Impact of Field-Grown Genetically Modified Maize on Native Rhizobacteria

By

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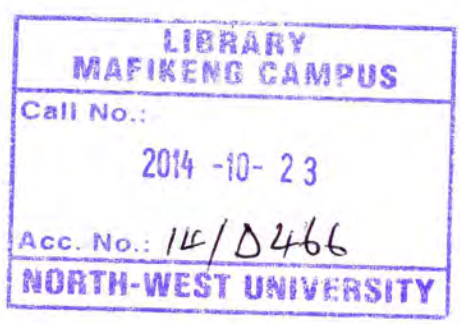

DECLARATION

I, the undersigned, declare that the thesis hereby submitted to the North-west University Mafikeng Campus for the degree of MSc. (Biology) and the work contained therein is my own original work and has not previously, in its entirely or in part, been submitted to any university for a degree. All materials used have been acknowledged.

Signed _____ this the 19 day of December 2013

DEDICATION

I dedicate this work to my loving parents Mr Bumunang Peter V and Mrs Bumunang Francisca A. for their help and support.

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The printed pages of this thesis hold far more than the culmination of years of study. These pages also reflect the relationships with many generous and inspiring people I have met since the beginning of my research work. The list is long, but I cherish each contribution to my development as a scholar.

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ABSTRACT

Plant-root interaction occurs in the rhizosphere, a region referred to as a biologically active zone of soil where microorganisms and plant roots interact. This study examined the impact of genetically modified (GM) maize (Bt) on functional community of rhizobacteria. Soil samples of field grown GM and non-GM maize were collected from an experimental field in Delmas, South Africa, at 30 days after sowing (DAS) and 1 day after harvest (DAH). Chemical analyses of soil properties in GM and Non-GM soil samples were performed. Quantitative analysis was achieved through soil dilution and plate count (colony forming units) using selective and non-selective media (tryptic soy agar, nutrient agar, Luria Bertani agar and *Pseudomonas* selective agar). Qualitative analysis was achieved using analytical profile index identification systems and sequence data of rhizobacterial isolates. Biolog GN2 microplate was used to compare community catabolic profile of rhizobacteria in GM and non-GM soils and denaturing gradient gel electrophoresis technique (DGGE) was used in comparing rhizobacterial community profiles in GM and non-GM soil samples.

Chemical analyses of GM and non-GM soil samples collected 30 DAS and 1 DAH indicated the same elements with similar percentages. The pH of GM and non-GM soil samples range from 6.12-7.03, indicating slightly acidic to slightly alkaline soil. Total count of rhizobacteria (cfu/g) in GM and non-GM maize soil samples collected 30 DAS and 1 DAH was not significantly different in the media. Similar rhizobacterial species from the rhizosphere of both GM and non-GM maize were identified using analytical profile index and sequence data. No significant difference was observed in the community catabolic profile among the rhizobacteria in GM and non-GM soil samples. Cluster analyses of DGGE bands indicated that band patterns of GM and non-GM samples 30 DAS and 1 DAH were similar to each other. These findings suggest that the

GM maize was not able to alter microbial community and activity and are significant to the investigation of the impact of GM maize on rhizobacteria.

Keywords: Genetically modified maize, Rhizosphere; Rhizobacteria, DGGE and Biolog.

LIST OF ABBREVIATIONS

AHL	: Acylated homoserine lactone.
API	: Analytical profile index.
AWCD	: Average well colour development.
$AWCD_G$	: Group-wise average well colour development.
BLAST	: Basic local alignment search tool.
Bt	: *Bacillus thuringiensis.*
DAH	: Day after harvest.
DAS	: Day after sowing.
DGGE	: Denaturing gradient gel electrophoresis.
DRB	: Deleterious rhizobacteria.
GMO	: Genetically modified organisms.
GMPs	: Genetically modified plants.
GN	: Gram-negative.
HCN	: Hydrogen cyanide.
ISAAA	: International service for the acquisition of agric-biotect applications.
LBA	: Luria Bertani agar.
NA	: Nutrient agar.
NADH	: Nicotinamide adenine dinucleotide plus hydrogen.
NTOs	: Non-target organisms.
OM	: Organic matter.
PCR	: Polymerase chain reaction.

PGPR	: Plant growth promoting rhizobacteria.
PSA	: *Pseudomonas* selective agar.
SCSUP	: Sole carbon substrate utilization patterns.
Ti	: Tumour inducing.
TSA	: Tryptic soy agar.

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CHAPTER ONE

INTRODUCTION

Genetically modified plants (GMPs) are plants carrying genetic traits that are not naturally present in them (Andrew et al., 2006). These plants are modified for specific reasons that include long life span, resistance to pest or diseases, nutritional improvement, herbicide tolerance or resistance to abiotic factors such as drought and nitrogen deficiency (Isik and Guenther, 2008). Since the first commercialization of GM crops in 1996 the planting of GM crops has increased by 10% or more each year worldwide (Isik and Guenther, 2008). In 2009, a total of 28.7 million hectares of GMPs were planted worldwide compared to 26.9 million hectares in 2008 (James, 2009). However, the possible effects of GMPs on human health and ecological functioning have been debated extensively (Sessitsch et al., 2004). Brusetti et al. (2004) studied exudates of *Bacillus thuringiensis* (Bt) maize (a genetically modified maize) and of its non-Bt counterpart and reported differences in the microbial communities in the rhizosphere of Bt maize. On the contrary, Devare et al. (2004) found that genetically modified maize had no effect on microbial communities in the rhizosphere. Nevertheless, the possible impact of GMPs on rhizobacteria should be considered. Some genetically modified plants have successfully targeted changes in microbial community composition, by inhibiting plant pathogenic organisms (Ahrenholtz et al., 2000). However, most studies show either minor non-target effects (Hopkins et al., 2001) or no detectable non-target effects (Saxena and Stotzky, 2001; Heuer et al., 2002);

Plants are major drivers of soil ecosystems and provide fundamental services like the regulation of water quality and quantity, nutrient cycling, carbon sequestration and the bioremediation of waste that supports plant growth (Andrew et al., 2006). Soil anchors plants and harbours a diverse range of microorganisms (bacteria, algae and fungi). Plant-root interaction occurs in the

rhizosphere. This region is referred to as a biologically active zone of soil, and is of major importance for plant growth as well as for nutrient cycling and ecosystem functioning (Singh et al., 2004). Root exudates in the root-soil interface create a unique microbial microenvironment, differentiating it from bulk soil not influenced by the roots (Hartman et al., 2007). Furthermore, root exudates quality and quantity vary with the plant developmental stage, plant species and contribute to microbial community structure (Berg et al., 2002). Different rhizosphere populations have been reported in different plant species at different plant growth stages (Somers et al., 2004). Maize root exudates are composed of 33% organic acid, 65% sugars and 2% amino acids, leading to different rhizobacterial community structures (Baudoin et al., 2003). Species of *Pseudomonas*, *Azospirilum*, *Azotobacter*, *Bacillus*, *Arthrobacter*, *Serratia*, *Rhizobium*, *Beijerinckia*, *Zoogloea*, *Sinorhizobium* and *Mesorhizobium* have been reported as native rhizosphere organisms (Esitken et al., 2003; Babalola, 2010a).

Bt maize contains insecticidal toxic proteins (Bt endotoxins) that have advantages for crop production, such as increased insect resistance, grain yield and plant growth (Obrycki et al., 2001). A good knowledge of the effects of GMPs on soil microorganisms is important for implementing assessments techniques that could reduce any negative impacts (Fang et al., 2007). In order to ensure a suitable choice of criteria and method for risk assessment and monitoring the effects of GMPs on bacteria community in soil, some keystone indicators have been recommended (Kowalchuk et al., 2003) such as soil natural antagonistic organisms like *Pseudomonas*. Species of *Pseudomonas* exhibiting plant growth-promoting traits and pathogen-suppressing characteristics could be harnessed as biofertilizers and biological control agents (Keel et al., 1992).

1 Problem statements

Much work has been done to investigate the environmental impacts of GMPs "above ground" compared to the limited research that has been directed towards the impact of genetically modified organisms (GMOs) on soil microorganism and processes (O'Callaghan and Glare, 2001). While molecular profiling techniques such as proteomics, transcriptomics and metabolomics have been used (Barros, 2010) to detect unintended effects in GM maize, the impact of GMPs on soil microorganisms is often neglected because of the difficulties involved in their study (Andrew et al., 2006). However, the role played by microorganisms such as mineralization and immobilization of nutrients, biological control of plant pest and food source for other organisms needs to be considered. Therefore, cultured-independent technique such as polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) method for evaluating rhizobacteria community profile will be employed in this study. Further information on rhizobacteria activity and population will be obtained by using cultured-dependent method such as Biolog plates coupled with general and selective agar isolation. These will surely:

- Give meaningful information to the public about GMPs' impact on rhizobacteria.
- Contribute to a more accurate assessment of the impact of GMPs on rhizobacteria.

2 Hypotheses

Field grown genetically modified maize affect rhizobacterial population, diversity and activity.

3 Aims

To assess the impact of field grown GM maize on native rhizobacteria.

4 Objectives

The objectives of the research were to:

- Assess and characterize rhizobacterial population in field grown GM and non-GM maize rhizosphere.

- Assess the rhizobacterial catabolic fingerprints and community profile in field grown GM and non-GM maize soil samples.

CHAPTER TWO
LITERATURE REVIEW

2.1 Genetic modification of plant

2.1.1 Plant transformation

Plant transformation is defined as the firm introduction and expression of foreign genes into plants (Van den Eede et al., 2004). This process involves several ways such as the use of *Agrobacterium* Ti (tumour inducing) plasmid by direct uptake of foreign DNA in to the protoplast serving as biological delivery system (Jian-Wei Liu, 2006). Ti plasmid of *Agrobacterium tumefaciens* is a circular and double-stranded ring form DNA with the size of 200kb. The size of DNA and the ability to induce tumour makes it the most widely used for plant transformation (Jian-Wei Liu, 2006). Ti plasmids carrying transfer DNA (T-DNA) is inserted in to the plant through exposed wound plant cells (hence integrated into plant chromosomes at random) and particle bombardment (Van den Eede et al., 2004).

Genes of interest used in plant transformation are genes encoding insecticidal toxins from *Bacillus thuringiensis*, chitinase from fungi, virulence factor of nematodes and mycoplasma, herbicide resistance enzymes from bacteria, avirulence factors of bacteria and fungi and viral coat proteins of plant viruses (Jian-Wei Liu, 2006). Inserted genes may be useful in providing resistance to bacterial, viral and fungal plant diseases, herbicide tolerance, insect diseases resistance, tolerance to abiotic factors and production of industrial chemicals (Sessitsch et al., 2004).

2.2 GMPs cultivation

2.2.1 Worldwide status

Genetic modification being the most recent technology is receiving greater attention worldwide and scepticisms as to it human and environmental impacts. Since the first commercialization of

GMPs in 1996, its cultivation worldwide has increased dramatically reaching 148 million hectares globally in 2010 (James, 2009). Canada, United States of America (USA), Argentina and Brazil are the leading exporters of GMPs in the world (Davison, 2010) with USA topping the list of GMPs exporters.

According to International Service for the Acquisition of Agric-Biotech Applications (ISAAA) report (2011) 19 out of 29 countries involved in GMPs cultivation worldwide are developing countries. As of 2011, the principal GMPs planted worldwide stood at soybean occupying 47% of global GMPs, maize second with 32%, thirdly by cotton with 15% and finally canola with 5% with main traits being herbicide tolerance 59%, stacked genes (combination of two genes) 26% and insect resistance 15% (ISAAA, 2011).

Table 2.1 Worldwide status of GMPs cultivation since 2006 and prediction for the year 2015 (James, 2011)

	Year		
	2006	**2010**	**2015**
Number of countries	22	29	About 40 million
Number of famers cultivating GMPs	10 million	15.4 million	About 20 million
Global GMPs area cultivated	100 million hectares	148 million hectares	About 200 million hectares

2.2.2 Africa

The growing problems of food security, poverty and environmental degradation, particularly in developing countries deem modern biotechnology application as the potential answer (Penalba et al., 2006). South Africa, Kenya, Burkina Faso and Egypt are the four African countries cultivating GMPs. According to James, (2011) by 2015 up to 10 African countries will be involved in GMPs cultivation i.e. South Africa, Burkina Faso, Egypt, Mali, Togo, Nigeria, Kenya, Uganda, Tanzania and Malawi. However, countries like Ghana and Benin are yet to approve biosafety bill (Black et al., 2011). Burkina Faso and South Africa have made a significant contribution to the income of small resource-poor farmers principally with GM cotton and maize (ISAAA, 2011).

