Influence of vermicompost application on rhizospheric microbial communities and Arbuscular mycorrhizal fungal colonisation of BT and non-BT maize in agricultural soil

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DEDICATION

Every challenging work needs self-efforts as well as guidance of elders especially those who were very close to our heart.

My humble efforts I dedicate to my sweet and loving

Mother and Father,

Whose affection, love, encouragement as well as prays of day and night make me able to get such success and honour

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ABSTRACT

Increasing crop production to ensure future food security while reducing environmental pressure on agro-ecosystems requires improved nutrient and water use efficiency. The soil microbial community directly and/or indirectly has important consequences on food security since these microorganisms participate in several soil processes. Genetically modified maize, a product of biotechnological advancement that addresses agricultural challenges related to yield losses and pests was adopted as a sustainable solution. Despite these advances, the insecticidal proteins expressed by Bt maize may alter soil functions and microbial communities associated with rhizosphere soil. The application of arbuscular mycorrhizal fungi and vermicompost, due to its innate biological, physiochemical and biochemical properties has been suggested as a possible solution to combat potential negative impact of Bt maize and can be indirectly involved in controlling plant pathogens, nematodes and other pests. This study investigated the impact of vermicompost application on rhizospheric microbial communities and arbuscular mycorrhizal fungal colonisation of Bt and non-Bt maize in agricultural soils. In addition, rhizosphere soil samples were also collected from Bt and non-Bt maize fields and analysed for chemical composition, enzyme activity and community ecology. It was observed that nitrate and phosphorus concentrations were significantly higher in non-Bt maize dryland soils, while organic carbon was significantly higher in non-Bt maize irrigated field soil. Acid phosphatase and β-glucosidase activities were significantly reduced in soils under Bt maize cultivation. The bacterial diversity analysis showed no differences in species abundance or richness between Bt and non-Bt maize treatments for all samples. Evaluation of microbial communities showed Actinobacteria, Proteobacteria, and Acidobacteria to be the dominant phyla. Differences in the abundance of some genera, including *Acidovorax*, *Bacillus*, *Flavobacterium*, *Paenibacillus* and *Pseudomonas*, whose species are known plant growth promoting bacteria were observed between Bt and non-Bt maize treatments. Redundancy analyses indicate that chemical properties, enzyme activities and bacterial diversity were mostly related to the different amendments and growth stages rather than the effect from genetic modification of maize. The differences were more pronounced between the diversity and abundance of particular species, rather than the species richness of the maize bacterial community.

Investigation of the potential effect of vermicompost application in the elimination or alleviation of the negative impact of genetic modification on the interaction between arbuscular mycorrhizal fungi and Bt maize showed that maize dry matter, chemical properties, enzyme activities and mycorrhizal root colonisation in maize were significantly improved by the co-application of arbuscular mycorrhizal fungi and vermicompost. The findings were in comparison to treatments without the addition of vermicompost. However, caution should be exercised in the interpretation of results obtained in this study because it is possible that the presence of the Cry protein in Bt maize plants could have contributed to the differences observed.

Keywords: Bt maize, rhizosphere soil, microbial communities, vermicompost, arbuscular mycorrhizal fungi

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LIST OF ABBREVIATIONS

Measuring units

cm: centimetre

°C: degree Celsius

E: East

g: gram(s)

g kg^{-1}: gram per kilogram

h: hour(s)

Kg: kilogram

L: litre

m: metre

mg: milligram

ng: nanogram

µg: microgram

µg ml^{-1}: microgram per millilitre

µl: microliter

µM: micromole

mM: millimolar

min: minute(s)

M: molar

nm: nanometre

%: percentage

pmol: picomole

S: South

v/v: volume to volume

General Abbreviations

ANOVA: analyses of variance

Bt: *Bacillus thuringiensis*

C: carbon

DNA: deoxyribonucleic acid

GM: genetically modified

HCℓ: hydrochloric acid

KOH: Potassium hydroxide

LSD: least significant difference

N: nitrogen

NH_2: nitrite

NH_3: nitrate

NH_4: ammonium

NRF: National Research Foundation

NGS: next generation sequencing

OTUs: operational taxonomic units

P: phosphorus

PCoA: Principal Coordinate Analyses

(Pty) Ltd: Propriety limited

PCR: polymerase chain reaction

RDA: redundancy analyses

RDP: Ribosomal Database Project

rRNA: Ribosomal ribonucleic acid

S: sulphur

USA: United States of America

CHAPTER 1

INTRODUCTION AND PROBLEM STATEMENT

1.1 Introduction

The world population grew dramatically from 2.6 to 6.7 billion people during the second half of the 20th century, and is expected to reach 9.2 billion by 2050 (Rodriguez and Sanders, 2015). Along with projected population growth in urban areas of less developed African and Asian countries are the challenges of food security, soil quality and environmental threats (United Nations, 2008). Consequently, intensive agriculture to meet demand has led to a trend where chemical fertilisers as well as high yielding, disease and drought resistant genetically modified crops have become popular (Gizaki *et al*., 2015).

Maize as an important staple food, was one of the first genetically modified crops to be produced globally (Joshi *et al*., 2005; Prasanna, 2012; Ranum *et al*., 2014). Genetically modified (GM) maize is generally engineered to express beneficial traits such as insecticidal properties, herbicide- or drought-tolerance (Yang *et al*., 2007). A popular example is the *cry1Ab* gene derived from *Bacillus thuringiensis* (Bt) for protection against insect pests and consequent yield enhancement (Prasanna, 2012; Ranum *et al*., 2014). Regardless of these benefits, the presence of insecticidal *cry1Ab* gene products in the environment may directly and/ or indirectly affect non-target organisms including the soil microbial community and associated soil processes (Feng *et al*., 2011; Fließbach *et al*., 2012).

Bt maize emerged as a result of a biotechnological advancement that addresses agricultural challenges related to pests and undernourished soils. Unfortunately, cultivation of Bt maize also has associated marked effects on soil nutrients and microbial ecology (Motavalli *et al*., 2004). With fewer technological advancements, potential effects of GM maize are of greater concern in developing countries where food security threats are linked to nutrient deprived soils (Buiatti *et al*., 2013).

1.2 Maize importance globally and in South Africa

Maize (*Zea mays* L.) is a key food security crop with high nutrient value, including elevated levels of starch and essential proteins and oils (Mboya *et al*., 2011). It is globally important and its consumption continues to rise in highly populated countries such as the United States of America (USA), China, and Brazil. These countries produce approximately 563 of the 717 million metric tons/year (Ranum *et al*., 2014). In the USA, Argentina, Australia, China and India maize is mainly produced as feed for livestock and poultry (FAO, 1997; Joshi *et al*., 2005; Mc Donald and Nicol, 2005; Meng *et al*., 2006; Capehart and Allen, 2013). However, more than half of maize production in South Africa, specifically white maize is primarily produced for human consumption (GrainSA, 2016).

South Africa was the 11th largest producer of maize in 2013 globally (FAOSTAT, 2015). Maize has been one of the most essential crops cultivated in South Africa since the 1950's (Van Rensburg *et al*., 1987), producing approximately eight million metric tons on three million hectares (ha) of land annually (Du Plessis, 2003; Anonymous, 2013). Currently on the African continent, South Africa holds the biggest maize production areas that are situated across four provinces. These are North West, Free State, Mpumalanga (Highveld) and KwaZulu-Natal (Midlands) Provinces (Du Plessis, 2003; Bekker, 2016). To improve quality and quantity of yield, maize was one of the first crops to be genetically engineered to incorporate traits for higher yields, herbicide tolerance and resistance to pest and disease (Prasanna, 2012; Ranum *et al*., 2014).

1.3 Overview of genetically modified crops

The genetic modification of crops confers certain traits that may enhance crop capabilities to deal with pest, weed and many environmental challenges (Yang *et al*., 2007). The first introduction of commercially available GM crops was in 1996 with only six countries adopting the technology to cultivate the transgenic crops (Bawa and Anilakumar, 2013). By 2015, 28 countries, of which 20 were developing and 8 industrial countries adopted the technology (James, 2015). Consequently, 179.7 million hectares were planted with biotech crops compared to only 1.7 million hectares in 1996 (James, 2015). In spite of the global adoption rates and potential advantages, only four African countries agreed to commercially cultivate GM crops. These include Burkina Faso, Egypt, South Africa and Sudan (James, 2013).

In terms of cultivation, South Africa is ranked ninth on the global scale (James, 2013; James, 2015) (Figure 1.1). Of the 2.7 million hectares of GM crops grown in South Africa in 2013, 78% represented the Bt maize (James, 2015; Iversen *et al*., 2014).

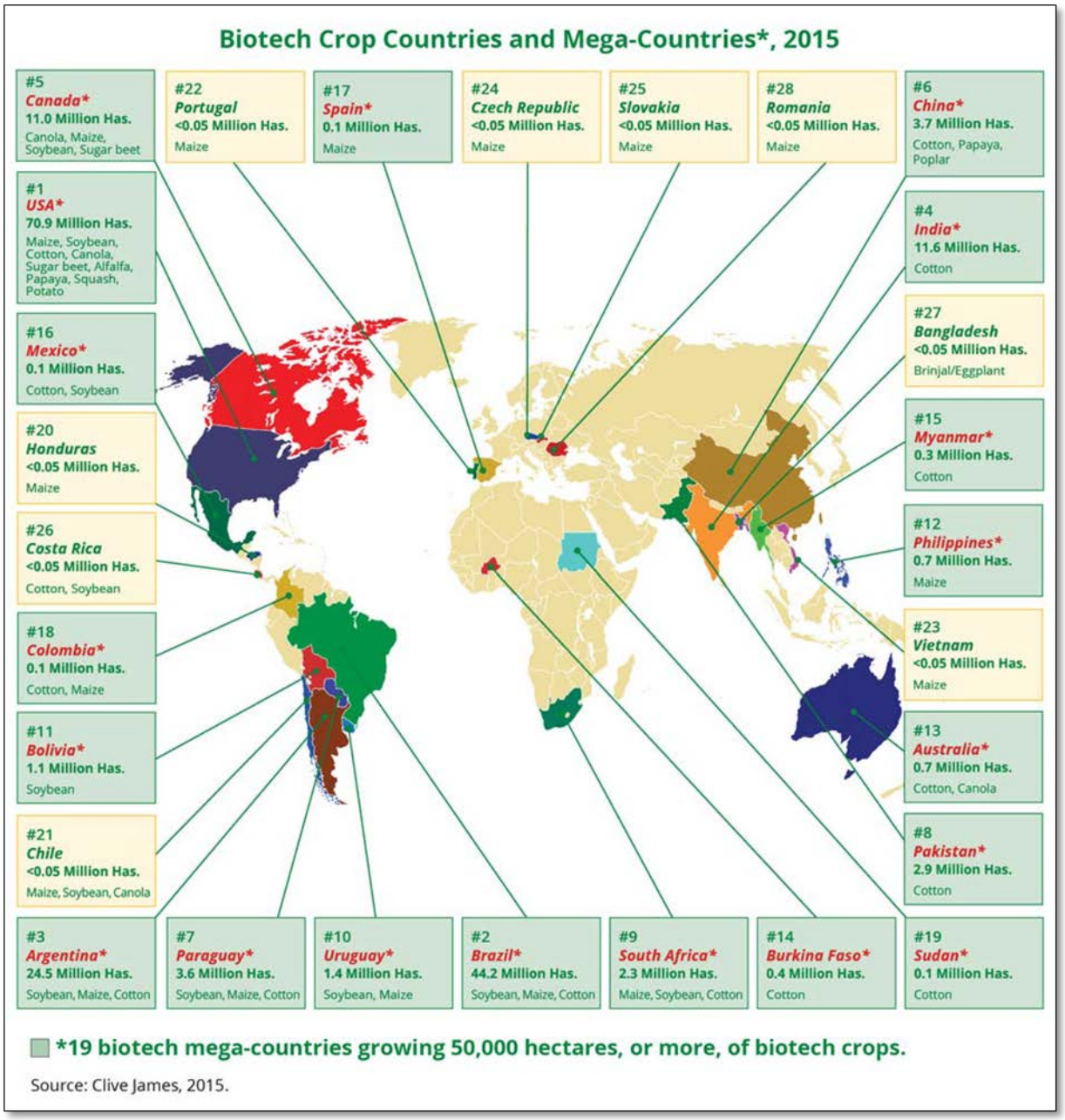

Figure 1.1: Nineteen mega-countries growing 50,000 hectares, or more, of biotech crops in the world (James, 2015).

Genetically modified crops offer several benefits, including protection of crops against pests, weeds, diseases and environmental stressors (Kfir *et al*., 2002; Lewis *et al*., 2010). One major benefit is reduced insecticide use and subsequent minimisation of impacts of these chemicals on non-target organisms (Barton and Dracup, 2000; Kruger, 2010). The use of GM crops has also been reported to reduce labour and maintenance costs (Ismael *et al*., 2001), as well as improved nutritional quality (De Groote *et al*., 2004). Nonetheless, the debate over GM crops is continuous in the European Union (EU) as a consequence of high public sensitivity and complexity of safety issues (Kostov *et al*., 2014). Other primary concerns are the effects on the environment associated with potential gene flow (Piñeyro-nelson *et al*., 2009), invasiveness of GM plants and possible interactions with non-target organisms (e.g., Icoz and Stotzky, 2008b).

1.3.1 Bt maize cultivation perspectives: Global and South African

Although there are many Bt crops, Bt maize stands out as the most widely grown Bt crop in the world. Transgenic Bt maize is maize that carries genes encoding insecticidal proteins derived from the spore-forming soil bacterium *Bacillus thuringiensis* (Bt) that are toxic to the larvae of insects (Castagnola and Jurat-Fuentes, 2012; Van den Berg *et al*., 2013). Bt maize was initially developed to control two North American lepidopteran stem borer species. These stem borers are *Diatraea grandiosella* (Lepidoptera: *Crambidae*) (Archer *et al*., 2001) and *Ostrinia nubilalis* (Lepidoptera: *Pyralidae*) (Ostlie *et al*., 1997). In South Africa, the target pests of Bt maize are the lepidopteran stem borers *Busseola fusca*, *Chilo partellus*, and *Sesamia calamistis* (Lepidoptera: *Noctuidae*) (Erasmus *et al*., 2010). These pests were effectively controlled by the Cry1Ab toxin expressed by the MON810 and Bt11 events (Van Rensburg, 1999).

Cultivation of Bt maize in South Africa, has made a substantial contribution in increasing crop production and reducing poverty. However, holistic views regarding agricultural sustainability indicate there may be negative impacts on the non-target soil organisms which will in turn disturb soil functions and processes (Motavalli *et al*., 2004). Such processes could be affected, for example, by the presence of Cry proteins in soils through cultivation of Bt crops (Holst-Jensen, 2009).

The biodiversity of an agro-ecosystem is not only important for its intrinsic value, but also because it influences ecological functions that are vital for crop production in sustainable agricultural systems (Hilbeck *et al*., 2006). Species assemblages (guild) in an agro-ecosystem fulfil a variety of ecosystem functions that may be harmed if changed (Dutton *et al*., 2003). For example, guild rearrangements due to the elimination of target or non-target organisms and subsequent changes in guild structure can lead to development of secondary pests (Van Wyk *et al*., 2007). For this reason, it is essential to address the potential environmental risks that the cultivation of GM crops may hold (Van Wyk *et al*., 2007).

1.4 Impact of Bt crops on soil ecosystems

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, 2008b), which could interfere with microbe-mediated processes and soil fertility. Several studies have reported that genetic modification of maize may result in possible unintended effects on plant structure and chemical compositions, which may have implications on decomposition processes resulting in nutrient recycling being affected (Poerschmann *et al*., 2005). Bt maize has been reported to have no effect on the mineralisation of nitrogen (Mungai *et al*., 2005; Devare *et al*., 2007). In a study by Mungai *et al*. (2005) no differences were observed in decomposition, chemical composition and nitrogen mineralisation of stem and leaves associated with Bt and non-Bt maize. However, in the field and laboratory experiments the nitrogen mineralised 2.7 times more in non-Bt maize roots than in Bt maize roots (Mungai *et al*., 2005). Furthermore, no adverse effects of Cry3Bb1 from Bt maize on microbial biomass carbon and nitrogen mineralisation over a three-year cropping cycle under field conditions were reported by Devare *et al*. (2007). In contrast, Poerschmann *et al*. (2005) and Daudu *et al*. (2009) reported that Bt maize cultivars containing Cry1Ab protein had higher lignin content than their near-isogenic lines, which could slow decomposition. This modification could cause a reduction in nutrient cycling attribute by the high lignin content observed in Bt maize (Motavalli *et al*., 2004; Mungai *et al*., 2005).

Soil enzymes produced by various soil microorganisms are important for catalysing a significant number of reactions necessary for decomposition of organic residues, cycling of nutrients and formation of soil structure (Bandick and Dick, 1999). These enzymes include acid and alkaline phosphatases, arylsulfatase dehydrogenase, β-glucosidase

and urease which have significant functions in P, S, C, N and nutrient cycling, respectively (Bandick and Dick, 1999). Enzymes have a critical biochemical function in organic matter decomposition as they catalyse several important reactions necessary for decomposition of organic waste, formation of organic matter and nutrient cycling (Griffiths *et al*., 2003). Lang *et al*. (2006) reported that no significant differences on microbial biomass and enzyme activities could be determined between soil with Bt and non-Bt maize. These findings were similar to that of Icoz *et al*. (2008), who also reported no consistent effects of Bt maize on microbial populations and activities of various soil enzymes. In general, outcomes of such studies are influenced by agricultural practices and specific environmental conditions.

1.5 Impact of Bt crops on non-target soil organisms

Several hypothesis have been developed as to how GM crops may exert direct and indirect effects on non-target soil microorganisms. The direct effects are produced by the activity of transgenic proteins; through root depositions, as exudates, cells and mucilage as well as through unintended changes in the plant due to the genetic modification (Kostov *et al*., 2014). Indirect effects may be ascribed to modifications occurring in GM crop plant metabolic pathways leading to changes in root exudate composition and altered expression in plant tissues that may affect these non-target microorganisms. In addition, the key concern is related to soil microbial ecology and that any effects of Bt crop cultivation on non-target microorganisms may affect soil ecosystem functioning (Kostov *et al*., 2014).

1.5.1 Potential effects of Bt maize on soil bacteria

Bacteria are by far the most abundant organisms in the soil and are important for nutrient mineralisation, decomposition of organic matter, protection against plant pathogens, degradation of chemicals/toxins in the environment and nutrient cycling. The total number of bacteria per gram of dry soil is *ca*. 1.5×10^{10} (Torsvik *et al*., 1990). In both natural and agro-ecosystems, bacterial abundance is highest in the rhizosphere - the narrow area of soil directly surrounding and influenced by plant roots. Plants support the development of microbial communities in the rhizosphere by producing root exudates that contain carbon-rich nutrients such as carbohydrates and proteins (Grayston *et al*., 1997; Morgan *et al*., 2005). Soil organisms take advantage of these carbon resources and plants benefit via increased nutrient availability, improved mineral

uptake, and enhanced soil fertility provided by the soil microbial community (Smith and Gianinazzi-Pearson, 1988).

While most of the studies reviewed by Icoz and Stoztky (2008b) indicated that Bt-expressing plants cause no or minor changes in microbial communities, in other studies, distinctions were established in both diversity and abundance of microorganisms between soils cultivated with Bt and non-Bt maize were demonstrated. In an attempt to discover the mechanism involved in the actual process, direct incorporation of Cry1Ab toxin into soil was tested but there were no adverse effects on culturable bacteria (Saxena and Stotzky, 2001). Similarly, Cotta *et al*. (2013) and Ondreičková *et al*. (2014), reported that there were no observable effects of GM maize on rhizospheric microbial communities. Moreover, a long-term field study also found no consistent differences in soil microbial communities between Bt and non-Bt maize during a four-year successive study (Barriuso *et al*., 2012). In contrast, Castaldini *et al*. (2005) reported consistent differences in rhizosphere heterotrophic bacteria and mycorrhizal colonisation (including *Glomus*. *mosseae*) between Bt maize (event Bt176) and its conventional counterpart. More recently, van Wyk *et al*. (2017) reported that there were differences in microbial community structures between Bt and non-Bt maize fields, however, the differences were not related to the genetic modification of the maize. These differences were more specific to agricultural practices (tillage, irrigation), cultivar type and environmental parameters. Furthermore, it is possible that the varying observations or reports are a function of variations in study environments, experimental designs and transgenic maize events used among others.

Overall, reported effects on microbial communities were considered spatially and temporally limited, and small compared with those induced by differences in geographic location, temperature, seasonality, plant variety and soil type (Fang *et al*., 2005, Fang *et al*., 2007; Griffiths *et al*., 2005, Griffiths *et al*., 2006; Filion, 2008; Icoz and Stotzky, 2008b). Factors such as plant growth stage and field heterogeneity produced larger effects on soil microbial community structure than MON810 maize (Baumgarte and Tebbe, 2005; Griffiths *et al*., 2007b).

1.5.2 Potential effects of Bt maize on arbuscular mycorrhizal (AM) fungi

Arbuscular mycorrhizal (AM) fungi represent an important group of non-target microorganisms, fundamental for soil fertility and plant nutrition. This is an ancient fungal group, which has coevolved with plants in the last 400 million years, assisting plants in the conquest of dry lands (Schüβler *et al*., 2001). Maize is one of the heavily mycorrhizal-dependent plant species (Tawaraya, 2003). Therefore, the importance of AM fungi in maize growth is expected to increase with the rise in frequency of extreme water events (droughts and floods) (Rillig *et al*., 2003).

This symbiosis is mutually beneficial: AM fungi improve the supply of water and nutrients, especially phosphorus, to their host plants; this in turn provides the AM fungal community with carbohydrates essential for growth (Hodge *et al*., 2010). In addition, AM fungi also improve host plant tolerance to disease and pathogens and promote the aggregate stability of soils (Singh *et al*., 2012; Steinkellner *et al*., 2012). AM fungi provide nutritional benefits to plants in exchange for carbon resources and protection by the host plant. Although ubiquitous, many soil organisms such as AM fungi are sensitive to a variety of agricultural practices, including pesticide applications, tilling, cultivation practices (e.g., compost versus chemical fertiliser), and even the type of plant grown. Due to the close association of AM fungi with the plant roots, they are more sensitive to changes in the physiology of the host plant as well as the composition of root exudates.

In addition to the debate spearheaded by the EU and non-government organisation, there is scientific evidence highlighting the potential negative influence of genetic modification on plant symbiosis with AM fungal communities (Glandorf *et al*., 1997; Anderson *et al*., 2005; Zeng *et al*., 2014). Hence, AM fungi are considered important soil microorganisms, which can be used to assess the effects associated with GM crops (Liu and Du, 2008; Liu, 2010). For instance, due to AM fungi reliance on plant host for reproduction and nutrition, they may be sensitive to changes in the physiology of the host plant, to biochemical changes associated with the Bt modification, or to alterations in root exudates released into the rhizosphere. Although Bt proteins are expressed in the roots of most Bt maize lines (Saxena and Stotzky, 2000; Saxena *et al*., 2002; Icoz and Stotzky, 2008a, b; Cheeke *et al*., 2014), the evidence that Cry proteins have a direct effect on AM fungi is contradictory. Various studies using a variety of Bt events could not demonstrate significant differences in mycorrhizal colonisation when compared to parent lines (Castaldini *et al*., 2005; De Vaufleury *et al*., 2007; Tan *et al*.,

2011; Cheeke *et al*., 2014). However, some studies have shown the contrary (Castaldini *et al*., 2005; De Vaufleury *et al*., 2007; Cheeke *et al*., 2012). The discrepancy of these studies may result from the differences in experimental designs, transgenic maize events used, the age of the growing plants, the species of AM fungi and fertilisers among other factors.

Having realised the role of AM fungi in agroforestry, farmers are now artificially introducing AM fungi into the soil environment to improve crop production. Such application is referred to as bio-fertilisation. However, whether AM fungi are artificially introduced or naturally present, their roles in the ecosystem could be affected by the genetic modification of their hosts.

1.6 Bio-fertilisers

Bio-fertilisers are a group of beneficial or a large population of specific microorganisms, which enhance the productivity of soil (Roychowdhury *et al*., 2017). These microorganisms enhance soil fertility through different processes. An example is their ability to fix atmospheric nitrogen, both, in association with or without plant roots. They may also solubilise insoluble soil phosphates and stimulate plant growth through synthesis of growth-promoting substances (Sadhana, 2014; Raimi *et al*., 2017). Bio-fertilisers are not harmful to crops or other plants and are environmentally friendly. In addition, the use of bio-fertilisers in the soil, makes the plants healthy as well as protect them from diseases (Sadhana, 2014; Raimi *et al*., 2017).

The main sources of bio-fertilisers are fungi and bacteria. Bio-fertilisers could be used for inoculating soil and/or seed under ideal conditions to increase the availability of plant nutrients (Raimi *et al*., 2017). Among them are the AM fungi inoculants that are important in the cultivation of many crops especially maize.

1.6.1 Arbuscular mycorrhizal fungi as bio-fertiliser

Arbuscular mycorrhizal (AM) fungi are obligate symbionts that are predominantly found in the roots and soils of agricultural crop plants. They are members of the subphylum Glomeromycotina (Spatafora *et al*., 2016) that are obligate biotrophs and obtain their nitrogen from the soil and can translocate it to the host plant (Smith and Read, 1996). The plants provide carbohydrate to the AM fungi while the fungi supply soil nutrients such as phosphorus, copper, zinc, and sulfur to the plants (Maji *et al*., 2017). They are

considered natural bio-fertilisers because they provide the host with nutrients, water, and pathogen protection, in exchange for photosynthetic products. Furthermore, AM fungi have been shown to improve the growth, health, nutrient uptake, flowering and drought tolerance of plant species (Young *et al*., 2015). Thus, AM fungi are primary biotic soil components which can lead to a less efficient ecosystem functioning when absent or impoverished.

