Characterization of *Rhizobacteria* from Field Grown Genetically Modified (GM) and Non-GM Maizes

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ABSTRACT

This study was done to examine the rhizobacteria from field grown Genetically Modified (GM) maize and its non-GM counterpart. Rhizospheric soil samples were collected at 30 days after sowing (DAS) and at post-harvest from two experimental fields in Gauteng, South Africa. Total rhizobacteria (cfu/g) in GM and non-GM soil samples was not significantly different across the different media 30 DAS and at post-harvest. Rhizobacterial isolates obtained were biochemically characterized using the analytical profile index. Species of Pseudomonas, Aeromonas, Sphingomonas, Burkholderia, Stenotrophomonas, Achromobacter, Ewingella *and* Bacillus *were screened in vitro for plant growth promoting traits such as, ammonia production, catalase activity, indole acetic acid production, phosphate solubilisation, hydrogen cyanide production and antifungal activity. All the 32 rhizobacterial strains tested in this study were positive for catalase activity, ammonia production and IAA production; 90.6% were positive for phosphate solubilisation, 34.3% for indicate antifungal activity but none for hydrogen cyanide production. These findings contributed to the quest for potential biofertilizers and biocontrol agents for sustainable agriculture.*

Key words: Genetically modified maize, Plant growth promoting traits, Rhizosphere, Rhizobacteria

INTRODUCTION

Plant-microbe interaction occurs in the rhizosphere, a region referred to as a biological active zone of soil where microorganisms and plant roots interact, and is of major importance for plant growth as well as for the nutrient cycling and ecosystem (Babalola 2010). The rhizoplane or the root surface provides a highly favorable nutrient base and attachment site for many bacteria. However, in response to this microbial habitat and the nutrients provided by the plant, some microbes assist the plant by making nutrients available, while some may cause harm to the plant by acting as root pathogens (Anton et al. 2006). Kloepper and Schroth (1978) introduced the term plant growth promoting rhizobacteria (PGPR), referring to the bacteria capable of colonizing the plant root, or rhizosphere promoting plant growth. The 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 the plant growth (Gray and Smith 2005). Species of *Pseudomonas*, *Azospirilum*, *Azotobacter*, *Klebsiella*, *Bacillus*, *Arthrobacter*, *Alcaligenes*, *Serratia*, *Rhizobium*, *Beijerinckia*, *Zoogloea*, *Sinorhizobium* and *Mesorhizobium* have been reported to promote plant growth directly or

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indirectly (Esitken et al. 2003; Babalola 2010). The mechanism of action of the PGPR in the promotion of plant growth are not fully understood, but are thought to include the synthesis of phytohormones and lytic enzymes at the level of plant root (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 2007), asymbiotic fixation of nitrogen (Sahin et al. 2004) and the synthesis of antibiotics (Debbelaere et al. 2002). *Bacillus thuringiensis* (Bt) maize (a genetically modified maize) contains insecticidal toxic proteins (Bt endotoxins) that has advantages for crop production, such as increased insect resistance, grain yield and plant growth (Obrycki et al. 2001). These Bt endotoxins could be introduced in the rhizosphere through root exudates (Saxena et al. 2002) and could possibly affect the rhizobacterial community structure. Root exudates in the root-soil interface create a unique microbial microenvironment, differentiating it from the bulk soil not influenced by the roots (Hartman et al. 2006; Babalola 2010). Furthermore root exudates quality and quantity vary with the plant developmental stage and plant species determining the microbial community structure (Berg et al. 2002; Babalola 2007). Different rhizosphere populations have been reported in different plant species at different plant growth stages (Somers et al. 2004). According to Brusetti et al. (2004), microbial community associated with the rhizospheres of Bt maize differs from its non-Bt maize. In contrast, Devare et al. (2004) found that genetic modification of maize had no effect on the microbial communities in the rhizosphere. However, concerns about possible effects of genetically modified (GM) maize root exudates on rhizobacteria species and population needs to be considered.