Biosafety initiatives programmes concerning GMPs in Africa are not aligned with each other on the socio-economic impact involved in risk assessment and adoption (Black et al., 2011). This could be resulting from the lack of local expertise in developing countries to perform environmental risk assessment and post-released monitoring programs for GMPs (Arpaia, 2010.) coupled with lack of baseline biological information and limited local funds. Nevertheless, developing countries adoption of GMPs cultivation in 2011 stood at 50% of all global GMPs cultivation (ISAAA, 2011).

2.2.3 South Africa

South Africa became the first African country in 1997, to commercially produce GMPs. According to African Center for Biosafety (2010) South Africa tops the list of GMPs cultivation in Africa and is ranked as the eight largest producers of GMPs in the world. The GMPs approved for planting and commercial release in South Africa are cotton, maize and soybean. Since the first commercialisation of GMPs in South Africa, they have been an increase in the different

varieties of GMPs genes from single to stack genes. Main companies involved in the production of GMPs seeds and chemicals in South Africa are Monsanto a USA corporation, Pioneer a USA chemical company and Syngenta a British/Swiss corporation.

Table 2.2 Sequence of GMPs traits approved in South Africa (Biosafety South Africa, 2013)

Plant	Trait	Variety	Company	Year approved
Maize	Insect resistant	MON810 / Yieldgard	Monsanto	1997
Cotton	Insect resistant	Line 531 / Bollgard	Monsanto	1997
Cotton	Herbicide tolerant	RR lines 1445	Monsanto	2000
Soybean	Herbicide tolerant	GTS40-3-2	Monsanto	2001
Maize	Herbicide tolerant	NK603	Monsanto	2002
Maize	Insect resistant	Bt11	Syngenta	2003
Cotton	Insect resistant	Bollgard II, line 15985	Monsanto	2003
Cotton	Insect resistant and Herbicide tolerant	Bollgard RR	Monsanto	2005
Maize	Insect resistant and Herbicide tolerant	MON810xNK603	Monsanto	2007
Cotton	Herbicide tolerant	MON88913 (RR flex)	Monsanto	2007
Cotton	Insect resistant and Herbicide tolerant	Bollgard II x RR flex (MON15985x MON88913)	Monsanto	2007
Maize	Insect resistance	MON89034	Monsanto	2010
Maize	Insect resistance and Herbicide tolerant	MON89034xNK603	Monsanto	2010
Maize	Herbicide Tolerant	GA21	Syngenta	2010
Maize	Insect resistance and Herbicide tolerant	Bt11X GA21	Syngenta	2010
Maize	Insect resistance and Herbicide tolerant	TC1507	Pioneer	2012

2.3 GMPs' effects on soil microorganisms

The effects of Bt maize Cry endotoxins on soil microorganisms have been studied, with different results obtained. This might be as a result of different study sites, climatic condition or methodologies. Brusetti et al. (2004) reported that the chemical composition and protein toxin released by Bt maize in the root exudate is completely different from root exudate of its non-Bt maize counterpart. However, Icoz and Stotzky (2008) indicated that Bt- expressing plants cause no or minor changes in microbial communities, meanwhile lower ratios between Gram-positive and Gram-negative bacteria in soil with Bt-maize compared to control soils has been reported (Xue et al., 2005) while the effect was reversed in Bt-expressing potato. Castaldini et al. (2005) also reported repeated differences in rhizosphere heterotrophic bacteria and mycorrhizal colonization between Bt maize and its control. According to these reports, Bt maize might have led to changes in plant physiology and composition of root exudates, which in turn may have affected symbiotic and rhizosphere microorganisms. No difference in population dynamics of pink-pigmented facultative methylotrophs in the rhizosphere of both Bt and non-Bt-cotton has been reported (Balachandar et al., 2008).

Bt maize Cry endotoxins can enter the soil through root exudates, sloughed off root debris and plant residues. Bt toxins incorporated with soil can remain bound for a very long time (Crecchio and Stotzky, 1998). Therefore, the possibility of Bt toxins been picked up by plants roots through reabsorption or by microorganisms through adsorption stands a great chance. Changes in the physiology of plant and microorganisms caused by possible uptake of Bt Cry toxins can affect plant-microbe interaction, herbivores-plant interaction, insect-plant interaction and even human population. On the other hand it can also affect microbial activity and diversity, hence destabilising the ecosystem.

Soil contains many different species of microorganisms (bacteria, fungi and algae). Bacterial communities are known to be the dominant group of microorganisms in the soil. Biological processes such as mineralisation and immobilisation carried out by microorganisms strongly affects soil nutrients and productivity. This process which leads to the recycling of soil organic matter maintains the soil structure. Consequently, soil fertility an important ecosystem service, requires constant risk assessment of GMPs effects to avoid possible future environmental negative impacts. Non-target organisms (NTOs) are all living organisms that are not meant to be affected by newly expressed compounds in GMPs, and that can be potentially exposed, directly or indirectly to GMPs and/ or its products in the agro-ecosystem where GMPs will be released or in adjacent habitats (Arpaia, 2010).

Bt maize contains insecticidal toxic proteins (Bt endotoxins) that have advantages for crop production, such as increased insect resistance. For this reason, Bt endotoxins incorporated into the plant should targets insects and not microorganisms. However, they are environmental concerns that these endotoxins might interfere with microorganism's activity when incorporated by them. The incorporation of Bt endotoxins by soil microorganism produced by *Bacillus thuriengensis* a soil microorganism can be naturally possible through normal cell to cell contact. In this regard, soil microorganisms all fall in the category of NTOs. Assessing GMPs effects on soil microorganisms therefore, requires an in depth knowledge about these organisms. Nevertheless, most soil microorganisms are not culturable (Amann et al., 1995), due to the fact that they may enter viable-but-non culturable state and unknown growth requirements (Van Overbeek et al., 1995). In this case, most soil microbial communities are excluded when assessing the possible effects of GMPs. Possible criteria have been proposed which select species based on characteristics such as: their abundance in the specific environment, their susceptibility

to known agents that causes stress, their practicability of conducting laboratory tests (Cowgil and Atkinson, 2003; Andow and Hilbeck, 2004). Kowalchuk et al. (2003) recommends wood lignin decomposing fungi, nitrogen fixing and denitrifying bacteria, arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria (PGPR). New molecular biological technique such as (PCR-DGGE) is now been used in investigating the possible impact of GMPs on soil microorganisms (Stephen and Kowalchuk, 2002), coupled with cultured-dependent method such as community catabolic profiling, carbon mineralization and cultured based isolation method have been widely used to study the effects of GMPs on soil microbial communities and processes (Bruinsma et al., 2003).

Plants, anchored by soil use the nutrients released by microbial activity. Any change in the nutrient content of soil can strongly affect plant growth. A microbial interaction within the soil and root interphase is termed rhizosphere effect (Darrah and Roose, 2007). Plant roots secrete root exudates which selectively regulates the type of organisms in the rhizosphere (Berg et al., 2002). Therefore, quantitative and qualitative changes in root exudates could influence the diversity and activity of soil microorganisms (Icoz and Stotzky, 2008). However, microbial composition, quantity, quality and activity does not depend solely on root exudates but could also be influenced by temperatures, pH, geographical location, plant variety, soil type, developmental stage of plant and anthropological activity.

2.4 GMPs debates and profiling techniques

Political leaders globally are increasingly viewing genetically modified crops as a key part of the solution to critical social issues of food security and sustainability (James, 2009). However, there are concerns of the regulation of GM products. The Cartagena Protocol on Biosafety was

adopted in Montreal on 29 January 2000 as an international agreement. It arises from the Convention on Biological Diversity (CBD). The objective of this protocol is to "ensure the adequate handling, transfer and use of living modified organisms resulting from modern biotechnology that may have adverse effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health" (Secretariat of the CBD, 2000). The Cartagena protocol entered into force on the 11 September 2003. Therefore, risk assessment of GM plants should aim at identifying any adverse effect.

Regulatory bodies such as the European Food Safety Agency, the Department of Agriculture and Environmental Protection Agency in USA and the South African Advisory committee (AC) (advisory board of GMO) and Executive Council (EC) (the GMO decision-making body) are all required to comply with the Cartagena Protocol. One hundred and sixty-four countries are member states of the Biosafety Protocol regulating the application and commercialization of GMOs. Interestingly, USA which is ranked first in the production and commercialization of GMOs, is not a member state to this protocol.

Available data on the safety of GMOs is a prerequisite for regulatory approval of GM products released into the environment. Data collection for regulatory bodies' approval therefore, requires efficient profiling techniques for risk assessment. In South Africa, the AC is made up of researchers appointed by ministerial decree to carry out risk assessments on GMP applications. Recommendations by the AC are then forwarded to the EC for approval or disapproval.

Regulatory bodies and pro-GMPs organisations face confrontation from anti-GMPs activists such as non-governmental organizations. The church, especially the Roman Catholic Church has opposed the cultivation of GM crops (Liborio, 2007). However, one of the resolutions accepted in an international workshop for Islamic scholars on GMPs held in Georgetown, Penang,

Malaysia 2010, were that "Islam and science are complementary and Islam supports beneficial scientific innovations for mankind. Modern biotechnology and genetic engineering are important developments that merit promotion in all organization of Islamic conference members. Regulatory measures should facilitate the acceptance and use of GM products particularly by Muslims" (Salleh, 2012). These contradictory views from the religious sector give more strength to the pro-GMPs activist who argue that GMPs technology will increase crop yield, improve nutrient contain in foods and contribute to the goal of increased food security for the poor in developing countries. On the contrary, anti-GM activist deem this technology as a means for the rich to grow richer, a threat to human health and environmental biodiversity. All arguments, be it pro or anti-GM, requires stringent scientific bases to prove their point.

2.5 GMPs risk assessment profiling techniques

Profiling techniques are employed to identify possible dangers posed by GMO on human or animal health. One of such technique called "omic technology" is been used in studying the transcriptome, proteome and metabolome of GMPs and GM food products (Barros, 2010). Transcriptomics analyses expressed genes by GMOs employing complementary DNA microarrays technique, proteomics measures all the proteins translated from expressed genes using two-dimensional gel electrophoresis or microarrays of antibodies methods and metabolomics analyses chemical components derived from the actions of enzymes and transport proteins using gas chromatography coupled to mass spectrometry. Other omic techniques such as lipomics and glycomics which studies lipid and carbohydrate fractions in plants are also been used for GMPs profiling with respect to non-GM counterparts. However, the applicability of these techniques for the safety assessment of GMOs and GM food products needs to be standardized by establishing a database with its non-GM counterpart. Omic technology focuses

much on GMOs impact above ground meanwhile little attention is directed to soil microbial community.

Molecular biological techniques are now shedding light on the possible impact of GMPs on soil microorganisms (Stephen and Kowalchuk, 2002). Muyzer et al. (1993) introduced the PCR-DGGE technique used in studying microbial diversity. This technique is capable of detecting differences between DNA fragments of the same size but with different sequences (Hovig et al., 1991). DNA fragments of about the same length are produced by amplification of a target gene by PCR. These fragments are electrophoresed on a polyacrylamide gel containing a linear gradient of DNA denaturant such as a mixture of urea and formamide (Muyzer et al., 1993). The electrophoretic separation of double stranded DNA fragments is based on the differences in their melting behaviour in a gradient of the denaturing agent. The DNA sequence consists of "melting domains", described by (Muyzer and Smalla, 1998) as stretches of base-pairs with an identical melting temperature. According to Danilo, (2004) DNA fragment becomes partially melted once the melting temperature of the lowest melting domain is reached creating branched ''breaking'' molecules which decreased migration through the gel. The base pair guanine and cytosine has three hydrogen bonds, and two between adenine and thymine. As a result, more denaturant is needed to separate sequence with higher guanine and cytosine content due to differences in the number of hydrogen bonds between complementary nucleotides holding DNA strands together. In this regard, this technique can be used to compare many different samples of microorganisms with different melting domains. Difference in sample profiles (many bands in the gel) reflects the microbial diversity of the sample.