Inoculation of plants like Bt maize with AM fungi has the potential to increase yields and arrest P limiting situations (Douds *et al*., 2007). The most frequently reported benefit of AM fungi is enhanced uptake of immobile nutrients for plants, notably P, from the soil solution (Douds *et al*., 2007). Here, fungal hyphae are able to mobilise and make P available to the plants. AM fungi form specialised structures inside root cells called arbuscules which are believed to be the main site for nutrient transfer between the plant and fungus (Mishra and Kizhakkepurakkal, 2014).

In addition to many advantages of AM fungi in the ecosystem, increased benefits have been reported when co-applied with vermicompost. The co-application of AM fungi with vermicompost can increase the growth and yield of maize as well as other components of maize plants like cob weight, leaf production, height, and weight (Roychowdhury *et al*., 2017). This co-application of AM fungi with vermicompost can also enhance root development, mycorrhizal colonisation and soil nutrient uptake (Shishehbor *et al*., 2013; Hussain *et al*., 2016). These beneficial effects of AM fungi with vermicompost on plants are attributed mainly to the additional soil nutrient supply.

1.7 Vermicompost

Vermicompost is a humus-like substance that is formed when organic matter is being broken down by the combined action of earthworms and microorganisms (Lazcano *et al*., 2008). Vermicompost are finer in structure and retain nutrients for a longer time. Vermicompost are highly porous, well-aerated, well-drained and have good water-holding capacity. In addition, vermicomposts also contains important nutrients like nitrogen, phosphorus and potassium (Edwards and Burrows, 1988; Shishehbor *et al*., 2013). One of the unique features of vermicompost is that during the process of conversion of various organic wastes by earthworms, many of the nutrients are changed to their available forms in order to make them easily utilisable by plants (Gopinathan and Prakash, 2014). Vermicompost contains nutrients in readily available form to plants such as nitrate, exchangeable, soluble potassium, calcium and magnesium (Edwards

and Burrows, 1988; Orozco *et al*., 1996) and have large particular surface area that provides many microsites for microbial activity and for the strong retention of nutrients (Shi-wei and Fu-zhen, 1991). Since vermicompost is a store house of almost all the nutrients required by plants for proper growth and development, its addition in soil enhanced availability of these nutrients. It has also been suggested that nutrients are released more gradually from vermicompost preventing problems such as nutrient loss, toxicity, and salinity, which may otherwise be associated with utilisation of organic materials under certain conditions.

1.8 Problem statement

An increasing number of crops commercially grown today are GM to resist insect pests and/or herbicides. Although Bt maize is one of the most commonly grown GM crops in South Africa, little is known about its effects on the health of soils and non-target beneficial microorganisms (Cheeke *et al*., 2012). There are many benefits associated with the cultivation of Bt maize. Examples include reduction of insecticide use, and protection against common agricultural pests such as the maize root worm and the lepidopteran stem borer *Busseola fusca* (Erasmus *et al*., 2010). However, apart from the pest problem, there are other challenges facing maize crop production. One of the persistent challenges is the fertility of soil, which is a multifaceted challenge that is often influenced by the microbial diversity and activity of the soil. Rhizosphere microbial communities are an important component of soil quality and fertility (Jangid *et al*., 2008). The cultivation of GM plants may alter these soil microbial communities, hence jeopardising agricultural sustainability. When the proteins are released from Bt maize in the root exudates or from decomposing plant tissue, organisms in the soil will come into contact with these transgenic Cry proteins and consequently pose a potential risk for non-target organisms, such as soil bacteria and fungi (Icoz *et al*., 2008; Tan *et al*., 2010). Ubiquitous microscopic soil fungi such as AM fungi, form symbiotic relationships with the roots of most plants. This mutualistic relationship involves the supply of carbon to the fungi by the plants, whereas the fungi aid the host plant's ability to uptake nutrients and water from the surrounding soil (Smith and Read, 2008).

Most studies conducted on the effects of Bt crops on AM fungi showed that Bt plants affect colonisation and symbiotic development of AM fungi (Liang *et al*., 2015; Turrini *et al*., 2005). Other studies also have indicated that Bt crops have no consistent significant impacts on AM fungi (De Vaufleury *et al*., 2007; Knox *et al*., 2008; Liu, 2010). In

conjunction with different cultivars, soil types possibly play a major role in these interactions. However, very little is known about these effects and particularly AM fungi in South African maize soils.

Possible solutions to deal with the potential negative impact of Bt maize on soil beneficial microbes (naturally present or artificially introduced) could be the application of vermicompost. The use of vermicompost has become a popular method of enhancing the performance of soil microbes. Vermicompost boosts soil biodiversity by enhancing the growth of beneficial microbes, and such microbes may in turn enhance plant growth directly by production of plant growth-regulating hormones and enzymes. In addition, vermicomposts can be indirectly involved in controlling plant pathogens, nematodes and other pests, thereby enhancing plant health and crop yield (Pathma and Sakthivel, 2012). Because of their innate biological, physiochemical and biochemical properties, vermicomposts may be used to promote sustainable agriculture and for the safe management of agricultural wastes, which may otherwise pose a threat to life and environment.

Having acknowledged the potential of altered effects of Bt maize on AM fungi in the soil, the proposed study intends to investigate if the application of vermicomposts could restore and maintain the symbiotic relationship between Bt maize and AM fungi. Vermicomposts are already shown to be able to stimulate mycorrhizal colonisation of roots (Cavender *et al*., 2003). The question now arises whether vermicomposts can stimulate arbuscular mycorrhizal colonisation of roots of Bt maize?

The aim of this study is thus to investigate the impact of vermicompost application on rhizospheric microbial communities and arbuscular mycorrhizal fungal colonisation of Bt and non-Bt maize in agricultural soils in South Africa.

Specific objectives were to:

i. assess the structure and enzymatic activity of rhizosphere soil microbial communities associated with field grown Bt and non-Bt maize
ii. evaluate the potential impacts of the genetic modification and soil amendments on rhizobacterial communities associated with Bt maize plants over 120 days
iii. evaluate the potential of vermicompost application in the elimination or alleviation of the negative impact of genetic modification on the interaction between AM fungi and Bt maize over 120 days.

1.9 Outline of thesis

Chapter 1 provides an introduction to the study, which describes the importance of maize globally and in South Africa. Specific focus was on Bt maize and its, benefits and possible effects on the soil ecosystem. Furthermore, capabilities of AM fungi and vermicompost to promote crop growth and soil quality are discussed. This chapter also includes a problem statement, aims, specific objectives and outline of the thesis chapters.

Chapter 2 describes the structure and enzyme activities of rhizosphere soil microbial communities associated with field grown Bt and non-Bt maize. In this chapter, information on how high-throughput sequencing was employed to analyse bacterial diversity is provided. In addition, further information on the correlations between the physico-chemical, enzymatic activities and abundance of bacterial genera is provided.

Title:	Ecological guild and enzyme activities of rhizosphere soil microbial communities associated with Bt maize cultivation under field conditions in North West Province of South Africa
Authors:	Van Wyk, D.A.B., Adeleke, R., Bezuidenhout, C.C., Rhode, O.H.J.
Journal:	Journal of Basic Microbiology (Published)

Chapter 3 describes the potential impacts of genetic modification and soil amendments on rhizobacterial communities of Bt maize. In this chapter, bacterial community structures were analyses using next generation sequencing of the Illumina MiSeq. Further information was provided on the correlations between the different treatments, physico-chemical properties and enzymatic activities.

Title:	Genetic modification or soil amendment: what factors drive the rhizobacterial communities of Bt maize plant?
Authors:	Van Wyk, D.A.B., Adeleke, R., Bezuidenhout, C.C., Rhode, O.H.J.
Target Journal:	Journal of Basic Microbiology

Chapter 4 reported the potential of vermicompost application in the elimination or alleviation of the negative impact of genetic modification on the interaction between AM fungi and Bt maize. In this chapter, information was provided on the correlations between the different treatments, physico-chemical properties and enzymatic activities. In addition, further information was provided on the dry matter of plants and mycorrhizal colonisation of maize roots.

Title:	Vermicompost application: A potential solution to challenges associated with interactions between Bt maize and arbuscular mycorrhizal fungi
Authors:	Van Wyk, D.A.B., Adeleke, R., Bezuidenhout, C.C., Rhode, O.H.J.
Target Journal:	Applied Soil Ecology

Overlaps in the thesis were unavoidable.

Chapter 5 is a summary of all previous chapters from which relevant conclusions are drawn and concludes with meaningful recommendations for future research in this field.

CHAPTER 2

Ecological guild and enzyme activities of rhizosphere soil microbial communities associated with Bt maize cultivation under field conditions in North West Province of South Africa

2.1 Introduction

Maize is one of the world's most important agricultural crops and it is a stable food for many developing countries such as South Africa. In 1997, genetically modified (GM) maize expressing insecticidal Cry proteins (Bt toxins) were among the first GM plants to be approved in South Africa. By 2013, South Africa had 2.3 million hectares of GM crops under cultivation, of which the majority was maize (representing 78% of the GM crops under cultivation) (Iversen *et al*., 2014). This crop either have resistance to insect pests or tolerance to broad range of herbicides, or both (Cheeke *et al*., 2012). The most dominant types of GM cultivars are insect-resistant (Bt maize) and herbicide-tolerant (Roundup Ready® soybean). However, new GM cultivars have been developed that offer stacked traits (herbicide tolerance plus resistance to multiple insect pests) and increased stress tolerance (e.g., salt stress or drought tolerant varieties) (Cheeke *et al*., 2012). This rapid and widespread adoption of GM crops has led to a dramatic shift in the agricultural landscape and has raised concerns about the impact of agricultural biotechnology on non-target microorganisms in the soil environment. Although some GM crops can provide a variety of benefits, there may also be negative impacts on the environments especially to non-target soil microorganisms such as bacteria and fungi (Dohrman *et al*., 2013).

Soil bacterial communities are relevant and good indicators for monitoring potential impacts of different agricultural practices such as farming practices, fertiliser applications as well as pesticide applications on the ecosystem functions. Soil microorganisms are a very important part of the environmental ecosystems, which could adjust energy flow and play a pivotal role in growth and development of agricultural crops (Philippot *et al*., 2013). They are also involved in soil biochemical processes such as production of enzymes which are responsible for catalytic reactions necessary for organic matter decomposition, energy transfer, environmental quality and crop productivity (Carpenter, 2011; Zhang *et al*., 2016). In addition, soil enzymes also play important roles in the nutrient cycling and are good indicators of soil quality (Zhang *et*

al., 2016; Pajares *et al*., 2011). Numerous studies have investigated the soil microbial properties using broad-scale or integrative methods such as enzyme activities, microbial biomass and microbial diversity associated with Bt maize. Typically, the results of such studies have shown significantly positive, negative and or sometimes transitory effects of Bt maize on essential microbial properties (Zhang *et al*., 2016; Chen *et al*., 2011; Griffiths *et al*., 2006; Ondreičková *et al*., 2014). However, the impacts of Bt maize may be masked by "functional redundancy" where overall soil functions are unaffected but microbial community composition is altered and key functions mediated by specific microbial populations are affected. Therefore, in-depth studies on the soil microbial communities associated with field grown Bt and non-Bt maize are essential understanding the microbial processes and changes in the chemical and biochemical processes in soil. Currently, metagenomic analysis of microbial ecology, such as next generation sequencing (NGS) based on 16S rRNA gene profiling, has been the focus of several environmental studies including soil (Lemos *et al*., 2011). Such profiling analyses provide extensive information on community structure and composition (Kakirde *et al*., 2010). In addition, phylogenetic and functional analyses of microorganisms can be determined at community level (Cowan *et al*., 2005). Our aim was to study the structure and enzymatic activities of rhizosphere soil microbial communities associated with field grown Bt and non-Bt maize.

2.2 Materials and methods

2.2.1 Study fields

The study was conducted in two localities in the North West Province of South Africa, where maize is intensively cultivated. These localities are situated between latitudes (26°22'45"S and 26°44' 0"S) and longitudes (26°48' 23"E and 27°4'52"E) and comprised of established fields under dryland (DL) and irrigation (IL) conventional cultivation where Bt maize had been grown. Transgenic Bt maize expressing the Cry1Ab protein (event MON 810) and a near-isogenic non-Bt line were used. Cultivars used under DL cultivation comprised of DKC 80-12 B and DKC 80-10 (Monsanto), while for IL PAN 6236B and PAN 6126 from Pannar were used.

2.2.2 Rhizospheric soil sampling

Soil samples were randomly collected from the rhizosphere of both Bt and non-Bt maize in all study fields. Sampling was done in a W-shaped pattern in all fields to obtain representative samples. A total of 16 soil samples were collected from the rhizosphere of Bt maize (8 each from DL and IL), while 14 soil samples were collected from non-Bt maize (7 each from DL and IL) rhizosphere. All maize plants were at the maturing stage at the time of sample collection. These samples were collected aseptically as described by Dick *et al*. (1996) and immediately transported on ice to the laboratory for further analyses.

2.2.3 Determination of soil enzymatic activities

The activities of acid phosphatase (EC 3.1.3.2) and β-glucosidase (EC 3. 2.1.21) were assayed using 1g of soil with the appropriate substrates and incubated for 1 h (37 °C) at an optimal pH as described by Tabatabai (1994) and Dick *et al*. (1996), respectively. Urease (EC 3.5.1.5) enzyme activity was estimated according to Kandeler and Gerber (1988). This method was based on the estimation of urea hydrolysis in soils. Briefly, this method involves mixing 5 g of soil with a urea solution and incubating it for 2 h at 37°C. Enzyme activities were assayed in duplicate with one control, to which substrate was added after incubation.

2.2.4 Chemical analysis

Standard chemical analyses of the soil were performed by the Agricultural Research Council-Institute for Soil Climate and Water (ARC-ISCW). The pH of the soil was determined as described by McLean (1982) with potassium chloride (pH [KCℓ]) by means of a calibrated pH meter (Radiometer PHM 80, Copenhagen). Ammonium (NH_4^+-N) concentrations were measured by means of the ammonia-selective electrode method (Banwart *et al*., 1972) and organic carbon was determined by the Walkley-Black method of Nelson and Sommers (1982). The anions nitrate (NO_3^--N), nitrite – (NO_2^--N), and phosphate – (PO_4^--P) were determined according to the method of Sonnevelt and van den Ende (1971). The P-Bray 1 was determined according to the procedure of Bray and Kurtz (1945).

2.2.5 Genomic DNA extraction

The Machery-Nagel Nucleospin Soil DNA Extraction kit (Machery-Nagel, Germany) was used to extract DNA from rhizospheric soil samples as described by the manufacturer. DNA quantity and quality were determined by using a NanoDrop 1000 Spectrophotometer (Thermo Fischer Scientific, California, USA).

2.2.6 Illumina MiSeq sequencing

Microbial genomic DNA from Bt and non-Bt maize soil samples were normalised to concentration ≤ 10 ng/µL. Sequencing library preparation guide was followed (Illumina Inc.). Locus-specific primers 341F (5´-CCTACGGGNGGCWGCAG-3´) and 805R (5´-GACTACHVGGGTATCTAATCC-3´) (Herlemann *et al*., 2011), targeting the hypervariable V3-V4 region (≈460 bp) of the bacterial 16S rRNA gene were used. Illumina forward and reverse overhang adapters (Illumina Inc., CA, USA) were attached to the 5`-end of forward and reverse primers, respectively. All polymerase chain reaction (PCR) components and protocols were exactly as reported in the library preparation guide (Illumina Inc., California, USA). Sequencing run on the Illumina MiSeq, de-multiplexing and secondary analyses of the reads were performed using the MiSeq reporter software (Illumina Inc., California, USA).

Raw data from Illumina sequencing of the 16S rRNA gene were processed on the Galaxy GVL 4.0.0 pipeline (http://galaxy-qld.genome.edu.au/galaxy) as previously described (Afgan *et al*., 2015). To improve the quality of next generation sequencing data and eliminate the effect of random sequencing errors, some unreliable data from the libraries were deleted, such as average q-value below 25, singletons, and reads shorter than 200bp. Sequences were classified into operational taxonomic units (OTUs) with 97% similarity for the 16S rRNA gene after excluding chimeric sequences by using the UCHIME method. Taxonomic information of sequences by the Ribosomal Database Project (RDP) classifier for the 16S rRNA gene were assigned at confidence cutoff of 0.5.

2.2.7 Statistical analyses and Bioinformatics

The data sets obtained from both chemical and biochemical analyses of both Bt and non-Bt maize soil samples were analysed with the Statgraphics software package version 5 (Statistical Graphics Corporation, USA). Redundancy analysis (RDA) was performed to measure chemical and enzymatic properties that influence microbial community variations. The significant correlations of the parameters were examined by a Monte Carlo permutation. The triplot was generated by CANOCO 4.5 (Biometrics Wageningen, The Netherlands). Graphs were generated by CanoDraw 4.0 (Biometrics Wageningen, The Netherlands).

The Alpha diversity parameters were calculated for each field under Bt and non-Bt maize cultivation comprising of OTUs richness, Shannon-Weiner (H`), Evenness, Inverse Simpson indexes, Chao1 richness estimator, and the rarefaction curve at 0.03 using gplot package of R on the relative abundance of each taxon. A principal coordinate analysis (PCoA) was carried out based on weighted beta diversity. In addition, a Venn diagram was constructed using the following online site [http://bioinfogp.cnb.csic.es/tools/venny/ date of access: 10 June 2016]. All multivariate and community analyses were conducted using the gplot and vegan, packages of R based on the relative abundance of each taxon.

2.3 Results

2.3.1 Chemical properties of Bt and non-Bt maize rhizosphere soil under DL and IL

In Table 2.1, the mean values of soil chemical characteristics comprising of Bt and non-Bt maize samples under DL and IL conditions are shown. Results of Bt and non-Bt maize fields under DL conditions showed a slightly acid pH, whereas fields under IL conditions of Bt and non-Bt maize soils indicated a slightly acid to neutral pH (Table 2.1). Nitrate (NO_3^+) and phosphorus (P) concentrations were significantly higher ($p<0.05$) in non-Bt maize soils under DL conditions compared to Bt maize soil. There was no significant difference ($p>0.05$) in values of nitrite (NO_2^-), ammonium (NH_4^+), and organic carbon (C) between Bt and non-Bt maize fields under DL conditions. No significant difference ($p>0.05$) in values of nitrate (NO_3^+), nitrite (NO_2^-), ammonium (NH_4^+), and phosphorus (P) were showed between Bt and non-Bt maize fields under IL conditions (Table 2.1). However, non-Bt maize soil under IL conditions did show a significantly higher ($p < 0.05$) organic carbon (C) percentage compared to Bt maize soil.

Table 2.1: Mean values of chemical properties of DL and IL under Bt and non-Bt maize fields.

Parameters	pH (KCℓ)	Organic Carbon (%)	NO_3^+ (mg/kg)	NO_2^- (mg/kg)	NH_4^+ (mg/kg)	P (mg/kg)
Dryland Fields						
DLNBt (n=8)	6.2[a]	1.0[a]	6.1[a]	0.4[a]	2.4[a]	41.2[a]
DLBt (n=7)	6.3[a]	0.9[a]	4.1[b]	0.4[a]	2.4[a]	31.7[b]
Irrigated Fields						
ILNBt (n=8)	6.4[a]	1.4[a]	29.5[a]	0.5[a]	2.8[a]	52.2[a]
ILBt (n=7)	6.6[a]	1.2[b]	26.4[a]	0.6[a]	2.8[a]	82.7[a]

Fields under DL and IL conditions with different combinations of superscript alphabetic letters in the same column indicate significant difference between each other.

2.3.2 Biochemical properties of Bt and non-Bt maize rhizosphere soil under DL and IL

The average activities of the enzymes assayed are presented in Figure 2.1. Results illustrated that there were significant differences in acid phosphatase and β-glucosidase activities between Bt and non-Bt maize soil samples under DL and IL conditions, while urease showed no significant differences. Significantly higher acid phosphatase ($p < 0.05$) and β-glucosidase activities ($p < 0.05$) were recorded for soils under non-Bt maize cultivation of DL and IL conditions.

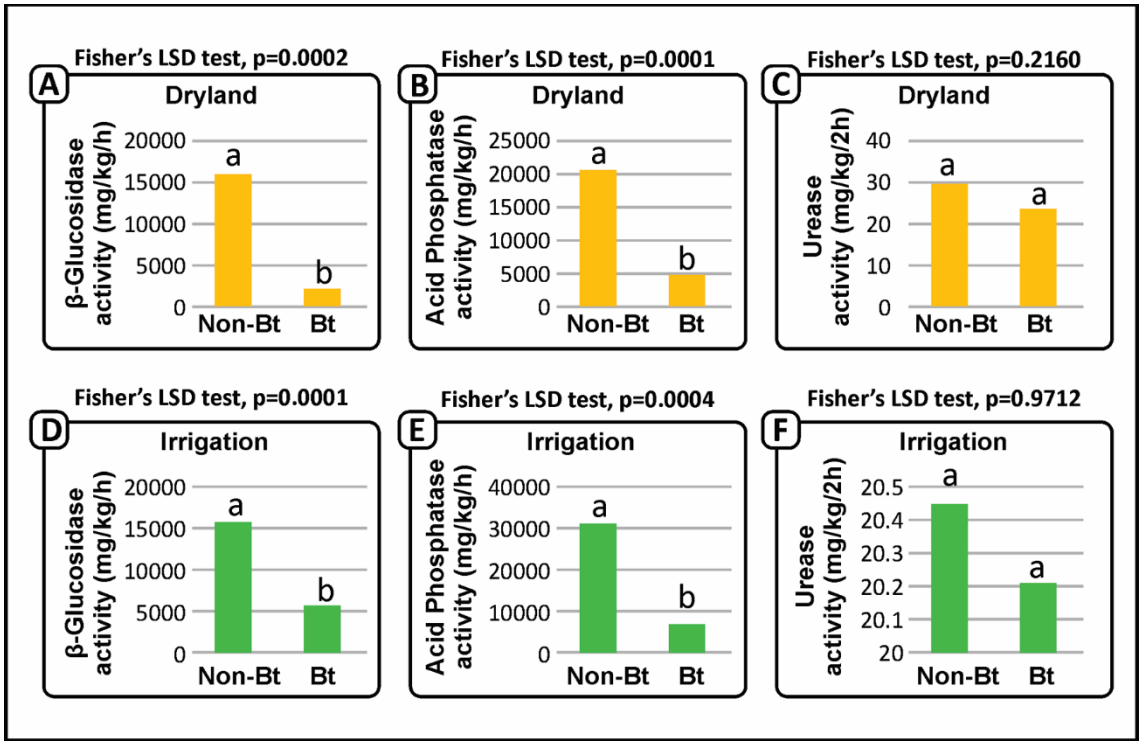

Figure 2.1: Activity of β-glucosidase (A, D), acid phosphatase (B, E) and urease (C, F) under dryland and irrigated conditions of Bt and non-Bt maize fields. The data are expressed as the means of two replicated. Different letters (a, b) indicates a significant difference at $p \leq 0.05$. Activity of β-glucosidase (A, D), acid phosphatase (B, E) and urease (C, F) under dryland and irrigated conditions of Bt and non-Bt maize fields. The data are expressed as the means of two replicates. Different letters (a, b) indicates a significant difference at $p \leq 0.05$.

2.3.3 Bacterial diversity and richness between Bt and non-Bt maize rhizosphere soil under DL and IL

The similarity based OTUs, species richness and diversity are shown in Figure 2.2 under DL and IL fields. A total of 306,979 and 238,594 OTUs were obtained from Bt and non-Bt maize fields under DL conditions respectively (Table 2.2), with number of sequences ranging from (33,850 to 68,201) and (25,790 to 51,605) at 3% distance, respectively. The Bt and non-Bt maize fields under IL conditions had a total of 326,952 and 216,489 OTUs, respectively (Table 2.2). The number of sequences ranged from (28,462 to 55,258) and (24,486 to 41,408) between Bt and non-Bt maize fields. The results indicate that Bt maize fields under DL and IL conditions had the highest number of species present, compared to non-Bt maize fields (Table 2.2).

Table 2.2: Similarity based OTUs and species richness estimates of the Bt and non-Bt maize dryland and irrigated fields.

Sample ID	Valid Reads	Cluster Distance (0.03)			
		OTU	ACE	Chao1	Shannon (H)
Dryland fields					
DLBt	2,066,107	306,979	304	306	20
DLNBt	1,740,647	238,594	307	318	16
Irrigated fields					
ILBt	2,119,423	326,952	312	313	21
ILNBt	1,578,565	216,489	311	311	20

All rarefaction curves approached a plateau, indicating that the number of sequences obtained was sufficient to describe the bacterial diversity within these soil fields (Table 2.2). Alpha diversity estimates shown in Figure 2.2, illustrated that the mean of the OTUs richness and Chao1 richness estimator of the non-Bt soils population under DL conditions were greater (Figures 2.2A and 2.2E), than DL non-Bt maize soils population. In contrast, under IL conditions, Bt maize soil populations had the higher richness (Figures 2.2A and 2.2E), while non-Bt maize soils had the lowest richness (Figures 2.2A and 2.2E) (Table 2.2). Furthermore, the mean of the evenness and Shannon and

Simpson indexes showed that DL Bt maize soils population exhibited the highest diversity (Figures 2.2B-2.2D), compared to non-Bt maize soils. While under irrigated conditions non-Bt maize exhibited the highest diversity, compared to IL non-Bt maize soils population (Figures 2.2B-2.2D). Overall, the OTUs (or species) are more evenly distributed in DL Bt maize soils (mean evenness value of 0.48) than in DL non-Bt maize soils (mean evenness value of 0.46) (Figure 2.2C). However, under IL conditions non-Bt maize soil showed the highest evenly distribution species (mean evenness value of 0.48), compared to Bt maize soils (mean evenness value of 0.45) (Figure 2.2C). Tukey HSD tests for differences in OTUs diversity measures between DLBt/DLNBt and ILBt/ILNBt maize soils populations indicated that the differences found were not significant ($p > 0.05$).

