Bacterial Community Profiling in the Rhizosphere of Field Grown GM and Non-GM maize

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This study examined the impact of genetically modified corn on bacterial functional community in the rhizosphere. Rhizospheric soil samples from GM and non-GM corn were collected at 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 at 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 was observed. Based on analytical profile index identification system, species of *Pseudomonas putida*, *P. stutzeri* and *Achromobacter denitrificans* were identified in GM and non-GM at 30 DAS. No *Pseudomonas* species was identified in 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.

Key words: Genetically modified maize, Rhizosphere, Biolog, Rhizobacteria.

Genetically modified plants (GMPs) are plants carrying genetic traits that are not naturally present in the plant[1]. 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[2]. Since the first commercialization of GM crops in 1996 the planting of GM crops has increased by 10% or more each year worldwide[2]. In 2009, a total of 28.7 million hectares of GMPs were planted worldwide compared to 26.9 million hectares in 2008[3]. However, the possible effects of genetically modified plants on human health and ecological functioning have been debated extensively[4]. Brusetti *et al*[5] 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. Meanwhile, Devare *et al*[6] found that genetic modification of 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

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targeted changes in microbial community composition, by inhibiting plant pathogenic organisms[7]. However most studies show either minor non-target effects[8,9] or no detectable non-target effects[10,11].

Plants are major drivers of the 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[1]. Soil anchors plants and harbours diverse range of microorganisms (bacteria, protista, and fungi). Plant-root 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 nutrient cycling and ecosystem functioning[12]. Root exudates in the root-soil interface create a unique microbial microenvironment, differentiating it from bulk soil not influenced by the roots[13]. Furthermore root exudates quality and quantity vary with plant developmental stage, plant species and determine microbial community structure[14]. Different rhizosphere populations have been reported in different plant species at different plant growth stages[15].

Maize root exudates are composed of 33% organic acid, 65% sugars and 2% amino acids, leading to different rhizobacterial community structure[16]. Species of *Pseudomonas, Azospirilum, Azotobacter, Bacillus, Arthrobacter, Serratia, Rhizobium, Beijerinckia, Zoogloea, Sinorhizobium and Mesorhizobium,* have been reported as native rhizosphere organisms[17,18,19].

Bt maize contains insecticidal toxic proteins (Bt endotoxins) that has advantages for crop production, such as increased insect resistance, grain yield and plant growth[20]. However, a good knowledge of the effects of exudates from GMPs on soil microorganisms is important for implementing assessments techniques that could reduce any negative impacts[21].

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[22]. While molecular profiling techniques such as proteomics, transcriptomics and metabolomics have been used[23] 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[1]. 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. 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 recomended[24] 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[25].

The objective of this study is to evaluate the effect of Bt maize exudates on the rhizobacterial community structure. To investigate this, we evaluated *Pseudomonas* plate count and sole carbon substrate utilization profile (SCSUP) of field grown GM maize with its non-GM counterpart.

MATERIALS AND METHODS

Soil **s**amples of field grown GM and non-GM maize were collected from 2 experimental fields in Gauteng, South Africa 30 days after sowing (DAS) and at post-harvest. Sixteen soil samples were collected in triplicate. Of the sixteen soil samples 8 were collected in the GM maize area and the other 8 from the non-GM maize area.

One gram of soil sample of field grown GM and non-GM maize was weighed and transferred into test tubes with 9 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 *Pseudomonas* selective media (Sigma Aldrich, South Africa). 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 that indicated oxidase activity were further identified using the analytical profile index API 20NE (Biomérieux, France) identification system.

Rhizobacterial isolates from GM and non-GM soil samples were grown overnight in nutrient broth and genomic DNA was extracted by the

hexadecyltrimethy-lammonium bromide (CTAB) method[26]. The 16S rDNA gene region about 989 bp was amplified[27]. The bacteria 16S rDNA primer Ps-for (5'GGTCTGAGAGGATGATCAGT3') and Ps-rev (5'TTAGCTCCACCTCGCGGG3') was used. Polymerase chain reaction (PCR) was done using a DNA Engine DYAD™ (MD Research, USA). 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 (Fermentas, South Africa) was used as a molecular size marker and loaded on the left lane of the gel. The gel was then stained with ethidium bromide and digital picture of amplified gene was taken under UV light (G: BOX SYNGENE, USA).