Additional information on soil microorganism activities and diversity can be obtained by studying the community catabolic profiles of soil microbes using Biolog plates technique

(Griffiths et al., 2002). The 96 wells of the microplate contain different carbon sources and the redox dye tetrazolium. Well one is the control well containing the redox dye with no substrate. Carbon compounds in the Biolog GN2 plates can be divided into carbohydrates (30), amino acids (20) and carboxylic acids (24), combined with other compounds in lower numbers such as polymers (5), amines/amides (6) and miscellaneous (10) (Preston-Mafham et al., 2002). The redox dye tetrazolium is soluble in water in its oxidised state and appears colourless. Microbial respiration produces nicotinamide adenine dinucleotide plus hydrogen which reduces tetrazolium dye to insoluble violet formazan complex. The rate of colour development in the wells gives information on the bacteria metabolic activity of the sample, while the diversity of colour in the wells gives information about microbial diversity of the sample (Stefanowicz, 2006).

2.6 Future prospects of GMPs

GMP cultivation has been in a high increased worldwide over the last 14 years with an exceptional increased rate in the United States of America (James, 2009). Tough political will coupled with appropriate and efficient regulatory bodies might lead to high adoption of GMPs cultivation in the future. This should include GMPs cultivation with improved genotype, higher yield potential associated with better tolerance to drought, salinity and nutrient limitation (Zhu and Ma, 2011). Although GMPs might be a potential means of promoting food security in the world, they are just one tool among many other technologies. Exploring the potentials of beneficial microbes for plant growth promotion is another option which can promote food security. These microbes are termed biofertilizers and biocontol agents and include a group of bacterial called plant growth promoting rhizobacteria (Juanda, 2005).

2.6.1 Plant growth promoting rhizobacteria

The rhizosphere is the region of the soil with plant roots, where by microorganisms are abundantly present (Ahemad and Mohammad, 2010). Root exudates in the root-soil interface create a unique microbial microenvironment, differentiating it from bulk soil not influenced by the root (Anton et al., 2007). The rhizoplane or root surface provides a highly favourable nutrient base and attachment site for many bacteria. However, in response to this microbial habitat and nutrients provided by the plant, some microbes assist the plant in making nutrients available, while others may cause harm acting as root pathogens (Anton et al., 2007). The (plant growth promoting rhizobacteria) PGPR can be divided into two groups according to their residing site, iPGPR (symbiotic bacteria) which live inside the plant cells, produce nodules and are localized inside those specialized structures, and ePGPR (free living rhizobacteria) which live outside the plant cells and do not produce nodules, but still prompt plant growth (Gray and Smith, 2005). Meanwhile bacteria that are able to colonize plant root or rhizosphere inhibiting plant growth directly or indirectly are called deleterious rhizobacteria (DRB).

Species of *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Bacillus*, *Arthrobacter*, *Alcaligenes*, *Serratia*, *Rhizobium*, *Beijerinckia*, *Zoogloea*, *Sinorhizobium* and *Mesorhizobium* have been reported to promote plant growth directly or indirectly (Esitken et al., 2003; Babalola, 2010a). The mechanism of action of PGPR in the promotion of plant growth are not fully understood, but are thought to include, synthesis of phytohormones and lytic enzymes at the level of plant root (Lucy et al., 2004; Gray and Smith, 2005), antagonism against pathogenic microbes at the level of roots by the production of siderophores (Dey et al., 2004), induce host resistance to pathogenic microbes (Van Loon and Glick, 2004; Van Loon, 2007), asymbiotic

fixation of nitrogen (Sahin et al., 2004), the synthesis of antibiotics (Dobbelaere et al., 2002; Lucy et al., 2004) and the aggregation of soil particles (Shetty et al., 1994).

Apart from the function of roots as anchor, water and nutrient uptake, plant root also contributes in rhizodeposition (root exudate) (Nicholas, 2007). Root exudates (carbon compounds) are secreted by plant in the rhizosphere. Root exudates quality and quantity vary with plant developmental stage, microbial degradation and plant species (Anton et al., 2007). Root exudates is not only been used by microbes as a source of nutrients and energy, but it also determines the quantity and quality of rhizobacteria in the rhizosphere. PGPR make up a diverse group of rhizosphere-colonizing bacteria and diazotrophic microorganism in association with plants roots, which promotes growth of the plant. Growth development of the plant can be affected directly or indirectly (Vessey, 2003). Root and microbial interaction are biotic factors that influence rhizosphere effect and plant growth. Meanwhile, soils play a major role in determining root exudation, activity and diversity of rhizobacteria in the rhizosphere. Soil pH, temperature, available nutrient, water availability, oxygen availability and anthropogenic effects also contribute greatly to rhizosphere effect and diversity of rhizobacteria.

Table 2.3 Organic compounds released by plant roots (Nicholas, 2007)

Sugars and polysaccharides	Arabinose, deoxyribose, fructose, galactose, glucose, maltose, mannose, mucilages of various composition, oligosaccharides, raffinose, rhamnose, ribose, sucrose, xylose.
Amino acids	α-alanine, β-alanine, amino adipic, gamma amino butyric, arginine, asparagines, aspartic, citrulline, cystathionine, cysteine, cystine, deoxymugineic, 3-epihydroxymugineic, glutamic, glycine, homoserine, histidine, leucine, lysine, methionine, mugineic, ornithine, phenylalanine, proline, serine, threonine, tyroptophan, tyrosine, valine.
Organic acids	Acetic, aconitic, aldonic, ascorbic, benzoic, butyric, caffeic, citric, p-coumaric, erythonic, ferulic, formic, fumaric, glutaric, glycolic, glyoxilic, lactic, malic, malonic, oxalacetic, p-hydroxy benzoic, piscidic, propionic, pyruvic, succinic, syringic, tartaric,tetronic, valeric, vaillic.
Fatty acid	Linoleic, linolenic, oleic, stearic.
Sterols	Campesterol, cholesterol, sitosterol, stigmasterol.
Growth factors	p-amino benzoic acid, biotin, choline, n-methyl nicotinic acid, niacin, pantothenic, vitamins B_1 (thiamine), B_2 (riboflavin), and B_6 (pyridoxine).
Enzymes	Amylase, invertase, peroxidase, phenolase, phosphatases, polygalacturonase, protease.
Flavonones and nucleotides	p-amino benzoic acid, biotin, choline, n-methyl nicotinic acid, niacin, pantothenic, vitamins B_1 (thiamine), B_2 (riboflavin), and B_6 (pyridoxine).

2.6.2 PGPR as biocontrol agents

Anton et al. (2007) defined biocontrol as the "suppression of plant pathogens without the use of chemical agents". The mechanism responsible for biocontrol activity includes competition for nutrients, antibiosis, and induced-systemic resistance by PGPR.

PGPR ability to compete with soil pathogens is seen in the production of siderophores which chelates iron rendering it soluble and available for plants, while unavailable for soil pathogens inhibiting their growth (Dey et al., 2004). Some *Pseudomonas* spp have been reported for siderophore production (Lugtenberg et al., 2001). Plants are also capable of resisting pathogenic infections when stimulated by O-antigen of lipopolysaccharides and salicylic acid produced by some *Pseudomonas* specie termed induced-systemic resistance (DeMeyer et al., 2001). "Suicidal seed germination" stimulation using ethylene-producing bacteria preventing seed infestation by *Striga* sp has been reported (Berner et al., 1999). Babalola, (2010b) quantified the level of ethylene production in three rhizobacteria, *Pseudomonas spp.*4MKS8, *Klebsiella oxytoca* 10MKR7 and *Enterobacter sakazakii* 8MR5 from sorghum and maize.

Antibiosis and antagonism by PGPR inhibits or eliminates soil pathogens either by the production of antibiotic compounds like phenazine, hydrogen cyanide or the synthesis of lytic enzymes which degrade cell walls of pathogenic bacteria or fungi (Lucy et al., 2004). Biocontrol therefore prevents or reduces the deleterious effects of pathogenic microbes' hence promoting plant growth indirectly. Hass and Keel (2003) found that quorum sensing ability of PGPR species coupled with the quantity and quality of available nutrients in the rhizosphere contributed to the release of biocontrol agents.

Table 2.4 Mechanism of microbial control of plant pathogens with some examples (Anton et al., 2007)

Mechanism	Biocontrol agent	Pathogen
Stimulation of plant systemic resistance		
Induced systemic resistance	*Pseudomonas fluorescence* *Pseudomonas putida*	*Pseudomonas syringae* *Fusarium oxysporum* *Colletotrichum orbiculare*
Systemic acquired resistance	*Pseudomonas syringae* (avirulent)	*Pseudomonas syringae*
Nutrient competition siderophore	*Pseudomonas putida* *Pseudomonas aeruginosa* *Serratia sp.*	*Fusarium oxysporum* *Pythium splendens* *Verticillium dahliae*
Carbon substrate	*Agrobacterium radiobacter*	*Agrobacterium tumefaciens*
Antibiosis Antibiotics	*Pseudomonas fluorescence* *Pseudomonas putida*	*Graminis var. tritici* *Rhizoctonia solani*
Hydrogen cyanide	*Pseudomonas fluorescence*	*Graminis var. tritici*

2.6.3 PGPR as biofertilizers

Biofertilizers are cells of different types of microorganisms, having the potential to convert important nutritional elements from unavailable state to available form through biological processes (Vessey, 2003). Mechanism of action of this process entails symbiotic nitrogen fixation, asymbiotic nitrogen fixation and phosphate solubilisation by PGPR. Symbiotic PGPR penetrate the cortex of the root, multiply and differentiate into bacteroides which secrets a complex enzyme nitrogenase. Plant roots create a low oxygen concentration within induced nodules which stimulate bacterial nitrogenase to convert atmospheric nitrogen into ammonia (Bloemberg and Lugtenberg, 2001). Asymbiotic nitrogen fixing PGPR also fixes atmospheric nitrogen using a nitrogenase complex under low oxygen concentration (Steenhoudt and Vanderleyden, 2000) available for plant uptake. Phosphate solubilizing bacteria secrete phosphatase an enzyme that converts insoluble phosphate to soluble form thereby making phosphorous available for plants uptake and growth (Gyaneshwar et al., 2002). Some species of *Bacillus* have been reported for phosphate solubilisation (Wu et al., 2004).

2.6.4 PGPR as phytostimulators

Plant root elongation or rooting could be influenced by phytohormones such as auxins (indole-3-acetic acid) (Leyser, 2002). Root elongation increases surface area for nutrient uptake by plants. Species of *Azospirillum*, *Azotobacter*, *Bacillus* and *Pseudomonas* have been reported in the production of plant growth promoting substance such as auxins, gibberellins ethylene and cytokines (Joo et al., 2009). Auxin is an important phytohormone produced by PGPR and according to (Vessey, 2003), treatment of plant with auxin-producing rhizobacteria increases growth. Babalola et al. (2002) reported the production of the hormone ethylene by *Pseudomonas* spp.4MKS8 that is responsible for the regulation of certain physiological processes in plants such as breaking of seed dormancy, increasing the number

of roots and adventitious root formation. In contrast, high levels of ethylene at the level of plant roots causes root growth inhibition. However, studies by Glick et al. (1998) revealed that PGPR produced 1-aminocyclopropane 1-carboxylate (ACC) deaminase an enzyme which cleaves ACC the immediate precursor molecule of ethylene at the level of plants root to ammonium and α-ketobutyrate leading to root growth. Therefore, PGPR capable of producing phytohormones at the level of plant roots could influence physiological processes such as root elongation, number of roots and tissue differentiation, hence promoting the growth of plant directly.

