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Analysis of Rhizobacterial Community in Field Grown GM and Non-GM Maize Soil Samples Using PCR-DGGE

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Abstract This study assessed the impact of *Bacillus thuriengensis* genetically modified maize (GM Bt maize) on functional community of rhizobacteria. Denaturing gradient gel electrophoresis technique (DGGE) was used. Results indicated that band patterns of GM and non-GM samples were similar to each other at 30 days after sowing (DAS) and 1 day after harvest (DAH). Sequence analysis showed that uncultured bacteria, *Actinobacterium*, *Bradyrhizobium* spp., and *Sphingomonas* spp., were common in the soil samples indicating that these were a stable community and species. These findings showed that the GM maize did not alter the soil microbial community which is significant in the assessment of the impact of GM maize on rhizobacteria.

Keywords: Genetically modified maize, Rhizobacteria, PCR-DGGE, Sequencing

Introduction

Maize (*Zea mays*) is one of the most important staple food crops cultivated worldwide. In South Africa, maize is one of the genetically modified (GM) crops approved for planting and commercialization (African Center For Biosafety 2010). According to James (2010) there is high prospect of GM crops cultivation in the future. Farmers are shifting to GM maize simply because it is reported to have higher yields because of its resistance to pest or diseases, herbicide tolerance or resistance to abiotic factors such as drought (Isik and Guenther, 2008). However, there are environmental concerns on the impact of GM crop on soil microorganisms in the soil. Rhizobacteria are a group of bacterial that colonize plant roots and can promote plant growth directly or indirectly (Babalola, 2010). Therefore, the impact of cultivating GM crops has been considered. Some

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studies have reported differences in rhizobacterial diversity in GM maize with its non-GM counterpart (Chelius and Triplett, 2000; Saxena and Stotzky, 2001; Schmalenberger and Tebbe, 2002; Brusetti *et al*., 2004; Lynch *et al*., 2004) while, the works of (Devare *et al*., 2004; Oger *et al*., 2004; Bumunang *et al*., 2013) reported no difference in rhizobacterial community. Molecular biological techniques are shedding light on the possible impact of GM crops on soil microorganisms (Stephen and Kowalchuk, 2002).

The use of the polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) technique to study microbial diversity was first introduced by Muyzer *et al*. (1993). 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 behavior 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 with decreased migration through the gel. Therefore, this technique can be used to compare many different samples of microorganisms with different melting domains. Difference in sample profiles (depicted by many bands in the gel) reflects the microbial diversity of the sample.

Objectives: Although there are a number of studies on the possible impact of GM maize on microorganisms, much data is required to determine any long term possible effects. In this regard, we used the PCR-DGGE technique to assess the rhizobacterial phylotypes in field grown GM and non-GM maize soil samples. These findings contributed to the evaluation of the possible impact of GM maize on rhizobacteria in the soil.

Materials and methods

Study site

Sixteen soil samples of field where GM Bt maize and non-GM maize were grown, collected from an experimental field in Delmas (26°09′S, 28°41′E), South Africa 30 days after sowing (DAS) and 1 day after harvest (DAH). Eight samples were collected from the soil where GM maize was

grown and eight samples from the soil where non-GM maize was grown 35 cm apart.

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. PCR amplification targeting bacterial 16S rDNA was performed with the primer set of 357F-GC (guanine cytosine) clamp (5′CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCCTACGGGAGGCAGCAG-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^{TM} Thermal Cycler (Bio-Rad, USA). The amplification conditions were 94℃ for 3 min, followed by 30 cycles of 94℃ for 1 min, 50℃ for 1 min, and 72℃ for 1 min, with a final extension at 72℃ for 10 min. The 200bp PCR products were checked by gel electrophoresis on 1% agarose gel, 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 the amplified gene taken under UV light using a BIO-RAD ChemiDoc^{TM} MP imaging system (Bio-Rad, USA).

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℃ and 100 V using a 16 x 16 x 0.1 cm BIO-RAD DCode^{TM} 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^{TM} 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).

