EFFECTS OF GENETICALLY MODIFIED MAIZE (MON810) AND ITS RESIDUES ON THE FUNCTIONAL DIVERSITY OF MICROORGANISMS IN TWO SOUTH AFRICAN SOILS

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Submitted in fulfillment of the requirements for the degree of Master of Science in Biochemistry

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June 2011

DECLARATION

I, Usanda Puta, declare that this dissertation being submitted to the University of Fort Hare for the degree of Master of Science in Biochemistry is my original work and has not been submitted to any other University for the award of any degree.

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ACKNOWLEGEMENTS

ABSTRACT

Genetically modified (GM) crops are commercially cultivated worldwide but there are concerns on their possible negative impacts on soil biodiversity. A glasshouse study was conducted to determine effects of Bt maize residues on soil microbial diversity. Residues of Bt maize (PAN 6Q-308B) and non-Bt maize (PAN 6Q-121) were incorporated into the soil and corresponding maize seeds planted. The treatments were replicated three times. Fertilizer and water application were similar for both treatments. Rhizosphere and bulk soil was destructively sampled from each treatment and analyzed for microbial community level physiological profiles using Biolog plates with 31 different carbon substrates. Absorbance in the Biolog plates was recorded after 72 h of incubation at 20°C. Arbuscular mycorrhizal fungi spore counts were also determined. Field studies were conducted at the University of Free State and University of Fort Hare Research Farms to determine the effects of growing Bt maize on soil microbial diversity. One Bt maize cultivar (PAN6Q-308B) and non-Bt maize (PAN6Q-121) were grown in a paired experiment at University of Free State farm, while two Bt maize (DKC61-25B and PAN6Q-321B) and their near-isogenic non-Bt maize lines (DKC61-24 and PAN6777) were grown in a randomized complete block design with three replicates. Fertilization, weed control and water application, were similar for both Bt maize cultivars and their non-Bt maize counterparts. Rhizosphere soil samples were collected by uprooting whole plants and collecting the soil attached to the roots. The samples were analysed for microbial diversity and for arbuscular mycorrhizae fungal spore counts. Principal

component analysis showed that soil microbial diversity was affected more by sampling time whereas genetic modification had minimal effects. Presence of residues also increased the diversity of microorganisms. Mycorrhizal fungal spores were not affected by the presence of Bt maize residues. Growing Bt maize had no effect on the soil microbial diversity in the rhizosphere.

Keywords: Bt maize, bulk soil, microbial diversity, rhizosphere, substrate utilization, arbuscular mycorrhizal fungi

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CHAPTER 1

INTRODUCTION

The production of genetically modified (GM) crops in South Africa has rapidly increased in the past few years, since their first commercialization in 1996 (Sanvido et al., 2006). Maize modified to improve resistance to herbicides (Round-Up-Ready) and insect pests (Bt crops) is the most cultivated GM crop (Hernandez et al., 2004). In 2007 GM maize was grown on 1.6 million ha which accounted for 57% of the maize produced in that year (Icoz and Stotzky, 2008).

Bt maize is genetically modified to produce the Cry1Ab protein, which is derived from the *Bacillus thuringiensis* bacterium, to protect it from the insect pests of the order Lepidoptera. The protein enters the soil through root exudates (Saxena and Stotzky, 2001) and by incorporation of the residues into the soil (Flores et al., 2005). Although these toxins quickly decompose in soil, when free, (Baumgarte and Tebbe, 2005), they resist microbial degradation when bound to active soil particles. The continued release of Bt proteins and their stabilization in the soil may lead to their accumulation, which will increase their exposure to non-target soil organisms (Koskella and Stotzky, 1997).

Cultivation of GM crops could result in addition, to the soil, of large amounts of the GM products and plant residues with modified chemical composition (Icoz and Stotzky, 2008), which could affect soil organisms and interfere with microbe-mediated processes and soil fertility. Release of Cry proteins into the rhizosphere of

Bt maize could affect microbial diversity and function (Icoz and Stotzky, 2008). Changes in chemical composition of the Bt maize residues could also alter soil microbial composition and activity (Stotzky, 2004).

Microorganisms are important in soil processes including decomposition of crop residues, fixation of nitrogen and uptake of phosphorus (Motavalli et al., 2004). Enzymes have a critical biochemical function in organic matter decomposition as they catalyze several important reactions necessary for decomposition of organic waste, formation of organic matter and nutrient cycling (Griffiths et al., 2003). Any negative effect on soil microbial community could result in decline of soil fertility and crop productivity. Soil microorganisms are important for the decomposition of organic matter and also increase the availability of nutrients (Flores et al., 2005).

While Lehman et al. (2008) found no changes in chemical composition of Bt maize residues, Poerschmann et al. (2005) found that higher lignin, could affect microbial diversity because microorganisms will take longer to decompose these residues (Icoz and Stotkzy, 2008). It is of importance to study the effects that Bt maize (MON810) and its residues might have on the diversity of soil microorganisms, which are vital for the decomposition of crop residues and nutrient cycling among other functions (Icoz and Stotzky, 2008).

Research on the impact of these crops on soil organisms has produced conflicting results, with some authors reporting effects on earthworms, nematodes, protozoa, bacteria and fungi (Icoz and Stotkzy, 2008; Flores, 2005; Fang et al., 2007). Blackwood and Buyer, (2004) also reported that Bt toxins did not have negative

effects on soil biological communities in the short term. Griffiths et al. (2007) reported minimum non-persistent and site specific effects of Bt maize (event MON810) on nematodes (fewer), protozoa (more) and amoeba (fewer) in work done in France and Denmark. Their work also showed differences in microbial community structure (community physiological profiles) as a result of Bt maize. Differences in the findings of the different studies could be a result of differences in the techniques used.

It is necessary to utilize sensitive techniques when studying the effects of Bt maize on soil microorganisms, including Denaturing Gradient Gel Electrophoresis (DGGE), Phospholipid Fatty Acids (PLFA), Fatty Acid Methyl Ester (FAME) and Community Level Physiological Profile (CLPP), have been used to determine the effects of Bt maize and its residues on soil microorganisms. Culture based techniques have the limitation that only 0.01% to 1% of microbes are culturable on selective artificial media (van Der Merwe et al., 2002)

Signature lipid biomarkers (SLBs) profiles use phospholipids as fingerprints of microbial communities. Specific patterns of phospholipids fatty acids (PLFA) are indicative of the physiological stress, nutritional status as well as the biomass of the microbial population, which other techniques do not reflect (Van de Merwe et al., 2002).

Community level physiological profiling (CLPP) determines the changes in substrate utilization patterns in soil (Goberna et al., 2005) and is useful in assessing temporal changes in the microbial community. It is based on the ability of the microbial

community to utilize a range of carbon substrates while reducing tetrazolium dye giving a violet color which indicates the presence of microorganisms that are able to use the substrates (Malosso et al., 2005). Recent literature suggests that CLPP approach is more sensitive than PLFA in determining changes in microbial diversity due to soil disturbance (Griffiths et al., 2007).

Vulnerable groups of microorganisms like mycorrhizae, nitrifying and biological nitrogen fixing bacteria have been suggested for assessing the impact of Bt maize (Icoz and Stotzky, 2008). A negative effect on mycorrhizal fungi would reduce the uptake of phosphorus in crops like maize while a negative effect on rhizobium would affect biological nitrogen fixation by legumes (Yanni et al., 2010).

It is essential to understand effects of Bt maize and its residues on the diversity of microorganisms in soil for the monitoring and minimization of the potential negative effects (Oliveira et al., 2008; Caldwell, 2005).

AIM

This study was aimed at evaluating the effects of GM maize (MON810) and its residues on microbial diversity and spore counts of arbuscular mycorrhizae in the soil.