These results indicate that soils with a large number of species showed a degree of evenness (equitability) among species abundance. If compared to fields that displayed low species richness, indicating that many individuals belonging to the same species were detected.

2.3.4 Relationship between bacterial communities among DL and IL Bt and non-Bt maize rhizosphere soil

To obtain an overall view on the identified linkages between DL and IL Bt and non-Bt maize soil samples, Bray-Curtis distance's principal coordinates analysis (PCoA) plots of the OTUs distributions (at 97% 16S rRNA sequence similarity) based on unweighted (absence/present of taxa) and weighted (absence/present and relative abundance of taxa) are shown in Figure 2.2F and Figure 2.2G. Permutational analysis of variance (PERMANOVA) of unweighted (PERMANOVA, $R^2= 0.22$, $p < 0.001$) and weighted (PERMANOVA, $R^2= 0.48$, $p < 0.001$) Bray- Curtis distance matrices suggests that the differences between Bt and non-Bt maize soils of DL and IL conditions are not largely influenced by Bt maize (Figures 2.2F and 2.2G). Nevertheless, the PCoA plots of both weighted and unweighted Bray-Curtis distance similarity matrices suggest that there are some differences between the OTUs richness and abundance between certain Bt and non-Bt maize fields under dryland and irrigated conditions (Figures 2.2F and 2.2G). For example, the DLBt, DLNBt and ILBt soil samples were dispersed between each other, while ILNBt soil sample clustered separately together (weighted measures) (Figure 2.2G). These results suggest that some of the bacterial species in DLBt, DLNBt and ILBt field samples were similar across fields, compared to ILNBt soil samples.

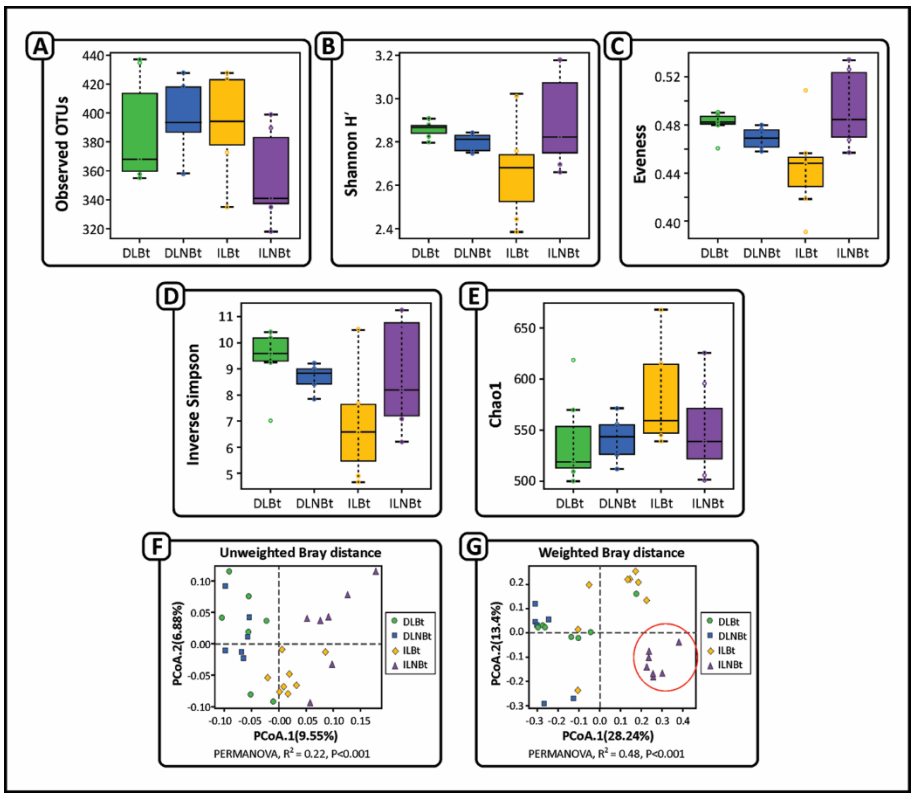

Figure 2.2: Similarity based OTUs and species richness estimates of the Bt and non-Bt maize dryland (DL) and irrigated (IL) fields. (A) Observed OTUs, (B) Shannon-Weiner index (H´), (C) Evenness, (D) Inverse Simpson and (E) Chao1 richness estimator. (F and G) Principal coordinate analyses (PCoA) of unweighted and weighted Bray-Curtis distance matrix showing microbial differences between Bt and non-Bt bacterial communities of dryland and irrigated fields. Relative abundance of OTUs obtained from clustering at 97% sequences similarity were used to compute PCoA. DLBt and DLNBt represent the dryland Bt and non-Bt maize samples, while ILBt and ILNBt represent the irrigated Bt and non-Bt maize samples.

2.3.5 Bacterial taxonomic community composition

2.3.5.1 Soil bacterial community composition between Bt and non-Bt maize rhizosphere soil under DL and IL cultivation

Dryland (DL) and irrigated (IL) Bt and non-Bt maize soils showed similarities in bacterial community composition at the phylum level with 36 bacterial phyla identified from both fields. Both fields of Bt maize soils comprises of 33 bacterial phyla respectively, while non-Bt maize soils under DL conditions represented 32 bacterial phyla and IL conditions 34 bacterial phyla. The Bt and non-Bt maize soil samples for both fields were predominated by members of the phyla Actinobacteria (14.4-37.0%), Proteobacteria (14.4-30.4%) and Acidobacteria (11.7-24.4%) (Figure 2.3). Furthermore, results indicated that Actinobacteria (Bt = 36.99% and non-Bt = 30.44%) was the dominant

phylum under DL fields. In contrast, Proteobacteria (30.35%) were predominant in soil under Bt maize conditions of IL, while non-Bt maize soil were dominated by Acidobacteria (24.37%) (Figure 2.3).

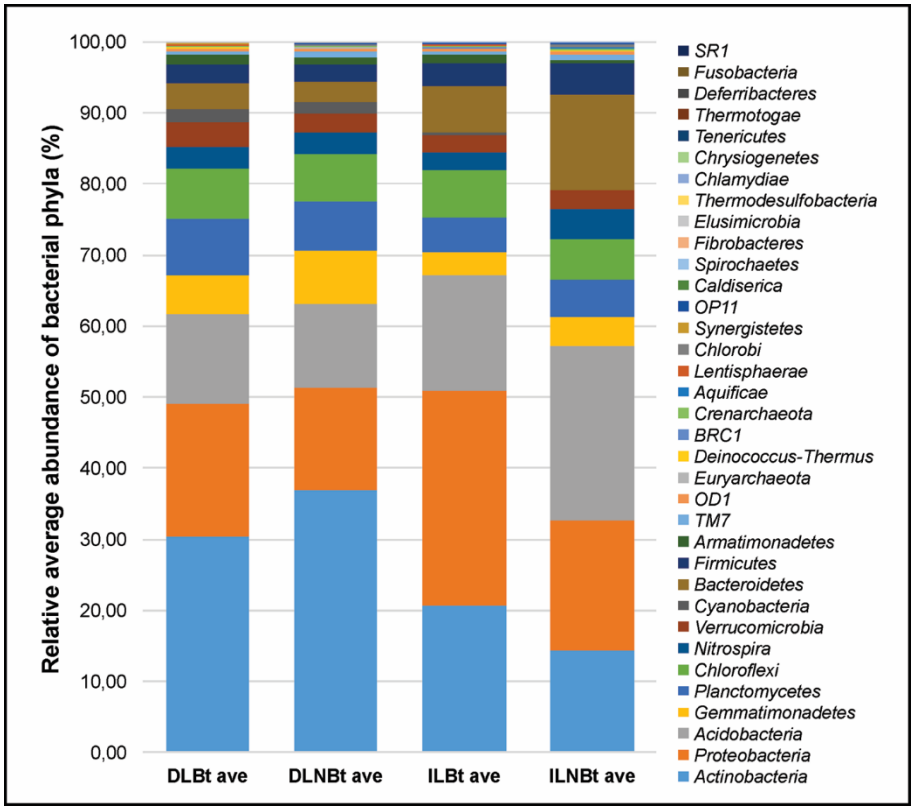

Figure 2.3: Relative average abundance of bacterial phyla present in dryland and irrigated fields of bacterial communities of Bt and non-Bt maize.

The Venn diagrams in Figure 2.4 illustrates the distribution of the soil bacterial communities between Bt and non-Bt maize soils under DL and IL conditions and the total shared richness. The number of species present in soils under Bt and non-Bt maize cultivation of DL were 303 and 297, respectively. Under IL condition, the number of species present in Bt maize soil is 310 and in non-Bt maize soil it is 305. Furthermore, results showed that the number of species shared between DL Bt and non-Bt maize soils was 285, whereas IL Bt and non-Bt maize soils shared 292 species between each other (Figure 2.4). Results also indicate that within Bt maize soils under DL and IL conditions *Arthrobacter*, *Gp, Rubrobacter* and *Sphingomonas* were the most dominant genera present in both fields (Figure 2.5). While, under non-Bt maize cultivation of DL and IL conditions *Gp* and *Rubrobacter* were the dominant genera. However, it was interesting to note that *Sphingomonas* and *Arthrobacter* were not

detected in non-Bt maize soils under DL and IL conditions, respectively. These results indicate that more than 90% of soil microorganisms found in Bt and non-Bt maize soils under DL and IL conditions were similar (Figure 2.4).

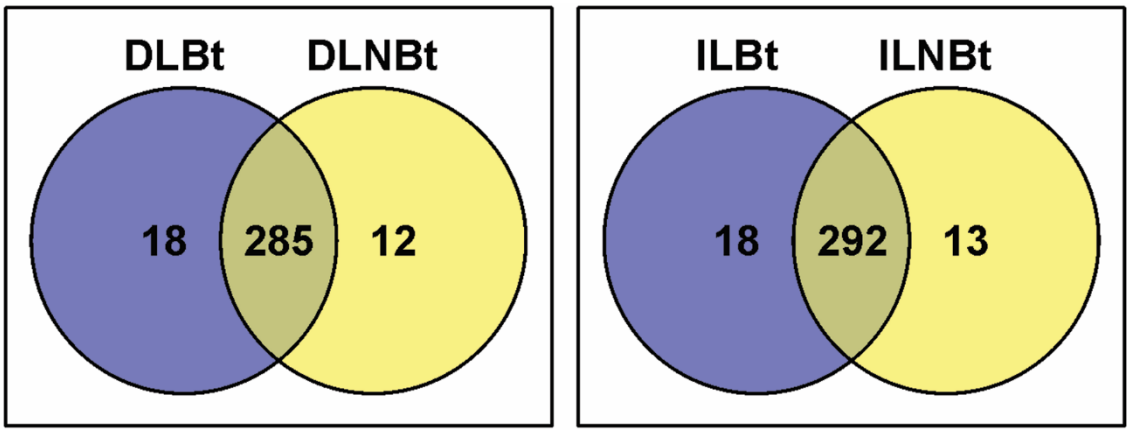

Figure 2.4: Venn diagrams signifying the number of unique and shared species between Bt and non-Bt maize DL and IL field soils at 3% distance level. DLBt and DLNBt represent the dryland Bt and non-Bt maize samples, while ILBt and ILNBt represent the irrigated Bt and non-Bt maize samples respectively.

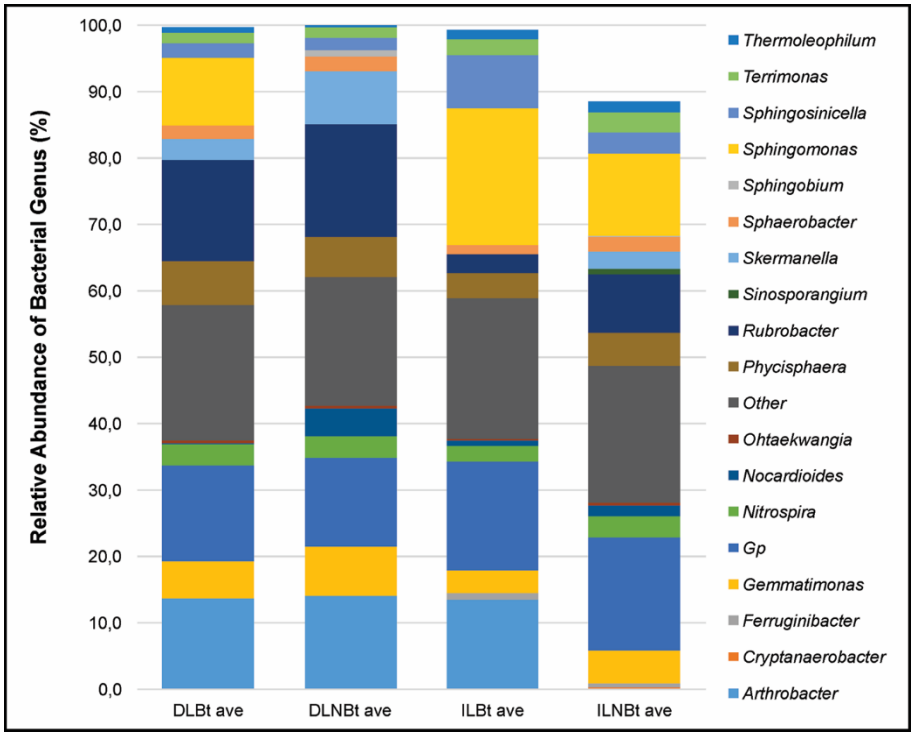

Figure 2.5: Relative abundance of predominant bacterial composition in the four treatments.

The heatmap plot depicted the relative percentage of each bacterial genus within each field (Figure 2.6). As shown in Figure 2.6, some soil genera in DLBt, DLNBt and ILBt maize fields overlapped, while ILNBt formed a separate cluster (Figures 2.2F and 2.2G). A similar trend was observed in the PCoA (Figures 2.2F and 2.2G). These fields gathered by decreasing order of similarity in soil genera. The relative abundance for each bacterial genus was depicted by colour intensity with the legend indicated in the figure on the right. In this study, a total of 24 genera and a group named "Other" (with OTU abundance percentage of less than 5%) were identified between DL and IL Bt and non-Bt maize field samples (Figure 2.5).

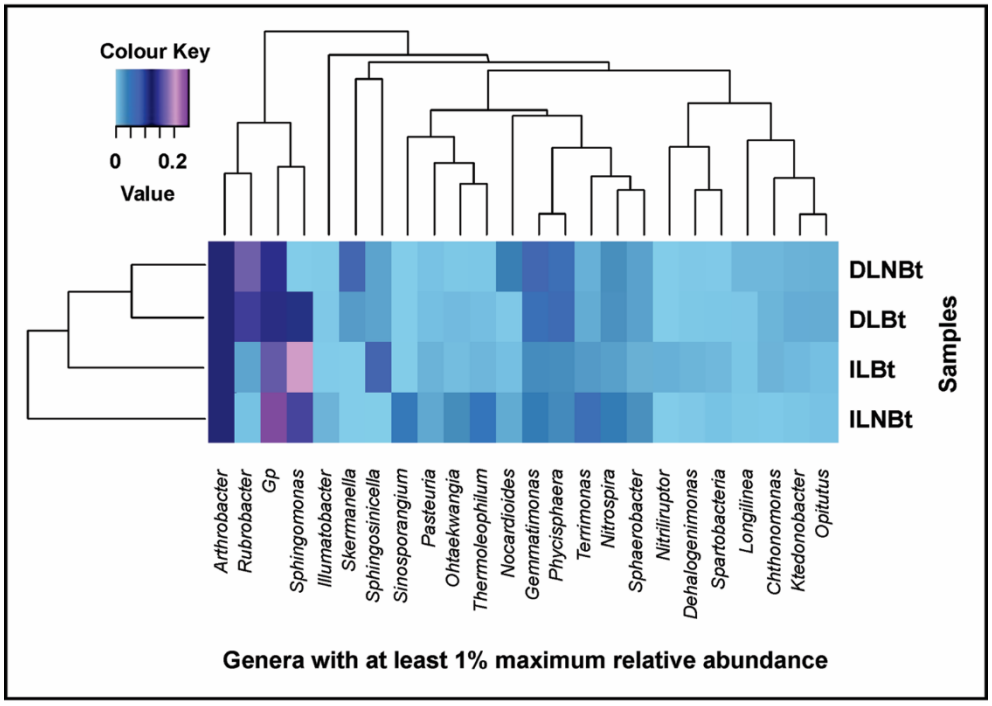

Figure 2.6: Effect of Bt maize on non-target soil organisms. Heat map of weighted Bray Curtis with hierarchal clustering of bacterial distribution of different communities from the dryland and irrigated Bt and non-Bt maize soil samples at the genus level. The relative abundance for each bacterial genus were depicted by colour intensity (clustering on the X-axis) with each field (Y-axis clustering). The higher values are represented by darker colours whereas lower ones are represented by lighter colours. DLBt and DLNBt represent the dryland Bt and non-Bt maize samples, while ILBt and ILNBt represent the irrigated Bt and non-Bt maize samples.

The four most abundant genera identified in DL Bt maize soil are, *Rubrobacter* (overall average 15.2%), *Gp* (14.4%), *Arthrobacter* (13.6%) and *Sphingomonas* (10.2%), while under DL non-Bt maize soil *Rubrobacter* (17.0%), *Arthrobacter* (13.98%), *Gp* (13.3%) and *Skermanella* (8.1%) were the top 4 genera. Under IL Bt maize field samples *Sphingomonas* (overall average 20.5%), *Gp* (16.4%), *Arthrobacter* (13.4%) and

Sphingosinicella (7.9%) were the four most abundant genera. Whereas *Gp* (17.0%), *Sphingomonas* (12.3%), *Rubrobacter* (8.8%) and *Gemmatimonas* (5.1%) were the top 4 genera under IL non-Bt maize field samples. However, there were some variances between DL and IL Bt and non-Bt maize soil fields in the relative abundance of these major genera. In DL and IL Bt maize fields' soil samples, the bacterial pairs *Sinosporangium*/*Sphingobium* as well as *Skermanella*/*Sphingobium* were not present, respectively. Furthermore, DL non-Bt maize field was the only soil where *Sphingomonas* was absent.

2.3.5.2 Correlation between environmental parameters and microbial community

The average values of the dominant soil chemical and enzymatic activities used in the RDA analysis are presented in Table 2.1 and Figure 2.1. Based on the results obtained, it is evident that pH (KCℓ) was the dominant chemical parameter in Bt maize field samples, while nitrate, organic carbon, phosphorus, nitrite and ammonium were the predominant parameters in non-Bt maize field samples under DL conditions (Figure 2.7). Furthermore, results showed that non-Bt maize field samples were positively associated with acid phosphatase, β-glucosidase and urease activities. However, a negative association was observed between Bt maize field samples and enzyme activities (Figure 2.1 and Figure 2.7). Results also indicated an association between chemical parameters and enzymatic activities. Ammonium was positively associated with urease and acid phosphatase (Figure 2.7), while β-glucosidase was positively associated with organic carbon, phosphorus, nitrate and nitrite. A negative association was also apparent between pH (KCℓ) and all the enzymatic activities assayed (Figure 2.7). Relative abundance in Bt maize soils of some genera, *Ohtaekwangia* and *Nitrospira* were correlated with pH (KCℓ), while the relative abundance in non-Bt maize soils of some genera, *Skermanella*, *Arthrobacter* and *Sphaerobacter* were correlated with nitrate, phosphorus, carbon and nitrite (Table 2.1 and Figure 2.7). Some other non-Bt maize genera such as *Sphingobium* and *Nocardioides* were correlated with ammonium (Figure 2.7). The relative abundance of most genera in non-Bt maize fields were strongly correlated to acid phosphatase, β-glucosidase and urease enzyme activities (Figure 2.7). Acid phosphatase, β-glucosidase and urease enzyme activities were relatively high and showed a positive association with the relative abundance of most genera in non-Bt maize fields as compared to the Bt maize field (Figure 2.1 and Figure 2.7).

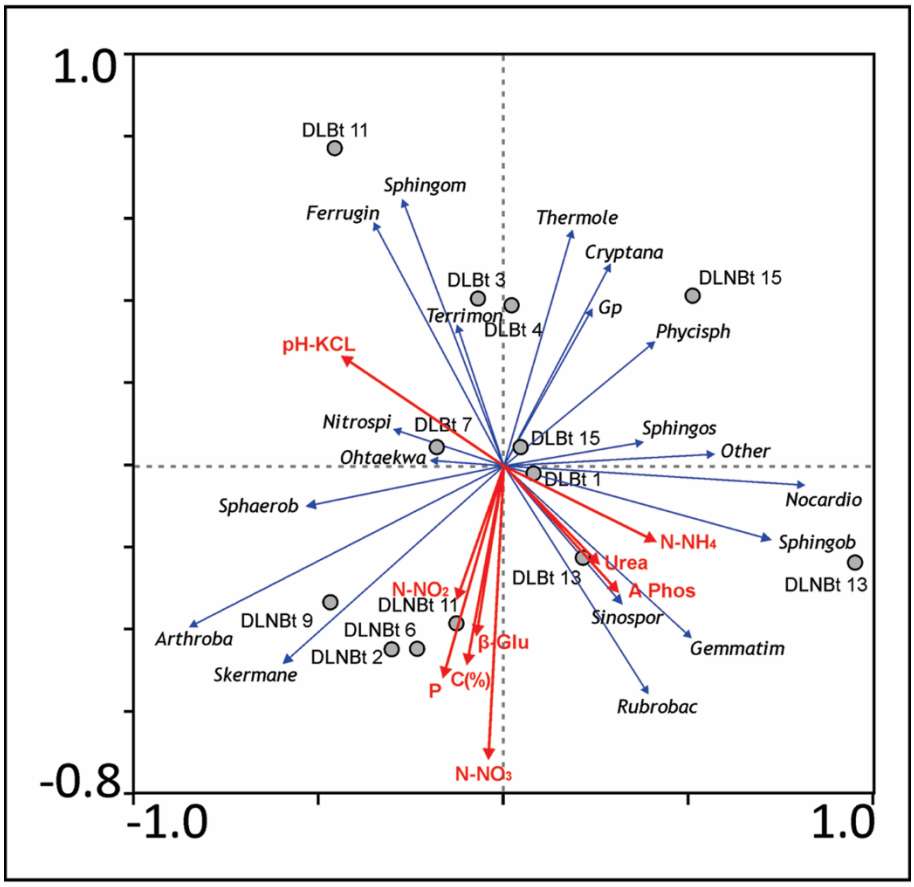

Figure 2.7: RDA triplot of dominant genera as affected by selected environmental variables. Genera are indicated by blue vectors and chemical and biochemical variables are represented by red vectors under DL conditions.

Results obtained, indicated that ammonium, pH (KCℓ), phosphorus and nitrite had a strong positive association with Bt maize field samples, whereas organic carbon and nitrate had a strong positive association with non-Bt maize field samples under IL conditions (Figure 2.8). Furthermore, results showed that non-Bt maize field samples were positively associated with acid phosphatase, β-glucosidase and urease activities, while Bt maize field samples had a strong negative association with enzyme activities (Figure 2.1 and Figure 2.8). In addition, acid phosphatase, β-glucosidase and urease activities were strongly associated with organic carbon in the non-Bt maize field. These enzymes were also positively associated with nitrate content of the IL non-Bt maize field, but to a lesser extent. Ammonium and pH (KCℓ) were moderately associated with urease activity (Figure 2.8), while phosphorus and nitrite concentrations showed a negative association with the enzyme activities (Figure 2.8).

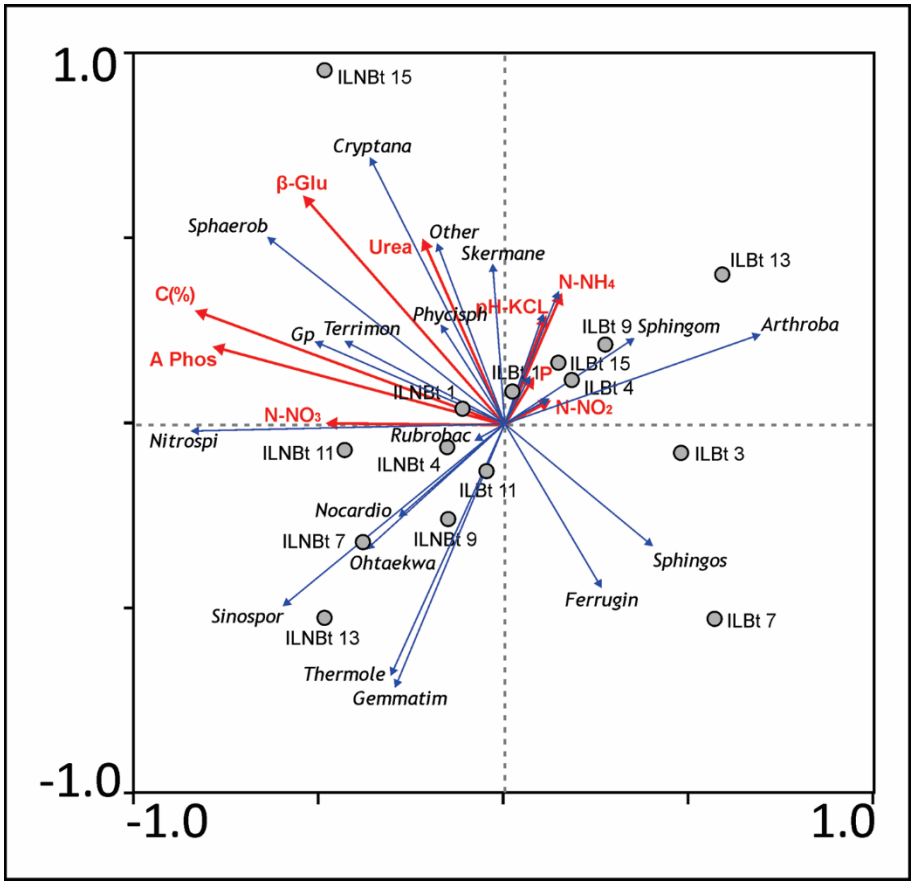

Figure 2.8: RDA triplot of dominant genera as affected by selected environmental variables. Genera are indicated by blue vectors and chemical and biochemical variables are represented by red vectors under IL conditions.