Various studies have been carried out globally to isolate and screen the rhizobacteria for plant growth trait. Literature with regards to PGPR from genetically modified maize and its non-GM counterpart is rare. Coupled with the idea that GM plants could release high root exudate due to increase rate of photosynthesis, this study attempted to isolate and characterize the PGPR in field grown GM maize and its non-GM counterpart. A good knowledge and understanding of the different species and their mechanism in plant growth promotion could be used in sustainable agriculture.

MATERIALS AND METHODS

Isolation and characterization
Soil samples of field grown GM and non-GM maize were collected from two experimental fields in Gauteng, South Africa 30 days after the sowing (DAS) and at post-harvest. Sixteen soil samples were collected in triplicate; eight from the GM maize area and the other eight from the non-GM maize area.

One gram of soil sample of field grown GM and non-GM maize was transferred into test tubes with 9.0 mL of distilled water. Serial dilutions up to 10^{-6} were made using distilled water. A volume of 0.5 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). The plates were incubated at 28°C for two to three days. Bacterial growth was recorded as colony forming units (cfu). Colonies were randomly selected and biochemically identified using the analytical profile index (API), followed by *in vitro* characterization for plant growth promoting traits.

Genomic DNA extraction
Rhizobacterial isolates from the GM and non-GM soil samples were grown overnight in nutrient broth and genomic DNA was extracted by the hexadecyltrimethy-lammonium bromide (CTAB) method (Doyle and Doyle 1990). The bacterial 16S rDNA primer Ps-for (5'GGTCTGAGAGGATGA-TCAGT3') and Ps-rev (5'TTAGCTCCACCTCGCGGG3') as well as universal primer 27F (5'AGAGTTTGATCCTGGCTCAG3') and 1492R (5'TGACTGACTGAGACTACCTTGTTACGA3') were used to amplify 989 bp and 1500 bp of the 16S rDNA gene region. Polymerase chain reaction (PCR) was done using a DNA Engine DYAD^{TM} Peltier Thermal Cycler. Thermal cycling were performed as follows: Initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min and final extension at 72°C for 8 min. The fragments obtained were analyzed 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 G: BOX SYNGENE. The PCR products were sent to Inqaba laboratory in Pretoria for sequencing. Sequence search for the alignment of nucleotides was performed using the BLAST (Basic Local Alignment Search Tool) web-based program (Altschul et al. 1990).

Production of Indole Acetic Acid (IAA)

IAA production was detected as described by (Brick et al. 1991). Freshly grown cultures in the nutrient broth amended with tryptophan and incubated at $36\pm2°C$ for 48-72 h were centrifuged at 7500 x g for 30 min. After which, 2.0 mL of the supernatant was mixed with two drops of 10 mM orthophosphoric acid and 4.0 mL of the Salkowski reagent (50 mL, 35% of perchloric acid, 1.0 mL 0.5 M $FeCl_2$ solution). The mixture was incubated at room temperature for 25 min. The development of pink colour indicated the production of IAA.

Production of Ammonia

Freshly grown cultures of the isolates were inoculated in 10 mL of peptone water in a tube and incubated for 48-72 h at $36\pm2°C$. After which, 0.5 mL of Nessler's reagent was added in the tube. The development of brown to yellow colour was a positive test for ammonia production (Cappuccino and Sherman 1992).

Catalase Production

Catalase test was done by adding a drop of 3% hydrogen peroxide to freshly grown bacterial isolate on a sterile slide using a sterile wire loop. The effervescence indicated catalase activity.

Antifungal Activity

Antifungal activity was tested using the method of Mehnaz and Lazarovits (2006). Test bacterial isolates were co-inoculated on potato dextrose agar (PDA) containing the fungal pathogen and incubated at 27°C for five days. Antibiotic production against the pathogen was observed as a zone of growth inhibition around the tested bacterial isolate.