SPECIFIC OBJECTIVES

1. To determine the effects of Bt maize cultivation on microbial diversity in the rhizosphere

2. To determine the effects of growing Bt maize on the number of spores of mycorrhizal fungi in the rhizosphere

3. To determine the effects of Bt maize residues incorporated in soil microbial diversity

4. To determine the effects of incorporated Bt maize residues on the number of spores of mycorrhizal fungi

HYPOTHESIS

1. Bt maize does not have an effect on microbial diversity in the soil

2. Bt maize does not have an effect on the number of mycorrhizal fungal spores

3. Bt maize residues does not have an effect on soil microbial diversity

4. Bt maize residues does not have an effect on the number of mycorrhizal fungal spores

CHAPTER 2

LITERATURE REVIEW

INTRODUCTION

Insect damage reduces maize yield in most agro-ecosystems. Stem borers' tunnell the maize stock, which increases the risk of stalk lodging leading to reduced yield, resulting in food shortages in many African countries (Butrón et al., 2009). Genetically modified (GM) crops are grown all over the world to improve the yield with the annual increase rising to the average of 10% (Icoz and Stotzky, 2008). The adoption of genetically modified crops increased dramatically in the last 11 years. However, the introduction of these plants into agriculture ecosystem has raised a number of questions (Bruisma et al., 2003). There are concerns that GM crops might have a negative effect on non-target organisms, soil arthropods, soil microorganisms and function (Holst-Jensen, 2009).

The use of GM crops in agriculture

GM crops possess genes that are transferred from different species. They carry traits that are different from the ones found in conventional crops (Sanvido et al., 2006). These crops are developed for longer shelf life, tolerant to herbicides, control pests, improve nutritional value and to be resistant against diseases and stresses such as drought or low nitrogen (Icoz and Stotzky, 2008). The first GM crop to be approved for commercial use was the FlavrSavr tomato in the USA in 1994 (Food and Drug Administration, 1994), which was developed for delayed ripening and longer shelf life. Herbicide tolerant soy bean is tolerant to Round-Up, a non-selective

herbicide which acts by entering the plant and inhibiting an enzyme necessary for building aromatic amino acids. The lack of these amino acids kills the plant (van Wyk et al., 2009). Herbicide tolerant crops are considered not to have a direct effect on non target organisms because the enzymes that codes for the herbicide tolerance are normally available in plants and do not have any toxic properties. As a result of these advantages genetically modified crops have increasingly been grown worldwide (Icoz and Stotzky, 2008).

To reduce the environmental problems associated with the cultivation of GM crops, *Bacillus thuringiensis* (Bt), a gram negative bacterium was used to modify crops. The spores of Bt can be isolated from the soil, fresh water and also from the insects that fed on the crops that are carrying the bacterium. This bacterium produces crystal inclusions that are made up of Cry proteins toxic to a number of insects (Santos et al., 2009). To protect the plant against insect pests Bt maize was developed. Insecticidal Cry proteins derived from this bacterium improves crop's life by reducing the damage that is made by insect pests. The Cry proteins include Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry3Bb1 (Sun et al., 2007).

Bacillus thuringiensis produces inactive form of Cry proteins called protoxins, which are activated when they are ingested by the insect. As a result, these proteins are highly specific and only kill the target insects of the orders Lepidoptera, Diptera and Coleoptera, depending on the protein. Bt crops produce the active protein, which do not need activation by the gut conditions when the insect larva ingests it. In cotton Cry1Ac is active against the cotton bollworm (Helicoverpa zea Boddie)

(Lepidoptera), Cry3A and Cry3C against the Colorado potato bettle (Leptinotarsa decemlineata Say) (Coleoptera). The Cry1Ab protein is active against Lepidoptera insects while Cry3Bb1 is active against the corn rootworm (Coleoptera) (Stotzky, 2004). Most Bt maize hybrids expresses the Cry1Ab protein with a few expressing Cry1Ac and Cry9C, all targeting the European corn borer (Ostrinia nubilalis Hübner) (Lepidoptera), a major insect pest of maize. Reduction of plant damage by insects due to these proteins has been important in increasing yields (Pigott et al., 2007).

In 2007 GMOs occupied more than 143 million ha in 23 countries (Icoz and Stotzky, 2008) with herbicide tolerance as the most dominant GM trait followed by insect resistance. Some of the crops that are widely cultivated are soy bean, cotton, maize and rapeseed (canola) (James, 2007). In the USA soybean and cotton with herbicide tolerant traits are the most dominant crops followed by insect resistant corn and cotton. In 2006 herbicide tolerant soybean, maize, cotton and canola occupied 68% (69.9 million hectares) of the total global GM crop area (Icoz and Stotzky, 2008). Insect-resistant Bt maize producing the Cry1Ab protein, is the second major trait that is used in commercial GM crops occupying 11.3 million hectares (James, 2005). A number of transformation events have been used in Bt maize, including Bt 11, Bt 176 and MON810.

Genetically modified MON810 maize was developed by introducing a modified DNA that codes for Cry1Ab protein, representing an active form of a protein. This event was authorized for cultivation in 1998 in European Union. *Bacillus thuringiensis* MON810 is active against *Ostrinia Nubilalis*, a European corn borer of the order Lepidoptera (Singh et al., 2005). The concentration of the protein has been shown to

be more in the leaves than in the roots (Baumgarte and Tebbe, 2005). This event has been reported to cause 100% mortality in *Chilo partellus* (Swinhoe) (Van Rensburg, 1998).

In South Africa GM crops were commercialized in 1996. In 2007 the cultivation of GMOs increased by 10% as compared to 1996 (Van Rensburg, 1998). Insect resistant maize (MON810 and Bt11) and herbicide tolerant cotton are cultivated in South Africa (van Rensburg, 2001). MON810 was reported to have a 100% mortality rate for *C. partellus* as compared to *B. fusca* which can survive in other plants (Singh et al. 2005). There are a number of environmental concerns with growing GM crops in South Africa.

Environmental concerns of Bt maize

Bt maize was initially developed in North America to control stem borers, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) and *Diatraea grandiosella* (Dyar) (Lepidopera: Crambidae) before it was introduced for the control of *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) and *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) in South Africa (Archer et al., 2001; Goberna et al., 2005). However, *B. fusca* has developed resistance to Bt maize in parts of South Africa (Kruger et al., 2009). Reasons for this resistance are still unknown with the suspicions that non compliance to refuge requirements played a role (van Rensburg, 2007; Kruger et al., 2009). During the introduction of GM crops for cultivation, farmers were mandated to use the high-dose/refuge strategy to delay pest resistance. This method required the

planting of a toxin-free crop near the Bt crop thereby promoting survival of pests and this method has been proved to reduce resistance (Chilcutt et al., 2004).

Coexistence of the GM and non-GM plants might cross pollinate organic plants and that might also cause problems in agricultural practices. Pollen has been proved to travel long distances in favorable conditions (Brookes and Barfoot, 2006).

There are also concerns that the cultivation of GM might have a negative impact on the non-target soil organisms which will in-turn disturb soil functions. These could be affected for example, by the presence of insecticidal Cry proteins in soils through cultivation of Bt crops (Holst-Jensen et al., 2009). The release of these proteins as components of root exudates could lead to high concentrations of Cry protein in rhizosphere than in bulk soil and possible accumulation and persistence of the protein in the soil (Saxena and Stotzky, 2001a). Saxena and Stotzky (2002) showed that insect resistant GM crops, such as Bt maize, potato, and rice, contributes to the presence and persistence of Cry proteins in soil via root exudation as compared to Bt cotton and canola.

Effects on soil organisms and function
Earthworms are one of the most important soil organisms. They are responsible for the decomposition of the above ground plant litter. A study conducted by Saxena and Stotzky (2001b), showed that the Cry1Ab protein does not have effects on the earthworms even though the earthworms (Lumbricus terrestris) ingested the protein. There was no difference in the mortality and weight of these earthworm between the earthworms that were in the Bt planted soil as compared to the non Bt planted soil.

Schrader et al. (2008) also reported no differences between earthworms (Lumbricus terrestris, Aporrectodea caliginosa) that were incubated in the MON810 maize variety and those incubated in the absence of the protein. There was no decrease in earthworm number but a reduction of weight (Zwahlen et al., 2003). Although Saxena and Stotzky. (2001) reported no effects of Bt toxins on nematodes, Flores et al. (2005) found that Bt toxins have deleterious effects.

Effects of genetic modification on chemical composition of Bt maize residues

Bt proteins are introduced into the soil via the decomposition of plant material and through root exudates in the rhizosphere (Saxena and Stotzky, 2000). There are conflicting results about the different effects of Bt maize in the environment. There are results that report that Bt toxin does not persist in soil, it decomposes quickly into undetectable concentrations after decomposition of the plant (Crecchio and Stotzky, 2001; Sims and Holden, 1996), this was later confirmed by Sun et al. (2007).

