THE EFFECT OF GLYPHOSATE AND GLYPHOSATE-RESISTANT MAIZE AND SOYBEANS ON SOIL MICRO-ORGANISMS AND THE INCIDENCE OF DISEASE

By

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This dissertation is dedicated to my late grandfather, best friend and life mentor,

Karel Retief Wolmarans

DECLARATION

I declare that the dissertation hereby submitted by me for the degree *Magister Scientae* at the University of the Free State is my own independent work and has not previously been submitted by me at another university/ faculty. I furthermore cede copyrights of the dissertation in favour of the University of the Free State.

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Karen Wolmarans

PREFACE

Glyphosate is the most widely used herbicide in agriculture. Previous studies have reported negative as well as stimulatory effects of glyphosate on soil microbes and plant pathogens. The glyphosate resistant gene inserted into crops has been demonstrated to change plant physiology and root exudates.

The aim of the present study was to determine the effect of glyphosate and glyphosate resistant (GR) maize and soybeans on rhizosphere microbes and the incidence of plant disease on these crops. In chapter 1, current literature on how herbicides and herbicide tolerant (HT) crops influence soil micro-biota was reviewed. Views on the topic remain controversial, with some researchers supporting the positive influence of genetically modified (GM) crops and herbicides on soil micro-organisms, while others differ. Emphasis was placed on negative and positive effects of herbicides *per se* on soil micro-biota in general with specific reference to glyphosate, and the effect of GR crops, either positive or negative, on soil micro-organisms and plant nutrition.

In Chapter 2 the effect of nine different herbicides on eight fungal species was investigated *in vitro.* The aim was to demonstrate the inhibitory effect of herbicides on soil-borne fungi by making use of agar amended with herbicides at different concentrations.

Chapter 3 focused on the toxic effects of 2,4-D-Dichlorophenoxyacetic acid (2,4-D), paraquat, and glyphosate on soil microbes. The aim was to determine the

effect of these three herbicides on total microbial and bacterial activity in soil microcosms.

Chapters 4 & 5 focused on the effect of GR maize and soybeans, respectively, on rhizosphere microbes. It was hypothesized that the GR gene inserted into GR maize and soybeans changes plant physiology and root exudates to such an extent that rhizosphere organisms decrease in terms of their activity.

Chapters 6 & 7 focused on the effect of GR maize and soybeans, as well as glyphosate application, on the incidence of disease. Two plant pathogens, *Fusarium verticillioides* (Sacc.) Nirenberg and *Sclerotinia sclerotiorum* (Lib.) de Bary, respectively were inoculated into soil in which the two crops were grown. It was hypothesized that GR maize and soybeans, and glyphosate application, will lead to an increase in the incidence of disease compared to conventional cultivars.

LIST OF ABBREVIATIONS

2,4-D	2,4-D-Dichlorophenoxyacetic acid
a.i	Active ingredient
ANOVA	Analysis of variance
AWCD	Average well colour development
BSA	Bovine serum albumin
BT	*Bacillus thuringiensis*
C	Carbon
Ca	Calcium
$CaCl_2$	Calcium chloride
cfu	Colony forming units
CLPP	Community-level physiological profiling
Cu	Copper
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
E	East
EPSP	5-enolpyruvyl shikimic acid-3-phosphate
FDA	Fluorescein diacetate
Fe	Iron
gDNA	Genomic DNA
GM	Genetically modified
GR	Glyphosate resistant
HPLC	High performance liquid chromatograph

HT	Herbicide tolerant
KMnO$_4$	Potassium permanganate
KOH	Potassium hydroxide
LSD	Least significant difference
Mg	Magnesium
MgCl$_2$	Magnesium chloride
Mn	Manganese
MnO$_4^-$	Permanganate
N	Nitrogen
Ni	Nickel
NW	North-west
OPS	Organically poor soil
ORS	Organically rich soil
P	Phosphorous
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
rDNA	Recombinant DNA
rpm	Revolutions per minute
S	South
SOP	Standard operating procedure
UFS	University of the Free State
UV	Ultra violet
WA	Water agar
Zn	Zinc
GW	Glifosaat weerstandbiedend

CHAPTER 1

The influence of herbicides and genetically modified herbicide resistant crops on soil micro-biota

ABSTRACT

Much controversy exists regarding the use of herbicides and herbicide tolerant (HT) crops in agro-ecosystems, particularly with regard to environmental and human safety. Concern has also been raised regarding the potential increase in crop disease incidence and severity caused by the increased cultivation of HT crops and use of herbicides. The use of herbicides and HT crops are said to adversely alter soil microbial biodiversity, thus negatively influencing the soil ecosystem. This practice could in turn lead to a decrease in soil fertility and plant nutrition, leading to weakened crops that are more susceptible to pathogen attack.

Keywords: *Glyphosate; Glyphosate resistant crops; Herbicides; Herbicide tolerant crops; Rhizosphere organisms.*

1.1.   INTRODUCTION

The soil ecosystem is extremely complex, containing many thousands of different species of bacteria, protozoa, fungi, micro- and macro-fauna (Young & Crawford, 2004; Turbé *et al*., 2010). This spatially and temporally variable soil community provides many key 'ecological services' to agriculture and the wider environment (Young & Crawford, 2004; Turbé *et al*., 2010). The maintenance of soil quality is therefore critical for ensuring the sustainability of food production and its positive effect on the environment (Bastida *et al.,* 2008).

Soil is a dynamic system in which physical, chemical and biological components interact (Bastida *et al.,* 2009). Within this system, micro-organisms perform an important task in the decomposition and transformation of organic soil materials, which is crucial for the functioning of the carbon, nitrogen and phosphorous cycles (Bastida, *et al.* 2009). Soil provides a complex medium for many positive and negative interactions with crop plants in the agro-ecosystem, thus affecting the productivity and sustainability of the cropping system above- and below-ground (Young & Crawford, 2004; Turbé *et al.*, 2010).

Unwanted side effects of xenobiotic compounds on non-target organisms are an environmental concern (Carlisle & Trevors, 1988). Unfortunately it is often difficult to measure and predict the impacts of toxic chemicals, such as herbicides, on natural communities (Marrs & Frost, 1997). Another important factor to keep in mind when assessing possible impacts of pesticides on the ecosystem is the fact that pesticides differ from each other with regard to their environmental behaviour and toxicological profile. Differences include chemical structure of the xenobiotic compound, different

dose-response relationships, the type of organisms sensitive to toxic effects and the nature of toxic effects caused by the pesticide (Van Eerd, Hoagland & Hall, 2003; Kleter *et al.*, 2008).

Since soil micro-organisms play critically important roles in soil ecosystem processes, it is important to examine the impact of herbicides, as well as genetically modified (GM) crops, on the dynamics of micro-organisms in the rhizosphere (Dunfield & Germida, 2004). Any impact that GM plants or herbicides may have on the rhizosphere, and associated microbes, could in turn have positive or negative effects on plant growth and health, and ecosystem sustainability. Transgenic or GM plants possess novel genes that can impart beneficial characteristics such as herbicide tolerance. The potential for interaction between transgenic plants and the soil microbial community is not well understood. Consequently, acknowledgement that these interactions could affect ecosystem functioning has initiated a number of studies in this area (Dunfield & Germida, 2004). Novel proteins have for example been shown to be released from transgenic plants into the soil ecosystem, eventually influencing the biodiversity of microbial communities by selectively stimulating the growth of organisms that can utilize them as nutrients (Andersen *et al.*, 2007; Partoazar, Hoodaji & Tahmourespour, 2011).

Changes in soil microbial communities associated with growing transgenic crops are less drastic and transient in comparison with agricultural practices such as crop rotation, tillage, herbicide usage, and irrigation (Dunfield & Germida, 2004). Yet, minor alterations in the diversity of the microbial community, such as the removal or appearance of specific functional groups of bacteria such as plant-growth-promoting

rhizobacteria, phytopathogenic organisms or key organisms responsible for nutrient cycling processes, can affect ecosystem functioning. The impact of plant genotype on the dynamics of rhizosphere microbial populations therefore requires further study (Dunfield & Germida, 2004).

Much controversy surrounds the use of glyphosate in agro-ecosystems for weed control. Some researchers believe that the use of glyphosate and glyphosate-resistant (GR) crops hold no threat for agricultural sustainability or soil and environmental quality (Araujo, Monteiro & Abarkeli, 2003; Duke & Cerdeira, 2007; Kleter *et al.*, 2008), while others choose to believe the opposite (Sanogo, Yang & Scherm, 2000; Neumann *et al.*, 2006; Huber, 2010). Interest in microbial functionality has grown in recent years as researchers seek to understand the relationship between microbial communities and their surrounding environment (Bastida, *et al.,* 2009). One approach toward studying the impact of GM plants on soil micro-organisms is to study the structure and functioning of the whole community, rather than to focus on a specific group of micro-organisms (Dunfield & Germida, 2004).

This review will focus on (i) the effects, both inhibitory and stimulatory, of herbicides *per se* on soil micro-biota in general, with specific reference to glyphosate; and (ii) the effect, either positive or negative, which herbicide resistant crops have on soil micro-organisms and plant nutrition.

1.2.   THE EFFECT OF HERBICIDES ON SOIL MICRO-BIOTA

Agrochemical manufacturers constantly pursue the development of agrochemicals that are: (i) effective against target organisms, (ii) not persistant in the

environment, (iii) and have low toxicities to non-target organisms (Carlisle & Trevors, 1988). However, the excessive use of agrochemicals in conventional crop management has caused serious environmental and health problems, including loss of biodiversity and certain human disorders (Liu *et al.*, 1999; Ghorbani *et al.*, 2008). Regardless, herbicides are widely used in modern agriculture to control weedy plant species (Liu, Punja & Rahe, 1997). High crop productivity requires protection of crops against competition from weeds and attack by pathogens, and herbivorous insects (Oerke & Dehne, 2004). The heavy utilization of pesticides and, their persistence and transfer into trophic food webs can however cause major environmental contamination (Imfeld & Vuillemier, 2012). Similarly, concern regarding their effect on non-target organisms has grown considerably (Nyström, Björnsäter & Blanck, 1999; Cedergreen & Streibig, 2005; Sebiomo, Ogundero & Bankole, 2011).

Serious questions are being raised about the potentially harmful effects of pesticides on consumers and the ecosystem (Zaltauskaite & Brazaityte, 2011). There is increasing concern that herbicides not only affect target organisms but also non-target organisms such as microbial communities present in the soil environment (Haney, Senseman & Hons, 2002; Partoazar *et al.*, 2011; Sebiomo *et al.*, 2011). These non-target effects may impact on many important soil functions such as organic matter degradation and the nitrogen cycle (Sebiomo *et al.*, 2011; Zaltauskaite & Brazaityte, 2011). Ignoring the potential non-target detrimental side effects of any agricultural chemical, may therefore have dire consequences for food security, such as rendering soils infertile, crops non-productive, and plants less nutritious (Altman & Campbell, 1977).

The soil ecosystem can be altered by herbicides through direct and indirect effects on various components of the soil microflora, including saprophytes, plant pathogens, pathogen antagonists or mycorrhizae (Lévesque & Rahe, 1992; Ghorbani *et al.*, 2008; Sanyal & Shrestha, 2008), which can result in increased or decreased disease incidence. Phytotoxicity, and disease enhancement, are two of the most commonly reported problems of herbicide use on crops. It is generally accepted that herbicide-induced weakening of a plant can predispose the plant to infection by facultative pathogens (Lévesque & Rahe, 1992).

1.2.1. Negative herbicidal effects

The usage of herbicides may have indirect impacts on the whole ecosystem. These indirect impacts may be relatively severe since herbicide effects on target as well as non-target organisms may disrupt community structure and ecosystem function (Zaltauskaite & Brazaityte, 2011). Applied pesticides ultimately reach the soil in large amounts where they accumulate, leading to pesticide residues which can be ingested by invertebrates, absorbed by plants or broken down into other toxic products (Subhani *et al.*, 2000). There is a significant response of soil microbial activity to herbicide treatment, either directly to the herbicide or to the breakdown products of the herbicide. Adaptation of microbial communities to increasingly higher herbicide concentrations and chemical residues can occur over weeks of continuous treatment (Sebiomo *et al.*, 2011).

1.2.1.1. *Microbial biomass*

Herbicides have been shown to affect microbial biomass in soil. For instance, the use of the uracil herbicide group, with the active ingredient (a.i) bromacil, reduces microbial biomass significantly, an effect that can last up to 11 months after application (Sanders, Wardle & Rahman, 1996). A significant reduction in microbial biomass can consequently delay the breakdown of this active ingredient (a.i). Furthermore, severe stress on soil microflora caused by bromacil may interfere with the ability of microbes to degrade the herbicide during repeated applications (Sanders *et al.*, 1996). Similarly, the application of imazethapyr to a silty loam and a loamy soil leads to a shift in the soil community structure. Soil microbial biomass carbon (C) is reduced after imazethapyr application (Zhang *et al.*, 2010).

1.2.1.2. *Fungi*

Plant-herbicide-pathogen interactions can have negative repercussions that should not be ignored (Altman & Campbell, 1977). For example, when the roots of plants that have been treated with herbicides die, they become colonized by facultative parasites such as *Pythium* spp., *Rhizoctonia solani* Kühn and *Fusarium* spp. as a result of the exudation of sugars and other carbon sources from the dead roots (Sullivan, 2004). *Rhizoctonia* root disease of wheat increased when a mixture of paraquat and diquat was applied close to the sowing date (Roger *et al.,* 1994). The problem was due to a lack of competing organisms, and was overcome by allowing a greater time between application and sowing date (Roger *et al.,* 1994), in order to allow for competition by soil micro-organisms. It has been observed that the application of

glyphosate or paraquat in bean fields also results in an increase of *Pythium* spp. in the soil (Descalzo *et al.*, 1998).

1.2.1.3. *Bacteria*

Herbicides have been shown to have negative impacts on soil bacterial populations, either directly or indirectly. For example, no decrease in bacterial numbers in soil treated with atrazine was observed, yet untreated soil showed an eightfold increase in bacterial numbers (Cole, 1976). Although repeated application of atrazine did not affect the abundance of bacteria producing hydrolytic enzymes, a transient inhibition of bacterial growth was observed during the first week of application (Cole, 1976). The mere observation that bacterial numbers did not increase nor decrease with atrazine application does not suggest that this herbicide has no effect on the bacterial populations. In fact, the increase in bacterial numbers in untreated soil suggests that the atrazine does in fact negatively affect bacterial populations.

Soil bacterial populations have also been shown to be much lower, during the first week after herbicide application, in soils treated with atrazine, primextra, paraquat and glyphosate respectively (Sebiomo *et al.*, 2011), while paraquat has also been shown to greatly stress and inhibit bacterial populations temporarily (Kopytko, Chalela & Zauscher, 2002). Glyphosate has also been observed to cause a decrease in pseudomonad populations, which antagonize fungal pathogens in soil (Kremer & Means, 2009). It has also been observed that alachlor and paraquat are toxic to bacteria (Sahid, Hamzah & Aris, 1992).

1.2.1.4. *Other micro-organisms*

Certain herbicides have been shown to be toxic to some soil fauna. For instance, paraquat has been shown to be toxic to non-target organisms, such as *Collembola* (Curry, 1970). Similarly, Zaltauskaite & Brazaityte (2011) observed that the application of three herbicides with different active ingredients, namely amidosulfuron, iodosulfuron, and sodium salt, caused 50-100% mortality of the micro-invertebrate *Daphnia magnal* due to runoff into drainage sites and rivers. Atrazine application to soil may also affect certain Collembola species, such as *Entomobrya musatica* Stach. (Al-Assiuty & Khalil, 1996). Effects include direct toxicity and negative effects on reproduction and the fecundity of the animals which could adversely affect abundance and development of the organism (Al-Assiuty & Khalil, 1996). In contrast, Sabatini *et al* (1998) observed no direct effect of the herbicide triasulfuron, at recommended field rate, on the Collembola species, *Onychiurus pseudogranulosus* Gisin. Atrazine may however be taken up through the body surface, even when applied at the recommended field rate, and lead to a direct lethal effect (Sabatini *et al.*, 1998). Atrazine and monuron have been shown to decrease the number of wireworms and springtails in grassland soils. In addition, atrazine has also been shown to reduce earthworm populations in grassland soils (Fox, 1964).

Any impact herbicides may have on soil fauna may adversely affect plant health due to a decrease in mineral and oxygen availability as a result of less channeling in soil. A further effect is less predation of potential plant pathogenic organisms by other soil fauna (Brown *et al.*, 2001). Whatever effect herbicides have on soil fauna, it can

result in a shift in the soil faunal community which will have a positive or negative impact on ecosystem functions.

1.2.2. Positive herbicidal effects

Most herbicides used at normal field rates are generally considered to have no major or long-term effect on gross soil microbial activities (Subhani *et al.*, 2000; Zabaloy, Garland & Gómez, 2008). However, some reports indicate that herbicide application to soil may lead to the proliferation of general or specific organisms which can utilize a particular chemical in the herbicide for nutrition (Audus, 1951; Brazil *et al.*, 1995; Paulin, Nicolalsen & Sørensen, 2011). This observation can be substantiated by the fact that certain herbicides, especially hormone-based types, can disappear from the soil due to microbial decomposition (Chandra, Furtick & Bollen, 1960). The degradation process by soil micro-organisms is probably the most important pathway responsible for the breakdown of herbicides (Curran, 1998; Subhani *et al.*, 2000). The synergistic interaction of the microbial community in the rhizosphere may also facilitate degradation of recalcitrant compounds (Costa, Camper & Riley, 2000). For instance, atrazine concentration decreases in the rhizosphere compared to non-vegetated areas (Costa *et al.*, 2000). The degradation of atrazine is higher in a rhizosphere dominated system, where the half-life is 7 days, compared to non-vegetated soil where the half-life is greater than 45 days (Costa *et al.*, 2000). Similarly, mesotrione, a selective herbicide used for maize crops, applied at the recommended field rate is quickly dissipated from a chernozem[1] soil type and has no consistent impact on soil microbial communities

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[1] Chernozem soil: Black, humus-rich grassland soil (Anonymous, 2012).

(Crouzet *et al.*, 2010). This suggests that the herbicide is degraded by soil micro-organisms. However, Crouzet *et al* (2010) also stated that mesotrione, at doses far exceeding the recommended field rates, has an impact on non-target soil organisms.

1.2.2.1. *Microbial biomass*

The amount of herbicide available to soil micro-organisms depends on various factors, including available nutrients, pH, temperature, and moisture, although these factors differ in importance depending on the pesticide involved (Weber *et al.*, 1993). For instance, the application of bentazon at the recommended field rate to soil does not significantly affect the microbial community, even in the absence of microbial degradation (Allievi *et al.*, 1996). The addition of atrazine to a semi-arid soil with low organic matter content, resulting in increased microbial activity, can be explained by adaptation of the resident microbial community to the xenobiotic (Moreno *et al.*, 2007)

1.2.2.2. *Fungi*

Fungi react differently to herbicides, even within the same genera. For instance, three different Basidiomycete species were reported to have different degradation rates on the herbicides chlortoluron, isoproturon and diuron. *Ceriporiopsis subvermispora* degraded chlortuloron 18%, isoproturon 60% and diuron 18%; *Coniophora puteana* 13%, 69% and 38% respectively, and *Phlebia radiate* 33%, 25% and 82%, respectively (Khadrani *et al.*, 1999). Claims have been made that repeated application of atrazine does not affect the number of viable fungi in any way (Cole, 1976), suggesting that herbicides can elicit different reactions by different fungi. Certain fungal species are

benefitted by herbicide addition, while others are inhibited. This could lead to the false perception of increased total microbial activity, while in actual fact only a specific population of organisms which are able to utilize the specific herbicide increased. For instance, herbicides may reduce the severity of plant diseases by stimulating certain microbial antagonists which can suppress soil pathogens (Katan & Eshel, 1973).

1.2.2.3. Bacteria

The degradation of atrazine in soils is a result of the activity of bacteria which are able to use the compound as a source of carbon (C) or nitrogen (N) (Mandelbaum, Wackett & Allan, 1993). An increase in soil microbial respiration observed after atrazine addition could thus be due its utilisation as a substrate for micro-organisms such as *Pseudomonas* spp. (Mandelbaum *et al.*, 1993). The stimulation of bacterial populations in soil by atrazine (Ros *et al.*, 2006) as well as the stimulation of aerobic heterotrophic bacterial populations by glyhosate, 2,4-D-Dichlorophenoxyacetic acid (2,4-D), and metsulfuron (Zabaloy *et al.*, 2008) has also been documented. Kremer & Means (2009) reported that glyphosate increases the proportion of bacteria able to oxidize manganese (Mn).

1.2.3. Negative effects of glyphosate

The introduction of GR crops has greatly increased the volume and scope of glyphosate usage (Cerdeira & Duke, 2006; Huber, 2010; Riley & Cotter, 2011). Glyphosate leaves a residue trail after application, thus an interaction between glyphosate and plant nutrition is obvious.   The extensive use of glyphosate has

intensified deficiencies of numerous essential micronutrients, and some macronutrients (Kremer & Means, 2009; Tesfamariam *et al.*, 2009; Huber, 2010; Riley & Cotter, 2011). Several enzymes function with Mn in the shikimate pathway and are responsible for plant responses to stress and defense against pathogens. Inhibition of the enzymes in the shikimate pathway of a plant renders it highly susceptible to various soil-borne pathogens, such as *Fusarium, Pythium, Phytophthora* and *Rhizoctonia* (Huber, 2010).

In contrast to microbial toxicity, glyphosate in soil stimulates oxidative soil microbes that reduce nutrient availability by decreasing their solubility for plant uptake (Huber 2010). An increase in the proportion of Mn-oxidizing bacteria and a decrease in the pseudomonad component that antagonize fungal pathogens has for example been reported in the rhizosphere of GR soybean and maize (Kremer & Means, 2009).

1.2.3.1. *Plant Pathogenic fungi*

Glyphosate blocks the synthesis of phenylalanine-derived phenols via the inhibition of the enzyme 5-enolpyruvyl shikimic acid-3-phosphate synthase (EPSPS), thereby inhibiting the production of phenolics, including lignin precursors and some classes of phytoalexins involved in resistance of plants to disease (Lévesque & Rahe, 1992). Glyphosate also stimulates soil borne pathogens and other soil microbes to reduce nutrient availability (Huber, 2010). It also reduces nutrient uptake and efficiency and increases drought stress in plants. Glyphosate is reportedly a potent micro-biocide and reduces beneficial organisms involved in the suppression of soil-borne diseases (Huber, 2010).

Glyphosate application increased *Pythium* populations in a muck soil after foliar application to bean seedlings, probably because the root residues of the dying plants caused a temporary elevation in populations of the pathogen which consequently increased the damping-off potential of the soil (Descalzo *et al.*, 1998). Glyphosate also increased *Fusarium solani* f. sp. *glycines* in the rhizosphere of GR soybean (Sanogo *et al.*, 2000) and disease caused by *R. solani* and *Fusarium oxysporum* Schlecht. F. sp. *Betae* Snyd. & Hans. in GR sugar beet (Larson *et al.*, 2006). However, Njiti *et al* (2003) showed that glyphosate and GR soybean did not increase *Fusarium solani* (Mart.) Sacc f.sp. *glycines* significantly. The effect was presumably due to a genotype interaction.

Glyphosate has been found to increase root disease of wheat (caused by various *Pythium* spp.) in a minimum tillage situation when it was used to kill weeds close to the date of sowing (Pittaway, 1995). The increase was attributed to the pathogens increasing their inoculum potential on the weed residues prior to sowing (Pittaway, 1995). This probably occurs because of the predisposition of weeds to *Pythium* infection (Lévesque & Rahe, 1992), availability of glyphosate as a nutrient source and a temporary reduction in populations of competing micro-organisms (Partoazar *et al.*, 2011).

1.2.3.2. *Other micro-organisms*

The application of glyphosate to unsterile soil is reported to decrease bacterial populations (Mekwatanakarn & Sivasithamparam, 1987), and in other instances increase populations (Partoazar *et al.,* 2011). There are also reports of increased populations of actinomycetes after treatment with glyphosate (Araujo *et al.,* 2003;

Carlisle & Trevors, 1988). In contrast, there are reports that the application of glyphosate and a mixture of diquat and paraquat, respectively, to unsterile soil had no effect on actinomycete numbers (Mekwatanakarn & Sivasithamparam, 1987). Glyphosate has also been shown to be toxic to earthworms (Huber, 2010).

1.2.4. *Positive effects of glyphosate*

In addition to increasing disease incidence (Descalzo *et al.*, 1998; Huber, 2010), glyphosate exhibits activity against some fungi, which provide disease control benefits (Anderson & Kolmer, 2005; Feng *et al.*, 2008). It has been shown to have both preventive as well as curative activity against *Puccinia striiformis* f. sp. *tritici* (Erikss) CO Johnston and *Puccinia triticina* Erikss in GR wheat (Anderson & Kolmer, 2005; Feng *et al.*, 2008). Glyphosate also reportedly reduces the incidence of Asian soybean rust, *Phakospora pachyrhizi* Syd & P Syd in GR soybeans (Feng *et al.*, 2008).

The degradation of glyphosate in most soils is slow or non-existent, since it is not "biodegradable" and degradation is primarily by microbial co-metabolism when it does occur (Huber, 2010). Araujo *et al* (2003) however, claimed that glyphosate is indeed biodegraded by soil micro-organisms and that this phenomenon has a positive effect on soil microbial activity in both the long- and short term. Soil microbial activity increases with the application of glyphosate. This could be due to the utilization of glyphosate as a potential C or nutrient source (Partoazar *et al.*, 2011). In addition, glyphosate may also serve as a more utilizable phosphorous (P) source to soil microbes rather than a C source (Partoazar *et al.*, 2011). An increase in microbial activity due to glyphosate application may be beneficial or detrimental toward soil quality. Beneficial effects

include optimum plant growth and production due to greater availability of nutrients, resulting from mineralization of glyphosate mediated by soil micro-organisms. On the other hand, increased microbial activity and high microbial populations may also sequester plant nutrients in microbial biomass, decrease crop growth and yields, and increase susceptibility to pests and disease (Yamada & Xe, 2000; Wolf & Wagner, 2005).

1.2.4.1. *Fungi*

Microbial activity can be stimulated by the presence of glyphosate (Busse *et al.,* 2001; Haney *et al.*, 2002; Partoazar *et al.*, 2011). Some studies report increased fungal populations following treatment with glyphosate (Carlisle & Trevors, 1988; Araujo *et al.,* 2003), as well as with a mixture of diquat and paraquat (Mekwatanakarn & Sivasithamparam, 1987). This might be due to the fact that certain fungi are able to use glyphosate as a nutrient and energy source (Araujo *et al.*, 2003). Krzysko-Lupicka & Orlik (1997) concluded that glyphosate added to a sandy clay soil with a history of repeated glyphosate treatment, appeared to select for specific fungal species that were able to use it as a nutrient source.

1.2.4.2. *Bacteria*

The heterotrophic bacterial population in a soil with a long history of glyphosate application increases significantly after glyphosate application. This could be due to the bacterial population using the herbicide as a nutrient source (Partoazar *et al.*, 2011). Busse *et al* (2000) also observed an increase in total and viable bacteria after

glyphosate application, with *Pseudomonas, Arthrobacter, Xanthomonas,* and *Bacillius* spp. increasing in population dominance.

1.3. THE EFFECT OF GENETICALLY MODIFIED HERBICIDE RESISTANT CROPS ON SOIL MICRO-BIOTA

The interaction of GM crops with soil biota is complex, requiring both specific and broad spectrum assessments (Birch *et al.*, 2007). The soil biotic structure is affected by most of the common variables in agricultural practices, including crop species, water stress, fertilization, soil tillage, pesticide regimes, soil type and depth. Thus it is not suprising that GM crops also have some effect on the soil ecosystem (Birch *et al.*, 2007; Duke & Cerdeira, 2007).

In 2005, almost 90% of the 100 million hectares of transgenic crops grown annually worldwide were GR or had GR genes stacked with *Bacillus thuringiensis* (BT*)* toxin-based insect resistant genes (Duke & Cerdeira, 2007). This state of affairs raised concern about GM crop-associated changes in crops and management practices (Birch *et al.*, 2007). Furthermore, the increasing use of GR crops has also increased concerns regarding the potential environmental impact of glyphosate (Haney *et al.*, 2002). Apparently no significant negative environmental effects have been documented in areas where these GR crops are grown (Duke & Cerdeira, 2007). Claims have, however, been made that GR crops may significantly alter rhizosphere communities (Hart *et al.*, 2009).

Pline-Srnic (2005) expressed concern among growers about GR crops which include perceptions of increased sensitivity to diseases and environmental stress.

Enhanced root colonization of GR crops by microbes could lead to the development of root disease, competition with roots for nutrients, or selection and enrichment in soils of specific micro-organisms that are either detrimental or beneficial for crop growth (Kremer, Means & Kim, 2005). Genetically modified crops can have direct negative effects through the toxicity of an expressed GM trait on key non-target species or broader functional groups of micro-organisms. There can also be indirect impacts via trophic interactions at multiple levels, and the soil ecosystem can be affected by unintended changes in the metabolism of the GM plant (Duke & Cerdeira, 2007). Furthermore, pathogenic fungi may build up in soil and become a potential problem for subsequent crops, especially GR crops, cultivated in the same field (Kremer *et al.*, 2005). Knowledge of the impact of transgenic crop residues on soil microbial ecology is therefore essential for understanding the long-term agronomic and environmental effects of GM crops. It can assist in developing appropriate management practices for minimizing potential negative impacts of herbicides (Fang *et al.*, 2007).

1.3.1. *Impacts on rhizosphere micro-organisms*

The potential impact of GM plants on the dynamics of the rhizosphere and root-interior microbial community can be either positive or negative in terms of plant health and ecosystem sustainability. Minor alterations in the diversity of microbial communities could affect soil health and ecosystem functioning (Dunfield & Germida, 2004). Based on field evaluations of micro-fauna and micro-organisms, Griffiths *et al* (2007) concluded that there are no negative soil ecological consequences for soil biota associated with the use of BT- or HT maize in place of conventional varieties. Other

land management options, such as tillage, crop species and a sound pest management regime, have a more significant effect on the biology of soil than GM maize (Griffiths *et al.,* 2007). Yet, certain reports do claim that (GR) cropping systems change the soil environment by introducing novel compounds and glyphosate into the soil environment. Soil microbial communities, in particular rhizosphere microbes, may therefore be particularly sensitive to the effects of transgenic crops because of their close proximity (Dunfield & Germida, 2004).