2.6.5 PGPR as biofilms

Biofilms are a collection of microorganisms and their extracellular products attached to living or non-living surfaces enabling gene regulations by microorganisms (Faqua and Greenberg, 2002). One of the reasons for biofilm formation by PGPR is nutrients acquisition. Biofilm in the rhizosphere has been directly linked to root exudates (Rovira and Campbell, 1974). Biofilm do not only benefit from root exudates (carbon substrate) as source of energy but also enhance nutrient supply to plants through root-microorganism symbiosis such as rhizobia and leguminous plant (Werner, 1992) and also protects roots from pathogens by *Pseudomonas* spp. which antagonize soil pathogens (Bianciotto et al., 2001; Morris and Monier, 2003). *Pseudomonas aeruginosa* biofilm development and population density is maintained by autoinducing molecules called *acylatedhomoserine lactone* (AHL) in an intercellular communication process called quorum sensing (Madigan et al., 2009). However, it has been reported that some rhizobacteria are capable of degrading AHL (Elasri et al., 2001) hence, suppressing PGPR that promote plant growth through quorum sensing.

2.6.6 Future prospects of PGPR

A better knowledge of PGPR mechanisms of action, diversity, host specificity coupled with description and field applications will correlate with high yield and production in the

agricultural industries (Podile and Kishore, 2007). Furthermore, it will also contribute to a sustainable environment through the reduction in chemical fertilizers and pesticides application which promote plant growth but not environmental-friendly. However, new technologies such as GMPs which promotes plant growth and yield coupled with doubts about their possible impact on these natural plant growth promoters needs to be constantly accessed in order to preserve these species and their natural processes.

2.6.7 Deleterious rhizobacteria

Deleterious rhizobacteria (DRB) differ from PGPR in that they are capable of colonizing the rhizosphere or plant root and inhibit plant growth directly or indirectly. Direct inhibition of plant growth includes the production of growth inhibitors which deform and reduce root elongation and defence system hence, increasing infection by root colonizing pathogenic fungi (Li and Kremer, 2006). Meanwhile, indirect plant inhibition include the production of phytotoxin at level of plant root inhibiting PGPR colonization (Tranel et al., 1993), siderophore chelation where they compete with PGPR for available iron (Fuhrmann and Wollum, 1989), production of extracellular metabolites which exert negative impact on root colonizing ability of PGPR through chemotaxis (Keel et al., 1992) and competition for available nutrients with PGPR at level of plant root or the rhizosphere.

Deleterious rhizobacteria should be characterized with respect to soil type and host plant (Preston, 2004). It is difficult to draw a line between PGPR and DRB, based on the report that characterized PGPR with growth promoting traits in one plant may also affect other plants species negatively (Nehl et al., 1997). Hydrogen cyanide (HCN) a plant growth promoting trait exhibited by some Pseudomonads which kills pathogens in one crop could also act as DRB on another crop if HCN affects it growth (Defago and Hass, 1990).

2.6.8 Some effects of DRB

Deleterious rhizobacteria rhizosphere interaction affects both plant and rhizosphere species. Tranel et al. (1993) found that *Pseudomonas fluorescence* D7 can produce phytotoxin at level of cheatgrass (an invasive plant) root restricting root ability to elongate. However, Kennedy et al. (2001) reported that *Pseudomonas fluorescence* D7 specie was specific to cheatgrass and does not have unintended effects on non-target organisms. Furthermore, Dooley and Beckstead (2010) dual application of *Pseudomonas fluorescence* D7 with *Pyrenophora semeniperda* (fungi) as a much more effective method on cheatgrass as biocontrol, concluded that both organisms all have potential as biocontrol agents. These biocontrol agents can be applied separately or used in association with traditional control methods such as tillage and herbicide application. However, the effects of these species on cheatgrass are considered as positive effects in controlling the invasiveness of cheatgrass, while its effects on crop plants will be deemed harmful.

Arbuscular mycorrhizae symbiotic colonizing ability in plant root which promotes plant growth can be inhibited by DRB decreasing root colonization, spores germination and hyphal length, hence indirectly inhibiting plant growth (Hodge, 2000). Furthermore, DRB may inhibit nitrogenase activity of leguminous plants by reducing nodulation capacity and rhizobial growth in the rhizosphere (Kremer, 2006). Berggren et al. (2001) found that growth inhibition of *Rhizobium leguminosarum. viceae* resulted from its direct contact with, as well as its exposure to extracellular metabolites of the DRB (*Pseudomonas putida* Å313). Furthermore, Berggren et al. (2005) observed that *Pseudomonas putida* also colonized the rhizoplane causing deformation in root hair which influenced rhizobial infection and poor nodulation implying that the production of phytoinhibitory metabolites possibly affects the roots membranes.

2.6.9 Future prospects of DRB

Deleterious rhizobacteria have great potential as bioherbicides and biopesticides if they are capable of restricting plant growth in a specie specific manner (Mazzola et al., 1995). In this regard, DRB producing phytotoxins that can target weeds growth and not crop plant can be reckoned in the production of biocontrol and biopesticides (Kremer, 2006). Such technology requires a better understanding of DRB mechanisms of action, diversity host specificity and application method. A breakthrough will help reduce the widespread use of herbicides and pesticides hence minimizing environmental contamination and degradation. Although DRB are deemed harmful with respect to crop plants and PGPR, they also play an important role in the ecosystem such as mineralization and immobilization of nutrients, biological control of plant pest and food source for other organisms. Therefore, DRB are to be considered when assessing possible impacts of GMPs cultivation on native rhizobacteria in order to maintain a sustainable environment.

2.7 Factors influencing rhizobacteria other than root exudates

2.7.1 Soil

Soil quality determines its fitness to perform certain ecosystem functions (Karlen et al., 2001) such as promotion of plant growth, biogeochemical cycling of nutrients, provide habitat for microbes (Brady and Weil, 2002). These functions are greatly influenced by the increase in organic matter (OM) content in soil. Soil texture, determines the precise site of nutrient in soil, where by strongly attached nutrient to soil particles are often less available for rhizobacteria decomposition (Knaebel, 1994), affecting their activity. Pores size of soil also serves as protective microhabitat for rhizobacteria against protozoa predation reducing the rate of predation (Van Overbeek and Van Elsas, 1997).

2.7.2 Nutrient availability

Soil OM composition is comprised of dead decaying remains of plant and animal, humic acid, fluvic acid, humin, sugars, amino acids and synthesized biochemical products of microorganisms (Weil and Magdoff, 2004). High level of soil OM correlates with enhanced soil aggregation, nutrient cycling improved filtration and water retention (Greenland and Szabolcs, 1994) Therefore, rhizobacteria activity and diversity may depend on available nutrients since they mineralized and immobilised nutrients in the rhizosphere.

2.7.3 Water availability in soil

Water availability in soil is influenced by soils OM capacity to hold plant available water and to absorb water from rain since much water is lost as runoff. Water provides moisture necessary for rhizobacteria metabolic activity implying that water deficit soils will pose a problem for rhizobacteria since metabolic activity will be affected negatively.

2.7.4 Soil temperature

Cell metabolic activities such as cell division, protein synthesis and respiration are affected by temperature from the optima state. Therefore, rhizobacteria activities may be inhibited in adverse conditions of temperature (too hot or cold) hence influencing rhizobacteria activity and diversity in the rhizosphere.

2.7.5 Soil pH

Soil pH play an important role in rhizobacteria activity with most bacteria striving well in neutral pH or slightly acidic/alkaline pH, not excluding microbes which strive well in high alkaline and low acidic soil conditions. Lauber et al. (2009) reported that, microbial composition in soil correlates with soil pH. Soil pH may vary with different species of plant. Soil pH is nutrient dependent, with nitrate and ammonium ion in soil leading to more alkaline

conditions due to influx of hydroxide ions in soil and as a result brings about a large change in rhizobacteria diversity and activity in the rhizosphere.

2.7.6 Oxygen availability in soil

Soils vary from sandy, clay to waterlog, with variation in air penetration. Well drained soil experience high penetration of air hence rhizobacteria respiration is enhanced. Meanwhile, waterlogged soil have little dissolved air in water and is rapidly used by microbes and roots leading to anaerobic conditions favouring anaerobic rhizobacteria.

2.7.7 Anthropogenic impact on rhizobacteria

Agricultural cropping based on pesticides not only control target pathogen but also affect the non-target natural enemies of the pathogen, leading to negative impact on rhizobacteria (Fischer, 2007). Furthermore, pathogen resistance to pesticide could require application of higher doses or frequent application of pesticide or introduction of new pesticide altering microbial population and diversity in the rhizosphere. Chemical fertilizers have been reported for variable effects on the biodiversity of the rhizosphere, with unpredictable effects on microbial growth and activity in the rhizosphere (Melissa et al., 2007). However, increase in rhizosphere biodiversity and activity can be achieved by enhancing organic farming (i.e. does not allow use of synthetic fertilizers or pesticides), minimum tillage, mulching and agro forestry, which contrasts bush burning, deforestation and deep tillage affecting rhizobacteria diversity and activity (Waid, 1999). Murata and Goh (1997) correlate high levels of OM and rhizobacteria biomass in organic farming than in conventional farming. Therefore, conclusions about the possible impact of GMPs on indigenous rhizobacteria should not be based only on root exudate but should involve all parameters affecting rhizobacteria activity and diversity in the rhizosphere.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study site and sampling

Soil samples of field grown GM (Bt) and non-GM maize were collected from an experimental fields in Delmas (26°09′S, 28°41′E), South Africa 30 days after sowing (DAS) and 1 day after harvest (DAH). GM and non-GM plants were grown 35 cm apart. The soil classification is lithic (Fey, 2010).

The rhizospheric soil samples were collected from the roots of maize (about 0 to 2 mm away from the root surface) using sterile collection bags and trowel spoon. The soil was collected from rhizosphere of four different maize plants and bulked together to represent one sample. Eight soil samples were collected from each of GM and non GM maize rhizosphere respectively (i.e four soil samples each for GM 30 DAS, GM 1 DAH, non-GM 30 DAS and non-GM 1 DAH). Samples were placed in an ice chest cooler with cold packs and transported to the laboratory.

3.2 Analyses of properties of GM and Non-GM soil samples

The quantity of macro and microelements in soil samples of field grown GM and non-GM maize were measured using a Ray EDX-720 Energy Dispersive X-RAY Spectrometer. Ten grams of soil samples was weighed and transferred into a 50 ml beaker containing 25 ml of distilled water. The pH meter and probe was calibrated according to the manufacturer's directions and pH measured (McLean, 1982). The pH (water) was determined according to the method of (McLean, 1982).

3.3 Microbial isolation and biochemical characterization

Serial dilutions up to 10^{-6} were made using distilled water from the sixteen soil samples collected. A volume of 0.3 ml of each dilution was plated by spread plate technique on tryptic soy agar (TSA), nutrient agar (NA), Luria Bertani agar (LBA) (non-selective media for

general isolation of culturable bacteria) and *Pseudomonas* selective agar (PSA). Plates were replicated three times and the experimental design was completely randomized design. Plates were incubated at 28°C for two to three days. Bacterial growth was recorded after two to three days as colony forming units (cfu). Colonies were randomly selected and purified in new plates of NA. Purified isolates obtained were Gram stained using standard methods (Cruiskshank et al., 1975). Finally, isolates were biochemically identified using the analytical profile index (API) (Biomeriux, France).

3.4 Community catabolic profiling of rhizobacteria from field grown GM and non-GM maize soil samples

An amount of 4 g of GM and non-GM soil samples was diluted in 36 ml of sterile 0.85% NaCl (sodium chloride), vortexed for 5 min, and serial dilutions up to 10^{-3} made. Amounts of 150 µl of 10^{-3} dilution for each sixteen soils were inoculated into each well of Biolog GN2 microplate. Optical density (OD) was read at time zero using microplate reader (Thermo Scientific Multiskan Ex, China) at 620 nm. Plates were incubated at 30°C for 120 h and utilization of sole compounds was measured by colour development and the OD reading taken at 620 nm. Visual observation (counting) of all positive wells was done, followed by overall expression of average well colour development (AWCD) indicating the sum of absorbance units of all 96 wells divided by the total number of wells. The controlled well (well one) optical OD was recorded. Group-wise average well colour development of the total per group of substrates ($AWCD_G$) which is the sum of absorbance units of individual substrate sets divided by the number of compounds belonging to the substrate group was also recorded.

3.4.1 Calculations and statistical analyses

Community catabolic profile analyses were done by calculating the net absorbance of each well. This was obtained by subtracting the absorbance value of the control well from the

absorbance value of each well. The AWCD, expressing the sum of absorbance units of all 96 wells divided by the total number of wells of single data was calculated. Furthermore, the $AWCD_G$, was calculated according to the method of (Sammar and Beatrix, 2009). Substrate utilization pattern for each (carbon substrate) was subjected to analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS).