Sole carbon substrate utilization profile (SCSUP) of GM and non-GM microbial community function was studied using Biolog GN2 microplates (Cabot Bouleward Hayward, California, USA)[28]. The 96 wells of the microplate contain different carbon source (except the blank well with no substrate) and the redox dye tetrazolium. 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)[29]. The redox dye tetrazolium is soluble in water in its oxidised state and appear colourless. Microbial respiration produces Nicotinamide Adenine Dinucleotide plus Hydrogen (NADH) 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[30].

An amount of 4 g of GM and non-GM soil samples was diluted in 36 ml of sterile 0.85% NaCl, vortexed for 5 min, and serial dilutions up to 10^{-3} made. Amounts of 150 µl of 10^{-3} dilution 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 and recoding 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). Group-wise average well colour development of the total per group of substrates ($AWCD_G$, indicating the sum of absorbance units of individual substrate sets divided by the number of compounds belonging to the substrate group) was also recorded.

SCSUP analysis was done by calculating the net absorbance of each well. This was obtained by subtracting the absorbance value of the control plate from the absorbance value of each plate. 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[31]. Substrate utilization pattern for each (carbon substrate) was subjected to one way analysis of variance (ANOVA) using statistical package for the social sciences (SPSS) software.

RESULTS AND DISCUSSION

In this study we evaluated the possible impact of field grown GM maize root exudate on rhizobacteria in contrast to root exudate of the non-GM counterpart. Considering the SCSUP analysis, AWCD pattern was similar in GM and non-GM soil samples 30 DAS, indicating no significant difference in rhizobacterial community function. A similar pattern was observed for soil samples at post-harvest, indicating AWCD did not increase nor significantly reduce throughout the growing season (Table 1). Furthermore, there was no difference in the rhizobacterial community between the two experimental sites. From results obtained on SCSUP through inoculation of Biolog microplates in this study, we can conclude that bacterial functional community in soil samples 30 DAS and at post-harvest were highly active (high optical density). Based on the similar pattern of SCSUP in the entire Biolog microplates 30 DAS and at post-harvest we hypothesised that there might have been no changes in bacterial functional community throughout the growing season. Changes in microbial functional community have

been attributed to variation of soil type and not due to genetic modification of maize[32]. SCSUP (substrate per group) and $AWCD_G$ on Biolog plates indicated that polymers, amino acids, carboxylic acids, miscellaneous, carbohydrates and amide/amines were all preferred by microbial community in both GM and non-GM 30 DAS and at post-harvest (Tables 2 and 3).

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

Soil samples	OD_{620nm}	Visual observation
Non-GM 30 DAS	$0.65^a \pm 0.03$	59
GM 30 DAS	$0.66^a \pm 0.01$	58
Non- GM post-harvest	$0.64^a \pm 0.05$	53
GM post-harvest	$0.64^a \pm 0.04$	54

OD: Optical density

Table 2. Biolog substrate utilization by microbial communities of GM and non-GM soil samples 30 DAS and at post-harvest

Group of compounds	Number of carbon source Per group in Biolog GN2 microplate	GM 30 DAS	Non-GM 30 DAS	GM post-harvest	Non-GM post-harvest
Carbohydrates	30	19 (63.3%)	20 (66.6%)	19 (63.3%)	18 (60%)
Carboxylic acid	24	20.5 (85.4%)	21.5 (89.5%)	19 (79%)	19 (79%)
Amino acids	20	16.5 (82.5%)	17 (85%)	16 (80%)	15.5 (77.5%)
Polymers	5	4 (80%)	4 (80%)	4 (80%)	4 (80%)
Amines/Amides	6	4 (66.6%)	4 (66.6%)	3.5 (58.3%)	3.5 (58.3%)
Miscellaneous	10	7.5 (75%)	7.5 (75%)	7 (70%)	6.5 (65%)

Number in the body of table: number of utilized carbon sources of the given substrate with their percentages