1.3.2. *Negative impacts of glyphosate resistant crops on plant nutrition*

The use of HT crops and herbicides, such as glyphosate, in agricultural production systems significantly changes nutrient availability and plant efficiency for a number of essential plant nutrients (Neumann *et al.*, 2006; Huber, 2010). Increased disease incidence, yield loss and a reduction in crop quality may be the consequence of micronutrient deficiencies. Glyphosate may cause some of these changes either through direct toxicity or indirectly through changes in populations of soil organisms that are important for nutrient access, availability, or plant uptake (Neumann *et al.*, 2006; Huber, 2010).

Unfortunately, very little research has examined the direct and indirect effects of transgenic crops and their management on microbial mediated nutrient transformation in soil. Despite widespread public concern, no conclusive research has yet been presented that current transgenic crops are causing significant stimulation or suppression of soil nutrient transformation in field environments (Motavalli *et al.*, 2004). Micronutrients play an essential role in plant protection by acting as regulators,

activators, and inhibitors of plant defense mechanisms that provide resistance to stress and disease (Huber, 2010). The chelation of micronutrients by glyphosate renders them unavailable to plants which may lead to a compromise in plant defenses and an increase in pathogenesis. An increase in the severity of many abiotic as well as infectious diseases of GR as well as non-GR crops has also been observed (Huber, 2010).

The micronutrient Mn acts as a cofactor which activates 35 different enzymes (Gordon, 2007). Some enzymes activated by Mn lead to the biosynthesis of aromatic amino acids such as tyrosine and secondary products such as lignin and flavonoids, which stimulate root nodulation in legumes. In Mn deficient plants, a lower concentration of lignin and flavonoids leads to a decrease in disease resistance (Gordon, 2007). Studies have found that GR soybeans had a Mn deficiency compared to conventional soybeans. Evidence also suggests that glyphosate may interfere with Mn metabolism and also adversely affect soil microbial populations responsible for the reduction of Mn to a plant available form (Gordon, 2007). Untreated micronutrient deficiencies can also lead to yield losses, reduced crop quality and increased disease incidence (Huber & Haneklaus, 2007). The addition of the herbicide resistant gene and the application of glyphosate may thus be a major contributor to nutrient deficiencies in soil (Huber, 2010).

Early applications of glyphosate to GR soybean has been shown to delay nitrogen fixation and decrease biomass as well as the accumulation of nitrogen in GR soybean cultivars (King, Purcell & Vories, 2001). An evaluation of different cultivar maturity groups on different soil types also revealed a significant decrease in macro-

and micronutrients in leaf tissue, and in photosynthesis with increased glyphosate use (Zobiole *et al.,* 2010). Calcium, Mg, Zn, Mn, and Cu were the most commonly reduced mineral nutrients. Glyphosate may interfere with uptake and translocation of Ca, Mg, Fe and Mn, by crops, possibly by binding and thus immobilizing them (Cakmak *et al.*, 2009). Most of the nutrients that were reduced by the GR gene were reduced further when glyphosate was applied (Zobiole *et al.,* 2010).

1.4.   CONCLUSIONS

From the aforegoing, it is clear that much uncertainty remains regarding the use of herbicides and HT transgenic crops and the possible harmful effects these practices may, or may not, have on soil micro-biota and soil fertility (Sahid *et al.*, 1992). Interactions in the soil environment between xenobiotics and soil biota should be viewed as a dynamic process, involving many complex mechanisms (Meharg, 1996). By not acknowledging these interactions when investigating the environmental behaviour of pesticides, gross misperceptions of their ecological implications will be fostered (Meharg, 1996).

The advent of genetic engineering presents opportunities for novel methods of plant protection against pests with decreased reliance on potentially dangerous chemical controls (Fischhoff, 1988). Generally, few negative impacts are observed with GR crops in comparison to conventional crops. Favourable environmental effects of the glyphosate-containing herbicide regimes on GR crops appear feasible, provided appropriate measures for maintaining biodiversity and prevention of volunteers and gene flow are applied (Kleter *et al.*, 2008). However, literature on the topic is sparse

and far more research is thus urgently required to investigate the effect which GR crops may or may not have on ecosystem functioning.

It is clear that the type of herbicide plays a major role with regard to how soil microbes react to it in the soil environment. Sublethal doses of herbicides may either protect or predispose crops to disease (Lévesque & Rahe, 1992). Herbicides can directly alter the nature of soil ecosystems through promotion or suppression of activities of plant pathogens or beneficial micro-organisms. Fungal colonization of roots rapidly follows the application of certain herbicides. Non-specialized facultative pathogens can increase their inoculum potential on weeds or volunteers treated with herbicide and subsequently cause crop disease. Soil-borne fungi can also act as synergists in the herbicidal action of glyphosate, possibly because glyphosate blocks the production of phenolics involved in disease resistance of plants to these pathogens (Lévesque & Rahe, 1992).

The literature reviewed clearly shows that many factors contribute to how free-living soil microbes, rhizosphere microbes and plant pathogens, will react to herbicide application, as well as the introduction of transgenic plants into the soil ecosystem. Soil type plays a role with regard to how soil organisms react, because microbial biomass varies significantly between soils (Krzysko-Lupicka & Orlik, 1997; Descalzo *et al.*, 1998; Crouzet *et al.*, 2010). Furthermore, the mineralization and degradation of certain herbicides is controlled by active C present in the soil (Willems *et al.*, 1996).

Glyphosate application may increase soil microbial activity which may be either beneficial or detrimental toward plant growth, and soil quality (Partoazar *et al.*, 2011). It should also be kept in mind that increased microbial activity could be perceived as a

positive effect. It could be ascribed to an increase in certain groups of microbes which are able to utilize the xenobiotic compound, thus still leading to a shift in the community structure which could in turn lead to negative side effects on crops (Macur *et al.*, 2007).

Different techniques for assessing microbial responses to xenobiotics should be used. For instance, high atrazine levels produced an increase in bacteria as measured by colony forming units (cfu), which showed different banding patterns during denaturing gradient gel electrophoresis analysis (DGGE) compared to no or low atrazine concentrations; but microbial communities at high atrazine levels showed less capacity to use different carbon sources (Ros *et al.*, 2006). Since much controversy remains around the usage of transgenic plants and herbicides *per se*, the potential negative or positive effects it may or may not have on non-target micro-biota and thus soil health are unknown. It is also clear that there is no consensus regarding their positive or negative effects in terms of plant health. Further research on this topic is therefore urgently required.

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CHAPTER 2

The effect of herbicides on soil-borne fungi *in vitro*

ABSTRACT

An *in vitro* study was conducted to determine the effect of nine herbicides on soil-borne fungi. Active ingredients were: (1) 2,4-D-Dichlorophenoxyacetic acid (2,4-D), (2) atrazine, (3) bendioxide, (4) S-Metolachlor, (5) 5-ethyl dipropyl-thiocarbamate (EPTC) (6) atrazine, terbuthylazine and S-metolachlor combination, (7) paraquat, (8) glyphosate, and (9) atrazine and terbuthylazine mixture. Water agar amended with three different concentrations of the active ingredient (a.i) of the herbicides was tested on eight fungal species. Species tested were *Cunninghamella elegans, Aspergillus niger* var. *awamori, Trichoderma konigii, Trichoderma viride, Trichoderma harzianum, Rhizopus oligosporus, Fusarium oxysporum* and *Phoma sorghina.* Radial mycelial growth was measured three days after plating seeded plugs onto agar media and results were documented and subjected to statistical analysis. It was concluded that the tested herbicides, used at recommended field rates, had significant *in vitro* inhibitory effects on the eight fungal species.

Keywords: Herbicides; Soil fungi.

INTRODUCTION

Herbicides are widely used in modern agricultural practices in many countries around the world (Marecik *et al.,* 2008). The manner in which microbes respond to these chemicals when introduced to their environment, depends on the type of micro-organism involved, the concentration and exposure time of the herbicide applied, as well as other chemical, physical, biological, and environmental factors (Levine & Rachakornkij, 1994). The environmental fate of herbicides is a matter of concern given that only a small fraction reaches the target organism (Pimental, 1995). Herbicide use leads to chemical residues which can impact negatively on human, animal and crop health (Zabaloy *et al.*, 2011). Differential toxicity of herbicides to soil micro-organisms may alter the microbial community structure of soil, including a potential increase in soil-borne pathogens. Herbicides may also cause changes in microbial community function, leading to altered soil health and ecosystem processes (Zabaloy *et al.,* 2011).

Most herbicides used at normal field rates are generally considered to have no major or long-term effect on soil micro-organisms or on gross soil microbial activities (Bollen, 1961). However, some reports indicate that herbicide application to soil may lead to the proliferation of specific organisms which can metabolize the chemical (Katan & Eshel, 1973). This selective action may result in direct and indirect qualitative changes in soil microbial populations which can influence soil fertility (Mekwatanakarn & Sivasithamparam, 1987).

Micro-organisms reportedly have a positive effect on xenobiotic degradation. This phenomenon can be explained in terms of a variety of different bacterial species that occur in soil, and the broad spectrum of enzymes produced by them. Thus, micro-

organisms can use different sources of carbon and nitrogen to adapt to different environments, including soil subjected to herbicide application (Struthers, Jajychandran & Moorman, 1998; Seffernick *et al.*, 2000). For example, 2,4-D-Dichlorophenoxyacetic acid (2,4-D), which is one of the most studied herbicide degradation pathways, can be readily used as a C and energy source by soil micro-organisms (Zabaloy *et al.*, 2011). In the case of atrazine, several strains of bacteria and fungi have been identified as having the ability to degrade atrazine. Many of these micro-organisms may perform either atrazine dealkylation, deamination or dechlorination, but not complete mineralization (Marecik *et al.*, 2008).

Contradictory views are especially prevalent regarding the effect of glyphosate on non-target organisms. Apparently glyphosate has no measured effect on soil respiration when added to soil at normal field concentrations, but at high concentrations it stimulates respiration (Busse *et al.*, 2001). Busse *et al* (2001) found little evidence that repeated field applications of glyphosate is detrimental to microbial populations and processes in soil, a finding consistent with that of Duke & Cerdeira (2007). Reports that glyphosate is utilized by soil micro-organisms as a source of nutrients resulting in stimulation of their activity have been documented (Partoazar, Hoodaji & Tahmourespour, 2011). The functional diversity of the cultivable portion of heterotrophic soil microbial communities is stimulated by the application of glyphosate, presumably because the microbial community utilizes the glyphosate as a source of carbon (C), nitrogen (N) and phosphorous (P) (Mijangos *et al.*, 2009). Most herbicide-degrading micro-organisms are bacteria, but fungi are also known for their capacity to

degrade complex substrates, and may be more important in herbicide degradation than is presently thought (Smith & Collins, 2007)

Contradictory reports also exist concerning the bio-availability of glyphosate residues in soil and the potential toxicity risk to non-target organisms (Tesfamariam *et al.*, 2009). Glyphosate has been found to be toxic to culturable fungi and bacteria in laboratory studies when added to artificial media (Busse *et al.*, 2001). Contrary to these laboratory results, field studies have revealed very few examples of effects on soil micro-organisms. Increases in bacteria and fungi (Carlisle & Trevors, 1988; Araujo, Monteiro & Abarkeli, 2003; Partoazar *et al.*, 2011), soil respiration (Carlisle & Trevors, 1988; Haney *et al.*, 2000), N mineralization (Haney *et al.*, 2000), and soil enzyme activity (Gianfreda, Sannino & Violane, 1995) are reported, while negligible changes in N cycling processes (Müller *et al.*, 1981; Carlisle & Trevors, 1988) and microbial biomass (Araujo *et al.,* 2003) have also been observed.

Discrepancies between laboratory and field studies can be explained partially by the unrealistically high herbicide concentrations used in many laboratory studies (Wardle, 1995) by inadequate techniques to measure and by herbicide chemistry (Busse *et al.*, 2001). Although the availability of glyphosate is unrestricted in artificial media, binding to soil particles and metal complexes reduces the pool of labile glyphosate and, consequently, the uptake rate by soil microbes (Busse *et al.*, 2001). This raises the question whether microbial communities in soils having low adsorption capacity are at greater risk due to higher concentrations of labile glyphosate (Busse *et al.*, 2001).

Unfortunately it is often difficult to measure and predict the impact of chemicals, such as herbicides, on natural microbial communities. Laboratory experiments are mostly artificial and difficult to carry out over long enough periods of time. Field experiments on the other hand are often difficult because of the high variability inherent in natural microbial populations (Marrs & Frost, 1997). However, regardless of the disadvantage of laboratory testing, a major advantage is that herbicide studies can be done under carefully controlled and repeatable conditions (Marrs & Frost, 1997).

The aim of the present study was to determine the *in vitro* effect of nine selected herbicides on eight species of fungi isolated from soil. It was hypothesized that herbicides will have an inhibitory effect on the fungal isolates at recommended field application rate of the active ingredient (a.i.). The stimulatory, or inhibitory effects of the herbicides on the selected fungi was determined by examining their radial growth on agar media amended with three concentrations of the a.i. of the respective herbicides (Johal & Rahe, 1984; Kawate *et al.*, 1992).

MATERIALS AND METHODS

Isolation of test fungi: Soil was collected from an experimental farm of the University of the Free State (UFS) (Kenilworth), near Bloemfontein (-29.020397 S; 26.145308 E). The soil had a history of annual tilling, and maize was being cultivated on the field during collection. The soil is categorized as Kenilworth–Bainsvlei ecotope and an example of a fine sandy loam soil in a semi-arid area (Soil Classification Working Group, 1991). A second batch of organic untilled soil (96 % organic matter)

was collected from a site 35 km NW of Bloemfontein (S 28° 53' 5.64"; E 25° 57' 33.48").

In both instances, soil was collected from the top 5 cm of the surface. Chemical

analysis was conducted on both batches of soil.

A dilution series was made by suspending 10 g of each soil type in 90 ml of

sterile water. A dilution series of 1:10, 1:100 and 1:1000 was made and a 100 µl aliquot

of the soil suspension was plated onto potato dextrose agar (PDA). Petri-dishes were

incubated at 24 °C in a Labcon growth chamber for 5 days after which transfers were

made onto sterile agar until uncontaminated cultures were obtained. Fungi were

identified based on morphological characteristics. Single spore colonies were

maintained on PDA slants and stored at 4 °C (Kremer, Means and Kim, 2005).

In vitro inhibition of fungi: Nine herbicides (Table 2.1) were selected based on

activity groups and those most frequently used by farmers. Herbicides were individually

incorporated into water agar (WA) at concentrations of 0, 0.1, 1.0 and 10 mg ml^{-1}a.i (Liu,

Punja & Rahe, 1997). Agar was cooled to a temperature of 45 – 50 °C before an aliquot

of each herbicide and 0.3 ml streptomycin (0.3 ml/ 1000ml) was added and mixed with a

magnetic stirrer. The amended agar was then poured into 90-mm-diameter Petri-dishes

and allowed to solidify. A concentration of 0.1 µg/ ml of the a.i. present in 1 ml agar

represented the concentration of a.i. present at the recommended field rate, except in

the cases of bendioxide (Basagran), 5-ethyl dipropyl-thiocarbamate (EPTC) (Eptam

Super) and paraquat (Gramoxone). For the latter, concentrations at recommended field

rates are 0.03 µg/ ml, 0.3 µg/ ml and 0.3 µg/ ml a.i. per 1 ml of agar, respectively.

The eight purified fungal isolates were plated onto PDA (Kremer *et al.*, 2005) and left to grow for 3 days (d) whereafter 5 mm agar plugs of mycelium were removed from the actively growing edge of respective colonies with a sterilized cork borer (Kremer *et al.*, 2005) and placed in the middle of 90-mm-diameter Petri-dishes containing herbicide amended agar. The Petri-dishes were then incubated at 24 ˚C in the dark in a Labcon growth chamber for 3 d (Busse *et al.*, 2001) whereafter fungal colony diameter was measured. The experiment was designed as a complete randomized block design with three replicates per treatment and conducted twice.

Statistical analysis: Data were subjected to analysis of variance (ANOVA) and, when F values were significant ($P<0.05$), means were separated using Fisher's protected least significant difference (LSD) test to determine the statistical significance of treatment differences.

RESULTS

In vitro inhibition of fungi. Fungal isolates reacted differently to the nine herbicides and the different concentrations of the respective herbicides. Although bendioxide showed stimulation of three isolates, at a higher concentration than the recommended field rate, the remaining eight herbicides were inhibitory at all times (Table 2.2). All nine herbicides were inhibitory over the three different concentrations. At the lowest concentration, all herbicides showed a mean inhibition higher than 47 %. Bendioxide was the least toxic to the isolates with a mean inhibition of 14 % at the lowest concentration, which is 3.3x higher than the recommended field rate.

Glyphosate and paraquat showed the highest mean inhibition (82.21 %) at the lowest concentration. At the two higher concentrations all herbicides were inhibitory, except for bendioxide. Although all fungal isolates responded differently to the same herbicide, *Trichoderma* spp. were most sensitive and showed the highest inhibition levels for all herbicides tested. An analysis of variance (ANOVA) proved that the main interaction between herbicides, herbicide concentration and fungal isolate, was statistically significant ($P<0.05$) (Table 2.3).

DISCUSSION

Although the toxicity of herbicides differed, six of the nine herbicides inhibited fungal growth at the recommended field rate, and none displayed growth stimulation except bendioxide. The latter stimulated three fungal isolates, in contrast to the findings of Ceballos, Quiroz & Palma (2011) who observed that bendioxide has no effect on fungal growth, even at high application rates. All eight isolates were inhibited at some stage and at the recommended field rate, glyphosate had the greatest inhibitory effect. This finding is consistent with that of Busse *et al* (2001), who observed that glyphosate was toxic to fungi when grown in soil-free media. Liu *et al* (1997) also observed inhibition of mycelial growth of *Pythium sylvaticum* at high concentrations of glyphosate in WA.

The inhibition of fungal isolates observed at recommended field rate applications in the present study is consistent with previous similar studies (Poprawski & Majchrowicz, 1995; Wilcox, 1996; Pakdaman *et al.*, 2002; Pakdaman & Goltapeh, 2007; Westerhuis, Vawdrey & Piper, 2007). Results obtained prove that some fungal species

are more sensitive to herbicide toxicity than others. For example *Trichoderma* spp. and *Cunninghamella elegans* were most sensitive in the present study. It is important to note that fungal species which play an important role in soil fertility were most sensitive to the herbicides. Reports claim that micro-organisms are capable of degrading herbicides in the soil by utilizing them as a supply of nutrients and energy (Kanissery & Sims, 2011; Partoazar *et al.,* 2011). Results obtained from the present study however suggest that some commonly used herbicides have an inhibitory effect on soil fungi when used at the recommended field rate. This could have far reaching consequences for soil fertility and ecosystem functioning. It is important to note, however, that *in vivo* and *in vitro* results will not necessarily correspond, because laboratory conditions differ significantly from field conditions (Busse *et al.*, 2001).

The results presented in this study suggest that a greater spectrum of the soil organisms should be tested to obtain a more complete picture of the impact of herbicides on soil fungi. Results also clearly indicated that fungal inhibition is dependant on the rate of herbicide application (Table 2.2). The two highest concentrations used in the experiment, i.e. 1.0 and 10 µg of a.i. per 1 ml agar respectively, have important implications in cases where herbicide spillage may occur. Maintaining the recommended dosage is very important if damage to the soil ecosystem is to be prevented. Literature on the effect of herbicides *in vitro* on saprophytic soil or mycorrhizal fungi is however scant. Most literature is based on *in vitro* toxic effects on plant pathogenic fungi (Wilcox, 1996; Pakdaman *et al.*, 2002; Westerhuis *et al.*, 2007; Ceballos *et al.*, 2011). However, plant pathogenic fungi interact with saprophytic and

mycorrhizal fungi in the soil environment, and future research therefore needs to take these interactions into account.

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Table 2.1. Herbicides selected for *in vitro* studies (Van Zyl, 2012).

Herbicide Trade name	Active ingredient(s)	Recommended dosage ha^{-1} (Maize) (11-20 % clay)	Recommended use	Herbicide group
2,4-D Amine Weed Killer	Dimethylamine salt of 2,4-D-Dichlorophenoxyacetic acid – 480 g/ L	3.3 L ha^{-1}	Selective broadleaf weed control	O
Atrazine 500 SC	Atrazine – 485 g/ L Other active triazines – 15 g/ L	3.25 L ha^{-1}	Annual broadleaf weed control	C1
Basagran	Sodium salt of bentazon – 44%	1 L ha^{-1}	Herbicide for post emergence use	E
Dual Gold	S-Metolachlor – 960 g/ L	2 L ha^{-1}	Controls certain annual grasses and broadleaf weeds	K
Eptam super	5-ethyl dipropyl-thiocarbamate (EPTC) – 800 g/ L	5.5 L ha^{-1}	Selective herbicide for use in mineral soils	8
Gardomil Gold 600 SC	Atrazine – 248.6 g/ L Terbuthylazine – 248.6 g/ L S-metolachlor – 102.8 g/ L	2.3 – 4.8 L ha^{-1}	Selective pre-emergence and early post-emergence control of most annual broadleaf weeds and some annual grasses in maize	C1 & K3
Gramoxone	Paraquat 200 g/ L (present as dichloride)	2 L ha^{-1}	Control of annual grasses and annual broadleaf weeds in crops. Inactivated on contact with soil	D (22)
Roundup®	Glyphosate	4.7 L ha^{-1}	Post-emergence control of annual and perennial weeds	G
Terbuzine 600 SC	Atrazine – 290 g/ L Related active triazines – 10 g/ L Terbuthylazine 290 g/ L	2.7 L ha^{-1}	Selective control of most annual broadleaf weeds as well as goose grass in maize and grain sorghum	C1

Table 2.2. The relative inhibition of eight different fungi isolated from soil by different herbicides *in vitro.*

Herbicide (Active ingredient) (Concentration of a.i.)	Fungus	Average Inhibition (%)			Mean
		Concentration			
		0.1 µg/ml ai	1.0 µg/ml ai	10 µg/ml ai	
2,4 D amine **(Dimethylamine salt of 2,4-D-Dichlorophenoxyacetic acid)** **(480 g/ L a.i.)**	*Cunninghamella elegans*	63.81	97.12	97.12	**86.02**
	Aspergillus niger var. awamori	56.45	96.19	96.19	**82.94**
	Trichoderma koningii	48.10	96.28	98.78	**81.05**
	Trichoderma viride	65.17	99.27	99.27	**87.90**
	Trichoderma harzianum	40.56	99.54	99.54	**79.88**
	Rhizopus oligosporus	52.17	99.56	99.56	**83.77**
	Fusarium oxysporum	57.09	96.64	96.64	**83.45**
	Phoma sorghina	62.94	95.94	95.94	**84.94**
Mean		**55.78**	**97.57**	**97.88**	
Atrazine 500 SC **(Atrazine)** **(485 g/ L a.i.)**	*Cunninghamella elegans*	54.62	73.84	85.81	**71.42**
	Aspergillus niger var. awamori	24.02	69.90	88.88	**60.93**
	Trichoderma koningii	64.29	84.79	88.99	**79.36**
	Trichoderma viride	71.60	92.08	94.43	**86.04**
	Trichoderma harzianum	97.54	98.94	99.29	**98.59**
	Rhizopus oligosporus	54.86	79.73	88.59	**74.39**
	Fusarium oxysporum	29.92	67.35	77.59	**58.29**
	Phoma sorghina	59.94	71.70	80.42	**70.69**
Mean		**57.10**	**79.79**	**88.00**	
Basagran **(Bendioxide)** **(44% a.i.)**	*Cunninghamella elegans*	-15.94	0.65	38.11	**7.61**
	Aspergillus niger var. awamori	7.72	34.50	86.66	**42.96**
	Trichoderma koningii	-2.04	17.74	48.78	**21.49**
	Trichoderma viride	22.20	50.86	78.77	**50.61**
	Trichoderma harzianum	45.38	46.35	47.58	**46.43**
	Rhizopus oligosporus	14.59	82.80	99.46	**65.62**
	Fusarium oxysporum	-14.12	16.78	43.00	**15.22**
	Phoma sorghina	55.95	60.37	63.80	**60.04**
Mean		**14.22**	**38.76**	**63.27**	

Fisher's LSD (P<0.05): Herbicide = 3.27; Fungus = 3.09; Herbicide x Fungus = 16.03

Table 2.2. *Continued*

Herbicide (Active ingredient) (Concentration of a.i.)	Fungus	Average Inhibition (%)			Mean
		Concentration			
		0.1 µg/ml ai	1.0 µg/ml ai	10 µg/ml ai	
Dual Gold Sc **(S- Metolachlor)** **(960 g/ L)**	*Cunninghamella elegans*	13.51	55.65	83.89	**51.01**
	Aspergillus niger var. awamori	10.14	68.30	96.59	**58.34**
	Trichoderma koningii	15.61	90.89	99.44	**68.65**
	Trichoderma viride	22.77	82.50	99.06	**68.11**
	Trichoderma harzianum	47.28	98.63	99.67	**81.86**
	Rhizopus oligosporus	15.67	87.30	99.46	**67.48**
	Fusarium oxysporum	6.66	30.25	93.62	**43.51**
	Phoma sorghina	46.56	56.82	95.73	**66.37**
Mean		**22.28**	**71.29**	**95.93**	
Eptam Super **(5-ethyl dipropyl-thiocarbamate(EPTC))** **(800 g /L)**	*Cunninghamella elegans*	40.60	99.17	99.17	**79.64**
	Aspergillus niger var. awamori	64.02	96.35	97.55	**85.97**
	Trichoderma koningii	52.87	97.95	99.44	**83.42**
	Trichoderma viride	53.86	97.73	99.06	**83.55**
	Trichoderma harzianum	39.04	98.89	99.67	**79.20**
	Rhizopus oligosporus	68.87	99.13	99.46	**89.15**
	Fusarium oxysporum	33.32	86.13	97.53	**72.33**
	Phoma sorghina	62.92	93.53	96.60	**84.35**
Mean		**51.94**	**96.11**	**98.56**	
Gardomil Gold **(Atrazine - 248.6 g/ L)** **(Terbuthylazine - 248.6 g/ L)** **(S- Metalochlor - 102.8 g/ L)**	*Cunninghamella elegans*	30.83	96.88	99.17	**75.63**
	Aspergillus niger var. awamori	7.97	67.84	92.34	**56.05**
	Trichoderma koningii	64.97	88.87	97.44	**83.76**
	Trichoderma viride	71.16	84.03	97.49	**84.23**
	Trichoderma harzianum	73.56	91.00	98.05	**87.54**
	Rhizopus oligosporus	27.75	92.93	99.46	**73.38**
	Fusarium oxysporum	36.63	78.96	91.76	**69.11**
	Phoma sorghina	70.53	81.07	95.55	**82.39**
Mean		**47.92**	**85.20**	**96.41**	

Fisher's LSD (P<0.05): Herbicide = 3.27; Fungus = 3.09; Herbicide x Fungus = 16.03

Table 2.2. *Continued*

Herbicide (Active ingredient) (Concentration of a.i.)	Fungus	Average Inhibition (%)			Mean
		Concentration			
		0.1 µg/ml ai	1.0 µg/ml ai	10 µg/ml ai	
Gramoxone **(Paraquat)** **(200 g/ L)**	*Cunninghamella elegans*	52.40	87.40	98.02	**79.28**
	Aspergillus niger var. awamori	86.64	94.97	96.19	**92.60**
	Trichoderma koningii	89.30	98.78	97.44	**95.17**
	Trichoderma viride	96.60	99.27	99.27	**98.38**
	Trichoderma harzianum	98.67	99.54	99.54	**99.25**
	Rhizopus oligosporus	93.68	99.56	99.56	**97.60**
	Fusarium oxysporum	64.15	94.07	96.64	**84.95**
	Phoma sorghina	76.23	88.60	95.94	**86.92**
Mean		**82.21**	**95.27**	**97.82**	
Roundup® **(Glyphosate)** **(360 g/ L)**	*Cunninghamella elegans*	60.64	69.81	83.93	**71.46**
	Aspergillus niger var. awamori	77.06	91.05	94.06	**87.39**
	Trichoderma koningii	33.93	75.95	98.78	**69.55**
	Trichoderma viride	89.87	97.02	98.96	**95.28**
	Trichoderma harzianum	80.67	88.19	98.96	**89.27**
	Rhizopus oligosporus	70.87	83.79	97.13	**83.93**
	Fusarium oxysporum	72.48	86.12	96.00	**84.86**
	Phoma sorghina	84.22	94.01	95.94	**91.39**
Mean		**82.21**	**95.27**	**97.82**	
Terbuzin 600 SC **(Atrazine - 290 g/ L)** **(Related active triazines - 10g/ L)** **(Terbuthylazine - 290g/ L)**	*Cunninghamella elegans*	22.38	60.11	99.17	**60.55**
	Aspergillus niger var. awamori	9.61	55.82	85.94	**50.46**
	Trichoderma koningii	43.24	79.45	89.10	**70.60**
	Trichoderma viride	65.08	65.12	86.92	**72.38**
	Trichoderma harzianum	71.21	92.01	94.35	**85.86**
	Rhizopus oligosporus	31.50	73.07	96.72	**67.10**
	Fusarium oxysporum	39.61	67.41	77.96	**61.66**
	Phoma sorghina	61.22	70.46	82.51	**71.40**
Mean		**71.22**	**85.74**	**95.47**	

Fisher's LSD (P<0.05): Herbicide = 3.27; Fungus = 3.09; Herbicide x Fungus = 16.03

Table 2.3. ANOVA table calculated for *in vitro* trial on the effect of herbicides on selected isolated fungi.