There were reports that Bt maize and non-Bt maize are chemically different with some farmers saying that Bt maize has a high lignin content than the non-Bt maize (Lehman et al., 2008). This is one of the factors that are effecting the decomposition of the residues which might also have an impact on the soil microbial community. The decomposition of residues might disturb soil functioning by affecting the most valuable enzymes and microorganisms in the soil (Austin and Ballare, 2010). Residues that have higher lignin content have a tendency of decomposing slowly leading to persisting longer in soil than residues with low lignin content (Poerschmann et al., 2005). Yanni et al. (2010) also reported that Bt maize has more lignin than the non-Bt maize and could take more time to decompose in soil.

Flores et al. (2005) are in support of these findings as they also discovered that Bt maize has indeed a high lignin and takes time to decompose in soil. Hopkins and Gregorich (2003) did not observe any detectable difference in the decomposition of plant material from Bt and non-Bt maize. Enzymes that are used for the decomposition of these treatments are not different (Flores et al., 2005). Studies of soil microbial communities were conducted using CLPP and it was observed that there are differences in the profiles of bacteria and fungi and also in the population of nematodes (Griffiths et al., 2005).

Effects of Bt maize on soil microorganisms and function

Agricultural crops have a major influence on the processes and functions of the soil such as nitrogen cycling, decomposition of wastes and mobilization of nutrients. They influence the functioning of the micro and other organisms such as earthworms and nematodes in the soil (Oliveira et al., 2008). The major carbon supply to soil systems is from plant litter incorporated after harvest and from root exudation (Icoz and Stotzky, 2008). GM crops such as Bt maize, potato and rice contributes to the presence and persistence of Cry protein in the soil as compared to Bt cotton and canola. These proteins enter the soil via root exudates. (Saxena et al., 2004) Cry proteins are released into the soil via root exudates throughout the growth of the plant but there is no relationship between the time of planting and plant growth (Baumgarte and Tebbe, 2005). Bt protein have been found to be present in the rhizosphere soil during the growth of the plant and months after the harvest (Saxena and Stotzky, 2001). Although they are present in soil after the harvest, they had no

effects on Collembolans (Heckmann et al., 2006, nematodes, algae, fungi and earthworms (Koskella and Stotzky, 2002).

All agricultural crops that are planted in the soil interact with the soil ecosystem and the effects of these interactions have a major influence in the microbial diversity. Microorganisms are responsible for the normal functioning of the soil as they are responsible for the soil processes such as the cycling of nitrogen, decomposition of waste and the distribution of nutrients in the soil. The disturbance of these processes will affect the number of organisms and their diversity.

Arbuscular mycorrhizal fungi are beneficial microorganisms that are important in soil fertility and plant nutrition. However they need a compatible plant host to complete their life cycle and produce their spores. Maize has been found to be a suitable host for them (Wenke and Lianfeng, 2008). Owing to their symbiosis relationship with the roots system, AMF are important in enhancing soil nutrient to cope with environmental stress (Brachmann and Parniske, 2006). Intra-radical mycelium of the AM fungi proliferates in the root cortex of the plant with the hyphae spreading around the root system by which it absorbs nutritional elements such as phosphorus (P), nitrogen (N), zinc (Zn), and copper. Deficiency of P and Zn are associated with the growth disorders in which early growth in the crop is stunted, crop maturity delayed and yield decreased (Rochester et al., 2001). AM fungi have been proved to be useful for the transportation of nutrients during drought seasons. GM cotton depends on the symbiosis with AMF for the uptake of nutrients in the soil in order to survive. GM crops were reported to have no effects on AM fungi, as they colonize both GM and non GM crops (Knox et al., 2008). Although Ferreira et al. (2003) demonstrated

no effects of Bt proteins on AMF, Turrini et al. (2005) showed that event Bt176 reduced hyphal growth and impaired the appressoria. Bt transgenic crops affected the colonization and symbiotic development of AMF in Bt176 as there were differences between the Bt and the non-Bt treatments. There is need to determine effects of Bt maize, with the MON810 event, on soil microbial functional diversity and AM fungi, which affect soil function.

Approaches for studying soil microbial diversity

Various methods, including Denaturing Gradient Gel Electrophoresis (DGGE), Phospholipid Fatty Acid Assay (PLFA) and Community Level Physiological Profiles (CLPP), have been used to determine effects of Bt maize and its residues on soil microorganisms.

Due to the selectivity of the culture based methods, molecular techniques are used. DGGE is one of the methods that are used to determine microbial genetic structure. When the DNA reaches its denaturing region, it melts and as the denaturing conditions reaches extremes, the melted fragment dissociates into single strands. The problem with this technique is that there is no assurance that the entire DNA is extracted and they are time consuming and very expensive to perform (van der Merwe et al., 2002). Compared to Biolog assay, DGGE is more sensitive because it is used to detect differences in melting behavior of small DNA fragments (200-700bp). To determine the microbial genetic structure this method is usually coupled with Polymerase Chain Reaction (PCR) (Demane`che et al., 2008).

PLFA is a quantitative method that is independent of cell culturability. It allows for the identification microorganisms that have distinctive phospholipid fatty acid (PLFA) profiles. Chemical decomposition of PLFA biomarkers differs depending on the type of organism. It can be used to generate a fingerprint of the microbial community. When the cell dies, PLFA decomposes quickly. Types of PLFAs include monoenoic (Sulfate or iron reducing bacteria), normal saturates which are found in all organisms and polynoics which are found in fungi, algae, protozoa and plants. It can be used to determine the changes in the composition of the microbial community such as stress and nutritional status none of which are indicated by other techniques (Baker et al., 2003).

Community Level Physiological Profiles (CLPP) are based on the ability of microbial communities to utilize a range of carbon substrates in Biolog microplate. The substrates include amines, amino acids, carbohydrates and carboxylic acids (Garland, 1996a). Reduction of the tetrazolium dye in the Biolog plates gives a violet color which indicates the presence of microorganisms that are able to use that substrate (Malosso et al., 2005). It has been found as a valuable method for assessing changes in microbial communities. A lot of data can be collected which help in the characterization and identification of the microbial population (Garland et al., 1996b). This approach has been found to be more sensitive in determining the functional diversity of microorganisms in the soil that the other methods which require expensive equipments and are time consuming (Griffiths et al., 2007). However, it does not reflect the actual microorganisms in the soil because some of the microorganisms are not able to utilize the substrates in the plate.

CHAPTER 3

MATERIALS AND METHODS

This work involved glasshouse and field experiments conducted at the University of Fort Hare Research Farm, Alice in the 2009/2010 season and University of Free State Research Farm, Bloemfontein, in the 2008/2009 and 2009/2010 seasons.

Effects of Bt maize (MON810) on soil microbial diversity under glasshouse conditions

The glasshouse study was carried out in pots, using soil sampled from the 0-20 cm depth of a sandy loam soil in the same site at the University of Fort Hare Research Farm.

The soil was air-dried and sieved to 4 mm before it was used in this study. The soil contained clay (176 g kg^{-1}), organic carbon (1.39%), pH 6.5 (water) (Muchaonyerwa and Waladde, 2007). Residues of Bt maize (PAN6Q-321B) and non-Bt maize (PAN6Q-121) produced at the University of Free State Farm (SA), Bloemfontein, in the 2008/2009 season were used in the study. The experiment was set up in a randomized complete block design with 3 replicates, for each sampling, to allow for destructive sampling. The treatments were Bt maize with or without residues and non Bt maize with or without residues.

The soil (5Kg) was either amended with 15 g of Bt maize or non-Bt maize residues or not amended. The amendments were equivalent to 6 t residues ha^{-1}, which is

expected dry matter production under field conditions. Bt and Non-Bt maize seeds were planted in pots amended with corresponding residues. Non-amended pots were also planted with either Bt maize or non Bt maize.

Plants were fertilized (0.45g) and irrigated to replenish any water loss. Destructive sampling was done on 7, 14, 28, 42 and 90 days after planting (DAP) from bulk and rhizosphere soils. Rhizosphere soils were sampled by removing the plant completely from the pot with the root system still attached to the plant. The soil that was attached to the root was removed from the roots. The bulk soil was mixed and a sample was taken. The samples were stored at -20 and 4°C for further analysis.

Analysis of soil microbial community level physiological profiles

Microbial community level physiological profiles (CLPP) were determined using Biolog EcoPlates ™. The plates contained 31 carbon sources with water as a control. The substrates were amines, amino acids, carbohydrates and carboxylic acids. Soil samples were pre-incubated at 25°C for 24 hours before they were analyzed. Soil sample (1g) was suspended to 1L with sterile distilled water (Garland, 1996) and the suspension was shaken for 20 minutes at 200 rpm and allowed to settle for 30 minutes at 4 °C. An aliquot of the suspension (150 µl) was dispensed into each well of the Biolog plates and incubated at 25 °C.

