nutrient agar medium was prepared with 1000/mL distilled water, plus an addition with 4.4 g of glycine, pH 7 and bacterial cultures were streaked on the modified agar medium. A Whatman filter paper no. 1 soaked in 2% sodium carbonate and 0.5% picric acid solution was placed on the top half of the Petri dish plate, the plates were sealed with parafilm to avoid releasing gas and incubated for 120 h at 25 °C. The colour change of the filter paper into orange to red indicated HCN production. Control tests were done using plates without cultured inoculum (Ahmad et al., 2008).

3.2.5.4 Siderophore production

Testing strains for the ability to produce siderophore by releasing Fe^{3+} chelates. Strains were grown in Lauria Bertani (LB) broth medium for 24 h at 25 °C. Then, the LB agar medium supplemented with Chrome Azurol S dye (CAS) was prepared (1000/mL distilled water: 6 g piperazine-N, N'-bis-2-ethane sulfonic acid, 15 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.6 g NaOH, 1.5 g K_2HPO_4 , 10 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 15 g proteose peptone, 72.9 mg cetrimonium chloride, 10 mL glycerol, 60.5 mg CAS, pH 7). Then, 100 μL of bacterial suspension was spot inoculated at the centre of the modified plates and incubated for 48 h at 25 °C. A colour change from blue to orange around inoculated spots indicated positive results for siderophore production, control tests were performed using CAS modified agar plate without bacterial inoculations (Szentes et al., 2013).

3.2.5.5 Phosphate solubilization

Testing the ability of strains to solubilize phosphate was performed using Pikovskaya's agar medium (1000/mL distilled water: 5 g $\text{Ca}_3(\text{PO}_4)_2$, 0.5 g $(\text{NH}_4)_2\text{SO}_4$, 0.1 mg FeSO_4 , 0.1 mg $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$, 0.2 g KCl, 0.1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g yeast extract, 15 g glucose, pH 7). Then, 100 μL of bacterial suspension was spot inoculated at the centre of the plates and incubated for 48 h at 25 °C. After incubation, the occurrence of clear zones around the inoculated spots indicated positive results for phosphate solubilization. Control tests were performed using Pikovskaya's agar medium without bacterial inoculations (Laslo et al., 2012).

3.2.5.6 Nitrogen-fixing bacteria

This test was done to determine the ability of strains to fix atmospheric nitrogen. Strains were grown in a TSB medium for 24 h at 25 °C, Yeast Extract Mannitol medium (YEM) (1000/mL distilled water: 0.5 g K_2HPO_4 , 0.1 g NaCl, 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20 g agar, 10 g mannitol, 1 g yeast extract, 10 mL of 250 g of bromothymol blue dissolved in 100 mL distilled water, pH 7) was prepared and poured into plates. Then left to cool, and 8 mm well (one well per plate) was punched at the centre of the plates and some 100 μL bacterial suspensions was filled in the wells of the solid medium, incubated for 48 h at 25 °C. A colour change from red to blue indicated positive results for nitrogen-fixing bacteria. Control tests were performed using YEM without bacterial inoculations (Grobelak et al., 2015).

3.2.5.7 Antibiotic sensitivity assay

This test was done to determine the susceptibility of the strains against different antibiotics at a 30 $\mu\text{g/mL}$ concentration which was uniform for all the antibiotics, namely, vancomycin (VA),

chloramphenicol (C), kanamycin (K), tetracycline (TET), erythromycin (E) and ampicillin (AMP) using the disc diffusion technique. The strains were grown in TSB medium for 24 h at 25 °C. The Mcfarland turbidity standard prepared with 1 mL of H₂SO₄ dissolved in 99 mL distilled water, and 0.5 g BaCl₂·2H₂O dissolved in 50 mL distilled water, mixed together to form a turbid/cloudy solution which was used as a control to regulate the turbidity of the bacterial suspensions. One hundred µL (100 µL) bacterial suspension was spread plated on MHA plates each using a cotton swab. Then, the antibiotic discs were placed on top of the bacterial cultures and incubated for 24 h at 37 °C. Plates showing a clear zone around antibiotic discs indicated bacterial sensitivity and the diameter of inhibition zone was measured in (mm), whereas, zero zones indicated strain resistance against the applied antibiotic (Grobelak et al., 2015).

3.2.6 Taxonomic characterization of bacterial strains

3.2.6.1 Extraction of bacterial genomic DNA

The extraction of genomic DNA from cultured bacteria was conducted using the Nucleospin® Soil (MACHEREY-NAGEL; genomic DNA from soil, Germany) extraction kit. Protocols were conducted according to the manufacturer's instructions.

3.2.6.2 Extraction of bacterial genomic Plasmid

The preparation and extraction of bacterial plasmid from cultured bacterial strains was performed using QIAGEN® Plasmid mini kit for purification of ultrapure, transfection-grade plasmid DNA, plasmid purification handbook (sample and assay technologies, Germany). The extraction steps were carried out according to the manufacture's guidelines.

3.2.6.3 Bacterial DNA detection and quantification

After DNA extraction, the detection and quantity of the extracted DNA was done. Distilled water (1.5 µL) was applied on the Nanodrop spectrophotometer as a control before adding bacterial DNA. Then, 1.5 µL of bacterial DNA was applied on the Nano-drop spectrophotometer to measure the DNA absorbance at 260 nm.

3.2.6.3.1 PCR amplification of genes

Table 3. 1: Targeted partial gene sequences, amplicon sizes and primers

Identification genes	Targeted primers (forward and reverse)	Primer sequences (5-3')	Sequence amplicon size (bp)
16S rDNA	907F	CCGTCAATTCMTTTRAGTTT	1500
	1492R	GGTTACCTTGTTACGACTT	
gyrB	gyrB1F	TTCAGCTGGGACATCCTGGCCAA	586
	gyrBr2R	TCGATCATCTTGCCGACRACCA	
rpoB	rpoB1F	GGTGATAACGAATACTTAAGC	321
	rpoB1R	AATGCGATCAAGTGTACGACG	
Tuf	tufGPF	ACGTTGACTGCCCAGGACAC	750, 1185
	tufGPR	GATACCAGTTACGTCAGTTGTACGGA	

The primers used for the PCR amplifications were listed in Table 3.1. PCR amplifications of the bacterial DNA was performed using a Thermal Cycler model C1000 Touch (BIO-RAD, USA) with an amplification reaction volume of 50 μ L. The prepared mixture contained 25 μ L Master mix (ThermoScientific), 2 μ L bacterial DNA, 22.5 μ L sterile Milli-Q water (ThermoScientific), 0.5 μ L of each forward and reverse primers. The thermal cycling conditions for the 16S rDNA gene sequence amplification for initial denaturation was 94 °C for 5 min, for denaturation with 30 cycles at 95 °C for 30 s, for annealing was 54 °C for 1 min, for extension at 72 °C for 2 min, and for the final extension at 72 °C for 2 min. Conditions for the MLSA gyrB and rpoB gene sequence amplifications for the initial denaturation was at 94 °C for 2.5 min, for denaturation with 30 cycles at 94 °C for 30 s, for annealing at 65 °C for 30 s, for extension at 72 °C for 60 s, and for the final extension was at 72 °C for 10 min. Conditions for the MLSA tuf gene sequence amplification for initial denaturation was at 95 °C for 5 min, for denaturation with 30 cycles at 94 °C for 30 s, for annealing at 55 °C for 30 s, for extension at 72 °C for 1 min, and for the final extension was at 72 °C for 10 min.

To prepare for the detection of amplified extracted bacterial DNA, 1% agarose gel was prepared by adding 1 g agarose powder (Merck, USA) into 100 mL 1X Tris- Acetate EDTA (TAE) buffer (1:1 v/v) (BIO-RAD, USA). The agarose solution was heated for 3 min in the microwave (DEFY) and allowed to cool, followed by the addition of 10 μ L of 0.5 μ g/ mL ethidium bromide into the agarose solution, then further poured into the electrophoresis tray.

The electrophoresis well combs were placed onto the trays before the gel was poured then after the gel solidified/hardened, they were taken out. The tray was transferred into a buffer electrophoresis chamber filled with 1X TAE. Five μL (5 μL) of each bacterial DNA solution was mixed with 5 μL loading dye to a concentration of 10 μL , about 3 μL of a 1 Kb ladder was loaded in the gel wells for the approximation of DNA sizes and electrophoresis was carried out using ELITE 300 PLUS (WEALTEC, USA) for 1 h at 80 V and 400 mA. The gel was viewed under a UV transilluminator 2000 (BIO-RAD, USA) to check for amplified genes with the expected targeted sequence amplicon sizes (bp) (Caamano-Antelo et al., 2015).

3.2.6.3 Phylogenetic analysis/Nucleotide sequence determination

The PCR amplified products of the 16S rDNA, *gyrB*, *rpoB* and *tuf* gene sequences for the bacterial strains were sent for partial sequencing using the ABI PRISM® 3500XL DNA Sequencer Applied Biosystems at Inqaba biotechnological Industries (Pty) Ltd, Pretoria, SA. The resultant subsequent forward and reverse chromatograms were corrected and trimmed with Chromas Lite version 4 software (Technelysium (Pty) Ltd, 2012). The sequences were further edited, ran through reverse complement, pairwise alignment, gaps examination, and created consensus sequences which were saved in FASTA format using BioEdit version 5 software (Hall, 2004). The consensus nucleotide sequences were analysed for identification similarities regarding related bacterial species contained in the NCBI GenBank database through BLAST searches available on the ncbi.nlm.nih.gov website (Altschul et al., 1997). Multiple alignments for the sequences were carried out through running ClustalW with related nucleotide sequences of corresponding genus species downloaded from NCBI GenBank database on the MEGA version 6 software. Phylogenetic analysis was carried out using MEGA version 6 software to determine the evolutionary distance relationships as reported by Tamura et al. (2013). The phylogenetic tree was constructed through the Neighbour-joining method, by bootstrap analysis, with 1000 replications for the data set (Felsenstein, 1985; Saitou & Nei, 1987). Tree editing and manipulation were done using TreeView (Page, 1996).

3.2.6.3.1 Nucleotide sequences accession numbers

The accession number for the partial gene sequence for 16S rDNA gene was obtained through deposition in the NCBI GenBank database, and the accession numbers for the partial protein-coding MLSA *gyrB*, *rpoB* and *tuf* gene sequences were obtained by deposition in the NCBI BankIt database.

3.2.7 Screen-house experiment (*in vivo* screening)

The screen-house experiment was done according to Zalila-Kolsi et al. (2016). Before preparing pot treatments, strains LBC4ME, LBC22HL, and LBC35MW were checked for antagonistic activity against each other in a dual culture method to test for synergic activity between the strains. One hundred μL (100 μL) of each bacterial strain suspensions ($\text{OD}_{600\text{ nm}} = 0.5$) was spot inoculated against each strain in an LBA medium for 24 h at 30 °C. After incubation, inhibition zones were observed. Based on the effectivity of the *in vitro* plant growth-promoting (PGP) tests results, LBC4ME, LBC22HL and LBC35MW bacterial strains were selected for the study to evaluate the biocontrol efficacy *in vivo* PGP assay against the phytopathogen *F. graminearum* FG1 strain on maize.

The experiment was conducted at the North-West University (Mafikeng campus) under screen-house conditions. Sand-loamy soil with a pH of 5.5 was used for the pot experiments, the soil was autoclaved three times at 121 °C before use for sterilization and allowed to rest. Harvesting was done after 90 days of planting, and the experiment was conducted from September to November 2016 and repeated from February to April 2017. Pots were irrigated every two days before harvesting. The study was conducted with a completely randomized block design (CRB) using single, binary, and consortia of strain applications. Nine (9) treatments (7 antagonists and 2 controls, which are listed below) with 3 replicates per treatment were used. Three (3) seeds per pot were initially planted and later thinned to one plant per pot after seedling emergence. In preparation for inoculation, the bacterial cultures were grown in LB broth at 37 °C and *F. graminearum* FG1 was grown in PDA broth for 120 h at 25 °C. The mycelium growth was filtered with sterile cloth and transferred to sterile distilled water to make a diluted growth of 10^5 CFU/mL. The resulted from bacterial and fungal suspensions were used to treat seeds under screen-house conditions. Maize seeds (Colorado yellow maize cultivar) obtained from Mahikeng town area at the (AFGRI Ltd, SA) store, North West province, SA, were surface sterilized in 70% ethanol for 2 minutes followed by 1.2% sodium hypochlorite for 8 minutes and rinsed 5 times with sterile distilled water. The seeds were soaked in 10 mL of each of the bacterial suspension as a treatment for 6 h and dried before use. The pots measurements were (25 cm in diameter and 30 cm in height) (Bakthavatchalu et al., 2012; Agbodjato et al., 2016).

Pot treatments included:

Control treatment 1. Inoculated with fungal pathogen suspension *F. graminearum* FG1 only (PO).

Control treatment 2. Without inoculation with any bacterial or fungal pathogen suspension (Negative control).

Antagonist treatments:

Treatment 3. Inoculated with strain LBC4ME plus pathogen *F. graminearum* FG1 (LBC4ME + P).

Treatment 4. Inoculated with strain LBC22HL plus pathogen *F. graminearum* FG1 (LBC22HL + P).

Treatment 5. Inoculated with strain LBC35MW plus pathogen *F. graminearum* FG1 (LBC35MW + P).

Treatment 6. Inoculated with strain LBC4ME and LBC22HL plus pathogen *F. graminearum* FG1 (LBC4ME + LBC22HL + P).

Treatment 7. Inoculated with strain LBC4ME and LBC35MW plus pathogen *F. graminearum* FG1 (LBC4ME + LBC35MW + P),

Treatment 8. Inoculated with strain LBC22HL and LBC35MW plus pathogen *F. graminearum* FG1 (LBC35MW + LBC22HL + P).

Treatment 9. Inoculated with strain LBC4ME, LBC22HL and LBC35MW plus pathogen *F. graminearum* FG1 (LBC4ME + LBC22HL + LBC35MW + P).

Plant parameters and the effect of the treatments for shoot length (mm), shoot fresh weight (g), shoot dry weight (g), root length (mm) root fresh weight (g), root dry weight (g), mean disease rating, disease reduction (%), root mean, disease rating, root disease reduction (%) were recorded at harvest.

The disease severity % was calculated using a 0-5 scale whereby values indicate symptoms:

0 = Healthy plants

1 = Appearance of a lesion at the collar region of 2-7 mm

2 = Large lesions of 8-12 mm

3 = Moderate rotting at the collar region (slight drooping)

4 = Extensive rotting at the collar region (wilting and drying of many leaves and shoots)

5 = Plant completely wilted, dead and dry

For root rot, the disease severity rating was scaled 0-4, whereby values indicate symptoms:

0 = Healthy root

1 = Less than 25% of root spoiled due to rotting

2 = 25-50% of root spoiled (drooping leaves)

3 = Up to 75% of root damaged (wilt and drying of leaves)

4 = Complete rotting of the root (completely wilted, dead and dry plant)

The formula was done according to Pal et al. (2001) for mean disease rating

$$\text{MDR} = \frac{ax0+bx1+cx2+dx3+ex4+fx5}{a+b+c+d+e+f}$$

Whereby a, b, c, d, e and f represent the number of plants with disease rating (0-5)

Per cent for disease reduction

$$\text{PDR} = \frac{\text{MDR of pathogen control} - \text{MDR of inoculated treatment}}{\text{MDR of pathogen control}} \times 100$$

3.2.7.1 Rhizosphere colonization ability

The isolation of the bacteria from the harvested maize roots with adhering soil was carried out through the serial dilution spread plate technique as described earlier according to Guiñazú et al. (2013) and Fan et al. (2015). The preliminary biochemical and physiological standard tests using the API®50CH ID strips kit (Biomérieux, USA) were conducted according to the manufacturer's guidelines to identify the present strains after screen-house experiments as mentioned above.

3.2.8 Statistical analysis

The data obtained from each of the experiments were repeated 3 times. Statistical analysis for the screen-house pot treatments data was subjected to the one-way analysis of variance (ANOVA) using the GEN STAT software and the LSD multiple comparison range test for ($p < 0.05$) was determined. Microsoft Excel 2013 software was used to draw graphs from tests and the determination of standard error (SE) for replications ($n = 3$).

3.3 Results

3.3.1 *In vitro* biocontrol plant growth-promoting traits

The results from isolation revealed a total of 46 rhizosphere associated bacterial strains. From Table 3.2, the presented results showed that overall of 3 bacterial strains (LBC4ME, LBC22HL and LBC35MW) had a high potential antagonistic activity against the fungal pathogen *F. graminearum* FG1. These strains showed potent antagonistic reactions compared to the rest of the isolated strains by being able to effectively reduce the growth of *F. graminearum* FG1 through the antifungal assay test which resulted in impressive fungal inhibition rates of above 50%. The strain LBC22HL (66.37%) had the highest antifungal activity followed by LBC4ME (64.33%) and LBC35MW (63.60%) compared to the rest of the isolated strains which had an inhibition rate below 50%, while the control plate which had the pathogen only showed no reaction of fungal reduction.

The results presented in Table 3.2 showed that all the LBC4ME, LBC22HL and LBC35MW strains had a positive test for production of proteases by having clear zones around the culture growth which indicated the activity of casein reduction. Cellulase production was demonstrated by the appearance of clear zones after addition of hexadecyltrimethylammonium bromide to the Petri dish plates.

The results presented in Table 3.2 showed that all the 3 (LBC4ME, LBC22HL and LBC35MW) strains managed to give positive results for antimicrobial metabolite production. The strains were able to produce NH_3 , and this was confirmed from the observed development of a colour in the flask from brown to yellow. IAA production achieved to occur on different application levels of L-tryptophan by the colour changing to pink which indicated a positive test, whilst a non-colour change indicated negative results for IAA production. The strain LBC4ME (0.39, 0.44 $\mu\text{g/mL}$) showed the highest production of IAA at 100 $\mu\text{g/mL}$, and the lowest production

at 200 µg/mL concentrations, followed by LBC22HL (0.47 µg/mL) which showed the highest growth at 200 µg/mL followed by LBC35MW (0.45 µg/mL). All the strains showed a positive test for phosphate solubilization by the appearance of clear zones around the culture organism's growth. A positive test for HCN production was demonstrated in the LBC4ME, LBC22HL and LBC35MW strains by developing a colour change on the filter paper from yellow to orange. A positive test for nitrogen fixation was observed through a colour change from red to blue which indicated positive results for nitrogen-fixing rhizobacteria. All the strains also showed a positive result for production of siderophores, indicating that Fe^{3+} was fixed from the Chrome Azurol S dye complex which was demonstrated through the colour change from blue to orange around the culture's growth.

The results presented in Table 3.2 showed that strain LBC4ME was sensitive to VA with an inhibition zone of 10 mm, the highest sensitivity was observed on E with an inhibition zone of 26 mm, and lower sensitivity was seen on AMP with an inhibition zone of 2 mm, whereas, the LBC22HL and LBC35MW strains showed to be resistant to the aforementioned antibiotics by showing no inhibition zones. All the LBC4ME, LBC22HL and LBC35MW strains showed sensitivity by inhibition zones on the K (12, 18, 10 mm, respectively) and TET (10, 22, 10 mm, respectively) antibiotics. The strains LBC4ME and LBC35MW showed to be sensitive to C by both displaying an inhibition zone of 14 mm, whereas, the strain LBC22HL showed resistance to this antibiotic.

Table 3. 2: Bacterial response to PGP traits, hydrolytic enzymes and antibiotic tests

Bacterial strains	Fungal inhibition rate (%)	Hydrolytic enzymes production	PGP traits								Antibiotic sensitivity (mm)				
		Protease	Cellulase	IAA (100/200)(µg/mL)	NH ₃	Phosphate solubilization	HCN production	Siderophores	Nitrogen fixation	VA	C	K	TET	E	AMP
LBC4ME	64.33 ± 0.13	+	+	0.39 ± 0.01 / 0.44 ± 0.04	+	+	+	+	+	10	14	12	10	26	2
LBC22HL	66.37 ± 0.12	+	+	0.38 ± 0.05 / 0.47 ± 0.03	+	+	+	+	+	-	-	18	22	-	-
LBC35MW	63.60 ± 0.09	+	+	0.35 ± 0.04 / 0.45 ± 0.03	+	+	+	+	+	-	14	10	10	-	-
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Keys: + = Positive test for production (enzyme production), - = negative test for production (no enzyme production). + = >1 mm diameter of antibiotic inhibition zone (mm), - = no inhibition zone = resistant to antibiotics. Mean values ± standard errors of the sum of three independent replications (n = 3)

3.3.2 Preliminary identification analysis

3.3.2.1 Morphological characterization

The results displayed on the cultured's strain plates, regarding the morphological characterization of the strains showed that the colony of strain LBC4ME was a light yellow colour, round shape, shiny surface, hard texture and sharp edges. The strain LBC22HL's colony had a milky white colour, bumpy shape, matte surface, slimy texture and smooth edges. The LBC35MW strain had a light brown colour, creamy texture, round shape, smooth flat surface and sharp edges.

The biochemical and physiological characteristics of the strains were tested and presented in Table 3.3. The bacterial strains LBC4ME, LBC22HL and LBC35MW were characterized to be Gram-positive rods, and also produced spores. The results also showed positive tests for catalase and oxidase detection. Nitrate reduction by the strains was demonstrated with the appearance of red colour in the presence of nitrite which indicated a positive reaction, a negative reaction occurred when the solution turned pink to red after the addition of nitrate reagent C.

The strains were tested for their ability to utilize carbon sources using the API®50CH strips, and from Table 3.3, strains LBC4ME, LBC22HL and LBC35MW showed a positive test for the listed test strips: GLY, LARA, RIB, GAL, GLU, FRU, MNE, DUL, MAN, SOR, MDG, ARB, ESC, SAL, CEL, MAL, LAC, SAC, TRE, MLZ, AMD, GLYG, GEN and LYX. All the strains tested negative for tests strips containing ERY, ADO, LXYL, MDX, MDM, SBE, NAG, INU, XLT, TUR, TAG, DFUC, LFUC, 2KG and 5KG. Strains LBC4ME and LBC35MW had negative tests for strips containing DARA, RHA, INO, AMY, DARL, DFUC, LARL and GNT, whereas strain LBC22HL showed positive tests. The strains LBC4ME and LBC22HL showed positive tests MEL, whereas, the strip was negative for strain LBC35MW. Tests strips containing RAF were positive for strains LBC35MW and LBC4ME but negative for strain LBC22HL. From the API®50 CH ID strips, strains LBC4ME, LBC22HL, and LBC35MW were evaluated to be *Bacillus* spp. with percentage range (90-98%), namely, *B. thuringiensis* (90%), *B. velezensis* (98%), and *B. thuringiensis* (97%), respectively, through the APILAB software.

Table 3. 3: Biochemical characterization of strains

Characterization test	Bacterial strains		
	LBC4ME	LBC22HL	LBC35MW
Spore production	+	+	+
Gram reaction	+	+	+
Catalase	+	+	+
Oxidase	+	+	+
Nitrate reduction	+	+	+
Glycerol	+	+	+
Erythritol	-	-	-
D-arabinose	-	+	-
L-arabinose	+	+	+
D-ribose	+	+	+
D-xylose	-	-	-
L-xylose	-	-	-
D-xylose	+	+	+
Methyl-beta-D-xylopyranoside	-	-	-
D-galactose	+	+	+
D-glucose	+	+	+
D-fructose	+	+	+
D-mannose	+	+	+
L-sorbose	-	-	-
L-rhamnose	-	+	-
Dulcitol	+	+	+
Inositol	-	+	-
D-mannitol	+	+	+
D-sorbitol	+	+	+
Methyl-alpha-D-mannopyranoside	-	-	-
Methyl-alpha-D-glucopyranoside	+	+	+
N-acetylglucosamine	-	-	-
Amygdalin	-	+	-
Aburbin	+	+	+
Esculin ferric citrate	+	+	+
Salicin	+	+	+
D-cellobiose	+	+	+
D-maltose	+	+	+
D-lactose (Bovine origin)	+	+	+
Melibiose	+	+	-
D-saccharose (sucrose)	+	+	+
D-trehalose	+	+	+
Inulin	-	-	-
D-Melezitose	+	+	+
D-raffinose	+	-	+
Amidon (starch)	+	+	+
Glycogen	+	+	+
Xylitol	-	-	-
Gentiobiose	+	+	+
D-turanose	-	-	-
D-lyxose	+	+	+
D-tagatose	-	-	-
D-fucose	-	-	-
L-fucose	-	-	-
D-arabitol	-	+	-
L-arabitol	-	+	-
Potassium gluconate	-	+	-
Potassium 2-ketogluconate	-	-	-
Potassium 5-ketogluconate	-	-	-

Keys: + = Positive test, - = Negative test

3.3.2.2 Physiological characteristics and their effect under different growth conditions for maximum antagonistic activity

The results presented on chart (i) from Figure 3.2 showed that all the strains managed to grow and exhibited antifungal activity when grown on a medium adjusted in the pH range 2, 4, 7, and 10. The strains LBC4ME, LBC22HL, and LBC35MW showed optimum growth and antifungal inhibition at pH 7 ($OD_{600\text{ nm}} = 0.547, 0.55, \text{ and } 0.549$: Inhibition 26, 28, 26 mm, respectively) followed by pH 4 ($OD_{600\text{ nm}} = 0.391, 0.457, \text{ and } 0.406$: Inhibition 13, 13, and 21 mm, respectively) and the lowest response was at pH 2 ($OD_{600\text{ nm}} = 0.117, 0.096, \text{ and } 0.117$: Inhibition 3, 2, and 4 mm, respectively). Among the strains, LBC4ME ($OD_{600\text{ nm}} = 0.369$: Inhibition 14 mm) showed the highest growth and antifungal inhibition at pH 10 compared to LBC22HL ($OD_{600\text{ nm}} = 0.278$: 10 mm) which showed lower values.