Source Term	DF	Sum of Squares	Mean Square	F-ratio	Probability level	Power (Alpha = 0.05)
A: Herbicide	8	294144.2	36768.03	183.22	0.000000*	1.000000
B: Concentration	2	383802.3	191901.2	956.28	0.000000*	1.000000
AB	16	60280.5	3767.531	18.77	0.000000*	1.000000
C: Fungi	7	58895.53	8413.646	41.93	0.000000*	1.000000
AC	56	86372.49	1542.366	7.69	0.000000*	1.000000
BC	14	29025.98	2073.285	10.33	0.000000*	1.000000
ABC	112	73528.54	656.5048	3.27	0.000000*	1.000000
D: Reps	5	6380.262	1276.052	6.36	0.000008*	0.989128
S	1075	215725.6	200.675			
Total (Adjusted)	1295	1208156				
Total	1296					

*Term significant at alpha = 0.05

CHAPTER 3

The effect of selected herbicides on soil microbes in soils with contrasting levels of organic matter

ABSTRACT

The aim of the present study was to determine the effect of 2,4-Dichlorophenoxyacetic acid (2,4-D), paraquat (Gramoxone) and glyphosate (Roundup®) on soil microbial populations from two soils with contrasting levels of organic matter content in microcosms. It was hypothesized that these herbicides will have an effect on soil microbial activity, and that soil with a high level of organic matter will have a buffering effect on micro-organisms when the herbicide is applied. The effect of the herbicides on the soil biota was assessed by measuring: (1) Active carbon (C) levels; (2) carbon source utilization patterns by means of community-level physiological profiling (CLPP) using the BiologEcoPlate® system; (3) microbial activity by means of fluorescein diacetate hydrolysis (FDA), and (4) ergosterol levels. Deoxyribonucleic acid (DNA) fingerprinting was also conducted for all the treatments by means of denaturing gradient gel electrophoresis (DGGE) and, terminal restriction fragment length polymorphisms (T-RFLP). Herbicides mostly had no significant effect ($P < 0.05$) on the soil microbial community. However, organic matter content and number of days post herbicide application greatly influenced the effect which herbicides had on soil biota.

Keywords: *Herbicides, Soil micro-organisms, Microcosms.*

INTRODUCTION

Concern regarding the increased use of pesticides in modern agriculture has increased over recent years (Ros *et al.*, 2006). The risks posed by agricultural chemicals such as herbicides for crop production are significant. Negative impacts on soil micro-organisms can render soils infertile, and lead to impaired crop growth, and yield (Chandra, Furtick & Bollen, 1960; Altman & Campbell, 1977). When a herbicide is introduced into the soil environment several types of interactions are possible that may directly or indirectly affect soil microbial activity (Liu, Punja & Rahe, 1997; Haney, Senseman & Hons, 2002; Johal & Huber, 2009).

Pesticide application can change the community structure of bacteria in soil, even though nitrogen and carbon metabolism appear to be largely unaffected (Johnsen *et al.*, 2001). Some micro-organisms may be suppressed while others proliferate in vacant ecological niches (Altman & Campbell, 1977; Johnsen *et al.*, 2001; Mijangos *et al.*, 2009). This may consequently lead to altered successions in the microbial community and thus changes in their activity at a later point in time, leading to changes in soil fertility (Johnsen *et al.*, 2001). The bio-availability of applied pesticides is largely determined by adsorption of the chemical to organic matter, solubility in water molecules between soil particles, leaching and chemical degradation (Johnsen *et al.*, 2001; Kopytko, Chalela & Zauscher, 2002).

Reports claim that most herbicides, when applied at normal field rates, are generally considered to have no major or long-term effect on microbial populations or gross microbial activity in soil (Altman & Campbell, 1977; Sahid, Hamzah & Aris, 1992;

Busse *et al.*, 2001; Sengupta *et al.*, 2009; Crouzet *et al.*, 2010). In contrast, however, there are reports that have shown proliferation of general or specific soil organisms due to the utilization of the introduced chemical (Altman & Campbell, 1977; Mijangos *et al.*, 2009). This selective action may result in direct and indirect, qualitative and quantitative, changes in soil microbial populations which can influence soil fertility (Mekwatanakarn & Sivasithamparam, 1987b).

Micro-organisms vary in their behaviour when a herbicide is introduced into their environment. Numerous reports reveal both toxic effects on, or the inhibition of actinomycetes, other bacteria and fungi (Cole, 1976; Mekwatanakarn & Sivasithamparam, 1987a, b; Sahid *et al.*, 1992; Busse *et al.*, 2001; Zobiole *et al.*, 2010) in addition to stimulatory effects on actinomycetes, bacteria and fungi by herbicides (Mekwatanakarn & Sivasithamparam, 1987a, b; Araújo, Monteiro & Abarkeli, 2003; Ros *et al.*, 2006; Mijangos *et al.*, 2009). A decrease in the capacity of bacterial populations to use different carbon sources has also been observed (Ros *et al.*, 2006). Some reports also indicate that certain herbicides have no apparent stimulatory or inhibitory effect on actinomycetes (Cole, 1976; Mekwatanakarn & Sivasithamparam, 1987b) or soil micro-organisms in general (Tu, 1993; Busse *et al.*, 2001; Duke & Cerdeira, 2007; Weaver *et al.*, 2007; Sengupta *et al.*, 2009).

Contradictory views are especially prevalent regarding the effect of glyphosate on non-target organisms. Glyphosate reportedly has no measured effect on soil respiration when added to soil at normal field concentrations, but at high concentrations it stimulates respiration (Busse *et al.*, 2001). Furthermore, according to Duke & Cerdeira (2007), no agriculturally significant effect of glyphosate on soil micro-

organisms has been documented. Glyphosate can also be degraded by soil micro-organisms (Araújo *et al.*, 2003), and utilized as a source of carbon (C), nitrogen (N) and phosphorous (P) by certain microbial communities present in the soil (Mijangos *et al.*, 2009). Contradictory reports also exist concerning the bio-availability of glyphosate residues in soils and the potential toxicity risk for non-target organisms (Tesfamariam *et al.*, 2009). Glyphosate has been found to be toxic to culturable fungi and bacteria in laboratory studies when added to artificial media (Busse *et al.*, 2001) but agricultural field studies have revealed either no affect, or slight stimulation of soil micro-organisms (Duke & Cerdeira, 2007)

Discrepancies between laboratory and field studies in terms of herbicide toxicity to bacteria or fungi can be explained partially by the abnormally high herbicide concentrations used in many laboratory studies (Wardle, 1995) and by herbicide chemistry (Busse *et al.*, 2001). Although the availability of glyphosate, and most other herbicides, is unrestricted in artificial media, in soil it binds to soil particles and metal complexes thus reducing the pool of labile glyphosate and, consequently, uptake by soil microbes. This raises the question whether microbial communities living in soils with low adsorption capacity are at greater risk due to higher concentrations of labile glyphosate (Busse *et al.*, 2001).

In recent studies the use of soil microcosms to mimic the natural soil ecosystem under a controlled environment has increased considerably. Microcosms are a valuable tool to study interactions between micro-organisms that take place in natural soil (Hill & Top, 1998). The use of soil microcosm experiments addresses the need by researchers to mimic soil systems by controlling key parameters which exist in a natural soil system.

Microcosm based experiments are thus intermediate between field and laboratory experiments (Verhoef, 1996; Marrs & Frost, 1997; Hill & Top, 1998). Soil microcosms have been used to assess the potential ecological effects which may occur as a result of the introduction of a foreign organism or compound into a particular habitat (Hill & Top, 1998). The use of microcosms in soil experiments for example, allows for simultaneous assessment of the overall effects of a single pesticide on a range of representative soil organisms (Burrows & Edwards, 2002). Studies where the effect of pesticides on soil microbial communities are investigated should, however, incorporate evaluation of microbial diversity. Immediate displacement of micro-organisms by a direct toxic effect as well as long term effects on processes caused by altered succession in the microbial community can then be ascertained (Johnsen *et al.*, 2001).

Unfortunately it is often difficult to measure and predict the impact of herbicides on natural microbial communities. Microcosm experiments are either artificial, or have not been carried out over a long enough period to be a true reflection of what really happens. Field experiments are often expensive and difficult because of the high variability inherent in natural populations (Marrs & Frost, 1997). The main advantage of microcosms is that the application of herbicides can be done under carefully controlled and repeatable conditions (Marrs & Frost, 1997). Problems in assessing the impact of herbicides on soil microflora are numerous and complicated. This is largely due to the highly complex nature of multiple interactions occurring simultaneously between the herbicide, soil, and micro-organisms (Sahid *et al.*, 1992).

Molecular tools can rapidly asess microbial diversity on a community scale, and have often been used because they provide information, which cannot be observed by

means of traditional techniques (Johnsen *et al.*, 2001). Terminal restriction fragment length polymorphisms (T-RFLP) analysis is a polymerase chain reaction (PCR)-fingerprinting method that is commonly used for comparative microbial analysis. T-RFLP can be used to analyze communities of bacteria, archae-bacteria, fungi, other phylogenetic groups or subgroups, as well as functional genes (Thies, 2007). The T-RFLP method is rapid, highly reproducible, and often yields a higher number of operational taxonomic units than other, commonly used PCR-fingerprinting methods (Kitts, 2001; Thies, 2007). This approach has been used successfully to analyze the composition of microbial community structure and dynamics in soil. A great advantage of the T-RFLP method is that it is a user-friendly molecular approach to microbial community analysis and easy to execute. The T-RFLP method has been used in a variety of ecological studies to provide useful information about the diversity and behavior of microbial populations in soil (Thies, 2007). The patterns produced by T-RFLPs are recognised as having better resolution than other DNA - based methods for evaluating community structure (Kitts, 2001).

The aim of the present study was to determine the effect of 2,4-Dichlorophenoxyacetic acid (2,4-D), paraquat and glyphosate on soil microbial populations in soils with significantly different organic matter content. The effect of the herbicides on the soil biota were assessed for: (1) Active carbon (C) levels; (2) carbon source utilization patterns by means of community-level physiological profiling (CLPP) using the BiologEcoPlate® system; (3) microbial activity by means of fluorescein diacetate hydrolysis (FDA), and (4) fungal biomass as reflected by ergosterol levels. Deoxyribonucleic acid (DNA) fingerprinting was also conducted for all the treatments by

means of denaturing gradient gel electrophoresis (DGGE) and, T-RFLP. It was hypothesized that herbicides will have an effect on soil microbial activity, and that soil with high organic matter content will buffer toxic effects on micro-organisms.

MATERIALS AND METHODS

Soil sampling. Soil was collected from an experimental farm, Kenilworth near Bloemfontein (-29.020397 S; 26.145308 E). The soil had a history of annual tilling, and at the time of sampling, non-GM maize was being cultivated on the field. The soil is categorized as Kenilworth–Bainsvlei ecotope and an example of a fine sandy loam soil in a semi-arid area (Soil Classification Work Group, 1991) and will henceforth be referred to as organically poor soil (OPS) due to its lower level of organic matter (Table 3.1). A second batch of soil was collected from an untilled site, 96 % organic matter and 35 km NW of Bloemfontein (S 28° 53' 5.64"; E 25° 57' 33.48"), and will henceforth be referred to as organically rich soil (ORS) due to its high level of organic matter (Table 3.1). In both localities, soil was collected from the uppermost 5 cm. Soil analyses were conducted on both samples (Table 3.1) by the Department Climate-, Crop – and Soil Sciences at the University of the Free State. Soil was stored at 4 °C until further use (Zabaloy, Garland & Gómez, 2008).

Soil preparation and herbicide treatments for soil microcosm trial. Soil was placed in 200 ml square plastic containers (microcosms), and weighed. The soil mass in each container was maintained throughout the experiment by adjusting moisture loss with the addition of sterilized water to keep the soil moist but not wet (Zabaloy *et al.*,

2008; Crouzet *et al.*, 2010). The plastic microcosms were loosely capped to avoid excessive carbon dioxide accumulation in the head space (Zabaloy *et al.*, 2008). The experiment was carried out as described by Zabaloy *et al* (2008) with some modification. The microcosms were pre-incubated for six days in a growth chamber (Labcon) in the dark at 27 °C. On day six of the pre-incubation period, aqueous solutions of the three herbicides (2,4-D, paraquat, and glyphosate) were prepared and applied at a rate 10x that of the recommended field rate to each soil type as described by Zabaloy *et al* (2008). A control treatment entailed the application of sterilized water in both soil treatments. Microcosms were incubated in the dark at 28 °C for 21 days (d) (Zabaloy *et al.*, 2008) in a growth chamber (Labcon). Soil treatments were sampled before herbicide treatment for laboratory analysis, and again on days 3 and 21 post-herbicide application. The experiment was conducted twice and comprised a complete randomized design with three replicates per treatment.

Active carbon determination. Active carbon was determined as described by Weil *et al* (2003), to measure soil organic matter that is in a form readily utilizable by micro-organisms. The procedure involves two main steps. Firstly, oxidation of the active carbon by a 0.02 M $KMnO_4$ solution, and secondly, measurement of the absorbance of the reduced MnO_4^- solution at 550 nm (Weil *et al.*, 2003). The presence of active carbon reduces manganese (Mn) from Mn^{7+} to Mn^{2+}, which leads to bleaching of the $KMnO_4$ solution and thereby reduces absorbance. Active C was determined as mg/ kg soil with reference to a calibration curve (Weil *et al.*, 2003). Soil from the microcosms was prepared by sieving approximately 10 g through a 2 mm sieve and drying it at 40 °C in an incubator (Labcon) overnight. Distilled water (18 ml) was added

to a 50 ml plastic centrifuge tube, and also 2 ml of a 0.2 M $KMnO_4$ in 0.1 M $CaCl_2$ stock solution. The dried soil sample (2.5 g) was added to this solution and the tube was agitated for 2 min on a Heidolph Multi Reax shaker at 300 rpm. Tubes were then placed on the bench and left undisturbed for 8 min, after which 500 µl of the suspension was transferred to 49.5 ml of distilled water in a clean 50 ml plastic centrifuge tube. Absorbance values were read on a Spec: T60 UV-visible spectrophotometer at 550 nm in 1 ml plastic cuvettes. The standard curve and calculations were determined as described by Weil *et al* (2003). Data from both trials were pooled and subjected to analysis of variance (ANOVA) and, when F values were significant ($P<0.05$), means were separated using Fisher's protected least significant difference (LSD) test.

Community-level physiological profiling (CLPP). Community-level physiological profiles (CLPP), based on carbon source utilization patterns of the treatments, were assessed using the BiologEcoPlates™ (Biolog, Hayward, CA, USA). A soil suspension was made by suspending 2 g of soil in 200 ml of sterilized water (Palmroth *et al.*, 2004) and shaking it for 30 min at room temperature to allow the soil to become totally suspended in the water. Each well of the microtiter plate was filled with 90 µl of the soil suspension and plates were incubated at 25 ˚C in a Labcon Growth chamber in the dark for 96 h (Palmroth *et al.*, 2004). The change in optical density (OD) was then measured at 590 nm using a multi-well plate reader (Akmal *et al.*, 2005). The overall colour development in each BiologEcoPlate™ was expressed as average well colour development (AWCD) (Garland & Mills, 1991; Cai *et al.*, 2010). Data from both trials were pooled and subjected to an analysis of variance (ANOVA) and, when F

values were significant (P<0.05), means were separated using Fisher's protected LSD test.

Determination of soil ergosterol.   Ergosterol is an important membrane sterol in almost all eumycotic fungi and has been postulated to be strongly associated with living cytoplasmic fungi in the soil (Chiocchio & Matković, 2011). In order to estimate fungal biomass in soil samples (Davis & Lamar, 1992), ergosterol was extracted as described by Jambunathan, Kherdekar & Vaidya (1991) with minor modifications. Methanol (50 ml) was added to 10 g of moist soil in a 50 ml centrifuge tube, and the solution was agitated in a Heidolph Multi Reax shaker for 40 min at 1600 rpm. The suspension was then allowed to settle for approximately 30 min after which 25 ml of the clean supernatant was removed and added to a clean 50 ml centrifuge tube containing 3 g of potassium hydroxide (KOH). The mixture was again shaken for 10 min to allow the KOH to dissolve, after which 10 ml of n-hexane was added followed by incubation in a 75 °C water bath for 30 min. The solution was then cooled to room temperature and 5 ml distilled water was added. The hexane layer was then removed and transferred to a clean 50 ml centrifuge tube. Ten millilitres of n-hexane was added to the rest of the aliquot in the tube and mixed well. The hexane layer was again removed and added to the previous hexane aliquot. The last two steps were repeated. The hexane extract was then placed in a hot water bath and evaporated until a dry residue was left. The residue was dissolved in 5 ml of methanol and the solution filtered through a 0.45 µm millipore filter and placed in a 2 ml high performance liquid chromatograph (HPLC) bottle. The filtrate was analyzed at an emission wavelength of 282 nm using a Perkin Elmer HPLC fitted with a Phenomene x C18.5 µm 4.6 x 150 mm analytical column.

Ergosterol concentrations were calculated and data from both trials were pooled and subjected to analysis of variance (ANOVA) and, when F values were significant (P<0.05), means were separated using Fisher's protected LSD test.

Fluorescein diacetate hydrolysis. Fluorescein diacetate (FDA) hydrolysis is used as a simple method for estimating microbial activity in soil environments (Adam & Duncan, 2001). Schnurer & Rosswall (1982) found that there is a good correlation between FDA hydrolysis and respiration in soil. The method has the advantages of being simple, rapid, and sensitive (Schnurer & Rosswall, 1982).

The method of Adam & Duncan (2001) as modified by Zabaloy *et al* (2008), was used with a few modifications. A 2 g soil sample from each treatment was mixed with 20 ml of a 60 mM sodium phosphate buffer, with pH 7.6 (Schnurer & Rosswall, 1982; Adam & Duncan, 2001), in a 50 ml plastic centrifuge tube and 0.2 ml of a 2mg/ ml FDA stock solution was added (Schnurer & Rosswall, 1982). The FDA stock solution was prepared by dissolving 200 mg of FDA in 100 ml of acetone. The stock solution was kept at -20 °C until further use (Schnurer & Rosswall, 1982). The 50 ml tubes were incubated for 20 min at 28 °C in a Labcon growth chamber, and the tubes were shaken 3x by hand during incubation. After incubation, the tubes were placed on a Heidolph Multi Reax shaker for 10 min at room temperature and vigorously shaken at 300 rpm. The hydrolysis reaction was terminated by adding 15 ml of 2:1 chloroform: methanol solution. Approximately 1.5 ml of the supernatant was then placed in an epindorph tube and centrifuged at 200 rpm for 3 min before placing 1 ml into a 1 ml plastic cuvette for measuring absorbance readings at 490 nm in a Spec: T60 UV-visible spectrophotometer. Two controls were prepared for each soil sample. Firstly, a control

containing 2 g of the soil sample containing phosphate buffer without the addition of FDA. The second control contained phosphate buffer and FDA, but no soil. Their absorbance values were subtracted from the absorbance values of the corresponding soil sample to account for any colour formation that might have occurred that was not associated with the soil sample (Anonymous, 2004). Results are reported as µg fluorescein ml^{-1} of the soil solution. The amount of FDA hydrolyzed was measured as absorbance at 490 nm and expressed as µg/ ml^{-1} FDA hydrolyzed as determined from a standard curve (Adam & Duncan, 2001; Anonymous, 2004). The standard curve was set up as described by Anonymous (2004). Data from both trials were pooled and subjected to analysis of variance (ANOVA) and, when F values were significant ($P<0.05$), means were separated using Fisher's protected LSD test.

DNA isolation and 16S rDNA PCR amplification for denaturing gradient gel electrophoresis (DGGE). Total genomic DNA (gDNA) was extracted from 0.25 g soil samples using the PowerSoil™ DNA isolation kit according to the manufacturers' instructions. The DNA extracts were visualized on a 1 % agrose gel with GoldView staining under UV-illumination. Concentration and purity of gDNA were determined fluorometrically with a NanoDrop 2000 spectrophotometer (Thermo Scientific) and expressed as ng/µl. Two 16S rDNA primers specifically designed for bacteria (27F 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R 5'-GGTTACCTTGTTACGACTT-3') were used to amplify a portion of the conserved 16S rDNA gene (Weisburg *et al.*, 1991). Each PCR reaction contained 1-50 ng gDNA, 10 µM of each primer set, 10 mM of deoxyribonucleotide triphosphate (dNTP's), 2 mM MgCl$_2$, 1x PCR reaction buffer, 1.25 U of Taq DNA Polymerase (SuperTherm), and distilled water up to 50 µl. The PCR

program was as follows: initial denaturation 95 ˚C for 5 min; 95 ˚C for 45 s, 49 ˚C for 45 s and 72 ˚C for 1 min (25 cycles); 72 ˚C for 10 min. This was conducted according to the standard operating procedure (SOP 29) at the University of the Free State developed in the Metagenomics Platform Laboratory. The DNA was visualized on a 1% agarose gel stained with ethidium bromide under UV illumination.

Nested PCR and Denaturing Gradient Gel Electrophoresis (DGGE). Nested PCR was performed using a fragment of the same 16S rDNA region. Recovered DNA was used in the nested PCR. A 50 µl nested PCR reaction was carried out composed of recovered DNA, 1 µl dNTP's (10 mM; KAPA BIOSYSTEMS), 1 µl Bovine serum albumin (BSA), 1 µl PCR buffer (1x), 1 µl GoTaq® Flexi DNA Polymerase (1.25 U), 1 µl (10.µM) reverse primer 517R (5'-ATT ACC GCG GCT GCT GG-3') (Muyzer & Smalla, 1998), 1 µl (10 µM) forward primer 341F (5'-CCT ACG GGA GGC AGC AG-3') with a GC clamp (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCCG-3') (Muyzer & Smalla, 1998) and distilled water. The PCR cycle was as follows: initial denaturation 95 ˚C for 5 min; 95 ˚C for 45 s, 49 ˚C for 45 s and 72 ˚C for 1 min (25 cycles); 72 ˚C for 10 min. The DNA fragments were verified by visualization on a 1 % agarose gel stained with ethidium bromide under UV illumination The amplified DNA fragments were exposed to a gradient of denaturant (urea/ formamide) at an elevated temperature (60 °C) within a polyacrylamide gel (Tzeneva *et al.,* 2008). The procedure was carried out according to the standard operating procedure (SOP 19) developed by the Metagenomics Platform Laboratory using a DCode™ Universal Mutaton Detection System (BioRad), followed by staining and visualization under UV transillumination. The DGGE banding profile of each sample was quantified and compared with Quantity

One® 1-D Analysis imaging software available from the Biorad Molecular Imager Gel Doc ™ XR system to determine relatedness.

Terminal Restriction Fragment Length Polymorphisms (T-RFLP): Denaturing gradient gel electrophoresis (DGGE) and T-RFLP produce a pattern or profile of nucleic acids amplified from a sample and that pattern reflects the microbial community structure (Kitts, 2001).

To reliably compare T-RFLP fingerprints between different soil samples, the following was standardized: (1) template DNA concentration before PCR amplification, (2) the quality of the PCR-amplified DNA before restriction digestion, and (3) digested PCR products before sizing the terminally labeled fragments (Thies, 2007).

DNA isolation from soil: Total genomic DNA was extracted from 0.25 g soil samples using the PowerSoil™ DNA isolation kit according to the manufacturers' instructions. The extracted DNA was finally re-suspended in 100 µl elution buffer. The concentration of the DNA was determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) and expressed as ng/ µl.

16S rDNA PCR amplification: Two bacterial 16S rDNA specific primers were designed according to Thies (2007). The primer sequences were as follows: 5'-/56 FAM/AGAGTTTGATCMTGGCTCAG-3' and 5'-ACCTTGTTA CGACTT-3' (IDT®). Each 100 µl PCR reaction contained 100 ng DNA, 0.2 µM of each primer, 0.2 mM dNTP's (KAPA Biosystems), 2.5 mM $MgCl_2$, PCR buffer, and 1.25 U GoTaq® Flexi DNA Polymerase (Promega). The amplification regime was as follows: one cycle of 94 ˚C for

3 min followed by 35 cycles of 94 °C for 30 s, specific annealing temperature for 15 s and 72 °C for 1 min followed by a final extension step at 72 °C for 5 min.

A gradient PCR reaction was initially done to determine the optimal annealing temperature of the primer pair. The temperature gradient ranged from 55 °C to 60 °C. An optimal annealing temperature of 56.3 °C was subsequently used for all PCR reactions. To quantify the PCR products, an internal *Escherichia coli* DNA standard was included in all PCR reactions. The optimal amount of *E. coli* DNA added to each PCR reaction was determined by running a concentration gradient from 0.0125 to 25 ng DNA. The optimal amount decided on was 0.37 ng *E. coli* DNA per PCR reaction. The success of the PCR reaction was determined by separating 5 μl of the PCR product on a 1 % (w/v) agarose gel.

Restriction digestion of amplified soil DNA: For T-RFLP analysis, 600 ng amplified DNA product was digested in a 50 μl reaction containing 0.3 U RsaI enzyme (Fermentas) and 1 x concentration of the RsaI buffer (33 mM Tris-acetate; pH 7.9), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/ ml BSA). Digestion was carried out for 3 h at 37 °C whereafter the reaction were terminated at 68 °C for 10 min. The success of digestion was confirmed by separating 5 μl of the digestion reaction on an agarose gel as previously described.

The digested product was precipitated before fragment analysis as follows: 11.25 μl of a 125 mM ethylene-diamine-tetra-acetic acid (EDTA) solution and 135 μl of 100 % ethanol were added to the remaining 45 μl digestion product and vortexed. The solution was incubated at room temperature for 15 min, after which it was centrifuged at 12000 *g* for 15 min at 4 °C. After centrifugation the supernatant was completely removed and

200 µl 70 % (w/v) ethanol was added to the pellet and re-centrifuged at 12000 *g* for 5 min at 4 °C. The supernatant was completely removed after which the pellet was dried. Fragment analysis of the samples was carried out by the Department of Microbiology, Biochemistry and Food biotechnology at the University of the Free State, Bloemfontein. After adding 9.5 µl Hi-Di formamide and 0.5 µl of GeneScan™ -1200 LIZ size standard (Applied Biosystems) to the precipitated product it was vortexed. The solution was transferred to a 96 well sequencer plate after which it was incubated for 3 min at 96 °C in a PCR machine. After being cooled at 4 °C, the sequencer plate was placed in a 3130xl Genetic Analyzer (Applied Biosystems) and the program for fragment analysis was run as standardized by the Department of Microbiology, Biochemistry and Food Biotechnology.

The T-RFLP data were analyzed using the GeneMarker® version 2.2.0 software. Two methods of multivariate analysis were applied to T-RFLP and DGGE results. Firstly cluster analysis represented as dendrograms and secondly principle component analysis (PCA). Cluster analysis was based on a binary matrix, representing the presence or absence of bands in each treatment.

RESULTS

Soil chemical characteristics: Distinct differences were observed between the two soils with regard to their respective chemical characteristics. The organically rich soil (ORS) was 96 % organic matter (OM) content, calcium (Ca), potassium (K), magnesium (Mg), sodium (Na), zinc (Zn), iron (Fe), copper (Cu), and phosphorous (P)

levels than OPS (Table 3.1). Furthermore, ORS had a higher pH (pH 7.01) than OPS (pH 6.02).

Active carbon determination: A distinct difference between the ORS and OPS was observed regarding active C concentration.   There was a significant ($P<0.05$) interaction between soil and incubation period (Table 3.3b).   The mean active C concentrations remained unchanged in the ORS during the 21 days post herbicide application incubation period, while mean active C concentration changed over the 21 day post herbicide application incubation period for the OPS in all treatments (Table 3.3a). Mean active C concentration was lowest for the control treatment in the OPS. Furthermore, active C concentrations were lower for the pre-herbicide treatment than at 21d post-treatment.

Community-level physiological profiling (CLPP):   Herbicide application showed no significant ($P<0.05$) effect for both soils (Table 3.4b.). The ORS differed significantly from OPS ($P<0.05$). This was refelected by the mean AWCD value which was consistently higher in the ORS than in the OPS (Table 3.4a). In the ORS, the AWCD value increased over the 21d post herbicide treatment incubation period, while in the OPS the AWCD value showed an initial decrease on 3d post herbicide treatment followed by an increase on 21d post herbicide treatment. The control treatment had the lowest AWCD value 21d post herbicide treatment for the ORS, but the highest AWCD for the OPS. The two main effects, soil organic matter content and incubation time post herbicide application, were statistically significant ($P<0.05$) (Table 3.4b), indicating that soil organic matter content and incubation time had a significant influence bacterial diversity.

Determination of soil ergosterol: A distinct difference was observed in terms of ergosterol concentration between ORS and OPS (Figures 3.1a & 3.1b). The ORS consistently had an approximate 600x higher ergosterol concentration than OPS. Furthermore, soil type was significantly ($P<0.05$) different from each other (Table 3.6). No significant ($P<0.05$) differences were observed between herbicide treatment or incubation period post herbicide application (Table 3.6).

Fluorescein diacetate hydrolysis (FDA): The interaction between soil type (organic matter content) and number of days elapsed after herbicide treatment was significant ($P<0.05$). No significant ($P<0.05$) effect was observed regarding herbicide treatment (Table 3.5b). Microbial activity was slightly stimulated by herbicide treatments over the 21d incubation period for ORS, including the control treatment. Microbial activity decreased over the 21d incubation period post herbicide application for the OPS for all four treatments (Table 3.5a).

Denaturing gradient gel electrophoresis (DGGE): Cluster analysis (Figures 3.2a & b, 3.4a & b) based on the DGGE data, for trials A and B, showed that the number of days after herbicide treatment and soil organic matter content in soil were the two main factors influencing bacterial populations.

Terminal restriction fragment length polymorphism (T-RFLP): Cluster analysis (Figures 3.3a & b, 3.9a & b) based on the T-RFLP patterns, for trials A and B, showed that the number of days after herbicide treatment and soil organic matter content were the two main factors influencing bacterial populations for the experiment.

DISCUSSION

Active C concentration remained stable in the ORS for the duration of the 21 days post herbicide application. Herbicides were possibly utilized by soil microbes as a nutrient source (Altman & Campbell, 1977; Qiu *et al.*, 2009), thus not depleting the resident pool of active C. This assumption is supported by FDA analysis (Table 3.5a) and CLPP (Table 3.4a) results which indicated a slight stimulation of microbial activity as well as AWCD values respectively. However, the control treatment also remained stable in terms of active C. In contrast, active C concentration increased in the OPS over the 21d incubation period post herbicide application, including the control treatment. This suggests that herbicides did not have any additional impact on soil microbes responsible for carbon mineralization.