Relative abundance in non-Bt maize field samples of *Skermanella* was associated with pH (KCℓ) and ammonium concentration, while *Gp*, *Terrimonas* and *Sphaerobacter* were strongly associated with organic carbon (Figure 2.8). *Nitrospira*, *Rubrobacter* and *Nocardioides* were strongly associated with nitrate concentrations (Figure 2.5). The relative abundance in IL Bt maize field of the soil microbial genera, *Sphingomonas* and *Arthrobacter* was positively associated with nitrite, phosphorus, ammonium and pH (KCℓ), but to a lesser extent. *Sphingosinicella* and *Ferruginibacter* were negatively associated with all six chemical parameters in the IL Bt maize field. The relative abundance of most genera in non-Bt maize field was strongly correlated to acid phosphatase, β-glucosidase and urease enzyme activities. A similar trend was observed in non-Bt maize field samples of DL (Figure 2.7). Results indicated that both non-Bt maize field under DL and IL conditions were positively associated with enzyme activities, while Bt maize fields were negatively associated with these enzymes.

2.4 Discussion

Soil microbial communities perform multifarious processes, which have major agricultural and ecological importance. In agriculture, it is pertinent to maintain healthy soils by regulating the physico-chemical and biochemical cycles along with the soil microbial communities present in soil (Ondreičková *et al*., 2014). Unfortunately, planting of Bt crops, could affect soil processes, due to the genetic modification and perhaps the Bt toxin.

Soil chemical composition does not only contribute to plant nutrition but also plays a role in microbial activities and overall soil fertility (Meliani *et al*., 2012). For the represented study, nitrate, phosphorus and organic carbon levels were significantly higher under non-Bt maize soil, except for phosphorus which was higher under IL Bt maize soil. This is similar to what Griffiths *et al*. (2007a) and Liu *et al*. (2010) previously observed for GM and non-GM maize soils. As explained by Powell *et al*. (2009), differences in chemical properties between Bt and non-Bt maize soils under DL and IL might be a result of the difference in nutrient utilisation by soil bacteria. In addition, agricultural practices and application of fertilisers could also contribute to the differences observed in chemical properties of these conventional fields (Esperschütz *et al*., 2007). These findings are further substantiated by the redundancy analyses (RDA) which indicate that organic carbon and nitrate were strongly associated with non-Bt maize field samples under DL and IL conditions (Figures 2.7 and 2.8). However, these correlations were not statistically significant. High organic carbon content favours water-holding capacity; root penetration and adsorption of microorganisms and nutrients (Meena *et al*., 2013). These factors in turn contribute to conditions that further favour microbial activity and nutrient cycling, making the aim of achieving sustainable ecosystems more viable (Meena *et al*., 2013).

Many studies have successfully matched presence and absence of soil enzymes to soil ecological processes, hence, they are accepted as indicators of soil microbial activities and fertility (Baćmaga *et al*., 2015; Cardoso *et al*., 2013; Luna *et al*., 2016). In our study, the activities of β-glucosidase and acid phosphatase in soil were significantly higher under non-Bt maize soil samples compared to Bt maize soil. The significant increase of these soil enzymes in non-Bt maize soils could be attributed to the positive association with organic carbon (Figures 2.7 and 2.8), which also plays an important role in the soil nutrient cycle (Meena *et al*., 2013). Enzyme activities of soils are usually correlated with

their organic carbon and available nitrate contents (Taylor *et al*., 2002). Similar results were obtained by Dick and Tabatabai (1984) and Frankenberger and Dick (1983), who also found organic carbon to be positively related to enzyme activities. Higher levels of organic carbon stimulate microbial activity, and therefore enzyme synthesis.

Furthermore, the lower soil enzyme activity shown in soils of Bt maize under DL and IL conditions indicated that some of the bacterial species were perhaps inhibited and did not participate in the metabolic activities of the soil (Beura and Rakshit, 2013). In previous studies involving Bt crops, there was no consistent trend between quantity levels of soil enzymes of GM and non-GM plants, nevertheless, there were differences among seasons and crop varieties (Icoz *et al*., 2008; Shen *et al*., 2006). These results are not consistent with our findings.

Overall, alpha diversity was higher under DL Bt and IL non-Bt maize soils. However, soil bacterial richness was greater under DL non-Bt and IL Bt maize soils (Figures 2.2A and 2.2E). The higher soil bacterial richness in DL non-Bt maize soil samples is not unexpected due to the high contents of essential nutrients in this soil. The observation of no significant differences between the DLBt/DLNBt and ILBt/ILNBt maize OTU richness of Bt and non-Bt maize soil populations suggest that the Bt maize (including cultivar type) do not have any effect on the soil species richness in these fields populations. Analysis of the DL and IL Bt and non-Bt maize soil microbiota indicated dominance by the members of the bacterial phyla Actinobacteria, Proteobacteria and Acidobacteria (Figure 2.3). With Actinobacteria being the most dominant phyla present in both Bt and non-Bt maize soil under DL conditions, Proteobacteria and Acidobacteria were the major phyla present in Bt and non-Bt maize soil under IL, respectively. All of these phyla contain taxa commonly found within the soil community (Philippot *et al*., 2013). These results were consistent with those of Barriuso *et al*. (2012) and Newman *et al*. (2016), who also found the above mentioned bacterial phylum's to be the predominant groups in the rhizosphere of Bt maize. The reason why the phyla composition of the soil bacterial community differed under IL conditions could be due to the water supply in the fields and type of maize variety used. Similar results as these were obtained by Baumgarte and Tebbe (2005), Fang *et al*. (2005) and Barriuso *et al*. (2012) who also found plant cultivars, soil structure and environmental factors (plant growth phase) to have an impact on soil bacterial microbial community structures. In IL Bt maize soil, the genus *Sphingomonas* belonging to the phylum Proteobacteria was

found to be dominant. It is known that species in this particular genus can respond to labile carbon sources and are considered r-selected, fast growing, and opportunistic bacteria (Barriuso *et al*., 2012). The detection of *Sphingomonas*, is in agreement with reports from Bumunang *et al*. (2015) and Dohrmann *et al*. (2013) who also found this genus to be the most commonly found in maize soil. Therefore, it had been suggested that this species can be used as a key indicator in monitoring GM effects on soil maize communities (Bumunang *et al*., 2015). However, in Bt and non-Bt maize fields under DL conditions, the genus *Rubrobacter* was the most enriched genus, originating from the phylum Actinobacteria, one of the major groups, which are mostly gram-positive microbes and with an important component of soil communities. Although unexplained, *Sphingomonas* and *Arthrobacter* were not detected in DL and IL non-Bt maize soils, respectively. The results of this study showed subtle variations in soil bacterial community composition between DL and IL Bt and non-Bt maize soils. The largest difference in relative abundance was observed for *Sphingomonas* and *Arthrobacter*. Based on our results, it was concluded that variances in soil microbial communities and differences observed in acid phosphatase and β-glucosidase activities were probably a result of farming practices and environmental factors. This is in accordance to a recent study in our laboratory where variations in diversity and abundance of endophytes associated with the phyllosphere of Bt and non-Bt maize could not be conclusively linked to Bt genetic modification of the maize plant (Mashiane *et al*., 2017).

In conclusion, it is recommended that future studies should expand number of samples collected to include pre-sowing, pre-harvest and post-harvest data as such approach will give more insights into the potential impacts of the genetic modification of the Bt maize. Nevertheless, the findings of our study have provided an insightful snapshot of the ecological guild and enzymatic activities of rhizosphere soil microbial communities in soil under Bt and non-Bt maize cultivations. However, further investigations are needed to elucidate whether these differences were transient, seasonal or over a period of time.

CHAPTER 3

Genetic modification or soil amendment: What factors drive the rhizobacterial communities of Bt maize plant?

3.1 Introduction

The rhizosphere is the biologically active zone of soil where plant roots and microorganisms interact. These interactions involve root exudates, which shape the structure and enhance the activity of microbial communities, as well as nutrients released by microorganisms. The variations in root exudate composition and abundance may alter soil microbial biodiversity and activities, hence have different effects on harmful or beneficial microbes (Icoz and Stotzky, 2008a). Microorganisms are important players in the mineralisation and recycling of key plant nutrients such as nitrogen, phosphorus and carbon (Adeleke *et al*., 2010; Adeleke *et al*., 2012; Adeleke *et al*., 2015; Adeleke, 2014; Adeleke *et al*., 2017). These plant nutrients are essential to analyse the plant species, genotype and fertiliser regime that have an impact on microbial communities in agricultural soils (Appuhn and Joergensen, 2006).

Although healthy rhizosphere soils are expected to support plant growth and health, advancement in biotechnological applications relating to plant development may alter this function. Examples are the negative impacts of genetic modification of maize on non-target rhizosphere microorganisms. The cultivation of GM Bt maize may alter these soil bacterial communities, hence endangering crop production and environmental sustainability. Transgenic crops have been genetically engineered to express the *cry1Ab* gene (Bt toxins) from *Bacillus thuringiensis* (Bt). It is inevitable, that Bt toxin will be introduced to soil in root exudates throughout the growth of the transgenic plant (Icoz and Stotzky, 2008b), through pollen deposition during tasseling, e.g., maize (Saxena and Stotzky, 2000; Zwahlen *et al*., 2003), and by incorporation of plant residues after harvest (Zwahlen *et al*., 2003; Stotzky, 2005). Consequently, posing a potential risk to non-target organisms, such as beneficial soil bacteria and fungi. These non-target organisms play a fundamental role in crop residue degradation and in biogeochemical cycles. Several studies have shown that soil microbes represent important key non-target organisms able to highlight unforeseen collateral effects of transgenic crops on natural and agricultural ecosystems (Han *et al*., 2013; Velasco *et al*., 2013; Guan *et al*.,

2016). These microorganisms are also sometimes used as organic –and bio-fertilisers in soil.

In agro-ecosystems, AM fungi are significant representatives of the soil microbiota. They colonise the roots of vast majority of plants, including most crop plants. By forming an extended, intricate hyphal network, AM fungi can efficiently absorb mineral nutrients from the soil and deliver them to their host plants in exchange for carbohydrates (Oehl *et al*., 2006; Garg and Chandel, 2010). One of the most common heavily mycorrhizal-dependent plant species is maize (Tawaraya, 2003). The importance of AM fungi in maize growth is expected to increase with the rise in frequency of extreme water events (droughts and floods) (Rillig *et al*., 2003). Arbuscular mycorrhizal fungi may be sensitive to changes in the physiology of the host plant because they rely on a plant host for nutrition and reproduction (Cheeke *et al*., 2012). Furthermore, AM fungi have been shown to improve the growth, health, nutrient uptake, flowering and drought tolerance of numerous plant species (Sadhana, 2014; Young *et al*., 2015).

Similarly, organic fertilisers such as vermicomposts, when applied to the soil, enhance soil microbial activities, which improve crop growth. Vermicomposting has become a popular method of promoting biological fertility by enhancing the growth of beneficial microbes (Pathma and Sakthivel, 2012), and such microbes may in turn enhance plant growth directly by production of plant growth-regulating hormones and enzymes (Oo *et al*., 2015). These beneficial microbes are also responsible for mineral cycling in the soil (Pathma and Sakthivel, 2012). Thus, the impact of these mineral cycles on plant health and subsequent crop production is important. These mineral cycles are to determine whether or not these functions are impaired as a result of the Cry proteins (genetic modification) released into the soil via root exudates (Zwahlen *et al*., 2003).

Previous studies regarding the effects of Bt maize cultivation on soil and rhizospheric bacterial communities, were either based on fingerprinting methods such as PCR-DGGE and 16S rRNA gene library construction (Barriuso *et al*., 2012; Cotta *et al*., 2013; Ondreičková *et al*., 2014; Bumunang *et al*., 2015). These methods are promising; however, they are complex, costly, time-consuming, and only detect certain dominant soil microbial groups. Recently, NGS technologies, such as Illumina MiSeq sequencing has dramatically reduced the cost and time of sequencing and enabled the generation of several thousand sequences per sample. Furthermore, the large number of 16S rRNA genes analysed per sample allow for a more accurate representation of the

relative abundance of the bacterial community present (Huse *et al*., 2008) and provides an opportunity for achieving a high-throughput and deeper insight into soil bacterial communities. Based on literature research, only a few reports associated with Illumina MiSeq on Bt maize bacterial community studies (Mashiane *et al*., 2017; van Wyk *et al*., 2017) are known.

Therefore, the aim of this aspect of the study was to investigate potential impacts of genetic modification of Bt maize and soil amendments on rhizobacteria using Illumina MiSeq 16S rRNA gene profiling.

3.2 Materials and methods

3.2.1 Soil collection and treatment

Soil used in the pot trial was collected from an uncultivated field of the Agricultural Research Council-Grain Crops (ARC-GC) Potchefstroom, South Africa. A portion of the collected soil sample was taken to the laboratory for physico-chemical analysis and the remaining portion was sterilised and prepared for the use as a growth medium in the greenhouse experiment. Soil was sterilised twice at 121°C for 1 hour using an autoclave (Hirayama HV-85, Japan). The physiochemical properties of the soil before the start of the experiment are shown in Table 3.1.

Table 3.1: Soil and vermicompost chemical properties used in the greenhouse experiment.

Parameters	pH (KCℓ)	NO_3 (mg/kg)	NO_4 (mg/kg)	(%) C	K (mg/kg)	P (mg/kg)	Sand (%)	Silt (%)	Clay (%)
Soil	5.62	24.70	3.45	0.98	203	17	56	13	31
Vermicompost	6.18	4040	50.00	27.35	4600	1435	-	-	-

3.2.2 Biological treatments and experimental design

Four seeds each of both Bt and non-Bt cultivars - DKC78-15B and DKC80-10 (Monsanto Seed [Pty] Ltd., Greytown, South Africa) were sown in a pot at a depth of 2-3 cm, respectively. Ten days after emergence, the two less viable seedlings were thinned from each pot. Furthermore, plants were fertilised weekly with 200 ml standard Hoagland solution (Hershey, 1994). These maize cultivars reached a physiological maturity stage in circa 115 days. Therefore, the experiment was conducted for 120 days.

The Mycoroot™ SuperGo product was used as bio-fertiliser. It consists of five different species of arbuscular mycorrhizal isolates, *Rhizophagus clarus*, *Gigaspora gigantea*, *Funneliformis mosseae*, *Claroideoglomus etunicatum* and *Paraglomus occulum*. This bio-fertiliser was obtained from Mycoroot™ (Pty) Ltd, South Africa.

Vermicompost used in this study was produced and supplied by organic vegetable waste and red worms (SoilSouls, South Africa). The chemical properties of the vermicompost used are shown in Table 3.1.

A pot trial experiment was carried out at the Agricultural Research Council-Grain Crops (ARC-GC) Potchefstroom, South Africa (26°44'9" S, 27°4'27" E). The experiment was a randomised complete block design and used to position 54 pots. Each of the pots used in the greenhouse experiment had a dimension of a 30 cm height and 25 cm diameter. The 54 pots consisted of six treatments of three replicates each at three sampling times (6x3x3). Treatment combinations consisted of:

1. Bt maize seeds in 10 kg soil (T1)
2. Bt maize seeds in 10 kg soil incorporated with 30 g of mycoroot (T2)
3. Bt maize seeds in 5 kg soil incorporated with 5 kg of vermicompost and 30 g of mycoroot (T3)
4. non-Bt maize seeds in 10 kg soil incorporated with 30 g of mycoroot (T4)
5. non-Bt maize seeds in 5 kg soil incorporated with 5 kg of vermicompost and 30 g of mycoroot (T5)
6. non-Bt maize seeds in 10 kg soil (T6)

Maize plants were grown for 120 days in the greenhouse under controlled conditions at 25°C during the day and 20°C during the night, with a 16 h photoperiod. Pots were watered to 75% water-holding capacity twice a week.

3.2.3 Soil sampling

Rhizosphere soil samples were collected at 60 (active growing stage), 90 (tasseling) and 120 (flowering) days after planting (DAP) of Bt and non-Bt maize treatments. A total of 54 soil samples were collected from both Bt maize treatments (27) and non-Bt maize treatments (27). Samples were immediately transported on ice to the laboratory and stored at -80 °C prior to analyses.

3.2.4 DNA extraction

Community DNA was extracted from 54 rhizospheric soil samples using the Power Soil Extraction Kit (MO BIO Laboratories, CA), with the protocol supplied by the manufacturers. The integrity of extracted DNA was verified by agarose gel electrophoresis, DNA was quantified using a Qubit 2.0 fluorimeter (Invitrogen, California, USA).

3.2.5 High-throughput sequencing and analyses of rhizospheric soil bacteria

3.2.5.1 Preparation of partial 16S rRNA gene library

Library preparation was carried out as described in the Illumina MiSeq 16S library preparation guide (Illumina, 2016). The primers for the 16S V3-V4 region (~460 bp) were 341F (5´-CCTACGGGNGGCWGCAG-3´) and 805R (5´-GACTACHVGGGTATCTAATCC- 3´) (Herlemann *et al*., 2011). Forward and reverse primers contained Illumina forward and reverse overhangs, respectively (Illumina Inc., California, USA). All PCR amplifications were conducted in 25 µl volumes containing; 12.5 µl of 2x KAPA HiFi Hot Start Ready Mix (KAPA Biosystems, Massachusetts, USA), 5 µM (volume) of each primer, 12.5 ng template DNA and nuclease-free water to a final volume of 25 µl. The thermal cycling parameters included initial denaturation at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s, with a final extension of 72°C for 5 min. The PCR amplicons were purified and indexed using the Nextera XT primers (Illumina Inc., CA, USA). Following indexing PCR, amplicon library was quantified, normalised and pooled prior to loading on the MiSeq flow cell for 2 × 300 paired-end sequencing.

3.2.5.2 Sequence processing, operational taxonomic units (OTUs) clustering and diversity analyses

Following sequencing, sequence reads were de-multiplexed using the on-system MiSeq reporter software. The quality of the reads was initially checked using the FastQC software (v 0.11.5, Babraham Bioinformatics, United Kingdom) prior to assembling forward and reverse reads using PANDAseq (Masella *et al*., 2012). Assembled reads were then clustered into OTUs using the “pick_open.reference_otus.py” script in QIIME (Caporaso *et al*., 2010b, Mashiane *et al*., 2017) and aligning it against the SILVA rRNA database (release 128) (Quast *et al*., 2012; Mashiane *et al*., 2017) using usearch61 (Edgar, 2010) and PyNAST aligner (Caporaso *et al*., 2010a). The OTU table generated from the clustering step was first rarefied prior to summarising the taxa, and computing

alpha and beta diversity in R software (R Core Team, 2013). The R packages; vegan, ape, labdsv and ggplot2 were used for statistical analyses and generation of plots. Alpha diversity results were statistically evaluated by Fisher's least significant difference (LSD) procedure (Statistical Graphics Corporation, USA) at the 95% confidence level.

3.3 Results

3.3.1 Diversity of bacterial OTUs between Bt and non-Bt maize

Following quality trimming of initial sequence reads, a total of 2,151,289 high-quality reads were obtained and assigned to OTUs. After rarefaction at a depth of 21,964 sequences per sample, a total of 211,994 OTUs were obtained in all Bt and non-Bt maize treatments (T1-T6). The number of OTUs observed in each Bt and non-Bt maize rhizosphere soil sample over time is presented in Table 3.2. The overall highest number of OTUs was observed in Bt maize treatments (T1-T3), whereas the least number of OTUs was observed in non-Bt maize treatments (T4-T6). At 60 and 120 DAP, the highest number of OTUs were mostly observed in Bt maize treatments, while at 90 DAP the highest number of OTUs were mostly observed in non-Bt maize treatments (Table 3.2). Furthermore, significantly ($p < 0.05$) higher numbers of OTUs were observed in T3 and T5 at 60, 90 and 120 DAP (Table 3.2). Significantly, ($p < 0.05$) lower numbers of OTUs were observed in T2 and T4 at 60 and 90 DAP, and in T6 at 120 DAP. However, there were no significant differences between Bt and non-Bt maize samples (LSD, $p > 0.05$). The rarefaction curves (Supplementary Figure 3.S1) for all samples approached a saturation plateau, which indicates that the current analysis had adequate depth to capture most bacterial diversity information.

Alpha diversity indices showed that non-Bt maize treatments (T4-T6) had a greater overall Shannon index compared to Bt maize treatments (T1-T3). There were no Simpson index differences between Bt and non-Bt maize treatments (Table 3.2). The Chao1 richness estimator indicated that non-Bt maize treatments (T4-T6) had a higher true OTU richness compared to Bt maize treatments (T1-T3) over time (Table 3.2). Fisher's LSD showed no significant differences between Bt and non-Bt maize bacterial community structures (LSD, $p > 0.05$). There were, however, significant differences (LSD, $p < 0.05$) between all treatments over time. Ultimately, the genetic modification of maize had no obvious effect on rhizosphere soil bacterial community structure of maize.

Table 3.2: Diversity indices of bacterial OTUs in different treatments of Bt and non-Bt maize soils.

Treatment	Diversity indices			
	Observed OTUs	Chao1	Shannon index (H`)	Simpson index (D)
D60T1	4151^{ab}	8836.91^{bc}	9.78^{b}	1.00^{a}
D60T2	3242^{b}	6629.18^{c}	9.16^{c}	0.99^{ab}
D60T3	4573^{a}	9331.27^{a}	10.08^{ab}	1.00^{a}
D60T4	4008^{b}	9130.42^{ab}	9.62^{bc}	1.00^{a}
D60T5	4490^{a}	8889.53^{b}	10.16^{a}	1.00^{a}
D60T6	3900^{b}	8600.65^{bc}	9.48^{ab}	0.99^{ab}
D90T1	3130^{ab}	6664.01^{bc}	9.20^{b}	0.99^{ab}
D90T2	2778^{b}	5576.93^{b}	8.88^{c}	0.99^{ab}
D90T3	4297^{a}	9454.00^{ab}	9.94^{ab}	0.99^{ab}
D90T4	2802^{b}	5268.42^{ab}	8.99^{bc}	0.99^{ab}
D90T5	4447^{a}	9674.43^{a}	10.23^{a}	1.00^{a}
D90T6	3622^{ab}	7352.71^{b}	9.48^{ab}	0.99^{b}
D120T1	4226^{ab}	8460.23^{ab}	10.13^{ab}	1.00^{a}
D120T2	3402^{b}	6740.52^{b}	9.36^{b}	0.99^{ab}
D120T3	4543^{a}	9933.96^{a}	10.18^{ab}	1.00^{a}
D120T4	2857^{bc}	5695.11^{bc}	9.13^{bc}	0.99^{ab}
D120T5	4413^{a}	8552.66^{ab}	10.27^{a}	1.00^{a}
D120T6	1806^{c}	2683.38^{c}	7.66^{c}	0.98^{b}

$^{\#}$Bt and non-Bt maize treatments with different combinations of superscript alphabetic letters in the same column indicate significant difference between each other.

The weighted (absence/presence and relative abundance of taxa) Bray-Curtis distance's principal coordinate analysis (PCoA) plot of the OTUs distributions (at 97% 16S rRNA sequence similarity) is shown in Figure 3.1. The soil bacterial community of T3 and T5 were distinctly separated from the controls (T1 and T6) and AM fungi (T2 and T4) treatments along the first component (PCoA2).