Table 3. Group-Wise Average Well Colour Development ($AWCD_G$) of Carbon Sources by microbial communities in GM and non-GM soil samples 30 DAS and at post-harvest. Overall values represent mean absorbance (OD_{620nm}) per group divided by number of carbon (C) sources per group during an observation period of 120 h

Group of compounds	Number of carbon source Per group in Biolog GN2 microplate	GM 30 DAS	Non-GM 30 DAS	GM post-harvest	Non-GM post-harvest
Carbohydrates	30	$0.68^a \pm 0.05$	$0.61^a \pm 0.09$	$0.67^a \pm 0.05$	$0.63^a \pm 0.09$
Carboxylic acids	24	$0.75^a \pm 0.02$	$0.75^a \pm 0.06$	$0.73^a \pm 0.02$	$0.73^a \pm 0.01$
Amino acids	20	$0.81^a \pm 0.02$	$0.81^a \pm 0.10$	$0.80^a \pm 0.05$	$0.72^a \pm 0.03$
Polymers	5	$0.64^a \pm 0.04$	$0.64^a \pm 0.06$	$0.68^a \pm 0.02$	$0.72^a \pm 0.01$
Amines/Amides	6	$0.31^a \pm 0.07$	$0.46^a \pm 0.02$	$0.30^a \pm 0.13$	$0.26^a \pm 0.03$
Miscellaneous	10	$0.37^a \pm 0.20$	$0.35^a \pm 0.02$	$0.31^a \pm 0.02$	$0.35^a \pm 0.02$

Means followed by same letters are not significantly different at Pe"0.05 using analysis of variance

Amongst the six polymers, Tween 80 and 40 showed high distinctive values for all soil samples at 30 DAS and at post-harvest. Carbohydrates such as D-galactose, D-glucose D-mannitol, Maltose, á-D- glucose, D-fructose, D-mannose N- Acetyl-D-galactosamine, N-Acetyl-D-glucsamine and Gentiobiose where highly utilized (OD greater than 1.5 nm) in both soil samples at 30 DAS and at post-harvest. Quinic acid, D-saccharic acid, Sebacic acid, D-L-lactic acid and á-keto Glutaric acid were the most preferred carboxylic acid substrates on all Biolog plates. Other carboxylic acids such as D-glutamic acid, D-glucoronic, D-saccharic acid and D-galacturonic acid where utilized. Amino acids such as L-proline, L-threonine, L-glutamic acid, L- serine, L-aspartic acid, and L-asparagine could be observed as most utilized Amino acids indicating high optical density in Biolog panel of both GM and non-GM soil samples. Amongst the ten miscellaneous compounds in Biolog plates, only urocanic acid indicated a high OD greater than 1 nm in all the soil samples. Finally microbial community function in amides/amine showed low utilization pattern in all the soil samples with OD of 1 nm observed only in glucuronamide and L- Alaninamide.

In this study we found that there was no significant difference in the pH levels of soil samples (GM and non-GM) 30 DAS and at post-harvest (Table 4). Bacterial functional community and structure could be affected by soil temperature, soil type, soil pH, nutrient status, water content and anthropogenic activity[33]. One soil type (loam soil) was used in this study. Therefore, similar pH and soil type was never considered as an interfering factor.

Similar bacterial population was observed in GM and non-GM at 30 DAS and GM and non-GM at post-harvest. However, post-harvest analysis for GM and non-GM, respectively showed an 80% and 76% decrease in bacterial population when compared to population at 30 DAS (Table 4). Similar bacterial population associated with GM and non-GM soil samples at 30 DAS and at post-harvest, did not comply with the suggestion that GM plants with high photosynthetic activity could release more root exudates resulting to high microbial population in the rhizosphere[34]. We hypothesised that root exudate quantity and quality might have changed throughout the growing season resulting in the decrease in bacterial population at post-harvest. These findings comply with Sommers et al[15] who observed changes in rhizosphere population during plant developmental stage.