The results presented on chart (ii) from Figure 3.2 showed that all the strains managed to grow and exhibited antifungal activity in media supplemented with glucose, D-fructose, sucrose, and D-galactose as carbon sources. LBC4ME, LBC22HL and LBC35MW strains showed optimum growth and antifungal inhibition when grown in the medium supplemented with glucose ($OD_{600\text{ nm}} = 0.509, 0.572, \text{ and } 0.504$: Inhibition 19, 21, and 17 mm, respectively) followed by the D-fructose ($OD_{600\text{ nm}} = 0.447, 0.497, \text{ and } 0.386$: Inhibition 13, 15, 12 mm, respectively) medium and the lowest activity was observed from the medium with D-galactose ($OD_{600\text{ nm}} = 0.142, 0.203, \text{ and } 0.113$: Inhibition 5, 7, 4 mm, respectively). Amongst the strains, LBC22HL showed the best growth rate and antifungal activity through all the carbon sources followed by strain LBC4ME, then least growth was strain LBC35MW.

The results presented on chart (iii) from Figure 3.2 showed the results on how bacterial strains LBC4ME, LBC22HL and LBC35MW were able to grow in medium cultures amended with KNO_3 , $(NH_4)_2SO_4$, NH_4NO_3 , and urea as nitrogen sources for growth rate and antifungal inhibition. Results showed that LBC4ME, LBC22HL, and LBC35MW strains per treatment had the highest growth rate and antifungal activity in the presence of $(NH_4)_2SO_4$ ($OD_{600\text{ nm}} = 0.489, 0.475, \text{ and } 0.306$: Inhibition 12, 11, and 9 mm, respectively) followed by urea ($OD_{600\text{ nm}} = 0.336, 0.292, \text{ and } 0.484$: Inhibition 13, 10, and 16 mm, respectively) and lowest in the medium with KNO_3 ($OD_{600\text{ nm}} = 0.308, 0.493, \text{ and } 0.226$: Inhibition 12, 20, and 11 mm, respectively). LBC4ME showed the highest growth among the strains in the medium with $(NH_4)_2SO_4$ followed by LBC22HL and LBC35MW showed the lowest growth rate and antifungal activity for the same medium. LBC35MW showed the highest growth in the medium

with urea followed by LBC4ME and LBC22HL showed the lowest growth in the same medium. LBC22HL ($OD_{600\text{ nm}} = 0.493$) showed the highest growth rate in the medium with NH_4NO_3 compared to strains LBC4ME ($OD_{600\text{ nm}} = 0.256$) and LBC35MW ($OD_{600\text{ nm}} = 0.26$).

The results presented on the chart (iv) from Figure 3.2 showed that all the strains managed to grow and exhibited antifungal activities after growth at temperatures 25 °C, 37 °C, and 45 °C, except at 4 °C. LBC4ME, LBC22HL and LBC35MW strains showed optimum growth and fungal inhibition when incubated at 37 °C ($OD_{600\text{ nm}} = 0.501, 0.532, \text{ and } 0.523$; Inhibition was 24, 26, 27 mm, respectively) followed by growth at 25 °C ($OD_{600\text{ nm}} = 0.391, 0.436, \text{ and } 0.394$; Inhibition 18, 20, and 17 mm, respectively). LBC22HL showed the highest growth rate at 37 °C followed by LBC35MW which exhibited the highest antifungal inhibition zone when incubated at the same temperature. LBC4ME, LBC22HL, and LBC35MW strains showed the lowest growth ($OD_{600\text{ nm}} = 0.098, 0.094, \text{ and } 0.095$, respectively) and antifungal inhibition (1, 0.9, 0.9 mm, respectively) at 45 °C. No growth and inhibition were observed at 4 °C by all the strains.

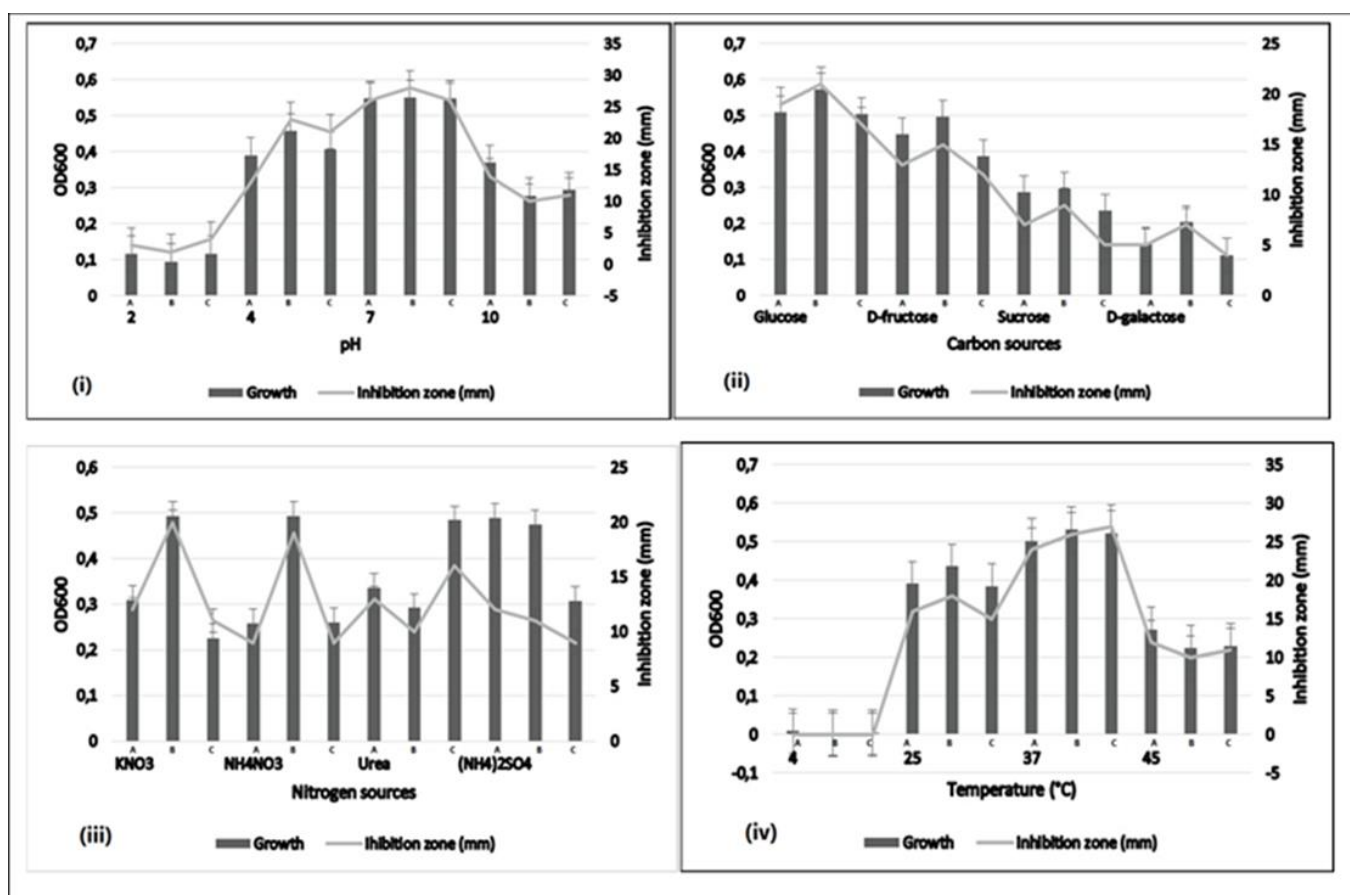


Figure 3. 2: Bar graph showing bacterial strains (letters A = LBC4ME, B = LBC22HL, C = LBC35MW) growth response and antifungal activity against *F. graminearum* FG1 at different temperatures, pH, carbon and nitrogen sources

3.3.3 Molecular identification of bacterial strains

The results presented in Figure 3.3 showed the PCR amplification of the molecular identification genes from the extracted bacterial genomic DNA of the strains. The gel electrophoresis showed PCR amplification of partial gene products for the 16S rDNA (lanes 4-6), rpoB (lanes 1-3), gyrB (lanes 7-9) and tuf (lanes 10-12) gene sequences. Whereby; M = 1 kb plus DNA ladder (molecular weight marker). Numbers representing bacterial strains in different lanes for LBC4ME (lanes 1, 4, 7, 10), LBC22HL (lanes 2, 5, 8, 11) and LBC35MW (lanes 3, 6, 9, 12). Number 13 = Nuclease free water. The applied primers for the 16S rDNA gene (907F/1492R), gyrB gene (gyrB1F/gyrB2R), rpoB gene (rpoB1F/rpoB1R) and tuf gene (tufGPF/tufGPR) yielded the targeted amplicon sizes at 1500 bp, 586 bp, 321bp and 750 bp, respectively.

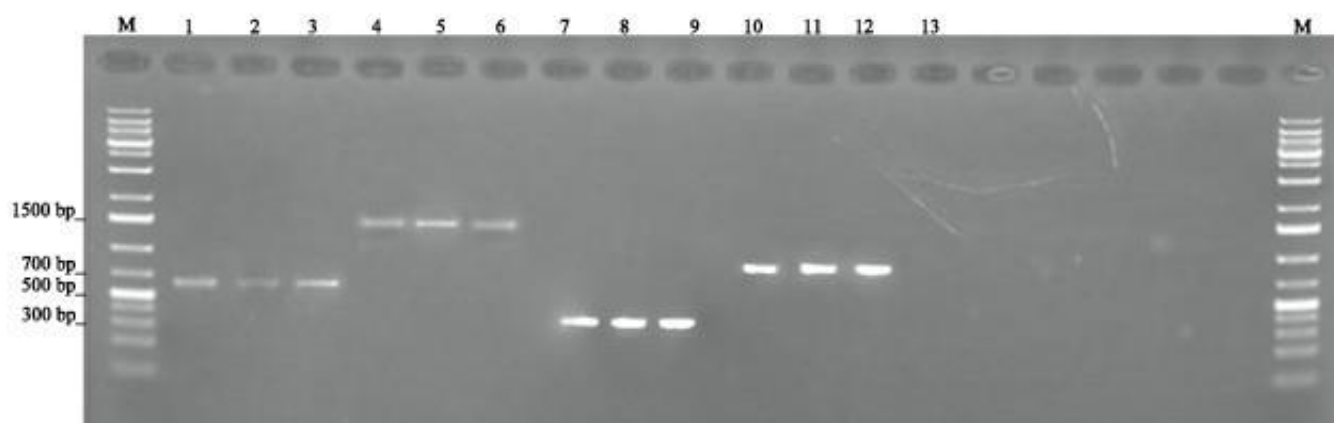


Figure 3. 3: Gel electrophoresis showing PCR amplifications of partial gene products for the 16S rDNA (lanes 4-6), rpoB (lanes 1-3), gyrB (lanes 7-9) and tuf (lanes 10-12) gene sequences. Whereby; M = 1 kb plus DNA ladder. Numbers representing bacterial strains in different lanes for LBC4ME (lanes 1, 4, 7, 10), LBC22HL (lanes 2, 5, 8, 11) and LBC35MW (lanes 3, 6, 9, 12). Number 13 = Nuclease free water

The results presented in Figure 3.4 showed the gel electrophoresis for the detection of a plasmid from each of the strains, the results showed that strain LBC35MW had a single band of plasmid DNA amplified at 20000 bp indicating the presence of a plasmid. The strains LBC4ME and LBC22HL showed to have a plasmid as well but did not show a clear approximation of the amplicon sizes (bp) in bands when using the specified 1 kb plus DNA ladder (molecular weight marker). The arrows are showing the detected presence of a plasmid without bands for the clear amplicon sizes (bp) approximation.

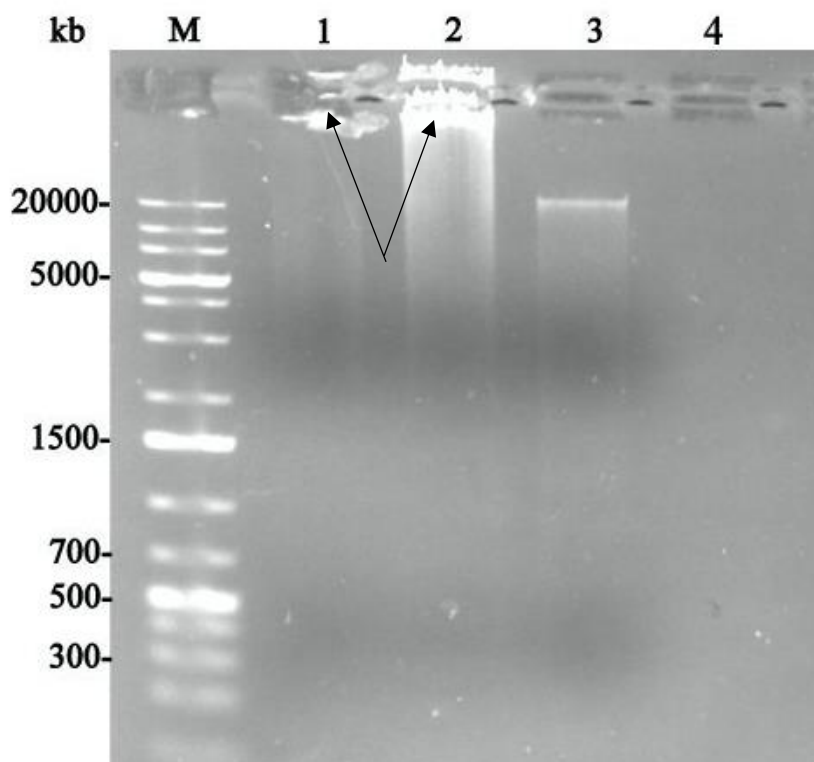


Figure 3. 4: Gel electrophoresis showing the detection of a plasmid from each of the strains, M = 1kb plus DNA ladder, numbers representing strains 1 = LBC4ME, 2 = LBC22HL, 3 = LBC35MW, 4 = Nuclease free water.

From the results presented in Table 3.4, the computational analysis was used as a confirmation tool to further identify the LBC4ME, LBC22HL and LBC35MW strains. After sequencing, the resultant partial nucleotide sequences of the 16S rDNA, *gyrB*, *rpoB* and *tuf* genes of the obtained strains were used for the taxonomic identification of the strains, these partial nucleotide sequences were compared with the nucleotide sequences in the NCBI BLAST tool database and confirmed that the strains' best pairwise alignment with the existing partial 16S rDNA, *gyrB*, *rpoB* and *tuf* gene sequences belonged to the phylum order of Firmicutes, genus *Bacillus* spp., consisting of varying E values and similar homology identities ranging from 90-100%. The results from the Table 3.4 showed that the BLASTN search tool used to analyse the partial 16S rDNA gene showed that the strains LBC4ME had 100% similarity with *B. cereus* (NR074540.1), LBC22HL had 100% similarity with *B. velezensis* (NR075005.2), and LBC35MW had 99% similarity with *B. thuringiensis* (NR112780.1) and they all had an E value = 0. The BLASTX search tool was further used to confirm the partial *gyrB* and *tuf* gene sequences. Results for the *gyrB* gene sequence showed that the strain LBC4ME had 96.49%

similarity with *B. thuringiensis* (PFU78677.1) with E value = $3e-29$, LBC22HL showed 91.18% similarity with *B. velezensis* (WP104842956.1) with E value = $9e-14$, LBC35MW showed 96.10% similarity with *B. thuringiensis* (WP144488915.1) with E value = $1e-40$. Results for the *rpoB* gene sequence showed that strain LBC4ME had 100% similarity with *B. thuringiensis* (AAK74099.1) with E value = $2e-48$, LBC22HL with 100% similarity with *B. velezensis* (QIW94476.1) with E value = $3e-65$, LBC35MW had 97.40% similarity with *B. thuringiensis* (WP088345021.1) with E value = $1e-40$. Results for the *tuf* gene sequence showed that the strain LBC4ME had 100% similarity with *B. thuringiensis* (AGH06016.1) with E value = $1e-152$, also LBC22HL had 100% similarity with *B. velezensis* (WP031379374.1) with E value = $3e-152$, LBC35MW had 99.61% similarity with *B. thuringiensis* (AGH06026.1) with E value = $7e-165$. The accession numbers given to bacterial strains obtained from this study were listed in the third column in Table 3.4 below together with the accession numbers of closely related strains on the fifth column. Strains LBC4ME and LBC35MW for the protein-coding *rpoB* gene sequence did not receive accession numbers due to unresolved errors reported when depositing gene sequences in GeneBank, the feedback reported that these nucleotide sequences might have problems with feature annotations or presence of internal stop codons.

Table 3. 4: NCBI BLAST search results for the identification of bacterial strains

Genes	Strains	Accession number	BLAST ID	Accession number	Per ID (%)	E-value
16S rDNA	LBC4ME	MG686564.1	<i>B. cereus</i>	NR074540.1	100.00	0.00
	LBC22HL	MG696227.1	<i>B. velezensis</i>	NR075005.2	100.00	0.00
	LBC35MW	MG686565.1	<i>B. thuringiensis</i>	NR112780.1	99.00	0.00
gyrB	LBC4ME	MN231255.1	<i>B. thuringiensis</i>	PFU78677.1	96.49	3e-29
	LBC22HL	MN231256.1	<i>B. velezensis</i>	WP104842956.1	91.18	9e-14
	LBC35MW	MN231257.1	<i>B. thuringiensis</i>	WP144488915.1	96.10	1e-40
tuf	LBC4ME	MN231258.1	<i>B. thuringiensis</i>	AGH06016.1	100.00	1e-152
	LBC22HL	MW161171.1	<i>B. velezensis</i>	WP031379374.1	100.00	3e-152
	LBC35MW	MN231259.1	<i>B. thuringiensis</i>	AGH06026.1	99.61	7e-165
rpoB	LBC4ME	NA	<i>B. thuringiensis</i>	AAK74099.1	100.00	2e-48
	LBC22HL	MN368611.1	<i>B. velezensis</i>	QIW94476.1	100.00	3e-65
	LBC35MW	NA	<i>B. thuringiensis</i>	WP088345021.1	97.40	1e-40

Keys: NA = did not receive accession numbers (does not appear in the NCBI database)

3.3.3.1 Phylogenetic tree analysis

The results from Figure 3.5 showed that from the 16S rDNA gene sequences analysis, the branch values less than 50% were hidden and the sum of each branch length = 0.77655553 was shown. The related taxa grouped in the bootstrap test with 1000 replicates had the replicate tree percentages shown next to the branches (Felsenstein, 1985). The branch lengths were in the same units as the evolutionary distances applied to deduce the phylogenetic tree drawn to scale. The maximum composite likelihood method was used to compute the evolutionary distances in unit numbers of the base substitutions per site (Tamura et al., 2004). Thirteen (13) nucleotide sequences were included in the analysis, the missing data and gaps contained in all the positions were deleted. The final data set contained a total of 652 positions.

As displayed from Figure 3.5, the neighbour-joining method showed that all the strains obtained from this study belonged to the genus *Bacillus* species. From the 16S rDNA gene sequence, the NCBI database reference strain *Bacillus proteolyticus* (MK418825.1) as an out-group also strains LBC4ME and LBC22HL did not cluster with any reference strains and showed to have a low (below 50) bootstrap value, also these strains showed close relatedness to each other and were an indication of distant clades to the other aligned *Bacillus* species as their closest relative. Strain LBC35MW showed high sequence similarity values of 100% with *B. cereus*, *B. thuringiensis*, *B. albus* and *B. luti* as their closest related strains. Overall, the

phylogenetic analysis revealed that all the LBC4ME, LBC22HL and LBC35MW strains have a distinct clade and their low sequence similarity values indicated that they belong to the *Bacillus* species and have a sequence similarity of 100% for the first group with *B. velezensis*, *B. subtilis*, *B. vallismortis*, *B. amyloliquefaciens*, *B. methylotrophicus* and *B. siamensis* as their closest related strains.

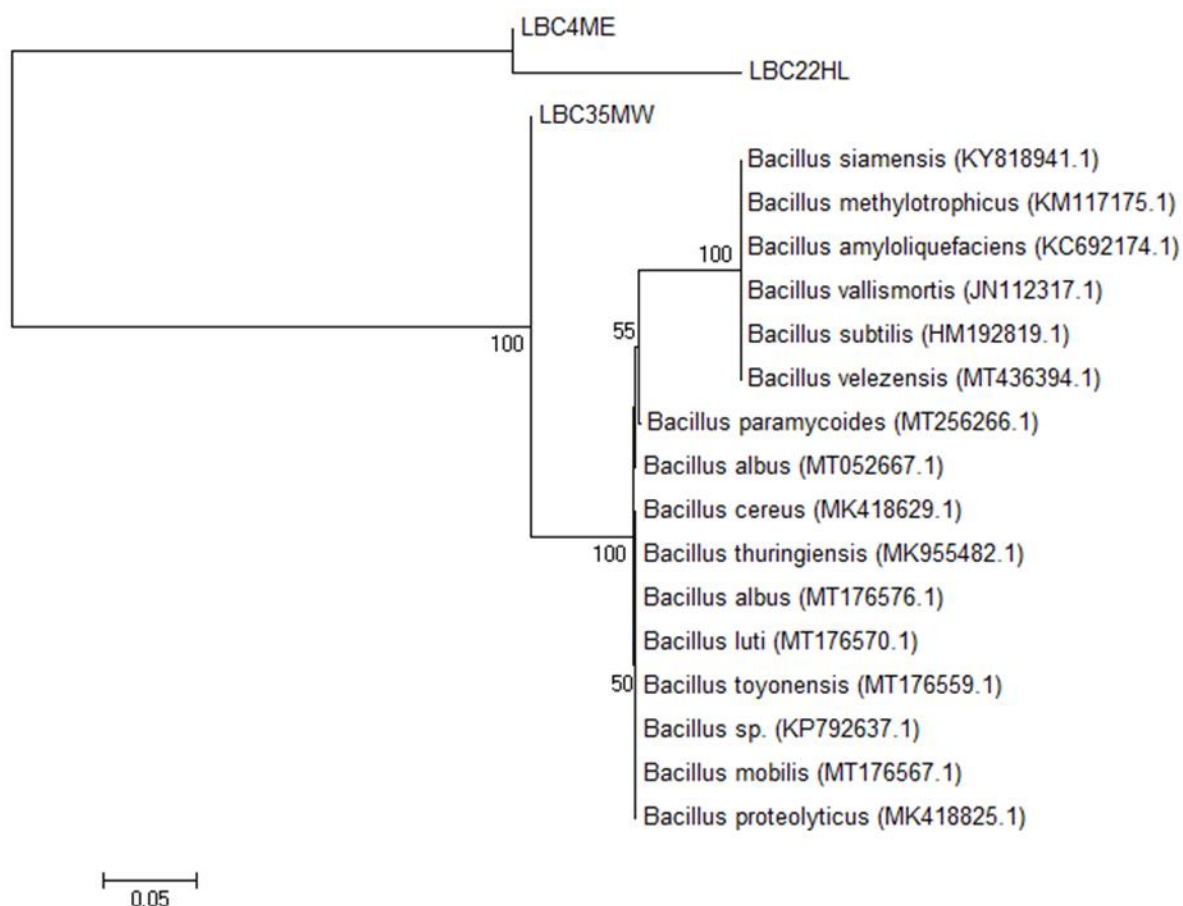


Figure 3. 5: Evolutionary relationships of taxa based on the neighbour-joining phylogenetic tree analysis based on the 16S rDNA gene sequence. 0.05 substitutions per nucleotide position are indicated by the scale bar

The results presented in Figures 3.6-3.8 showed the Neighbour-joining method that was used to conclude the evolutionary history of the *gyrB*, *rpoB* and *tuf* gene sequences of the LBC4ME, LBC22HL and LBC35MW strains obtained from this study. The tree at optimum from each analysis showed a branch length sum = 4.08566094, 144.58000930 and 3.55227287, respectively. The numbers at the branches show the replicate trees' percentage for the related taxa clustered together in the bootstrap test based on 1000 replications. The branch lengths are in the same units as the evolutionary distances applied to conclude the phylogenetic tree and

drawn to scale. In the same units as the number of base substitutions per site, the maximum composite likelihood method was used to compute the evolutionary distances. Respectively, 14, 8 and 13 amino acid sequences were evaluated in this phylogenetic analysis. There was overall 88, 186 and 79, respectively, positions from the finishing data set and all the missing data and gaps were deleted from each of the positions.