Phosphate Solubilization

Qualitative assay for phosphate solubilization was performed using Pikovskaya medium containing tricalcium phosphate (Pikovskaya 1948). Bacterial culture were streaked on the medium and incubated at 30°C for 48 h. The presence of a clear halo zone around the culture spot indicated phosphate solubilization capacity of the isolate.

Production of Hydrogen Cyanide (HCN)

Freshly grown isolates were screened for HCN production by adapting the method of (Lorck 1948). Isolates grown in nutrient broth amended with 4.4 g glycine/L were streaked on the modified nutrient agar plate. A sterile Whatman filter paper no.1 soaked in 2.5% sodium carbonate in 0.5% picric acid solution was placed at the top of the plate. Plates were sealed with parafilm and incubated at $36\pm2°C$ for four days. Change of yellow colour on Whatman filter paper to orange-red colour indicated HCN production.

RESULTS AND DISCUSSION

Total rhizobacteria cfu/g of GM and non-GM soil samples across the different media is presented in Figure 1. There was no significant difference in the rhizobacterial population between the GM and non-GM soil sample either at 30 DAS, or at post-harvest. However, plate count for *Pseudomonas* selective medium at post-harvest indicated a significant decrease in the rhizobacterial population when compared to the population at 30 DAS. Based on the API identification, similar bacterial species were identified in the GM and non-GM maize rhizosphere (Table 1). However, different rhizosphere species were identified at 30 DAS and at post-harvest from PSA. *P. putida*, *P. stutzeri* and *A. denitrificans* were identified in the soil samples at 30 DAS. The post-harvest identification indicated species of *Burk. cepacia* and *A. denitrificans* in the GM and non-GM with no species of *Pseudomonas*.

In this study, species of *Pseudomonas*, *Aeromonas*, *Sphingomonas*, *Burkholderia*, *Stenotrophomonas*, *Achromobacter*, *Bacillus* and *Ewingella* isolated from maize rhizosphere (GM and non-GM) were tested *in vitro* for the plant growth promoting traits (Table 2). Of the 32 rhizobacterial strains tested in this study, all strains were positive for catalase activity, ammonia production and IAA production; 90.6% were positive for phosphate solubilisation, 34.3% indicated antifungal activity but none were positive for hydrogen cyanide production. There was no significant difference in the pH levels of soil samples (GM and non-GM) 30 DAS and at

post-harvest (Table 3). Loam soil was used in this study.

The amplification of the 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 1.5 and 1.0 kb (Figs.

2 and 3). The sequence analysis of Gram-positive rhizobacterial isolates from the GM and non-GM maize rhizosphere are shown in Table 4. The rhizobacteria isolates EW51, EW62, EW66, EW68, EW6, EW5, and EW35 belonged to *B. cereus*, *Bacillus* spp, *B. aryabhattai*, *B. pumilus*, *B. megaterium*, *B. thuriengiensis* and *B. safensis*.
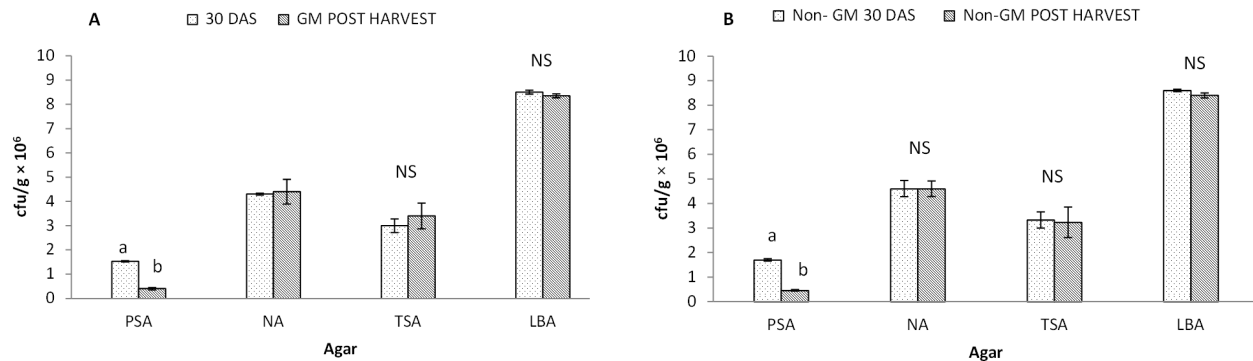