Using the API identification system, species of *Pseudomonas putida*, *P. stutzeri*, *Achromobacter denitrificans* and *Burkholderia*

Table 4. pH and plate count of soil samples

Samples (soil)	p		*cfu/g	
	30 DAS	Post-harvest	30 DAS	Post-harvest
GM	$6.74^a \pm 0.04$	$6.78^a \pm 0.16$	$1.5^a \times 10^3$	$0.3^b \times 10^3$
Non-GM	$7.03^a \pm 0.06$	$6.12^a \pm 0.59$	$1.7^a \times 10^3$	$0.4^b \times 10^3$

*cfu- colony forming units.
Means followed by same letters are not significantly different at Pe"0.05 using analysis of variance. For cfu, values followed by different letters are significantly different.

Table 5. Species in GM and non-GM soil samples 30 DAS
and at post-harvest based on API 20 NE identification system

Samples (soil)	Species
Non-GM 30 DAS	*Pseudomonas stutzeri, Achromobacter denitrificans*
GM 30 DAS	*Pseudomonas putida, Achromobacter denitrificans*
Non-GM post-harvest	*Burkholderia cepacia, Achromobacter denitrificans*
GM post-harvest	*Burkholderia cepacia, Achromobacter denitrificans*

cepacia were identified in soil samples at 30 DAS and at post-harvest (Table 5). *P. putida*, *P. stutzeri* and *A. denitrificans* were all identified in soil samples at 30 DAS. Meanwhile, post-harvest identification indicated species of *B. cepacia* and *A. denitrificans* in both samples GM and non-GM with no species of *Pseudomonas*. Persistence of

B. cepacia throughout the maize developmental stages in our studies correlates with the findings of Di Cello *et al*[35] who reported an increase in *B. cepacia* population throughout the season in maize rhizosphere at different plant growth stages. Furthermore, we found that, *Pseudomonas* species did not persist throughout the growing season in
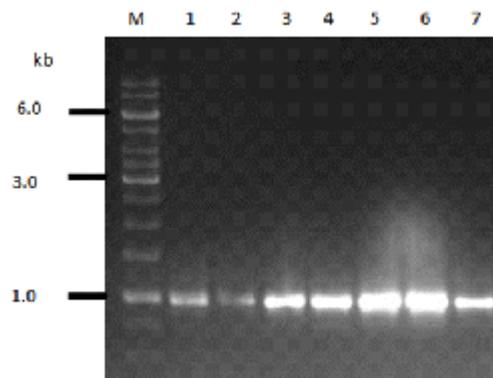
Fig. 1. PCR products amplified from GM and non-GM soil samples from *Pseudomonas* selective media. Lane M, 1-kb DNA ladder, lane 1-3 *P. putida* and lane 4-7 *P. stutzeri*

both GM and non-GM soil samples, suggesting that root exudate quantity and quality might have changed throughout the growing season with respect to the plant developmental stage resulting in change in the rhizosphere species at post-harvest. Changes in rhizosphere species throughout developmental stages of plants have been documented and attributed to change in root exudate[14].

Amplification of genomic DNA from the rhizobacterial isolates using *Pseudomonas* primers (Ps-for and Ps-rev) yielded DNA fragments with band size of 1.0 kb (Figure 1). Results confirmed the specificity of the primer targeting the 16S rDNA *Pseudomonas*' genes.

CONCLUSIONS

Based on the results obtained in our studies, the changes observed in the rhizobacterial population and in the different soil species throughout the growing season, cannot be attributed to genetic modification of maize with respect to control (non-GM). Our findings comply with reports that changes in rhizobacterial

population in the rhizosphere correlated with soil texture and not to genetic modification of maize[36,37]. Biolog GN2 technology, like any other technology has benefits and drawbacks. One drawback is the difficulty in interpreting the results and the method of application. Despite these disadvantages, it has contributed, and still continues to contribute to the understanding of the microbial functional community and structure between GM and non-GM crops. Bacterial population variation is not a sensitive indicator for the assessment of the effects of GM crops, considering the fact that other factor such as predation can reduce the number of soil bacteria[38]. However, the use of bacterial population to assess changes remains important because they provide basic assessment data and highlight any change compared with controls[1]. In conclusion, *Pseudomonas* species ubiquitous nature made it a focus in our studies to assess the impact of GM maize on rhizoshphere bacterial functional community. Nevertheless, this does not exempt the entire rhizobacterial community in soil samples but, offers different risk assessments criteria which can contribute to the data base of safety techniques.

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