Soil bacterial activity as measured by CLPP and expressed as AWCD, was higher in the ORS than in OPS. Results showed an increase in AWCD in the glyphosate treated microcosm for both soil types. In the ORS, herbicides yielded higher AWCD values than in the control treatment, indicating that herbicide treatments had a stimulatory effect on the bacterial activity, supporting the hypothesis that the soil micro-organisms are buffered by higher organic matter content and probably utilize herbicides as a nutrient source (Altman & Campbell, 1977; Qiu *et al.*, 2009). In the case of the OPS, AWCD values obtained were lower for the herbicide treatments than the control treatment, indicating less adaptation by the soil bacterial population to the herbicide disturbance than in the ORS due to significantly lower organic matter or mineral content in the OPS. This suggests that soil with a higher organic matter or mineral content will have a buffering effect (Chandra *et al.*, 1960) against herbicide application.

The higher bacterial and microbial activity in ORS was consistent with ergosterol concentrations. This can be attributed to the higher organic matter content in the ORS, and thus higher fungal populations than OPS with low organic matter (Grandy *et al.,* 2009). Soil organic matter content and pH play a definitive role in terms of soil microbial populations (Grandy *et al.,* 2009; Rousk, Brookes & Bååth, 2009; Rousk *et al.,* 2010). Soil pH between 4.5, and 7 favours bacterial as well as fungal growth (Rousk, Brookes & Bååth, 2009; Rousk *et al.,* 2010), which is consistent with pH levels for both soils. The ORS had 96 % organic matter, while the OPS had 0.2 % organic matter. High organic matter increases microbial populations due to increased decomposition and nutrient availability (Grandy *et al.,* 2009), an observation which is consistent with those of the present study.

The ORS and OPS were significantly different in terms of microbial activity as measured by FDA hydrolysis. This observation can be attributed to a higher level of organic matter in the ORS, which buffered the effect of the herbicides, as indicated by CLPP results.   In the ORS, microbial activity showed a stimulatory tendency by herbicide addition based on FDA results. Although not statistically significant, this finding is consistent with that of Mijangos (2009) where glyphosate was utilized by the soil micro-organisms as a source of nutrients, leading to stimulation of the soil microbial community. Similarly, Zabaloy *et al* (2008) found that soil microbial populations reacted differently to herbicide treatment in different soils. Certain soils showed an increase or decrease in microbial activity following herbicide application, while others showed no effect. For instance, in microbial active soil, the mean half-life of atrazine is 2.4 times lower than in sterile soil (Accinelli *et al.*, 2001). There is a positive correlation between

the size of soil microbial populations and soil organic matter content. Microbial activity in turn represents the capacity of soil micro-organisms to respond to herbicide treatments (Sebiomo, Ogundero & Bankole, 2011). In ORS, the positive effect of micro-organisms in the process of degrading this xenobiotic compound can possibly be attributed to higher diversity and a broad spectrum of produced enzymes. Micro-organisms can utilize different sources of carbon and nitrogen to adapt to different soil environments (Altman & Campbell, 1977; Struthers, Jajychandran & Moorman, 1998; Seffernick *et al.*, 2000). For OPS, results suggest that herbicides did not influence soil microbial activity due to the fact that the control treatment reacted similarly to the herbicide treatments. Clearly other factors were responsible for a decrease in microbial activity observed during the incubation period.

The two soils differed in terms of chemical characteristics and pH (Table 3.1). Soil pH can directly influence soil micro-organisms and is therefore an important factor in terms of soil fertility (Ghorbani *et al.*, 2008). Soil pH can determine the amount of herbicide which will be available to the soil micro-organisms (Ortiz-Hernández *et al.*, 2011), as well as the persistence of herbicides (Curran, 1998). Microbial degraders work in natural environments and some alterations are imperative to encourage the organisms to degrade the herbicide at a faster rate in a limited time frame (Kanissery & Sims, 2011), thus leading to higher microbial activity and stable active carbon levels. The OPS had a clear disadvantage over the ORS in terms of initial microbial activity, active carbon concentrations, fungal biomass and microbial diversity which resulted in lower resilience of the microbial community.

The effects of pesticides on soil micro-biota can be assessed at the whole or sub-community level. The use of molecular tools has greatly improved the ability to detect pesticide induced changes in soil, as they allow better resolution of the microbial community structure (Zabaloy *et al.*, 2011). The DNA patterns represent a highly sensitive measure of microbial diversity and changes which may occur therein following herbicide application (Johnsen *et al.*, 2001). In the present study, the T-RFLP technique was standardized regarding (1) concentration of template DNA before PCR amplification, (2) PCR-amplified DNA quality before restriction digestion, and (3) hydrolyzed PCR products before sizing the terminally labelled fragments (Thies, 2007). During standardization, DNA concentration was increased and *E. coli* was included in the 16S PCR template as an internal standard. It was evident that the ORS had a much higher DNA concentration than OPS, and the protocol was thus adjusted accordingly. Results obtained from the present study from DGGE and T-RFLP data, clearly show that soil organic matter content and number of days post herbicide addition played a more significant role in how the soil micro-organisms reacted, than the type of herbicide used. Results obtained from DGGE and T-RFLP data were consistent.

The present study confirmed that soil organic matter plays a role in terms of how soil micro-organisms react to herbicide application (Busse *et al.,* 2001; Haney *et al.*, 2002; Zabaloy *et al.*, 2008). Results strongly suggest that certain micro-organisms can utilize herbicides as a source of C and nutrients (Araújo *et al.,* 2003; Marecik *et al.,* 2008). Due to the control treatments reacting the same as the herbicide treatments in terms of active carbon, carbon source utilization patterns and microbial activity, it can be concluded that there where other factors that had greater impact on the soil biota than

the herbicide treatments. The two most significant factors were soil organic matter content, and incubation period. The great difference in soil chemical properties could also have contributed to the change in microbial populations present in the two soils. This is supported by the view that microbial response to herbicides manifests itself in a variety of ways depending on: the herbicide itself, inherent micro-organism populations, herbicide concentration, exposure time, and chemical and physical characteristics of the soil (Levine & Rachakornkij, 1994).

It was clearly evident that the ORS remained more stable during the incubation period than the OPS soil which experienced greater change over the incubation period. Thus, the hypothesis that high organic matter content in soil reacts as a buffer to xenobiotic disturbances was confirmed. Herbicides *per se*, however, did not have a significant impact on soil micro-organisms as measured by the techniques used in this study.

The present study suggests that one cannot rely on one biochemical or molecular technique to provide an accurate indication of soil microbial biomass (Zhao, Lin & Brookes, 2005), diversity and activity, since each technique brings different insights to the bigger picture. Thus, future studies need to incorporate a number of techniques to analyse soil microbial populations in order to make accurate and reliable conclusions as to how xenobiotics influence these sensitive micro-organisms.

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Table 3.1. Chemical characteristics of soils tested.

Soil type	Organic matter (OM) %	pH	Ca (mg/kg)	K (mg/kg)	Mg (mg/kg)	Na (mg/kg)	Zn (mg/kg)	Fe (mg/kg)	Cu (mg/kg)	P (mg/kg)
Organically poor soil (OPS) (Bainsvlei soil)	0.232	6.02	474.0	120.0	76.0	44.0	1.78	16.36	1.0	19.76
Organically rich soil (ORS)	96	7.01	14813.0	1800.0	3525.0	275.0	41.8	12050.0	15.3	1110.0

Table 3.2. Herbicides selected for microcosm assays (Van Zyl, 2012).

Herbicide Trade name	Active ingredient(s)	Recommended dosage ha^{-1} (Maize) (11-20 % clay)	Recommended use	Herbicide group
2,4-D Amine Weed Killer	Dimethylamine salt of 2,4-D-Dichlorophenoxyacetic acid – 480 g/ L	3.3 L ha^{-1}	Selective broadleaf weed control	O
Gramoxone	Paraquat 200 g/ L (present as dichloride)	2 L ha^{-1}	Control of annual grasses and annual broadleaf weeds in crops. Inactivated on contact with soil.	D (22)
Roundup®	Glyphosate	4.7 L ha^{-1}	Post-emergence control of annual and perennial weeds	G

Table 3.3a. The effect of herbicides on soil microbes in terms of the amount of active carbon present in the soil.

| Herbicide | Soil | Average Active carbon (mg/ g soil) | | | Mean |
| | | DAY | | | |
		0	3	21	
Control	Organically rich soil	1339.96	1339.96	1339.96	**1339.96**
2,4-D-Dichlorophenoxyacetic acid	Organically rich soil	1339.96	1339.96	1339.96	**1339.96**
Paraquat	Organically rich soil	1339.96	1339.96	1339.96	**1339.96**
Glyphosate	Organically rich soil	1339.96	1339.96	1339.96	**1339.96**
Control	Organically poor soil	985.539	1046.981	1028.549	**1020.36**
2,4-D-Dichlorophenoxyacetic acid	Organically poor soil	999.768	1039.867	1055.066	**1031.57**
Paraquat	Organically poor soil	986.832	1048.922	1062.504	**1032.75**
Glyphosate	Organically poor soil	992.33	1028.225	1043.101	**1021.22**
Mean		**1165.54**	**1190.48**	**1193.63**	

Fisher's LSD (P<0.05): Herbicide = 26.97; Soil = 19.07; Day = 23.36; Soil x Day = 33.03;

Herbicide x Soil x Day = 66.07

Table 3.3b. ANOVA table as calculated for the effect of herbicides on active carbon concentration.

Source Term	DF	Sum of Squares	Mean Square	F-ratio	Probability level	Power (Alpha = 0.05)
A: Soil	1	1179301	1179301	1156.56	0.000000*	1.000000
B: Days	2	7579.875	3789.938	3.72	0.039929*	0.372203
AB	2	7579.875	3789.938	3.72	0.039929*	0.372203
C: Herbicide	3	391.1998	130.3999	0.13	0.942586	0.060976
AC	3	391.1998	130.3999	0.13	0.942586	0.060976
BC	6	658.2881	109.7147	0.11	0.994695	0.062628
ABC	6	658.2881	109.7147	0.11	0.994695	0.062628
D: Reps	1	4282.321	4282.321	4.20	0.052003	0.294112
S	23	23452.17	1019.66			
Total (Adjusted)	47	1224294				
Total	48					

*Term significant at alpha = 0.05

Table 3.4a. The average well colour development (AWCD) as determined using BiologEcoPlates™ of herbicide treatments, for organically rich and poor soil.

Herbicide	Soil	AWCD			Mean
		DAY			
		0	3	21	
Control	Organically rich soil	1.04	1.61	1.72	**1.46**
2,4-D-Dichlorophenoxyacetic acid	Organically rich soil	1.42	1.48	1.59	**1.50**
Paraquat	Organically rich soil	1.65	1.55	1.68	**1.63**
Glyphosate	Organically rich soil	1.01	1.69	1.80	**1.50**
Control	Organically poor soil	1.34	0.70	1.66	**1.23**
2,4-D-Dichlorophenoxyacetic acid	Organically poor soil	0.81	1.02	1.01	**0.95**
Paraquat	Organically poor soil	1.04	0.97	1.38	**1.13**
Glyphosate	Organically poor soil	0.79	1.14	1.28	**1.07**
Mean		**1.14**	**1.27**	**1.51**	

Fisher's LSD (P<0.05): Herbicides (H) = 0.32; Soil (S) =0.22; Day (D) = 0.27; HxDxS =0.77

Table 3.4b. ANOVA table as calculated for average well colour development (AWCD) to evaluate the effect of herbicides on soil bacteria community level physiological profiles as determined by BiologEcoPlates™.

Source Term	DF	Sum of Squares	Mean Square	F-ratio	Probability level	Power (Alpha = 0.05)
A: Soil	1	2.163846	2.163846	15.50	0.000659*	0.776552
B: Reps	1	$8.333334e^{-10}$	$8.333334e^{-10}$	0.00	0.999939	0.415370
C: Days	2	1.175067	0.5875336	4.21	0.027724*	0.415370
AC	2	0.2516285	0.1258142	0.90	0.420044	0.120198
D: Herbicide	3	0.1687216	$5.624054e^{-02}$	0.40	0.752387	0.086250
AD	3	0.1799648	$5.998826e^{-02}$	0.43	0.733755	0.088832
CD	6	0.6880007	0.1146668	0.82	0.565065	0.169099
ACD	6	0.6614441	0.1102407	0.79	0.587363	0.163687
S	23	3.211876	0.1396468			
Total (Adjusted)	47	8.500549				
Total	48					

*Term significant at alpha = 0.05

Table 3.5a. The effect of herbicides on soil microbial activity as determined by fluorescein diacetate (FDA) hydrolysis.

Herbicide	Soil	Average FDA hydrolyzed (µg/ml^{-1} soil solution)			Mean
		DAY			
		0	3	21	
Control	Organically rich soil	0.602	0.582	0.655	**0.61**
2,4-D-Dichlorophenoxyacetic acid	Organically rich soil	0.610	0.623	0.653	**0.63**
Paraquat	Organically rich soil	0.588	0.610	0.660	**0.62**
Glyphosate	Organically rich soil	0.593	0.628	0.660	**0.63**
Control	Organically poor soil	0.220	0.049	0.084	**0.12**
2,4-D-Dichlorophenoxyacetic acid	Organically poor soil	0.213	0.048	0.044	**0.10**
Paraquat	Organically poor soil	0.174	0.081	0.030	**0.09**
Glyphosate	Organically poor soil	0.171	0.085	0.069	**0.11**
Mean		**0.40**	**0.34**	**0.36**	

Fisher's LSD (P<0.05): Herbicides (H) = 0.92; Soil (S) = 0.65; Day (D) = 0.80; SxD = 1.12; HxSxD = 2.25

Table 3.5b. ANOVA table as calculated for the effect of herbicides on soil microbial activity as determined by fluorescein diacetate hydrolysis.

| Source Term	DF	Sum of Squares	Mean Square	F-ratio	Probability level	Power (Alpha = 0.05)
A: Soil	1	3.200234	3.200234	2708.08	0.000000*	1.000000
B: Herbicide	3	$7.631666e^{-04}$	$2.543889e^{-04}$	0.22	0.884787	0.068772
AB	3	$1.85975e^{-03}$	$6.199167e^{-04}$	0.52	0.669735	0.098121
C: Days	2	$2.819017e^{-02}$	$1.409508e^{-02}$	11.93	0.000279*	0.862523
AC	2	$8.210316e^{-02}$	$4.105158e^{-02}$	34.74	0.000000*	0.999670
BC	6	$8.078833e^{-03}$	$1.346472e^{-03}$	1.14	0.371546	0.225862
ABC	6	0.0022785	$3.7975e^{-04}$	0.32	0.919040	0.090680
D: Reps	1	$8.500833e^{-04}$	$8.500833e^{-04}$	0.72	0.405097	0.090302
S	23	$2.717992e^{-02}$	$1.181736e^{-03}$			
Total (Adjusted)	47	3.351538				
Total	48					

*Term significant at alpha = 0.05
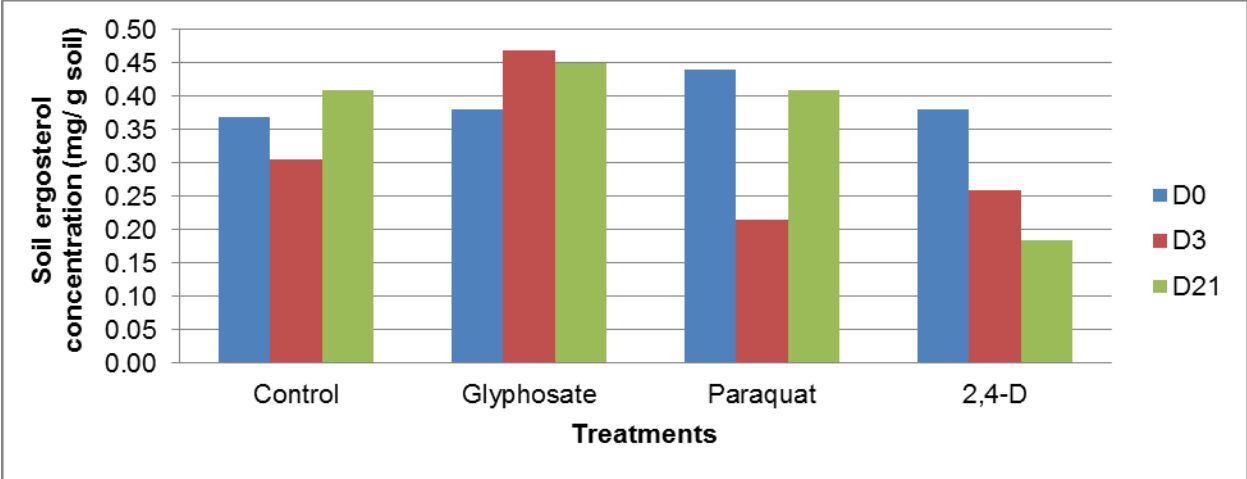
Figure 3.1a. Soil ergosterol concentrations for organically poor soil. (D0 = Day 0 (pre-herbicide application); D3 = Day 3 (post-herbicide application); D21 = Day 21 (post-herbicide application). *Fisher's LSD (P<0.05): Soil = 32.618.*

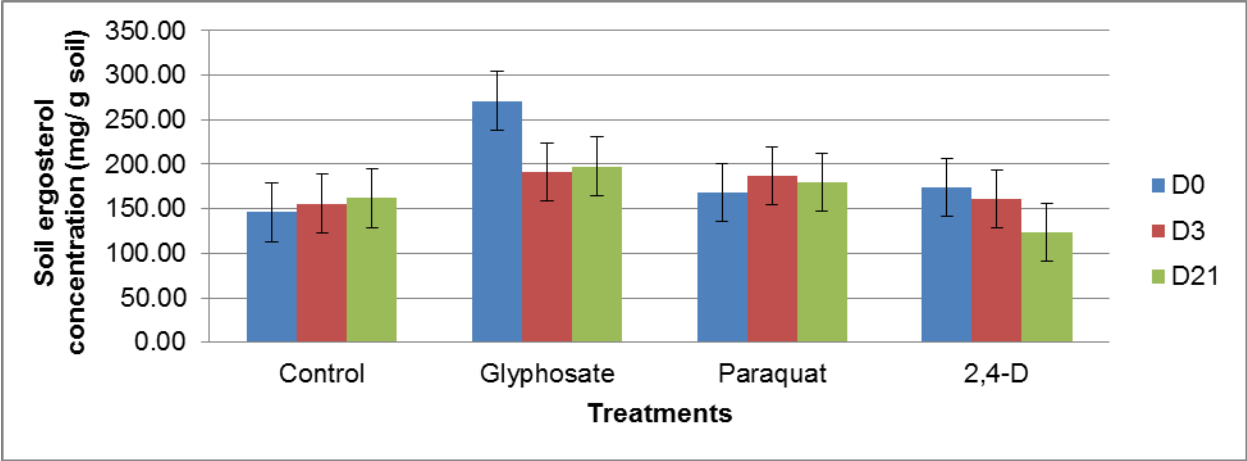**Figure 3.1b.** Soil ergosterol concentrations for the organically rich soil. (D0 = Day 0 (pre-herbicide application); D3 = Day 3 (post-herbicide application); D21 = Day 21 (post-herbicide application). *Fisher's LSD (P<0.05): Soil = 32.618.*

Table 3.6. ANOVA table as calculated for the effect of herbicides on soil ergosterol concentration.

Source Term	DF	Sum of Squares	Mean Square	F-ratio	Probability level	Power (Alpha = 0.05)
A: Soil	1	372509.7	372509.7	124.89	0.000000*	1.000000
B: Herbicide	3	8897.264	2965.755	0.99	0.413057	0.147065
AB	3	8835.201	2945.067	0.99	0.416099	0.146310
C: Days	2	1198.47	599.2351	0.20	0.819408	0.064678
AC	2	1190.235	595.1177	0.20	0.820521	0.064575
BC	6	4362.108	727.018	0.24	0.956982	0.080061
ABC	6	4375.409	729.2349	0.24	0.956670	0.080161
D: Reps	1	5206.042	5206.042	1.75	0.199444	0.149834
S	23	68600.75	2982.641			
Total (Adjusted)	47	475175.2				
Total	48					