Displayed in Figure 3.6, the neighbour-joining method revealed that strains LBC4ME, LBC22HL and LBC35MW did not cluster with any other reference strains and had sequence similarity of 100% with the group of *Bacillus* species, having *B. thuringiensis* and *B. velezensis* as their closest relatives with *Bacillus* sp. (WP094032016.1) as the out-group. The strains LBC4ME and LBC22HL had a low bootstrap value (59) which indicated distinct clades between these strains. The strain LBC35MW showed to have a sequence similarity of 99% with the strains LBC4ME and LBC22HL as close relatives.

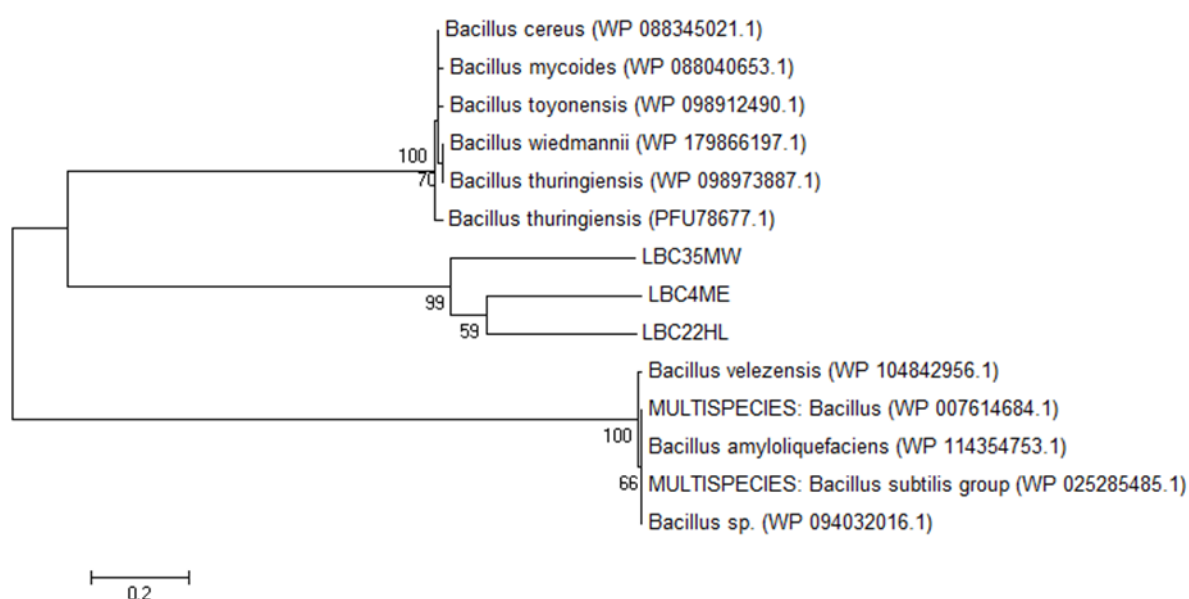


Figure 3. 6: Evolutionary relationships of taxa based on the Neighbour-joining phylogenetic analysis based on the *gyrB* gene sequence. The scale bar of 0.2 is showing the substitutions per nucleotide position

Displayed in Figure 3.7, the evaluation of the *rpoB* gene sequence using the neighbour-joining method displayed that strains LBC4ME, LBC22HL and LBC35MW showed close similarity to the genus *Bacillus* sp. reference strain as a close relative, with *B. thuringiensis* (AF205348.1) as an out-group. The strains LBC4ME and LBC35MW had very low bootstrap values (below 50), indicating reserved clades less than 50% with *Bacillus* sp. as their closest relative. The

bacterial strain LBC2HL clustered equally with the reference strains *Bacillus* sp. (AF205352.1, JF692783.1), *B. cereus* (AF205337.1) and *B. thuringiensis* (AF205347, AF20548.1) showing a high similarity index of 97%.

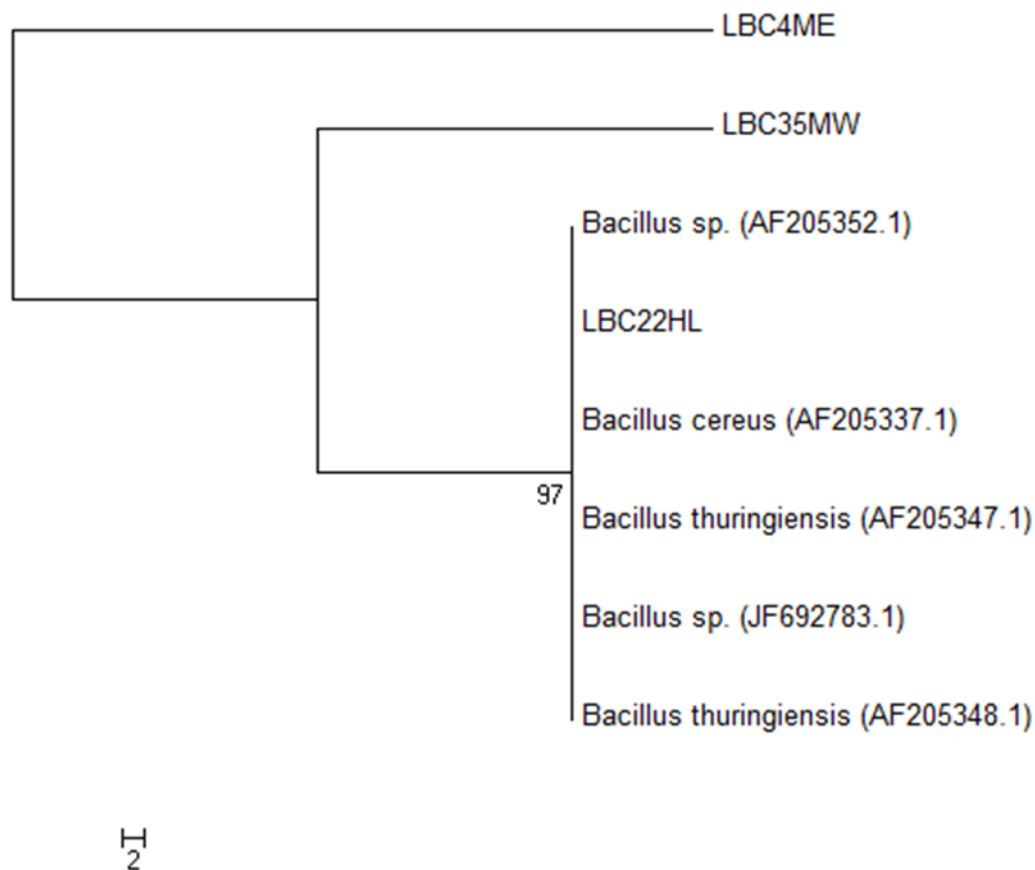


Figure 3. 7: Evolutionary relationships of taxa based on the Neighbour-joining phylogenetic analysis based on the *rpoB* gene sequence. The scale bar is showing the substitutions per nucleotide which are positioned at 2

Displayed in Figure 3.8, the analysis of the *tuf* gene sequence using the neighbour-joining method placed the reference strain *Bacillus toyonensis* (WP098912490.1) as an outgroup, the strains LBC4ME, LBC22HL and LBC35MW did not cluster with any reference strains from NCBI. However, they had an equally close similarity of 100% with the NCBI database reference strains *B. velezensis*, *Bacillus* sp. and *B. amyloliquefaciens* as their closest relatives. Also, the strains obtained from this study had a bootstrap value of 100 indicating that they have close clades with each other (close relatives).

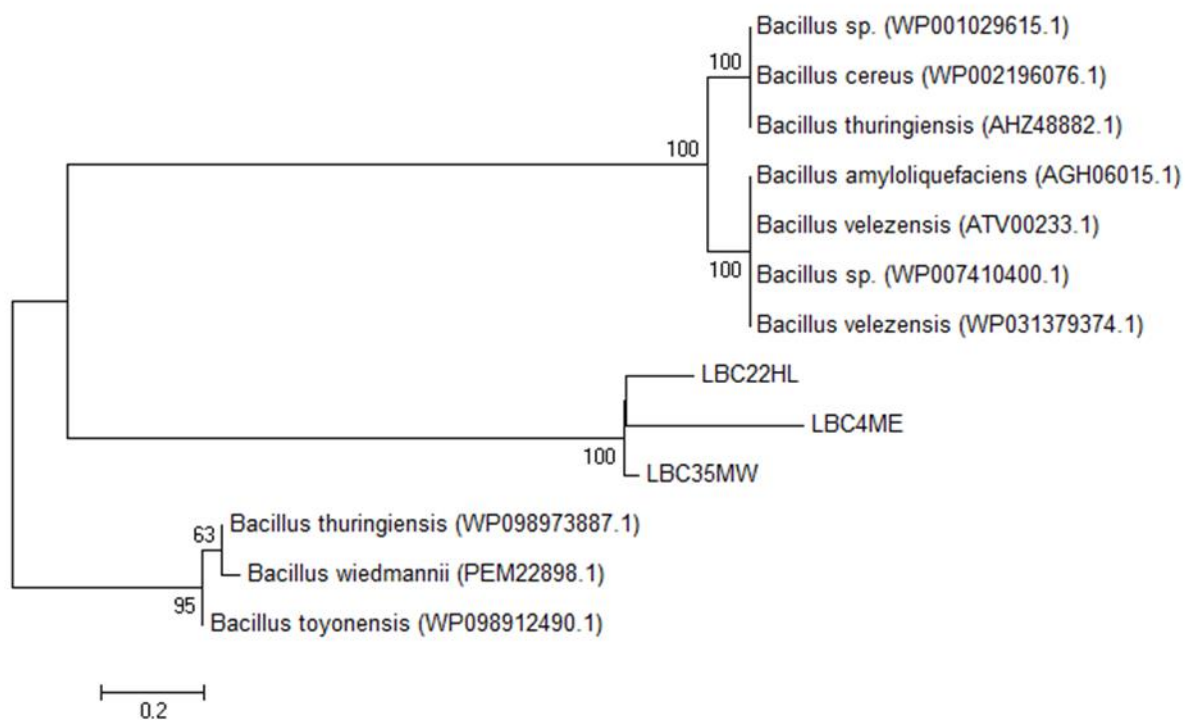


Figure 3. 8: Evolutionary relationships of taxa based on the Neighbour-joining phylogenetic analysis based on the *tuf* gene sequence. The scale bar with 0.2 represents substitutions per nucleotide position

3.3.4 Screen-house assessment (*in vivo* biocontrol)

The results for the synergy activity between the bacterial strains showed that all the strains were able to grow in the presence of each other without any inhibition zones. Displayed in Table 3.5, the results were measured and recorded at harvest time (90 days). The findings showed that the pot experiment on maize inoculated with treatment LBC4ME+LBC22HL+LBC35MW+P showed the highest ($P<0.05$) percentage for plant and root disease reduction (RDR) with a mean value of 50% and 54.50% reduction capacity, respectively, compared to other treatments. Maize inoculated with LBC4ME + P treatment showed the least ($P<0.05$) percentage (25%). Maize inoculated with the pathogen only (PO) did not show any disease reduction. Maize inoculated with LBC4ME+LBC22HL+LBC35MW+P treatment produced maize which had the highest ($P<0.05$) shoot length (191.20 cm), shoot fresh weight (189.60 g), shoot dry weight (22.33 g), root length (82.50 cm), root fresh weight (23.50 g), and root dry weight (12.40 g) which was significantly different ($P<0.05$) from other treatments, whereas maize inoculated with LBC4ME + P treatment had the lowest ($P<0.05$) yield of the agronomy parameters. Maize inoculated with LBC4ME + LBC22HL + P and LBC22HL + LBC35MW + P treatments showed similar ($P>0.05$) shoot length values. Maize inoculated with PO had the lowest ($P<0.05$) shoot length (133.43 cm), shoot fresh weight (69.37 g), shoot dry weight (8.00 g), root length (38.90 cm), root fresh weight (10.80 g), and root dry weight (3.60 g) which was significantly lower ($P<0.05$) from other treatments. The maize inoculated with the pathogen only as treatment was negatively impacted compared to the other treatments. There was no significant difference ($P>0.05$) between root length of maize inoculated with LBC4ME + LBC22HL + P and LBC22HL + LBC35MW + P treatments but the maize inoculated with treatment LBC4ME + LBC22HL + LBC35MW + P produced root lengths that were significantly different ($P<0.05$) compared to the other pot treatments.

Table 3.5: Effect of bacterial strains and *F. graminearum* inoculation on maize from the screen-house pot experiment

Treatments	SL (cm)	SFW (g)	SDW (g)	RL (cm)	RFW (g)	RDW (g)	MDR	DR (%)	RMDR	RDR (%)
PO	133.43 ^g	69.37 ^e	8.00 ^e	38.90 ^f	10.80 ^g	3.60 ^f	3.67	0.00	4.00	0.00
Control	161.50 ^f	111.20 ^d	15.53 ^d	61.90 ^e	16.20 ^f	8.07 ^e	0.00	0.00	0.00	0.00
LBC4ME + P	165.40 ^e	139.43 ^c	15.60 ^d	63.73 ^{de}	18.13 ^e	8.47 ^{de}	2.67	27.25	3.00	25.00
LBC35MW + P	165.83 ^e	139.60 ^c	16.37 ^d	64.27 ^d	18.33 ^e	8.83 ^d	2.33	36.51	2.67	33.25
LBC22HL + P	175.80 ^d	147.90 ^c	17.60 ^c	69.47 ^c	19.47 ^d	10.07 ^c	2.18	40.60	2.51	37.25
LBC4ME + LBC35MW + P	179.63 ^c	162.43 ^b	19.30 ^b	71.43 ^c	19.47 ^d	10.07 ^c	2.00	45.50	2.33	41.75
LBC4ME + LBC22HL + P	185.50 ^b	162.77 ^b	19.37 ^b	74.43 ^b	20.40 ^c	10.50 ^c	2.00	45.50	2.33	41.75
LBC22HL + LBC35MW + P	187.40 ^b	164.17 ^b	19.58 ^b	74.80 ^b	21.67 ^b	11.20 ^b	1.85	49.59	2.18	45.50
LBC4ME + LBC22HL + LBC35MW + P	191.20 ^a	189.60 ^a	22.33 ^a	82.50 ^a	23.50 ^a	12.40 ^a	1.67	54.50	2.00	50.00

Keys: PO = Pathogen only, P = Pathogen, SL = Shoot length, SFW = Shoot fresh weight, SDW = Shoot dry weight, RL = Root length, RFW = Root fresh weight, RDW = Root dry weight, MDR = Mean disease rating, DR = Disease reduction, RMDR = Root mean disease rating, RDR = Root disease reduction

3.3.4.1 Plant treatment effectivity/Disease severity observations

The numbers 1-9 from the Figures 3.9-3.12 represented the applied pot treatments, please refer to section 3.2.7 stating the specific treatment mixtures. The results from Figure 3.9 showed the effectivity of the applied pot treatments on the maize tassels. The tassel collected from the pots treated with treatment 1 represents the treatment with pathogen only; the tassels appeared to be dry, thin, wilted and had no sprouts compared to the tassels collected from treatments 2-9; tassels from pot treatments 3, 4 and 5 looked moderately dry and wilted compared to the tassel collected from the pot treatment 1 and also appeared thicker. Tassels collected from the pot treatments 6-9 appeared less dry, thicker and greener compared to the tassels collected from the other pot treatments.

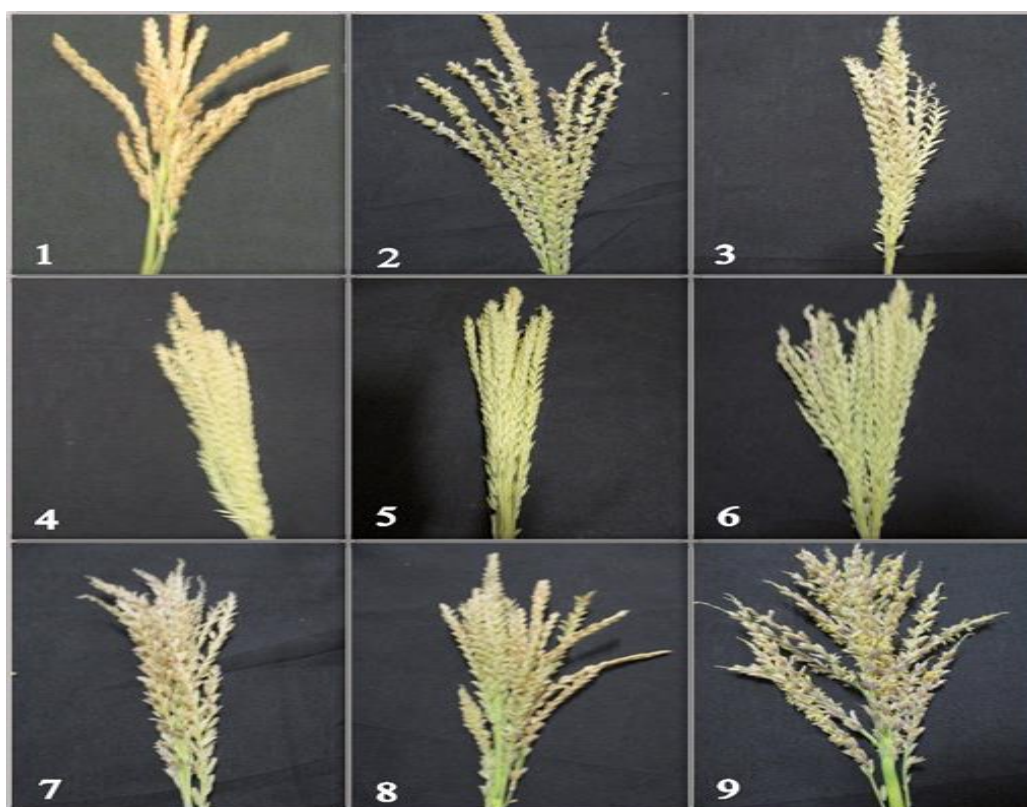


Figure 3. 9: Effect of treatments (1-9) on maize plant tassel at harvest (at 90 days harvest). Number 1 = PO, 2 = Control, 3 = LBC4ME + P, 4 = LBC35MW + P, 5 = LBC22HL + P, 6 = LBC4ME + LBC35MW + P, 7 = LBC4ME + LBC22HL + P, 8 = LBC22HL + LBC35MW + P, 9 = LBC4ME + LBC22HL + LBC35MW + P

The results from Figure 3.10 showed that the maize stalks collected from pots applied with pot treatment 1 only had brownish-purple lesions, black spots and rotting. Maize stalks collected from pots applied with pot treatment 3, 4, 5 and 6 had brownish-purple lesions, black spots and

rotting that were fewer in number compared to the stalks collected from pots with treatment 1. Maize stalks collected from the control treatment 2 did not show any colour change, they were green and did not have any lesions, black spots or rotting. Stalks collected from pots with treatment 7, 8 and 9 appeared purple and had the least number of lesions and lesser rotting, no black spots were observed from these pot treatments.



Figure 3. 10: Effect of treatments (1-9) on maize stalk (at 90 days harvest). Number 1 = PO, 2 = Control, 3 = LBC4ME + P, 4 = LBC35MW + P, 5 = LBC22HL + P, 6 = LBC4ME + LBC35MW + P, 7 = LBC4ME + LBC22HL + P, 8 = LBC22HL + LBC35MW + P, 9 = LBC4ME + LBC22HL + LBC35MW + P

The results from Figure 3.11 showed the effectivity of treatments (1-9) on the studied maize leaves, those collected from pots with treatment 1 appeared to have different sizes of lesions, leaves decolourization and wilting which showed to have a zero % DR compared to pot treatments (3-9) which showed to have a DR of about 27.25-54.50%; the maize leaves collected from the control pot treatment 2 appeared to be free from spots and lesions and did not have any colour change (remained green); maize leaves collected from pots applied with treatments 3, 4, 5, 6, 7, 8 with a DR of about 27.25-49.59%, respectively, and 9 which showed a DR of about 54.50%, appeared to have a moderation of leaf decolourization, wilting and the presence of lesions different in sizes compared to maize leaves applied with pot treatment 1.

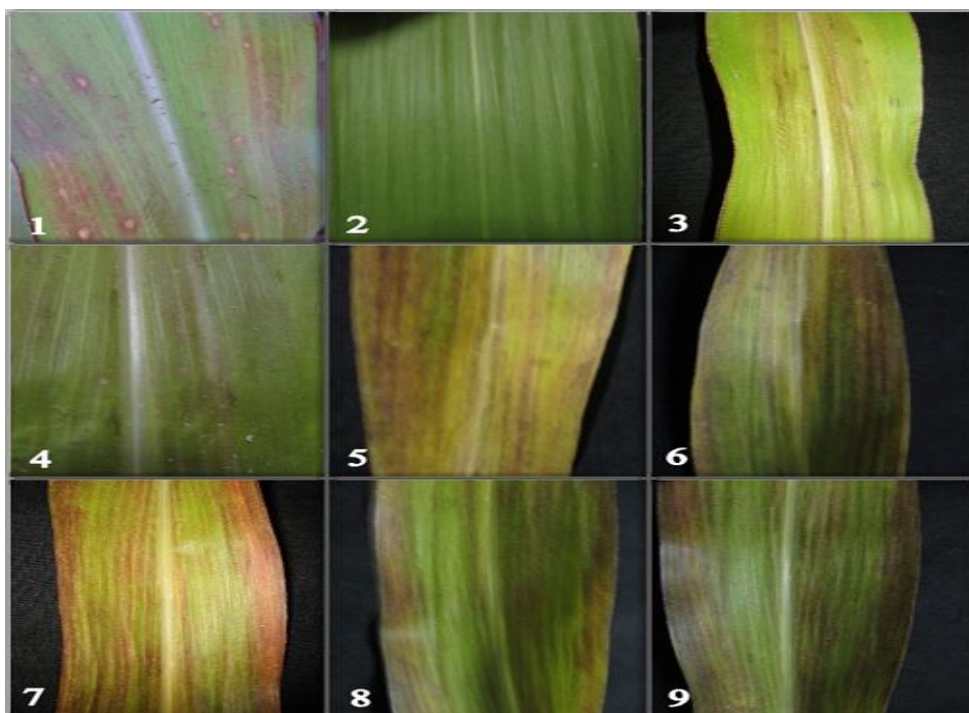


Figure 3. 11: Effect of treatments (1-9) on maize leaves (at 90 days harvest). Number 1 = PO, 2 = Control, 3 = LBC4ME + P, 4 = LBC35MW + P, 5 = LBC22HL + P, 6 = LBC4ME + LBC35MW + P, 7 = LBC4ME + LBC22HL + P, 8 = LBC22HL + LBC35MW + P, 9 = LBC4ME + LBC22HL + LBC35MW + P

The results from Figure 3.12 showed the response of pot treatments (1-9) on maize roots, maize roots collected from pots treated with treatment 1 showed to have the smallest root length indicating a zero % RDR, compared to the roots collected from pot treatments (3-9) which showed to have about 25-50% RDR. The longest maize roots were observed from maize collected from pots with treatment 9 which showed about 50% RDR, followed by those from pot treatment 4 (37.25% RDR) to 8 (45.50% RDR); maize roots collected from pots applied with treatments 2, 3, 5 and 7 did not show a greater difference in length.