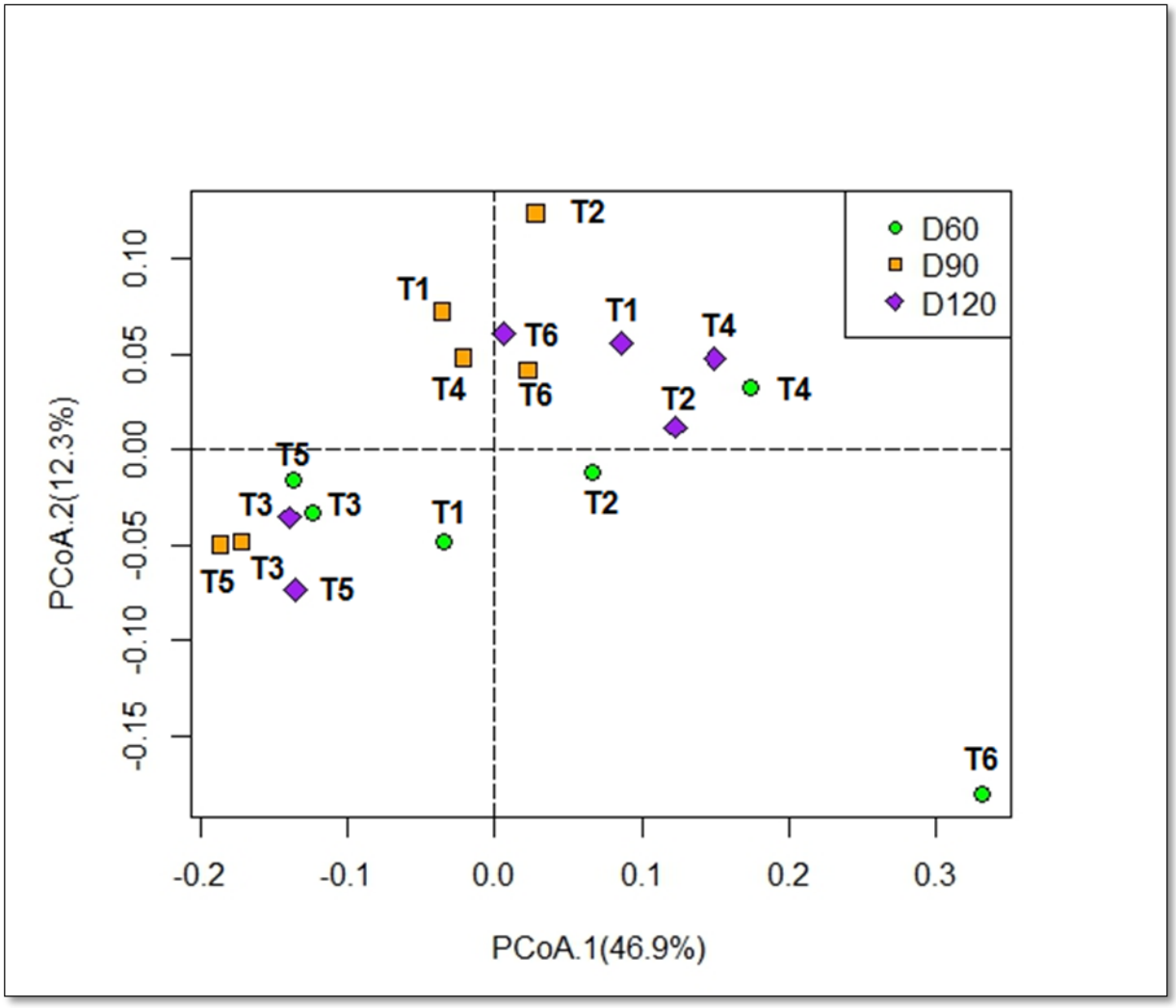

Figure 3.1: Principal coordinate analysis based on the distance matrix calculated using the weighted UniFrac algorithm showing bacterial differences between Bt and non-Bt maize plants grown in AM fungi and vermicompost amended soils. D60, D90 and D120 represent the different days after planting. Different treatments are represented by T1 to T6.

Furthermore, soil bacterial communities of T2 and T4 were separated from T1 and T6. However, at 90 DAP the soil bacterial community of treatments T2 and T4 were closely related to the control bacterial community (Figure 3.1). Overall results demonstrated that growth stage and co-application of AM fungi and vermicompost had an effect on both Bt and non-Bt rhizosphere soil microbiome.

3.3.2 Taxonomic diversity of OTUs associated with Bt and non-Bt maize cultivated in AM fungi and vermicompost

The total OTUs observed in all Bt and non-Bt maize treatments were taxonomically assigned into phylum, class, order, family, and genera levels using QIIME. A total of 29 different bacterial phyla were detected and a group named Unassigned (number of OTUs unclassifiable at phylum level) (Figure 3.2). Based on relative abundance, Proteobacteria (37.32%) was the most abundant bacterial phylum in all Bt and non-Bt maize treatments. Verrucomicrobia (11.22%) and Firmicutes (10.82%) had higher relative abundances in comparison to other phyla (Figure 3.2). Proteobacteria was more dominant in non-Bt maize treatments at 60 and 90 DAP than in Bt maize treatments at 120 DAP. A similar trend was observed for all the other phyla (Figure 3.2). A prominent trend observed in the phyla was the variations in proportions between different treatments, over time.

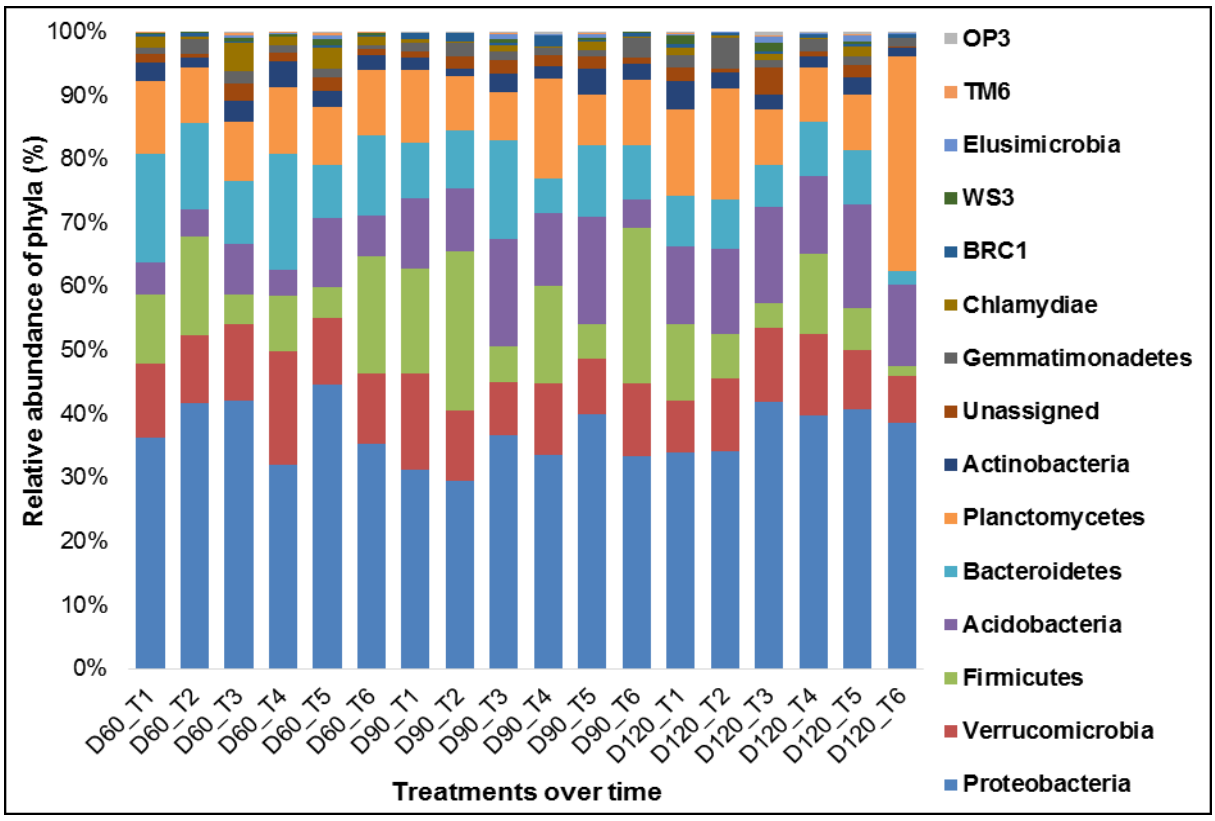

Figure 3.2: Relative abundance of the fifteen dominant bacteria in Bt and non-Bt maize soils at phyla levels.

A total of 219 genera was identified across all Bt and non-Bt maize treatments and the "Unassigned" group (Figure 3.3). However, only 43 genera constituted 1% abundance in one maize (Bt and non-Bt) sample (Figure 3.3). Of these genera, *Bacillus*, *Fluviicola*, *Lysobacter*, *Methylibium*, *Chitinophaga* and *Aliayclobacillus* were the most abundant in both Bt and non-Bt maize soil treatments (Figure 3.3). There were differences between Bt and non-Bt maize treatments in the relative abundance of these major genera. The abundance of *Bacillus* was lower in Bt maize treatments compared to non-Bt maize treatments at 90 DAP. While at 60 and 120 DAP, abundance of *Bacillus* was lower in non-Bt maize compared to Bt maize treatments. *Bacillus* was more abundant in Bt and non-Bt maize soils grown in control (T1 and T6) and AM fungi (T2 and T4) treatments at 60 and 90 DAP (Figure 3.3). Furthermore, *Bacillus* was less abundant in the Bt and non-Bt maize treatments amended with AM fungi and vermicompost, except for non-Bt maize control (treatment, T6).

The genus *Fluviicola* was more abundant in Bt maize treatments overall compared to non-Bt maize treatments over time. However, at 90 DAP *Fluviicola* was more abundant in non-Bt maize treatments compared to Bt maize treatments. Furthermore, results also showed that *Fluviicola* was more abundant in T1 and T6 as well as T2 and T4 over time (Figure 3.3). However, *Fluviicola* was less abundant in T3 and T5 (Figure 3.3). A similar trend was also observed for the genera *Lysobacter*, *Methylibium* and *Alicyclobacillus* (Figure 3.3). *Lysobacter*, *Methylibium* and *Alicyclobacillus* were more abundant in T1 and T6 as well as T2 and T4 over time (Figure 3.3). However, the genus *Chitinophaga* was more abundant in Bt and non-Bt maize of T3 and T5, but less in T1 and T6 as well as T2 and T4 (Figure 3.3). Moreover, the genus *Chitinophaga* reduced in abundance between 60 and 120 DAP in all treatments.

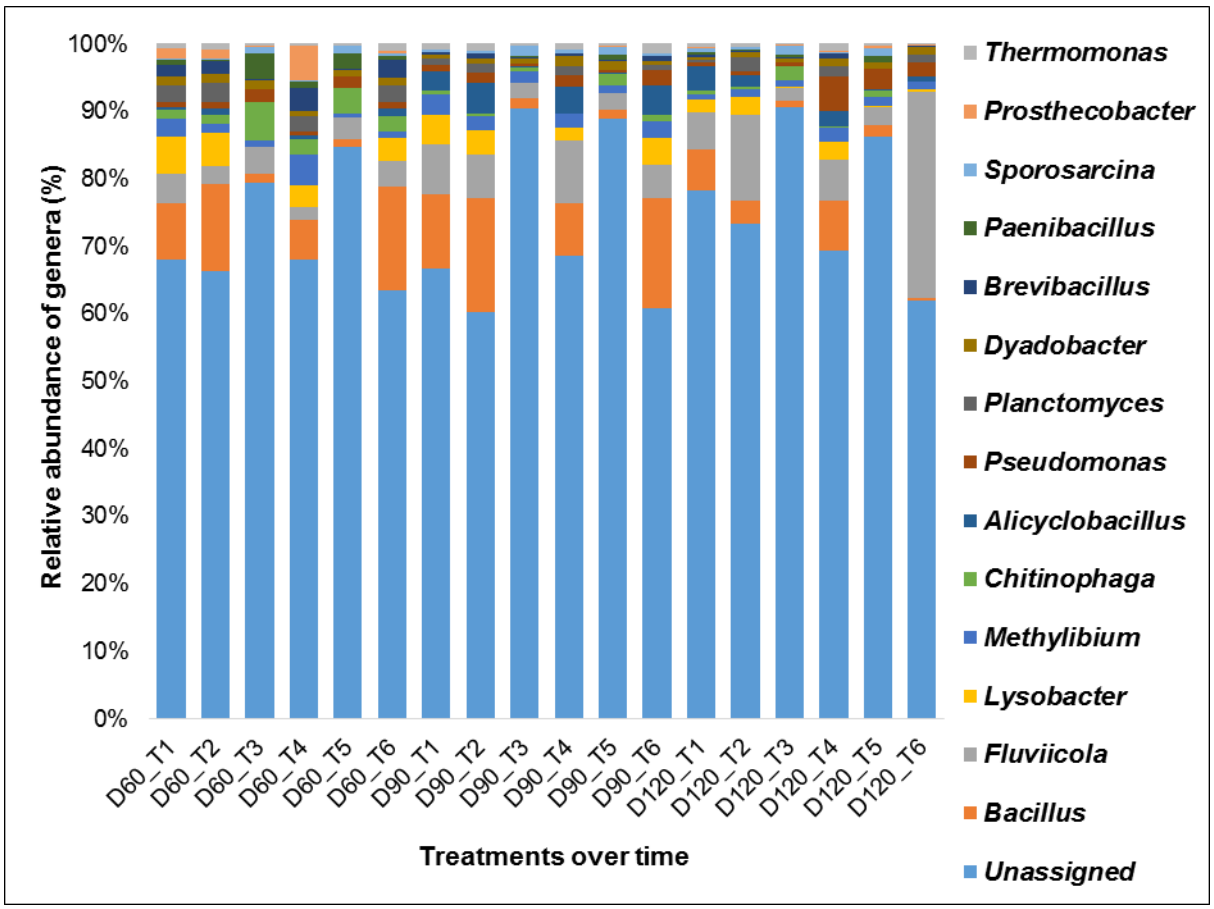

Figure 3.3: Relative abundance of the fifteen dominant bacteria in Bt and non-Bt maize soils at genus levels.

Further comparisons of genera with a maximum relative abundance of ≥ 1% in all Bt and non-Bt maize treatments by using hierarchal clustering of the Bray-Curtis dissimilarity matrix (Figure 3.4) suggest that the bacterial genera community of Bt and non-Bt maize associates differently with respect to the different treatments and growth stages. The shifts of the bacterial community compositions were further illustrated by clear clustering of the dominant bacterial genus corresponding to different treatments in the heatmap plot. Treatments T3 and T5 were clustered and largely dominated by *Chitinophaga*, *Pseudomonas*, *Dyadobacter*, *Paenibacillus*. Furthermore, T1 and T6 as well as T2 and T4 grouped together and showed overlap in genera. The predominant genera observed in this overlap were *Bacillus*, *Fluviicola*, *Lysobacter*, *Methylibium* and *Alicyclobacillus*. A similar trend was observed in the PCoA (Figure 3.1). These results suggest that some of the bacterial species in the different treatments were similar throughout the growth stages, but varied in abundance (Figures 3.3 and 3.4).

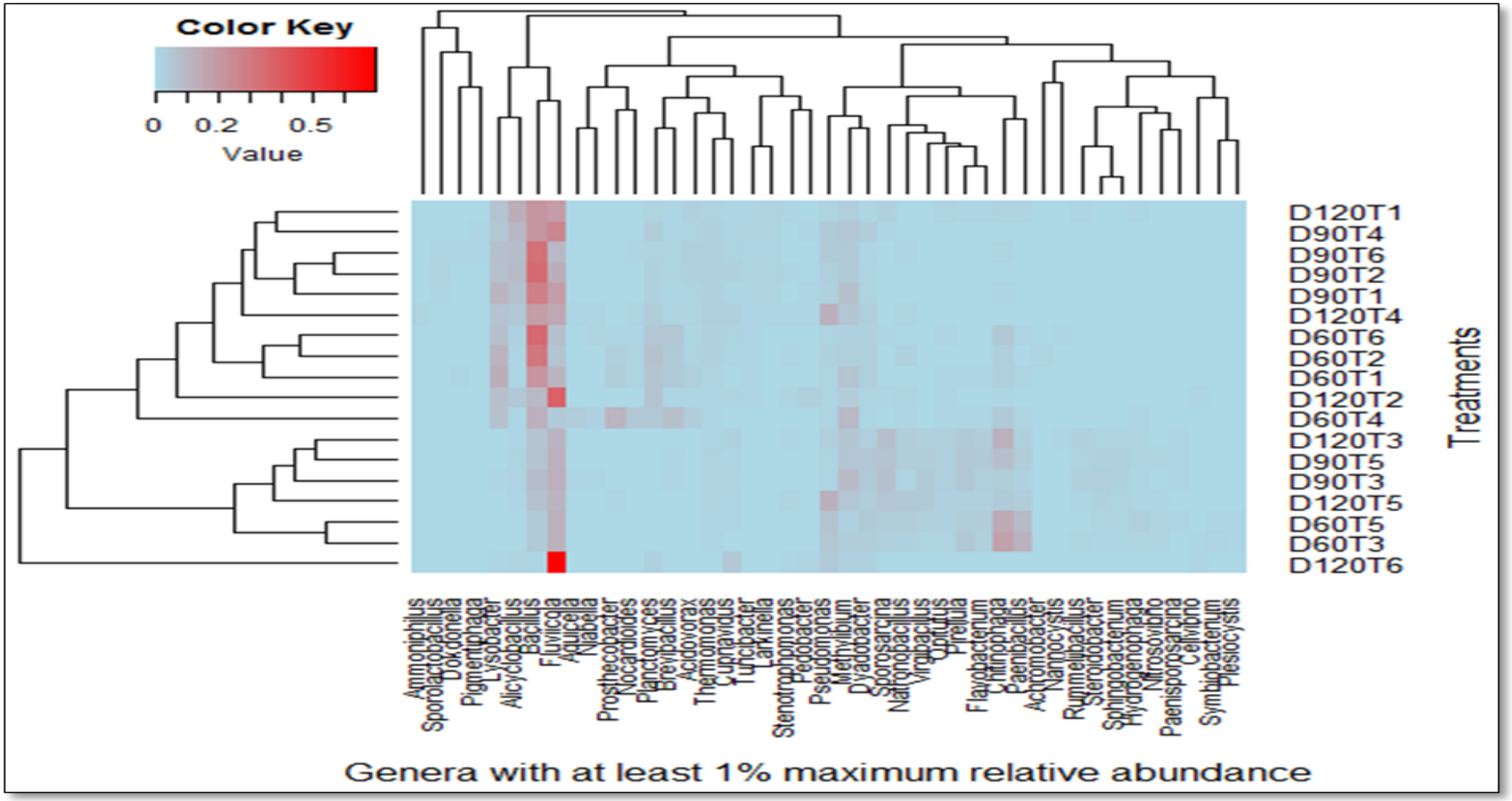

Figure 3.4: Hierarchically clustered heat map analysis of the highly represented bacterial taxa (at the genus level) found in Bt and non-Bt maize treatments grown in AM fungi and vermicompost amended soils (relative abundance > 1%) at 3 sampling times. The relative abundance for each bacterial genus were depicted by colour intensity (clustering on the X-axis) with each Bt and non-Bt maize treatment (Y-axis clustering). The higher values are represented by darker colours whereas lower ones are represented by lighter colours.

3.3.3 Effect of soil chemical properties and enzymatic activities on abundant genus

Average values of the dominant soil chemical properties and enzyme activities used in the RDA triplot are presented in Figures 4.2-4.4 of chapter 4 (section 4.3). From the results obtained it is evident that the chemical properties (Figures 4.2 and 4.3) and enzyme activities (Figure 4.4) were the most dominant in T3 and T5 (Figure 3.5). The relative abundance of *Sporosarcina*, *Pseudomonas*, *Paenibacillus*, *Chitinophaga* and *Dyadobacter* in T3 and T5 were positively associated with all chemical properties and enzyme activities over time (Figure 3.5). While, *Thermomonas*, *Bacillus*, *Acidovorax*, *Lysobacter*, *Planctomyces*, *Methylibium*, *Brevibacillus* and *Prosthecobacter* in T1 and T6 as well as T2 and T4 were negatively associated with chemical properties and enzyme activities at 60 DAP (Figure 3.5). A similar trend was observed for *Alicyclobacillus* and *Fluviicola* at 90 DAP, where chemical properties (Figures 4.2 and 4.3) and enzyme activities (Figure 4.4) were also negatively associated with these genera (Figure 3.5). Overall, results indicate that chemical properties, enzyme activities and genera were mostly related to amendments and growth stages rather than the effect from genetic modification of maize.

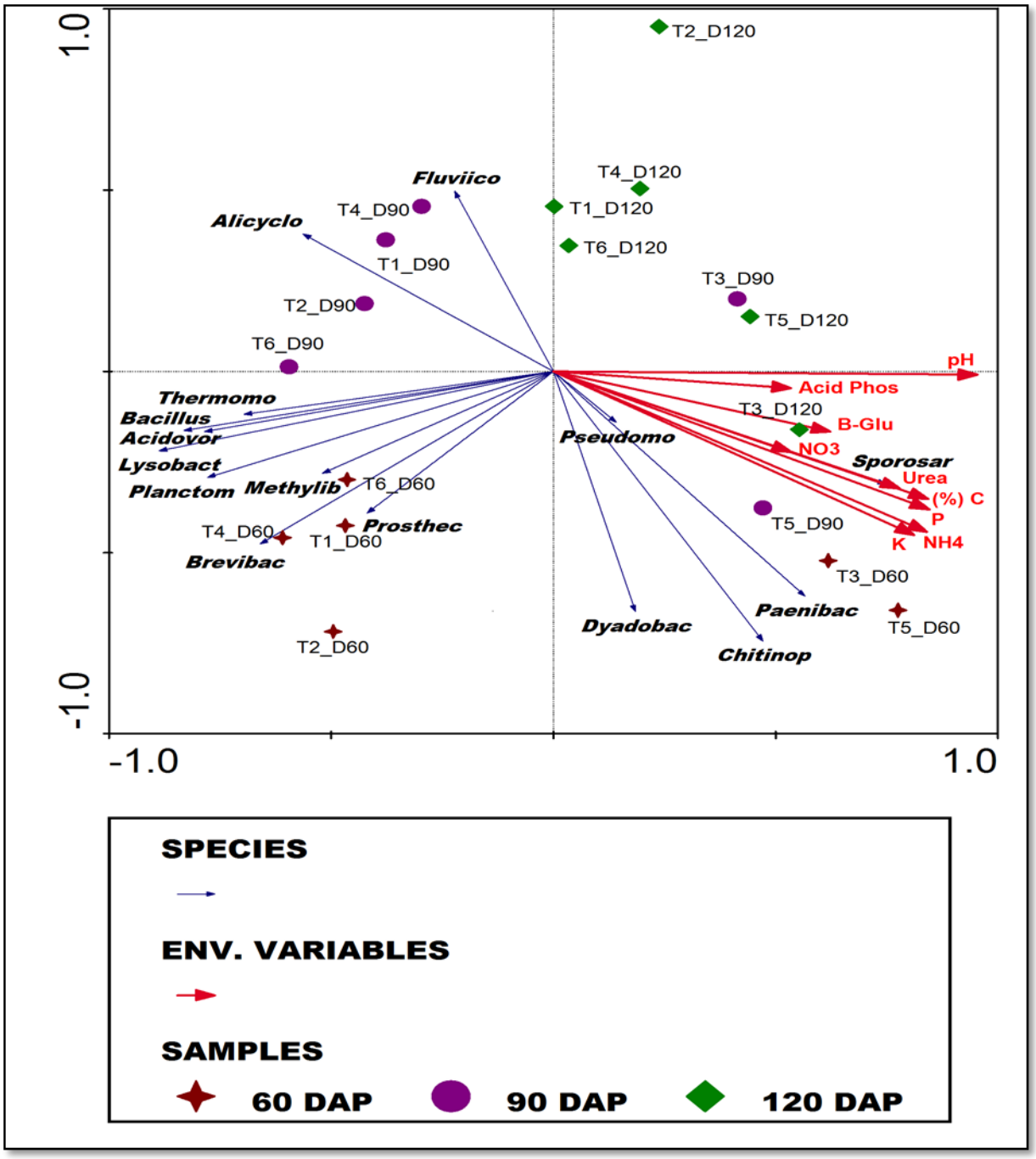

Figure 3.5: Redundancy analysis (RDA) based on the relative abundance of bacterial genera and selected soil chemical properties in Bt and non-Bt maize soils amended with AM fungi and vermicompost (T3 and T5), AM fungi (T2 and T4) and the controls (T1 and T6).

3.4 Discussion

Cultivation of GM crops might expose non-target beneficial soil microbes to certain risks relating to physiological and metabolic changes in GM plants and the release of their foreign expression products (e.g., Bt protein) into the soil ecosystem (Liu *et al*., 2005; Sanchis, 2010; Sanahuja *et al*., 2011; Hannula *et al*., 2014). This may adversely affect soil ecosystem functions and agricultural production. This is of particular importance in soil amended processes that rely on beneficial microorganisms. Hence, the focus of investigation on this aspect of the study is to investigate potential impact of genetic modification of Bt maize on rhizosphere microbes.

Fewer OTUs/species in both Bt and non-Bt maize treatments suggested that bacterial species richness in maize may be related to soil nutrients, root exudates and plant growth stages (Baumgarte and Tebbe, 2005; Yang *et al*., 2017). A study by Baumgarte and Tebbe (2005) presented that the structure of bacterial community in the rhizosphere of Bt maize was less affected by Cry1Ab toxin than by environmental factors such as the plant growth phase and native soil diversity.

However, the different degrees of reduction in OTUs/species in Bt and non-Bt maize treatments suggested that the AM fungi and vermicompost amendments had an influence on species diversity. The influence of organic amendments on the formation of bacterial communities have been previously reported (Hartmann *et al*., 2009; Hardoim *et al*., 2011). The Shannon index suggest higher richness of species diversity in non-Bt maize than in Bt maize (Table 3.2). Furthrmore, the Simpson index value suggest that the species are more evenly distributed between Bt and non-Bt maize soil and the different treatments (Table 3.2). The PCoA results showed that the soil bacterial community structures observed under treatment with AM fungi and vermicompost for both Bt and non-Bt maize differed from those found in AM fungi treated soils and controls. These results are corroborated by several previous investigations showing that the microbial community structure in soil amended with organic fertiliser applied over a longer period is significantly different from that in soil amended with chemical fertiliser as revealed by PCR-DGGE or 454 pyrosequencing methods (Ge *et al*., 2008; Chaudhry *et al*., 2012; Poulsen *et al*., 2013).

Of the 29 different phyla detected in both Bt and non-Bt maize treatments, phylotypes belonging to the phyla Proteobacteria, Verrucomicrobia, Firmicutes, Acidobacteria and Bacteroidetes dominated the microbial communities. These phyla contain taxa

commonly found within soil rhizospheres that are capable of exerting various effects on plant health including beneficial and pathogenic interactions (Berendsen *et al*., 2012; Philippot *et al*., 2013). For instance, the *Pseudomonas* species fall within the phylum Proteobacteria and have been reported to benefit plants by stimulating plant growth and exhibiting traits involved in biological control of plant diseases (Lugtenberg and Kamilova, 2009). Furthermore, Proteobacteria was the most abundant and largest phylum detected in both Bt and non-Bt maize treatments. This observation is in agreement with Barriuso *et al*. (2012), Dohrmann *et al*. (2013) and Ondreičková *et al*. (2013) whereby Proteobacteria were reported to be the dominant bacterial phyla associated with the rhizosphere soil of Bt and non-Bt maize. The overall relative abundance distribution of these phyla did not change from the non-Bt to the Bt maize at any sampling time, rather between treatments over time (Figure 3.2). This observation is supported by Sradnick *et al*. (2013) who reported that differences observed in microbial community structures were the result of different organic amendment applications. The overall relative abundance of these phyla did not change from the non-Bt to the Bt maize at any sampling time, rather between treatments over time (Figure 3.2).