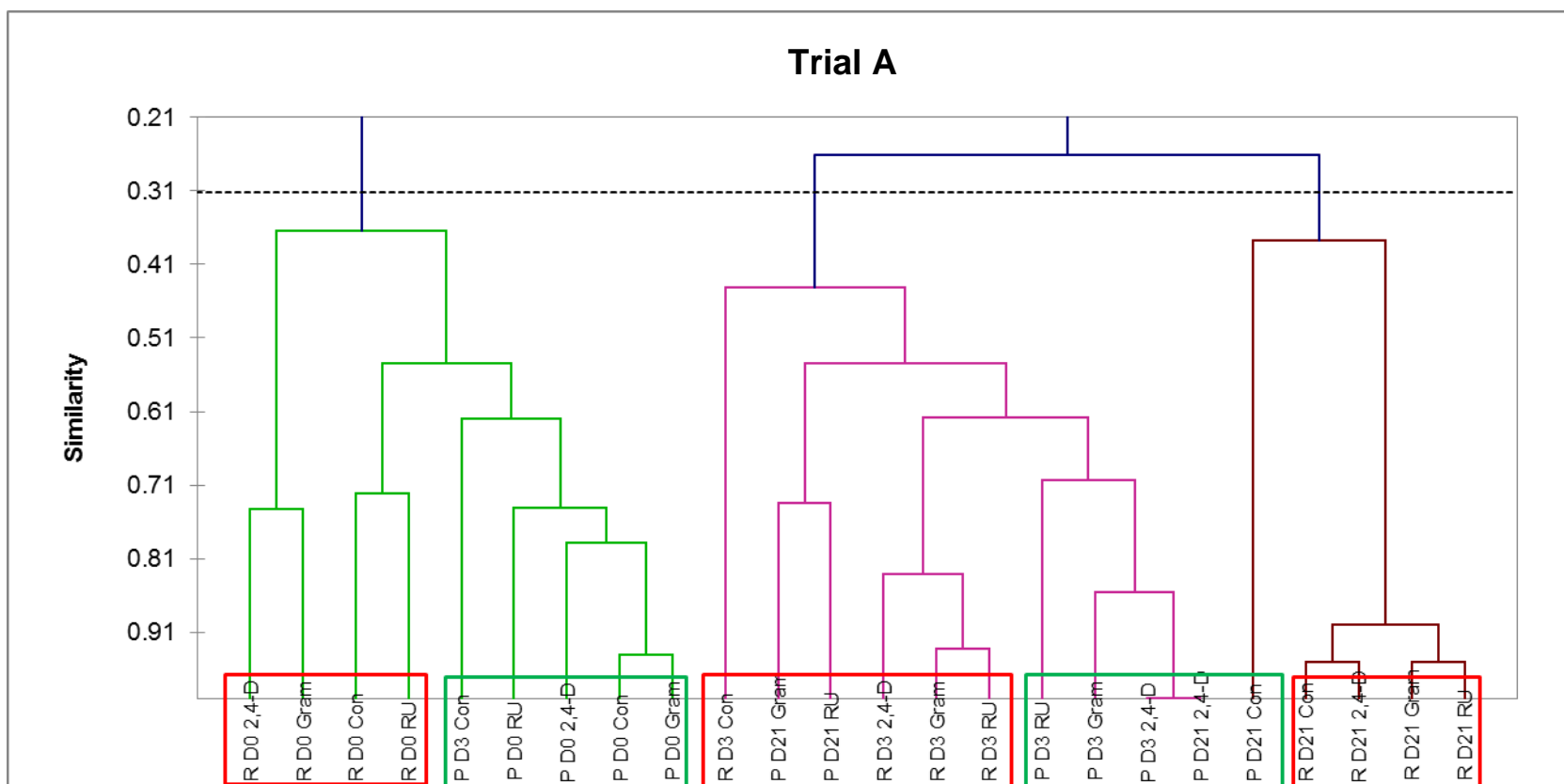*Term significant at alpha = 0.05

Figure 3.2a. The effect of 2,4-D, glyphosate and paraquat on soil bacterial diversity as determined by DGGE in two replicate trials (A and B). The above dendrogram represents data from trial A. (R = Organically rich soil; P = Organically poor soil; D0 = Day 0 (pre-herbicide application); D3 = Day 3 (post-herbicide application); D21 = Day 21 (post-herbicide application; 2,4-D = 2,4-D-Dichlorophenoxyacetic acid; Gram = Gramoxone (paraquat); RU = Roundup®(glyphosate)).
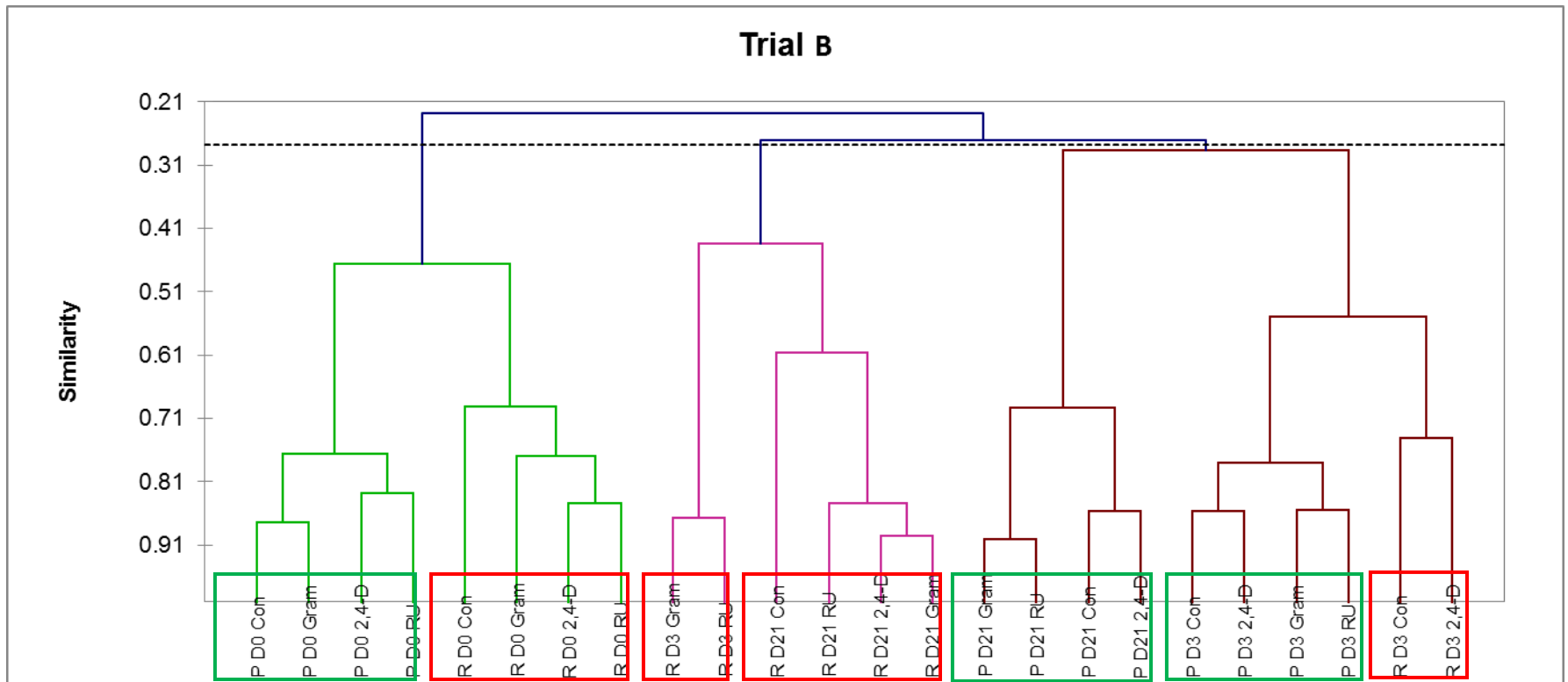
Figure 3.2b. The effect of 2,4-D, glyphosate and paraquat on soil bacterial diversity as determined by DGGE in two replicate trials (A and B). The above dendrogram represents data from trial B. (R = Organically rich soil; P = Organically poor soil; D0 = Day 0 (pre-herbicide application); D3 = Day 3 (post-herbicide application); D21 = Day 21 (post-herbicide application; 2,4-D = 2,4-D-Dichlorophenoxyacetic acid; Gram = Gramoxone (paraquat); RU = Roundup[®](glyphosate)).
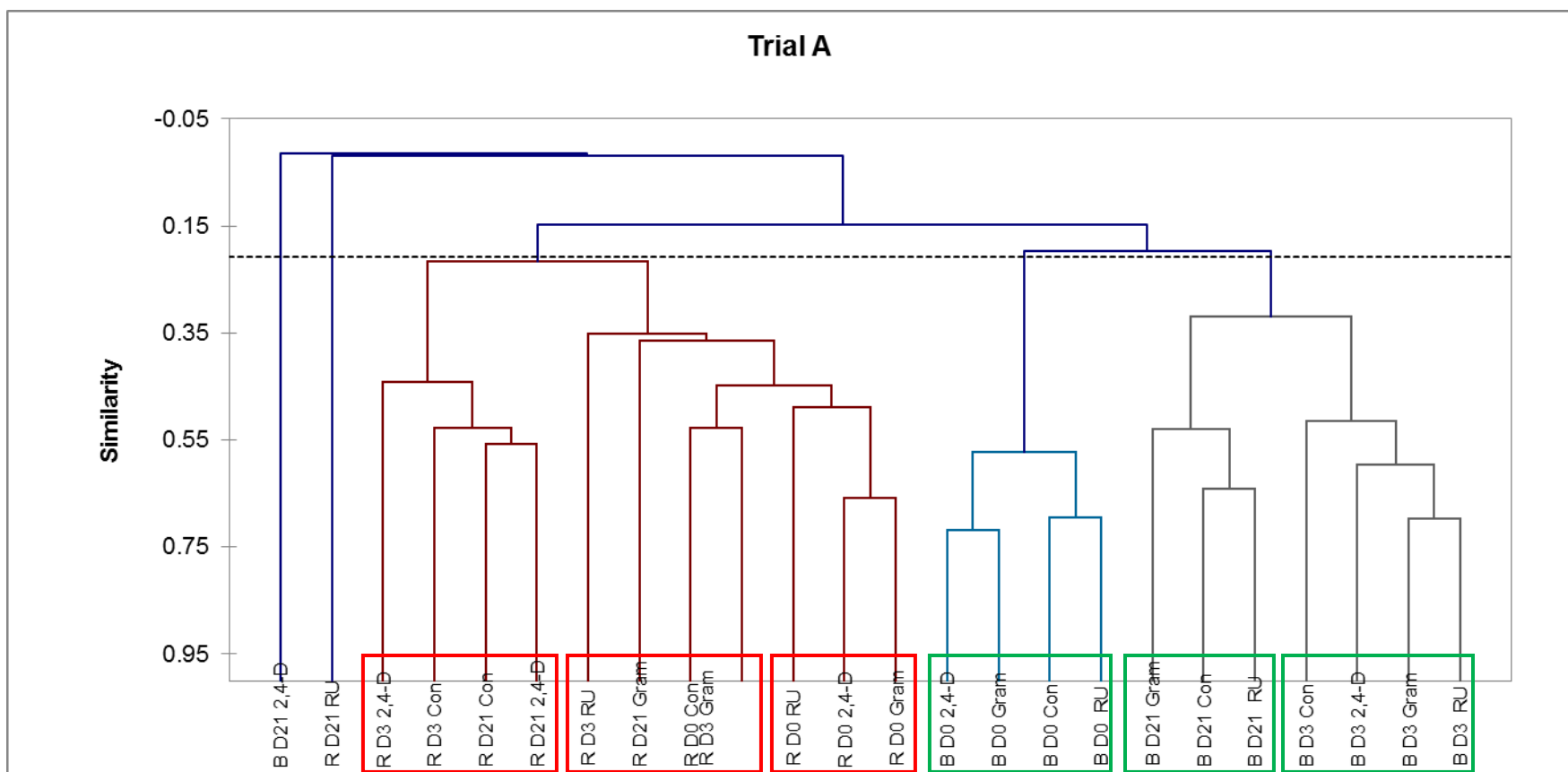
Figure 3.3a. The effect of 2,4-D, glyphosate and paraquat on soil bacterial diversity as determined by T-RFLP in two replicate trials (A and B). The above dendrogram represents data from trial A. (R = Organically rich soil; P = Organically poor soil; D0 = Day 0 (pre-herbicide application); D3 = Day 3 (post-herbicide application); D21 = Day 21 (post-herbicide application; 2,4-D = 2,4-D-Dichlorophenoxyacetic acid; Gram = Gramoxone (paraquat); RU = Roundup®(glyphosate)).
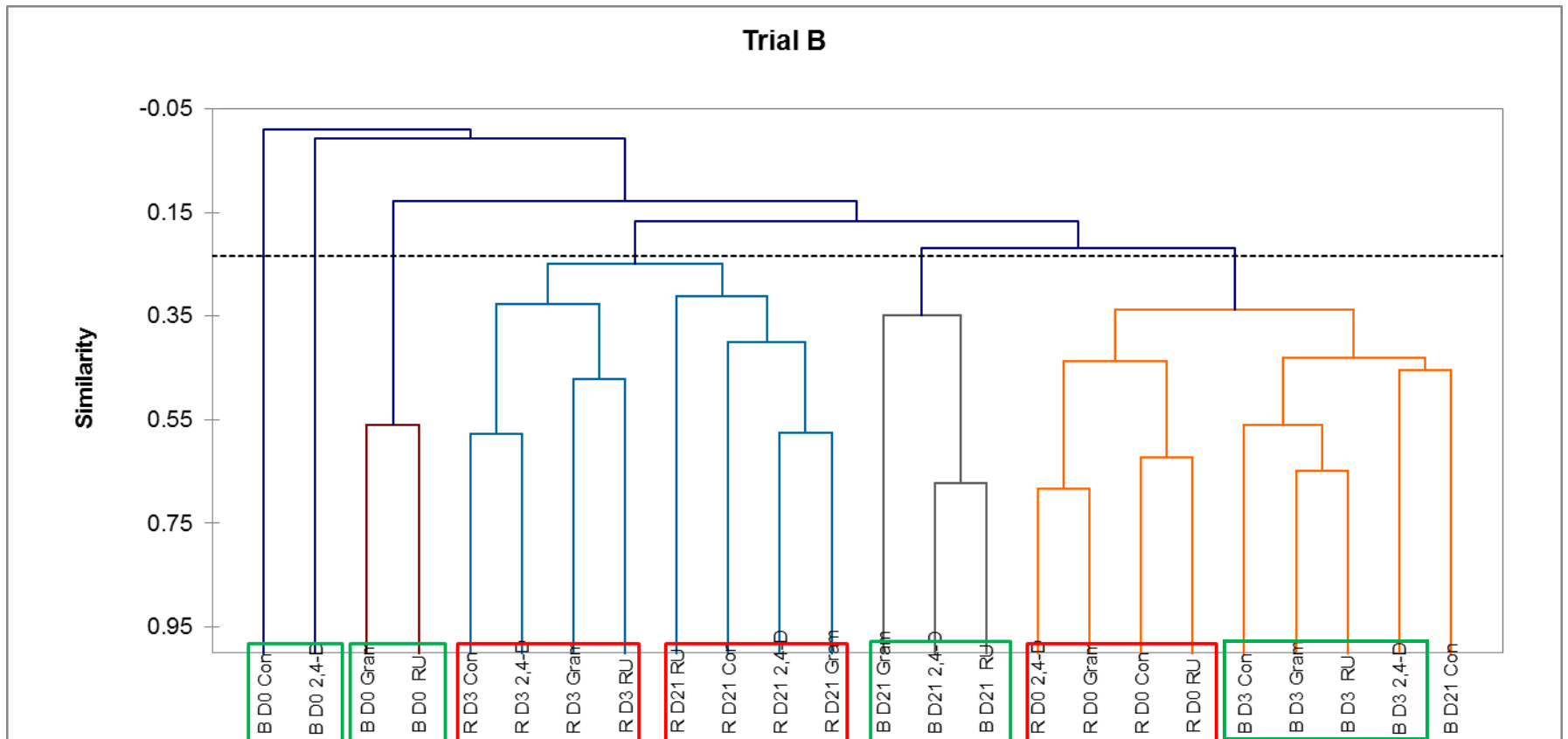
Figure 3.3b. The effect of 2,4-D, glyphosate and paraquat on soil bacterial diversity as determined by T-RFLP in two replicate trials (A and B). The above dendrogram represents data from trial B. (R = Organically rich soil; P = Organically poor soil; D0 = Day 0 (pre-herbicide application); D3 = Day 3 (post-herbicide application); D21 = Day 21 (post-herbicide application; 2,4-D = 2,4-D-Dichlorophenoxyacetic acid; Gram = Gramoxone (paraquat); RU = Roundup[®](glyphosate)).
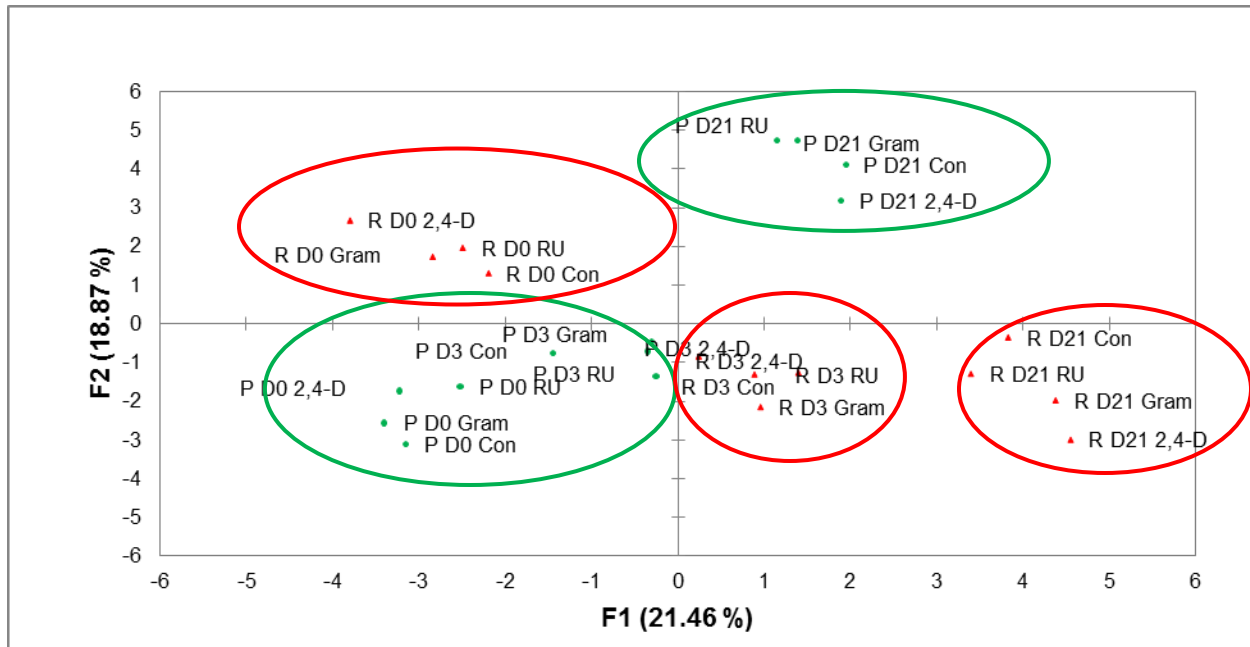
Figure 3.4a. The effect of 2,4-D, glyphosate and paraquat on soil bacterial diversity as determined by DGGE in two replicate trials (A and B). The above PCA represents data from trial A. (R = Organically rich soil; P = Organically poor soil; D0 = Day 0 (pre-herbicide application); D3 = Day 3 (post-herbicide application); D21 = Day 21 (post-herbicide application; 2,4-D = 2,4-D-Dichlorophenoxyacetic acid; Gram = Gramoxone (paraquat); RU = Roundup$^{®}$(glyphosate)).

Figure 3.4b. The effect of 2,4-D, glyphosate and paraquat on soil bacterial diversity as determined by DGGE in two replicate trials (A and B). The above PCA represents data from trial B. (R = Organically rich soil; P = Organically poor soil; D0 = Day 0 (pre-herbicide application); D3 = Day 3 (post-herbicide application); D21 = Day 21 (post-herbicide application; 2,4-D = 2,4-D-Dichlorophenoxyacetic acid; Gram = Gramoxone (paraquat); RU = Roundup®(glyphosate)).

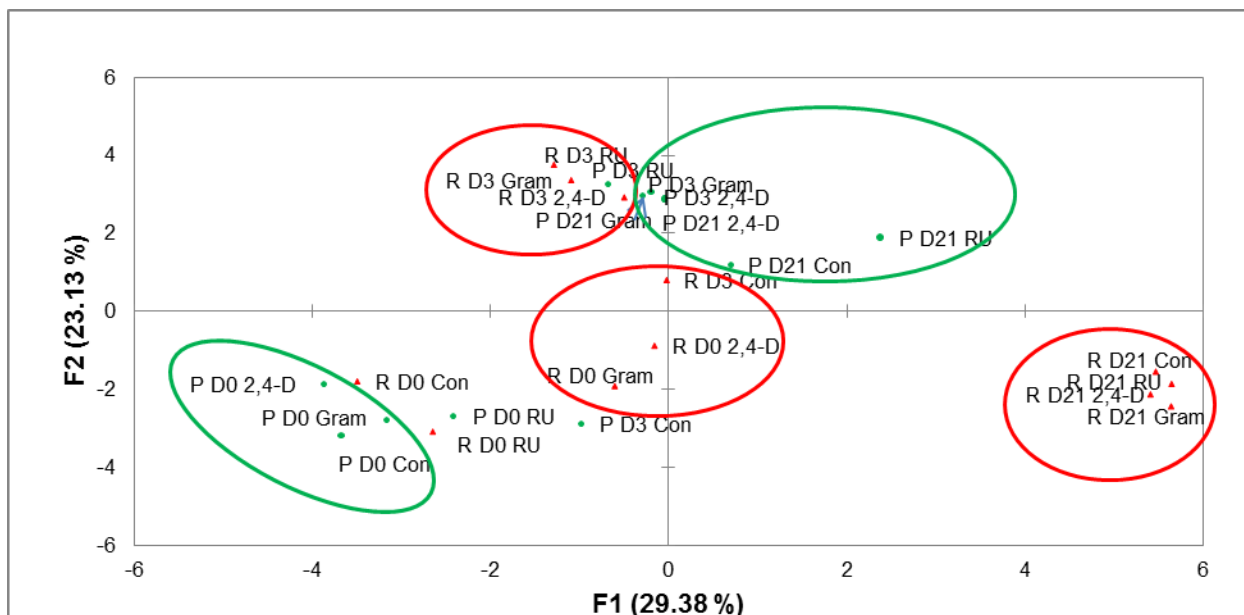**Figure 3.5a.** The effect of 2,4-D, glyphosate and paraquat on soil bacterial diversity as determined by T-RFLP in two replicate trials (A and B). The above PCA represents data from trial A. (R = Organically rich soil; P = Organically poor soil; D0 = Day 0 (pre-herbicide application); D3 = Day 3 (post-herbicide application); D21 = Day 21 (post-herbicide application; 2,4-D = 2,4-D-Dichlorophenoxyacetic acid; Gram = Gramoxone (paraquat); RU = Roundup[®](glyphosate)).
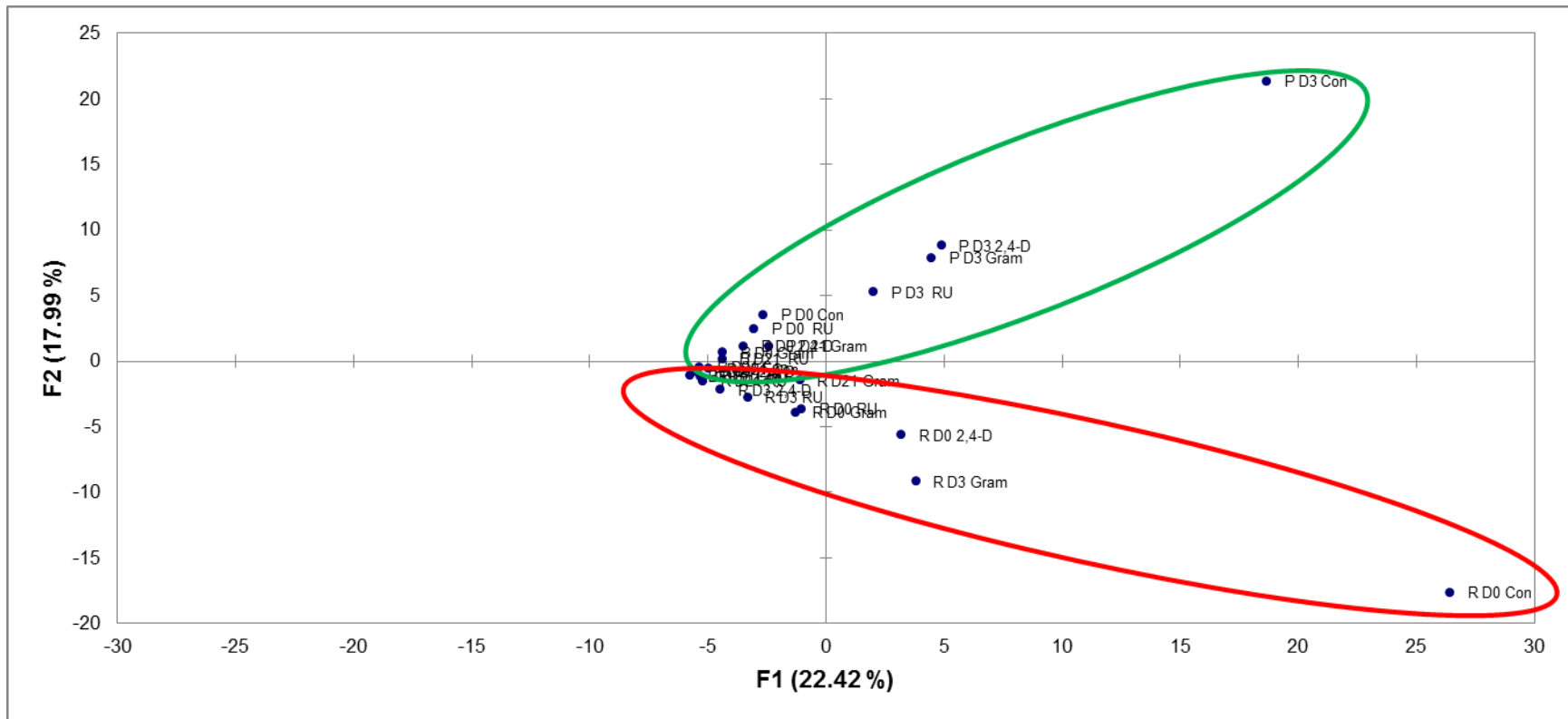
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Figure 3.5b. The effect of 2,4-D, glyphosate and paraquat on soil bacterial diversity as determined by T-RFLP in two replicate trials (A and B). The above PCA represents data from trial B. (R = Organically rich soil; P = Organically poor soil; D0 = Day 0 (pre-herbicide application); D3 = Day 3 (post-herbicide application); D21 = Day 21 (post-herbicide application; 2,4-D = 2,4-D-Dichlorophenoxyacetic acid; Gram = Gramoxone (paraquat); RU = Roundup®(glyphosate)).
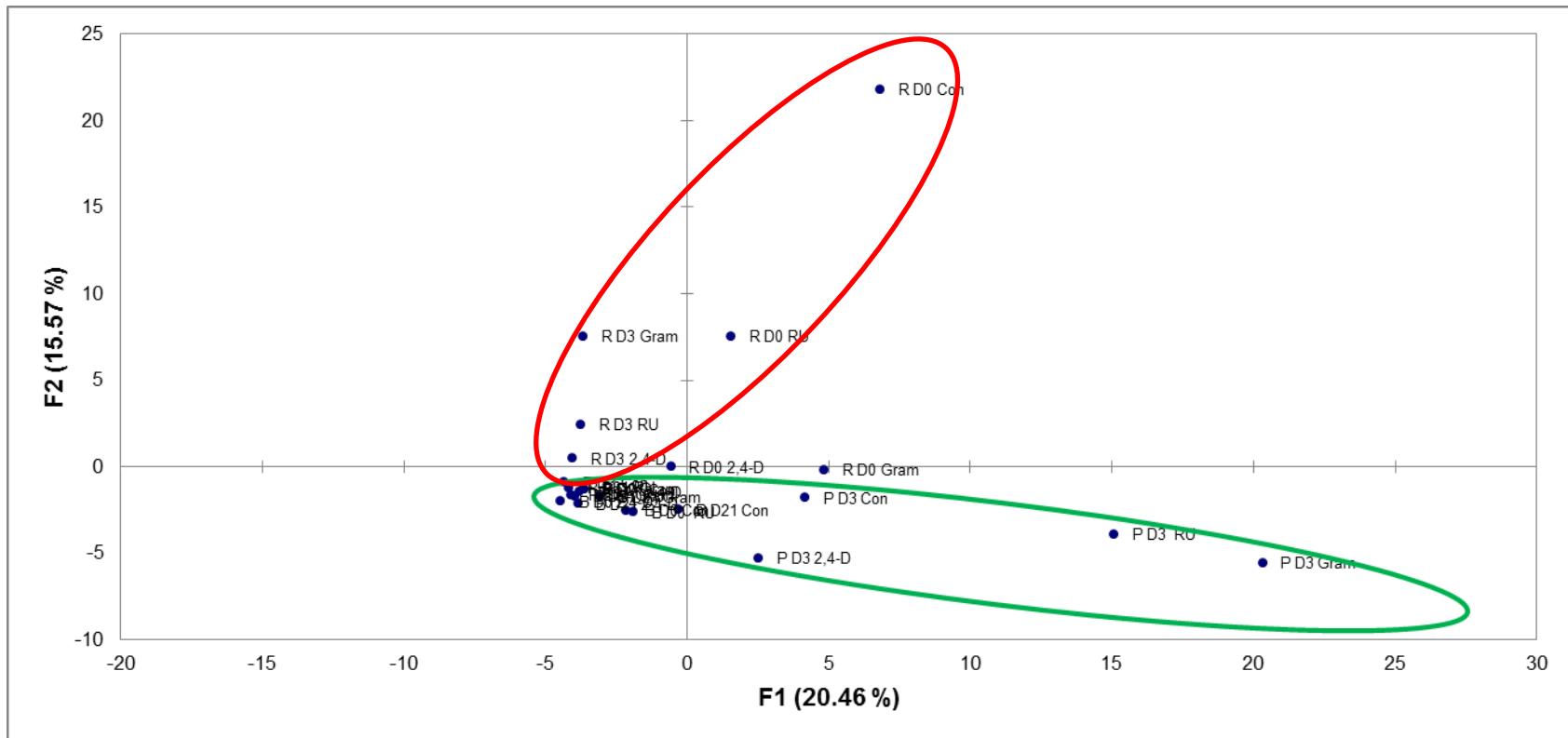
CHAPTER 4

The influence of glyphosate resistant and BT maize on rhizosphere microbes

ABSTRACT

The effect of glyphosate resistant (GR) and BT maize was investigated to determine whether rhizosphere microbes are influenced by root exudations. Microbial activity in soil collected from the rhizosphere of the different maize genotypes growing in two different soil types, one having high clay content (HCC), the other low clay content (LCC), was studied using various biochemical and molecular techniques. Eight maize hybrids of isogenic origin were assessed after cultivation in pots. No significant quantitative or qualitative effect of the GR or BT gene on rhizosphere microbes was observed using community-level physiological profiling (BiologEcoPlates™), active carbon determination, ergosterol extraction, denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphisms (T-RFLP). Fluorescein diacetate (FDA) hydrolysis did, however, show significant differences between rhizosphere microbial populations from the different genotypic backgrounds for both soil types. This study suggests that the root exudations from different genetically modified maize genotypes does have an influence on rhizosphere microbial populations.

Keywords: *Rhizosphere microbes, GR maize; BT maize, high clay content soil, low clay content soil.*

INTRODUCTION

Soil is a dynamic system in which physical, chemical and biological components interact. Within this system, micro-organisms perform an important task in the decomposition and transformation of soil materials, and are involved in carbon, nitrogen and phosphorous cycles, as well as the mobilization of nutrients (Icoz & Stotzky, 2008; Bastida *et al.*, 2009). Rhizosphere micro-organisms play a major role in nutrient transformations and element cycling, and any impact that genetically modified (GM) crops may have on the dynamics of the rhizosphere could affect both plants and soil (Dunfield & Germida, 2004). Thus, any alteration to the soil environment that affects soil micro-organisms will consequently affect soil health and ecosystem functioning (Dunfield & Germida, 2004).

Genetically modified (GM) crops have been promoted to posess a longer shelf life, enhanced disease and pest resistance, herbicide tolerance, nutritional improvement and, resistance to abiotic stresses such as drought or nitrogen starvation (Icoz & Stotzky, 2008). Since they also may have an opposite effect, the introduction of GM crops into an agricultural ecosystem has, however, raised a number of questions regarding the ecological impact these plants may have on the soil. Root exudates govern which organisms reside in the rhizosphere, and any change to the quality of rhizosphere exudations could modify soil dynamics and the activity of soil organisms (Savka & Farrand, 1997; Castaldini *et al.,* 2005; Icoz & Stotzky, 2008).

Genetically modified crops may potentially have adverse effects on non-target soil organisms which may lead to a change in microbe-mediated processes and functions in soil (Savka & Farrand, 1997; Castaldini *et al.,* 2005; Icoz & Stotzky, 2008).

Glyphosate resistant (GR) cropping systems can change the soil environment by introducing novel compounds (including glyphosate) into the soil environment. Rhizosphere microbes may be particularly sensitive to the effects of GM crops because of their intimate proximity with plant roots and thus root exudates (Hart *et al.*, 2009).

It is important to understand the impact of transgenic crop residues on soil microbial ecology. This is because of the long-term agronomic and environmental effects of GM crops for which appropriate management practices have to be developed for minimizing potential negative effects (Fang *et al.*, 2007). Such effects may occur because of the differences in soil chemical composition, quantity, and botanical form of BT corn residues, soil properties, temporal variation, and changes in management practices (Castaldini *et al.,* 2005; Fang *et al.,* 2007). Generally, the incorporation of residues from BT maize, containing a high lignin content and an elevated lignin/ nitrogen ratio, alters soil functional activity (substrate metabolism) and the structure of microbial communities compared with non-BT isolines (Fang *et al.*, 2007). Residues from BT maize have been shown to affect soil bacterial community function as measured by substrate utilization assays (Fang *et al.*, 2007).

In the present study, the effect of GR and BT maize and conventional maize from isogenic origin, on rhizosphere organisms were investigated. It was hypothesized that root exudates of GR and BT maize cultivars will lead to qualitative or quantitative changes in the activity of rhizosphere microbes. Biochemical and molecular techniques were used to investigate the effects on rhizosphere microbial activity.

MATERIALS AND METHODS

Soil sampling.   Soil was collected from an agricultural experimental farm, Kenilworth near Bloemfontein (-29.020397 S; 26.145308 E).   The soil has a 10 year history of annual tilling, and while sampling, non-GM maize was being cultivated on the field.   The soil is categorized as a Kenilworth-Bainsvlei ecotype and is an example of a fine sandy loam soil in a semi-arid area according to the Soil Classification Working Group (1991), and will henceforth be referred to as low clay content (LCC) soil (Table 4.1).   A second soil sample was from a another experimental farm, Paradys (-29.222950 S; 26.210747 E) which is catergorized as Tukulu according to the Soil Classification Working Group (1991), and will henceforth be referred to as high clay content (HCC).   In both instances, bulk soil samples were collected from the top 5 cm of the surface.   Chemical and textural analyses were conducted for both soils (Table 4.1 & 4.2).

Trial layout.   Eight maize hybrids from two near isogenic families were used, PAN 4P-116, PAN 4P-316B (BT), PAN 4P-516 R (GR), PAN 4P-716BR (BT + GR), PAN 6P-110, PAN 6Q-308B (BT), PAN 6Q-508 R (GR), and PAN 6Q-708BR (BT + GR).   Four to five seeds were planted in each batch of soil contained in 2L plastic bags. After germination, plants were thinned to three per bag.   The experiment was completely randomized with three replicates per treatment, and conducted twice. Plants were maintained in the glasshouse at 24˚C day temperature and 19˚C night temperature.   Plants were watered daily using filtered municipal water to eliminate chlorine.   Plants were fertilized weekly using Multifeed® Classic (19: 8: 16; N: P: K)

according to the manufacturer's instructions. Plants were sampled 8 weeks after planting.

Rhizosphere sampling. Soil was sampled by shaking off excess soil from plant roots and sampling soil clinging to roots. Plastic tubes containing the rhizosphere soil were placed in an isolated container containing ice-packs.

Community-level physiological profiling (CLPP). Community-level physiological profiles (CLPP) of the two soil samples for each hybrid were assessed using the BiologEcoPlates[TM] (Biolog, Hayward, CA, USA). One BiologEcoPlate was used per treatment. Each plate contains three replicate substrate sets. These plates provide specific substrates in each well to indicate nutrient utilization. A soil suspension was made by suspending 2 g of soil in 200 ml of sterilized water (Palmroth *et al.*, 2004) and shaking it for 30 min at room temperature to allow the soil to become totally suspended in the water. Each well of the microtiter plate was filled with 90 µl of the soil suspension and plates were incubated at 25 °C in a Labcon Growth chamber in the dark for 96 h (Palmroth *et al.*, 2004). The change in optical density (OD) was then measured at 590 nm using a multi-well plate reader to indicate nutrient activity (Akmal *et al.*, 2005). The overall colour development in each BIOLOG EcoPlate[TM] was expressed as average well colour development (AWCD) (Garland & Mills, 1991; Cai *et al.*, 2010).

Determination of soil ergosterol. Ergosterol is an important membrane sterol in almost all eumycotic fungi and has been postulated to be strongly associated with living cytoplasmic fungi in the soil (Chiocchio & Matković, 2011). In order to estimate fungal biomass in soil samples (Davis & Lamar, 1992), ergosterol was extracted as described by Jambunathan, Kherdekar & Vaidya (1991) with minor modifications.

Methanol (50 ml) was added to 10 g of moist soil in a 50 ml centrifuge tube, and the solution was agitated in a Heidolph Multi Reax shaker for 40 min at 1600 rpm. The suspension was then allowed to settle for approximately 30 min after which 25 ml of the clean supernatant was removed and added to a clean 50 ml centrifuge tube containing 3 g of potassium hydroxide (KOH). The mixture was again shaken for 10 min to allow the KOH to dissolve, after which 10 ml of n-hexane was added followed by incubation in a 75 °C water bath for 30 min. The solution was then cooled to room temperature and 5 ml distilled water was added. The hexane layer was then removed and transferred to a clean 50 ml centrifuge tube. Ten millilitres of n-hexane was added to the rest of the aliquot in the tube and mixed well. The hexane layer was again removed and added to the previous hexane aliquot. The last two steps were repeated. The hexane extract was then placed in a hot water bath and evaporated until a dry residue was left. The residue was dissolved in 5 ml of methanol and the solution filtered through a 0.45 µm millipore filter and placed in a 2 ml high performance liquid chromatograph (HPLC) bottle. The filtrate was analyzed at an emission wavelength of 282 nm using a Perkin Elmer HPLC fitted with a Phenomene x C18.5 µm 4.6 x 150 mm analytical column. Ergosterol concentrations were calculated and data from both trials were pooled and subjected to analysis of variance (ANOVA) and, when F values were significant ($P<0.05$), means were separated using Fisher's protected LSD test.

Fluorescein diacetate hydrolysis. Fluorescein diacetate (FDA) hydrolysis is widely accepted as an accurate and simple method for measuring total microbial activity in soil environments (Adam & Duncan, 2001). Schnurer & Rosswall (1982) found that

there is a good correlation between FDA hydrolysis and respiration in soil. The method has the advantages of being simple, rapid, and sensitive (Schnurer & Rosswall, 1982).