Figure 1 - Total rhizobacterial cfu/g of soil on *Pseudomonas* selective agar (PSA), nutrient agar (NA), tryptic soy agar (TSA) and Luria Bertani agar (LBA) from field grown Genetically Modified (GM) and non-GM maize soil samples 30 DAS and at post-harvest. Bars represent standard error of the mean of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

Table 1 - Identification of rhizobacterial isolates recovered at 30 DAS (days after sowing) and at post-harvest using analytical profile index (API).

Source	Time of collection	Medium	Species obtained
GM	30 DAS	NA	*S. paucimobilis, Sten. maltophilia, P. luteola*
		TSA	*S. paucimobilis, Burkholderia cepacia.*
		LBA	*S. paucimobilis, P. luteola, Burkholderia cepacia*
		PSA	*P. putida, A. denitrificans*
	Post-harvest	NA	*S. paucimobilis, Sten. Maltophilia, Burkholderia cepacia*
		TSA	*S. paucimobilis, P. stutzeri*
		LBA	*S. paucimobilis, Aeromonas 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, Burkholderia cepacia*
		PSA	*P. stutzeri, A. denitrificans*
	Post-harvest	NA	*S. paucimobilis, Burkholderia cepacia*
		TSA	*S. paucimobilis, E. americana*
		LBA	*S. paucimobilis, Ent. coacae*
		PSA	*Burkholderia cepacia, A. denitrificans*

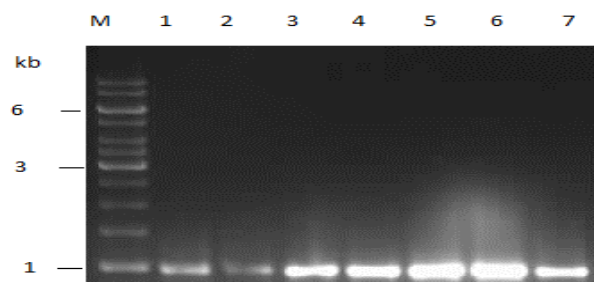GM = genetically modified; Non-GM = Non genetically modified

Table 2 - Plant growth promoting traits of rhizobacterial isolates from genetically modified (GM) and non-GM maize rhizosphere.

Source	Bacteria (no of strain)	Antifungal activity	Catalase activity	Ammonia production	IAA production	HCN production	Phosphate solubilization
GM	*P. luteola* (3)	+	+	+	+	-	+
	Aero. hyrophila (1)	-	+	+	+	-	-
	Sten. maltophilia (1)	-	+	+	+	-	+
	B. thuringiensis (1)	+	+	+	+	-	-
	P. putida (2)	-	+	+	+	-	+
	S. paucimobilis (3)	+	+	+	+	-	+
	Burk. cepacia (3)	-	+	+	+	-	+
Non-GM	*A. xylosoxidans* (1)	-	+	+	+	-	+
	S. paucimobilis (3)	+	+	+	+	-	+
	Bur. cepacia (3)	-	+	+	+	-	+
	Sten. maltophilia (1)	-	+	+	+	-	+
	E. americana (1)	-	+	+	+	-	+
	P. stutzeri (3)	-	+	+	+	-	+
	P. putida (3)	-	+	+	+	-	+
	B. thuringiensis (1)	+	+	+	+	-	-
	B. safensis (2)	-	+	+	+	-	+

+ = Positive reaction, - = Negative reaction

Table 3 - pH results of Genetically Modified (GM) and non-GM maize rhizosphere soil samples. The results represent the mean of three replicates ± standard error.