Figure 3. 12: Results of treatments (1-9) on maize roots (at 90 days harvest). Number 1 = PO, 2 = Control, 3 = LBC4ME + P, 4 = LBC35MW + P, 5 = LBC22HL + P, 6 = LBC4ME + LBC35MW + P, 7 = LBC4ME + LBC22HL + P, 8 = LBC22HL + LBC35MW + P, 9 = LBC4ME + LBC22HL + LBC35MW + P

3.3.4.2 Rhizosphere colonization

The results presented in Table 3.3 was referred to for the findings regarding bacterial root colonization because this was only done to detect the presence of the inoculated strains and due to similarities and identity in the conducted API®50CH strip tests, the results were not shown. The results showed that all the studied strains were found to be present in the root adhering soil from the treatment pots containing antagonistic strains (LBC4ME + P, LBC35MW + P, LBC22HL + P, LBC4ME + LBC35MW + P, LBC4ME + LBC22HL + P, LBC22HL + LBC35MW + P, LBC4ME + LBC22HL + LBC35MW + P) and this confirmed the ability of the studied strains to colonize the maize root, therefore, there were possibilities the strains could have influenced disease control.

3.4 Discussion

3.4.1 Selection of indigenous biocontrol PGPR

Isolation of 46 bacterial strains was successfully conducted to investigate and evaluate potential antagonistic activity against the fungal pathogen *F. graminearum* FG1 strain. Amongst them, three strains (LBC4ME, LBC22HL and LBC35MW) showed great antagonistic activity by being able to effectively reduce the growth of *F. graminearum* FG1 through the antifungal assay test results with impressive fungal inhibition rates which were above 50% as shown from

Table 3.2. The strain LBC22HL (66.37%) showed the highest antifungal activity followed by LBC4ME (64.33%) and LBC35MW (63.60%) compared to the rest of the isolated strains which had an inhibition rate below 50%, while the control plates with the pathogen only did not show any antifungal activity. Previous studies by Yamamoto et al. (2015) have also reported several *Bacillus* spp. that inhibited *F. graminearum* with an above 50% inhibition effectivity and even higher (up to 95%).

A study by Palazzini et al. (2016) also reported similar results showing *Bacillus* spp. to be efficiently powerful at reducing fungal mycelial growth under *in vitro* antifungal assay tests. Antifungal activity was successfully achieved by the bacterial strains LBC4ME, LBC22HL, and LBC35MW and this was demonstrated through the appearance of zones of inhibition on the *in vitro* antifungal antagonistic tests and inhibition rates in percentage as shown in Table 3.2. A study by Fan et al. (2015) reported that a high percentage of antagonistic bacteria against some soil-borne *Fusarium* spp. was found to be present from the isolation of healthy soil (48.1%) compared to uncultivated (38.6%) and diseased (18.2%) soil. Their study showed that collecting PGPR from the rhizosphere soil of a healthy plant is key to obtaining potent indigenous strains compared to other soil areas. Hence, the current study also isolated bacterial samples from healthy maize's rhizosphere. Santiago et al. (2015) stated that the selection of a good BCAs depends on the host plant of interest, implying that BCAs found in a particular preferred host plant's environment are going to perform better under similar conditions compared to a foreign host plant's environment due to the new living conditions.

3.4.2 Biochemical characterization of bacterial strains

The bacterial strains LBC4ME, LBC22HL and LBC35MW obtained from this study were characterized to be Gram-positive rods, that produced spores. Studies by Hyakumachi et al. (2013) reported that the genus *Bacillus* spp. can be applied effectively as stable BCAs due to its production of spores which can withstand very harsh environmental conditions and have a long lifespan. However, for these spores to be in a metabolically active stage safe for effective application, they require some time after their application to activate their vegetative cell (Santiago et al., 2015).

The results presented in Table 3.3 showed that strains LBC4ME, LBC22HL and LBC35MW had positive tests for catalase, oxidase and nitrate reduction. Bacterial strains that have been previously reported to test positive regarding the above mentioned tests have been previously

reported to attain some indirect PGP traits known to influence plant growth (Patel & Desai, 2015).

The Table 3.3 presented the results for the API®50CH ID strips and based on the physiological activities, identification of the strains LBC4ME, LBC22HL and LBC35MW were deemed to be *Bacillus* spp. with similarity percentage ranges (90-98%), namely, *B. thuringiensis* (90%), *B. velezensis* (98%), and *B. thuringiensis* (97%), respectively, through the APILAB software. These results are in agreement with Agrawal et al. (2015) who also successfully used the API®50CH system as an identification tool for PGPR species.

3.4.3 Biocontrol PGP traits

The results presented in Table 3.2 showed that in terms of PGP traits, all the 3 strains (LBC4ME, LBC22HL and LBC35MW) gave positive results on the tests for antimicrobial metabolite productions. Particularly, IAA production was observed from these strains showing values ranging between 0.35-0.39 µg/mL and 0.44-0.47 µg/mL with different application levels of L-tryptophan 100 and 200 µg/mL, respectively. The production of the IAA hormone by PGPR is considered an essential trait to boost plant nutrient absorption by assisting with the construction of the root structure system (Gull, 2016).

The strains from this study successfully produced IAA with different concentrations of L-tryptophan applications, and therefore, our study agrees with that of Bailly et al. (2014) who also reported that the majority of their studied *Bacillus* spp. strains were able to produce IAA in the presence of different L-tryptophan concentrations. Results from this study showed that there is a difference in the amount of IAA produced by each strain and this could be due to the variation in IAA production ability by different strains reported to be based on two genetic factors which are the location of the genes responsible for the regulation of IAA and the multiple pathways found to regulate IAA in one strain which may differ to the next strain in the same genus-group (Passari et al., 2016).

The current study exhibited the ability of selective *Bacillus* spp. strains LBC4ME, LBC22HL and LBC35MW presented in Table 3.2 to possess hydrolytic enzyme production of proteases and cellulases. Agbodjato et al. (2016) stated that the production of these enzymes by PGPR contributes to plant growth, thus, assists with the suppression of soil-borne pathogens. The fermentation of sugars through depolymerization of cellulose relies on the functioning of cellulase enzyme, and this enzyme is also an active catalyst in PGPR during inoculation to the

host plant and enables survival through the plant's defence system, additionally, the production of hydrolytic enzymes assists the rhizobacteria to penetrate cell walls and compete for nutrients (Ahmad et al., 2013).

The current study showed the ability of *Bacillus* spp. strains LBC4ME, LBC22HL and LBC35MW presented in Table 3.2 to have phosphate solubilization ability, nitrogen fixation, production of HCN and siderophores, which are an effective trait for plant growth and microbial disease control. According to Tokpah et al. (2016), the production of these traits reflects an effective competitive behaviour (biological control and bio-fertilization) by PGPR when present in the rhizosphere of a preferred plant host, consequently, suggesting these strains may be potential BCA inoculants against *F. graminearum* on maize as the desired host (Bakthavatchalu et al., 2012). Studies by Patel and Desai (2015) and Ahmad et al. (2013) have reported PGPR with similar production of multi PGP traits, and further added that the above-mentioned traits play a positive role aiding in both direct and indirect mechanisms.

The strains LBC4ME, LBC22HL and LBC35MW presented in Table 3.2 showed positive results on tests for phosphate solubilization. Enabling phosphorus absorption through phosphate solubilization by PGPR is reported to be beneficial in sustainable agricultural systems to assist with plant growth promotion and crop production. Several bacterial strains are known to avail insoluble soil phosphorus to their host plant through the solubilization of mineral phosphates from produced phosphatases or organic acids (Liu et al., 2014). Zhang et al. (2016) have previously reported that *F. graminearum* utilizes phosphorus-free membrane lipids that strived in a phosphorus-deficient environment, thus, retain pathogenicity against infected host maize, therefore, from (Table 3.2) suggesting PGPR strains with phosphate solubilization activities may assist with phosphorus absorption by the plant, consequently, inhibiting *F. graminearum* proliferation.

The strains LBC4ME, LBC22HL and LBC35MW presented in Table 3.2 showed positive HCN production. This is similar to the findings of Ghodsalavi et al. (2013) who also reported a majority of *Bacillus* spp. strains with HCN production. The HCN production trait is reported to act in disease control through the cyanide's role to repel competition and predation as a universal metabolic inhibitor (Farag et al., 2013). Passari et al. (2016) also reported a *B. thuringiensis* strain that was able to produce multiple volatile compounds with fungal growth inhibition activities.

A positive test for nitrogen fixation was shown in (Table 3.2). Inoculation with PGPR which can fix nitrogen and produce secondary metabolites are reported to not cause any harm to plants or humans when applied with restrictive recommended dosages to avoid toxicity and therefore, may function as effective safe BCAs whilst fixing nitrogen on the inoculated environment (Owen et al., 2015). The results from this study showed production of NH_3 from the studied bacterial strains. This trait is reported to aid in plant biomass by the accumulation and supply of nitrogen for the plant, thus, supporting root and shoot elongation (Oberson et al., 2013).

The strains LBC4ME, LBC22HL and LBC35MW presented in Table 3.2 also showed positive results for the production of siderophores, indicating that Fe^{3+} was fixed from the Chrome Azurol S dye complex. Siderophore production is regarded to be the most common functional activity for most *Bacillus* spp. strains to utilize as an antagonist against pathogens on the host plant (Laslo et al., 2012; Patel et al., 2015). Kaki et al. (2013) have reported that *Bacillus* spp. assist with plant growth by depriving pathogens of Fe through chelation by the production of siderophores and they assist through stimulation of plant growth by Fe competition.

Zhou et al. (2016) stated that it is possible to have one strain retain a broad spectrum of PGP traits including the production of lytic enzymes, NH_3 , IAA, phosphate solubilization and more which are associated with fungal antagonism when researching most *Bacillus* species. Thus, the activity of the above mentioned secondary antimicrobial metabolites (HCN, NH_3 , IAA, phosphate solubilization, and siderophores) from *Bacillus* spp. as shown from Table 3.2, have also been previously presented to facilitate heavy metal absorption by the roots, thus, increase the bioavailability of nutrients and enhance plant stress tolerance (Lakshmanan, 2015).

The results presented in Figure 3.2 showed the effect of different growth conditions for maximum antagonistic activity against *F. graminearum*. The results affirmed that bacterial strains LBC4ME, LBC22HL and LBC35MW had optimum growth and antifungal inhibition zones at pH 7, temperature 37 °C, carbon source as glucose and $(\text{NH}_4)_2\text{SO}_4$ as a nitrogen source. These findings are similar to the results reported by Singh and Cameotra (2013) who highlighted the necessity to determine the optimum growth conditions for important microorganisms attaining various productions of bioactive metabolites and antimicrobial activities.

The determination of the ideal growing conditions are recommended to be a starting point to obtain potent properties at an increased rate, therefore, to determine the optimum growth plus inhibition ability, from this study, under the different pH, temperature, carbon and nitrogen

source conditions were assessed and presented in Figure 3.2. The *Bacillus* spp. is known to be a chemoheterotrophic bacteria able to utilize many nutritional substrates, therefore, the aforementioned tests clarified which growth conditions are best suited for the studied strains. This suggested that poor metabolic activities and a reduced disease inhibition rate may result if the conditions are altered (Zhou et al., 2016).

The results from Table 3.2 in terms of antibiotic sensitivity displayed that, strain LBC4ME was sensitive to VA (10 mm), the highest sensitivity to E (26 mm), and lower sensitivity to AMP (2 mm) whereas LBC22HL and LBC35MW strains showed resistance to these antibiotics. Strains LBC4ME, LBC22HL, and LBC35MW showed sensitivity to K (12, 18, 10 mm, respectively) and TET (10, 22, 10 mm, respectively). Strains LBC4ME and LBC35MW showed sensitivity to C (both at 14 mm) whereas LBC22HL showed resistance to the antibiotic. Previous studies by Mnif et al. (2016) have reported the use of bacterial strains which are resistant to the above-mentioned antibiotics as microorganisms with multi-drug resistant profiles. Zhou et al. (2016) have also used multi-drug resistant bacterial strains in root colonization studies to exclude susceptible strains from the experiment. Therefore, bacterial strains LBC4ME, LBC2HL and LBC35MW as presented in Table 3.2 affirmed that they may be applied as either resistant or susceptible control strains under specified desired experimental conditions (e.g. LBC4ME strain may be used as a control bacterial strain susceptible to E or vice versa with the other antibiotics).

3.4.4 Molecular characterization of bacterial strains

Displayed in Figures 3.5-3.8, results for the 16S rDNA gene and MLSA for the molecular classification of the strains obtained from this study showed that they belonged to the taxonomic group of the phylum Firmicutes, genus *Bacillus* spp., from Figures 3.7 and 3.8, when using the partial *tuf* and *rpoB* gene sequencing, the strains LBC4ME and LBC22HL showed 100% similarity with *B. thuringiensis* and *B. velezensis*, respectively, with existing strains found in NCBI BLAST database, whereas, strain LBC35MW showed 99% similarity with *B. thuringiensis*; from Figure 3.6, when using the partial *gyrB* gene sequencing, the strains LBC4ME, LBC22HL and LBC35MW showed a close similarity of 96.49% (*B. thuringiensis*), 91.18% (*B. velezensis*) and 96.10% (*B. thuringiensis*), respectively. The above-mentioned strains did not show very high similarity compared to the existing gene sequences, according to Deepak and Jayapradha (2015) this may indicate that these strains are conceivably novel,

when using 16S rDNA gene sequencing, strains LBC4ME, LBC22HL and LBC35MW showed a close similarity of 100% with *B. cereus*, *B. velezensis* and *B. thuringiensis*, respectively.

Palazzini et al. (2016) stated that there are inconsistencies regarding the taxonomy of the genus *Bacillus* spp. strains' characterization, therefore, it is necessary to prioritize this area for proper strain identification. It also emphasised that some genus *Bacillus* spp. are considered to be conspecific, namely, *B. velezensis*, *B. thuringiensis*, *B. cereus*, and therefore, their discrimination may be confusing (Rosselló-Móra & Amann, 2015). Baruzzi et al. (2011) also reported encountering difficulties in terms of identifying between *B. cereus* and *B. thuringiensis* strains when using the 16S rDNA gene sequencing and suggested that they are phylogenetically similar. Due to the difficulties associated with the discrimination of some closely related *Bacillus* spp. strains, this study showed some of the additional molecular identification techniques (Protein-coding gene) which can be used to differentiate such species and made it possible to establish a relationship between PGPR or biocontrol activity and the phylogeny of the studied strains as shown in Figures 3.5-3.8 (Cordero et al., 2012).

Another study by Agrawal et al. (2015) has reported that highly conserved protein-coding genes encoding *gyrB* (Figure 3.6) and *rpoB* (Figure 3.7) gene sequences may be reliable for taxonomic identification among closely related species such as *B. thuringiensis* and *B. cereus* strains. Caamano-Antelo et al. (2015) also affirmed the successful application of the protein-coding genes *gyrB*, *rpoB*, and *tuf* together with the 16S rDNA gene sequences to differentiate among closely related species, and evaluated that the above mentioned genes had higher specificity oligonucleotide probes and higher molecular evolution compared to the 16S rDNA-based probes.

Ali et al. (2014) also reported homolog similarities of less than 100% for *Bacillus* spp. identification using the 16S rDNA gene sequence and also suggested the use of additional species-specific phylogenetic gene markers. It was noteworthy that for strain LBC4ME, the 16S rDNA gene sequence identified it as species *B. cereus* name which was different compared to the *tuf*, *gyrB* and *rpoB* gene sequences which assigned it as a *B. thuringiensis*, indicating that the 16S rDNA managed to identify the bacterial strain at the species level, whereas, the protein-coding gene sequences identified the bacterial strain at the strain level, thus, the identity results displayed by the above-mentioned protein-coding gene sequences were considered as the most reliable to discriminate between the closely related species (Zalila-Kolsi et al., 2016). Figure 3.4 showed the detection of a present plasmid in the studied strains (LBC4ME,

LBC22HL and LBC35MW). A study by Munees and Mulugeta (2014) have shown that the presence of a plasmid in a bacterial strain enhances its genetically competitive role and survival in natural environments due to the availability of an insertion sequence element (plasmid), and also added that, the location (e.g. Chromosome or plasmid) of the biosynthesis genes have an influence on the mode of expression which can be either induced or constitutive.

3.4.5 Screen-house analysis

The results presented on Table 3.5 and Figures 3.9-3.12 displayed results from the pot experiments conducted from this study, the studied *Bacillus* spp. managed to reduce the disease incidence of *F. graminearum* FG1 with the antagonistic treatments containing bacterial strains LBC4ME, LBC22HL and LBC35MW. It was noteworthy to observe that a higher percentage for disease incidence reduction was obtained through the consortia treatment containing the mixed strains compared to individual strain applications. Ali et al. (2014) also reported native *Bacillus* spp. strains which were capable of inhibiting a targeted fungal pathogen (*F. graminearum*) in the desired host plant, thereby, promoting their use in agro-systems.

The genus *Bacillus* spp. has previously been reported to exert biological control activity against *F. graminearum* and other *Fusarium* spp. which indicates its potential to control a range of soil-borne pathogens, hence, numerous mechanisms have been reviewed to participate in the ability of PGPR to have antifungal traits such as the production of antibiotics, hydrolytic enzymes, the competition for nutrients plus iron and more (Tokpah et al., 2016).

In terms of colonization, all the studied strains were found to be present in the root adhering soil from the treatment pots containing antagonistic strains (LBC4ME + P, LBC35MW + P, LBC22HL + P, LBC4ME + LBC35MW + P, LBC4ME + LBC22HL + P, LBC22HL + LBC35MW + P, LBC4ME + LBC22HL + LBC35MW + P) and this confirmed the ability of these strains to colonize the plant root. Kurabachew and Wydra (2013) stated that the ability of rhizobacteria to colonize the roots is an essential step to determine microbial interactions with plants in the rhizosphere and their biological control efficiency, this suggested that the inoculated strains used in this study could've influenced plant growth through biological control. However, according to Shi et al. (2014), it is challenging to evaluate the mode of action used by PGPR, and also environmental conditions may influence colonization ability by different strains.

In this study, the indigenous LBC4ME, LBC22HL and LBC35MW strains were predominant isolates found to be potential rhizobacteria that suppressed the growth of *F. graminearum* on maize as shown in Table 3.5 and Figures 3.9-3.12, concluding, that these strains can be potential BCAs for the targeted disease-causing pathogen.

The varied IAA production levels have been reported by Bailly et al. (2014) to be effective on the development of plant growth, however, the low concentrations produced possibly play a part in the growth of secondary roots and radical length. A study by Moustaine et al. (2017) has reported 3 strains (2025-1, 2027-2 and 2066-7) from the genus *Bacillus* spp. to have a stimulatory effect on shoot, root, stem length, and mean yield. Rayees et al. (2014) also reported that the strains from the genus *Bacillus* spp. were effective for plant growth promotion and suppress fungal pathogens through their broad spectrum of PGPR mechanisms and this led them to be important targets in terms of plant-microbe interactions.

The results presented in Table 3.5 and the Figures 3.9-3.12 showed that maize applied with pot treatments containing antagonistic strains were found to have reduced disease severity and healthier plant growth parameters compared to control and pathogen only pot treatments. These results indicated that strains LBC4ME, LBC22HL and LBC35MW inoculated on maize seeds had a positive effect on plant growth and disease control compared to the negative control and pathogen only pot treatments. Between the pot treatments, the consortia/ combined treatment (LBC4ME + LBC22HL + LBC35MW + P) showed the highest plant growth and reduced disease severity through improved maize shoot length, shoot fresh/dry weight, root length, root fresh/dry weight and also showing disease control on the roots and the plant by having a low mean disease rating (Table 3.5), which indicated disease reduction. This results confirmed that the consortia pot treatment had the most effective disease control efficiency (Ahmad et al., 2013).

These results are similar to the findings of Gupta et al. (2015) who also reported high biocontrol efficiency from treatments containing antagonistic individual strains having approximately above 30% disease reduction as well as with combined/ strains having more than 50%. Also, positive plant growth was reported overall (roots, shoot length, leaves) compared to treatments containing pathogen only. The biocontrol efficiency observed from the screen-house pot experiments demonstrated a positive correlation with the presence of inoculated bacterial strains LBC4ME, LBC22HL and LBC35MW in the treatments compared to the pot treatment without bacterial strain inoculations. The aforementioned findings could be explained

according to Jung and Park (2015) who stated that quorum sensing permits a coordinated microbial behaviour and facilitates gene expression through collective communication, thus, the more the number of different organisms (microbial count) with similar biocontrol traits encourages an improved biocontrol outcome compared to just one bacterium.

3.5 Conclusions

This research study evaluated the indigenous *Bacillus* spp. strains isolated from different areas in the North West province showed to possess the ability to affect *F. graminearum* under laboratory conditions. The strains exhibited efficient nitrogen fixation ability, solubilization of phosphate, production of IAA, NH₃, siderophores, and HCN antimicrobial secondary metabolites, as well as the production of cellulase and protease hydrolytic enzymes, indicating that these strains may be ideal for the production of the above mentioned antimicrobial metabolic traits. The isolated strains were identified to be *B. thuringiensis* (LBC4ME and LBC35MW) and *B. velezensis* (LBC22HL) through molecular and biochemical techniques. The identification for bacterial strain LBC4ME indicated classification errors with closely related species, thus, indicating the need to further use additional identification tools such as species-specific housekeeping gene sequences as an identification tool to distinguish between closely related species. This study also displayed that the selected bacterial strains had an effective disease reduction of *F. graminearum* FG1 on the maize tassel, stalk, leaves and roots from the screen-house pot treatment experiment. The pot experiment results also showed that consortia inoculated pot treatments had higher disease reducing ability compared to individual bacterium and pathogen only inoculations.

CHAPTER 4

Production, characterization and application of lipopeptide biosurfactants as a biocontrol against *F. graminearum* using *Bacillus* spp.

ABSTRACT

The use of synthetic pesticides is warned against as it is accompanied by degenerative health effects, thus, alternative supplementation is constantly acquired. An alternative is the production of lipopeptide biosurfactants which are sought as beneficial bioactive secondary microbial metabolites commonly produced by members of the PGPR including *Bacillus* spp. with a diversity of biotechnological and agricultural applications. This study aimed to extract lipopeptide biosurfactants from three novel *Bacillus* spp. and test their efficiency against a soil-borne pathogen *F. graminearum* FG1 strain. The objectives of this study were to screen and carry out tests for the production of antifungal biosurfactant lipopeptides from novel PGPR genus *Bacillus* spp. capable of inhibiting *F. graminearum* FG1 *in vitro*. The methods were to screen strains for potential biosurfactant production assay tests (Drop collapse method, oil displacement test, lipase test and E24% emulsification index), detect the presence of biosynthesis genes for surfactins, fengycins, iturin A and bacillomycin D by PCR and to confirm the functionality of these bioactive compounds through TLC, FTIR and ESI-MS techniques, and further evaluate their antagonistic stability and efficiency through different diluted concentrations *in vitro*. The results showed that the lipopeptide biosurfactants extracted from the *Bacillus* spp. strains exhibited efficient inhibition zones against the fungus using the disk diffusion assay. The detection, extraction and evaluation of lipopeptides were achieved from the novel *B. thuringiensis* LBC4ME, *B. velezensis* LBC22HL and *B. thuringiensis* LBC35MW strains previously isolated from healthy maize rhizosphere. The three strains showed positive tests when screened through biosurfactant production assay tests (Drop collapse method, oil displacement test, lipase test and E24% emulsification index). They also showed a positive detection of the lipopeptides when further confirmed through the amplification of molecular lipopeptide biosynthesis genes [surfactins (As1f/Ts2r), fengycins (Af2f/Tf1r), Iturin A (ItuDf/ItuD1r) and Bacillomycin D (Bacc1f/Bacc1r) by PCR. Extracted crude products were successfully characterized by chromatographic and spectroscopic analysis. The lowest inhibitory concentration was found to be at 10^{-8} µg/L by showing inhibition zones from strains LBC4ME (1.4 ± 0.19 mm), LBC22HL (1.5 ± 0.09 mm) and LBC35MW (1.3 ± 0.17 mm) using the serial dilution method to check the minimum inhibitory

concentration (MIC) against *F. graminearum* FG1. All the studied *Bacillus* spp. strains exhibited efficient inhibition zones against the targeted fungus, and therefore, these results showed that the production of these molecules by the PGPR strains may be considered as an ideal trait for the selection of molecules with biocontrol antagonistic activity against *F. graminearum* infections.