3.5 DNA extraction of culturable rhizobacteria in GM and non-GM maize soil samples

The cetyltrimethylammonium bromide (CTAB) method was used to extract the DNA from culturable rhizobacterial isolates (Doyle and Doyle, 1990). The concentration of genomic DNA was measured using a nanodrop at 260 nm and stored at 4°C.

3.5.1 PCR amplification of 16S rRNA gene fragments of culturable rhizobacteria

Amplification of 16S rRNA gene portions from different bacterial isolates was performed in a 25 µl reaction volume containing 0.1 mM of each primer, 1.5 mM $MgCl_2$ (Invitrogen), 10 mM of each dNTP (Amersham Biosciences), 1 U *Taq* DNA polymerase (Invitrogen) and 1µl of DNA template (20-50 ng/µl). The bacteria 16S rDNA primer Ps-for (5'GGTCTGAGAGGATGATCAGT3') and Ps-rev (5'TTAGCTCC-ACCTCGCGGG3') as well as universal primer 27F (5'AGAGTTTGATCCTGGCTCAG3') and 1492R (5'TGACTGACTGAGACTACCTTGTT-ACGA3') were used to amplify 989 bp and 1500 bp of the 16S rDNA gene region. Polymerase chain reaction (PCR) was done using a BIO-RADC1000 Touch™ Thermal Cycler (Bio-Rad, USA). Thermal cycling were performed as follows: Initial denaturation at 94°C for 5 min, followed by 30 cycles of 95°C for 30sec, 54°C for 1 min, 72°C for 2 min and final extension at 72°C for 5 min. The fragments obtained were analysed by gel electrophoresis (24 × 12 cm) with 1% agarose, and carried out at 80 V for 2 h. A 1-kb gene ladder loaded on the left lane of the gel was used as a molecular size marker. The gel was then stained with ethidium bromide and digital picture of amplified

gene was taken under UV light using a BIO-RAD ChemiDoc™ MP imaging system (Bio-Rad, USA). PCR products were sent to Inqaba laboratory in Pretoria for sequencing.

3.5.2 Sequence analysis of cultured Gram-positive rhizobacteria isolates

Sequence search for the alignment of nucleotides was performed using the basic local alignment search tool (BLAST) web-based program (Altschul et al., 1990).

3.6 Direct soil DNA extraction of rhizobacteria in GM and non-GM maize soil samples

Power soil extraction kit (MoBio Laboratories, Solana Beach, CA) was used to extract DNA from GM and non-GM maize soil samples as described in the protocol of the manufacturer. The extracted DNA was quantified by using a nanodrop at 260 nm and stored at 4°C.

3.6.1 PCR amplification of 16S rDNA gene segment for DGGE analysis

PCR amplification targeting bacterial 16S rDNA was performed with the primer set of 357F-GC (guanine cytosine) clamp (5′CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCA-CGGGGGGCCCTACGGGAGGCAGCAG-3′) and 518R (5′-ATTACCGCGGCTGCTGG-3′) (Muyzer et al., 1993). Each 50 µl of PCR mixture contained 1 µl of forward and reverse primers, 2 µl of DNA template (30-50 ng/µl), 25 µl of master mix and was filled up to the required volume with free nuclease water. PCR amplification was performed using a BIO-RADC1000 Touch™ Thermal Cycler (Bio-Rad, USA). The amplification conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR products about 200 bp were checked on 1% agarose gel, and carried out at 70 V for 2 h. A 1-kb plus gene ladder loaded on the left lane of the gel was used as a molecular size marker. The gel was stained with ethidium bromide and digital picture of amplified gene taken under UV light using a BIO-RAD ChemiDoc™ MP imaging system (Bio-Rad, USA).

3.6.2 DGGE analysis (clustering) of rhizobacterial community

DGGE was performed using 8% (w/v) acrylamide gel with a 40% to 60% denaturant gradient, where 100% denaturant was defined as 7 mol l^{-1}urea plus 30% formamide. Low and high denaturing solutions were prepared, mixed with the acrylamide solution, and poured in a gel casting by using a gradient former in order to generate a linear denaturing gradient. About 5 µl of loading dye was added to 20 µl of PCR products and applied on the denaturing gradient gel. The DGGE was run in 1 × TAE (Tris acetate ethylenediaminetetraacetic acid) buffer for16 h at a constant temperature of 60°C and 100 V using a 16 x 16 x 0.1 cm BIO-RAD DCode™ universal mutation detection system (Bio-Rad, USA). After the electrophoresis, the gel was stained in ethidium bromide solution for 45 min. The image of the gel was obtained using a BIO-RAD ChemiDoc™ MP imaging system (Bio-Rad, USA). After which, cluster analysis of band patterns was performed using the unweighted-pair group method using arithmetic average (UPGMA).

3.6.3 Band excision and sequencing

Twelve bands were carefully excised from the DGGE gel using sterile razor blade. Excised band was briefly washed with 50 µl of free nuclease water in a 1.5 ml microfuge tube to remove extra ethidium bromide. Finally, DNA was eluted by incubating the band in 30 µl of free nuclease water over night at 4°C. One µl of eluted DNA was used as a template for PCR amplification with a second set of primer 357F (5'-CCTACGGGAGGCAGCAG-3') without GC clamp and 518R (5′-ATTACCGCGGCTGCTGG-3′). The amplification conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min. The resulting amplicons were electrophoresed on a 1.5% agarose gel at 70 V for 2 h. A 200 bp gene ladder loaded on the left lane of the gel was used as a molecular size marker. The gel was stained with ethidium bromide and digital

picture of amplified gene taken using a BIO-RAD ChemiDoc™ MP imaging system (Bio-Rad, USA). PCR products were sent to Inqaba laboratory in Pretoria for sequencing.

CHAPTER FOUR
RESULTS

4.1 Soil properties of GM and Non-GM maize fields

Macro and microelements such as P, K, Ca, S and Si, Al, Fe, Ti, Cr, Mn, Cu were detected in the different soil samples. Percentages obtained in both GM and non-GM soil samples collected 30 DAS and 1 DAH was similar (Table 4.1). The pH of GM and non-GM soil samples ranges from 6.12-7.03, indicating slightly acidic to slightly alkaline soil (Table 4.1).

Table 4.1 Chemical analyses of soil properties in GM and Non-GM soil samples 30 DAS and 1 DAH

pH and Soil properties (%)	GM		Non GM	
	30 DAS	1 DAH	30 DAS	1 DAH
pH	6.74±0.04	6.78±0.16	7.03±0.06	6.12±0.59
Si	57.33±0.23	57.56±0.40	57.74±0.69	57.89±1.02
Al	23.020±0.27	23.22±0.43	23.53±0.55	23.34±0.40
Fe	13.33±0.13	13.32±0.15	13.21±0.15	13.28±0.13
K	2.04±0.01	1.99±0.06	1.91±0.03	1.97±0.07
Ti	1.34±0.01	1.35±0.01	1.30±0.01	1.34±0.01
P	0.71±0.07	0.77±0.03	0.69±0.06	0.69±0.05
Ca	0.68±0.008	0.68±0.009	0.67±0.01	0.67±0.002
Mn	0.28±0.005	0.28±0.006	0.27±0.002	0.28±0.008
S	0.15±0.01	0.16±0.01	0.15±0.001	0.16±0.005
Cr	0.06±0.004	0.06±0.001	0.06±0.004	0.06±0.008
Cu	0.03±0.002	0.02±0.001	0.03±0.002	0.03±0.0007

The values represent the mean of four replicates ± standard deviation

Fe = Iron, K = Potassium, Ca = Calcium, Mn = Manganese, S = Sulphur, Cr = Chromium, Cu = Copper, Si = Silicon and Al = Aluminium,

4.2 Plate count

Total rhizobacterial count (cfu/g) of GM and non-GM maize soil samples across the media is presented in (Figure 4.1). There was no significant difference in rhizobacterial population among GM and non-GM soil samples collected 30 DAS and 1 DAH. However, *Pseudomonas* selective medium indicated similar bacterial population in GM and non-GM soil samples collected 30 DAS and 1 DAH. Meanwhile, post-harvest plate count results for GM and non-GM, respectively showed a significant declined in rhizobacterial population when compared to population in soil samples collected 30 DAS.

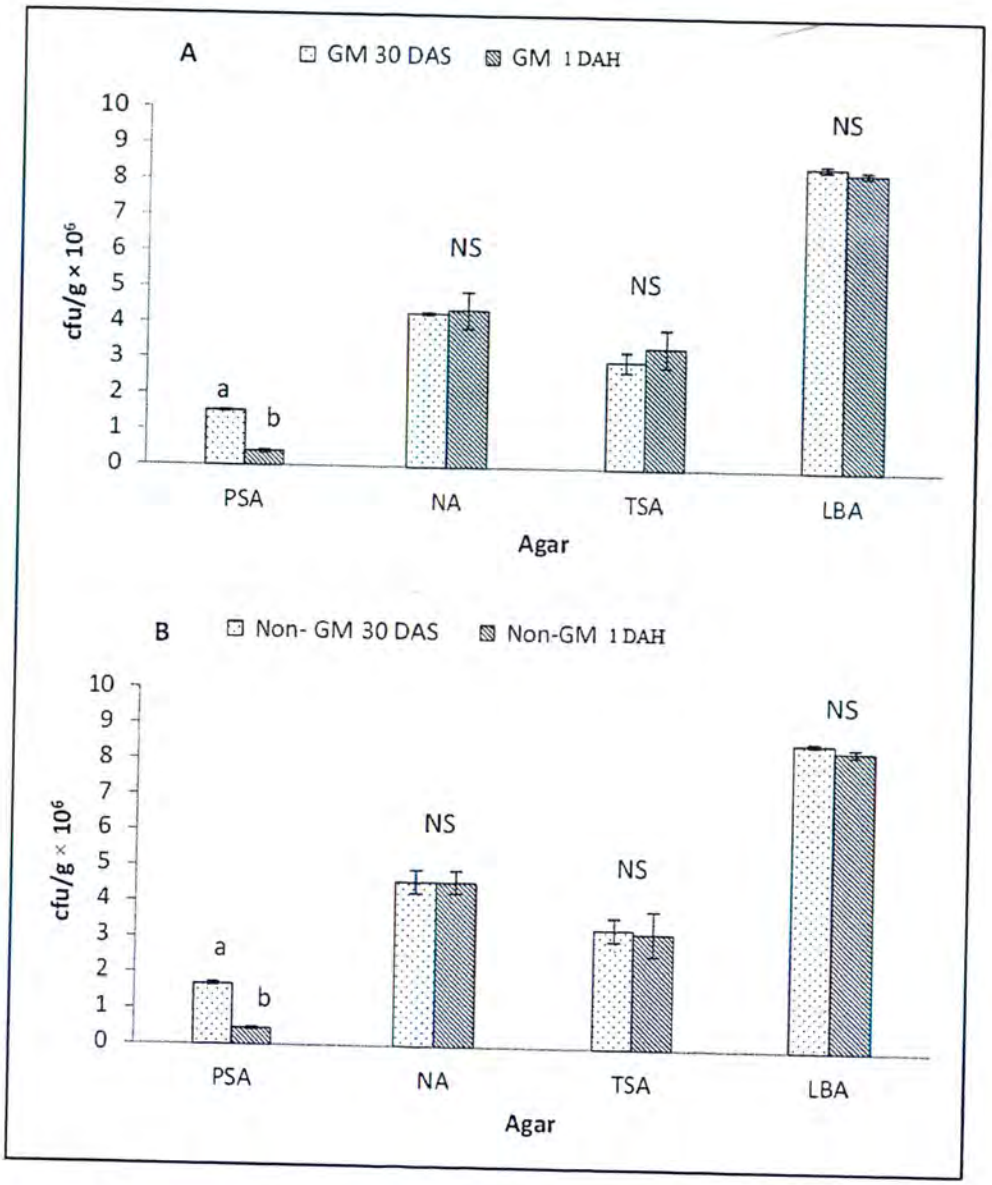

Figure 4.1 Total rhizobacterial cfu/g of soil on PSA (*Pseudomonas* selective agar), NA (nutrient agar), TSA (trypticase soy agar) and LBA (Luria Bertani agar) from field grown GM and non-GM maize soil samples collected 30 DAS and 1 DAH. Bars represent standard error of the mean of three replicates. The bars with different letters are significantly different at $P \geq 0.05$ using analysis of variance. NS: means not significant.