Source	Period of collection	pH values mean ±standard error
GM	30 DAS	$6.74^a \pm 0.04$
	Post-harvest	$6.78^a \pm 0.16$
Non-GM	30 DAS	$7.03^a \pm 0.06$
	Post-harvest	$6.12^a \pm 0.59$

Means followed by same letters are not significantly different at $P \geq 0.05$ using analysis of variance.
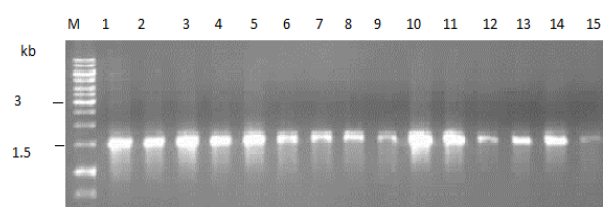
Figure 2 - Electrophoresis gel in agarose (1%) of PCR products amplified from GM and non-GM soil samples from nutrient agar (NA), *Pseudomonas* selective agar (PSA), trypticase soy agar (TSA) and Luria Bertani agar (LBA). Lane M, 1-kb DNA ladder, lane 1-3 *Bacillus thuringiensis*, lane 4-5 *B. safensis*, lane 6-9 *Sphingomonas 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*.

Figure 3 - Electrophoresis in agarose gel (1%) of PCR products amplified from Genetically Modified (GM) and non-GM soil samples from *Pseudomonas* selective media. Lane M, 1-kb DNA ladder, lane 1-3 *Pseudomonas putida* and lane 4-7 *Pseudomonas Stutzeri*.

Table 4 - Sequence analysis of rhizobacterial isolates from GM and non-GM maize rhizosphere.

Source	Isolate number	Description	% Identity	Accession n°.
GM	EW51	*B. cereus*	100	JX317637
	EW62	*Bacillus* spp.	99	AB736322
	EW66	*B. aryabhattai*	100	JX293286
	EW68	*B. pumilus*	100	HQ625388
Non-GM	EW6	*B. megaterium*	100	JX393073
	EW5	*B. thuriengiensis*	99	JX283457
	EW35	*B. safensis*	99	JX094951

The PGPR make up a diverse group of rhizosphere-colonizing bacteria in association with the plant roots, which promote growth of the plant. According to Cattelan et al. (1999), PGPR should be able to survive and colonize the rhizosphere. Their colonizing ability has been attributed to plant species (Garbeva et al. 2004) and antagonistic potentials (Berg et al. 2002).

In this study, identified species were able to survive in the maize rhizosphere 30 DAS and at post-harvest. The no significant difference in rhizobacterial population in the maize rhizosphere of GM and non-GM at 30 DAS and at post-harvest did not comply with the idea that GM plants could exhibit high photosynthetic activity and as a result release more root exudates resulting to higher microbial population in the rhizosphere.

S. paucimobilis, *Sten. maltophilia*, *Burk. Cepacia*, *P. putida*, and *Bacillus* spp obtained in this study have been reported in the maize rhizosphere (Chelius and Triplett 2000; Mehnaz and Lazarovits 2006; Mehnaz et al. 2007). These findings could be of significance because species of *B. cepacia* and *Sten. maltophilia* isolated in this study have been described as opportunistic human pathogens (Berg et al. 2005) where they could cause lung infections and bacteraemia, Therefore, future prospects in biotechnological applications needs further considerations.

Changes in the rhizosphere species throughout the developmental stages of the plants have been documented and attributed to changes in root exudates quality and quantity (Berg et al. 2002) According to Kapoor et al. (2012), plant age and nutrient exhaustion throughout the plant developmental stage could lead to a decrease in *Pseudomonas* population in the rhizosphere. These were in agreement with the present results, which indicated different rhizobacteria species be at 30 DAS, or at post-harvest using *Pseudomonas* selective medium. The persistence of *Burk. cepacia* throughout the maize developmental stages in this study correlated with the findings of Di Cello et al. (1997) who reported an increase in *Burk. cepacia* population throughout the season in maize rhizosphere at different plant growth stages.