Analysis of Cry1Ab protein concentration in soil under glasshouse conditions

The Cry1Ab protein levels were analyzed using Double Antibody Sandwich Enzyme linked Immunosorbent Assay using a Cry1Ab/Cry1Ac kit from Envirologix products (Bt-Cry1Ab/1Ac ELISA protein, Envirologix, Maine, USA). A gram of fresh soil was

added to 1ml PBST buffer provided in the kit and mixed with a vortex mixer for 5 minutes at 5000 rpm. The suspension was allowed to settle at 25 °C, before Cry1Ab/Cry1Ac enzyme conjugate (50µl) was dispensed in each well of the plate. Extraction buffer (50 µl) was added to the first well of the plate as a negative control. Positive control (50 µl) was added as follows: 0.025, 5.0, 10, 15, 20 ng Cry1Ab protein ml^{-1} to obtain a standard curve. The plate was incubated in a humid box at room temperature for 2 hours. The wells were then washed with PBST buffer and the TMB substrate was added into each well and incubated at room temperature for 20 minutes. The absorbance was recorded at a wavelength of 650 nm and the concentration the Cry1Ab protein was calculated based on a linear standard curve from the negative control (0), 0.025, 5.0, 10, 15, 20 ng Cry1Ab protein ml^{-1} (dilutions of 40 ng Cry1Ab protein ml^{-1} in the positive control).

Effects of Bt maize and its residues on mycorrhizal fungal spore counts in the rhizosphere soil under glasshouse conditions

Mycorrhizal spore counts were determined by wet sieving and decantation as described by Sylvia (1994). Soil samples (50g) from the different treatments were suspended in water and vigorously mixed with a warring blender to free the spores from soil particles. The suspensions were allowed to settle for 30 minutes and the supernatant were decanted through a stack of sieves (425µm and 45 µm). The particles retained on the 45 µm sieve were then transferred to 50 ml centrifuge tubes with water (30 ml) and centrifuged at 1300 g for 3 minutes. The supernatant was decanted and the pellet was suspended in chilled 1.17 M sucrose solution mixed with a spatula, and centrifuged at 1300 g for 1.5 minutes. The supernatant was then

poured through a small sieve (45 μm) and the spores held on the sieve were carefully rinsed with tap water and washed into plastic Petri dishes scribed with parallel lines spaced 0.5 cm apart. Spores were counted by scanning the dish under a dissecting microscope.

Effects of growing Bt maize (MON810) on soil microbial diversity under field conditions at the University of Free State

A study was established to determine gene flow of the yellow Bt maize to white non-Bt maize cultivars at the University of Free State Farm in November 2008. Soil samples (50g) were collected for initial characterization before planting. It was a paired trial without replication and was carried out in 2008/2009 and 2009/ 2010 seasons. The maize cultivars were PAN 6Q-308B (Yellow Bt) (3ha), PAN 6Q-121 (4ha). The maize was planted at a density of 20 000 plants per ha, on the 27th of November 2008 following about 100 mm of the rain the previous week. Cultivar PAN 6Q-321B was planted 2 weeks later. Fertilizer, irrigation, and weed management were the same for both treatments. This trial was also used to study microbial diversity in the Bt and non-Bt maize plots. Rhizosphere samples were collected after 11, 12, 18 and 28 weeks after planting (WAP) which corresponds to 9 February, 23 February, 3 April and 15 June 2009. The samples were collected by digging up the maize plants and soil around the root system was collected stored at 4 °C before analysis of Cry1Ab protein, microbial diversity and mycorrhizal spore counts.

The field trial was repeated in the 2009/10 planting season. The experiment was managed in the same way as the previous season. Soil samples were collected from the plots before planting and thereafter rhizosphere samples were collected after 4, 8, 12 and 16 (WAP). The samples were collected by uprooting the maize plants and

the roots and soil attached them were stored at 4 °C. The samples were analyzed for Cry1Ab protein, microbial diversity and mycorrhizal spore counts as described in the glasshouse study.

Effects of growing Bt maize on soil microbial diversity and mycorrhizal spore counts under field conditions at the University of Fort Hare

A field study was established at the University of Fort Hare Research Farm in Alice to determine the effects of growing Bt maize on microbial diversity and arbscular mycorrhizae in the rhizospere. The site receives mean annual rainfall of 575 mm and a mean annual temperature of 18.1 °C (Van Averbeke and Marais, 1991). The soil according to the South African classification is an Oakleaf soil form (Van Averbeke and Marais, 1991) with 48% sand, 38% silt, 14% clay and pH 6.2 (1:2.5, soil: water). The soil contains high mica and low kaolinite hematite and quartz. The field was previously cropped with maize and potatoes over the previous seven years.

Prior to planting, the field was a bush cut, harrowed and then ploughed. The experiment was established as a randomized complete block design (RCBD) with four treatments with three replications. The treatments were two Bt maize hybrids (both MON810), PAN6Q-321B (white, medium season) and DKC61-25B (yellow, short season), and their near-isolines, PAN 6777 and DKC61-24. The crops were planted on the 18 December, 2009 at 40 000 plants ha^{-1}. The plot size for each treatment was 12 m * 7.2 m and the distance between plots was 1m and replicates were separated by a distance of 2 m.

Planting stations were holes were made using hoes and six maize seeds were placed per hole to cater for damage by birds. Basal fertilizer (2:3:2; N:P:K) was applied at 10 g hole^{-1} (Muchaonyerwa and Waladde, 2007). Immediately after planting, the field was irrigated to facilitate seed germination, and the plots were thinned to two seedlings per hole at three weeks after planting. Topdressing (LAN) was applied at a rate of 50 kg N ha^{-1} at knee height and at flowering. Chemical weed control was done, whenever there was need, using Basagran® (bentazon) and Atrazine® at 2 L ha^{-1} applied post emergent for sedges and broad leaves, respectively. Bulldock 050EC was applied at a rate of 150 ml ha^{-1} in all treatments to control stem borers that were infesting the isolines. Supplementary irrigation was applied when necessary using the sprinkler system, throughout the growing season.

Rhizosphere soil samples were collected at 3, 7 and 12 WAP which corresponds to 09 January, 09 February, 09 March 2010. The samples were collected by uprooting the maize plants and the roots and soil attached to them were stored at 4 °C before they were analyzed for Cry1Ab protein, microbial diversity and mycorrhizal spore counts, as described in the glasshouse study.

Data handling and statistical analysis

Substrate utilization was measured by determining absorbance of color developed (tetrazolium dye) after 24, 48, 72, 96 and 120 h of incubation at a wavelength of 590 nm. The data for substrate utilization at 72 h was analyzed by Principal Component Analysis (PCA) after correction for control absorbance using the JMP for Windows

software. Substrate utilization data were also used to calculate Average Well Color Development (AWCD) and Shannon Weaver Index (H) (Gomez et al., 2006).

The AWCD and H for substrate utilization and arbscular mycorrhizal spore count data were subjected to analysis of variance (ANOVA) and the means were separated using the least Significant Differences (LSD) at p‹0.05.

CHAPTER 4

RESULTS

Effects of Bt maize (MON810) on soil microbial diversity under glasshouse conditions

Soil microbial community level physiological profiles in the rhizosphere

Principal Component Analysis showed that over 85% of the variation in CLPP of microorganisms in the rhizosphere soils could be explained by PC1 (77%) and PC2 (8%). These principal components (PC 1 and PC2) were related to sampling time and presence or absence of residues, respectively (Figure 1).

Analysis of soil microbial community level physiological profiles in the bulk soil

Principal Component Analysis showed that over 70% of the variation in CLPP of microorganisms in the bulk soils could be explained by PC1 (55%) and PC 2 (14%). The PCs (PC 1 and PC 2) appeared to be governed by sampling time and presence and absence of residues (Figure 3).