4.3 API characterization

API identification system identified the Gram-negative culturable maize rhizosphere species from the soil samples collected 30 DAS and 1 DAH (Table 4.2). Similar bacterial species were found in GM and non-GM soil samples at both sampling times using non-selective media. However, different rhizosphere species were identified in soil samples 30 DAS and 1 DAH using PSA. Species of *Pseudomonas putida*, *Pseudomonas stutzeri*, *Achromobacter denitrificans* and *Burkholderia cepacia* were identified in soil samples collected 30 DAS and 1 DAH. *Pseudomonas putida*, *Pseudomonas stutzeri* and *Achromobacter denitrificans* were all identified in the soil samples collected at 30 DAS. Meanwhile, post-harvest identification indicated species of *Burkholderia cepacia* and *Achromobacter denitrificans* in both samples (GM and non-GM).

Table 4.2 API identification of Gram-negative rhizobacterial isolates recovered from soil samples collected 30 DAS and 1 DAH

Source	Time of collection	Medium	Species obtained
GM	30 DAS	NA	*S. paucimobilis, Sten. maltophilia, P. luteola.*
		TSA	*S. paucimobilis, Burk. cepacia.*
		LBA	*S. paucimobilis, P. luteola, Burk.cepacia.*
		PSA	*P. putida, A. denitrificans.*
	1 DAH	NA	*S. paucimobilis, Sten. Maltophilia, Burk. cepacia*
		TSA	*S. paucimobilis, P. stutzeri*
		LBA	*S. paucimobilis, Aero. hydrophila/caviae*
		PSA	*Burk.cepacia, A. denitrificans*
Non-GM	30 DAS	NA	*S. paucimobilis, P. luteola, Sten. maltophilia*
		TSA	*S. paucimobilis, Sten. maltophilia*
		LBA	*S. paucimobilis, Burk. cepacia*
		PSA	*P. stutzeri, A. denitrificans*
	1 DAH	NA	*S. paucimobilis, Burk. cepacia*
		TSA	*S. paucimobilis, E. americana*
		LBA	*S. paucimobilis, Ent. coacae*
		PSA	*Burk.cepacia, A. denitrificans*

Burkholderia (*Burk.*), *Sphingomonas* (*S.*), *Pseudomonas* (*P.*), *Achromobacter* (*A.*), *Stenotrophomonas* (*Sten.*), *Ewingella* (*E.*), *Enterobacter* (*Ent.*), *Aeromonas* (*Aero*).

4.4 Catabolic fingerprint

In these studies, average well colour development (AWCD) pattern was similar in GM and non-GM soil samples collected 30 DAS and 1 DAH (Table 4.3). Sole carbon substrate utilization patterns (SCSUP) on Biolog plates was similar. That is, polymers, amino acids, carboxylic acids, miscellaneous, carbohydrates and amide/amines were all preferred by microbial community in both GM and non-GM collected 30 DAS and 1 DAH (Tables 4.4 and 4.5).

Table 4.3 Average well colour development (AWCD) in Biolog microplates after incubation at 30°C for 120 h for GM and non-GM soil samples collected 30 DAS and 1 DAH

Soil samples	**Period of collection**	**OD_{620nm}**	**Visual observation (count)**
GM	30 DAS	$0.66^{a} \pm 0.01$	58
	1 DAH	$0.64^{a} \pm 0.04$	54
Non-GM	30 DAS	$0.65^{a} \pm 0.03$	59
	1 DAH	$0.64^{a} \pm 0.005$	53

OD: Optical density

The values are means of three replicates and the means followed by same letters are not significantly different at $P \geq 0.05$ using analysis of variance.

Table 4.4 Biolog substrate utilization by microbial communities of GM and non-GM soil samples collected 30 DAS and 1 DAH

Group of compounds	**Number of carbon source Per group**	**GM 30 DAS**	**GM 1 DAH**	**Non-GM 30 DAS**	**Non-GM 1 DAH**
Carbohydrates	30	19 (63.3%)	19 (63.3%)	20 (66.6%)	18 (60%)
Carboxylic acid	24	20.5 (85.4%)	19 (79%)	21.5 (89.5%)	19 (79%)
Amino acids	20	16.5 (82.5%)	16 (80%)	17 (85%)	15.5 (77.5%
Polymers	5	4 (80%)	4 (80%)	4 (80%)	4 (80%)
Amines/Amides	6	4 (66.6%)	3.5 (58.3%)	4 (66.6%)	3.5 (58.3%)
Miscellaneous	10	7.5 (75%)	7 (70%)	7.5 (75%)	6.5 (65%)

Numbers in header: number of carbon sources in each substrate group; Numbers in the body of the table: number of utilized carbon sources of the given substrate group by GM and non-GM samples; numbers in parenthesis: percentage of utilized carbon sources of the given substrate group by GM and non-GM samples.

Table 4.5 Group-Wise Average Well Colour Development ($AWCD_G$) of Carbon Sources by microbial communities in GM and non-GM soil samples collected 30 DAS and 1 DAH

Group of compounds	**Number of carbon source per group**	**GM 30 DAS**	**GM 1 DAH**	**Non-GM 30 DAS**	**Non-GM 1 DAH**
Carbohydrates	30	$0.68^a \pm 0.05$	$0.67^a \pm 0.05$	$0.61^a \pm 0.09$	$0.63^a \pm 0.09$
Carboxylic acids	24	$0.75^a \pm 0.02$	$0.73^a \pm 0.02$	$0.75^a \pm 0.06$	$0.73^a \pm 0.01$
Amino acids	20	$0.81^a \pm 0.02$	$0.80^a \pm 0.05$	$0.81^a \pm 0.10$	$0.72^a \pm 0.03$
Polymers	5	$0.64^a \pm 0.04$	$0.68^a \pm 0.02$	$0.64^a \pm 0.06$	$0.72^a \pm 0.01$
Amines/Amides	6	$0.31^a \pm 0.06$	$0.30^a \pm 0.13$	$0.46^a \pm 0.03$	$0.26^a \pm 0.06$
Miscellaneous	10	$0.37^a \pm 0.02$	$0.31^a \pm 0.02$	$0.35^a \pm 0.02$	$0.35^a \pm 0.02$

The values are means of three replicates and the means followed by same letters are not significantly different at $P \geq 0.05$ using analysis of variance.

4.5 Molecular characterization of culturable rhizobacteria in GM and non-GM maize soil samples

Amplification of genomic DNA from the rhizobacterial isolates using the universal 16S primers (25F and 1492R) and *Pseudomonas* primers (Ps-for and Ps-rev) yielded DNA fragments with band sizes of 1.0 and 1.5 kb (Figure 4.2 and 4.3).

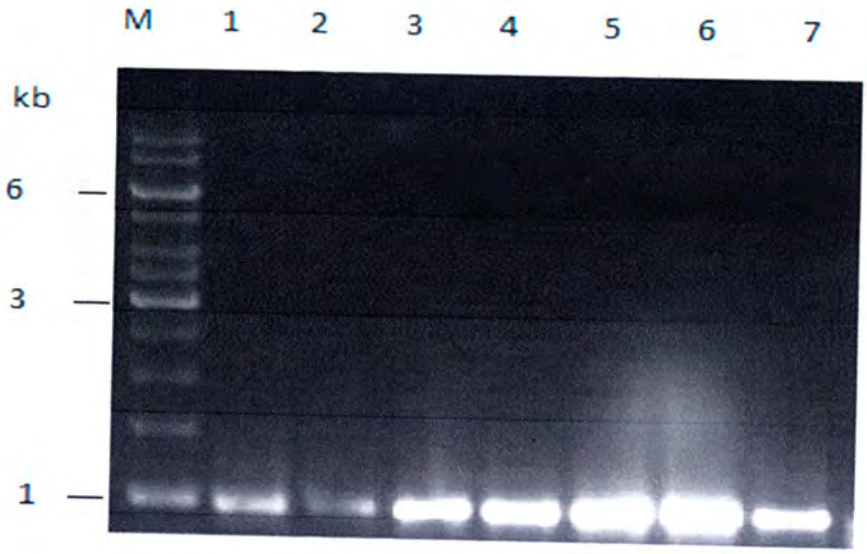

Figure 4.1 PCR products amplified from GM and non-GM soil samples from *Pseudomonas* selective media. Lane M, 1-kb DNA ladder, lane 1-3 *P. putida* and lane 4-7 *P. Stutzeri*.

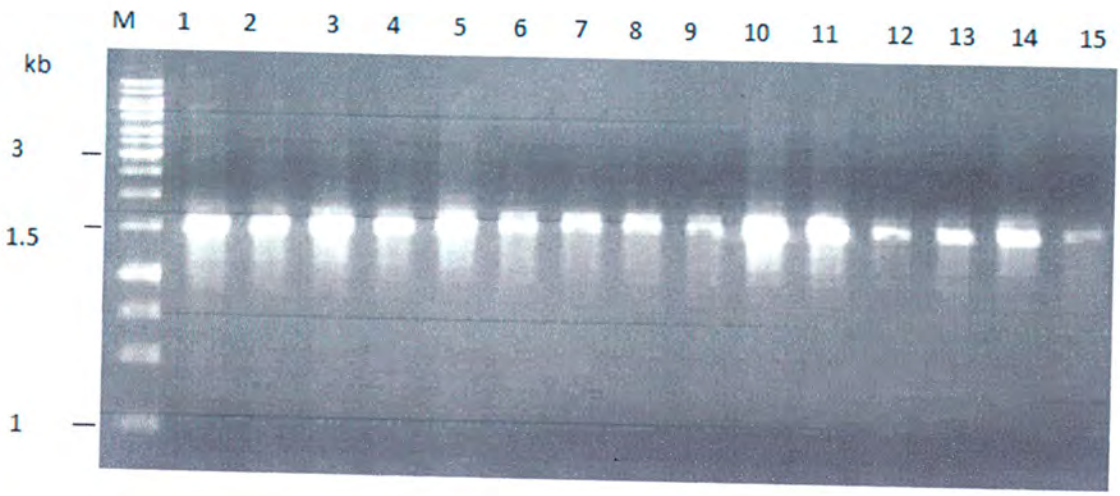

Figure 4.1 PCR products amplified from GM and non-GM soil samples from NA (nutrient agar), PSA (*Pseudomonas* selective agar), TSA (tryptic soy agar) and LBA (Luria Bertani agar). Lane M, 1-kb DNA ladder, lane 1-3 *B. thuringiensis*, lane 4-5 *B. safensis*, lane 6-9 *S. paucimobilis*, lane 10 *Sten. maltophilia*, lane 11 *Aero. hyrophila*, lane 12 *A. xylosoxidans*, lane 13 *E. americana*, lane 14 *Burk. Cepacia* and lane 15 *P. luteola*.

4.6 Sequence analysis of cultured Gram-positive rhizobacterial isolates

Based on sequence analysis, Gram-positive rhizobacteria isolates EW51, EW62, EW66, EW68, EW6, EW5, and EW35 belong to a group of *B. cereus*, *Bacillus* spp., *B. aryabhattai*, *pumilus*, *megaterium*, *thuriengiensis* and *safensis* (Table 4.6).

Table 4.6 Results of sequence analysis of Gram-positive rhizobacterial isolates from GM and non-GM maize rhizosphere

Source	Isolate number	Isolate Accession	Description	% Identity	Accession no.
GM	EW51	KC113513	*B. cereus*	100	JX317637
	EW62	KC113514	*Bacillus spp.*	99	AB736322
	EW66	KC113515	*B. aryabhattai*	100	JX293286
	EW68	KC113516	*B. pumilus*	100	HQ625388
Non-GM	EW6	KC113511	*B. megaterium*	100	JX393073
	EW5	KC113512	*B. thuriengiensis*	99	JX283457
	EW35	KC113510	*B. safensis*	99	JX094951

Bacillus (*B*).