Keywords: Antimicrobial agents, antimicrobial-lipopeptides, bioactive biosurfactants, mass spectroscopy analyses, surface activity

4.1 Introduction

Most soil-borne microbes such as *F. graminearum* are commonly known as being plant pathogenic and are a leading cause of numerous crop diseases including *Fusarium* head blight (FHB) and rotting, resulting in plant damage, defoliation and wilting of the infected crop (Dunlap et al., 2013). The use of synthetic chemicals to manage fungal pathogens has become a growing concern over the years, due to the development of microbial resistance, consequently, environmental pollution and health issues associated with the use of chemicals is alarming (Rincon-Florez et al., 2013). *Fusarium* spp. infections are a worldwide problem for crops and pose a threat to food security. It is, therefore, necessary to attain alternative practices that can assist in controlling the infections (Pandin et al., 2017).

Biological control is increasingly considered an alternative practice which comprises the use of microbe-microbe relationships to act as pathogen control agents (Anburajan et al., 2015). This includes PGPR which has previously been used in several direct and indirect processes to favour the survival of the host plant by reducing diseases (Bakthavatchalu et al., 2012). Thus, BCAs are constantly required for the development of commercial disease control products in the agricultural market because they are known to be eco-friendly alternatives as antifungal agents by producing several antibiotics which act against pathogens (Guo et al., 2015).

Many PGPR genera have been used in the application of BCAs (Agrawal et al., 2015). However, only *Bacillus* spp. were investigated in this study as they are well known for using several direct and indirect mechanisms to antagonize pathogens (Ali et al., 2016). In addition to the beneficial traits, *Bacillus* spp. are also capable of producing biosurfactants classified as lipopeptides, phospholipids, fatty acids, glycolipids, neutral lipids, and polymeric compounds (Christova et al., 2015). These complex amphipathic chemical molecules have a hydrophilic and hydrophobic entity which enables them to be soluble in polar and non-polar solvents, as a result, reduce surface tension (Ayed et al., 2014).

Also, these molecules have effective applications in several industries (e.g. Pharmaceuticals, chemical, agriculture, food processing, cosmetic, bioremediation, biotechnology and oil recovery), this is due to their lower toxicity, bioavailability, high selectivity, environmental compatibility, biodegradation, mild production from cheaper and renewable substrates, mobility, stability under diverse temperature, salinity and pH conditions (Alajlani et al., 2016). The hydrophobic fragment of the molecule is composed of hydroxyl fatty acids or α -alkyl- β -hydroxyl fatty acids and long-chain fatty acids, whereas, the hydrophilic fragment is made up

of either carbohydrates, phosphates, polysaccharide-protein complex, amino acids, alcohol, carboxylic acid or cyclic peptides (Singh & Cameotra, 2013).

The current study investigated lipopeptide compounds with antifungal properties produced by *Bacillus* species. These lipopeptide biosurfactants are secondary metabolites with antifungal activities, and for the *Bacillus* spp., the currently main known lipopeptide families are iturins, surfactins, and fengycins which are low molecular weight chemicals that have amphipathic (hydrophilic and hydrophobic) groups including biocontrol attributes against fungal pathogens (Palazzini et al., 2016).

Among these products; surfactins are known as a cyclic lipopeptide having a chain length with 13-15 carbon atoms with a -hydroxyl-fatty acid. Fengycins are cyclic lipodecapeptides having a chain length of 15-18 carbon atoms with a -hydroxy-fatty acid. Iturins are cyclic lipopeptides having a chain length of 14-17 carbon atoms with a -hydroxyl-fatty acid, also, these are non-ribosomal peptide synthetases (NRPSs) with multifunctional enzymes responsible for the biosurfactant production of non-ribosomal lipopeptides produced by some *Bacillus* strains (Gond et al., 2015). These biosurfactants are reported to have antifibrinolytic, antimicrobial, and antitumor activities and they also exhibit antimicrobial activities especially against fungi (Mnif et al., 2016).

These products possess several inherent advantages including their ability to persist through different extreme conditions, cost-effectiveness, solubility in solvents (polar and non-polar), low toxicity, and that they are biodegradable (Whillenbacher et al., 2014). In the present study, the objectives were to extract lipopeptide biosurfactants from novel LBC4ME, LBC22HL and LBC35MW strains, identify, characterise and assess them for antifungal biocontrol activity against the *F. graminearum* FG1 strain.

4.2 Materials and methods

The bacterial strains previously obtained (Please refer to CHAPTER 3, section 3.2.2 above) from healthy maize rhizosphere in local fields from the North-West province, SA, were selected and identified as *B. thuringiensis* LBC4ME, *B. velezensis* LBC22HL and *B. thuringiensis* LBC35M strains, and stored at -80 °C as a master stock until further use. Pathogenic *Fusarium graminearum* FG1 was obtained from the UMR-CNRS 5557 Ecologie Microbienne, France (Dr. Claire Prigent-Combaret) and sub-cultured on the Potato dextrose agar medium (PDA) plates at 25 °C for 5 days, and then kept at 4 °C as master stock until

further use. All media were maintained at pH 7 and autoclaved for 15 min at 121 °C. All the below-mentioned experiments were conducted in 3 independent replicates. All the microbial growth at OD_{600 nm} mentioned in the tests were determined by using a UV spectrophotometer: Spectronic 20 D+, USA. About 6 mol/L HCL and 2 mol/L NaOH were used to balance the pH of the medium on all the conducted experiments, except the ones specified differently.

4.2.1 Screening assays for biosurfactant production

The native bacterial strains LBC4ME, LBC22HL and LBC35MW isolated and characterized as part of this study, were used to determine the biosurfactant activity under different production tests methods, namely; oil displacement test, drop collapsing test, emulsification assay and lipase production which were conducted below:

4.2.1.1 Lipase production

The lipase activity by the three (3) novel strains was determined using the plate assay test. The strains were sub-cultured in LB broth for 24 h at 25 °C. After incubation, the cultures were further centrifuged (Benchtop centrifuge ECH-TYP, USA) for 30 min at 3000 revolutions per minutes (rpm) to obtain cell-free supernatants. The lipase production medium composition (per 100 mL distilled water: 2.5% agar, Tween 80, 0.5% methyl red) was used to spot inoculate 50 µL of each bacterial supernatant and incubated for 24 h at 25 °C. After incubation, the plates were checked for clear zones around the inoculated spots, while distilled water was used for negative control (Sriram et al., 2011).

4.2.1.2 Drop collapsing test

A Petri dish lid was used as a platform, using a dropper. About 1 drop of mineral oil was spot inoculated on the lid, left at room temperature for 1 h, then 5 µL of cell-free bacterial supernatant was added on top of the oil and a reaction was observed after 1 min. The test was scored negative if the oil droplet remained beaded on the plate, and scored positive if the droplet collapsed on the plate after application. Distilled water was used as a negative test (Bezza & Chirwa, 2015).

4.2.1.3 Oil displacement test

A Petri dish (25 cm diameter) filled with 40 mL of distilled water was used as a container, then 20 mL of crude oil was added to the Petri dish containing water. The oil was allowed to spread

on the water surface for 15 min. About fifty μL (50 μL) of each cell-free bacterial supernatant was spot inoculated on top of the oil surface and plates were observed for a reaction. The positive production test was scored on a plate showing clear oil zones from the inoculated suspension and a negative test showed no clear zones. A negative control test was performed using distilled water (Sriram et al., 2011).

4.2.1.4 Emulsification activity test

This experiment was conducted according to Sriram et al. (2011). Four mL (4 mL) of each cell-free bacterial supernatant was placed in 50-mL falcon tubes, and 6 mL of different hydrocarbons were tested separately and added to each tube and vortexed for 2 min at 3000 rpm, then the mixture was left for 24 hours at room temperature. The calculations were done according to Sriram et al. (2011) for the determination of the emulsion stability carried out after 24 h by calculating the percentage ratio for the emulsification index (E24) as:

$$\text{E24\%} = \frac{\text{Height of emulsion layer}}{\text{Total height of mixture}} \times 100$$

4.2.2 Molecular detection for biosynthetic genes with antifungal traits

Gene-specific primers for biosurfactant were used for PCR identification for the detection of bioactive molecules that produce antibiotics were listed in the following Table 4.1 below:

Table 4. 1: PCR primers and genes with corresponding sequences for lipopeptide biosurfactant biosynthesis

Lipopeptide biosurfactant	Targeted primers	Primer sequences (5'-3')	Amplicon size (bp)	References
Surfactins	As1-F	CGCGGMTACCGVATYGAGC	422	(Ayed et al., 2014)
	Ts2-R	ATBCCTTTBTWDAATGTCCGCC		
Fengycins	Af2-F	GAATAYMTCGGMCGTMTKGA	443	(Ayed et al., 2014)
	Tf1-R	GCTTTWADKGAATSBCCGCC		
Bacillomycin D	Bacc1F	GAAGGACACGGAGAGAGTC	875	(Gond et al., 2015)
	Bacc1R	CGCTGATGACTGTTCATGCT		
Iturin A	ItudD1F	GATGCGATCTCCTTGATGT	647	(Gond et al., 2015)
	ItuD1R	ATCGTCATGTGCTGCTTGAG		

The PCR amplifications were done according to the authors listed in Table 4.1, to detect the genes encoding the enzymes for the iturin A, surfactins, bacillomycin D and fengycins lipopeptide biosurfactants. The primers used for the PCR amplifications were listed in Table 4.1. PCR amplification from isolated bacterial DNA was performed using a Thermal Cycler C1000 Touch (BIO-RAD) with an amplification reaction volume of 50 μ L, prepared mixture contained 25 μ L Master mix (ThermoScientific), 2 μ L bacterial DNA, 22.5 μ L sterile Milli-Q water (ThermoScientific), 0.5 μ L of each forward and reverse primers. The thermal cycling conditions for the surfactin gene's amplification included an initial denaturation at 94 °C for 3 min, 30 cycles of 94 °C for 1 min, annealing at 43 °C for 30 s, the extension was at 72 °C for 45 s, and the final extension at 72 °C for 10 min. Conditions for fengycin genes included an initial denaturation at 94 °C for 3 min, 30 cycles at 94 °C for 1 min, annealing at 45 °C for 30 s, extension at 72 °C for 45 s, and the final extension 72 °C for 10 min. Conditions for iturin A and bacillomycin D genes included an initial denaturation at 95 °C for 5 min; 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and a final extension of 72 °C for 10 min.

To prepare for the detection of amplified extracted bacterial DNA from the strains. About 1% agarose gel was prepared by adding 1 g agarose powder (Merck, USA) into 100 mL 1X Tris-Acetate EDTA (TAE) buffer (1:1, v/v) (BIO-RAD, USA). The agarose solution was heated for 3 min in a microwave (DEFY) and allowed to cool, followed by the addition of 10 μ L of 0.5 μ g/ mL ethidium bromide into the agarose solution which was later poured into the electrophoresis tray with the electrophoresis combs in place and allowed to harden before taking out the combs. Then the agarose gel in the tray was overlayed with a 1X TAE buffer. About 5 μ L of each bacterial DNA solution mixed with 5 μ L of loading dye and 3 μ L 1 Kb ladder for the approximation of DNA sizes were loaded into the gel wells. The electrophoresis was run using ELITE 300 PLUS (WEALTEC) power supply for 1 h at 80 V and 400 mA. The gel was viewed under UV transilluminator 2000 (BIO-RAD, USA) to check for amplified genes with the expected target sequence amplicon sizes (bp).

4.2.2.1 Nucleotide sequence determination

The PCR amplified products of the surfactins, fengycins, bacillomycin D, and iturin A genes for the strains were then sent for partial sequencing using the ABI PRISM® 3500XL DNA Sequencer Applied Biosystems at Inqaba biotechnological Industries (Pty) Ltd, Pretoria, SA. The subsequent gene nucleotide sequences were then deposited into the NCBI GenBank

database. Nucleotide sequences' accession numbers were obtained after depositing nucleotide sequences in the BankIt database.

4.2.3 Production media, cultivation and purification of biosurfactant crude products

The fermentation medium was used to produce biosurfactant crude extracts. About 5% of LB broth containing each bacterial strain's inoculum was added to a fermentation broth medium (1000/mL distilled water: 25 g NaNO₃, 1 g Na₂HP0₄·12H₂O, 0.333 g KH₂P0₄, 0.15 g MgSO₄·7H₂O, 0.0075 g CaCl₂, 0.006 g FeSO₄·7H₂O, 0.006 g MnSO₄·H₂O, 1 g yeast extract, 30 g glucose, pH 7), the inoculated fermentation medium was incubated in a shaker (Labcon SPL-MP 3000, USA) at 200 rpm with a temperature of 37 °C for 48 h, centrifuged (Benchtop centrifuge ECH-TYP, USA) at 12 000 rpm for 20 min, the supernatant was adjusted to pH 2 with 6 M HCl and stored overnight at 4 °C. The formed acidic precipitate was collected by centrifugation again for 20 min at 15 000 rpm to yield solid crudes. The solid crudes were further dissolved in distilled water and freeze-dried to receive the final biosurfactant crude product in a powdered form (Sriram et al., 2011).

4.2.4 Characterization of the biosurfactants

4.2.4.1 TLC

The characterization of the crude products was analysed using TLC on 60 plate's silica gel (F254; Merck). Five mg (5 mg) of the crude products were dissolved in methanol for TLC analysis and were further developed in solvents; Chloroform: Methanol: Distilled water (65:15:4, v/v) to develop chromatograms. The compounds were identified by using 0.35% Ninhydrin (w/v, in acetone) to detect free amino groups and water to detect hydrophilic groups when applied on the plates, then the appearance of visible spots was achieved by heating the plate for 5 min at 110 °C. The separation of the spots was observed under a UV light (Bezza & Chirwa, 2015).

$$R_f = \frac{\text{Distance moved by spot (mm)}}{\text{Distance moved by solvent (mm)}}$$

4.2.4.2 FTIR spectroscopy

The FTIR spectroscopy (PerkinElmer 1600, USA), was used to determine the chemical structures of the crude sample product. About 1 mg of each strain's crude powder was placed on the metal and pressed for analysis. The IR spectra scans conditions for the wavenumber range was collected at 400-4000/cm, and the resolution was 2 cm, 32 scans for the reflectance spectra, and the BRUKER software was used to view spectra (Bezza & Chirwa, 2015).

4.2.4.3 ESI-MS analysis

The mass spectrometry analysis was carried out using the MicrOTOF-Q112010 390 instrument, USA. About 1 mg of each crude product was infused using a syringe 5 μ L/Min flow rate. The source voltage and capillary voltages were 5 kV and 45 V in positive ion mode and 5kV/15 V in negative ion mode. The capillary temperature was 180 °C. The total ion scan mode range was 50-3000 m/z (Ali et al., 2016).

4.2.5 Surface tension determination

The method described by Sriram et al. (2011) was followed to determine the surface tension. Some 5 mL of cell-free supernatant was added to a glass tube placed in a water bath at a temperature of 30 °C. A capillary tube was inserted inside the glass tube and monitored the final height of the rising liquid. A supernatant free broth was used as a control test. The final supernatant height that had risen in the column was used to calculate the surface tension using the formula according to Sriram et al. (2011):

$$y = \frac{rh\delta g}{2}$$

Whereby y = Surface tension (mN/m), r = Capillary radius (0.05 cm), h = Supernatant height inside the column (cm), δ = Density (g/mL), g = Gravity (980 cm/s²).

4.2.6 Determining biosurfactant antifungal activity

The Kirby-Bauer disk diffusion method on MHA plates was used to test lipopeptide biosurfactant of bacterial strains for antifungal activity. The fungus *F. graminearum* FG1 was grown on an MHA medium for 96 h at 25 °C. The mycelium spores were scraped from the plate and suspended in 10 ml of sterile normal saline solution (NSS) for about 1 h to a concentration of approximately 10⁵ CFU/mL. About 100 μ L of diluted spore suspensions were

streaked on the plates with a cotton swab. Paper discs (5 mm) previously drenched for 12 h in 100 μ L of the lipopeptide biosurfactant solution with a measured absorbance ($OD_{600\text{ nm}} = 0.5$) were placed on the plates. An MHA plate streaked with the spore suspension without a lipopeptide biosurfactant solution was used as a control plate. The plates were incubated for 120 h at 25 °C and the antifungal activity was evaluated by measuring the size of zone of inhibition against the fungal growth. For the calculation of the inhibition rate, please refer to CHAPTER 3, section 3.2.3.1 (Ahmad et al., 2008; Sharma et al., 2014).

4.2.7 Determination of MIC

A serial dilution method was used to determine the minimum concentration of the biosurfactant required to inhibit the growth of *F. graminearum* FG1 on a growth plate. About 100 μ L ($OD_{600\text{ nm}} = 0.5-0.9$) of biosurfactant crude product dissolved in methanol was diluted with distilled water serially from 10^{-1} to 10^{-8} . Then paper discs (5 mm) were soaked in each dilution for 3 h. *F. graminearum* FG1 diluted in distilled water to a concentration of approximately 10^5 CFU/mL was spread over MHA plates using a sterile cotton swab, then the discs containing different concentrations of biosurfactant were spot inoculated on the plates for 120 h at 25 °C. Methanol only was used as a control. The MIC value was determined after incubation and inhibition zones (mm) were measured from the observed antifungal activity (Sharma et al., 2014).

4.2.8 Statistical analysis

Each of the *in vitro* experiments was repeated 3 times and due to similarity in the replicated set, only data from one result was presented. The Microsoft excel 2013 software was used for the determination of standard error (SE) for replications ($n = 3$).

4.3 Results

4.3.1 Screening assays for biosurfactant production

The results presented from Table 4.2 showed the biosurfactant assays; the lipase production test showed that the cell-free supernatants from the strains LBC4ME, LBC22HL and LBC35MW had a change of colour to mild yellow where the supernatants were spot inoculated, whilst, the plates with distilled water did not show any inhibition or clear zones, thus, confirming an active production of the lipase enzyme. The results from the drop collapsing test

showed that the cell-free supernatants from the strains LBC4ME, LBC22HL and LBC35MW flattened/collapsed the mineral oil drop on the Petri dish whereas the distilled water control did not affect the oil drop and it remained beaded/round. The collapsing of the drop indicated the activity of biosurfactants in the cell-free supernatants. From the oil displacement test, the cell-free supernatants from all the strains were able to create hallow zones on the surface of the crude oil whereas there were no hallowed zones created by the distilled water, the positive results obtained from the above biosurfactant production assays positively confirmed active biosurfactant production by these studied strains. The emulsification activity measured after 24 h indicated that the cell-free supernatants from LBC4ME, LBC22HL and LBC35MW strains managed to produce stable emulsions with different hydrocarbons as shown in Table 4.2 below, Motor oil had the highest E24 index percentages range (46-55%) compared to crude oil (43-50%) and vegetable oil (42-49%).

Table 4. 2: Results from the biosurfactant production assays

Bacterial strains	Lipase test	Drop collapsing test	Oil displacement test	E24% for biosurfactant of strains		
				Hydrocarbons		
				Crude oil	Motor oil	Vegetable oil
LBC4ME	+	+	+	43.33 ± 0.03	46.79 ± 0.04	42.01 ± 0.02
LBC22HL	+	+	+	50.66 ± 0.04	55.70 ± 0.03	49.03 ± 0.04
LBC35MW	+	+	+	47.93 ± 0.02	48.76 ± 0.03	45.81 ± 0.03
Control	-	-	-	-	-	-

Keys: + = positive test, - = negative test. Mean values ± standard errors of the sum of three independent replications (n = 3)

4.3.2 Molecular characterization of biosynthetic genes with antifungal traits

The results from Figure 4.1 showed that the strains LBC4ME, LBC22HL and LBC35MW had positive amplifications for the targeted genes when using the primer pairs As1-F/Ts2-R at 422 bp which is known for the biosynthesis of surfactins; amplification of primer pairs Af2-F/Tf1-R at 443 bp for the biosynthesis of fengycins; amplification of primer pairs ItuD1F/ItuD1R at 647 bp for the biosynthesis of iturin A, and primer pairs Bacc1F/ Bacc1R at 875 bp for the biosynthesis of bacillomycin D which indicated biosynthesis from the strains.



Figure 4. 1: PCR product amplifications for the biosynthesis of lipopeptide biosurfactants biosynthesis genes for surfactins (As1-F/Ts2-R (lanes 1-3)), bacillomycin D (Bacc1F/Bacc1R (lanes 4-6)), fengycins (Af2-F/Tf1-R (lanes 7-9)) and iturin A ItuD1F/ItuD1R (lanes 10-12)) gene primer pairs. M = 1 kb plus DNA ladder (molecular weight marker). Numbers representing bacterial strains under different lanes for LBC4ME (lanes 1, 4, 7, 10), LBC22HL (lanes 2, 5, 8, 11) and LBC35MW (lanes 3, 6, 9, 12). Number 13 = Nuclease free water

The accession numbers given to the lipopeptide biosurfactant genes from the bacterial strains obtained from this study were assigned for strains LBC4ME (MN231263.1, MH492758.1), LBC22HL (MN231266.1, MN231264.1) and LBC35MW (MN231267.1, MN231265.1) for iturin A and bacillomycin D genes, respectively.

The accession numbers for the surfactins and fengycins genes under the LBC4ME, LBC22HL and LBC35MW strains were not received due to unresolved errors reported when depositing gene sequences in BankIt database, the feedback reported that these nucleotide sequences might have problems with feature annotations or presence of internal stop codons.

4.3.3 Characterization of the biosurfactants

The extracted biosurfactant crude products were produced with the fermentation medium, a brown powder was obtained as a maximal final biosurfactant product extract (crude extract), the products were measured with a scale (g) and obtained the final concentrations for strains LBC4ME (3.925 g), LBC22HL (2.905 g) and LBC35MW (3.838 g) after 48 h of cultivation.

4.3.3.1 TLC

TLC was performed using crude methanolic products extracted from bacterial strains LBC4ME, LBC22HL and LBC35MW with acid-precipitated suspensions. Figure 4. 2 showed the characterization of the extracted crude biosurfactant by TLC which revealed three spots from each of the LBC4ME, LBC22HL and LBC35MW strains when viewed under UV light, these spots had R_f values 0.75, 0.40 and 0.20. These spots also developed and bulged on the silica gel after spraying with water. Thus, indicating the presence of lipophilic and peptide bonds in the compounds.

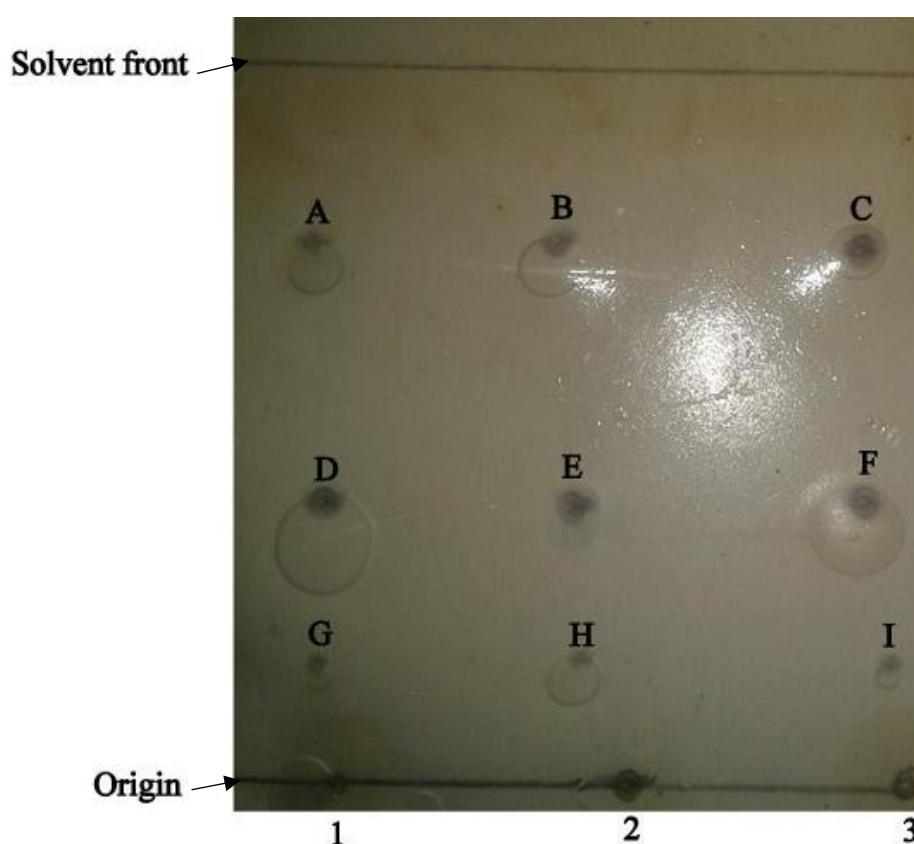


Figure 4. 2: TLC silica plate. Numbers (1-3) represented bacterial strains LBC4ME = 1, LBC22HL = 2 and LBC35MW = 3. Letters (A-I) represented developed spots and R_f values; letters A-C represented R_f values = 0.75, D-F for R_f values = 0.40 and G-I for R_f values = 0.20

The results presented in Figure 4.3 also showed the development of spots with R_f values 0.75 and 0.20 from each of the strains which also developed after being sprayed with ninhydrin, however the spot with R_f value of 0.40 did not develop from each of the strains when using the above mention solvent.