There were no effects of sampling time and treatments on both average well color development (AWCD) and Shanon-Weaver Index (H). All treatments had similar AWCDs whereas all sampling times had similar H values and AWCD except for 7 DAP samples which had greater diversity (AWCD) (Table 2). Non Bt maize treatments with or without residues had similar H indices. Bt maize treatments (with or without residues) had similar H indices. However, only the non Bt maize treatment with residues had greater H index than the Bt treatments.
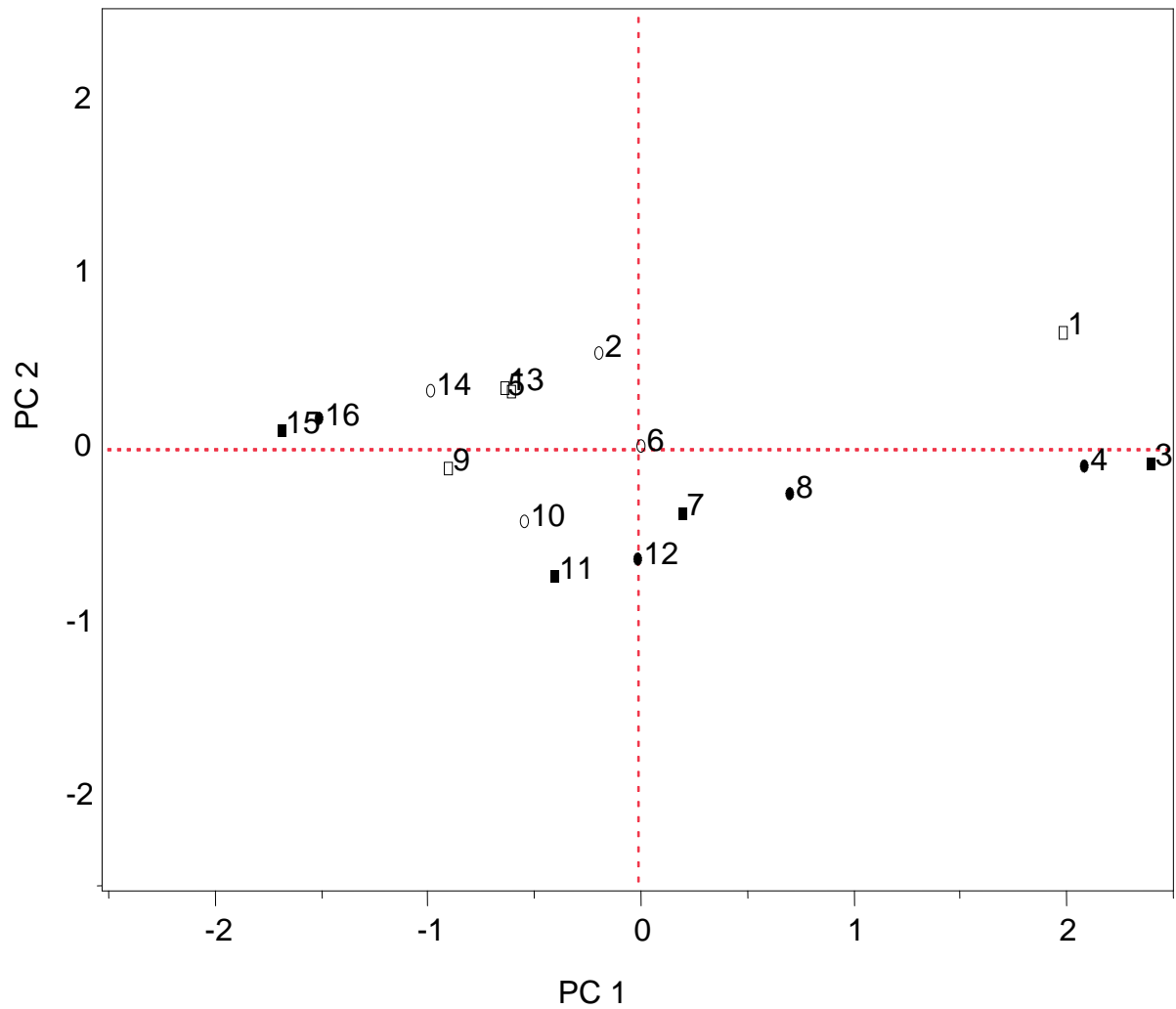
Figure 1: Community level physiological profiles of rhizosphere of maize planted on soils amended or unamended with residues of Bt and non-Bt maize residues. The numbers, 1-4, 5-8, 9-12, and 13-16 represent 14, 28, 42 and 90 DAP. For 14 DAP: 1= non Bt maize without residues, 2= Bt maize without residues, 3= non-Bt maize with residues, 4= Bt maize with residues. The order remains the same for all other sampling times.

Table 1: Average well color development (AWCD) in the rhizosphere of maize in the glasshouse study.

Source of variation	AWCD	Shannon-Weaver Index (H)
Sampling time (DAP)		
14	0.77[a]	3.00[a]
28	0.65[ab]	3.32[a]
42	0.56[bc]	3.34[a]
90	0.46[c]	3.21[a]
Treatment		
Bt residues with Bt plant	0.64[a]	3.28[a]
Non Bt residues with non Bt plant	0.51[a]	3.09[a]
Bt plant with no residues	0.58[a]	3.16[a]
Non Bt plant with no residues	0.71[a]	3.35[a]

- Means followed by the same letter are not significantly different
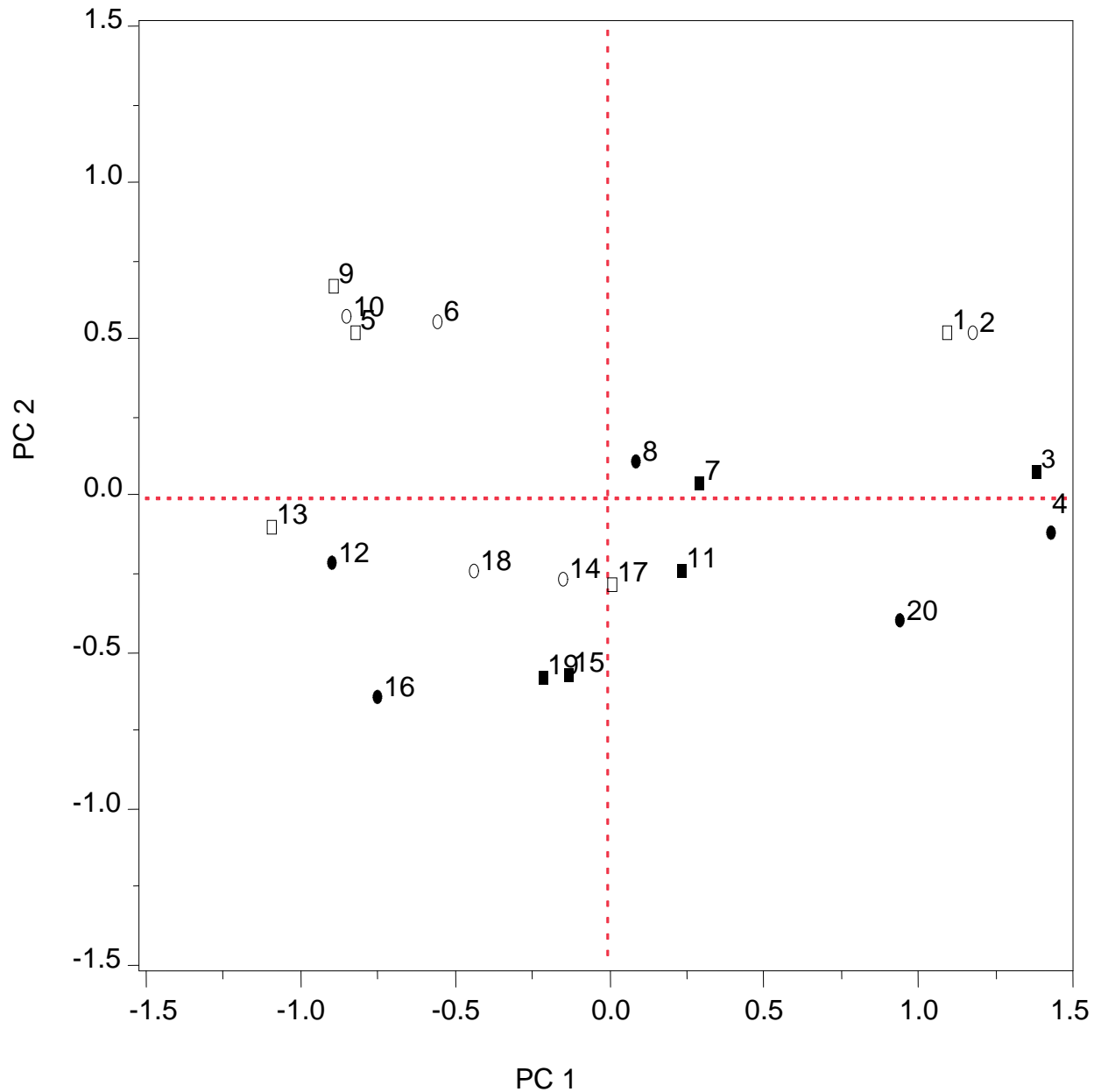
Figure 2: Principal component analysis of community level physiological profiles of bulk soils amended or unamended with residues of Bt and non-Bt maize residues. The numbers, 1-4, 5-8, 9-12, 13-16 and 17-20 represent 7, 14, 28, 42 and 90 WAP. For 7 WAP: 1 = non Bt maize without residues, 2= Bt maize without residues, 3 = non-Bt maize with residues, 4 = Bt maize with residues. The order remains the same for all other sampling times.