4.7 Molecular characterization of uncultured rhizobacteria in GM and non-GM maize soil samples

4.7.1 PCR amplification of 16S rDNA gene segment and DGGE analyses (clustering) of rhizobacterial community

Amplification of bacterial 16S rDNA with the primer set of 357F-GC clamp (5′CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCCTACGGGAG GCAGCAG-3′) and 518R (5′-ATTACCGCGGCTGCTGG-3′) yielded DNA fragments of about 200 bp (Figure 4.4). Visual observation indicated that a total of about eighty two intense and faint DGGE bands profiles were obtained in GM and non-GM maize 30 DAS and 1 DAH. Bands 2, 3, 4 and 8 were common in all profiles and several bands were unique in the soil samples (1, 9 and 10). Bands 6 was similar in non-GM and GM soil samples 30 DAS but absent at 1 DAH. Band 1 and 12 were highly intense. Results of DGGE profile are presented in (Figure 4.5). A 200 bp fragments were obtained from re-amplification of selected bands on acrylamide gel (Figure 4.6).

Cluster analyses indicated that band patterns of GM and non-GM samples 30 DAS and 1 DAH were closely related to each other (Figure 4.7), lane 12 and 13 (GM 1 DAH and Non-GM 1 DAH), 8 and 10 (GM 30 DAS and Non-GM 1 DAH), 3 and 7 (Non-GM 30 DAS and GM 30 DAS), 4 and 5 (Non-GM 30 DAS and GM 30 DAS) and 9 and 15 (GM 1 DAH and Non-GM 1 DAH). Furthermore, cluster analyses of banding patterns indicated that the dendrogram was divided into clusters (I and II) starting from the point five. Each cluster consisting of soil samples from GM and non-GM maize.

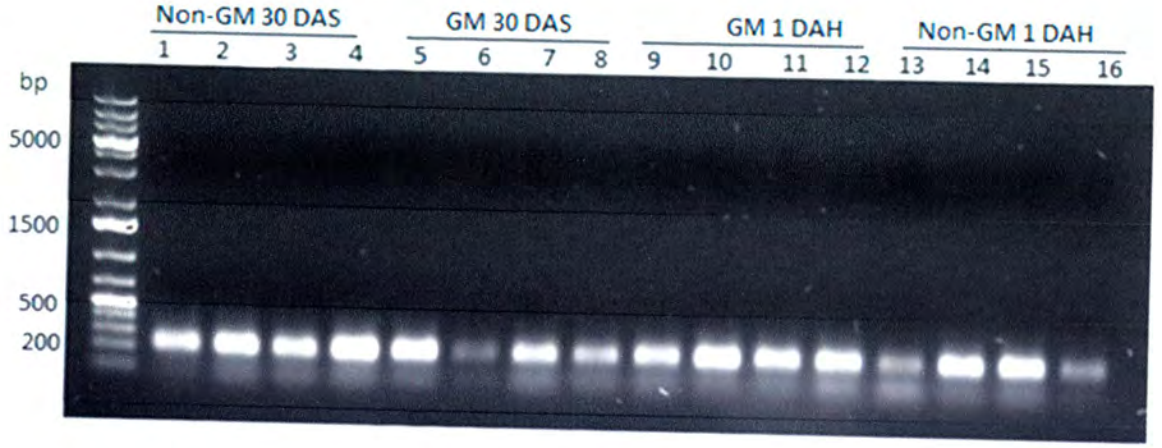

Figure 4.1 PCR products of DNA isolated directly from soil samples. M = 1kb plus ladder.

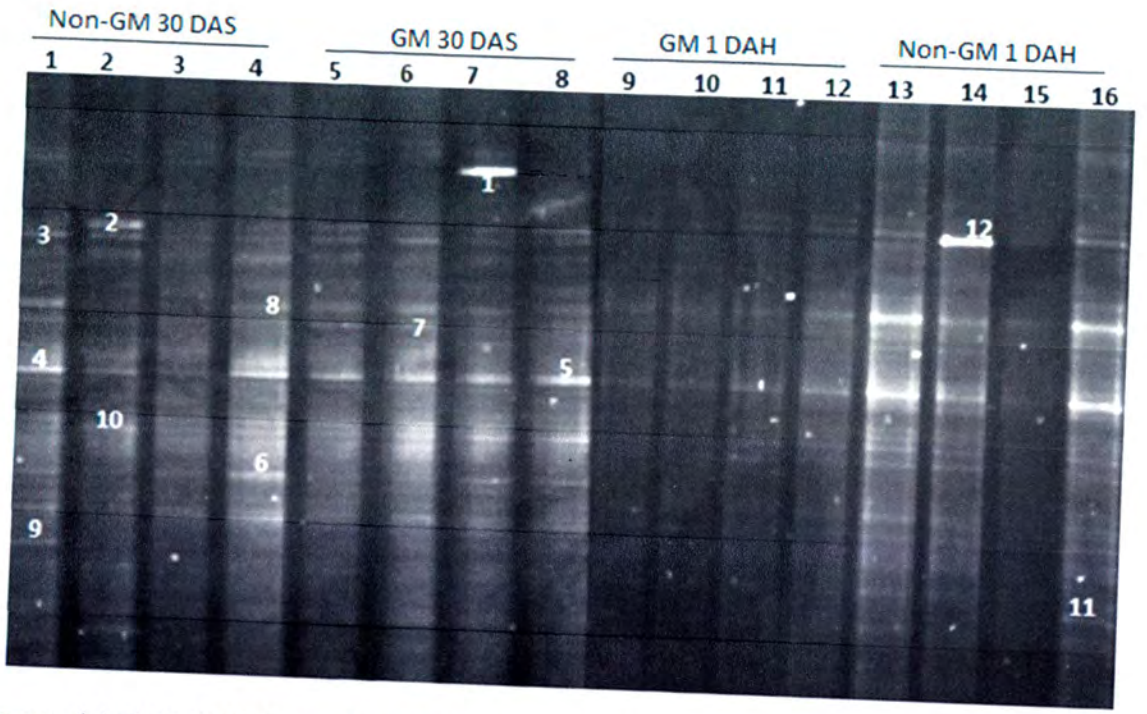

Figure 4.1 PCR-DGGE profiles representing the rhizobacterial diversity in GM and Non-GM soil samples 30 DAS and 1 DAH.

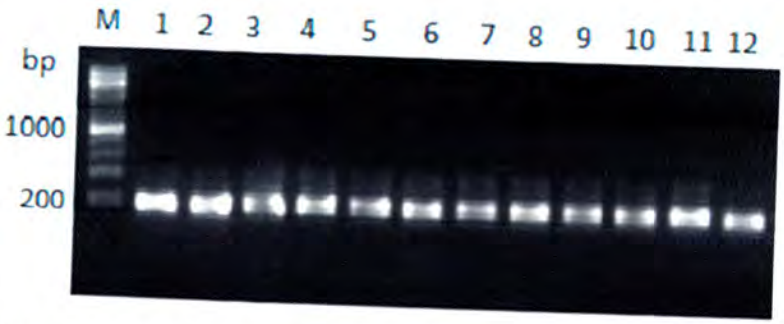

Figure 4.1 PCR products of excised bands from acrylamide gel. M = 200 bp ladder, lane 1 *Vitis vinifera subsp*, lane 2 uncultured bacterium, lane 3 *Bradyrhizobium sp*, lane 4 lane uncultured Actinobacterium, lane 5 uncultured Actinobacterium, lane 6 uncultured beta Proteobacterium lane 7 lane *Burkholderia* sp. lane 8 *Sphingomonas* sp, 9 lane uncultured *Beijernnckiaceae* sp, lane 10 *Erwinia chrysanthemi*, lane 11 *Ipomoea setosa* and lane 12 uncultured Methylobacterium.

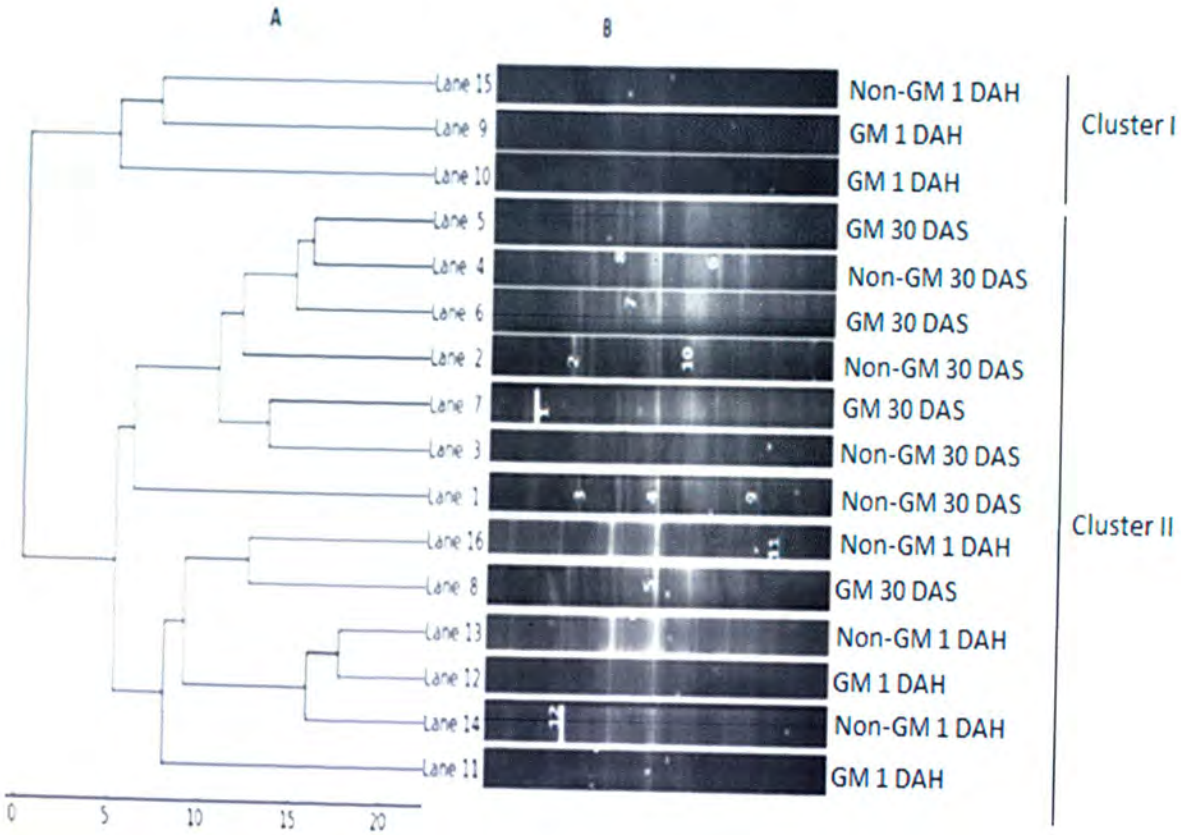

Figure 4.1 (A) Cluster analysis based on UPGMA of DGGE profiles shown in panel B. (B) DGGE profiles of unculturable rhizobacterial in GM and Non-GM soil samples 30 DAS and 1 DAH. Scale bar numbers indicate similarities among profiles.

4.7.2 Sequencing of uncultured rhizobacteria in GM and non-GM maize soil samples

Results of 16S rDNA gene sequences of uncultured rhizobacterial submitted to BLAST search are presented in (Table 4.7). Band 1, 2, 3, 4, 6, 7, 8, 9, 10, 11 and 12 represents uncultured *Vitis vinifera* subsp, uncultured bacterium *Bradyrhizobium* sp., uncultured Actinobacterium, uncultured beta proteobacterium, *Burkholderia* sp., *Sphingomonas* sp., uncultured *Beijernnckiaceae* sp., *Erwinia chrysanthemi* and uncultured *Methylobacterium* respectively.