The method of Adam & Duncan (2001), as modified by Zabaloy *et al* (2008), was used with a few modifications. A 2 g rhizosphere soil sample from each treatment was mixed with 20 ml of a 60 mM sodium phosphate buffer, with pH 7.6 (Schnurer & Rosswall, 1982; Adam & Duncan, 2001), in a 50 ml plastic centrifuge tube and 0.2 ml of a 2mg/ ml FDA stock solution was added (Schnurer & Rosswall, 1982). The FDA stock solution was prepared by dissolving 200 mg of FDA in 100 ml of acetone. The stock solution was kept at -20 °C until further use (Schnurer & Rosswall, 1982). The 50 ml tubes were incubated for 20 min at 28 °C in a Labcon growth chamber, and the tubes were shaken 3x by hand during incubation. After incubation, the tubes were placed on a Heidolph Multi Reax shaker for 10 min at room temperature and vigorously shaken at 300 rpm. The hydrolysis reaction was terminated by adding 15 ml of 2:1 chloroform: methanol solution. Approximately 1.5 ml of the supernatant was then placed in an epindorph tube and centrifuged at 200 rpm for 3 min before placing 1 ml into a 1 ml plastic cuvette for measuring absorbance readings at 490 nm in a Spec: T60 UV-visible spectrophotometer. Two controls were prepared for each soil sample. Firstly, a control containing 2 g of the soil sample containing phosphate buffer without the addition of FDA. The second control contained phosphate buffer and FDA, but no soil. Their absorbance values were subtracted from the absorbance values of the corresponding soil sample to account for any colour formation that might have occurred that was not associated with the soil sample (Anonymous, 2004). Results are reported as µg fluorescein ml^{-1} of the soil solution. The amount of FDA hydrolyzed was measured as

absorbance at 490 nm and expressed as µg/ ml^{-1} FDA hydrolyzed as determined from a standard curve (Adam & Duncan, 2001; Anonymous, 2004). The standard curve was set up as described by Anonymous (2004). Data from both trials were pooled and subjected to analysis of variance (ANOVA) and, when F values were significant ($P<0.05$), means were separated using Fisher's protected LSD test.

   DNA isolation and 16S rDNA PCR amplification for denaturing gradient gel electrophoresis (DGGE). Total genomic DNA (gDNA) was extracted from 0.25 g soil samples using the PowerSoil™ DNA isolation kit according to the manufacturers' instructions. DNA extracts were visualized on a 1% agrose gel with GoldView staining under UV-illumination.   Concentration and purity of gDNA were determined fluorometrically with a NanoDrop 2000 spectrophotometer (Thermo Scientific) and expressed as ng/µl. Two 16S rDNA primers specifically designed for bacteria (27F 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R 5'-GGTTACCTTGTTACGACTT-3') were used to amplify a portion of the conserved 16S rDNA gene (Weisburg *et al.*, 1991). Each PCR reaction contained 1-50 ng gDNA, 10 µM of each primer set, 10 mM of deoxyribonucleotide triphosphate (dNTP's), 2 mM MgCl$_2$, 1x PCR reaction buffer, 1.25 U of Taq DNA Polymerase (SuperTherm), and distilled water up to 50 µl. The PCR program was as follows: initial denaturation 95 ˚C for 5 min; 95 ˚C for 45 s, 49 ˚C for 45 s and 72 ˚C for 1 min (25 cycles); 72 ˚C for 10 min. This was conducted according to the standard operating procedure (SOP 29) at the University of the Free State developed in the Metagenomics Platform Laboratory. The DNA was visualized on a 1% agarose gel stained with ethidium bromide under UV illumination.

Nested PCR and Denaturing Gradient Gel Electrophoresis (DGGE). Nested PCR was performed using a fragment of the same 16S rDNA region. Recovered DNA was used in the nested PCR. A 50 µl nested PCR reaction was carried out composed of recovered DNA, 1 µl dNTP's (10 mM; KAPA BIOSYSTEMS), 1 µl Bovine serum albumin (BSA), 1 µl PCR buffer (1x), 1 µl GoTaq® Flexi DNA Polymerase (1.25 U), 1 µl (10 µM) reverse primer 517R (5'-ATT ACC GCG GCT GCT GG-3') (Muyzer & Smalla*,* 1998), 1 µl (10 µM) forward primer 341F (5'-CCT ACG GGA GGC AGC AG-3') with a GC clamp (5'-CGC CCG CCG CGC CCC GCG CCC GTC CGG CCG CCC CCG CCCG-3') (Muyzer & Smalla*,* 1998) and distilled water. The PCR cycle was as follows: initial denaturation 95 ˚C for 5 min; 95 ˚C for 45s, 49 ˚C for 45s and 72 ˚C for 1 min (25 cycles); 72 ˚C for 10 min. The DNA fragments were verified by visualization on a 1% agarose gel stained with ethidium bromide under UV illumination The amplified DNA fragments were exposed to a gradient of denaturant (urea/ formamide) at an elevated temperature (60 °C) within a polyacrylamide gel (Tzeneva *et al.,* 2008). The procedure was carried out according to the standard operating procedure (SOP 19) developed by the Metagenomics Platform Laboratory using a DCode^{TM} Universal Mutaton Detection System (BioRad), followed by staining and visualization under UV transillumination. The DGGE banding profile of each sample was quantified and compared with Quantity One® 1-D Analysis imaging software available from the Biorad Molecular Imager Gel Doc ^{TM} XR system to determine relatedness.

Terminal Restriction Fragment Length Polymorphisms (T-RFLP):
Denaturing gradient gel electrophoresis (DGGE) and T-RFLP produce a pattern or profile of nucleic acids amplified from a sample and that pattern reflects the microbial community structure (Kitts, 2001).

To reliably compare T-RFLP fingerprints between different soil samples, the following was standardized: (1) template DNA concentration before PCR amplification, (2) the quality of the PCR-amplified DNA before restriction digestion, and (3) digested PCR products before sizing the terminally labeled fragments (Thies, 2007).

DNA isolation from soil: Total genomic DNA was extracted from 0.25 g soil samples using the PowerSoil™ DNA isolation kit according to the manufacturers' instructions. The extracted DNA was finally re-suspended in 100 µl elution buffer. The concentration of the DNA was determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) and expressed as ng/ µl.

16S rDNA PCR amplification: Two bacterial 16S rDNA specific primers were designed according to Thies (2007). The primer sequences were as follows: 5'-/56 FAM/AGAGTTTGATCMTGGCTCAG-3' and 5'-ACCTTGTTA CGACTT-3' (IDT®). Each 100 µl PCR reaction contained 100 ng DNA, 0.2 µM of each primer, 0.2 mM dNTP's (KAPA Biosystems), 2.5 mM $MgCl_2$, PCR buffer, and 1.25 U GoTaq® Flexi DNA Polymerase (Promega). The amplification regime was as follows: one cycle of 94°C for 3 min followed by 35 cycles of 94°C for 30 s, specific annealing temperature for 15 s and 72°C for 1 min followed by a final extension step at 72°C for 5 min.

A gradient PCR reaction was initially done to determine the optimal annealing temperature of the primer pair. The temperature gradient ranged from 55°C to 60°C.

An optimal annealing temperature of 56.3°C was subsequently used for all PCR reactions. To quantify the PCR products, an internal *Escherichia coli* DNA standard was included in all PCR reactions. The optimal amount of *E. coli* DNA added to each PCR reaction was determined by running a concentration gradient from 0.0125 to 25 ng DNA. The optimal amount decided on was 0.37 ng *E. coli* DNA per PCR reaction.   The success of the PCR reaction was determined by separating 5 µl of the PCR product on a 1% (w/v) agarose gel.

Restriction digestion of amplified soil DNA: For T-RFLP analysis, 600 ng amplified DNA product was digested in a 50 µl reaction containing 0.3 U RsaI enzyme (Fermentas) and 1 x concentration of the RsaI buffer (33 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/ ml BSA). Digestion was carried out for 3 h at 37°C whereafter the reaction was terminated at 68°C for 10 min. The success of digestion was confirmed by separating 5 µl of the digestion reaction on an agarose gel as previously described.

The digested product was precipitated before fragment analysis as follows: 11.25 µl of a 125 mM ethylene-diamine-tetra-acetic acid (EDTA) solution and 135 µl of 100 % ethanol were added to the remaining 45 µl digestion product and vortexed. The solution was incubated at room temperature for 15 min, after which it was centrifuged at 12000 *g* for 15 min at 4 °C. After centrifugation the supernatant was completely removed and 200 µl 70 % (w/v) ethanol was added to the pellet and re-centrifuged at 12000 *g* for 5 min at 4 °C. The supernatant was completely removed after which the pellet was dried. Fragment analysis of the samples was carried out by the Department of Microbiology, Biochemistry and Food biotechnology at the University of the Free State, Bloemfontein.

After adding 9.5 µl Hi-Di formamide and 0.5 µl of GeneScan™ -1200 LIZ size standard (Applied Biosystems) to the precipitated product it was vortexed. The solution was transferred to a 96 well sequencer plate after which it was incubated for 3 min at 96 °C in a PCR machine. After being cooled at 4 °C, the sequencer plate was placed in a 3130xl Genetic Analyzer (Applied Biosystems) and the program for fragment analysis was run as standardized by the Department of Microbiology, Biochemistry and Food Biotechnology.

The T-RFLP data were analyzed using the GeneMarker® version 2.2.0 software. Two methods of multivariate analysis were applied to T-RFLP and DGGE results, namely cluster analysis and principle component analysis (PCA). Cluster analysis was based on a binary matrix, representing the presence or absence of bands in each treatment.

RESULTS

Soil analysis. Chemical and textural characteristics indicated clear differences between HCC and LCC soils. The HCC soil consistently had higher K, Fe, Cu, N and C levels than the LCC soil, while LCC soil consistently had higher levels of Ca, Mg, Na, Zn and P than the HCC soil. High clay content soil had a lower pH and higher organic matter content than LCC soil (Tables 4.1 & 4.2).

Community-level physiological profiling (CLPP). The HCC (Tukulu) soil consistently displayed less bacterial activity than the LCC (Bainsvlei) soil under cultivation for all hybrids (Figure 4.1 & 4.2). Bacterial activity was higher in rhizosphere soil of GR hybrids cultivated in HCC soil compared to conventional hybrids for both isolines, while the BT hybrid had higher bacterial activity than the conventional hybrid

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for isoline 1, but lower bacterial activity for isoline 2. Under cultivation in LCC soil the isolines reacted differently. Bacterial activity was lower in rhizosphere soil samples of GR hybrids compared to the conventional hybrid for isoline 1, while bacterial activity in the rhizosphere soil of the BT hybrid remained the same as the conventional hybrid. For isoline 2 cultivated in the LCC soil, bacterial activity in the rhizosphere soil from PAN 6Q-508R was lower than the conventional hybrid, while PAN 6Q-708BR was higher. Bacterial activity in rhizosphere soil of the BT hybrid remained the same as the conventional hybrid.

Determination of soil ergosterol. Fungal biomass based on soil ergosterol was consistently lower in the LCC soil than for the HCC soil under cultivation of all hybrids (Figures 4.3 & 4.4). However, for the HCC soil under cultivation of PAN 4P-116, PAN 4P-316B and PAN 4P-516R no ergosterol were detected. No significant differences ($P<0.05$) was observed between any of the treatments (Table 4.3); however a tendency was observed regarding rhizosphere soil of GR hybrids from isoline 2, where rhizosphere soil had lower microbial activity than the conventional and BT hybrids. Microbial activity in rhizosphere soil varied between hybrids from isoline 1.

Fluorescein diacetate hydrolysis (FDA). Microbial activity was consistently higher in the HCC soil than in the LCC soil, regardless of maize genotype cultivated in it (Figure 4.5 & 4.6). The main interaction was significant ($P<0.05$) (Table 4.4). For hybrids cultivated in the LCC soil, rhizosphere soil from GR and BT cultivars had higher microbial activity for hybrids from isoline 1, but lower microbial activity for isoline 2 than the conventional hybrids (Figure 4.5). The same tendency was observed for both isolines cultivated in the HCC soil (Figure 4.6).

Denaturing gradient gel electrophoresis (DGGE) and Terminal Restriction Fragment Length Polymorphisms (T-RFLP). Data obtained from DGGE and T-RFLP data clustered into two main groups in trial 1, the first group being the HCC and LCC soil groups, and the second grouping based on GR vs non-GR hybrids, but not for trial 2 (Figures 4.7 - 4.14). For trial 2, treatments clustered based on either HCC or LCC soil that the hybrids were cultivated in.

DISCUSSION

Results obtained from the present study suggest that rhizosphere soil from microbial populations of GR and BT maize hybrids differ from that of conventional isogenic hybrids. Microbial activity, as measured by FDA hydrolysis, suggested that the root exudates from different maize genotypes affect microbial activity differently, as shown by the significant interaction between microbial activity, maize genotype and soil type. The alteration in the soil microbial activity caused by GR and BT cultivars compared to their conventional counterpart could potentially affect soil health and ecosystem functioning (Dunfield & Germida, 2004).

Although results obtained from ergosterol extractions did not show significant (P<0.05) differences, a finding consistent with the findings of other workers (Mina, Chaudhary & Kamra, 2011), a clear relationship between ergosterol concentration and maize genotype was observed. The BT and GR hybrids tended to have lower ergosterol concentrations than their conventional counterparts in the HCC soil. Similarly, BT and GR hybrids from isoline 2 tended to have lower ergosterol

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concentrations than their conventional counterpart when cultivated in LCC soil. These findings are consistent with those of Castaldini *et al* (2005) who observed lower levels of mycorrhizae in BT maize roots compared to their conventional counterparts, but inconsistent with Hannula, de Boer and van Veen (2012) who observed that GM-traits in potatoes had no affect on soil fungal communities.

Results clearly indicate that root exudates from BT and GR maize and non-BT and non-GR differ in terms of their rhizosphere populations. These differences could be from either the plant species (maize versus potato) or their interaction with soil type. Root exudates from BT and GR maize had the potential to either stimulate or inhibit rhizosphere microbes in comparison to their conventional counterparts, depending on genotype. This is consistent with the findings of other workers on various crops (Savka & Farrand, 1997; Siciliano & Germida, 1999; Griffiths, Geoghegan & Robertson, 2000; Mansouri *et al.,* 2002; Brusetti *et al.,* 2004; Oger *et al.,* 2004; Castaldini *et al.,* 2005). Other workers however claimed that root exudates from transgenic crop roots have no effect on rhizosphere microbes (Marschner *et al.,* 2001; Saxena & Stotzky, 2001; Koskella & Stotzky, 2002; Fang *et al.,* 2005; Park *et al.*, 2006; Griffiths *et al.,* 2007; Hart *et al.*, 2009). Furthermore, results obtained from DGGE and T-RFLP analysis clearly discriminated between GR and non-GR hybrids, similar to results obtained from T-RFLP analysis by Lukow, Dunfield & Liesack (2000) on potatoes. Thus it can be concluded that although BT and GR maize root exudates do not necessarily inhibit rhizosphere microbial populations, they do alter the profile of rhizosphere soil microbial populations.

Soil pH, organic matter and clay content also influenced rhizosphere activity as indicated by molecular and biochemical data. Soil type, in terms of its textural and

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chemical properties, also influenced rhizosphere microbial communities. Neutral or slightly alkaline soil conditions generally favour oxidative bacterial growth, and the higher bacterial activity observed in the LCC soil having a higher pH is consistent with findings of Rousk, Brookes & Bååth (2009). Furthermore, the higher fungal biomass, as determined by ergosterol extraction, from the HCC soil with five genotypes is consistent with (Rousk *et al.,* 2009; Rousk *et al.,* 2010) who observed higher fungal activity in low pH soils with optimum fungal activity at pH 4.5. For three of the eight hybrids (Figure 4.2), ergosterol could not be extracted from the rhizospheres of plants in the HCC soil. In contrast, the remaining 5 hybrids displayed very high levels of ergosterol.

A positive relationship was observed between soil organic matter content, soil clay content and microbial biomass. This is consistent with the findings of Müller & Höper (2004) and Grandy *et al* (2009), and especially evident in the HCC soil, which had a higher clay content as well as higher organic matter than the LCC soil. Müller & Höper concluded that the clay dependent capacity of soil protect microbial biomass and that clay content has a significant influence on rhizosphere microbial populations (Marschner *et al.,* 2001; Müller & Höper, 2004).

Griffiths *et al* (2007) concluded that there is no soil ecological consequence for micro-fauna and micro-organisms associated with the use of BT- or herbicide tolerant (HT) maize in place of conventional varieties. Rather, it is other management options, such as tillage, crop type and pest management regimes that have larger effects on the soil biology than the genotype of maize grown. However, results from the present study indicated that root exudates from different maize hybrids from different genotypical backgrounds clearly differed in their effect on rhizosphere microbes. In certain

instances rhizosphere microbial activity might be either stimulated or inhibited, depending on maize genotype. Molecular and biochemical analysis clearly discriminated between GR and non-GR hybrids. Although microbial activity clearly differed between the HCC and LCC soils, the tendencies in terms of results obtained from the different techniques suggested that the BT, GR and non-GR root exudates affected microbial populations irrespective of the soil it was cultivated in. Future research should therefore include yield parameters to determine whether these differences are of economical importance.

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Table 4.1. Soil chemical characteristics.

Soil type	pH	Ca (mg/kg)	K (mg/kg)	Mg (mg/kg)	Na (mg/kg)	Zn (mg/kg)	Fe (mg/kg)	Cu (mg/kg)	P (mg/kg)	N (mg/kg)	C (mg/kg)
Bainsvlei (LCC)	6.02	474	120	76	44	1.78	16.36	1	19.76	359.2	1390
Tukulu (HCC)	4.98	334	168	66	28	1.44	110.6	1.44	15.03	492.3	2600

Table 4.2. Soil textural characteristics.

Soil type	Organic matter (OM) %	Slit %			Sand %		
		Coarse	Fine	Clay	Coarse	Medium	Fine
Bainsvlei (LCC)	0.232	3.33	3.33	3.33	0.67	8	81.17
Tukulu (HCC)	0.399	1.670	4.000	9.330	2.070	6.470	73.770

Table 4.3. ANOVA as calculated for fungal biomass obtained from ergosterol concentration data.

Source Term	DF	Sum of Squares	Mean Square	F-ratio	Probability level	Power (Alpha = 0.05)
A: Soil	1	134586.8	134586.8	4.04	0.079376	0.424189
B: Hybrid	7	132378.7	18911.24	0.57	0.765314	0.138677
AB	7	132135.8	18876.54	0.57	0.766036	0.138490
C: Reps	1	134955.6	134955.6	4.05	0.079038	0.425128
ABC	7	132062.5	18866.08	0.57	0.766254	0.138433
S	8	266713.3	33339.16			
Total (Adjusted)	31	932832.7				
Total	32					

*Term significant at alpha = 0.05

Table 4.4. ANOVA as calculated for microbial activity as obtained from fluorescein diacetate (FDA) hydrolysis data.

Source Term	DF	Sum of Squares	Mean Square	F-ratio	Probability level	Power (Alpha = 0.05)
A: Soil	1	6.874751E-02	6.87471E-02	37.41	0.000000*	0.999969
B: Hybrid	7	3.611457E-02	5.159225E-03	2.81	0.017823*	0.863345
AB	7	1.019374E-02	1.456248E-03	0.79	0.597958	0.295483
C: Reps	5	4.028955E-02	8.05791E-03	4.38	0.002819*	0.942146
ABC	35	0.1282675	3.664784E-03	1.99	0.017969*	0.975841
S	40	7.350617E-02	1.837654E-03			
Total (Adjusted)	95	0.357119				
Total	96					

*Term significant at alpha = 0.05
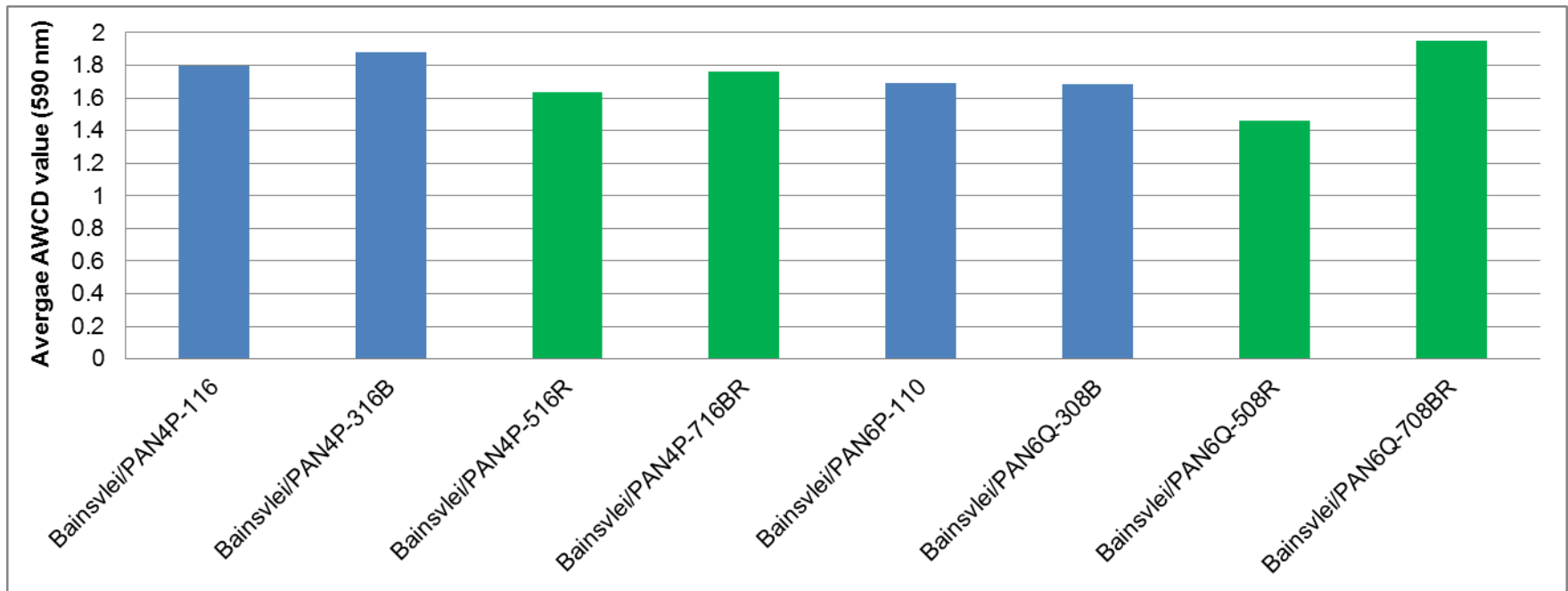
Figure 4.1. Differences observed between glyphosate resistant (GR), BT- and conventional maize hybrids for both isogenic lines cultivated in the LCC (Bainsvlei) soil in terms average well colour development (AWCD) as measured by BiologEcoPlates™. (B = BT; R = glyphosate resistant; BR = BT + glyphosate resistant).
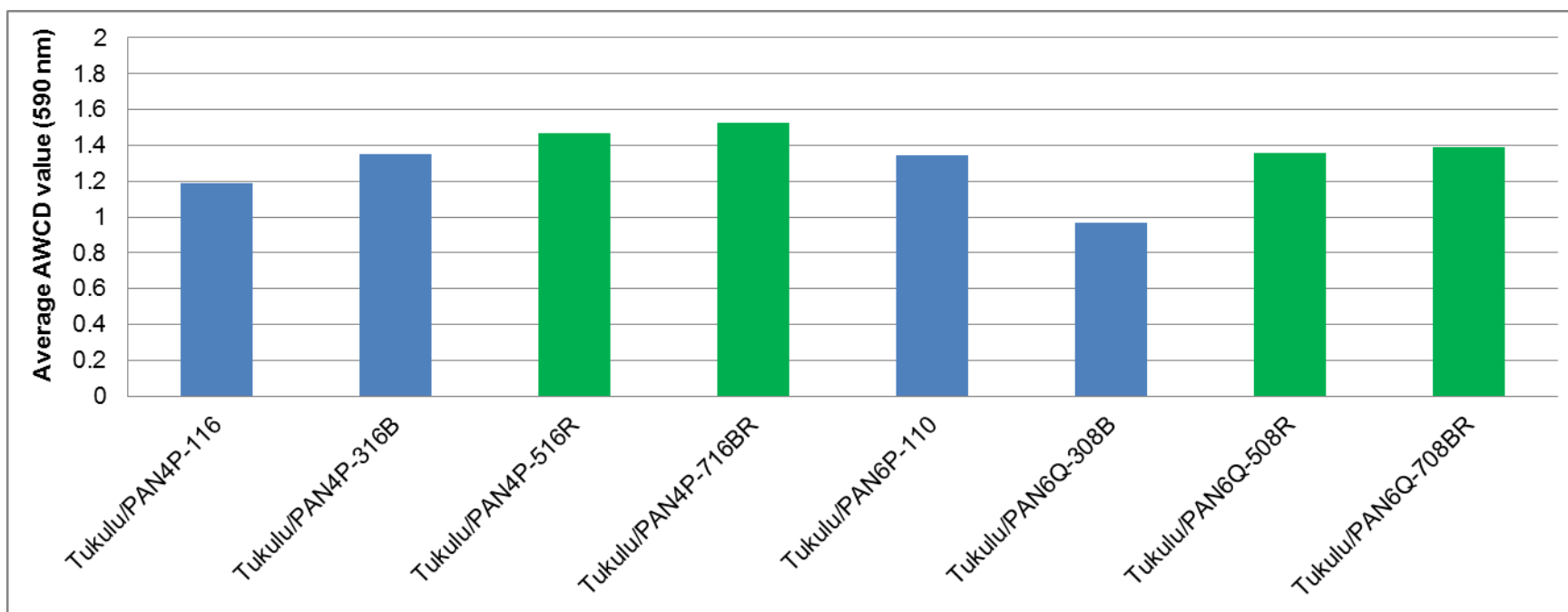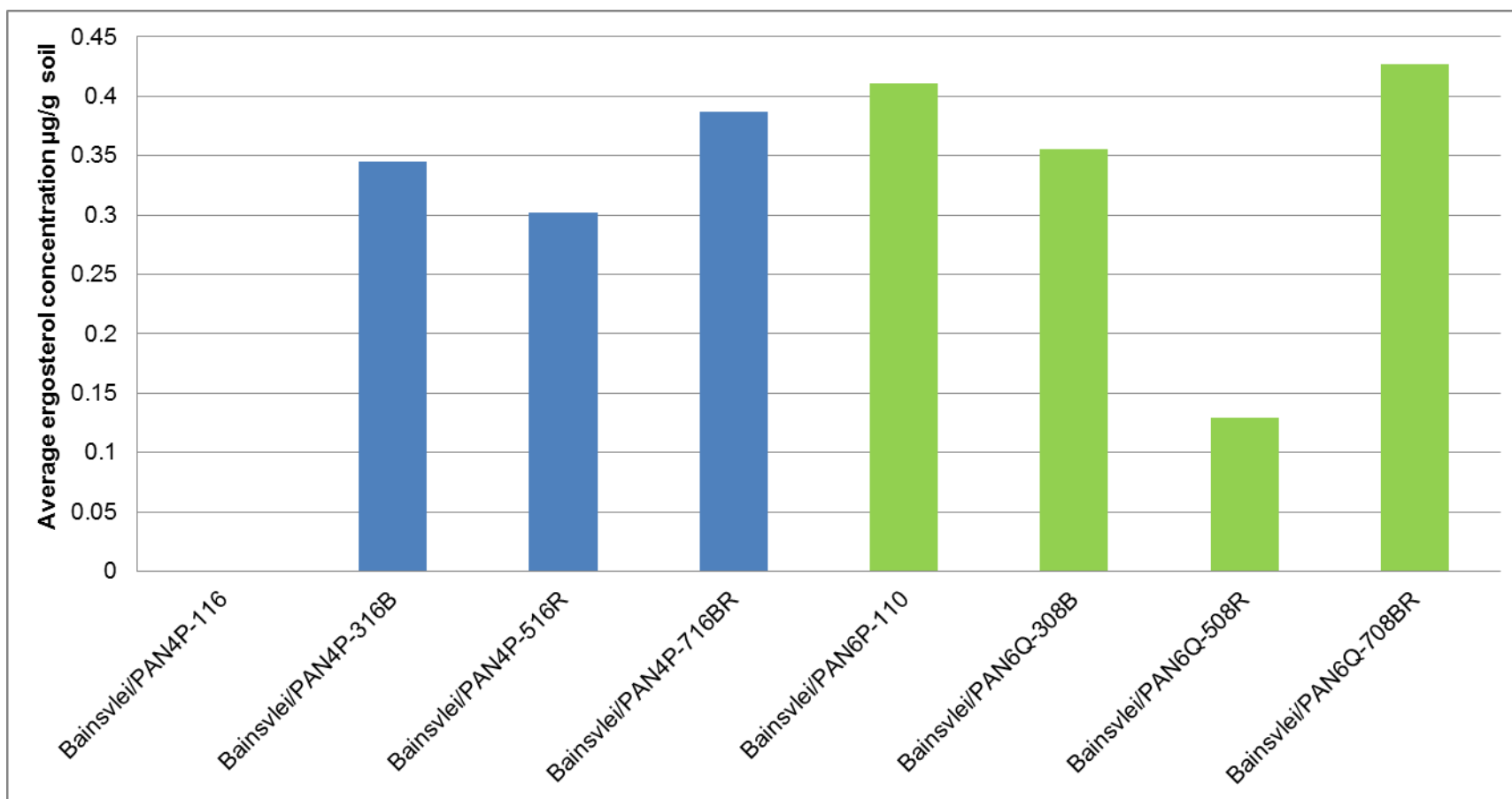
Figure 4.2. Differences observed between glyphosate resistant (GR), BT- and conventional maize hybrids for both isogenic lines cultivated in the HCC (Tukulu) soil in terms of average well colour development (AWCD) as measured by BiologEcoPlates™. (B = BT; R = glyphosate resistant; BR = BT + glyphosate resistant).