Twelve bands were carefully excised from the DGGE gel using sterile razor blade. Each 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 overnight at 4 ℃. One microliter of eluted DNA was used as a template for PCR amplification with a second set of primers 357F (5'-CCTACGGGAGGCAGCAG-3') without GC clamp and 518R (5´-ATTACCGCGGCTGCTGG-3´). The amplification conditions were 94 ℃ for 3 min, followed by 30 cycles of 94 ℃ for 1 min, 50 ℃ for 1 min, and 72 ℃ for 1 min, with a final extension at 72 ℃ 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^{TM} MP imaging system (Bio-Rad, USA). PCR products were sent to Inqaba laboratory in Pretoria for sequencing.

Results and discussion

DGGE is a fingerprinting technique used to compare bacterial community profiles. In this study, rhizobacterial phylotypes were compared between soil samples obtained from field grown GM and non-GM maize. The soil samples were analysed using DGGE. Amplification of bacterial 16S rDNA with the primer set of 357F-GC clamp (5´CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCCTACGGGAGGCAGCAG-3´) and 518R (5´-ATTACCGCGGCTGCTGG-3´) yielded DNA fragments of about 200 bp when separated in a 1% agarose gel (Figure 1). Visual observation indicated that a total of about eighty two intense and faint DGGE band profiles were obtained in DAS and DAH soils from both GM and non-GM maize fields suggesting a diverse rhizobacterial phylotype (Figure 2).

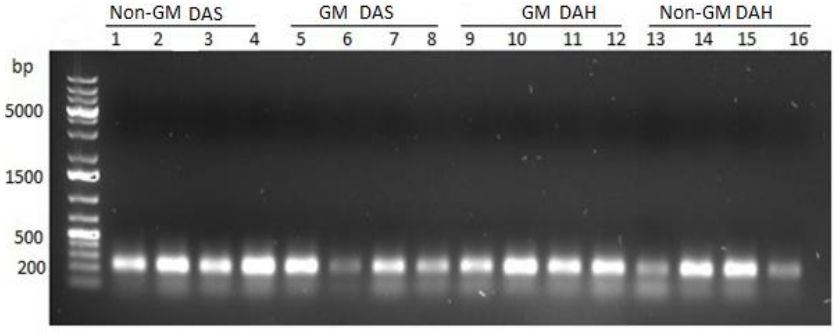

Figure 1. PCR products of DNA isolated directly from soil samples. M = 1kb plus ladder

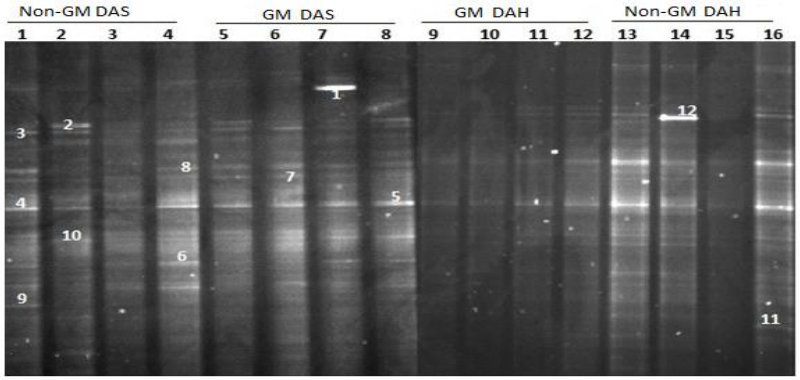

Figure 2. PCR-DGGE profiles representing the rhizobacterial diversity in GM and Non-GM soil samples DAS and DAH.

Bands 2, 3, 4 and 8 were common in all profiles and bands 1, 9 and 10 were unique in the soil samples (Figure 2). Band 6 was similar in DAS non-GM and GM soil samples but absent in DAH samples. Bands 1 and 12 were very intense in GM DAS and non-GM DAH. According to Muyzer *et al*. (1993) DGGE generated band profiles represent a dominant microbial phylotype or bacterial species, with more bands indicating higher diversity. The similarity in the band profiles generated in this study between GM and non-GM soil samples suggested a similar dominant rhizobacterial phylotype in GM and non-GM DAS and DAH. Furthermore common band profiles indicated a stable rhizobacterial phylotype in GM and non-GM. These bands when excised and sequenced can be used as reference species in future studies of rhizobacterial diversity in GM and non-GM maize.