Catalase activity is an important trait of bacteria as it helps the bacteria to protect itself against hydrogen peroxide, a poisonous compound to bacteria and plant roots. Therefore, PGPR with catalase activity will survive in the rhizosphere and promote plant growth indirectly. All the rhizobacterial strains in this study were catalase positive, correlating with earlier studies (Babu et al. 2007; Chaiharn et al. 2008). IAA a phytohormone, which controls root elongation and cell division in the plants, is produced from tryptophan by the process of decarboxylation and transamination (Khan et al. 2009). Sources of IAA other than those produced by the plants can also contribute to the morphological changes at the level of root system influencing the uptake of nutrients by the plant (San Francisco et al. 2005). In this study, all the rhizobacterial strains tested produced IAA in nutrient broth amended with tryptophan, and therefore, complied with the studies of Ahemad and Mohammad (2010) and Marques et al. (2010).

Similarly ammonia production was evident by all the bacteria isolates. Ammonia production is an important trait of PGPR, which promotes plant growth indirectly. Only the strains of *S. paucimobilis* and *P. luteola* showed antifungal activity against *F. oxysporum* a plant pathogen. This activity was read as zone of inhibition and could have contributed the dominance ability of *S, paucimobilis* in the rhizosphere of the GM and non-GM maize throughout the growing season. *P. luteola* antifungal activity in this study complied with that of Bano and Musarrat, (2003) and in combination with *Sphingomona* species could serve as potential biocontrol agents in the promotion of plant growth.

Phosphorous (P) is the second most important nutrient required by the plants for the growth compared to nitrogen (Pradhan and Sukla 2006). Podile and Kishore (2007) referred to phosphate solubilising bacteria as the bacteria capable of secreting phosphatases and organic acids, which converts the insoluble phosphate to soluble monobasic and dibasic ions. Rhizobacteria exhibiting this process makes phosphorous available for the plant uptake at the level of plant root (Gyaneshwar et al. 2002). In this study, the rhizobacterial isolates from the rhizosphere of the GM and non-GM maize were screened for phosphate solubilizing activity by the spot plate inoculation method on Pikovskaya's medium containing tricalcium phosphate. Phosphate solubilization was expressed in terms of clear zone produced around the bacterial strain. Of the 32 rhizobacterial strains tested, 90.6% were positive for phosphate solubilization. Therefore, rhizobacteria strains in this study were capable of solubilizing the mineral P in the rhizosphere hence, provided soluble P to plants.

Cynogenic activity (HCN production) by the rhizobacteria has been suggested to play a significant role in the biological control of pathogens (Voisard et al. 1989). The production of HCN by the rhizobacterial strains into the rhizosphere has been reported (Devi et al. 2007). In this study, all the rhizobacterial strains tested for cynogenic activity were negative. Results indicated that the bacterial strains could not induce resistance to plant from the antibiosis.

In this study, most rhizobacterial isolates showed four plant growth promoting traits, confirming that the PGPR strains were capable of expressing the multiple beneficial functions (Kloepper and Schrot 1978). However, *in vivo* screening and synergic biotechnological application are required for further confirmation as plant growth promoters.

CONCLUSIONS

In this study, most rhizobacterial isolates from the GM and non-GM maize rhizosphere showed four plant growth promoting traits, confirming that the PGPR strains were capable of expressing the multiple beneficial functions (Kloepper and Schrot 1978). However, *in vivo* screening and synergic biotechnological application of the PGPR as plant growth promoters are required for further confirmation. Therefore, considering the fact that PGPR are eco-friendly with regards to the misuse of chemical fertilizer and pesticide, which are not friendly to native soil rhizobacteria and environment puts PGPR as a better alternative to chemical fertilizers and pesticides.

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