A number of genera were associated with the rhizosphere soil of Bt and non-Bt maize treatments in the present study. Among these genera, the genus *Sphingomonas* has been consistently reported to be associated with Bt maize soil (Dohrmann *et al*., 2013; Bumunang *et al*., 2015; van Wyk *et al*., 2017). The differential abundances of *Bacillus*, *Fluviicola*, *Lysobacter*, *Methylibium*, *Alicyclobacillus*, *Chitinophaga*, *Pseudomonas*, *Dyadobacter* and *Paenibacillus* in both Bt and non-Bt maize treatments are prominent, suggesting that there are clear shifts in functional diversity of these species between treatments and across plant growth stages. Furthermore, genera analysis showed that *Bacillus* had the highest abundance overall among the treatments, which according to previous work, showed that organic fertiliser-treated soil can increase the abundance of *Bacillus*, for example in cucumber rhizosphere soil as shown by 454 pyrosequencing (Qui *et al*., 2012). As *Bacillus* can form a stable and extensive biofilm (Bais *et al*., 2004) and secrete many antifungal compounds, such as surfactin, bacillomycin and macrolactin, that protect plants against attack by soil-borne pathogens (Bais *et al*., 2004; Yuan *et al*., 2012), it is the most promising genus for use as biocontrol agents.

Some detected genera are known plant growth promoting bacteria and are important for plant health, productivity and soil fertility, through functions such as nutrient

mineralisation, production of phytohormones and antagonism of plant pathogens (Egamberdiyeva, 2007; Compant *et al*., 2010). Specific examples of genera with known beneficial species in the present study include *Acidovorax*, *Pseudomonas*, *Flavobacterium* and *Paenibacillus* among others. Species of *Bacillus* and *Pseudomonas*, also possess traits of biological control agents that protect plants against phytopathogenic organisms (Sinha *et al*., 2010; Megali *et al*., 2014), while the genus *Flavobacterium*, has been reported to be active in the solubilisation process of phosphorus (Pindi *et al*., 2012). *Paenibacillus* spp. are well-known plant-growth promoters, capable of promoting plant nutrient uptake, controlling phytopathogens and producing phytohormones (Grady *et al*., 2016). In addition to agricultural applications, *Paenibacillus* produces antimicrobial compounds that are useful as pesticides or in medicine. These species also produce many yield enzymes that could be utilised for bioremediation as well as valuable chemicals (Grady *et al*., 2016).

In conclusion, the rhizosphere soil bacterial community of Bt maize differed from those of non-Bt maize across plant growth stage and between bio-inoculants as well as bio-fertilisers. These differences were more pronounced between the diversity and abundance of particular species, rather than in the species richness of the maize bacterial community. Results from this study confirm that application of AM fungi and vermicompost had an enhancing effect on the total number of bacterial community of both Bt and non-Bt maize treatments. In addition, growth-promoting microorganisms beneficial for the plant growth appeared to have been more abundant in the current study than in the field study (Chapter 2).

CHAPTER 4

Vermicompost application: A potential solution to challenges associated with interactions between Bt maize and arbuscular mycorrhizal fungi

4.1 Introduction

There is contradictory scientific evidence about the impact of genetic modification (GM) of plants on essential ecosystem functions such as soil enzymatic activities, nutrient mineralisation, microbial population and plant growth (Motavalli *et al*., 2004; O'Callaghan *et al*., 2005; Beura and Rakshit, 2013; Cheeke *et al*., 2014; Shu *et al*., 2017; van Wyk *et al*., 2017). For this reason, pre-market environmental risk assessment of GM plants has become a global norm to ensure proper environmental protection (Arpaia *et al*., 2017). This approach is important for the evaluation of any immediate or long-term effects of GM plants on both target and non-target organisms in the environment (Arpaia *et al*., 2017). A common example of genetic modification of plants is the GM maize that carries genes encoding insecticidal proteins that are toxic to the larvae of insects (local example, *Bussesola fusca*) (Castagnola and Jurat-Fuentes, 2012). These insects cause some of the most important economic losses associated with maize cultivation (James, 2015; Ranjekar *et al*., 2003). Despite the popularity, it is not clear whether cultivation of transgenic Bt maize may have adverse effects on ecosystem functioning especially non-target organisms. Hence, there have been genuine concerns about the potential impacts of Bt maize on soil microbe-mediated processes and functions in soils due to the presence of the insecticidal Cry protein (Feng *et al*., 2011, Fließbach *et al*., 2012).

Soil plant growth promoting microorganisms such as AM fungi represent an important group of the non-target microorganisms that could be affected by the genetic modification of maize. Arbuscular mycorrhizal fungi are widespread in soils and form symbioses with most plant species including major crops such as wheat, maize, soybean and sorghum (Smith and Read, 1996). By establishing associations with plant roots, they provide more efficient uptake of nutrients through an extended soil mycelial network in exchange for carbon from the plant (Minaxi *et al*., 2013). This extended hyphal network can also improve water uptake to attenuate plant water stress (Augé, 2001). Thus, AM fungi can improve nutrient use efficiency from fertiliser and soil thereby positively influencing plant growth, reproduction and yield (Miller, 2000; Hussain *et al*.,

2016). Such beneficial microorganisms are applied as bio-fertiliser in different forms to promote plant growth, control root pathogens and sometimes for bioremediation of contaminated soils (Bello-Akinosho *et al*., 2016; Raimi *et al*., 2017). Furthermore, AM fungi can also have a direct effect on the ecosystem, as they improve the soil structure and aggregation (Rillig and Mummey, 2006; Rillig *et al*., 2015). Mycorrhizal fungi also play significant roles in plant ecophysiology and microbial activities (Hussain *et al*., 2016). Arbuscular mycorrhizal fungi in symbiotic relationship with plants also improves plant nutrition, enhances resistance to soil-borne pests and diseases (Awasthi *et al*., 2011), resistance to drought and tolerance to heavy metals (Gosling *et al*., 2006; Bhattacharyya and Jha, 2012). However, many of the aforementioned benefits of AM fungi could be threatened by the adoption of genetic modification of plants. For instance, studies had already reported reduced colonisation rate/percentage of the popular Bt maize (Castaldini *et al*., 2005; Turrini *et al*., 2005; Cheeke *et al*., 2011). Even though these studies showed no direct effect of the Bt proteins on AM fungi, the genetic engineering within the plant may have altered the plant's relationship with AM FUNGI. These genetic changes within the host plant resulted in alterations of plant root exudates (Broeckling *et al*., 2008), which may in turn affect mycorrhizal colonisation.

To reap the full benefits associated with the cultivation of Bt maize, it is important to eliminate or alleviate any potential associated risk(s). This is essential to ensure that benefits of genetic modification are holistic and sustainable. In order to achieve this, studies are usually focussed on solution that will involve additional genetic modification (James, 2010; Monsanto, 2011). Although, evidence of the negative impact of genetic modification on the interaction of AM fungi and Bt maize may not be conclusive, it is clear that using the same technology to seek solutions may not be acceptable. Hence, an integrated farming approach may offer a suitable and environmentally friendly solution to this challenge. For instance, there is an increasing number of farmers globally (Raei and Aghaei-Gharachorlou, 2015) that are now using combination of different soil amendments to improve quality of soil as well as quality and quantity of yield. As an organic fertiliser, vermicompost is one of the most popular in this category.

Application of vermicompost has many benefits but the one that is most relevant to the current study is the capability of this organic fertiliser to improve mycorrhizal colonisation of plants (Cavender *et al*., 2003; Carrenho *et al*., 2007; Hussain *et al*., 2016). This is important for agricultural sustainability as it offers farmers the opportunity to increase yield and at the same time ensure their agricultural practices are

sustainable. It is on this premise that the aim of the present study is designed to investigate the potential of vermicompost application in the elimination or alleviation of the negative impact of genetic modification on the interaction between AM fungi and Bt maize. In addition, the study further investigates any potential effect of Bt modification on the direct and indirect functions of vermicompost in the soil.

4.2 Materials and methods

4.2.1 Study site and experimental design

Refer to the greenhouse trial described in chapter 3 of section 3.2.

4.2.2 Sampling

Maize plants were carefully removed from the pots and rhizospheric soils were collected by gently shaking the roots to dislodge small clumps of soil adhering to the roots. Three replicates of the soils from each pot were sampled at 60 (active growing stage), 90 (tasseling) and 120 (flowering) DAP for maize plants. Soil samples were stored immediately at −80°C for further analysis. Furthermore, fine roots were also collected, washed and stored in 50% (v/v) ethanol for further analysis. Subsequently, at termination (120 DAP) maize plants were cut off at the first internode above the soil and plant material were weighed fresh as well as oven (Memmert, Germany) dried at 45°C for ten days to calculate biomass from each pot.

4.2.3 Soil chemical analyses

The pH of the soil was determined with potassium chloride (pH [KCl]) by means of a calibrated pH meter (Radiometer PHM 80, Copenhagen) (McLean, 1982). Organic carbon was determined by the Walkley-Black method of Nelson and Sommers (1982). Furthermore, ammonium (NH_4^+-N) and nitrate (NO_3^--N) concentrations were determined according to the methods of Banwart *et al*. (1972) and Sonnevelt and van den Ende (1971), respectively. Extractable phosphorus was determined by the Bray I method following the procedure of Bray and Kurtz (1945). Exchangeable potassium (K^+), was extracted with 1-N ammonium acetate (pH 7) (Schollenberger and Simon, 1945).

4.2.4 Determination of enzyme activities

Acid phosphatase activity was determined in a reaction mixture containing 1.0 g of soil, 1.0 ml 25 mM nitrophenyl phosphate (p-NPP) as substrate, 4.2 ml modified universal buffer at pH 6.5 (Tabatabai, 1994). The reaction mixture was incubated at 37°C for 60 min after which the reaction was terminated by adding 1.0 ml of 0.5 M calcium chloride and 4.0 ml of 0.1 M, pH 12, tris (hydroxymethy1) amino methane buffer. Enzyme activity was then determined at 405 nm with a digital UV-Vis spectrophotometer. Furthermore, the method for the determination of β-glucosidase was similar to that of acid phosphatase activity except for the 1.0 ml 25 mM p-nitrophenyl-β-D-glucopyranoside (p-NG) substrate, and the pH 6.0 (Tabatabai, 1994). Acid phosphatase and β-glucosidase activities were expressed as µg p-NPP and p-NG g $soil^{-1}$ h^{-1}, respectively.

Urease activity was determined by the non-buffered method of Kandeler and Gerber (1988). This method entails a mixture of 9 ml Tris (hydroxymethyl) aminomethane (THAM) buffer 0.05 M, pH 9, and 1 ml urea 0.2 M was added to 5 g soil and incubated at 37°C for two hours. The amount of ammonium released was determined and activity expressed as µg ammonium (NH_4^+-N) g^{-1} $2h^{-1}$.

4.2.5 Arbuscular mycorrhizal fungi colonisation in maize roots

A modified protocol of Koske and Gemma (1989) was used to stain root materials. The fine preserved roots stored in 50% (v/v) ethanol were cut into one cm long pieces for staining. Root samples of maize were then cleared by heating in 10% (w/v) potassium hydroxide (KOH) at 90°C for five min to prevent background staining of plant tissues. This was followed by soaking of root samples in 1% (v/v) hypochlorite (HCℓ) for 15 minutes. Root tissues were then stained with 0.05% (w/v) Trypan blue in acid-glycerol at 90°C for 30 min. After staining, the roots were de-stained and stored in acid-glycerol solution. De-stained roots were mounted on slides in an acid glycerol solution (Koske and Gemma, 1989) and gently squashed using a coverslip before visualisation under a light microscope (Nikon, Japan) at 400X magnification. The percentage colonisation was estimated using the slide-intersect method (McGonigle *et al*., 1990). Briefly, this method entails the presence/absence of any fungal structure (hyphae, arbuscules, and/or vesicles) per sample that are being observed per 100 intersects. From these results, the total percentage AM fungi colonisation is recorded as the total number of intersects out of 100 showing presence/absence of any fungal structure.

4.2.6 Statistical analysis

All data were tested for normality (Shapiro and Wilk, 1965) and were found to be normally distributed. Data were then subjected to an analysis of variance (ANOVA) using the General Linear Model procedure of SAS 9.4 (SAS Institute Inc. 1999). The two-way ANOVA was performed to determine the effects of treatments (T1-T6) and time (days) on chemical and biochemical properties in soil, as well as on root colonisation of maize. Student's *t* least significant difference (LSD) values were calculated to facilitate comparison between treatment means. Means that differed at the 5% probability level were significantly different (SAS Institute Inc. 1999). Multivariate ordination analysis such as Redundancy Analysis (RDA) using CANOCO (Canoco for Windows Version 4.5, Biometris-Plant Research International, Wageningen, The Netherlands) were conducted on the data to identify the correlations between the various treatments and the soil chemical parameters.

4.3 Results

4.3.1 Total dry matter of plants

The mean (n=3) total dry matter yield at termination (120 DAP) is shown in Figure 4.1. From the results, it is evident that Bt maize plants (T1-T3) had a significantly higher total dry matter ($p < 0.05$) ranging from 63.7 to 318.78 g, compared to non-Bt maize plants (T4-T6) ranging from 32.42 to 278.16 g (Figure 4.1). Furthermore, application of vermicompost with AM fungi in soils resulted in higher total dry matter when compared to the other treatments (T1, T2, T4 and T6) (Figure 4.1). Treatments T3 (318.78 g/pot) and T5 (278.16 g/pot) produced significantly higher total dry matter, compared to the rest of the treatments (Figure 4.1). Furthermore, results also indicated that total dry matter yield differed significantly between treatments (Figure 4.1). These results indicated that Bt maize plants (T1-T3) performed better in terms of total dry matter in control, AM fungi and co-application of AM fungi and vermicompost amended soils. Results also showed that AM fungi applied in combination with vermicompost contributed significantly towards the dry matter yield of maize (Bt and non-Bt) plants.

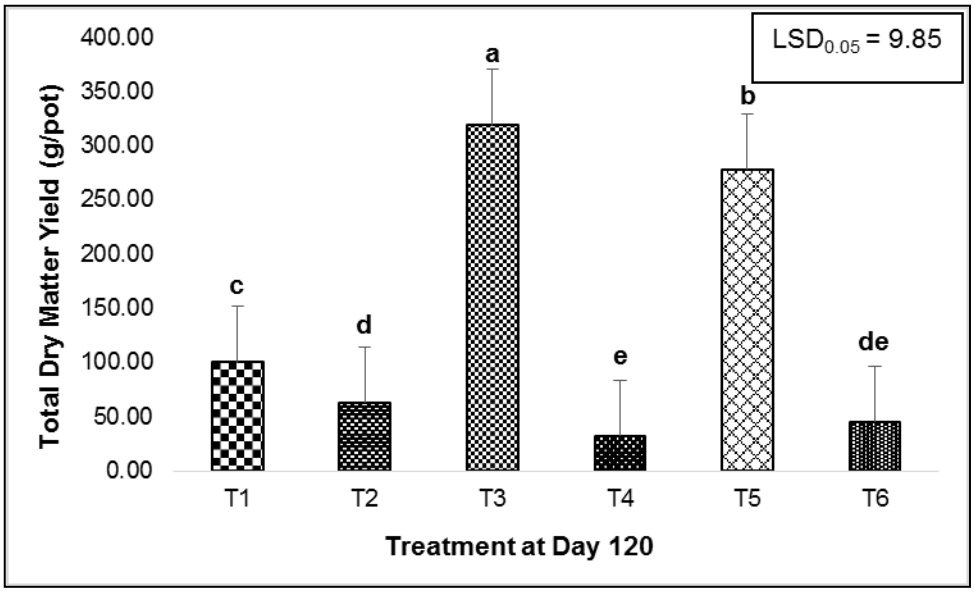

Figure 4.1: Total dry matter (g/pot) of Bt and non-Bt maize rhizosphere soil treatments at 120 DAP. Means of three replicates followed by different letters indicating a significant difference at $p \leq 0.05$ according to the Student's *t* LSD. Treatments; AM fungi (T2 and T4), AM fungi and vermicompost (T3 and T5) and controls (T1 and T6).

4.3.2 Soil chemical properties

The main soil chemical characteristics of the different treatments over time are depicted in Figures 4.2 and 4.3. Differences in the chemical characteristics between Bt and non-Bt maize soil treatments were noted over three sampling times. There was no significant difference ($p > 0.05$) in chemical properties between Bt and non-Bt maize soils amended with sole AM fungi (treatments, T2 and T4) and controls (treatments, T1 and T6). Significantly lower pH ($p < 0.05$) was observed in Bt maize control soils (T1) (Figure 4.2A), at 120 DAP. In addition, current results also illustrated that T5 had a significantly higher potassium concentration compared to T3 soil at 90 and 120 DAP (Figure 4.3C). Furthermore, the co-application of AM fungi and vermicompost showed an enhancing effect on the chemical parameters of maize (Bt and non-Bt) soils over the three sampling times. The soil pH of T3 and T5 was higher than 6.4, whereas without vermicompost addition (T1, T2, T4, T6), the pH ranged from 5.79 to 6.38 (Figure 4.2A). These results showed that the pH increased significantly ($p < 0.05$) in pots amended with AM fungi and vermicompost. Contrary to the high nitrate concentrations observed at 90 DAP, the ammonium was lower at 90 DAP (Figures 4.2B-C). Results showed that nitrate increased significantly from 60 to 90 DAP and then decreased from 90 to 120 DAP for all treatments. Furthermore, ammonium results showed a different trend where ammonium decreased from 60 to 90 DAP and increased from 90 to 120 DAP for all

treatments. These results are indicative that the growth period might have influenced nitrate and ammonium concentrations. Results also showed that nitrate and ammonium concentrations were significantly affected by T3 and T5. Treatments T3 and T5 had the highest percentage carbon (%C) compared to T2 and T4 and controls (T1 and T6) (Figure 4.3A). The overall highest percentage carbon was recorded for T5 (11.74%) at 120 DAP. Whereas, the lowest percentage carbon was recorded at T4 (0.84%) at 90 DAP. Furthermore, the phosphorus and potassium concentrations were significantly ($p < 0.05$) affected by T3 and T5 (Figures 4.4B and 4.4C). The highest phosphorus concentrations (Figure 4.3B) were recorded at T5 (669 mg/kg) and T3 (633 mg/kg) at 60 DAP. A similar trend was observed for the potassium where T5 (1700 mg/kg) and T3 (1630 mg/kg) also recorded the highest at 60 DAP (Figure 4.3C), whereas, the lowest phosphorus and potassium concentrations were recorded in T2 and T4 as well as T1 and T6 over time. A trend was observed for phosphorus and potassium concentrations where a decrease was observed over time (Figures 4.3B and 4.3C). Although there are no clear-cut differences between the chemical properties of Bt and non-Bt maize soil treatments, statistically significant differences were recorded for pH and potassium at 120 DAP- non-Bt maize soils had higher pH and potassium. Furthermore, the co-application of AM fungi and vermicompost and growth stages had the most significant effects on the soil chemical parameters shown in this study.

4.3.3 Soil enzyme activities

The activities of soil enzymes (acid phosphatase, β-glucosidase and urease) during the different maize (Bt and non-Bt) growth stages of the six treatments are shown in Figure 4.4. Differences in the activities of acid phosphatase, β-glucosidase and urease between Bt and non-Bt maize rhizospheric soil treatments over the three growth stages were observed (Figure 4.4). A prominent trend observed was that Bt maize treatments (T1-T3) had the highest enzyme activities at 60 DAP. Whereas non-Bt maize treatments (T4-T6) had the highest enzyme activity at 90 DAP (Figure 4.4). Results also showed that there were no significant differences in enzyme activities between Bt and non-Bt maize soils grown in control (T1 and T6) and AM fungi (T2 and T4) treatments over the three sampling points (Figure 4.4).

Nonetheless, maize (Bt and non-Bt) grown in AM fungi and vermicompost amended (T3 and T5) soils showed significant differences in urease and acid phosphatase activities at 60 and 120 DAP (Figures 4.4A and 4.4B). Urease activity at 60 DAP was significantly higher ($p < 0.05$) T3 compared to T5 soils (Figure 4.4A). At 120 DAP, acid phosphatase activity was significantly higher ($p < 0.05$) in T5 compared to T3 soils (Figure 4.4B). Furthermore, soils amended with AM fungi and vermicompost showed significantly ($p < 0.05$) higher amounts of, acid phosphatase, β-glucosidase and urease activities than T2 and T4 as well as controls (T1 and T6) at the three growth stages. Results also indicated that acid phosphatase and β-glucosidase activities exhibited a similar trend for all treatments (Figures 4.4B and 4.4C), while urease activity exhibited a decrease from 60 to 90 DAP and with a subsequent increase from 90 to 120 DAP (Figure 4.4A). At 120 DAP, enzyme activities at T3 and T5 were higher than at 60 and 90 DAP (Figure 4.4). Generally, enzyme activities showed no differences between Bt and non-Bt maize soil treatments. However, differences were observed for urease and acid phosphatase in soils amended with AM fungi and vermicompost. It was also evident that the addition of AM fungi and vermicompost into the soil, as an additional nutrient source, had a stimulating effect on all the enzyme activities studied (Figure 4.4).

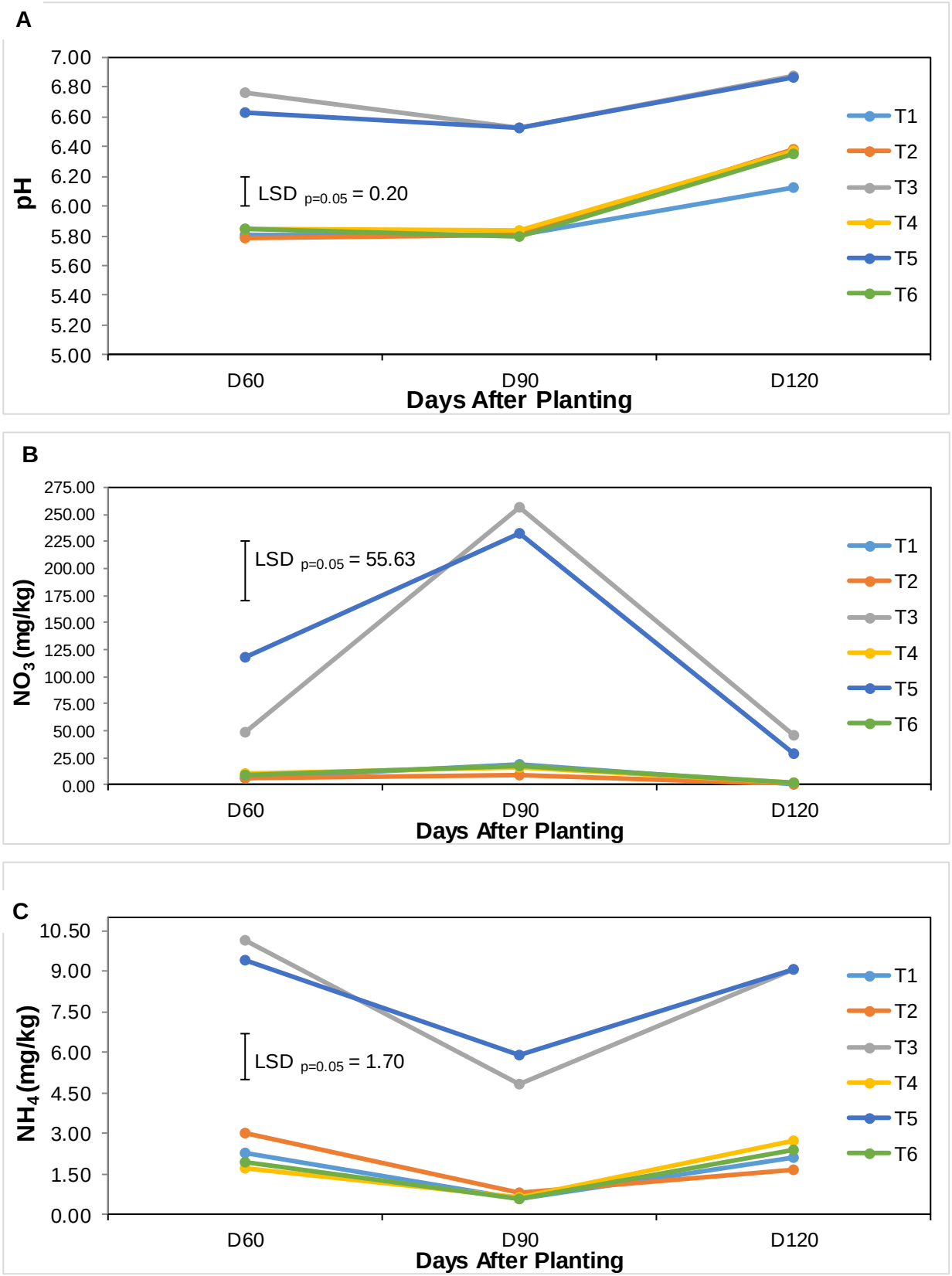

Figure 4.2: pH (A), nitrate (NO_3^--N) (B), and ammonium (NH_4^+-N) (C) in Bt and non-Bt maize soil at different growth stages. Each point represents the mean of three replicates. Treatments; AM fungi (T2 and T4), AM fungi and vermicompost (T3 and T5) and controls (T1 and T6)