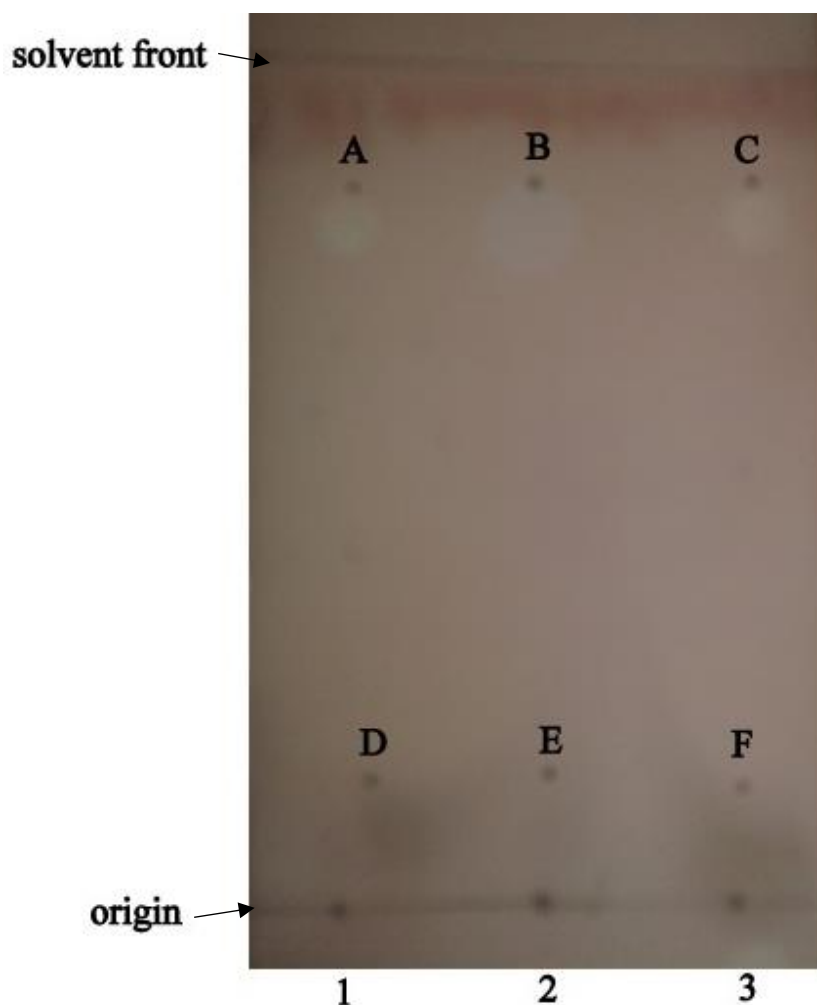


Figure 4. 3: TLC silica plate. Numbers (1-3) represented/represents/showing bacterial strains LBC4ME = 1, LBC22HL = 2 and LBC35MW = 3. Letters (A-F) represented developed spots with Rf values; Letters A-C represented Rf values = 0.75, D-F for Rf values = 0.20

4.3.3.2 FTIR

The results presented in Figures 4.4-4.6 showed the FTIR scans from each of the strains revealing wavelengths with high peaks. Figure 4.4 showed results for the LBC4ME strain at 3275.24/cm, 3078.48/cm, 2956.35/cm, 2923.47/cm, 2853.62/cm, 2358.27/cm, 2327.74/cm, 2196.80/cm, 2005.47/cm, 1984.32/cm, 1719.78/cm, 1625.76/cm, 1522.03/cm, 1444.96/cm, 1370.57/cm, 1221.56/cm, 1033.39/cm, 893.86/cm, 830.04/cm, 830.04/cm, 629.21/cm, 558.71/cm, 522.16/cm and 461.82/cm: From these major peaks, the characteristics of peptides (N-H and O-H stretching mode) was revealed at 3275.24/cm; stretching mode of the CO-N bond at 1625.76/cm; aliphatic chains (-CH₃, -CH₂-) of the fraction were revealed at 2956.35, 2923.47, 2853.62, 1444.96 and 1370.57/cm; a carbonyl group/ hydrophilic group was revealed

at 1719.78/cm and 1221.56/cm; a strong peptide (amide) N-H and C-N bond at 1522.03/cm. Aldehydes, ketones and esters were revealed at peaks 629.21-461.82/cm.

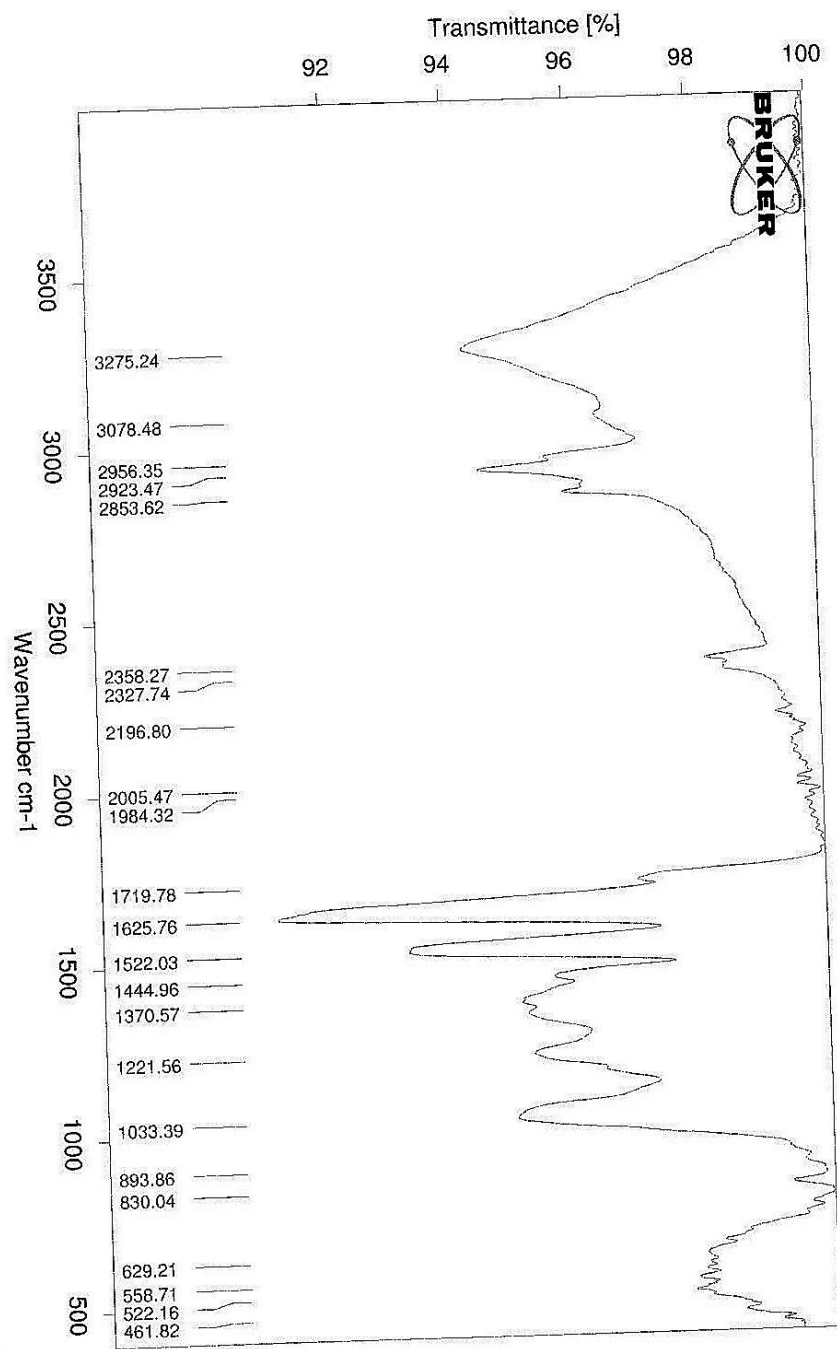


Figure 4. 4: FTIR scans for LBC4ME strain

The findings presented in Figure 4.5 showed results for LBC22HL strain at 3269.42/cm, 2958.37/cm, 2923.49/cm, 2854.88/cm, 2359.03/cm, 2327.67/cm, 2196.86/cm, 1625.31/cm, 1539.29/cm, 1329.29/cm, 1027.38/cm, 925.52/cm, 894.66/cm, 826.01/cm, 770.33/cm,

635.38/cm, 557.87/cm, 519.87/cm and 450.59/cm: From these strong peaks, structures of peptides (N-H and O-H bond stretching mode) were displayed at 3269.42/cm; CO-N bond stretching mode was at 1625.31/cm; aliphatic chains (-CH₃, -CH₂-) were shown at 2958.37, 2923.49, 2854.88 and 1329.29/cm; a strong peptide (amide) N-H and C-N bond at 1539.39/cm. Aldehydes, ketones and esters were revealed at peaks (635.38-450.59/cm)

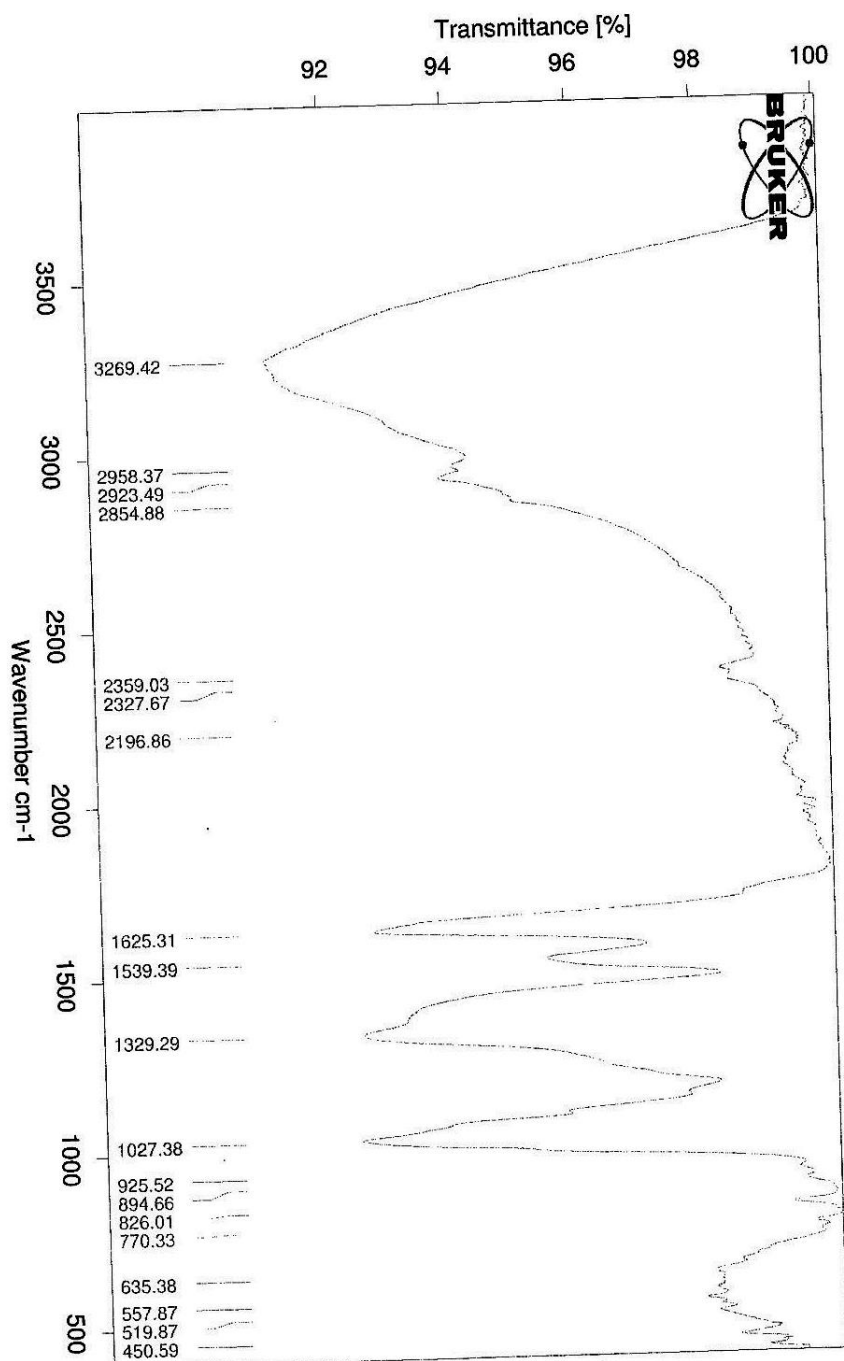


Figure 4. 5: FTIR scans for LBC22HL strain

The findings presented in Figure 4.6 showed results for LBC35MW strain at 3274.92/cm, 3069.70/cm, 2955.51/cm, 2923.35/cm, 2853.66/cm, 2359.78/cm, 2338.87/cm, 2189.34/cm, 1985.35/cm, 1719.38/cm, 1641.04/cm, 1536.96/cm, 1443.53/cm, 1370.06/cm, 1221.92/cm, 1043.19/cm, 890.65/cm, 829.05/cm, 666.71/cm, 611.45/cm, 582.31/cm, 554.65/cm, 521.87/cm, 486.94/cm and 458.35/cm: From these sturdy points, peptides (N-H and O-H stretching mode) were presented at 3274.92/cm; CO-N bond stretching mode was observed at 1641.04/cm; aliphatic chains (-CH₃, -CH₂-) at 2955.51, 2923.35, 2853.66, 1443.53 and 1370.06/cm; a strong peptide (amide) N-H and C-N bond at 1536.96/cm; a hydrophilic/ carbonyl group at 1719.38/cm. Aldehydes, ketones and esters were revealed at peaks (666.71-458.35)

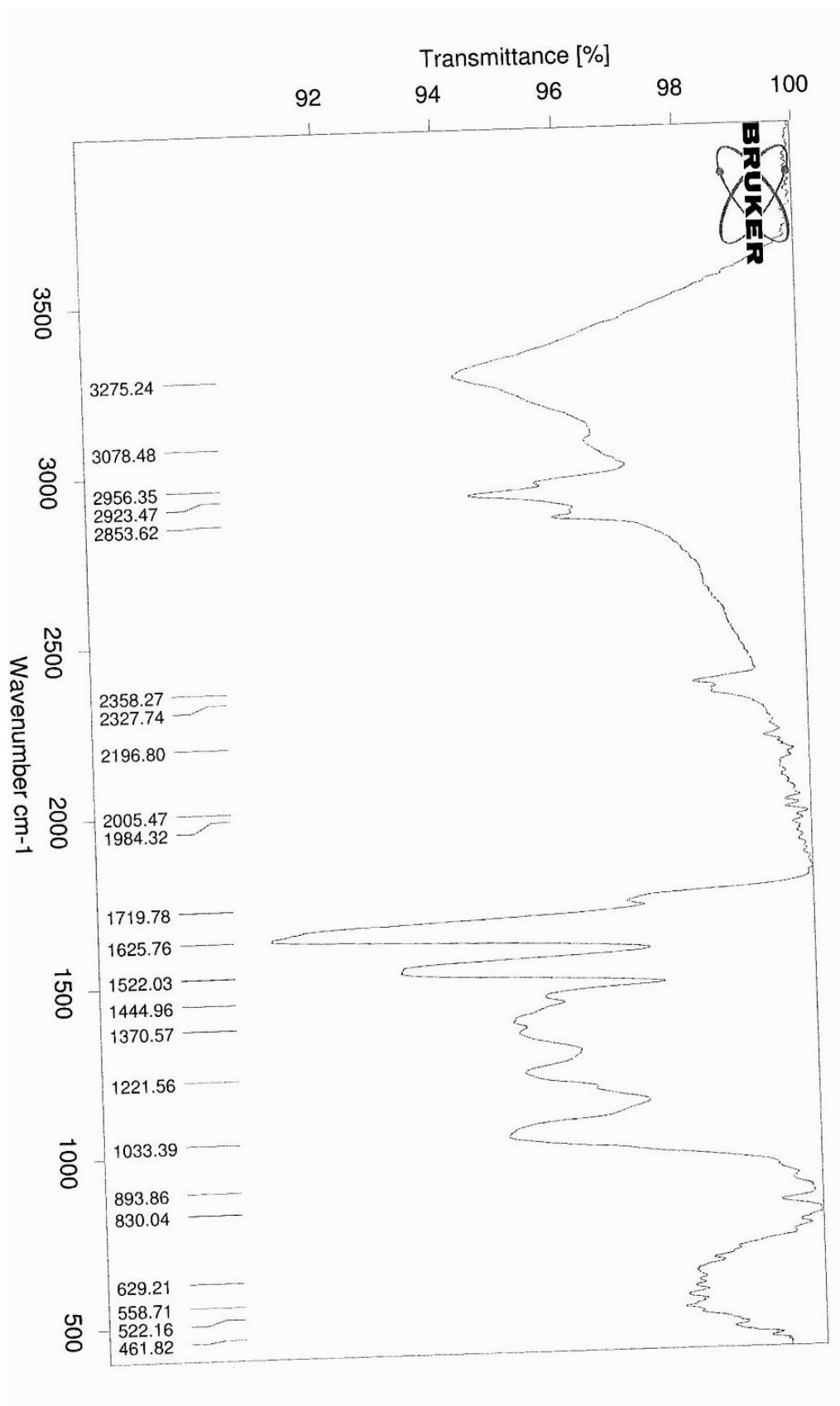


Figure 4. 6: FTIR scans for LBC35MW strain

4.3.3.3 ESI-MS

The ESI-MS analysis findings displayed in Figure 4.7 showed abundant peak $[M+H]^+$ at m/z 1463.8087 with product ions m/z 1074.6452 $[M+H]^+$, $[M+2H]^{2+}$ at m/z 804.6060 and 425.2889 and from Figure 4.8 showing abundant peak m/z 1060.6335 with product ions $[M+2H]^{2+}$ at m/z 751.3923, m/z 530.8187 and m/z 248.9704 from the LBC4ME strain; The ESI-MS analysis displayed from Figure 4.9 showed the abundant peak $[M+H]^+$ at m/z 1463.8104 with product ions at m/z 1058.6686 $[M+H]^+$, $[M+2H]^{2+}$ at m/z 743.3952, m/z 530.8157 and m/z 248.9686 and from Figure 4.10 showing abundant peak $[M+H]^+$ at m/z 1044.6613 with product ions $[M+2H]^{2+}$ at m/z 803.5414, m/z 751.3989 and m/z 657.3718 and m/z 613.3473 from the LBC22HL strain.

Findings displayed on Figure 4.11 showed abundant peak $[M+H]^+$ at m/z 1074.6452 with product ions $[M+2H]^{2+}$ at m/z 804.6060, m/z 751.3925, m/z 537.8258, m/z 425.2889 and Figure 4.12 showed abundant peak $[M+H]^+$ at m/z 1060.6335 with product ions $[M+2H]^{2+}$ at m/z 751.3923, m/z 530.8187 and m/z 248.9709, from LBC35MW strain. The presence of these ion products from the studied peaks could indicate losses of fatty acids with a molecular weight difference of 14 Da.

The ESI-MS analysis from Figure 4.7 and 4.8 revealed groups of abundant protonated molecular ion peaks $[M+H]^+$ at m/z 1463.8087, m/z 1074.6452 and m/z 1060.6335 for LBC4ME strain, these homologues had a 389 Da and 14 Da difference in molecular weight to each other; results from Figure 4.9-4.10 showed ion peaks $[M+H]^+$ at m/z 1463.8104, m/z 1058.6686 and m/z 1044.6613 for LBC22HL strain with a molecular weight difference of 419 Da and 2 Da; results from Figure 4.10. ESI-MS showing mass spectra from LBC22HL strain crude products under retention time (3-4 min) showed abundant peak $[M+H]^+$ at m/z 1044.6613 with product ions $[M+2H]^{2+}$ m/z 803.5414, m/z 751.3989, m/z 657.3718 and m/z 613.3474 from the LBC22HL strain.

The ion peaks m/z 1463.8087 from the LBC4ME strain detected in Figure 4.7 and m/z 1463.8104 from LBC22HL strain detected in figure 4.9 are approximately similar to the molecular weight of fengycin A fatty acid chain length, however, this ion peak was not revealed from the LBC35MW strain.

The findings displayed on Figure 4.7-4.8, also revealed sodiated molecular ion peaks $[M+Na]^+$ at m/z 1060.6335, m/z 1074.6452 from LBC4ME strain with a molecular weight difference

of 14 Da and Figure 4.9-4.10 showed m/z 1058.6686, m/z 1044.6613 from LBC22HL strain with 14 Da as molecular weight difference to each other and Figure 4.11-4.12 showed m/z 1074.6452 and m/z 1060.6335 from LBC35MW strain as main peaks with a molecular weight difference of 14 Da. The above mentioned molecular weights are approximately similar to the molecular weight of surfactin, fenycin, and iturin like lipopeptides.