Table 2: Average well color development (AWCD) in the bulk soil of maize in the glasshouse study

Source of variation	AWCD	Shannon-Weaver Index (H)
Sampling time (DAP)		
7	0.92[a]	3.38 [a]
14	0.64[b]	3.33[a]
28	0.62 [b]	3.27 [a]
42	0.65 [b]	3.35 [a]
90	0.72 [b]	3.39 [a]
Treatment		
Bt plant with residues	0.66 [a]	3.31 [b]
Non Bt plant with residues	0.75 [a]	3.89 [a]
Bt plant with no residues	0.7 [a]	3.32 [b]
Non Bt plant with no residues	0.74 [a]	3.35 [c]

- Means followed by the same letter are not significantly different

Effects of Bt maize and its residues on mycorrhizal fungal spore counts in the rhizosphere soil under glasshouse conditions

Bt maize treatments had high numbers of spores than the non-Bt treatments, except at the end of the experiment, where the treatments without residues has similar numbers of spores. The highest number of fungal spores was recorded in samples collected 14 days after planting (DAP) (Table 3). The number of fungal spores found in the rhizosphere decreased between 14 and 28 days of planting in all treatments. There was a slight increase between 28 and 42 DAP and a slight decrease at 90 days.

Table 3: Number of mycorrhizal spores in Bt and non Bt maize treatments with or without residues

Treatment	Number of spores kg⁻¹ soil (Mean±SEM)			
	14 DAP	28 DAP	42 DAP	90 DAP
Bt plant with residues	140±12	20±0	47±13	67±29
Non-Bt plant with residues	53±24	0±0	33±13	7±7
Bt plants without residues	120±30	60±20	80±12	47±13
Non-Bt plants without residues	53±13	13±13	13±7	40±20

Effects of growing Bt maize on microbial diversity in rhizosphere soil under field conditions at the University of Free State Research Station

Microbial community level physiological profiles in the rhizosphere of maize

In the 2008/2009 season over 78% of the variation in CLPP of microorganisms in the rhizosphere soils could be explained by PC 1 (65.4%) and PC 2 (13.1%) (Figure 4). PC1 appeared to be related to time of sampling and PC2 to genetic modification.

In the 2009/2010 season over 66.7% of variation in CLPP of microorganisms in the rhizosphere soils could be explained by PC 1 (40.6%) and PC 2 (26.3%) and (Figure 5). PC1 appeared to be related to genetic modification and PC2 to time of sampling.
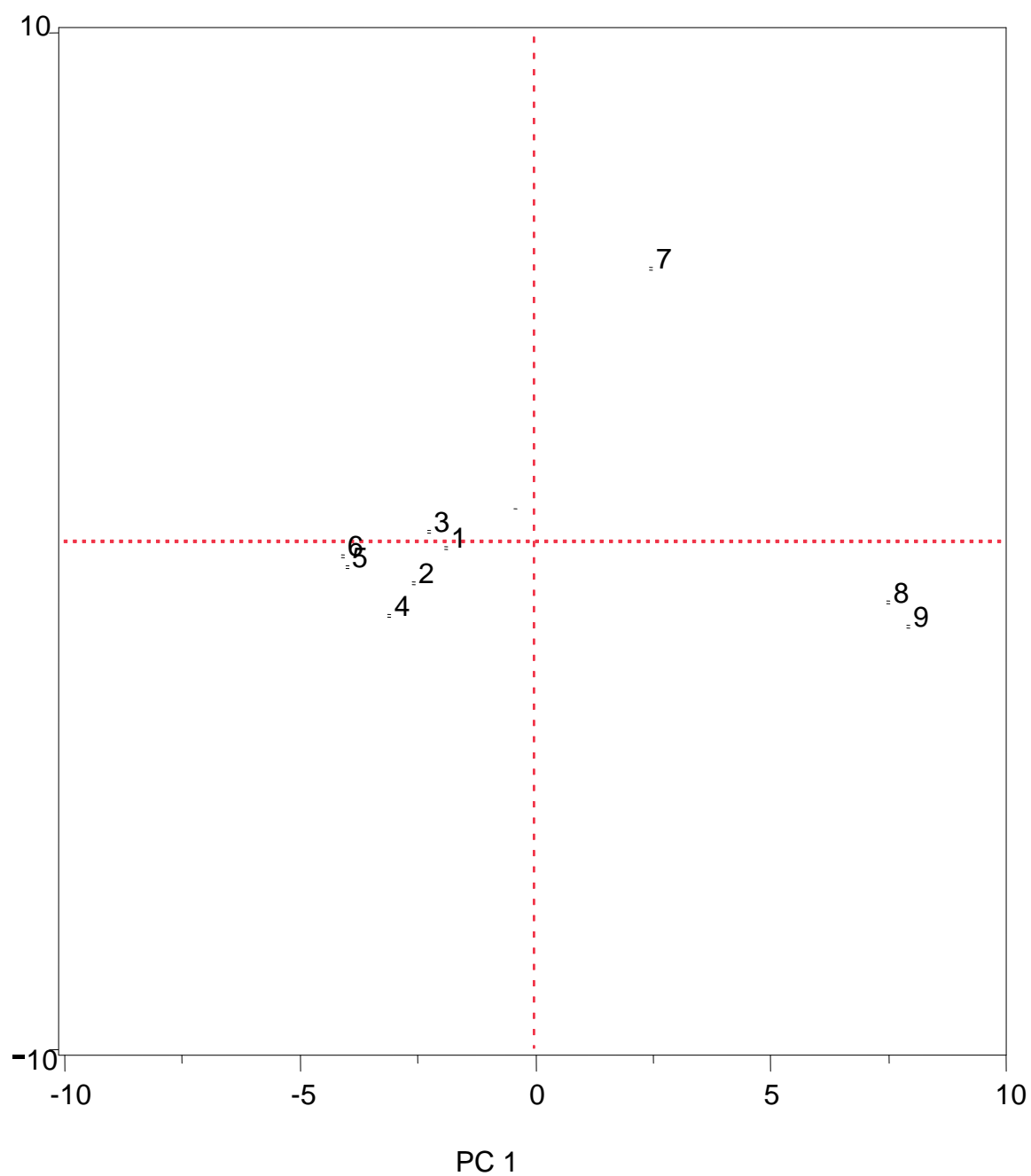
Figure 3: Community level physiological profiles of rhizosphere of Bt and non-Bt maize grown in Bloemfontein in the 2008/09 season. The numbers, 1 represents before planting, 2 and 3, 4 and 5, 6 and 7, 8 and 9, represent non Bt and Bt maize at 11, 12, 18, and 28 WAP respectively.
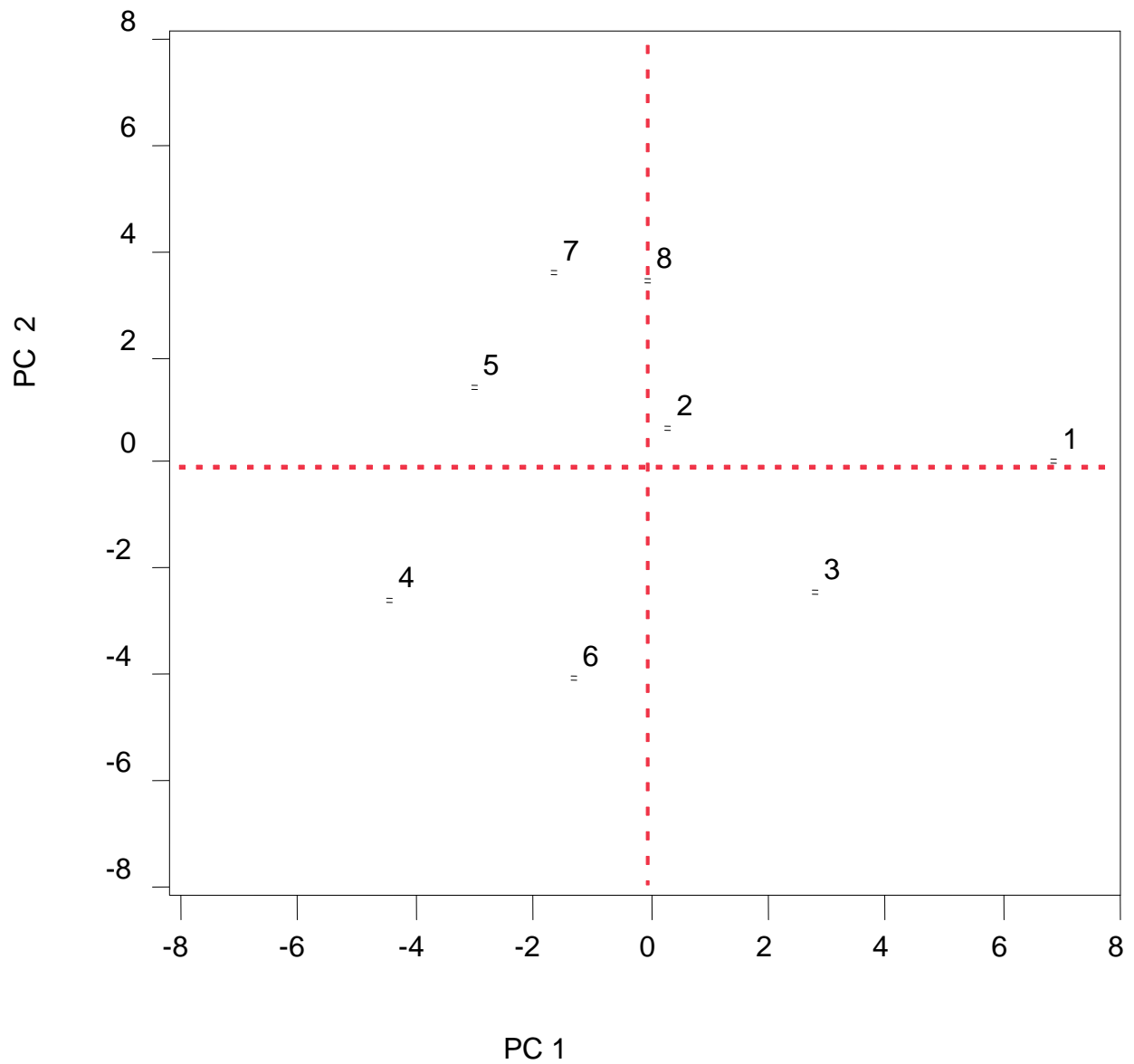
Figure 4: Community level physiological profiles of rhizosphere of Bt and non-Bt maize grown in Bloemfontein in 2009/10 season. The numbers, 1-2, 3-4, and 5-6, 7-8 represent 4, 8, 12 and 16 WAP respectively. For 4 WAP: 1 = non Bt maize, 2= Bt maize. The order remains the same for all other sampling times.