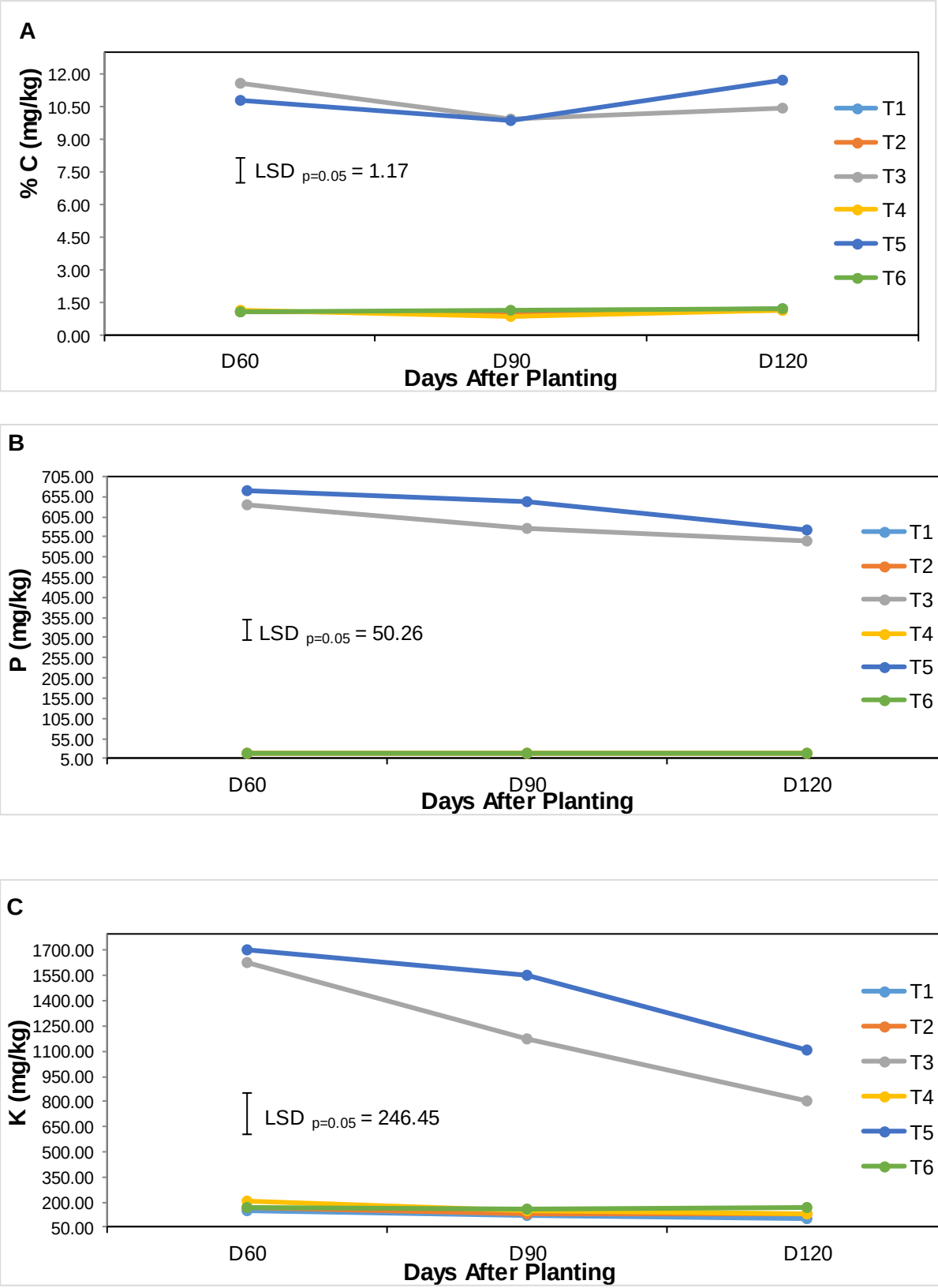

Figure 4.3: Percentage organic carbon (%C) (A), phosphorus (P) (B) and potassium (K) (C) in Bt and non-Bt maize rhizosphere soil at different growth stages. Each point represents the mean of three replicates. Treatments; AM fungi (T2 and T4), AM fungi and vermicompost (T3 and T5) and controls (T1 and T6)

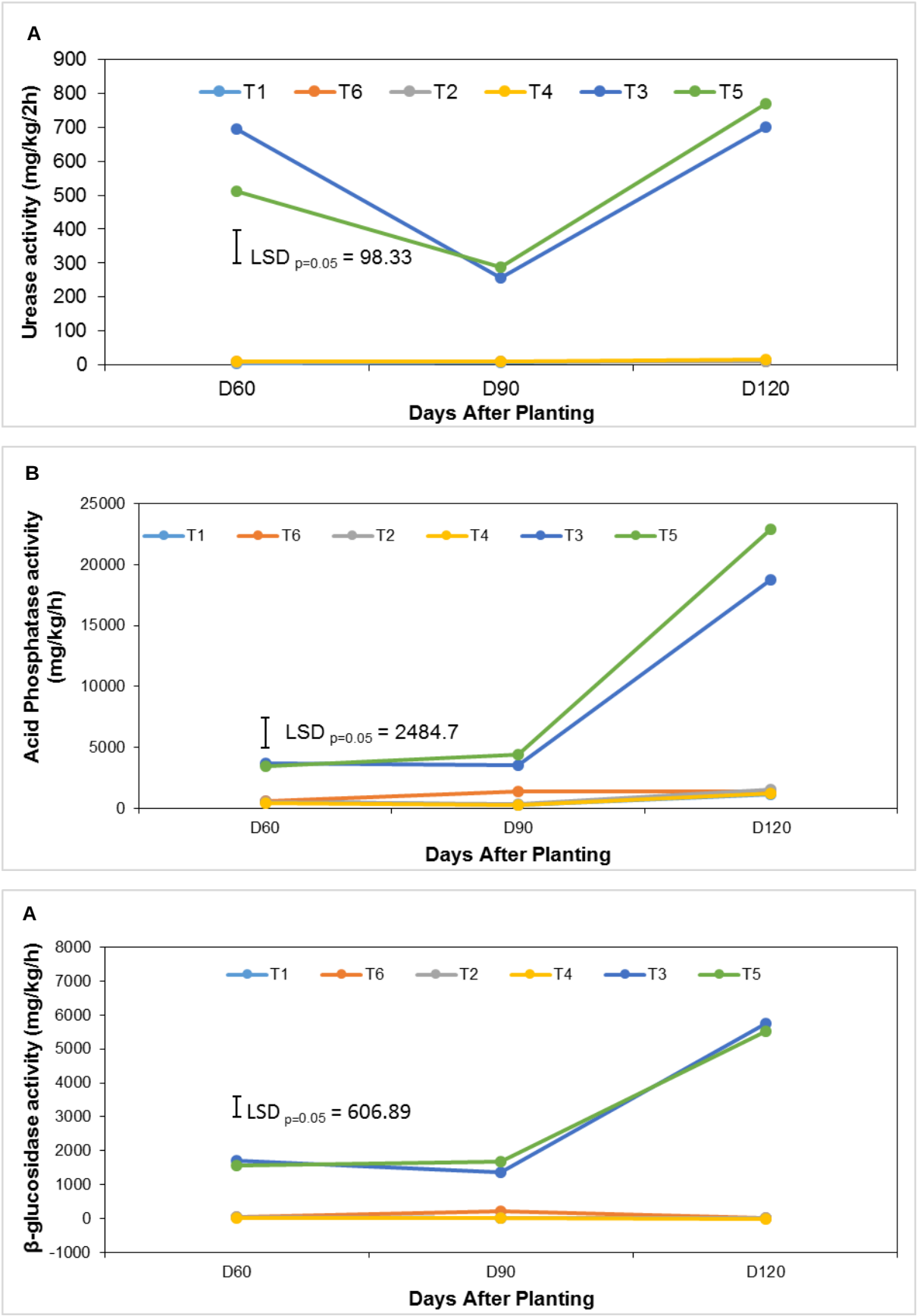

Figure 4.4: Acid phosphatase (A), β-glucosidase (B) and urease activities (C) of Bt and non-Bt maize at different growth stages of maize development. Each point represents the mean of three replicates. Treatments; AM fungi (T2 and T4), AM fungi and vermicompost (T3 and T5) and controls (T1 and T6).

4.3.4 Arbuscular mycorrhizal fungal root colonisation

Bt and non-Bt maize roots exhibited reduced and increased AM fungi root colonisation percentages respectively (Figure 4.5). These effects were most pronounced in T3 and T5. Results showed no significant differences in AM fungi colonisation percentage between Bt and non-Bt maize roots at 60 and 120 DAP in all the treatments (Figure 4.5). However, a prominent trend observed was the significantly higher ($p < 0.05$) AM fungi colonisation percentage in T5 in comparison to T3 at 90 DAP. Nevertheless, Bt maize roots in T1 and T2 showed significantly higher ($p < 0.05$) AM fungi colonisation percentage compared to T4 and T6 at 90 DAP. Furthermore, T3 and T5 as well as T2 and T4 had significantly ($p < 0.05$) higher root colonisation percentages compared to T1 and T6 (Figure 4.5) over time. Low levels of AM fungi colonisation of roots were detected in controls (T1 and T6) over time. Maximum root colonisation percentages of 75.00 and 87.50% were observed in T3 and T5 at 90 DAP, respectively. Minimum root colonisation percentages of 8.33 and 4.17% were recorded in T1 and T6 respectively at 60 DAP. Arbuscular mycorrhizal fungi colonisation of roots was significantly ($p < 0.05$) lower for all treatments at 60 DAP when plants were actively growing (Figure 4.5). Contradictory results were confirmed for the percentage AM fungi colonisation in both Bt and non-Bt maize roots. Furthermore, growth stage and co-application with AM fungi and vermicompost had significant impacts on root colonisation.

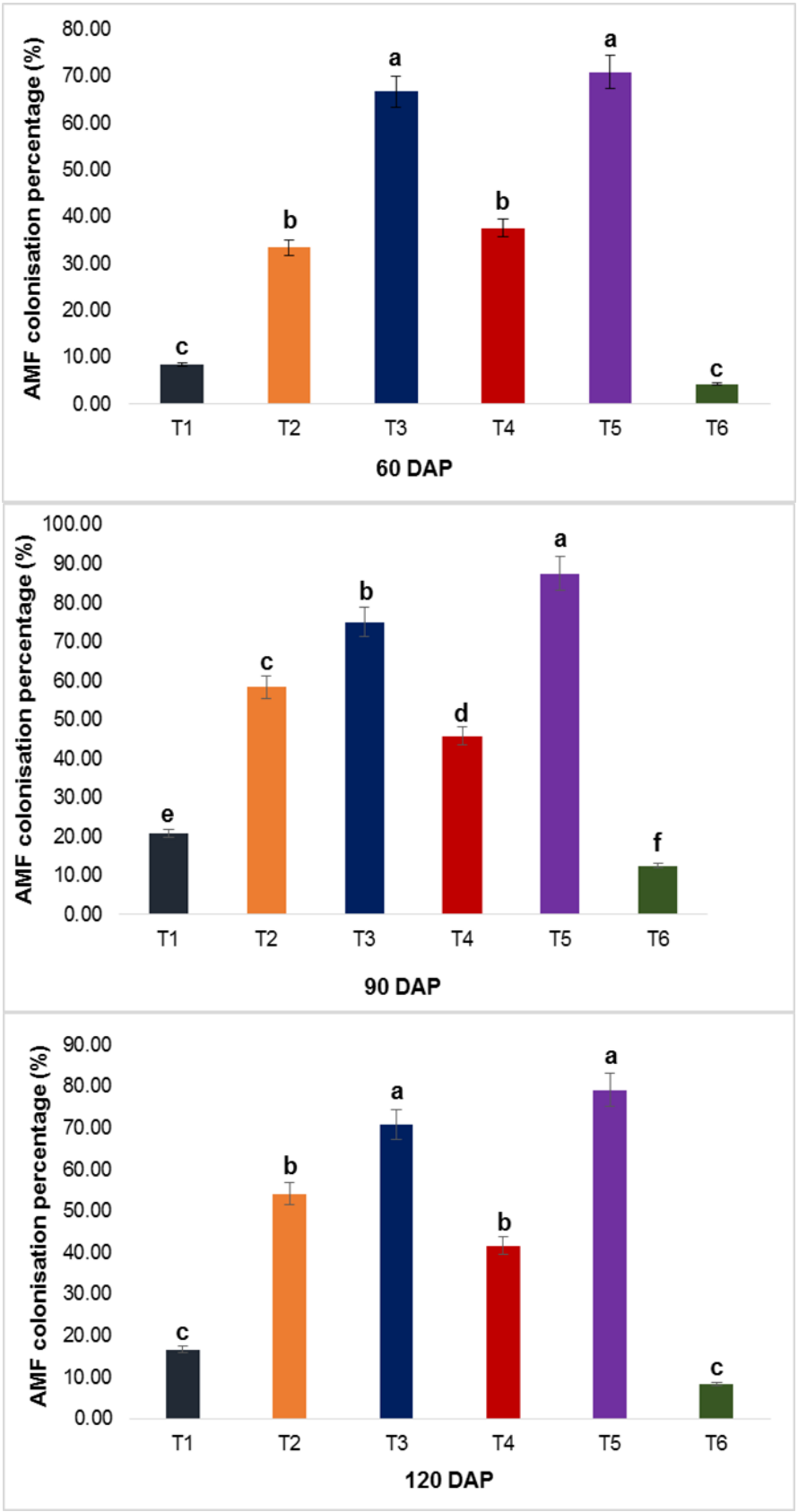

Figure 4.5: AM fungi colonisation percentage (%) in maize (Bt and non-Bt) roots at 60, 90 and 120 DAP. Means of three replicates followed by different letters indicating a significant difference at $p \leq 0.05$ according to the Student's *t* LSD. Treatments; controls (T1 and T6), AM fungi (T2 and T4), and AM fungi with vermicompost (T3 and T5).

4.3.5 Correlations between chemical properties and enzyme activities

As depicted in Figure 4.6, the association between the soil chemical properties and enzyme activities is described using redundancy analysis (RDA) triplot. Eigenvalues for the first two axes were 0.902 and 0.050 respectively. Total observed variance of the first two canonical axes was 95.6%. According to a Monte Carlo permutation test conducted with 499 permutations, the first canonical axis as well as the overall effect of the chosen environmental variables on the enzyme activities were statistically significant (P = 0.002). From the results, it is evident that the chemical characteristics and enzyme activities varied significantly between treatments and growth stages (Figure 4.6). Treatments T3 and T5 at 60 DAP had a strong positive relationship with potassium and phosphorus, while T5 at 90 DAP exhibited a strong positive relationship with nitrate (Figure 4.6). However, no association was observed for chemical parameters and enzyme activities between T2 and T4 as well as T1 and T6. Furthermore, acid phosphatase, β-glucosidase and urease activities were strongly associated with pH of the soil (Figure 4.6). Acid phosphatase activity was negatively associated with carbon, ammonium, phosphorus and nitrate percentage concentrations. The percentage carbon and ammonium was positively associated with urease activity. However, no association was observed between β-glucosidase activity and percentage carbon in the soil (Figure 4.6). A negative association was also apparent between T1-T2, T4 and T6 and all the enzymatic activities assayed. Furthermore, these enzymes were also positively associated with T3 and T5 (Figure 4.6). Overall, these results indicate that maize (Bt and non-Bt) grown in sole AM fungi and control treatment had negative correlations with chemical properties and enzymatic activities. Maize (Bt and non-Bt) grown in AM fungi and vermicompost showed a strong positive correlation with chemical properties and enzymatic activities.

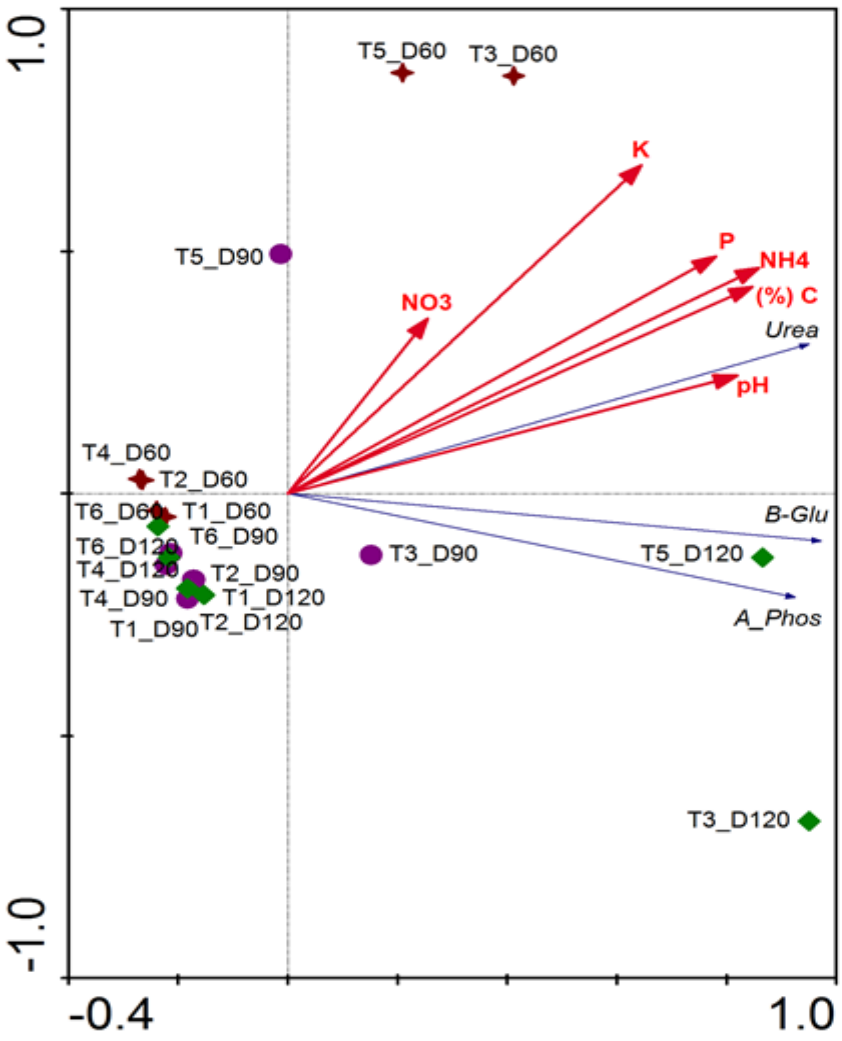

Figure 4.6: Redundancy analyses (RDA) triplot of the correlations between soil chemical properties and enzyme activities. The bold red arrows indicate the soil parameters that had strong and significant impact on enzyme activities (blue arrows) ($p \leq 0.05$). Six treatments at 60, 90 and 120 DAP are indicated by maroon stars, purple dots and green diamonds, respectively.

4.4 Discussion

The application of genetic modification may lead to the transfer of genetic materials from one organism to another (Prakash *et al*., 2011). The introduction of foreign genes into GM plants may interfere with other gene functions possibly resulting in potential risks (Dennis, 2002). Hence, there could be a number of unpredictable and predictable risks related to the release of GM plants into the environment. For instance, foreign genes inserted in GM plants may affect the amount of root exudates and soil ecosystem functions (Guan *et al*., 2015).

There are however, several contentious issues regarding Bt maize production conditions. For example, contradictions exist with regards to the potential impact that these plants could have on soil ecosystems (Motavalli *et al*., 2004; Beura and Rakshit, 2013; Cheeke *et al*., 2014; Shu *et al*., 2017; van Wyk *et al*., 2017).

Under conventional maize production conditions, impacts on soil quality is generally experienced (Chaparro *et al*., 2012; Franco-Otero *et al*., 2012). Most investigations on such impacts had been about the pre-existing microbial communities in the rhizosphere.

As a potential solution, the application of organic –and bio-fertilisers such as vermicompost and AM fungi has attracted enormous attention worldwide because they offer an entire range of agronomic and environmental benefits. Thus, in this study ecophysiological responses of both Bt and non-Bt maize genotypes to AM fungi and vermicompost amendments were assessed.

4.4.1 Total dry matter of maize plants

An important trend observed was the significantly higher dry matter ($p<0.05$) observed when maize (Bt and non-Bt) plants were produced in AM fungi and vermicompost amended soils (Figure 4.1, T3 and T5). The increased ($p < 0.05$) dry matter observed in T1-T3, in comparison to T4-T6, could be attributed to the conjoint effect of the genetic modification of Bt maize. Genetic modification of Bt maize is known for its consequent indirect yield enhancement, whereas the addition of organic matter and bio-fertilisers is known to increase the water-holding capacity of soil, as well as improving macro- and micro-nutrients availability as well as improving soil physical properties (Singh, 2003; Oo *et al*., 2015). Furthermore, the significant increase in dry matter attained by treatments T3 and T5 in this study can be explained by the plant roots secretion of exudates by plant roots, which attract and encourage the multiplication of microbes in rhizosphere. This in turn could benefit the plant with improved germination, nitrogen fixation, synthesis of growth promoting hormones, phosphorus solubilisation and improved nutrients and water uptake (Kumar *et al*., 2001; El-Komy, 2004; Vessey, 2003). Such growth enhancements as reflected in dry matter values has been reported in previous studies when AM fungi and vermicompost are co-applied (Maheswerі *et al*., 2006; Shishehbor *et al*., 2013; Hussian *et al*., 2016). These observations can be explained by the fact that vermicomposts contain plant growth regulators, such as auxins, cytokinins (Singh *et al*., 2008) and humic acids (Atiyeh *et al*., 2002). The regulators are produced through the action of earthworms and beneficial microbes with

capabilities to increase the plant growth and yield of many crops (Pezeshkpour *et al*., 2014; Atiyeh *et al*., 2002).

4.4.2 Soil chemical parameters

It is well-known that the application of organic -and bio-fertilisers such as vermicompost and AM fungi improves soil physical, chemical and biological properties (Oo *et al*., 2015). These enhancing effects were particularly observed in chemical properties of T3 and T5 soils over the three growth stages. This observation agrees with previous studies, which have shown that soils amended with vermicompost have significant increased effects on chemical properties (Arancon *et al*., 2006; Lazcano *et al*., 2013; Doan *et al*., 2014). In addition, the high organic carbon present in the soils from vermicompost could also contribute positively to soil properties, which in turn enables it to play a key role in establishing and maintaining the level of soil fertility. According to Tejada *et al*. (2006), the effect of organic amendments on soil total organic carbon depends on the chemical composition of the amendments, which determines the rate of their mineralisation by soil microorganisms (Hahn and Quideau, 2013). The highest percentage carbon was found in T3 and T5 at 60 and 120 DAP and might be due to the increased organic carbon content in the soil from the binary application of AM fungi and vermicompost. In particular, soil amended with AM fungi and vermicompost had higher percentage carbon than soil amended with AM fungi alone and controls.

Soil pH is an important chemical property which influences biological activities in the soil. The soil pH varied among different treatments, as different treatments showed an increasing trend towards neutral. Other studies also reported differences in soil pH after vermicompost addition (Gopinath *et al*., 2008; Nada *et al*., 2011). The pH values of T3 and T5 were very close to neutral, indicating biological stabilisation in soil (Papathanasiou *et al*., 2012). Decrease in pH at 90 DAP might be attributed to the nitrification process (Zhang *et al*., 2015) that took place. This result is corroborated by the significant increase in nitrates and decrease in ammonium at 90 DAP (Figures 4.2B-4.2C). The significantly lower ($p < 0.05$) pH observed in T1 soil may be due to the difference in nutrient utilisation (Powell *et al*., 2009; van Wyk *et al*., 2017). Nitrogen, potassium and phosphorus are the three most essential nutrients required for plant growth. These three essential nutrients are also important constraints in crop production in developing countries (Pharudi, 2010).

Significantly higher nitrate, ammonium, phosphorus and potassium in soils were also found in T3 and T5, compared with T2 and T4 as well as T1 and T6. The significantly lower potassium observed in T3 soils could be ascribed by the efficient uptake of this nutrient by Bt maize plants, which resulted in higher dry matter yield. Furthermore, the decreasing effect of potassium and phosphorus observed over time, could be due to the uptake of nutrients by the plant, which causes a depletion of these nutrients in the soil (Butt *et al*., 2017). For a maize plant to reach optimum yield potential, maize plants need readily available nitrogen during their key growth stages. The greatest demand for nitrogen during maize growth occurs at tasseling (75-90 DAP). At day 90 significantly ($p < 0.05$) higher nitrate concentration during the tillering stage and the low nitrate concentrations at maturing stage (120 DAP) were observed in all treatments. A possible reason could be the nitrification process at day 90, whereby nitrates get converted to ammonia (Zhang *et al*., 2015). Furthermore, the significant higher nitrate and significant lower ammonium observed in T3 and T5 soils at 90 DAP could also be explained by this result. Moreover, this is because maize plants require readily available nitrogen to reach optimum yield during their key growth stages (tillering) and this is also the time when the biggest nitrogen loss occurs in soil.

In addition, there were no significant differences in chemical parameters between Bt and non-Bt maize soils. Nevertheless, the observed differences could be due to variation in nutrient utilisation by soil bacteria (Powell *et al*., 2009; van Wyk *et al*., 2017). The application of fertilisers such as AM fungi and vermicompost could also contribute to this difference (Esperschütz *et al*., 2007). These findings are further substantiated by the RDA which indicated that maize (Bt and non-Bt) grown in AM fungi and vermicompost amended soils were strongly associated with the aforementioned chemical properties (Figure 4.6).