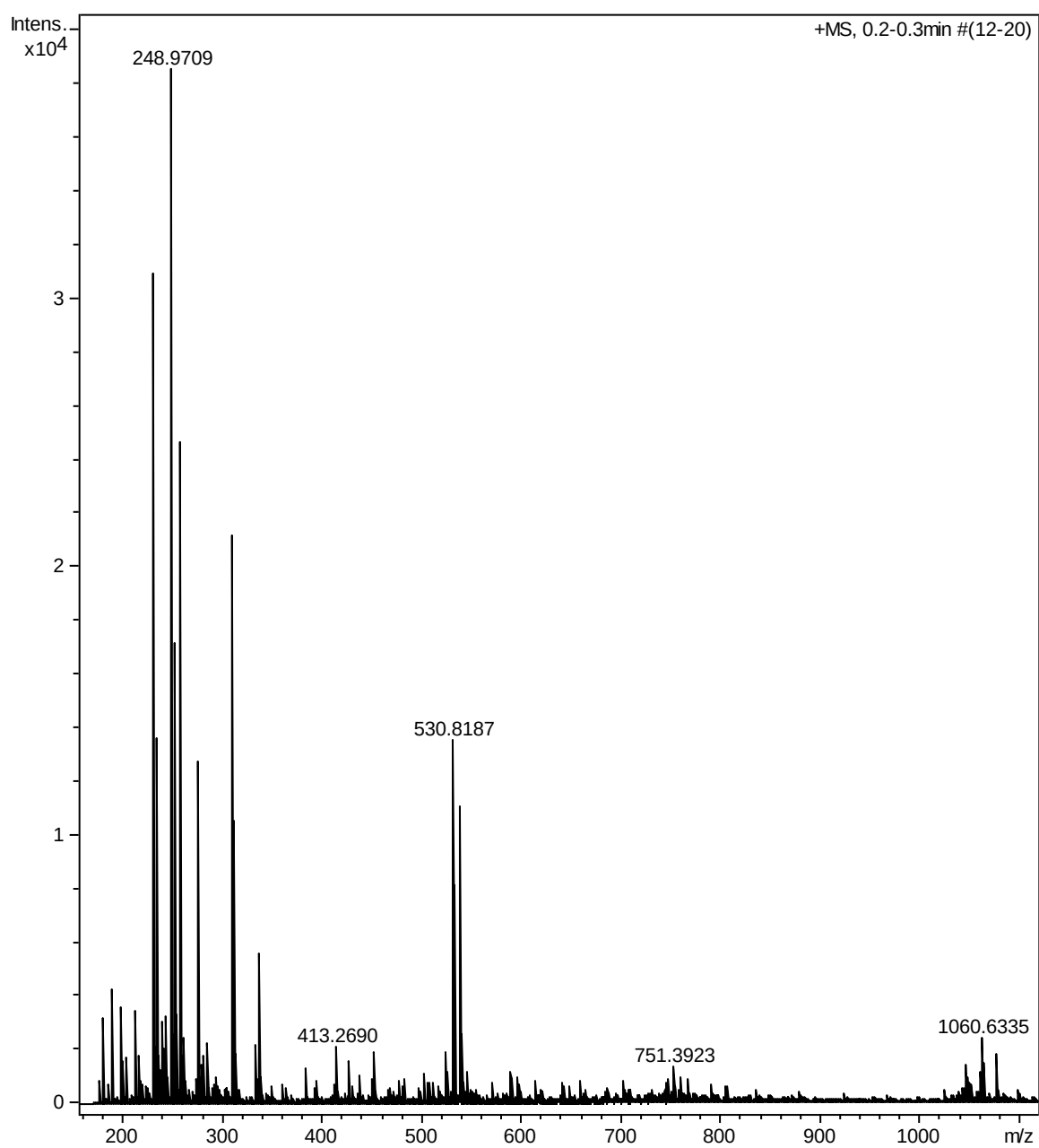


Figure 4. 7: ESI-MS showing mass spectra from LBC4ME strain crude products under retention time (2-3 min)

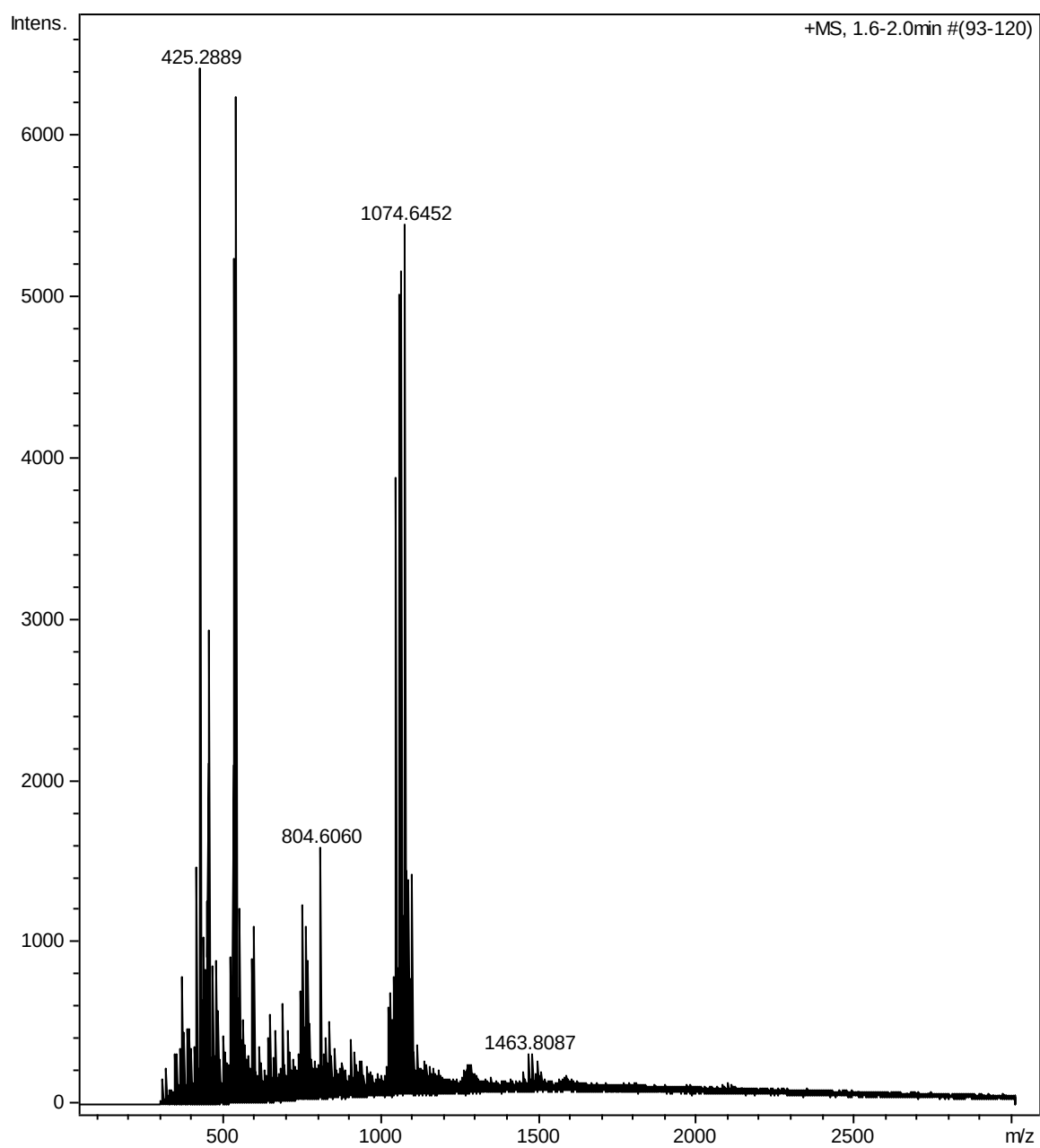


Figure 4. 8: ESI-MS showing mass spectra from LBC4ME strain crude products under retention time (1-2 min)

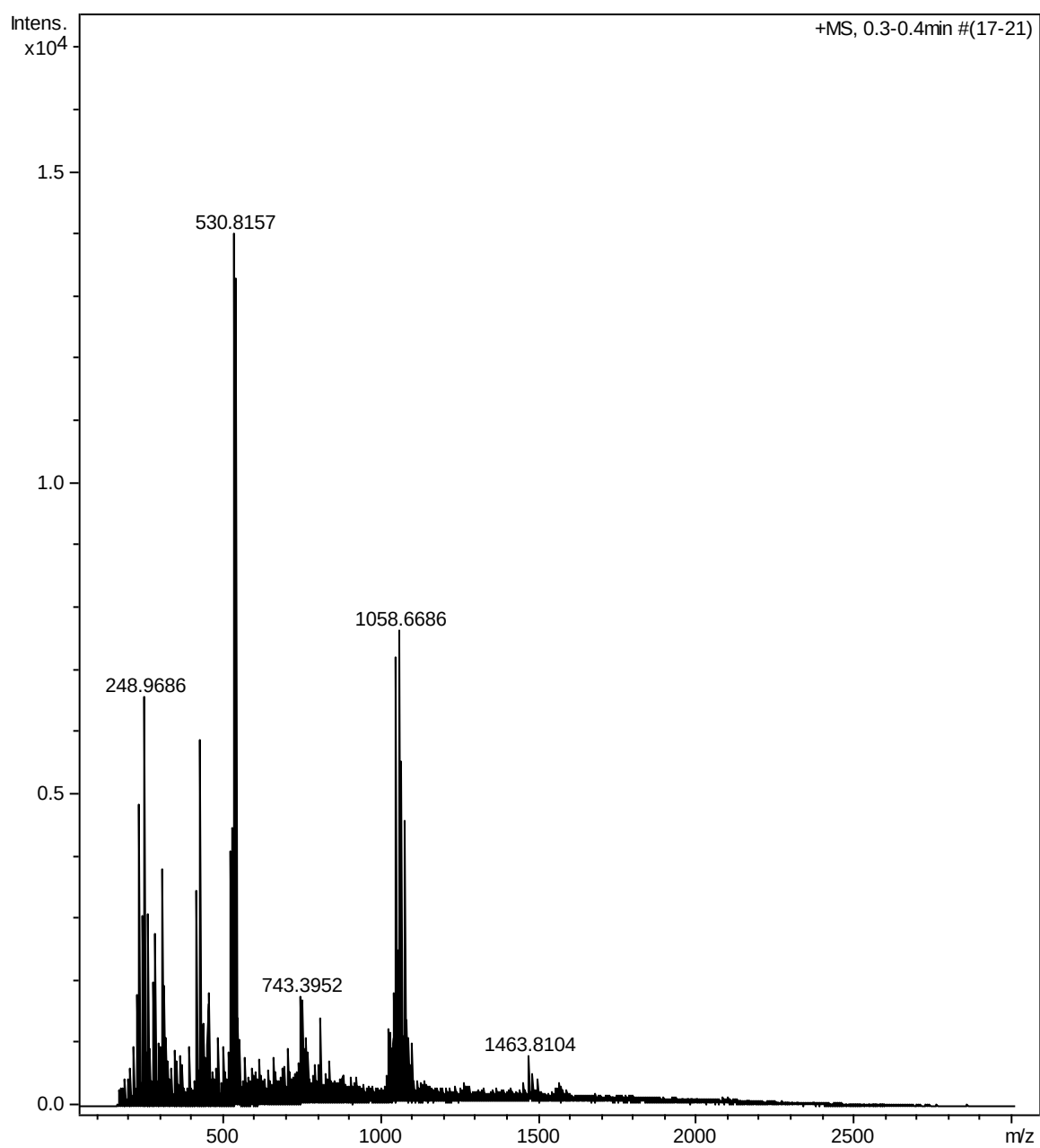


Figure 4. 9: ESI-MS showing mass spectra from LBC22HL strain crude products

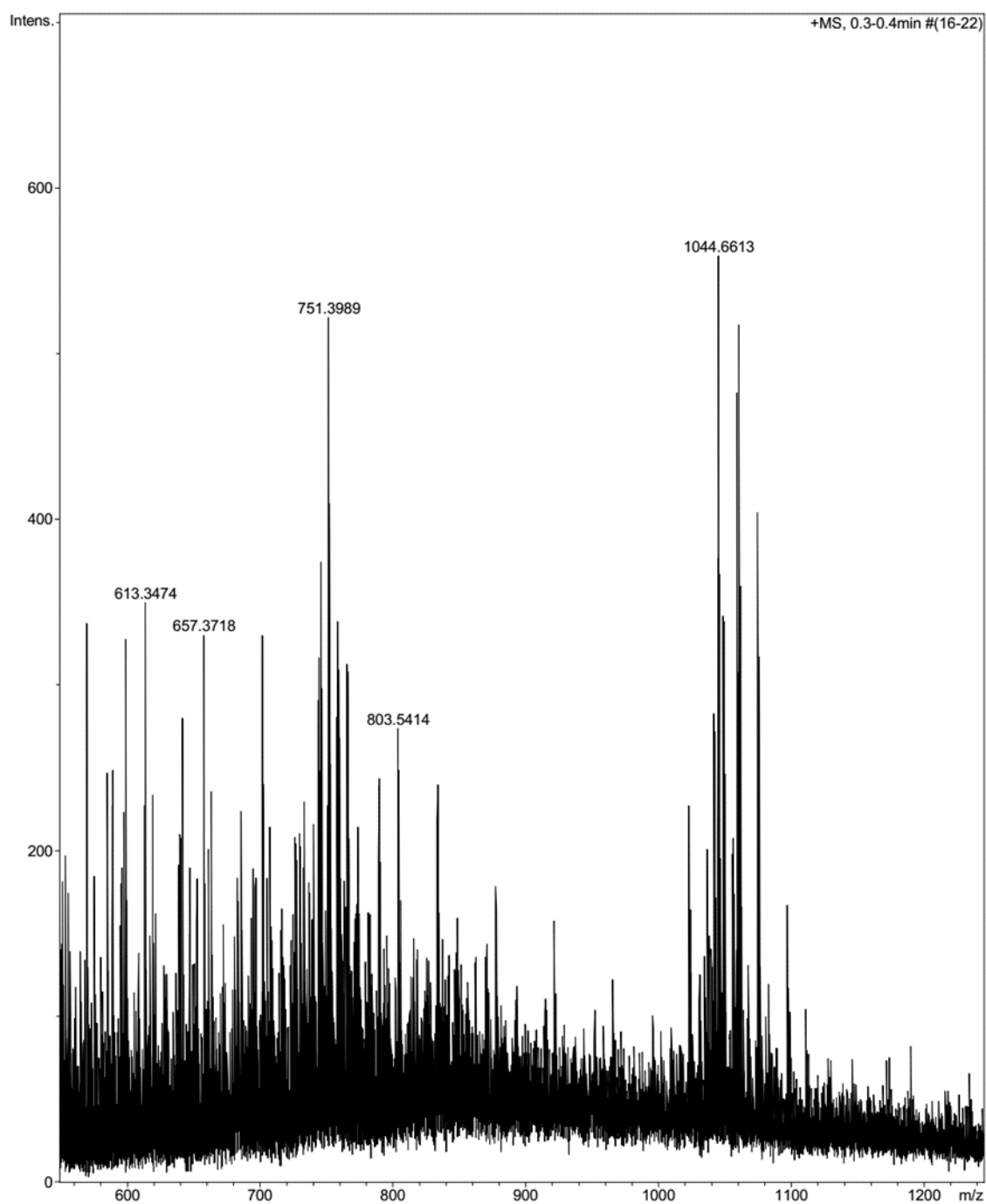


Figure 4. 10: ESI-MS showing mass spectra from LBC22HL strain crude products under retention time (3-4 minutes)

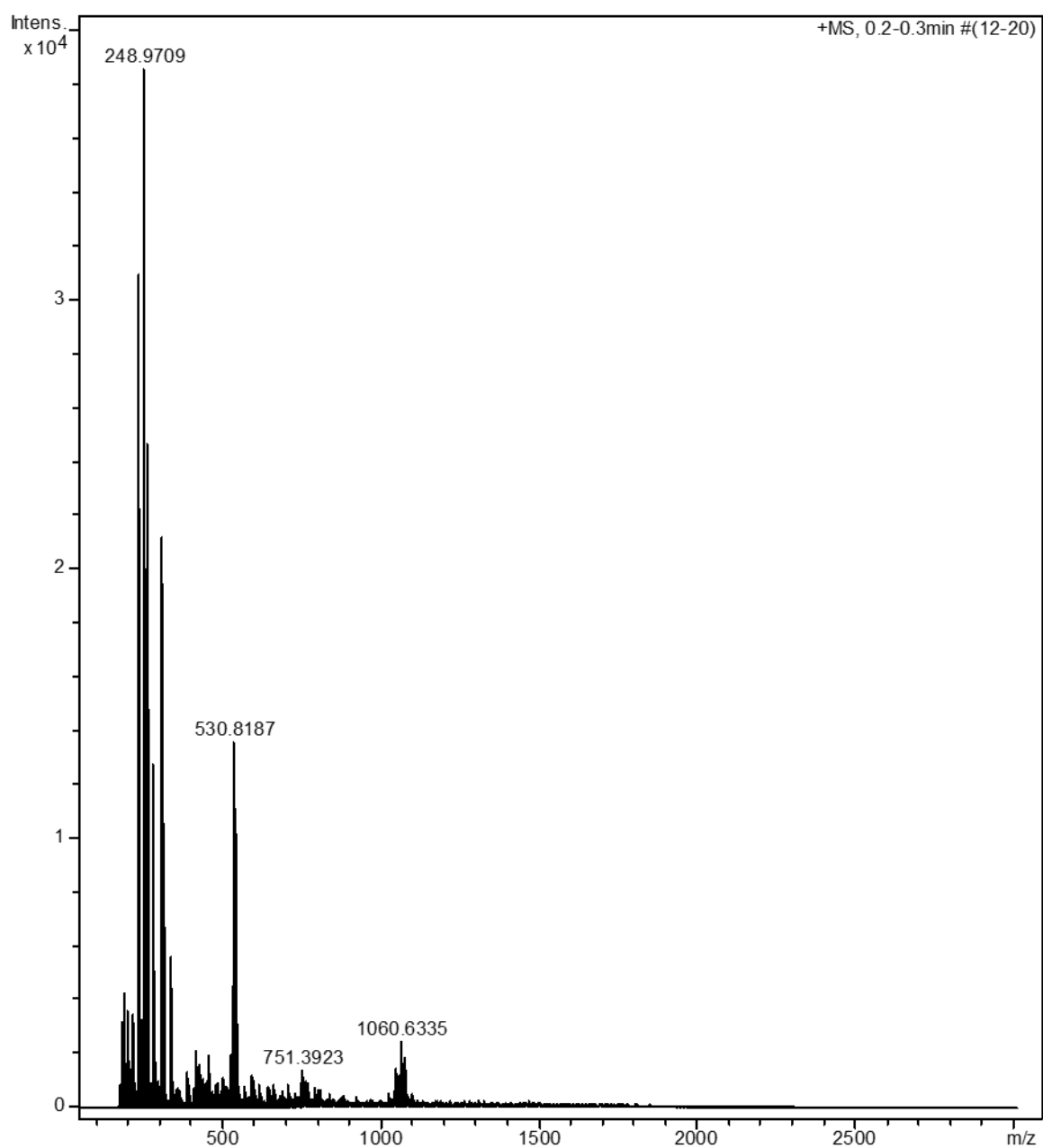


Figure 4. 11: ESI-MS showing mass spectra from LBC35MW strain crude products under retention time (2-3 min)

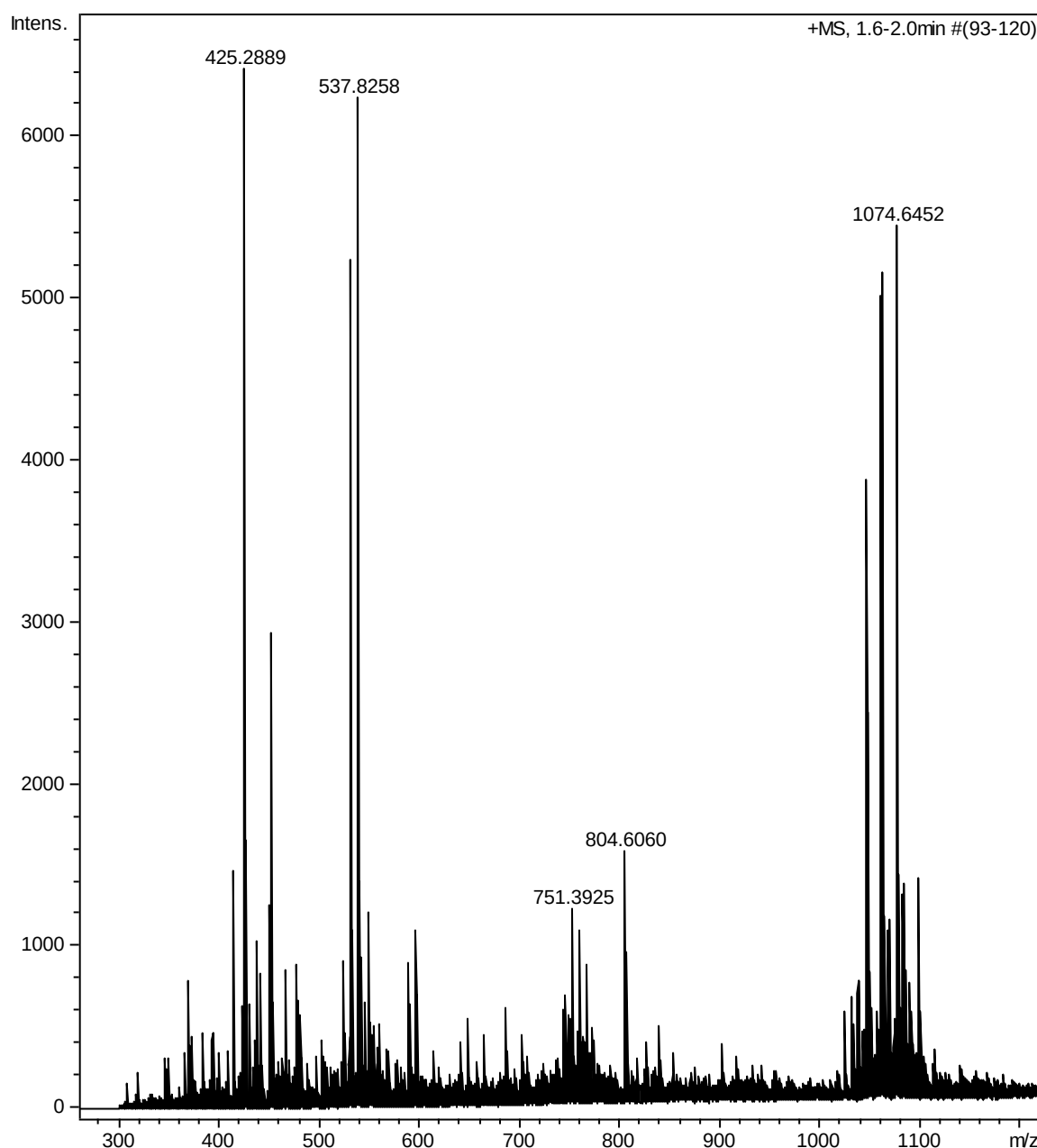


Figure 4. 12: ESI-MS showing mass spectra from LBC35MW strain crude products under retention time (1-2 min)

4.3.4 Antifungal activity and surface tension by lipopeptide biosurfactant

The results presented in Table 4.3 showed that the lipopeptide biosurfactants extracted from bacterial strains had inhibition rates of about 65.31% for LBC4ME, 65.22% for LBC35MW and 66.20% for LBC22HL strains indicating effective antifungal activity rating above 50% against *F. graminearum* FG1. The results from Table 4.3 showed that the surface tension of the lipopeptide biosurfactants from LBC4ME, LBC22HL, LBC35MW strains at 30 °C was (31.85, 29.40 and 34.30 mN/M, respectively).

Table 4. 3: Showing results for the lipopeptide biosurfactant surface tension and antifungal inhibition rate (%) against *F. graminearum* FG1 by each bacterial strain

Bacterial strains	Surface tension (mN/M)	Lipopeptide biosurfactant antifungal inhibition rate (%)
LBC4ME	31.85 ± 0.25	65.31 ± 0.23
LBC22HL	29.40 ± 0.29	66.20 ± 0.12
LBC35MW	34.30 ± 0.28	65.22 ± 0.12
Control	-	-

Keys: - = showed no reaction. Mean values ± standard errors of the sum of three independent replications (n = 3)

4.3.5 MIC

The results presented in Table 4.4 showed that the highest inhibition zones were observed from strains LBC4ME (16.2 ± 0.10 mm), LBC22HL (16.3 ± 0.15 mm) and LBC35MW (16.1 ± 0.09 mm) at the concentration of 10^{-1} µg/L and the lowest inhibitory concentration was found to be at 10^{-8} µg/L by showing inhibition zones from strains LBC4ME (1.4 ± 0.19 mm), LBC22HL (1.5 ± 0.09 mm) and LBC35MW (1.3 ± 0.17 mm). No (-) inhibition zones were observed from the control plate.