Figure 4.3. Average fungal biomass as determined from ergosterol concentrations for the LCC (Bainsvlei) soil. An ergosterol concentration of 0 µg/ g soil was obtained for PAN 4P-1116. (B = BT; R = glyphosate resistant; BR = BT + glyphosate resistant). No significant differences were observed.
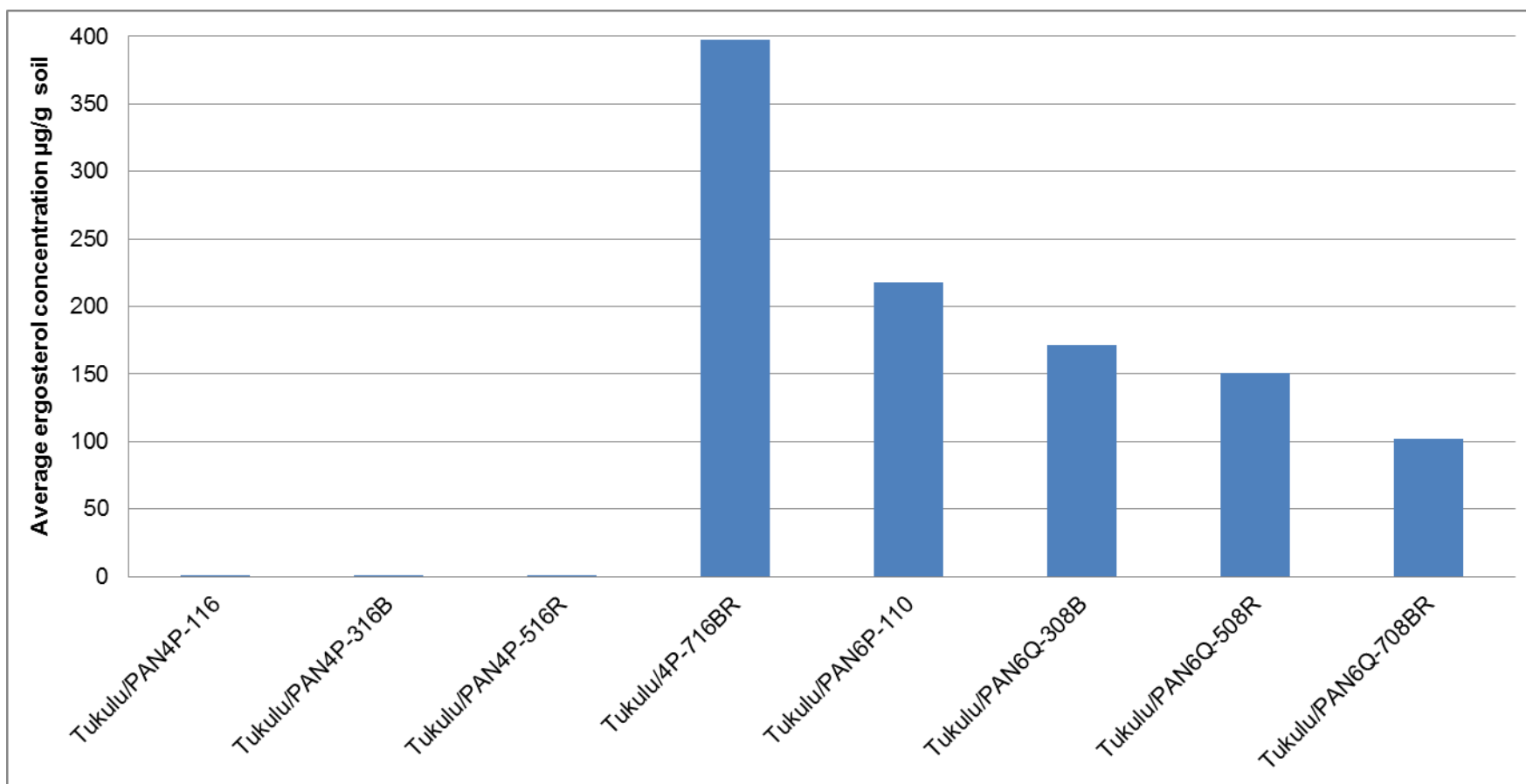
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Figure 4.4. Average fungal biomass as determined from ergosterol concentrations for the HCC (Tukulu) soil. An ergosterol concentration of 0 μg/ g soil was obtained for PAN 4P-1116, PAN 4P-316B and PAN 4P-516R. (B = BT; R = glyphosate resistant; BR = BT + glyphosate resistant). No significant differences were observed.
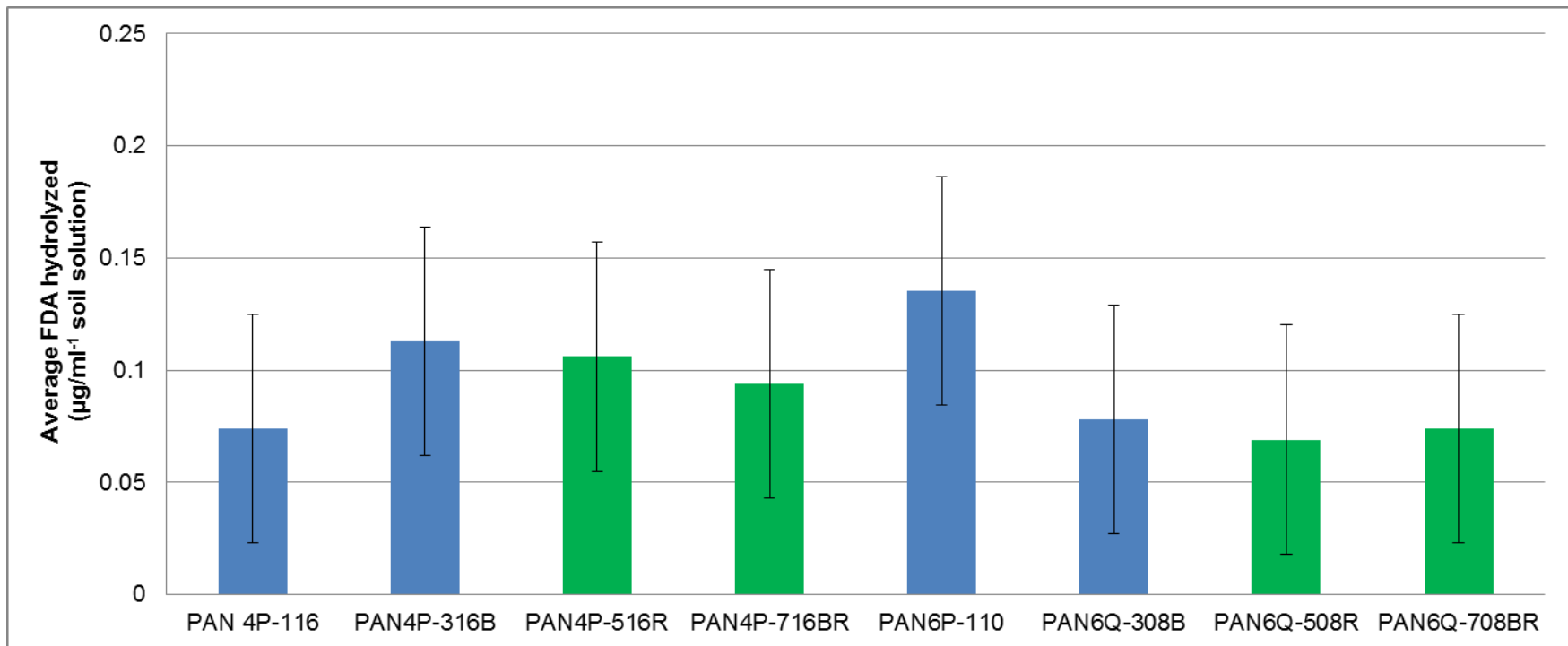
Figure 4.5. Microbial activity, as determined by fluorescein diacetate (FDA) hydrolysis for the LCC (Bainsvlei) soil. (B = BT; R = glyphosate resistant; BR = BT + glyphosate resistant).

Fisher's LSD (P<0.05): Soil = 0.018; Hybrids = 0.036; Soil x Hybrid = 0.051. The LSD value for the main interaction, soil x hybrid, is illustrated in the above figure.

Figure 4.6. Microbial activity, as determined by fluorescein diacetate (FDA) hydrolysis for the HCC (Tukulu) soil. (B = BT; R = glyphosate resistant; BR = BT + glyphosate resistant).

Fisher's LSD (P<0.05): Soil = 0.018; Hybrids = 0.036; Soil x Hybrid = 0.051. The LSD value for the main interaction, soil x hybrid, is illustrated in the above figure.
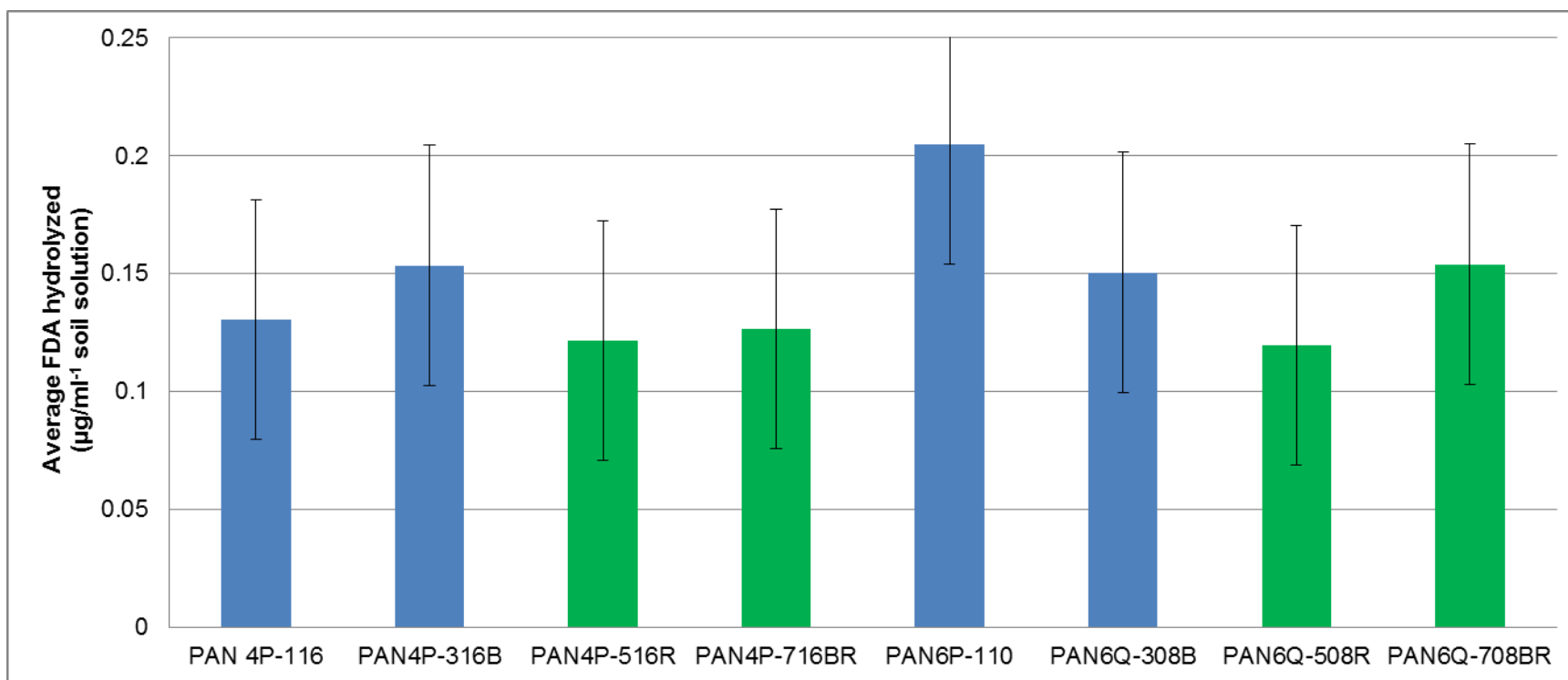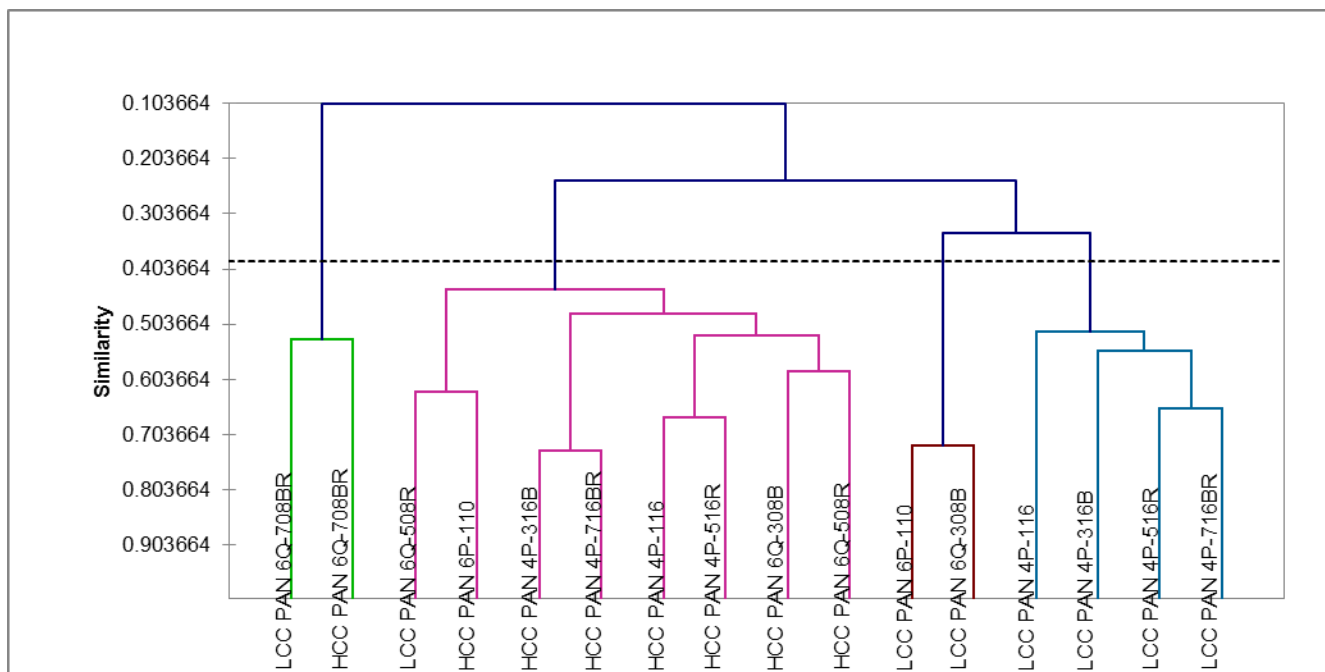
Figure 4.7. Cluster analysis of DGGE data for trial 1. (LCC represents low clay content soil and HCC high clay content soil. B = BT; R = GR; BR = BT + GR).
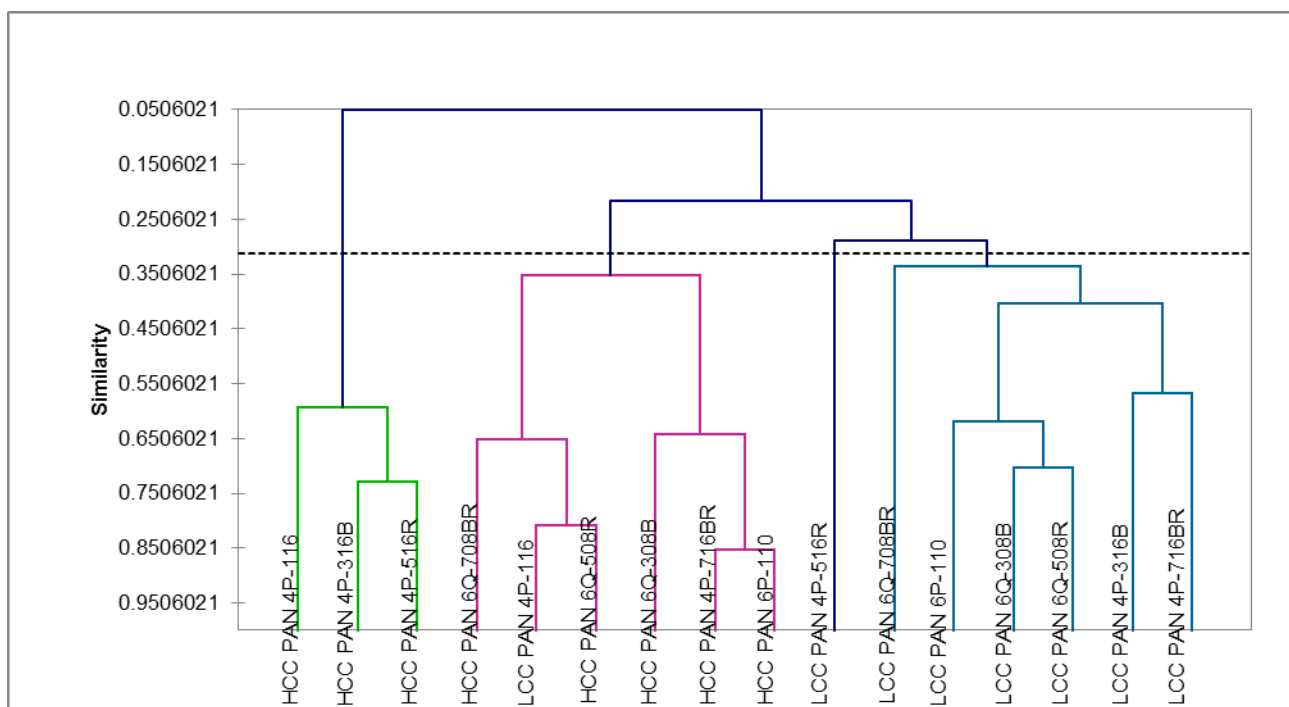

Figure 4.8. Cluster analysis obtained of DGGE data for trial 2. (LCC represents low clay content soil and HCC high clay content soil. B = BT; R = GR; BR = BT + GR).

Figure 4.9. Cluster analysis of T-RFLP data for trial 1. (LCC represents low clay content soil and HCC high clay content soil. B = BT; R = GR; BR = BT + GR).
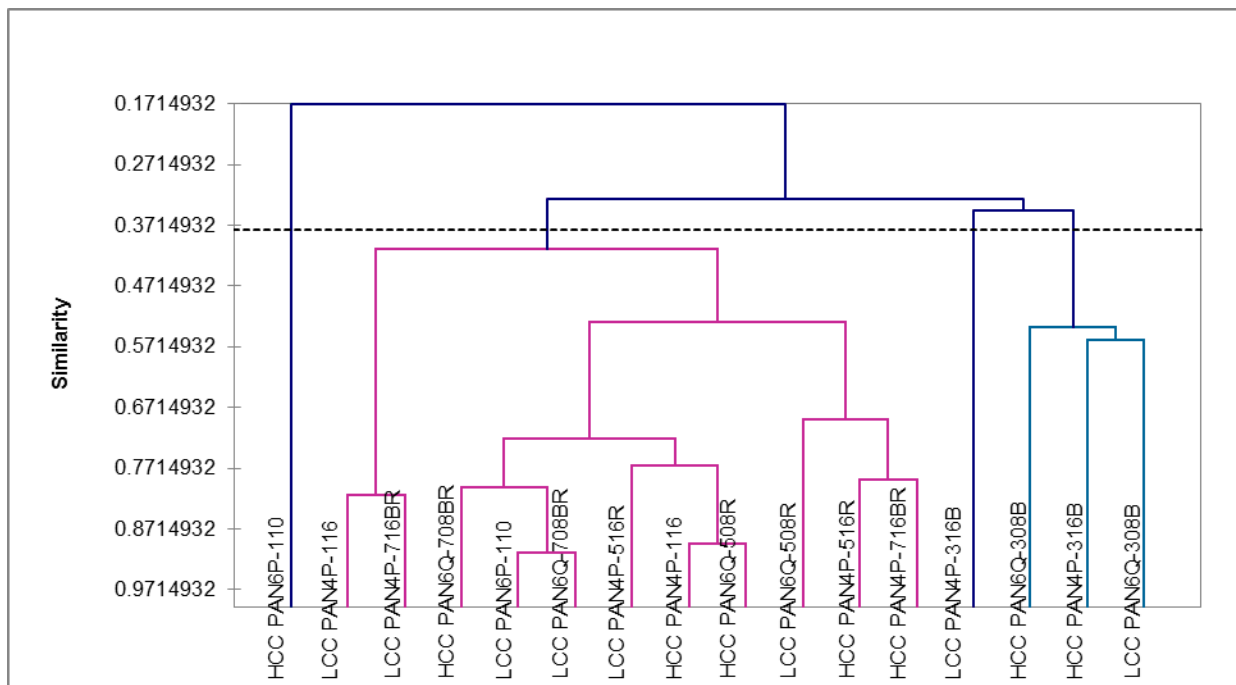
Figure 4.10. Cluster analysis of T-RFLP data for trial 2. (LCC represents low clay content soil and HCC high clay content soil. B = BT; R = GR; BR = BT + GR).
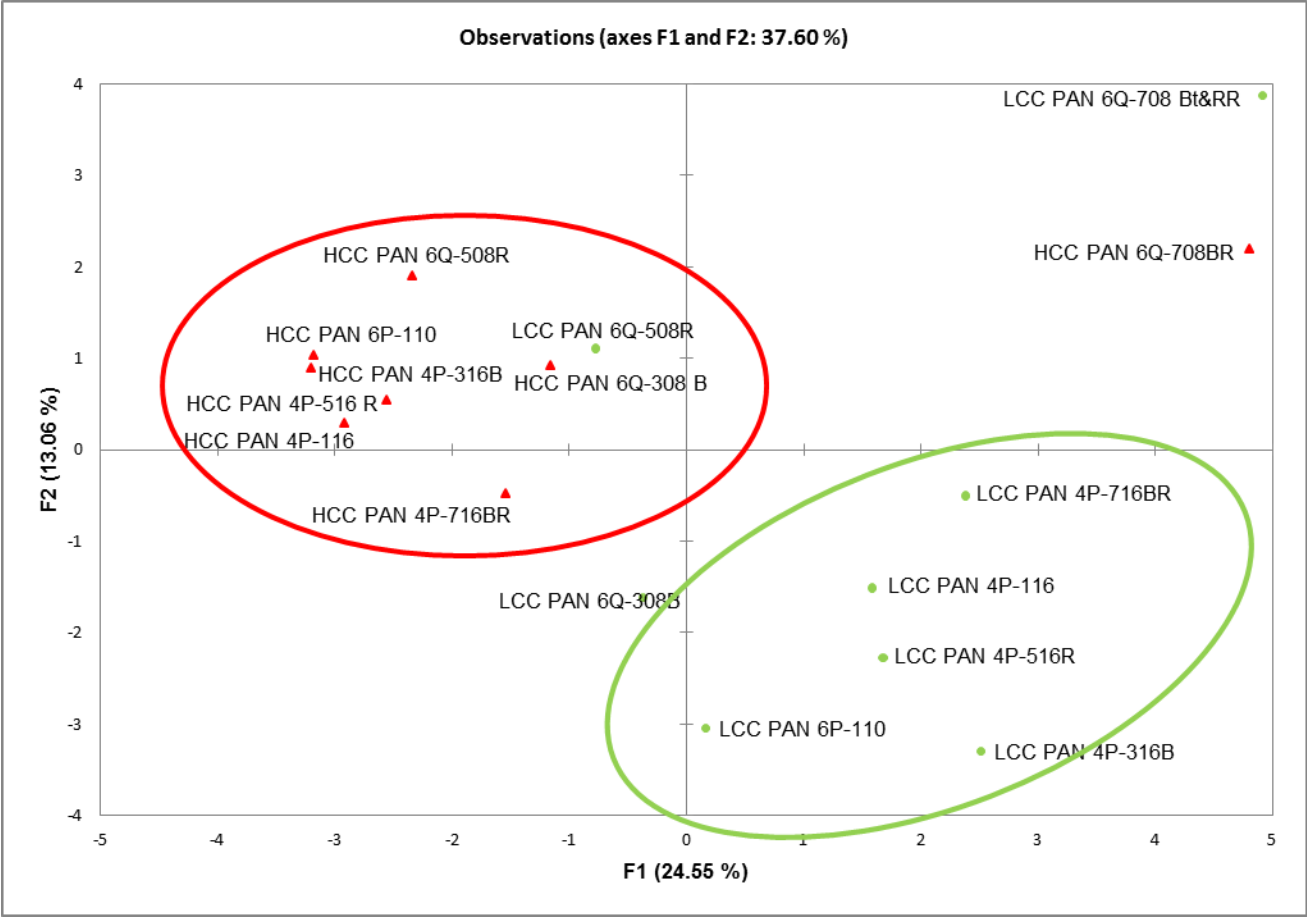
Figure 4.11. Principal component analysis obtained from DGGE data for trial 1. (LCC represents low clay content soil and HCC high clay content soil. B = BT; R = GR; BR = BT + GR).

Figure 4.12. Principal component analysis obtained from DGGE data for trial 2. (LCC represents low clay content soil and HCC high clay content soil. B = BT; R = GR; BR = BT + GR).
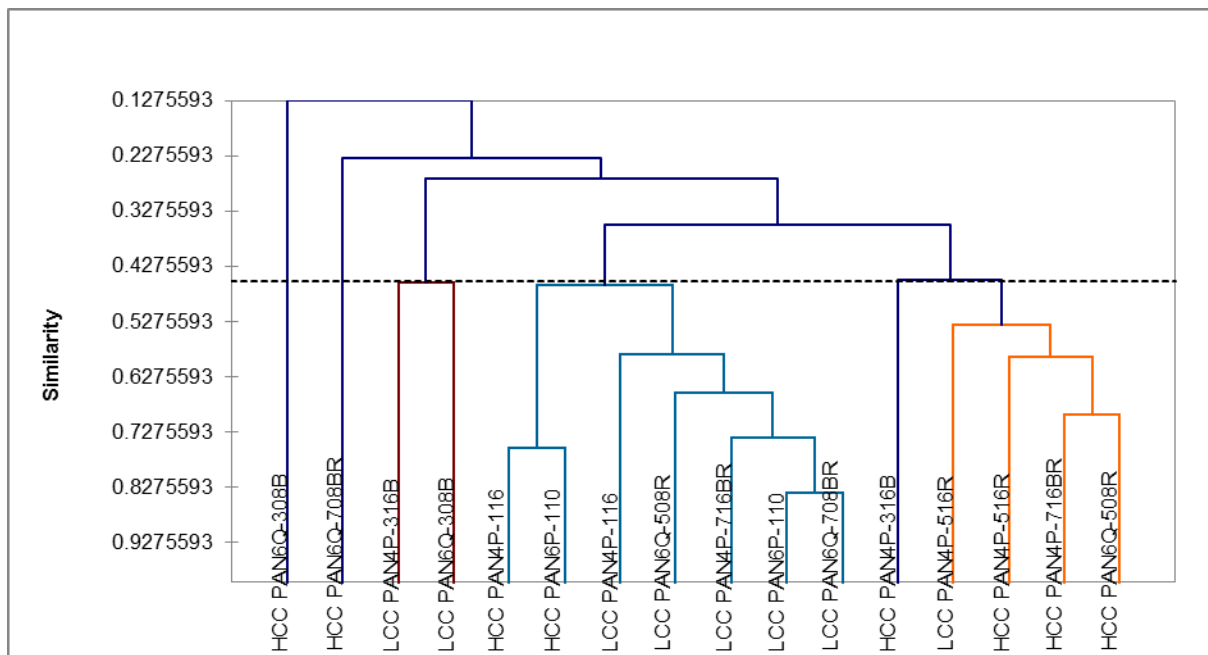
Figure 4.13. Principle component analysis of T-RFLP data for trial 1. (LCC represents low clay content soil and HCC high clay content soil. B = BT; R = GR; BR = BT + GR).
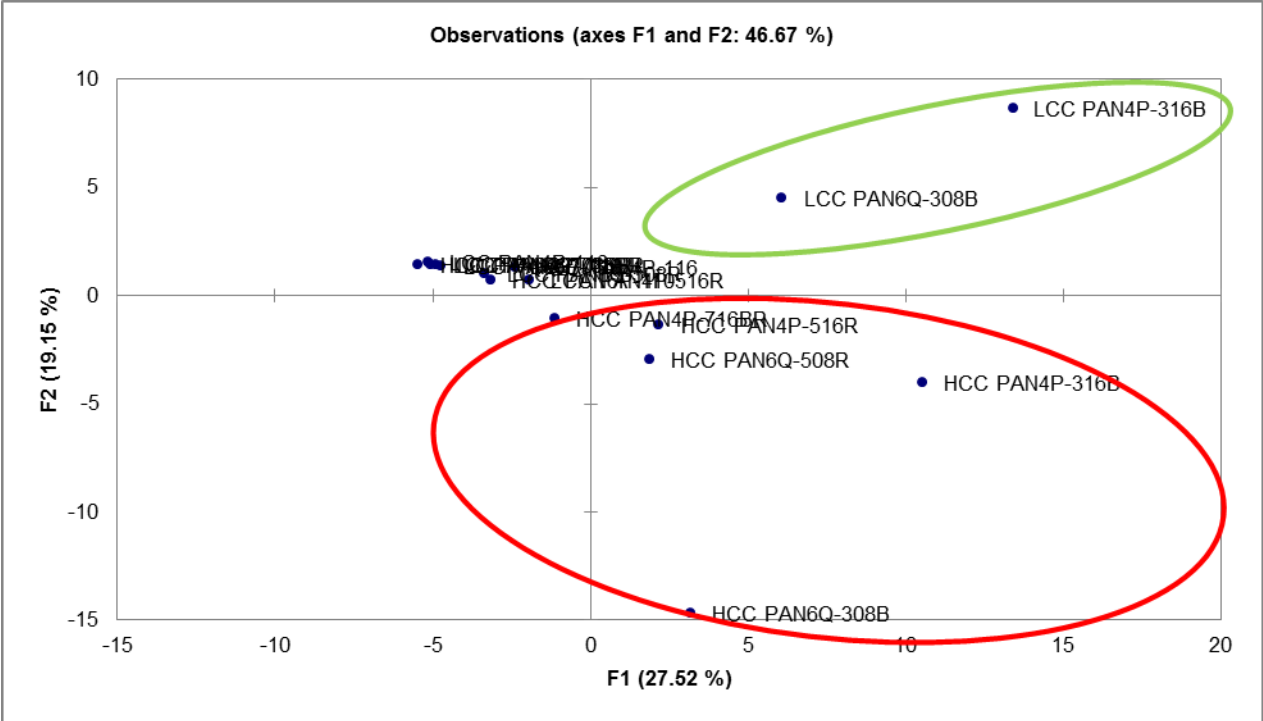
Figure 4.14. Principle component analysis of T-RFLP data for trial 2. (LCC represents low clay content soil and HCC high clay content soil. B = BT; R = GR; BR = BT + GR).