There was a decrease in the AWCD and H indices over time with samples collected at 8 WAP then an increase from 14 WAP until the end of the season. There were interaction effects of time of planting and maize treatments on AWCD and H indices (Table 4). The treatments had an effect on both AWCD and H indices that depended on the time of sampling.

Table 4: Average well color development (AWCD) in the rhizosphere of maize in the Bloemfontein field study during the 2008 /2009 planting season

Source of variation	AWCD	Shannon-Weaver Index (H)
Sampling time (WAP)		
6	0.35^c	3^c
8	0.19^a	2.5^a
14	0.36^c	2.8^b
24	0.56^e	3.02^c
Treatment		
Bt	0.45^b	3^a
Non Bt	0.3^a	3^a

- Means followed by the same letter are not significantly different

There was an increase in the AWCD at 8 WAP with a constant decrease until at 24 WAP where there was an increase. The latter is true for H indices which increased with the sampling time with a decrease at 24 WAP. There were interaction effects of time of planting and maize treatments on AWCD and H indices (Table 5). The treatments had an effect on both AWCD and H indices that depended on the time of sampling.

Table 5: Average well color development (AWCD) in the rhizosphere of maize in the Bloemfontein field study during the 2009 /2010 planting season

Source of variation	AWCD	Shannon-Weaver Index (H)
Sampling time (WAP)		
6	0.66^d	2.53^a
8	0.44^b	4.79^b
14	0.4^a	6.51^d
24	0.52^c	5.45^c
Sampling time (WAP)		
Bt	0.43^a	4.71^a
Non-Bt	0.57^b	4.93^b

- Means followed by the same letter are not significantly different

Effects of Bt maize on mycorrhizal fungal spore counts in the rhizosphere soil under field conditions at the University of Free State

Mycorrhizal spore counts were not determined for the 2008/2009 samples. Mycorrhizal fungal spore counts were lower in rhizosphere soil of Bt maize than on the non-Bt maize but the difference decreased with time up to the end of the season (Table 6).

Table 6: Recovery of mycorrhizal fungal spores in the rhizosphere of the plants during the 2009/2010 season

Treatment	Number of spores kg^{-1} soil (Mean±SEM)			
	4 WAP	8 WAP	12 WAP	16WAP
Bt plants	130±10	110±10	90±30	90±50
Non Bt plants	60±20	60±0	40±20	30±10

Effects Bt maize on microbial diversity in rhizosphere soil under field conditions at the University of Fort Hare Research Farm

Principal component analysis showed that over 75% of variation in CLPP of microorganisms in the rhizosphere soils could be explained by PC 1 (50.7%) and PC 2 (24.3%). Analysis of PCs showed that PC1 was related to the treatments (plant materials and genetic modification) and PC 2 to time of sampling. Both Bt maize cultivars (PAN6Q-321B and DKC 61-25B) had more positive loadings to PC1 than their near-isolines during the growing season but the difference decreased at the end of the season.