Table 4.7 Results of amplified 16S rDNA gene sequences excised from DGGE gel

Source	Band number	Description	% Identity	Accession no.
GM	1	*Vitis vinifera* subsp	100	AB856290
	5	Uncultured Actinobacterium	82	EF662966
	7	*Burkholderia* sp.	89	JF826038
	8	*Sphingomonas* sp.	84	FR692003
Non-GM	2	Uncultured bacterium	100	JQ381011
	3	*Bradyrhizobium* sp.	95	KC113622
	4	Uncultured Actinobacterium	82	KC442539
	6	Uncultured beta Proteobacterium	86	GQ863473
	9	Uncultured Beijernnckiaceae sp.	97	JQ178015
	10	*Erwinia chrysanthemi*	80	KF058034
	11	*Ipomoea setosa*	88	KF242499
	12	Uncultured Methylobacterium	78	KC493060

CHAPTER FIVE
DISCUSSION

5.1 Quantitative analyses of soil properties in GM and Non-GM soil samples

The soil properties do not only contribute to plant nutrition but also play a role in microbial diversity and activity. Therefore, any changes in the properties of GM maize field soil samples compared with its non-GM counterpart could serve as an indicator when studying the impact of GM on soil microorganisms. Powell et al. (2009) suggested that, variations in soil properties can possibly arise from management practices associated with growing plants and microbial activity other than with GM of plant. However, the soil nutrient status might be affected; either directly through for example roots exudates or indirectly through effects on soil biota. Furthermore, differences between before sowing and after harvesting might be expected as a result of plant uptake of nutrients and release of organic matter. The same chemical properties with similar percentages obtained in GM and non-GM soil samples 30 DAS and 1 DAH suggest that there was no difference in the soil properties. These results are in agreement with the suggestions of Powell et al. (2009) and do not comply with the findings of Liu et al. (2010) who reported differences in chemical soil properties of GM maize with its non-GM counterpart. The reason for similarity in soil chemical properties was probably as a result of no difference in nutrient utilisation of rhizobacteria in GM and non-GM soil samples and soil type.

Soil pH is a measure of soil acidity or alkalinity. Levels that are too high or too low may lead to deficiency of many nutrients and decline in microbial activity and diversity. Our results indicate soil samples buffering capacities were similar. Considering the fact that soil samples are of the same soil type, this might have contributed to a similar pH. Lauber et al. (2009) reported that, microbial composition in soil correlates with soil pH. Therefore, it would be expected that the organisms occupying the rhizosphere would be similar.

5.2 Quantitative and qualitative analysesof culturable rhizobacteria in GM and non-GM maize soil samples

5.2.1 Quantitative analyses

Factors such as predation (Wardle et al., 1995), soil temperature, soil type, soil pH, nutrient status, water content and anthropogenic activity (Baise et al., 2006) can affect the population of soil bacteria. Therefore, quantitative and qualitative changes in the composition of rhizobacterial communities could serve as important and sensitive indicators of both short and long-term possible impact of GMPs on rhizobacteria. The results of this suggest that GM of maize did not bring about any changes with respect to microbial population. This might probably be due to the fact that the properties of soil and pH in GM and non GM maize soil samples were similar.

5.2.2 Qualitative analysis

Among the identified species in this study, species of *Sphingomonas*, *Stenotrophomonas*, *Burkholderia*, *Pseudomonas* and *Bacillus* have been reported in the rhizosphere of maize (Chelius and Triplett, 2000; Mehnaz et al., 2007; Mehnaz and Lazarovits, 2006). Similar bacterial species obtained in both GM and non-GM maize rhizosphere 30 DAS and 1 DAH suggested that GM did not affect rhizobacterial community structure. There was a change in the population of rhizobacteria as *Pseudomonas* that were present at 30 DAS were absent 1 DAH in both GM and non-GM soil samples. This change was probably due to non-availability of resources that support the growth of *Pseudomanas* after the plants have been harvested.

5.2.3 Sequence analyses of culturable Gram-positive rhizobacterial in GM and non-GM soil samples

Sequence analysis of Gram-positive isolates from GM and non-GM soil samples 30 DAS and 1 DAH in these studies placed all isolates in the genus *Bacillus*. Fewer Gram-positive isolates

recovered from the maize rhizosphere was in agreement with report that Gram-negative microbes are dominant in the rhizosphere of maize (Olsson and Persson, 1999). *Bacillus* genus form endospore, produce antibiotics and have ability to degrade rhizodeposits. These characteristics might have probably contributed to *Bacillus* dominance in the rhizosphere of GM and non-GM maize as the only Gram-positive specie.

5.3 Community catabolic finger print

Garland and Mills, (1991) described sole carbon substrate utilization patterns (SCSUP) technique as a suitable method for comparing functional diversity and activity of whole microbial communities. Similar pattern of AWCD in GM and non-GM soil samples both at 30 DAS and 1 DAH in this study suggested that rhizosphere bacterial functional community was diverse and did not differ in both samples. Similarly, the substrate utilization pattern in Biolog plates of GM and non-GM maize soil revealed that the rhizobacterial community function or metabolic response in both GM and non-GM was active and did not differ in the two samples. Based on these, it was suggested that the GM maize was not able to alter microbial community and activity. These findings are in agreement with the studies of Devare et al. (2004) who observed that GM of maize had no effect on microbial communities in the rhizosphere.

Substrate utilization percentage per group of substrate (carbohydrate, amino acids, amines/amides, carboxylic acid, miscellaneous and polymers) ranged from 58.3% to 80% indicating that all these substrates were preferred by microbial community in both GM and non-GM 30 DAS and 1 DAH and the rhizobacterial functional community were highly active at both the sampling times. These substrates have also been reported as root exudates of maize (Baudoin et al., 2003). The Biolog plates technology, has contributed, towards the better understanding of the microbial functional community and diversity in the rhizosphere.

5.4 Rhizobacteria community analysis in GM and non-GM maize soil samples

5.4.1 DGGE analysis (clustering)

DGGE is a fingerprinting technique used in comparing bacterial community profiles. In this study, rhizobacterial community profile was compared in field grown GM and non-GM maize soil samples. The presence of soil samples profile from GM and non-GM maize in two clusters (I and II) in this study suggested that rhizobacterial community did not change. These results are in agreement with those of (Schmalenberger and Tebbe, 2002; Saxena and Stotzky, 2001) who observed no changes in bacterial community of GM plants with respects to its non-GM counterpart. Therefore, it can be suggested that the GM maize was not able to alter microbial community.

About eighty two intense and faint bands in DGGE gel suggested that rhizobacterial community in GM and non-GM soil samples was diverse. According to Muyzer et al. (1993), DGGE band represents a dominant phylotype, with more bands indicating higher diversity. Similar band profiles suggested similar dominant phylotype in GM and non-GM 30 DAS and 1 DAH. These further confirmed results obtained using Biolog plates in this study. Common band profiles in this study suggested a stable phylotype in GM and non-GM. These bands when excised and sequenced can be used as reference species in subsequent study of rhizobacterial diversity in GM and non-GM maize. PCR-DGGE compared with traditional methods such as plate counts and Biolog, provides more detailed information on rhizobacterial community profile. This is due to the fact that, many samples can be analysed at once.

5.4.2 Sequence analysis of uncultured rhizobacteria in GM and non-GM maize soil samples

One advantage of DGGE technique is that, microorganisms can be studied right down to specie level due to the fact that bands can be excised and sequenced. Uncultured bacteria, *Bradyrhizobium* sp., *Actinobacterium* and *Sphingomonas* sp., were common in the sixteen

soil samples (GM and non-GM 30 DAS and 1 DAH) indicating that these unidentified and closely related species were a stable community and species in the study site. Aislabie et al. (2006) defined Actinobacteria as a group of Gram-negative bacteria mostly found in soil and are responsible in the degradation of organic material. The detection of *Sphingomonas* and *Burkholderia spp* was in agreement with the reports of Chelius and Triplett (2000), Mehnaz et al. (2007) and Mehnaz and Lazarovits (2006) that these species are commonly found in the rhizosphere of maize. Therefore, we suggest that these species can be used as key stone indicators in monitoring GM effects on maize rhizobacterial community. However, most previously isolated species could not be detected on DGGE. This can be linked to the suggestions of (Muyzer et al., 1993), that DGGE profiles represent the dominant microbial community or species. It also probably indicates that most cultured species were either the fast growing species or less dominant in soil samples 30 DAS and 1 DAH.

5.5 Conclusions and recommendations

In this study, soil properties analyses, plate count method, Biolog and DGGE technique were used to evaluate the impact of GM maize on native rhizobacteria. Similar rhizobacterial population, activity and diversity in GM and non-GM soil samples revealed that GM maize did not bring about any significant changes in rihizobacterial community. These findings provided insights regarding the impact of GMPs on rhizobacterial.

There is need to do a long term assessment of GM impact on rhizobacterial population and activity. Selected soil microorganisms based on their ecological importance and how they respond to changes may also be employed to monitor possible effects of GMPs using polyphasic molecular approach for comparism of different methods.

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APPENDIX A

MEDIA COMPOSITION

Nutrient Broth (Merck, Biolab South Africa).

Preparation: Suspend 16g in 1L demineralised water, mix well and dispense into final containers. Autoclave at 121°C for 15 minutes.

Composition	g/L
Meat extracts	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	8.0

Pikovskaya's Agar (Sigma Aldrich, South Africa).

Preparation: Suspend 31.3 grams of Pikovskaya's Agar in 1000 ml of distilled water. Boil to dissolve the medium completely and sterilize by autoclaving at 15 lbs. pressure 121°C for 15 minutes.

Composition	g/L
Yeast Extract	0.50
Dextrose	10.00
Calcium Phosphate	5.00
Ammonium Sulphate	0.50
Potassium Chloride	0.20
Magnesium Sulphate	0.10
Manganese Sulphate	0.0001
Ferrous Sulphate	0.0001

Agar	15.00

Luria Bertani Agar (Merck, Biolab South Africa).

Composition	g/L
Tryptone	12.0
Sodium Chloride	12.0
Yeast Extract	6.0
Final pH	7.5 (±0.2) at 25°C

***Pseudomonas* Selective Agar (Sigma Aldrich, South Africa).**

Composition	g/L
Gelatine peptone	20.0
Magnesium chloride	1.4
Potassium sulphate	10.0
Cetrimide	0.3
Agar	13.0
Final pH	7.2 (± 0.2) at 37°C

Tryptic Soy Agar (Merck, Biolab South Africa).

Composition	g/L
Tryptone	15
Soytone	5
Sodium Chloride	5
Agar	15

Nutrient Agar (Merck, Biolab South Africa).

Composition	%
Beef Extract:	0.3%
Peptone:	0.5%
Agar:	1.5%
Final pH	

PCR Master Mix (Fermentas, South Africa).

Composition	
Taq DNA polymerase	0.05u/µl
Reaction buffer	
$MgCl_2$	4 mM
dNTP (dATP, dCTP, dGTP and dTTP)	0.4 mM

Biolog GN2microplates (Cabot Bouleward Hayward, California, USA).

Composition	Substrate/microplate
Carbohydrates	30
Amino Acids	20
Carboxylic Acids	24
Polymers	5
Amines/Amides	6
Miscellaneous	10

API AUX Medium (BioMérieux® France).

Composition	
Agar	1.5 g
Ammonium sulfate	2 g
Vitamin solution	10.5 mL
Trace elements	10 mL
Monosodium phosphate	6.24 g
Potassium chloride	1.5 g
Demineralized water to make	1000 ml
Final pH	7.0 (±0.2)

APPENDIX B
MANUSCRIPT (PUBLISHED)

JOURNAL OF PURE AND APPLIED MICROBIOLOGY

Bacterial community profiling in the rhizosphere of field grown GM and non-GM maize

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Abstract

This study examined the impact of genetically modified maize on bacterial functional community in the rhizosphere. Rhizospheric soil samples from GM and non-GM corn were collected 30 days after sowing (DAS) and at post-harvest from two experimental fields in Gauteng, South Africa. Plate count results obtained on *Pseudomonas* selective media indicated 1.5×10^3 cfu in GM and 1.7×10^3 cfu in non-GM soil samples collected 30 DAS. At post-harvest, there was 80% decline in the rhizobacteria population in GM sample and 76% in the non-GM. To compare bacterial functional community in GM and non-GM soil, Biolog GN2 microplate, a sole carbon substrate utilization profile, was used and no significant difference

wasobserved. Based on analytical profile index identification system, species of *Pseudomonas putida*, *P. stutzeri* and *Achromobacter denitrificans* were identified in GM and non-GM soil samples collected 30 DAS. No *Pseudomonas* species was identified in soil samples at post-harvest. These findings are of great significance with regards to the investigation of possible impact of GM maize on bacterial functional community in the rhizosphere.

Keywords: Genetically modified maize, Rhizosphere, Biolog, Rhizobacteria.