Cluster analyses indicated that banding patterns of GM and non-GM samples DAS and DAH were closely related to each other (Figure 3), lane 12 and 13 (GM DAH and Non-GM DAH), 8 and 16 (GM DAS and Non-GM DAH), 3 and 7 (Non-GM DAS and GM DAS), 4 and 5 (Non-GM DAS and GM DAS) and 9 and 15 (GM DAH and Non-GM DAH). Furthermore, cluster analyses of banding patterns indicated that the dendrogram was divided into two clusters (I and II) starting from the point five. Each cluster groups soil samples from GM and non-GM maize. However the presence of the GM and non-GM maize soil samples in the two clusters (I and II) suggested that the rhizobacterial phylotype remained unchanged between the two from the time of planting till the time of harvest. These results are in agreement with those of Stephen and Kowalchuk, 2002; Schmalenberger and Tebbe, (2002) who observed no changes in bacterial community of GM crops with respects to its non-GM counterpart. Therefore, it can be suggested that the GM maize was not able to alter microbial phylotype. 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, DNA that persist in soil for long time can be detected, coupled to the fact that just a small percentage of microorganisms are culturable.

Results of 16S rDNA gene sequences of uncultured rhizobacterial submitted to the basic local alignment search tool web-based program Altschul *et al*. (1990) are presented in (Table 1). Band numbers 1, 2, 3, 4, 6, 7, 8, 9, 10, 11 and 12 represent 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. One advantage of DGGE technique is that, microorganisms can be studied right down to species level due to the fact that bands can be excised and sequenced. Uncultured bacteria, *Bradyrhyzobium* sp., *Actinobacterium* and *Sphingomonas* sp. (bands 2, 3, 4 and 8 respectively) were common in the soil samples (GM and non-GM DAS and DAH) indicating that these species were a stable phylotype in the study site. According to Aislabie *et al*. (2006) Actinobacteria are a group of Gram-negative bacteria mostly found in soil and are responsible for the degradation of organic material. We therefore suggest the significance of these species for evaluation of possible impact of GM crops on soil microorganisms. The detection of *Sphingomonas* and *Burkholderia* spp. are in agreement with the reports of Chelius and Triplett, 2000; Mehnaz and Lazarovits, 2006; Mehnaz *et al*. (2007) 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. The PCR-DGGE technique used in this study shaded light on the possible impact GM maize DAS and DAH on rhizobacteria. Similar rhizobacterial phylotypes in both GM and non-GM soil samples suggest they might have been no change at the level of the rhizosphere. Based on our results, we conclude that there was no change in rhizobacterial diversity in GM and non-GM maize.

Conclusion

Similar rhizobacterial phylotypes in GM and non-GM soil samples revealed that GM maize did not bring about any significant changes in rihizobacterial phylotype. These findings provided insights regarding the impact of GMPs on rhizobacterial. However, there is need to do a long term assessment of GM impact on rhizobacterial phylotype. 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.

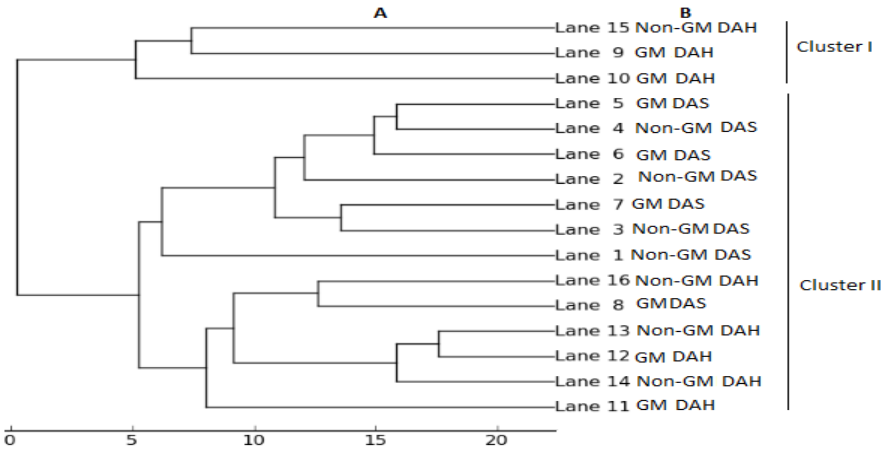

Figure 3. (A) Cluster analysis based on UPGMA of DGGE profiles shown in panel B. (B) DGGE profiles of yet unculturable rhizobacterial in GM and Non-GM soil samples 30 DAS and 1 DAH. Scale bar numbers indicate similarities among profiles.

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