Table 4. 4: Presenting results for MIC of extracted crude products from studied strains capable of inhibiting *F. graminearum* FG1

Diameter of inhibition zones (mm)				
Minimum inhibitory concentration ($\mu\text{g/mL}$)	Bacterial strains			
	LBC4ME	LBC22HL	LBC35MW	Control
10^{-1}	16.2 ± 0.10	16.3 ± 0.15	16.1 ± 0.09	-
10^{-2}	13.0 ± 0.13	15.0 ± 0.18	13.3 ± 0.11	-
10^{-3}	11.2 ± 0.11	12.2 ± 0.13	11.5 ± 0.19	-
10^{-4}	8.3 ± 0.15	9.1 ± 0.12	8.4 ± 0.19	-
10^{-5}	6.1 ± 0.18	8.1 ± 0.16	7.2 ± 0.12	-
10^{-6}	4.2 ± 0.12	7.1 ± 0.09	5.1 ± 0.14	-
10^{-7}	3.0 ± 0.16	3.9 ± 0.17	3.5 ± 0.15	-
10^{-8}	1.4 ± 0.19	1.5 ± 0.09	1.3 ± 0.17	-

Keys: - = Did not show any antifungal activity. Mean values $\pm$ standard errors of the sum of three independent replications (n = 3)

4.4 Discussion

4.4.1 Screening assays for biosurfactant production

The application of an efficient fermentation medium is found to aid potent bioactive metabolites production such as lipopeptide biosurfactants from most PGPR strains including the *Bacillus* species (Pandini et al., 2017). The results presented on Table 4.2 showed that the strains LBC4ME, LBC22HL and LBC35MW were able to produce surface-active substances by testing positive from the conducted experiments. These results are similar to the findings of Bezza and Chirwa (2015) who also researched *Bacillus* spp. that tested positive for biosurfactant production assay tests (lipase, drop collapse, oil displacement and E24% index). The results from Table 4.2 also showed that motor oil had the highest emulsification index percentage range (46-55%) measured from the LBC4ME, LBC22HL and LBC35MW strains and vegetable oil had the lowest percentage index range (42-49%). These results are in agreement with the findings of Sriram et al. (2011) who also reported higher emulsification index percentages from using motor oil (80%) and lower index percentages from the vegetable oil (50%) when researching surface activity from biosurfactants produced by *Bacillus* spp. using the same method applied in this study. The emulsification formation is stated as the stable interaction of hydrophobic and hydrophilic phase, thus, indicating that the higher E24 index amount shows, is a higher ability of the surfactant production potential, therefore, the values

obtained for the emulsifying index (Table 4.2) suggested that the bacterial strains LBC4ME, LBC22HL and LBC35MW expressed functional activity of the attained biosurfactant. Anburajan et al. (2015) stated that in terms of surface activity, a high E24 index amount promotes an effective additional emulsifying property to enhance stability through environmental applications.

4.4.2 Molecular characterization for biosynthetic genes with antifungal traits

The results presented in Figure 4.1 for PCR analysis showed positive gel electrophoresis amplifications of bands, which indicated that strains LBC4ME, LBC22HL and LBC35MW encoded the genes for the production of lipopeptide biosurfactant antibiotic biosynthesis (fengycins, iturin A, surfactins and bacillomycin D). These results suggested that the above-mentioned strains could produce surfactins, iturin A, fengycins and bacillomycin D lipopeptides commonly found to be produced by most studied *Bacillus* species.

These results are also similar to the findings of Ayed et al. (2014) who also reported *Bacillus* spp. that had positive amplifications for the above-mentioned genes and further evaluated that the detection of these genes by PCR proved positive to the functionality and production of this lipopeptide biosurfactants, thus, enabling them to exhibit a broad spectrum of antifungal activities (Gond et al., 2015). There are several molecules with similar structures and biosynthesis like fengycins, iturins and surfactins, however, these are reportedly known to be the three main family members of the lipopeptide biosurfactants commonly produced by *Bacillus* species (Christova et al., 2015).

4.4.3 Characterization of the biosurfactant

4.4.3.1 TLC

The results presented in Figure 4.2 and 4.3 showed the TLC analysis using crude extracts from strains LBC4ME, LBC22HL and LBC35MW representing the existence of lipids and peptide on the stained spots with R_f 0.75, 0.40 and 0.20 which are commonly known to represent the presence of surfactins, iturin A and fengycins, respectively. Thus, suggesting the presence and nature of the lipopeptide biosurfactants from the extracted crude products. These results are similar to the findings of Balan et al. (2017) who also detected spots with R_f values 0.45 and 0.73 known to be commonly reported to represent the presence or production of lipopeptide biosurfactants from previous studies researching *Bacillus* spp. with lipopeptide biosurfactant properties.

Alajlani et al. (2016) reported eight different spots with different R_f (0.08, 0.12, 0.21, 0.37, 0.49, 0.53 and 0.57) values and associated them with lipopeptide biosurfactants when using the same method with similar conditions like the current study to characterize lipopeptide biosurfactants produced by *Bacillus* species. Based on the laboratory production scale, the most widely used procedure to extract lipopeptide biosurfactants from microorganisms is acid precipitation. This procedure is reported to enable the solubility of the long fatty acid chains with organic solvents, thus, extraction using methanol, chloroform and distilled water displayed the presence of lipopeptide compounds (Christova et al., 2015).

4.4.3.2 FTIR

The results presented in Figures 4.4-4.6 showed that the studied strains LBC4ME, LBC22HL and LBC35MW had strong peaks detected between the range 3269.42-3275.24/cm. According to Bezza and Chirwa (2015), the detection of values between these ranges often indicated the presence of peptides (NH stretching mode).

From the strong peaks detected from crude extracts obtained from strains LBC4ME (2923.47/cm and 1625.76/cm) showed in Figure 4.4, LBC22HL (2923.49/cm and 1625.31/cm) showed in Figure 4.5, and LBC35MW (2923.35/cm and 1641.04/cm) showed in Figure 4.6 represented the occurrence of sp³ hybridized carbon and peptide bonds. According to (Jasim et al., 2016) these results suggested the presence of lipopeptides structures from the three studied strains. Peaks detected from strains LBC4ME (1719.78/cm and 1221.56/cm) showed in Figure 4.4, and LBC35MW (1719.38/cm and 1221.92/cm) showed in Figure 4.6 represented the existence of hydrophilic or carbonyl groups (-OH and -C=O) from the crude extracts of the studied strains.

Peaks detected from strain LBC4ME (1522.03/cm, 1625.76/cm and 1444.96/cm) in Figure 4.4, LBC22HL (1625.31/cm, 1539.29/cm) in Figure 4.5, LBC35MW (1641.04/cm, 1536.96/cm, and 1443.53/cm) in Figure 4.6 represented characteristics of surfactin like lipopeptides and also indicated the activity of biosurfactant production through the presence of peptide-like moiety and aliphatic hydrocarbons (-CH₃, -CH₂-) (Biniarz & Łukaszewicz, 2017). Therefore, from the above-mentioned results, it is suggested that the peaks are mostly similar to most findings reported for *Bacillus* spp. that tend to produce lipopeptide biosurfactants (Pandan et al., 2017).

The structural identification of lipopeptides biosurfactants is composed of amphiphilic anionic cyclic compounds with peptidic moieties connected to the tails of β -fatty acids, they also have

short linear chains of amino acids joined to a fatty acid through either amides or an ester bond, the lipopeptide molecule is known to be conferred through a lactone bridge between the carboxy-terminal function of the peptide and the β -hydroxyl function of the acid, thus, explains the structures observed from analysis of the strains obtained from this study (Deepak & Jayapradha, 2015).

4.4.3.3 ESI-MS

The mass spectrometry of iturins and surfactins is reported to display a pattern of masses ranging between m/z 1000-1200, and fengycins ranging between m/z 1400-1600 (Ali et al., 2016). Therefore, the observed ion peaks m/z 1463.8087 from LBC4ME strain detected in Figure 4.7 and m/z 1463.8104 from LBC22HL strain detected in Figure 4.9 were approximately similar to the molecular weight of fengycin A fatty acid chain length, however, this ion peak was not revealed from the LBC35MW strain. Ion peaks m/z 1074.6452 and m/z 1044.6613 (Figures 4.8, 4.10 and 4.12) from strains LBC4ME, LBC22HL and LBC35MW, respectively, are reported to belong to the surfactin family (Ali et al., 2014). Ion peaks m/z 1060.6335 (Figures 4.7 and 4.11) from strains LBC4ME and LBC35MW, and m/z 1058.6686 (Figures 4.9) from strain LBC22HL belong to the iturin family, thus, suggesting these lipopeptide biosurfactants are potentially harboured by these strains. The results from Figures 4.7-4.12 with molecular weight ions having a difference of 2 Da are suggested to be a combination of unsaturated and saturated fatty acids of the fengycin family (Jasim et al., 2016).

The results reported for the ESI-MS analysis showed in Figures 4.7-4.12 revealed that each of the molecular ion peaks from all the studied strains LBC4ME, LBC22HL and LBC35MW were detected under different retention times ranging between (1-4 min) and they differed from each other by several molecular weight differences which indicate different lipopeptide isomers and homologues with different numbers of CH_2 groups, thus, showing a complex mixture of lipopeptide biosurfactant family (Ali et al., 2016). These results are in accordance with the results reported by Sharma et al. (2014) who also detected molecular ions with different molecular weights under different retention times, thus, affirming the production of three lipopeptide biosurfactant families known to be produced by some *Bacillus* spp. with PGP attributes.

Lipopeptide biosurfactants represent an attractive class of bioactive secondary metabolites with antibiotic properties, this includes, iturin A, as it is reported to have acyl chain lengths of C14-C17, fengycins have fatty acyl chain lengths with a double bond of C14-18 and C15-17 and

surfactins have fatty acyl chain lengths of C12-C16 (Mnif et al., 2016). Deepak and Jayapradha (2015) reported the detection of molecular ions separation times between 6-25 min, which was more time compared to the current study which displayed separation times between 1-4 min (Figures 4.7-4.12).

A previous study has also successfully showed that some bacterial strains attain surface-active metabolites with hydrophobic and hydrophilic substrates, whereas, others can only attain one (Singh & Cameotra, 2013). This study has displayed the detection of cyclic lipopeptide biosurfactant attaining both hydrophobic and hydrophilic substrates produced by the studied bacterial strains as shown in Figures 4.7-4.12. Ayed et al. (2014) highlighted that each isoform group can have a difference in length of the fatty acid chain and still belong to the same family group, thus, suggesting that this isoform may contain different molecular weights. This explains the detection of different isoforms with molecular weight differences (Da) obtained from this study as shown in Figures 4.7-4.12.

Many microorganisms such as bacteria, fungi, and actinobacteria are capable of producing different biosurfactants with a variety of chemical structures, namely, glycolipids, lipopeptides, polymeric surfactants, and phospholipids (Biniarz & Łukaszewicz, 2017).

4.4.4 Antifungal activity by lipopeptide biosurfactant

The extracted lipopeptide biosurfactants showed effective antifungal activity through inhibition zones as shown in Table 4.3 which were observed from plates after incubation. Similar to this study, Liu et al. (2014) reported the extraction of lipopeptide biosurfactants iturins, surfactins and fengycins from *Bacillus* spp. with antifungal properties against plant pathogens, and also demonstrated that these molecules had the potential to be inhibitors of phytopathogenic growth. Mnif et al. (2016) together with Whillenbacher et al. (2015) also carried out a research and classified lipopeptide biosurfactants that were produced by *Bacillus* spp. and evaluated that they have a production of antimicrobial substances, consequently, the above mentioned lipopeptide biosurfactants were evaluated as fundamental characteristic features for biological control agents.

The results shown in Table 4.3 from the determination of the surface tension showed efficiency from the lipopeptide biosurfactants extracted from the strains LBC4ME, LBC22HL and LBC35MW. The surface tension values obtained from this study in the range 29-34 mN/m, fell within the expected range $\pm$ 27-36 mN/m commonly reported for studies on lipopeptides

biosurfactants surface tension for *Bacillus* species (Sharma et al., 2014). According to Liu et al. (2014) the higher the reduction value by surface-active compounds from 72 mN/m, is considered to have an effective surface activity ability, thus, suggesting the studied strains were able to efficiently produce surface-active compounds.

4.4.5 MIC

The MIC determination presented in Table 4.4 showed that the strains LBC4ME, LBC22HL and LBC35MW were able to produce lipopeptide biosurfactants with high stability and antifungal effectivity at the lowest concentration of 10^{-8} , thus, indicating strong antifungal properties. Zalila-Kolsi et al. (2016) reported a minimum concentration of about 0.6% under the same conditions when researching the minimum concentration required to inhibit *F. graminearum*.

4.5 Conclusions

The results from this study successfully showed that *Bacillus* spp. strains LBC4ME, LBC22L and LBC35MW were able to produce lipopeptide biosurfactants which had effective antifungal properties against *F. graminearum* FG1. The findings obtained from preliminary screening for biosurfactant production showed positive results for production ability. The TLC, FTIR and ESI-MS analysis also detected successfully the presence of spots, peaks and mass spectra, respectively, which are commonly reported to represent the presence and biosynthesis of lipopeptide biosurfactants from most studied *Bacillus* spp. The results from determining the MIC showed that the extracted biosurfactants have antifungal activity even at minute concentrations, thus, suggesting each strain as a potent potential BCAs for *F. graminearum* and other applications. The results from this study indicated that lipopeptide biosurfactants produced by the *Bacillus* spp. LBC4ME, LBC22HL and LBC35MW bacterial strains had antifungal activity against the targeted pathogen *F. graminearum* FG1. Thereby suggesting that these strains may be applied as an effective BCAs alternative to synthetic pesticides. Regarding the constant search for novel antibiotics against microbial pathogens, this study has presented a systematic approach to detect and apply such microorganisms.

CHAPTER 5

GENERAL DISCUSSION

Phytopathogen *F. graminearum* is a prevalent concern in food safety and security by causing a wide range of diseases resulting in reduced crop yield and product quality in agriculture, plus, the applied synthetic pesticides are posing distressing toxic effects to humans and the environment, therefore, safety and effective inoculant alternatives are necessary to improve the current applied practises (Yamamoto et al., 2015). The results obtained from this study showed that bacterial strains isolated from the rhizosphere of a healthy desired host plant may be used effectively as BCAs against specific soil-borne pathogen *F. graminearum* species as shown in Table 3.2. These findings are in agreement with Ali et al. (2014) who also reported that PGPR applications, in terms of *in vitro* treatments for screening strains against a fungal pathogenic mycelial growth proved to be highly effective and consistent among the studied *Bacillus* spp. strains and also displayed competitive attributes and survival under extreme growth conditions. The predominance of the genus *Bacillus* spp. as BCAs is considered an effective strategy for pathogen control (Moussa et al., 2013).

In another study by Wong et al. (2015) researching antifungal activity using *Bacillus* spp., about 50-85% of the studied strains gave potent inhibition rate (60-90%) results and efficiency as BCAs inoculants. The results of this study showed that *Bacillus* spp. strains (LBC4ME, LBC22HL and LBC35MW) showed about 63-66% fungal inhibition against *F. graminearum*. These values were similar to the values (28-74%) reported by Shi et al. (2014) when showing inhibition against a targeted fungal pathogen.

A previous study by Liu et al. (2013) also reported that their studied *Bacillus* spp. were evaluated to possess a wide variety of antagonistic mechanisms which included secretion of hydrolytic enzymes, phytohormones, PGP traits, volatile and organic compounds through different direct and indirect active pathways compared to the other studied genus of bacterial species which did not have a wide variety. The results from this study showed that the studied strains (LBC4ME, LBC22HL and LBC35MW) were able to inhibit *F. graminearum* FG1 strain growth under varied growth (pH, temperature, nitrogen and carbon sources) conditions as shown in Figure 3.2, this is similar to the study carried out by Zhang et al. (2016) who have shown that most *Bacillus* spp. can reduce their targeted fungal mycelial cells under varied growth conditions such as acidic (1-5) or alkaline (8-14) pH, high temperature (45-100 °C) and

low temperature (0-4 °C), with different carbon sources (Glucose, D-fructose, sucrose, and D-galactose), and nitrogen sources (KNO₃, NH₄NO₃, (NH₄)₂SO₄, and urea).

According to Ghyselinck et al. (2013) the biological control traits expressed by most PGPR may be used as an environmentally useful alternative practice to improve plant growth and protection against microbial invaders due to their natural occurrence in the soil. The authors also stated that there may be some inconsistencies in terms of PGPR performance in the screen-house compared to field experiments, further, highlighting the impact of available nutrients in the soil and a lack of competition from other microbes. From this current study, it could be suggested to test the LBC4ME, LBC22HL and LBC35MW strains for further field tests to compare disease severity performance with the screen-house results as shown in Table 3.5.

According to Jha and Saraf (2015) most PGPR studies have managed to obtain *Bacillus* spp. as an often found/ regular genus presently culturable from the soil. These findings indicated that this genus is an easily accessible and a natural antimicrobial candidate. Additionally, findings from this study also found *Bacillus* spp. as the most impressive genus to reduce fungal mycelial growth through antifungal assay tests as shown in Table 3.2 compared to the other isolated strains which were not further identified due to their lower antifungal activity. Also due to the development of fungal antibiotic resistance, the constant search for novel BCAs is necessary to prevent and manage persisting occurrences and further improve the already practised disease control strategies, and hence, the current study contributes to the constant journey of attaining potent indigenous BCAs (Al-Munjahindy et al., 2013).

According to Ghyselinck et al. (2013) the 16S rDNA gene sequence analysis fell short to distinguish beyond the genus *Bacillus* spp. identification, thus, leaving an unresolved gap for closely related species like the *B. thuringiensis* and *B. cereus* strains. Results from this study also showed difficulties in discriminating between the *B. cereus* and *B. thuringiensis* strains as shown in Figures 3.5-3.8 and Table 3.4, and therefore, further species specific-gene sequence analysis proved to be a necessity and a recommendation to identify closely related strains.

The 16S rDNA gene nucleotide sequences of the other reference strains were obtained by BLASTN search and the *gyrB*, *rpoB*, *tuf*, surfactins, bacillomycin D, fengycins and iturin A protein-coding gene sequences of other reference strains were obtained by BLASTX. The results from the Figures 3.5-3.8 and Table 3.4 showed that all the selected reference strains were selected based on high similarity (%) and a low E value, and therefore, the overall similarity (%) for the queried gene sequences of the above-mentioned gene sequences showed

the best pairwise alignment was with the *Bacillus* species, the BLAST search concluded that the isolated rhizobacterial strains were members of the GC-rich *Bacillus* species, in phylum order of Firmicutes. The selected molecular approach has displayed additional clarification and discrimination to further identify phenotypically close strains using protein-coding gene sequences (Caamano-Antelo et al., 2015; Rosselló-Móra & Amann, 2015).

The detection of the lipopeptide biosurfactant biosynthesis genes (iturin A, surfactins, bacillomycin D and fengycins) applied in this study was relatively strains specific (Figure 4.1), hence, the utilisation of protein-coding genes specific for the identification of lipopeptides biosurfactants produced by *Bacillus* spp. was followed (Balan et al., 2017). The control of plant diseases using unconventional practises is recommended to be a preferred alternative to pesticide usage, due to previous effective applications of bacterial inoculants which proved an effective control of numerous pathogens both *in vivo* and *in vitro* conditions (Beukes et al., 2017). The search for potent PGPR strains with biocontrol properties against soil-borne plant pathogens is crucial to explore as alternative natural product applications (Liu et al., 2013).

The results from this study showed the detection of a present single plasmid in the studied strains (LBC4ME, LBC22HL and LBC35MW) as shown in Figure 3.4, this suggested that the above mentioned strains exhibited antimicrobial metabolites which may be chromosomally or plasmid-mediated traits. Another study has reported the lipopeptide biosurfactants (surfactins, iturin A, bacillomycin D and fengycins) by several *Bacillus* spp. to be plasmid encoded, whereas, IAA was found to be encoded in the chromosome and the other secondary metabolites were not reported (Munees & Mulugeta, 2014).

Considering the disadvantages and manufacturing processes that are accompanied by making synthetic products, PGPR are diverse, exist freely and unlimited in the soil, hence, a gap for further exploration is always open (Courty, 2015). The rhizosphere region of the soil highly influences plant growth due to the presence of a diverse microbial community (Bishnoi, 2015). Therefore, in this study, rhizosphere soil was collected to obtain host-specific bacterial strains that can be used effectively as antimicrobial inoculants. As microorganisms are known to exist in similar colonies and other diverse communities, it is necessary to obtain operative formulations of BCAs to create a synergic environment between a plant host and bacterial inoculants (Kurabachew & Wydra, 2013).

A synergy between the inoculants could enable fast growth and reduce pathogen proliferation through the cell to cell communication and promote optimum living conditions (Hyakumachi

et al., 2013). Hence, the results in Table 3.5 and Figures 3.9-3.12, presented from this study showed reduced plant disease severity in pots treated with a combination of the bacterial cells from strains LBC4ME, LBC22HL and LBC35MW which did not inhibit each other when cultivated on the same growth medium plate. This indicated that the above-mentioned strains had a synergistic relationship and could've communicated a biocontrol attributed behaviour, and therefore, thrived better under new growth conditions (Zhang et al., 2016). Other studies have also reported similar successful suppression of plant disease incidences when using treatment containing combined bacterial strain mixtures compared to individual strains, pathogen only and control treatments in pot experiments under the same growth conditions that were conducted in this study (Wong et al., 2015; Jasim et al., 2016).

Szentes et al. (2013) also reported on the genus *Bacillus* spp. using bacterial strains (*B. thuringiensis* and *B. subtilis*) which were able to exhibit several antimicrobial activities against a fungal pathogen. The properties included the production of surface-active lipopeptide biosurfactants (iturin A, surfactins, bacillomycin D and fengycins), and further suggested that many of the isolated *Bacillus* spp. strains presented results which indicated similarities between the *Bacillus* spp. strains. As shown in Figures 4.2-4.12, using chromatography TLC and spectrometry (FTIR and ESI-MS) analysis and from Figure 4.1 using molecular (surfactins (As1f/Ts2r), fengycins (Af2f/Tf1r), Iturin A (ItuDf/ItuD1r), Bacillomycin D (Bacc1f/Bacc1r] gene sequencing by PCR) techniques applied in this study, the results showed successful detection of commonly detected surface-active substances and similar results were obtained by other authors who used similar detection methods (Anburajan et al., 2015; Gond et al., 2015).

Georget et al. (2015) and Balan et al. (2017) stated that successful extraction, purification and classification of bioactive metabolites that are produced by PGPR may lead to a clearer understanding of the main operative systems of the organisms and exploiting their chemical benefits to gain insights involving pathogen control. From the results presented in Table 3.2, Table 3.3 and Figures 4.2-4.12, the broad spectrum production of metabolites through both direct and indirect mechanisms observed in this study was thought to potentially contribute with different developmental, physiological, molecular and cellular modes of actions against plant pathogen *F. graminearum* sp. when present in the host plant, and therefore, evaluating that the aforementioned metabolites are a basic foundation for the initiation of selecting effective biocontrol candidates in the genus *Bacillus* spp. against the above mentioned disease-causing agent (Hyakumachi et al., 2013; Lee et al., 2015; Jasim et al., 2016).

GENERAL CONCLUSIONS

The results generated from this study showed that the maize rhizosphere soil from local North-West province areas in SA contained PGPR which was able to suppress *F. graminearum* under *in vitro* antifungal assay results. This study successfully achieved its aim by performing the specific objectives through the collection, screening, and selection of effective bacterial strains which were capable of inhibiting *F. graminearum* FG1 strain on maize under *in vivo* pot treatments. The results obtained from this study reported novel *Bacillus* spp. strains (*B. thuringiensis* LBC4ME, *B. velezensis* LBC22HL and *B. thuringiensis* LBC35MW) which remarkably exhibited numerous antifungal characteristics through different direct and indirect mechanisms against the fungal pathogen *F. graminearum* FG1 strain under both *in vitro* and *in vivo* conditions, and therefore, the selection of the potential biocontrol rhizobacteria inoculant strains was successfully carried out. Using conventional and molecular techniques, the taxonomic identity of the strains was found and characterized effectively to belong in the Firmicutes phyla, genus *Bacillus* species. Using spectrophotometric and chromatography techniques, the bioactive molecular compounds were successfully detected and characterized for the selection of antimicrobial characters that were revealed on the spectral scan analysis. This study positively presented the use of antifungal assays, detection of PGP traits, hydrolytic enzyme production, antibiotic susceptibility tests, biochemical, morphological, physiological, molecular, screen-house experiments, spectrophotometric chromatographic approaches to attain the taxonomy and the molecular selection of bacterial strains isolated from the rhizosphere of maize which has antifungal activities against *F. graminearum*. The methodology applied in this study has displayed a rapid system of the selection process and obtaining biocontrol PGPR against the plant pathogen *F. graminearum*. This study validated that the indigenous bacterial strains LBC4ME, LBC22HL and LBC35MW harboured a broad spectrum of antifungal activities which may be explored for further antimicrobial activity, thus, suggesting the bacterial strains obtained from this study to be prominent BCAs with anti fungicidal attributes.

FUTURE RECOMMENDATIONS

Future studies may be focused on testing the antifungal efficiency of novel strains through *in vitro*, screen-house and further under field conditions. Further studies may also be done to compare the biological control efficiency of the LBC4ME, LBC22HL and LBC35MW strains as antimicrobials against other plant disease-causing agents. Future work can be done using the *Bacillus* spp. strains to further research the cytotoxicity of the produced lipopeptide biosurfactants for safety applications in the food, mine and medical industries. The obtained crude products may be studied for further antimicrobial properties using different groups of microorganisms (e.g. Protozoa or viruses).

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