There were no interaction effects of time of planting and maize treatments on AWCD and H indices (Table 7). The treatments did not have an effect on both AWCD and H indices. There were decreases in AWCD and H indices over time with samples collected at 3 WAP having higher levels than those collected at 7 and 12 WAP.
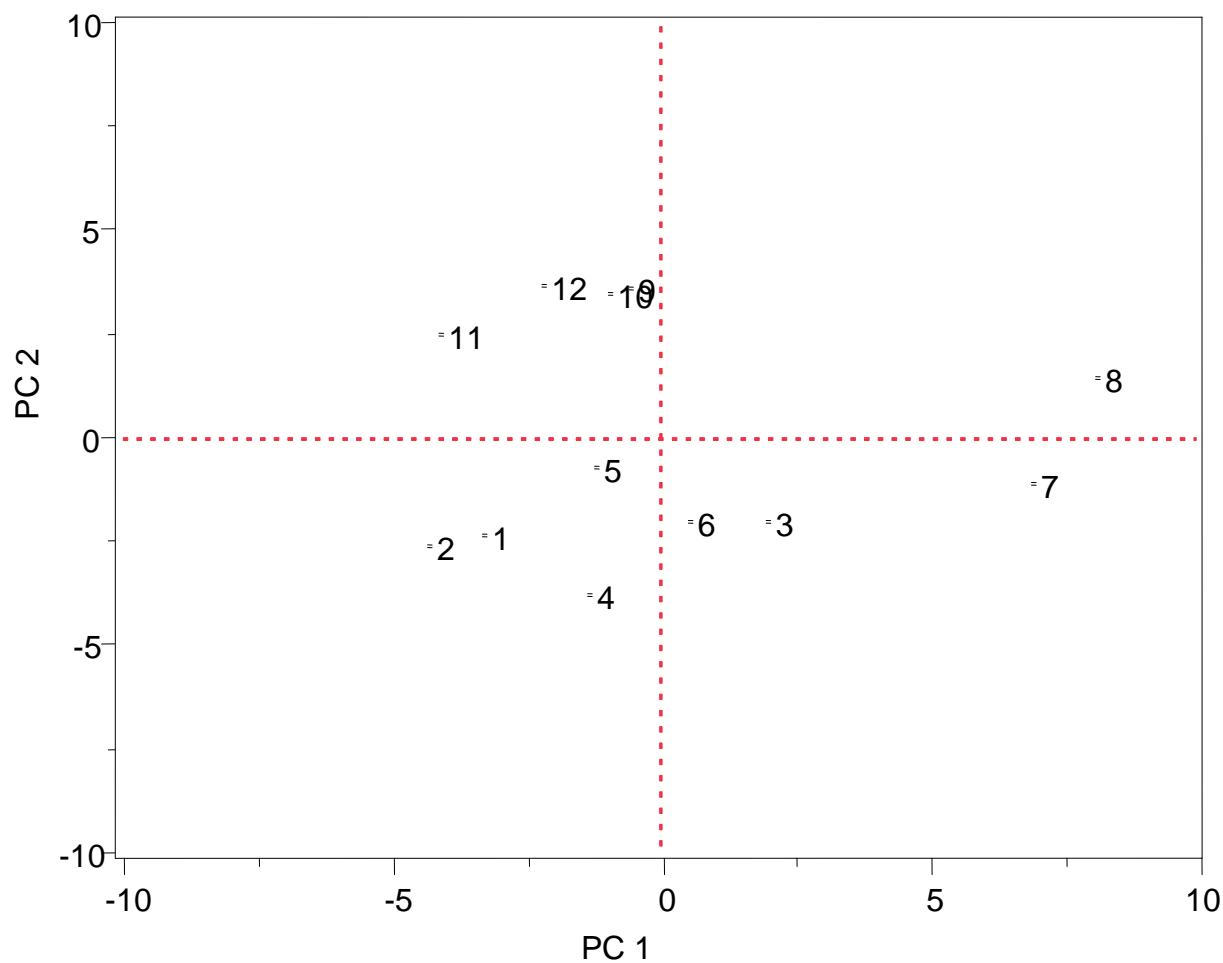
Figure 5: Community level physiological profiles of rhizosphere of Bt and non-Bt maize grown in UFH in 2009/10 season. The numbers, 1-4, 5-8, and 9-12 represent 3, 7 and 12 WAP. For 3 WAP: 1 = PAN 6777, 2= DKC 61-24, 3 = PAN6Q-321B, 4 = DKC 61-25B. The order remains the same for all other sampling times.

Table 7: Average well color development (AWCD) in the rhizosphere of maize in the UFH field study

Source of variation	AWCD	Shannon-Weaver Index (H)
Sampling time (WAP)		
3	1.03[a]	3.36[a]
7	0.74[b]	3.11[b]
12	0.68[b]	3.08[b]
Treatment		
DKC Bt	0.94[a]	3.23[a]
DKC N	0.93[a]	3.19[a]
PAN Bt	0.72[a]	3.17[a]
PAN N	0.66[a]	3.13[a]

- Means followed by the same letter are not significantly different

Mycorrhizal results for the UFH trial season 2009/10

Spore count were not determined for samples collected at 3 (WAP) because the rhizosphere samples were too small since the maize plants were still too young. PAN cultivars had similar spore counts at both sampling times (Table 8). Only DKC 61-24 had lower count at 7 WAP and higher at 12 WAP than the rest of the treatments, including its related Bt maize cultivar (DKC 61-25B).

Table 8: Rhizosphere mycorrhizal spore numbers in Bt and non Bt maize treatments in the UFH research trial

Cultivar	Number of spores kg^{-1} soil (Mean±SEM)	
	7 WAP	12 WAP
PAN6Q-321B	100±20	47±7
PAN 6777	133±18	73±27
DKC 61-24	67±18	120±20
DKC 61-25B	106±7	73±13

CHAPTER 5

DISCUSSION

Effects of Bt maize (MON810) on soil microbial diversity in rhizosphere and bulk soil

The results of PCA, AWCD and H index suggest that microbial diversity in the rhizosphere and bulk soil was more affected by sampling time and genetic modification of the maize had minimal effects. The presence of the residues also affected microbial diversity. This shows that functional diversity of CLPP does not depend on genetic modification but depended more on the presence or absence of maize residues (Figure 1, 3, 8). Microbial diversity was high in treatments that had residues whether they were Bt or non-Bt because of the decomposition of the residues in the soil which increases organic matter (Malosso et al. 2005). There are no differences between soil microbial diversity from the bulk and rhizosphere whether they are amended with Bt or not. Microorganisms that utilized the substrates were the same in both treatments, and this is in agreement with the results found by Fang et al., (2007), who reported that there are no differences between Bt and non-Bt planted soils in terms of microorganisms that utilize the carbon substrates. In the study that was conducted by Saxena and Stotkzy (2007), it was observed that there are no differences in the bacteria and fungi in the soil planted with Bt compared to the soil planted with non-Bt plants.

Time of sampling appeared to influence microbial diversity in all the experiments tested. This could be because of changes in the production of root exudates into the

rhizosphere as the plant grows. This is in agreement with the findings of Griffiths et al. (2003), who reported that factors like soil nutrients, pH, topsoil depth, water content, temperature and agricultural practices have an influence on the function of the soil microbial communities. The activity and diversity of soil microorganisms are often affected by the quality of residues that are incorporated in the soil depending on the amount of nutrients available in the residues (Bending et al., 2002). Daudu et al. (2009) reported no difference in the level of decomposition in Bt and non Bt residues even though Bt maize leaves had higher lignin.

Based on the results of the glasshouse study genetic modification has little to no influence on microbial diversity. These results are in agreement with Oliveira et al. (2009) who reported that no effects of genetic modification of maize (Bt 11 event 176, MON810) on microbial diversity in the rhizosphere. This could also be because changes in chemical composition of Bt maize has not been found to affect its decomposition. In contrast Flores et al. (2004) conducted a study which reported that the decomposition of Bt decompose less in soil than non-Bt maize.

Whereas there were minimal effects of genetic modification on rhizosphere community level physiological profiles under glasshouse conditions, it had some pronounced during the season but decreased with time until the treatments were similar at the end of the season under conditions at the University of Free State. These results are in agreement with those of Sun et al. (2007) who found that, effects of genetic modification on microbial diversity is inconsistent, as it appears to depend on the season. These findings appear to suggest that the chemical composition of the root exudates is different between Bt and non-Bt maize during the

growing season whereas at the end of the season the maize roots are no longer producing exudates resulting in similar microbial diversity.

The findings of the field experiment at the University of Fort Hare showed that genetic modification appeared to improve the biodiversity during the season. These findings imply that Bt maize may improve microbial diversity in the rhizosphere under field conditions. However the significance of these results a bit compromised because they are based on one season's data.

Based on the different applied approaches used, PCA appeared to be more sensitive than AWCD and H indices in detecting the effects of genetic modification on soil microbial diversity.

Effects of Bt maize (MON810) on mycorrhizal spore counts in soil

Whereas Bt maize treatments had higher spore counts in the rhizosphere soil than non Bt maize under glasshouse conditions, the reverse was observed under field conditions at the University of Free State Farm whereas there were no consistent effect on genetic modification at the University of Fort Hare. The findings are in agreement with Fladlung et al. (1999b) who reported that mycorrhizal spore counts decreased towards the end of the season. Ferreira (1999) also demonstrated that AMF colonization was not affected by Bt maize. However Turrini et al. (2005) reported that event Bt 176 reduced the symbiotic hyphal growth and endangered the development of appressoria. Infections were also lower in Bt maize that in non-Bt maize but there was no statistical difference. The reliability of the field results was a

bit compromised since they are based on one season's data at the two sites. Further research needs to be done to ascertain these findings over more seasons.

Implications of Bt maize (MON810) in the industry

Based on the findings from this work, seed companies that have marketed Bt maize (MON810) are assured that their product does not pose a risk to soil microbial diversity at least in the short term. However, any other product with a different transformation event needs to be tested.

At policy level, from a soil health perspective, it should be stated that the use of Bt maize (MON810) would not have a negative effect and post-release monitoring of bt maize (MON810) may not need to place emphasis on diversity of soil microorganisms.

CONCLUSIONS AND RECOMMENDATIONS

Growing Bt maize did not have an effect on the soil microbial diversity and mycorhizal spore counts in rhizosphere and bulk soils. Soil incorporated Bt maize residues did not affect oil microbial diversity and mycorhizal spore counts. Field soils on which Bt maize has been grown in the medium to long term, need to be studied to further establish the medium to long term effects on soil microbial diversity.

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