4.4.3 Enzymatic activities in soil

Enzymes such as urease, acid phosphatase and β-glucosidase play a pivotal role in the biogeochemical cycles of important elements such as nitrogen, phosphorus and carbon conversion, respectively (Makoi and Ndakidemi, 2008). Current results showed that the enzymatic activities analysed generally reacted differently depending on the treatment used. These changes are dependent on the maize growth stages and the change in root secretions during the different maize growth stages (Xu *et al*., 2009). The phosphate cycle enzyme activities are inversely related to phosphorus availability in soil

(Tadano *et al*., 1993) and when phosphorus is a limiting nutrient its demand increases, resulting in an increase in phosphatase activity, as usually found in natural AM fungi colonised rhizospheres. These results corroborate with the phosphorus results, which indicate a decrease over time (Figure 4.3B). The increase of acid phosphatase activity in soils of non-Bt maize could be attributed to the positive association with organic carbon, which also plays an important role in the soil nutrient cycle (Meena *et al*., 2013). According to Taylor (2002), enzyme activities of soils are usually correlated with their organic carbon and available nitrate contents. These results are corroborated by van Wyk *et al*. (2017), who also found percentage organic carbon to be positively correlated to urease, acid phosphatase and β-glucosidase activities in non-Bt maize fields. In previous studies involving Bt crops, differences among seasons, growth stages and crop varieties were independent of the presence of the Cry proteins in the plants and soils. Although there were some statistically significant differences in soil enzymes between Bt and non-Bt maize, they were temporary and varied among treatments (Icoz *et al*., 2008b; Shen *et al*., 2006; van Wyk *et al*., 2017). Hence, results from the present study are consistent with previous studies. Furthermore, the significant increase in acid phosphatase observed particularly at maturing stage was possibly due to the proliferation of phosphatase-producing microbes present in vermicompost (Perucci *et al*., 1988).

The significant increase in β-glucosidase attained particularly at maturing stage could be due to organic compounds such as cellulose present in vermicompost boost indigenous soil microorganisms to produce β-glucosidase (Uz and Tavali, 2014). In addition, the production of β-glucosidase and acid phosphatase could also be due to the presence of these enzyme substrates and high numbers of microorganisms (Uz and Tavali, 2014). When vermicompost is added to soil, resident β-glucosidase in the material remains active in soil and microorganisms added to soil may continue enzyme secretion resulting in elevated β-glucosidase activity (Albiach *et al*., 2000; Lai *et al*., 2002; Parthasarathi and Ranganathan, 2000; Aira *et al*., 2007; Doan *et al*., 2013). This significant ($p < 0.05$) increase found in urease activity during active growth (60 DAP) and at maturity stages (120 DAP), may be attributed to an increase in soil organic matter content. This finding is further substantiated by the RDA, which indicate that organic carbon was strongly associated with urease activity. In addition, the development of microbial populations favoured by root exudates of plants may also be responsible for the high enzyme activity present in the soil.

Current findings alongside Carpenter-Boggs *et al*. (2000) suggest that application of vermicompost with AM fungi could favour soil pH especially in acidic soils. Soil pH impacts on plant response by altering the availability of nutrients, the composition and diversity of the microbial communities present in soil and subsequent enzymatic activities (Dick *et al*., 2000). The observed positive relationship between urease activity and ammonium is explicable in terms of the positive effects of base cations on mineralisation (Brady, 1974). Urease activity correlated positively with soil percentage carbon, supports Schaller (2009) who reported a positive relationship between microbial enzyme activities and soil carbon. In addition, soil enzyme activities are strongly correlated to co-application of AM fungi and vermicompost. These correlations also suggest that although each enzyme depends on a specific substrate and takes part in specific reactions, the measurement of different enzyme activities in soil might be a valid tool for estimating the overall soil microbiological activity (García *et al*., 1997).

4.4.4 Arbuscular mycorrhizal colonisation in maize roots

Arbuscular mycorrhizal fungi (AM) fungi play a very crucial role in a growing maize plant as it improves phosphorus and micro-nutrient uptake by the roots and plant growth (Subramanian *et al*., 1997). It was evident from the data that root colonisation was significantly ($p < 0.05$) higher in T3 and T5, compared to T2 and T4. Furthermore, the increase in root colonisation was related to the slow release of nutrients from the vermicompost, mainly phosphorous and probably due to a change in the chemical properties and enzymatic activities of the soil (Pathma and Sakthivel, 2012). The release of inorganic P from vermicompost is slow, so no inhibition of AM fungi root colonisation occurred (Gosling *et al*., 2006). It was evident from the data that AM fungi root colonisation was higher ($p < 0.05$) in both Bt and non-Bt maize roots. This could be due to the nutrients supply from vermicompost to the maize plants. In addition, there is a possibility of vermicompost enhancing the plant's capacity to absorb water and nutrients, while at the same time increasing mycorrhizal colonisation (Carrenho *et al*., 2007; Jan *et al*., 2014; Hussain *et al*., 2016). Comparable results on increased root mycorrhizal colonisation in the presence of AM fungi and organic compost (vermicompost) on various non-transgenic crops such as maize (Carrenho *et al*., 2007), wheat (Jan *et al*., 2014 and Husssain *et al*., 2016), common millet (Shishebor *et al*., 2013), and coffee (Gómez-Valasco *et al*., 2014) have also been reported. Furthermore, results from the present study are consistent with previous findings by Sousa *et al*. (2012), suggesting that transport and absorption in mycelia of AM fungi were favoured

by humic substances like fulvic acids that result from the decomposition of organic fertilisers such as vermicompost. In addition, Cavender *et al*. (2003) have shown that nutrients present in vermicompost stimulated fungal colonisation in sorghum roots. Another important observation was the significant increase observed in AM fungi colonisation percentages observed at tillering (90 DAP) and decrease at maturity (120 DAP). This was reported in a study conducted by Abbott and Robson (1991), who observed that an increase in AM fungi root colonisation of crops takes place during the active root growth stages, followed by a decline when roots reached the biological ageing stage (senescence).

No significant differences were detected in AM fungi colonisation percentages between Bt and non-Bt maize roots at 60 and 120 DAP for all treatments. However, at 90 DAP significant differences in AM fungi colonisation percentage were observed between Bt and non-Bt maize. Furthermore, the reduced AM fungi colonisation percentages observed in Bt maize, may be ecologically significant. This could lead to a decrease in the abundance of AM fungi propagules in the soil over time, potentially impacting on soil structures and functions in areas where Bt crop cultivation is high. In contrast, sole AM fungi amendment showed significantly higher AM fungi colonisation percentages. These results reflect the nature of reports in literature regarding the effects of Bt maize plants on AM fungi colonisation (Castaldini *et al*., 2005; Cheeke *et al*., 2012; Cheeke *et al*., 2014; De Vaufleury *et al*., 2007; Tan *et al*. 2011; Verbruggen *et al*., 2012). For example, in a microcosm study on mycorrhiza colonisation, results showed that colonisation was significantly lower in Bt176 roots, compared to Bt11 maize roots (Castaldini *et al*., 2005). On the contrary, percentages of (not significant) AM fungi colonisation in Bt11 and MON810 were higher than in non-Bt maize roots (Tan *et al*., 2011). More recently, Cheeke *et al*. (2014) reported that there were no differences observed in AM fungi colonisation between field soils with a history of Bt maize cultivation and non-Bt cultivation. It is possible that such contradictory results are due to the differences in experimental designs, transgenic maize events used, the age of the growing plants, the species of AM fungi and fertilisers among others.

Additionally, studies have shown that variations in AM fungi colonisation are not directly linked to the presence of the Bt proteins (Cheeke *et al*., 2011; Cheeke *et al*., 2012). Thus, these results may be caused by other factors provided by the crops and different treatments or effects of the environmental conditions (Gavito *et al*., 2003). Perhaps, an

indirect effect of the genetic insertion within Bt plants (Naef *et al*., 2006; Prakash *et al*., 2011) may affect their ability to respond to or recruit AM fungi in the rhizosphere.

4.5 Conclusion

In this study, the impact of the genetic modification on mycorrhizal colonisation of Bt maize grown in organic –and bio-fertilisers were determined through a 120-day greenhouse study. The findings have demonstrated that maize dry matter, chemical properties and enzyme activities were significantly improved by the co-application of AM fungi and vermicompost. Significantly improved mycorrhizal root colonisation in maize was also observed due to the co-application of AM fungi and vermicompost. Despite no effect of the genetic modification being recorded in this study, it is possible that the presence of the Cry protein in Bt maize from roots may have ended up in the soil. However, this study did not investigate this possibility. Further long-term field trials are needed to establish a proper dose and frequency of applications of AM fungi and vermicompost in maize production in order to obtain the most beneficial influence on yield and its quality. In addition, the accumulating effect of Bt toxin in the rhizosphere should also be investigated. This accumulating effect has been reported to affect composition, activity of soil microbes as well as the plant growth and health (Sun *et al*., 2007; Chen *et al*., 2011).

CHAPTER 5

5.1 Conclusion and Recommendations

With increasing global population, crop production and environmental threats, ensuring future food security while reducing environmental pressure on agro-ecosystems requires improved application of biotechnology. The agricultural landscape has become more dependent on chemical fertilisers and GM crops to sustain food supply. This impacts directly and/or indirectly on the soil microbial community. Soil amendment processes such as application of organic and bio-fertilisers have the potential to boost nutrient and water efficiency. Thus, it is important to evaluate holistic solutions to these challenges to ensure various agricultural practices are sustainable. It is on this premise that the present study investigated the potential influence of vermicompost and AM fungi applications on rhizospheric microbial communities of maize (Bt and non-Bt) in agricultural soil.

A field study and greenhouse experiments were conducted for this investigation.

For the study, dryland and irrigated fields cultivated with Bt and non-Bt maize in the North West Province (MON810) were used. Furthermore, a greenhouse study was designed to investigate the potential of vermicompost application in the elimination or alleviation of the negative impact of genetic modification on the interaction between AM fungi and Bt maize. In addition, the study further investigates any potential effect of Bt modification on the direct and indirect functions of vermicompost in the soil. The potential impacts of genetic modification of Bt maize or soil amendments on rhizobacterial communities. Summary of the investigations are as provided below:

Ecological guild and enzyme activities of rhizosphere soil microbial communities associated with Bt Maize cultivation under field conditions in North West Province of South Africa

A scoping study was conducted to assess the structure and enzymatic activity of rhizosphere soil microbial communities associated with field grown Bt and non-Bt maize in North West Province of South Africa. Bacterial diversity was analysed using Illumina MiSeq sequencing and correlations between the physico-chemical, enzymatic properties and community structures were assessed by multivariate analysis.

The results from this study has shown that Bt and non-Bt maize fields had no apparent effect on the chemical properties, enzyme activities and the rhizospheric soil bacteria. However, nitrate and phosphorus concentrations were significantly ($p > 0.05$) higher in non-Bt maize soil samples of DL conditions, while organic carbon was significantly higher in non-Bt maize soil samples of IL conditions. Differences observed in acid phosphatase and β-glucosidase activities were significantly higher in both non-Bt maize fields, while urease showed no significant differences. This is important because these chemical properties and enzymes have implications on the decomposition processes resulting in nutrient recycling being affected (Poerschmann *et al*., 2005). Soil enzymes are important for catalysing a significant amount of reactions necessary for biological processes involving microorganisms in the soil, decomposition of organic residues, cycling of nutrients, and soil structure (Bandick and Dick, 1999). Furthermore, the variance observed in microbial community structures between Bt and non-Bt fields was also not related to the genetic modification of maize, but rather environmental factors and agricultural practices including, rain, pesticide applications, tilling, cultivation practices (e.g., compost versus chemical fertiliser).

Based on the overall observations, there was insufficient evidence to indicate any adverse effects of Bt maize on soil ecosystem function of the North West Province in South Africa. However, it may be mentioned that since the results reported in this paper represent a snapshot, because samples were not collected over the whole plant growth stage, which would have given more insight into the potential impacts of Bt maize over time or season. Hence, there is a need to conduct further long-term field studies over time and season.

Genetic modification or soil amendment: what factors drive the rhizobacterial communities of Bt maize plant?

Following the field study, high-throughput sequencing and multivariate analysis were used to assess which factors drive the rhizobacterial community of Bt maize (e.g. soil amendments or genetic modification of Bt maize) on rhizobacteria. This may identify possible bacterial indicators to predict biogeochemical changes in response to genetic modification disturbances.

The results provide a comprehensive understanding into the abundance of rhizobacterial communities, species richness and diversity in both Bt and non-Bt maize treatments. MiSeq Illumina sequencing data and multivariate analyses demonstrated that changes in physico-chemical properties as well as enzyme activities, due to addition of AM fungi and vermicompost, impacted Bt and non-Bt maize rhizosphere soil by changing the species abundances of communities. The results suggest that bacterial diversity and the abundances of taxa were significantly impacted by the different treatments.

The predominant bacterial phylum in all Bt and non-Bt maize rhizosphere soil treatments detected in the present study was Proteobacteria, followed by Verrucomicrobia, Firmicutes, Acidobacteria and Bacteroidetes. This observation is in agreement with Barriuso *et al*. (2012), Dohrmann *et al*. (2013) and Ondreičková *et al*. (2013) who reported Proteobacteria to be the dominant bacterial phyla associated with the rhizosphere soil of Bt maize and conventional maize. All of these phyla contain taxa commonly found within soil rhizospheres that are capable of having various effects on plant health including beneficial and pathogenic interactions (Berendsen *et al*., 2012; Philippot *et al*., 2013).

The detection of *Sphingomonas* have been consistently reported to be associated with maize soil (Mehnaz *et al*., 2007; Dohrmann *et al*., 2013; Bumunang *et al*., 2015; van Wyk *et al*., 2017). Therefore, it is suggested that these species could be used as key stone indicators in monitoring GM effects on maize rhizobacterial communities (Bumunang *et al*., 2015). The presence of *Acidovorax*, *Bacillus*, *Flavobacterium*, *Paenibacillus* and *Pseudomonas* in Bt maize treatments are of great importance for plant health, productivity and soil fertility, through functions such as nutrient mineralisation, production of phytohormones and antagonism of plant pathogens (Compant *et al*., 2010; Egamberdiyeva 2007). In addition, growth-promoting

microorganisms beneficial for the plant growth appeared to have been more abundant in the current study than in the field study (chapter 2).

The rhizosphere soil bacterial community of Bt maize differed from those of non-Bt maize across plant growth stage and between bio-inoculants as well as bio-fertilisers. When compared to the field study (Chapter 2) the rhizosphere soil bacterial community differences were related to agricultural practises, cultivar type and environmental parameters. These differences were more pronounced between the diversity and abundance of particular species, rather than in the species richness of the maize bacterial community. However, further research is necessary to classify the taxa up to species level to determine the species indicators in monitoring GM effects on maize rhizobacterial communities.

Vermicompost application: A potential solution to challenges associated with interactions between Bt maize and arbuscular mycorrhizal fungi

Although GM maize crops have become one of the most important agricultural crops globally, which ensure improved nutritional composition, higher yields, and resistance to pest, microbe-meditated processes and functions in soils may be at risk. Accordingly, the use of biological fertilisation including the use of arbuscular mycorrhizal fungi and vermicompost has been suggested as a useful method of fertilisation.

Differences between Bt and non-Bt maize treatments were observed for dry matter, chemical properties, enzyme activities and AM fungi root colonisation. The high dry matter yield observed for Bt maize treatments was possibly due to the conjoint effect of the genetic modification of Bt maize and the co-application of AM fungi and vermicompost. Moreover, the significantly lower ($p < 0.05$) pH in Bt maize control soils (T1) and significantly higher potassium concentration observed in non-Bt maize soils amended with both AM fungi and vermicompost (T5) were more related to plant growth stages and bio-inoculants and bio-fertilisation, rather than the genetic modification of maize. This was also true for the significantly higher ($p < 0.05$) urease activity at 60 DAP in Bt maize soils (T3) and acid phosphatase activity at 120 DAP in non-Bt maize soils (T5). Furthermore, AM fungi colonisation at 90 DAP was the only difference observed between Bt and non-Bt maize roots showed.

The current study endorses the co-application of AM fungi with vermicompost to significantly increase mycorrhizal root colonisation of in both Bt and non-Bt maize.

Besides, farmers would also be benefitted economically by using less chemical fertilisers. Furthermore, this co-application of AM fungi with vermicompost can also enhance root development, mycorrhizal colonisation and soil nutrient uptake (Shishehbor *et al*., 2013; Hussain *et al*., 2016). These beneficial effects of AM fungi with vermicompost on plants are attributed mainly by the additional soil nutrient supply

Future studies would benefit from examining bacterial community structure in a variety of Bt and non-Bt maize genotypes grown in a range of soil conditions amended with AM fungi and vermicompost in the field. Effects would likely be most pronounced in low input agricultural systems, areas where nutrients have been depleted, or during conditions of drought stress especially in countries such as South Africa.

5.2 Recommendations

The following recommendations are proposed:

- Future research should evaluate continuous growing of Bt maize for several years in the same field monitoring yield and soil functions, which might affect adversely the soil microbial function over time. It is also important to measure laccase activities of soils to determine if there are changes in the degradation of lignin whose content is sometimes affected by genetic manipulation of plants.

- Future studies should focus on the polyphasic approach. This approach exploits both conventional and molecular tools. The approach aims to generate all genotypic, phylogenetic and phenotypic information of a microbial taxon (Vandamme *et al*., 1996; Das *et al*., 2014). By selecting beneficial soil microorganisms based on ecological importance and their response to changes may also be employed to monitor possible effects of Bt plants using polyphasic molecular approach for comparison of different methods.

- Further field trails are needed to establish a proper dose and frequency of applications of AM fungi and vermicompost in Bt crop production in order to obtain the most beneficial influence on yield and nutrients uptake under various agro-ecological conditions. The co-application of AM fungi with vermicompost could improving plant growth, physico-chemical parameters and soil enzyme activities. Thus improving soil fertility and may pave newer ways to develop methods for production of higher agricultural outputs in an organically and ecologically sustainable manner.

- Extended field observations (trials) investigating additional maize varieties over more years need to be included in future studies. With this information, we would be able to determine whether the altered composition is attributable to the presence of transgenic crops, or is simply part of the variation driven by the presence of different genotypes. These studies should also involve different soil types and long-term monitoring to account for the variability of the natural environment. In addition, the accumulating effect of Bt toxin in the rhizosphere should also be investigated. This accumulating effect has been reported to affect

composition, activity of soil microbes as well as the plant growth and health (Sun *et al*., 2007; Chen *et al*., 2011).

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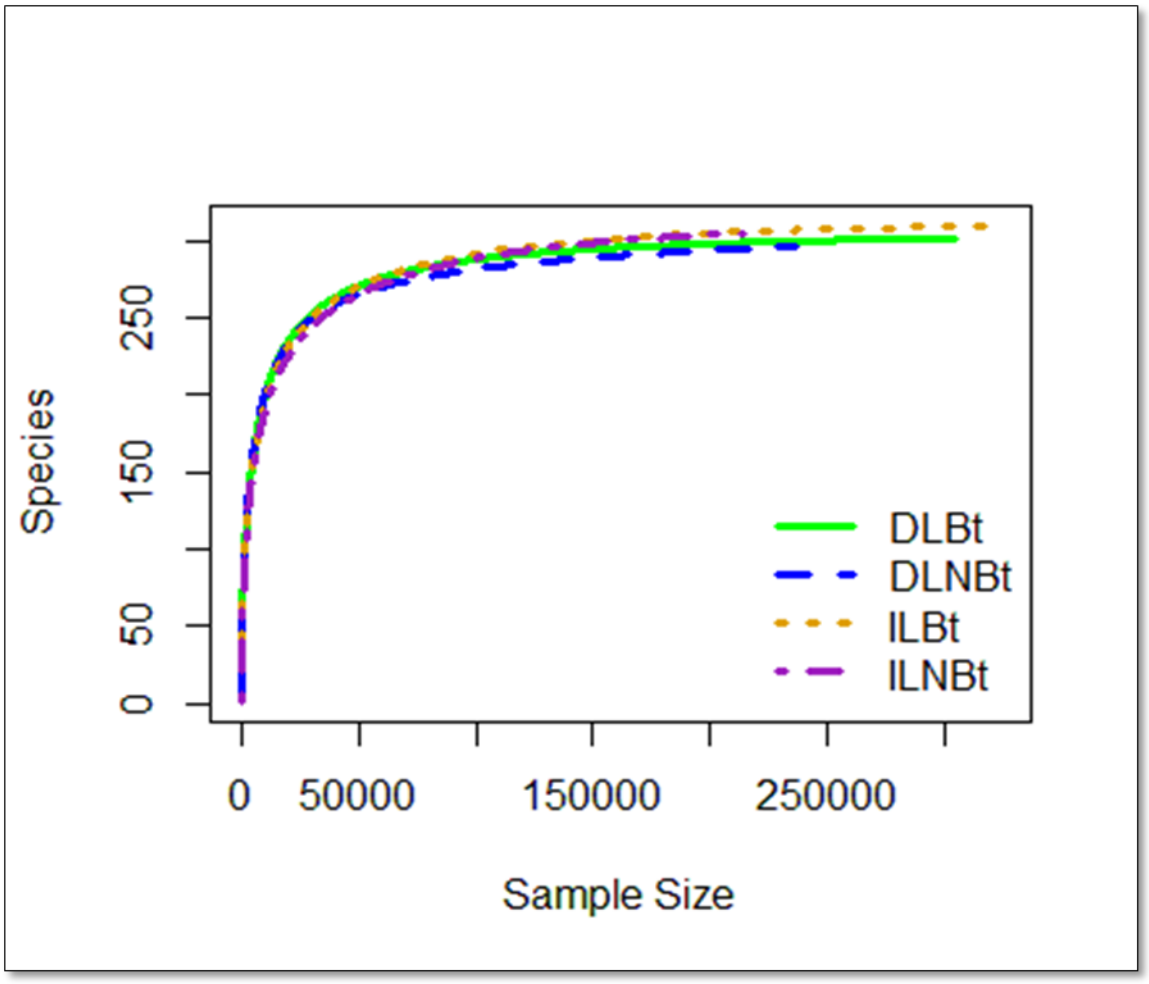
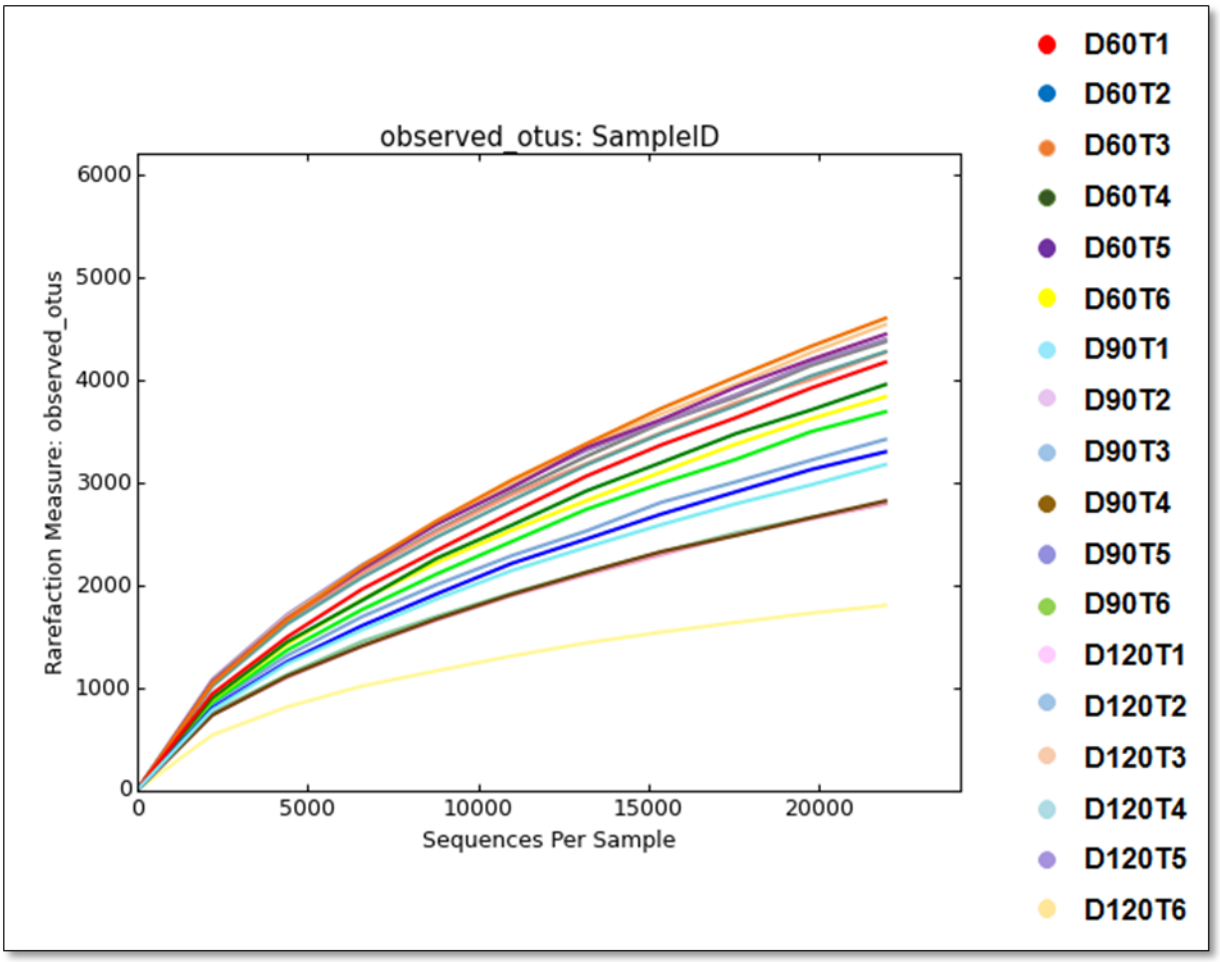
ANNEXURES

Supplementary Figures



Supplementary Figure 2-S1: Rarefaction curve for each field. DLBt and DLNBt represent the dryland Bt and non-Bt maize samples, while ILBt and ILNBt represent the irrigated Bt and non-Bt maize samples.



Supplementary Figure 3-S1: Rarefaction curve for each Bt and non-Bt maize treatment (T1-T6) over time field.