CHAPTER 5
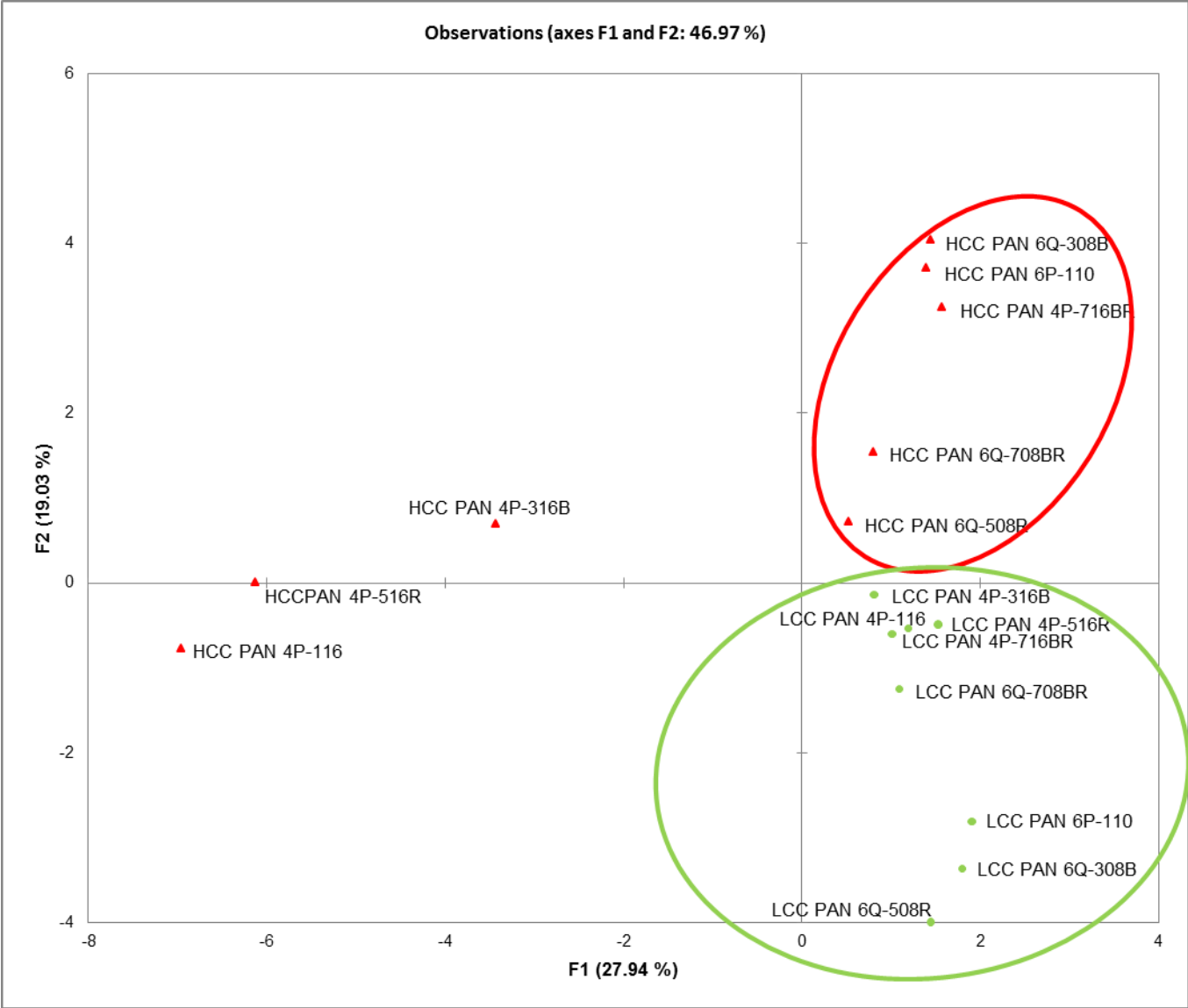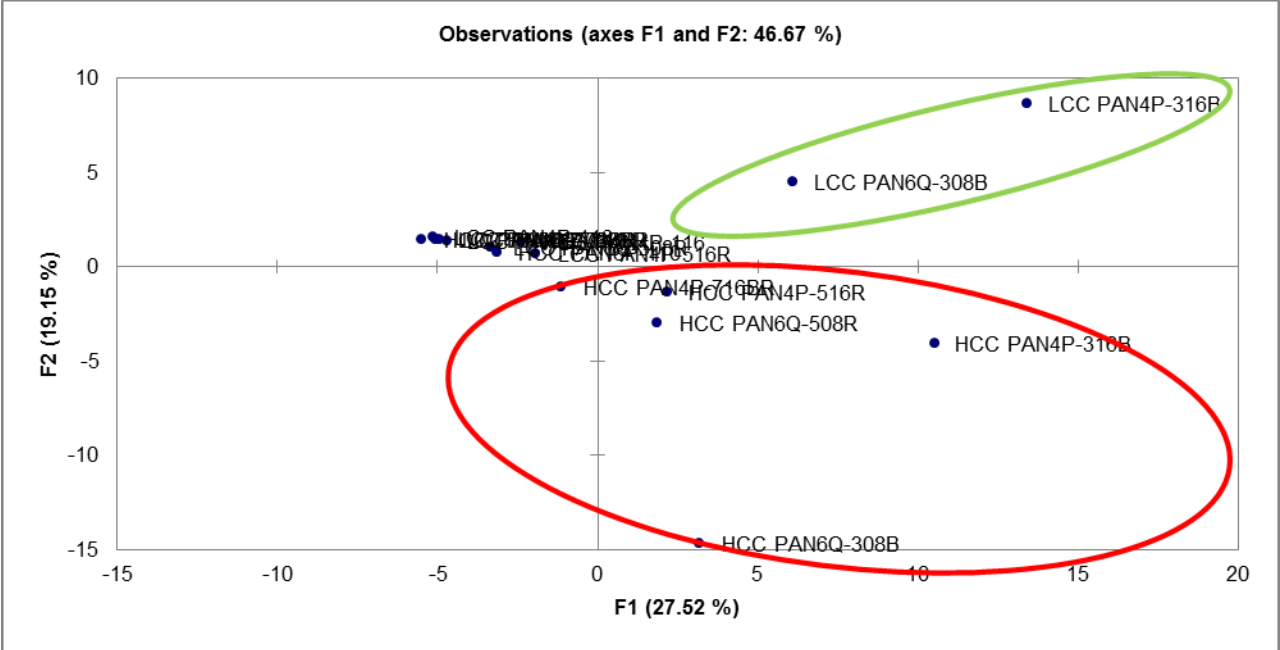
A quantitative and qualitative analysis of rhizosphere populations of soybeans as influenced by soil and plant genotype

ABSTRACT

The effect of glyphosate resistant (GR) soybeans on microbial activity in the rhizosphere was compared in soil having a high clay content (HCC) and low clay content (LCC) respectively. Microbial activity in rhizosphere soil was determined by means of biochemical techniques (active carbon, community level physiological profiling, ergosterol quantification and fluorescein diacetate hydrolysis) as well as molecular techniques (denaturing gradient gel electrophoresis and terminal restriction fragment length polymorphisms). Four non-isogenic soybean cultivars, two GR and two conventional, were assessed. Results obtained with the generalized techniques used, showed no effect of the GR gene on rhizosphere microbes, either qualitatively or quantitatively in either soil.

Keywords: *Rhizosphere microbes, GR soybean, high clay content soil, low clay content soil.*

INTRODUCTION

Soil is a dynamic system in which physical, chemical and biological components interact. Within this system, micro-organisms perform an important task in the decomposition and transformation of soil materials, and are involved in carbon (C), nitrogen (N) and phosphorous (P) cycles, as well as the mobilization of nutrients (Icoz & Stotzky, 2008; Bastida *et al.*, 2009). Rhizosphere micro-organisms play a major role in nutrient transformation and element cycling, and any impact that genetically modified (GM) plants may have on the dynamics of a plant's rhizosphere could indirectly affect that plant's health (Dunfield & Germida, 2004). Similarly, any alteration to the soil environment which affects soil micro-organisms will affect the functioning of an agroecosystem (Dunfield & Germida, 2004).

Genetically modified plants possess one or more genes that have been transferred from a different species (Icoz & Stotzky, 2008). Reasons for developing GM plants include longer shelf life, disease resistance, pest resistance, herbicide tolerance, nutritional improvement and, resistance to abiotic stresses such as drought or nitrogen starvation (Icoz & Stotzky, 2008). The introduction of GM plants into agroecosystems has raised a number of questions regarding the ecological impact of GM plants on the soil ecosystem, whether positive or negative. Root exudates govern which organisms reside in the rhizosphere of those plants. Any change to the quality of crop residues and rhizosphere dynamics could thus modify the composition and activity of soil organisms (Castaldini *et al.,* 2005; Icoz & Stotzky, 2008), resulting in an ecological shift in the soil microbial populations which could have negative consequences for crop yield.

Genetically modified crops may potentially have adverse effects on non-target soil organisms which may lead to a change in microbe-mediated processes and functions in soil (Savka & Farrand, 1997; Castaldini *et al.,* 2005; Icoz & Stotzky, 2008). Interactions between plants and the soil ecosystem indicate that GM crops can influence processes and functions in the soil, in a similar manner to which other non-GM crops do (Icoz & Stotzky, 2008). Glyphosate resistant (GR) cropping systems can change soil properties by introducing novel compounds, in addition to glyphosate, into the environment. Soil microbial communities, in particular rhizosphere microbes, may be particularly sensitive to the effects of GM crops because of their intimate proximity to the plant's physiology (Hart *et al.*, 2009).

The effect of GR soybean and conventional soybean on rhizosphere organisms was compared in potted plants. Unfortunately soybean isolines could not be obtained and four random cultivars were therefore selected. It was hypothesized that root exudates of GR soybean cultivars will lead to a decrease in rhizosphere microbial activity compared to conventional cultivars. To evaluate the effect of GR soybeans on rhizosphere populations, microbial activity was determined using various biochemical and molecular techniques. Techniques used to determine general effects were active carbon and ergosterol quantification, community level physiological profiling (CLPP) and fluorescein diacetate (FDA) hydrolysis. Molecular techniques used to evaluate the bacterial community structure were denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphisms (T-RFLP).

MATERIALS AND METHODS

Soil sampling. Soil was collected from an agricultural experimental farm, Kenilworth near Bloemfontein (-29.020397 S; 26.145308 E). The soil has a 10 year history of annual tilling, and during sampling, maize was being cultivated on the field. The soil is categorized as Kenilworth-Bainsvlei ecotype, a fine sandy loam soil in a semi-arid area (Soil Classification Working Group, 1991). It will henceforth be referred to as low clay content (LCC) soil (Table 5.1). A second batch of soil was from another experimental farm, Paradys (-29.222950 S; 26.210747 E) which is catergorized as Tukulu according to the Soil Classification Working Group (1991). It will henceforth be referred to as high clay content (HCC). In both instances, bulk soil samples were collected from the top 5 cm of the surface. Chemical and textural analyses were conducted for both soils (Table 5.1 & 5.2) by the Department Climate-, Crop- and Soil Sciences at the University of the Free State.

Trial layout. Four non-isogenic soybean cultivars were used, A 5409R (GR), PAN 535R (GR), PAN 660 (conventional) and PAN 1652 (conventional). Four to five seeds were planted in 2L plastic bags, containing the respective soil types. After germination, plants were thinned to three plants per bag. The experiment was a complete randomized design with three replicates per treatment and conducted twice. Plants were maintained in the glasshouse at 24 ˚C day temperature and 19 ˚C night temperature, until sampling of rhizosphere soil before flowering. Plants were watered daily using filtered municipal water to eliminate chlorine pollution. Plants were fertilized weekly using Multifeed® Classic (19: 8: 16; N: P: K) according to the manufacterer's instructions.

Rhizosphere sampling. Plants were removed from soil by lifting them out carefully with a pitch-fork. Soil was sampled by shaking off the excess soil from around plant roots and then sampling rhizosphere soil from the roots using a small brush. The samples were then immediately placed in 50 ml centrifuge tubes that were stored on ice-packs in a closed container.

Community-level physiological profiling (CLPP). Community-level physiological profiles (CLPP) of the two soils were assessed using BiologEcoPlates[TM] (Biolog, Hayward, CA, USA). A soil suspension was made by suspending 2 g of rhizosphere soil in 200 ml of sterilized water (Palmroth *et al.*, 2004) and shaking it for 30 min at room temperature to allow the soil to become totally suspended in the water. Each well of the microtiter plate was filled with 90 µl of the soil suspension and plates were incubated at 25 °C in a Labcon Growth chamber in the dark for 96 h (Palmroth *et al.*, 2004). The change in optical density (OD) was then measured at 590 nm using a multi-well plate reader (Akmal *et al.*, 2005). The overall colour development in each BiologEcoPlate[TM] was expressed as average well colour development (AWCD) (Garland & Mills, 1991; Cai *et al.*, 2010). Data from both experiments were pooled and subjected to analysis of variance (ANOVA) and, when F values were significant ($P<0.05$), means were separated using Fisher's protected least significant difference (LSD) test to determine significance of treatment.

Determination of soil ergosterol. In order to estimate fungal biomass in soil samples (Davis & Lamar, 1992), ergosterol was extracted as described by Jambunathan, Kherdekar & Vaidya (1991) with minor modifications. Methanol (50 ml) was added to 10 g of moist soil in a 50 ml centrifuge tube, and the solution was agitated

in a Heidolph Multi Reax shaker for 40 min at 1600 rpm. The suspension was then allowed to settle for approximately 30 min after which 25 ml of the clean supernatant was removed and added to a clean 50 ml centrifuge tube containing 3 g of potassium hydroxide (KOH). The mixture was again shaken for 10 min to allow the KOH to dissolve, after which 10 ml of n-hexane was added followed by incubation in a 75 °C water bath for 30 min. The solution was then cooled to room temperature and 5 ml distilled water was added. The hexane layer was then removed and transferred to a clean 50 ml centrifuge tube. Ten millilitres of n-hexane was added to the rest of the aliquot in the tube and mixed well. The hexane layer was again removed and added to the previous hexane aliquot. The last two steps were repeated. The hexane extract was then placed in a hot water bath and evaporated until a dry residue was left. The residue was dissolved in 5 ml of methanol and the solution filtered through a 0.45 μm millipore filter and placed in a 2 ml high performance liquid chromatograph (HPLC) bottle. The filtrate was analyzed at an emission wavelength of 282 nm using a Perkin Elmer HPLC fitted with a Phenomene x C18.5 μm 4.6 x 150 mm analytical column. Ergosterol concentrations were calculated and data from both trials were pooled and subjected to analysis of variance (ANOVA) and, when F values were significant (P<0.05), means were separated using Fisher's protected LSD test.

   Fluorescein diacetate hydrolysis. Fluorescein diacetate (FDA) hydrolysis is widely accepted as an accurate and simple method for measuring total microbial activity in soil environments (Adam & Duncan, 2001). Schnurer & Rosswall (1982) found that there is a good correlation between FDA hydrolysis and respiration in soil. The method has the advantages of being simple, rapid, and sensitive (Schnurer & Rosswall, 1982).

The method of Adam & Duncan (2001) as modified by Zabaloy *et al* (2008), was used with a few modifications. A 2 g rhizosphere soil sample from each treatment was mixed with 20 ml of a 60 mM sodium phosphate buffer, with pH 7.6 (Schnurer & Rosswall, 1982; Adam & Duncan, 2001), in a 50 ml plastic centrifuge tube and 0.2 ml of a 2mg/ ml FDA stock solution was added (Schnurer & Rosswall, 1982). The FDA stock solution was prepared by dissolving 200 mg of FDA in 100 ml of acetone. The stock solution was kept at -20 °C until further use (Schnurer & Rosswall, 1982). The 50 ml tubes were incubated for 20 min at 28 °C in a Labcon growth chamber, and the tubes were shaken 3x by hand during incubation. After incubation, the tubes were placed on a Heidolph Multi Reax shaker for 10 min at room temperature and vigorously shaken at 300 rpm. The hydrolysis reaction was terminated by adding 15 ml of 2:1 chloroform: methanol solution. Approximately 1.5 ml of the supernatant was then placed in an epindorph tube and centrifuged at 200 rpm for 3 min before placing 1 ml into a 1 ml plastic cuvette for measuring absorbance readings at 490 nm in a Spec: T60 UV-visible spectrophotometer. Two controls were prepared for each soil sample. Firstly, a control containing 2 g of the soil sample containing phosphate buffer without the addition of FDA. The second control contained phosphate buffer and FDA, but no soil. Their absorbance values were subtracted from the absorbance values of the corresponding soil sample to account for any colour formation that might have occurred that was not associated with the soil sample (Anonymous, 2004). Results are reported as μg fluorescein ml^{-1} of the soil solution. The amount of FDA hydrolyzed was measured as absorbance at 490 nm and expressed as μg/ ml^{-1} FDA hydrolyzed as determined from a standard curve (Adam & Duncan, 2001; Anonymous, 2004). Data from both trials were

pooled and subjected to analysis of variance (ANOVA) and, when F values were significant (P<0.05), means were separated using Fisher's protected LSD test.

DNA isolation and 16S rDNA PCR amplification for denaturing gradient gel electrophoresis (DGGE). Total genomic DNA was extracted from 0.25 g soil samples using the PowerSoil™ DNA isolation kit according to the manufacturers' instructions. The DNA extracts were visualized on a 1 % agrose gel with GoldView staining under UV-illumination. Concentration and purity of gDNA were determined fluorometrically with a NanoDrop 2000 spectrophotometer (Thermo Scientific) and expressed as ng/µl. Two 16S rDNA primers specifically designed for bacteria (27F 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R 5'-GGTTACCTTGTTACGACTT-3') were used to amplify a portion of the conserved 16S rDNA gene (Weisburg *et al.*, 1991). Each PCR reaction contained 1-50 ng gDNA, 10 µM of each primer set, 10 mM of deoxyribonucleotide triphosphate (dNTP's), 2 mM $MgCl_2$, 1x PCR reaction buffer, 1.25 U of Taq DNA Polymerase (SuperTherm), and distilled water up to 50 µl. The PCR program was as follows: initial denaturation 95 ˚C for 5 min; 95 ˚C for 45 s, 49 ˚C for 45 s and 72 ˚C for 1 min (25 cycles); 72 ˚C for 10 min. This was conducted according to the standard operating procedure (SOP 29) at the University of the Free State developed in the Metagenomics Platform Laboratory. The DNA was visualized on a 1% agarose gel stained with ethidium bromide under UV illumination.

Nested PCR and Denaturing Gradient Gel Electrophoresis (DGGE). Nested PCR was performed using a fragment of the same 16S rDNA region. Recovered DNA was used in the nested PCR. A 50 µl nested PCR reaction was carried out composed of recovered DNA, 1 µl dNTP's (10 mM; KAPA BIOSYSTEMS), 1 µl Bovine serum

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albumin (BSA), 1 µl PCR buffer (1x), 1 µl GoTaq® Flexi DNA Polymerase (1.25 U), 1 µl (10.µM) reverse primer 517R (5'-ATT ACC GCG GCT GCT GG-3') (Muyzer & Smalla, 1998), 1 µl (10 µM) forward primer 341F (5'-CCT ACG GGA GGC AGC AG-3') with a GC clamp (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCCG-3') (Muyzer & Smalla, 1998) and distilled water. The PCR cycle was as follows: initial denaturation 95 °C for 5 min; 95 °C for 45 s, 49 °C for 45 s and 72 °C for 1 min (25 cycles); 72 °C for 10 min. The DNA fragments were verified by visualization on a 1 % agarose gel stained with ethidium bromide under UV illumination The amplified DNA fragments were exposed to a gradient of denaturant (urea/ formamide) at an elevated temperature (60 °C) within a polyacrylamide gel (Tzeneva *et al.,* 2008). The procedure was carried out according to the standard operating procedure (SOP 19) developed by the Metagenomics Platform Laboratory using a DCode[TM] Universal Mutaton Detection System (BioRad), followed by staining and visualization under UV transillumination. The DGGE banding profile of each sample was quantified and compared with Quantity One[®] 1-D Analysis imaging software available from the Biorad Molecular Imager Gel Doc [™] XR system to determine relatedness.

Terminal Restriction Fragment Length Polymorphisms (T-RFLP): Denaturing gradient gel electrophoresis (DGGE) and T-RFLP produce a pattern or profile of nucleic acids amplified from a soil sample which reflects the microbial community structure in the soil (Kitts, 2001). To reliably compare T-RFLP fingerprints between different soil samples, the following was standardized: (1) template DNA concentration before PCR amplification, (2) the quality of the PCR-amplified DNA before

restriction digestion, and (3) digested PCR products before sizing the terminally labeled fragments (Thies, 2007).

DNA isolation from soil: Total genomic DNA was extracted from 0.25 g soil samples using the PowerSoil™ DNA isolation kit according to the manufacturers' instructions. The extracted DNA was finally re-suspended in 100 µl elution buffer. The concentration of the DNA was determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) and expressed as ng/ µl.

16S rDNA PCR amplification: Two bacterial 16S rDNA specific primers were designed according to Thies (2007). The primer sequences were as follows: 5'-/56 FAM/AGAGTTTGATCMTGGCTCAG-3' and 5'-ACCTTGTTA CGACTT-3' (IDT[®]). Each 100 µl PCR reaction contained 100 ng DNA, 0.2 µM of each primer, 0.2 mM dNTP's (KAPA Biosystems), 2.5 mM $MgCl_2$, PCR buffer, and 1.25 U GoTaq[®] Flexi DNA Polymerase (Promega). The amplification regime was as follows: one cycle of 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, specific annealing temperature for 15 s and 72 °C for 1 min followed by a final extension step at 72 °C for 5 min.

A gradient PCR reaction was initially done to determine the optimal annealing temperature of the primer pair. The temperature gradient ranged from 55 °C to 60 °C. An optimal annealing temperature of 56.3 °C was subsequently used for all PCR reactions. To quantify the PCR products, an internal *Escherichia coli* DNA standard was included in all PCR reactions. The optimal amount of *E. coli* DNA added to each PCR reaction was determined by running a concentration gradient from 0.0125 to 25 ng DNA. The optimal amount decided on was 0.37 ng *E. coli* DNA per PCR reaction. The

success of the PCR reaction was determined by separating 5 µl of the PCR product on a 1 % (w/v) agarose gel.

Restriction digestion of amplified soil DNA: For T-RFLP analysis, 600 ng amplified DNA product was digested in a 50 µl reaction containing 0.3 U RsaI enzyme (Fermentas) and 1 x concentration of the RsaI buffer (33 mM Tris-acetate; pH 7.9), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/ ml BSA). Digestion was carried out for 3 h at 37 °C whereafter the reaction were terminated at 68 °C for 10 min. The success of digestion was confirmed by separating 5 µl of the digestion reaction on an agarose gel as previously described.

The digested product was precipitated before fragment analysis as follows: 11.25 µl of a 125 mM ethylene-diamine-tetra-acetic acid (EDTA) solution and 135 µl of 100 % ethanol were added to the remaining 45 µl digestion product and vortexed. The solution was incubated at room temperature for 15 min, after which it was centrifuged at 12000 *g* for 15 min at 4 °C. After centrifugation the supernatant was completely removed and 200 µl 70 % (w/v) ethanol was added to the pellet and re-centrifuged at 12000 *g* for 5 min at 4 °C. The supernatant was completely removed after which the pellet was dried. Fragment analysis of the samples was carried out by the Department of Microbiology, Biochemistry and Food biotechnology at the University of the Free State, Bloemfontein. After adding 9.5 µl Hi-Di formamide and 0.5 µl of GeneScan™ -1200 LIZ size standard (Applied Biosystems) to the precipitated product it was vortexed. The solution was transferred to a 96 well sequencer plate after which it was incubated for 3 min at 96 °C in a PCR machine. After being cooled at 4 °C, the sequencer plate was placed in a 3130xl Genetic Analyzer (Applied Biosystems) and the program for fragment analysis

was run as standardized by the Department of Microbiology, Biochemistry and Food biotechnology.

The T-RFLP data were analyzed using the GeneMarker® version 2.2.0 software. Two methods of multivariate analysis were applied to T-RFLP and DGGE results. Firstly cluster analysis represented as dendrograms and secondly principle component analysis (PCA). Cluster analysis was based on a binary matrix, representing the presence or absence of bands in each treatment.

RESULTS

Soil analysis. Chemical and textural characteristics indicated clear differences between HCC and LCC soils. The HCC soil consistently had higher K, Fe, Cu, N and C levels than the LCC soil, while LCC soil consistently had higher levels of Ca, Mg, Na, Zn and P than the HCC soil. High clay content soil had a lower pH and higher organic matter content than LCC soil (Tables 5.1 & 5.2).

Community-level physiological profiling (CLPP): The two soil types, HCC and LCC, significantly differed ($P<0.05$) from each other in terms of AWCD values (Table 5.3). Soil bacterial activity did not differ significantly in terms of GR soybeans vs non-GR soybeans. Cluster analysis discriminated between samples based mainly on soil type and not in terms of soybean cultivar (Figure 5.4).

Determination of soil ergosterol: No significant ($P<0.05$) differences between treatments were observed (Table 5.4). The LCC soil had a significantly lower ergosterol concentration than the HCC soil for all the cultivars used (Figure 5.2).

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Fluorescein diacetate hydrolysis (FDA): The HCC and LCC soils were significantly different (P<0.05) in terms of microbial activity (Table 5.5). The LCC soil had significantly lower microbial activity than the HCC soil under all four cultivar cultivations (Figure 5.3). Microbial activity did not differ significantly in terms of GR soybeans vs non-GR soybeans.

Denaturing gradient gel electrophoresis (DGGE) and Terminal Restriction Fragment Length Polymorphisms (T-RFLP): In both cases, clustering in dendrograms as well as PCA (Figures 5.5 -5.12), showed that all samples mainly clustered in terms of HCC (Tukulu) and LCC (Bainsvlei) soil groups. Glyphosate resistant vs non-GR did not differ in terms of bacterial community structure.

DISCUSSION

Results obtained in the present study clearly indicated that root exudates from GR soybean and non-GR soybeans do not differ significantly in terms of their effect on rhizosphere microbes. These results are consistent with Hannula, de Boer and van Veen (2012) who also observed that GM-traits in potatoes had no affect on soil fungal communities. Results are also consistent with other workers on various other crops (Marschner *et al.,* 2001; Park *et al.*, 2006; Griffiths *et al.,* 2007; Hart *et al.*, 2009).

The present study did however show that soil pH, organic matter % and clay content influenced rhizosphere microbial activity. Data indicated that soil type, in terms of its textural and chemical properties, influenced rhizosphere microbial communities. Neutral or slightly alkaline soil conditions generally favour bacterial growth (Rousk *et al.,*

2009; Rousk, *et al.,* 2010), and the higher bacterial activity observed in the LCC soil, having a higher pH, was thus consistent with findings of Rousk, Brookes & Bååth (2009). Furthermore, a high fungal biomass, as determined by ergosterol extraction, in the HCC soil was consistent with Rousk *et al* (2009) and Rousk *et al* (2010) who observed higher fungal activity in low pH soils with optimum fungal activity at pH 4.5.

A consistent relationship was observed between soil organic matter content, soil clay content and microbial biomass. This is consistent with the findings of Müller & Höper (2004) and Grandy *et al* (2009), which were evident from the higher microbial activity in the HCC soil, which has a higher clay content as well as higher organic matter than the LCC soil. Müller & Höper (2004) concluded that the clay dependent capacity of soils protects microbial biomass from disturbances. The present study demonstrated that soil properties had a more significant influence on rhizosphere microbial populations, than soybean cultivar (Marschner *et al.,* 2001; Müller & Höper, 2004).

Results from the present study were inconsistent with our findings in chapter 4 where maize genotype did have an effect on soil micro-organisms. This suggests that the effect which the inserted gene into a specific crop will have on soil microbial populations will depend on crop species. Clay and organic matter content of soil is also an important consideration to be kept in mind by producers when deciding on crop genotype for cultivation.

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Table 5.1. Soil chemical characteristics.

Soil type	pH	Ca (mg/kg)	K (mg/kg)	Mg (mg/kg)	Na (mg/kg)	Zn (mg/kg)	Fe (mg/kg)	Cu (mg/kg)	P (mg/kg)	N (mg/kg)	C (mg/kg)
Bainsvlei (LCC)	6.02	474	120	76	44	1.78	16.36	1	19.76	359.2	1390
Tukulu (HCC)	4.98	334	168	66	28	1.44	110.6	1.44	15.03	492.3	2600

Table 5.2. Soil textural characteristics.

Soil type	Organic matter (OM) %	Slit %			Sand %		
		Coarse	Fine	Clay	Coarse	Medium	Fine
Bainsvlei (LCC)	0.232	3.33	3.33	3.33	0.67	8	81.17
Tukulu (HCC)	0.399	1.670	4.000	9.330	2.070	6.470	73.770

Table 5.3. ANOVA as calculated for average well colour development (AWCD) values obtained from BiologEcoPlate™ data.

Source Term	DF	Sum of Squares	Mean Square	F-ratio	Probability level	Power (Alpha = 0.05)
A: Soil	1	0.020292	0.020292	7.95	0.047876*	0.568950
B: Cultivar	3	1.579887E-02	5.266292E-03	2.06	0.247898	0.240345
AB	3	2.941718E-02	9.805728E-03	3.84	0.113263	0.407575
C:Reps	1	1.442401E-02	1.442401E-02	5.65	0.076261	0.441628
ABC	3	2.480122E-02	8.267074E-03	3.24	0.143062	0.352653
S	4	1.021387E-02	2.553467E-03			
Total (Adjusted)	15	0.1149472				
Total	16					

*Term significant at alpha = 0.05

Table 5.4. ANOVA as calculated for fungal biomass obtained from ergosterol concentration data.

Source Term	DF	Sum of Squares	Mean Square	F-ratio	Probability level	Power (Alpha = 0.05)
A: Soil	1	10.08062	10.08062	2.03	0.227037	0.197645
B: Cultivar	3	7.084475	2.361492	0.48	0.715729	0.090348
AB	3	11.07987	3.693292	0.74	0.578890	0.114530
C:Reps	1	0.49	0.49	0.10	0.768951	0.057036
ABC	3	22.3433	7.447767	1.50	0.342420	0.186036
S	4	19.8317	4.957925			
Total (Adjusted)	15	70.90997				
Total	16					

*Term significant at alpha = 0.05

Table 5.5. ANOVA as calculated for microbial activity as obtained from fluorescein diacetate (FDA) hydrolysis data.

| Source Term	DF	Sum of Squares	Mean Square	F-ratio	Probability level	Power (Alpha = 0.05)
A: Soil	1	0.1764381	0.1764381	155.67	0.000000*	1.000000
B: Cultivar	3	2.781138E-03	9.270458E-04	0.82	0.492474	0.123162
AB	3	3.826038E-03	1.275346E-03	1.13	0.351740	0.154272
C:Reps	9	4.104876E-02	4.560973E-03	4.02	0.001247*	0.829943
ABC	27	2.375559E-02	8.798366E-04	0.78	0.750302	0.380644
S	36	4.080175E-02	1.133382E-03			
Total (Adjusted)	79	0.2886514				
Total	80					

*Term significant at alpha = 0.05
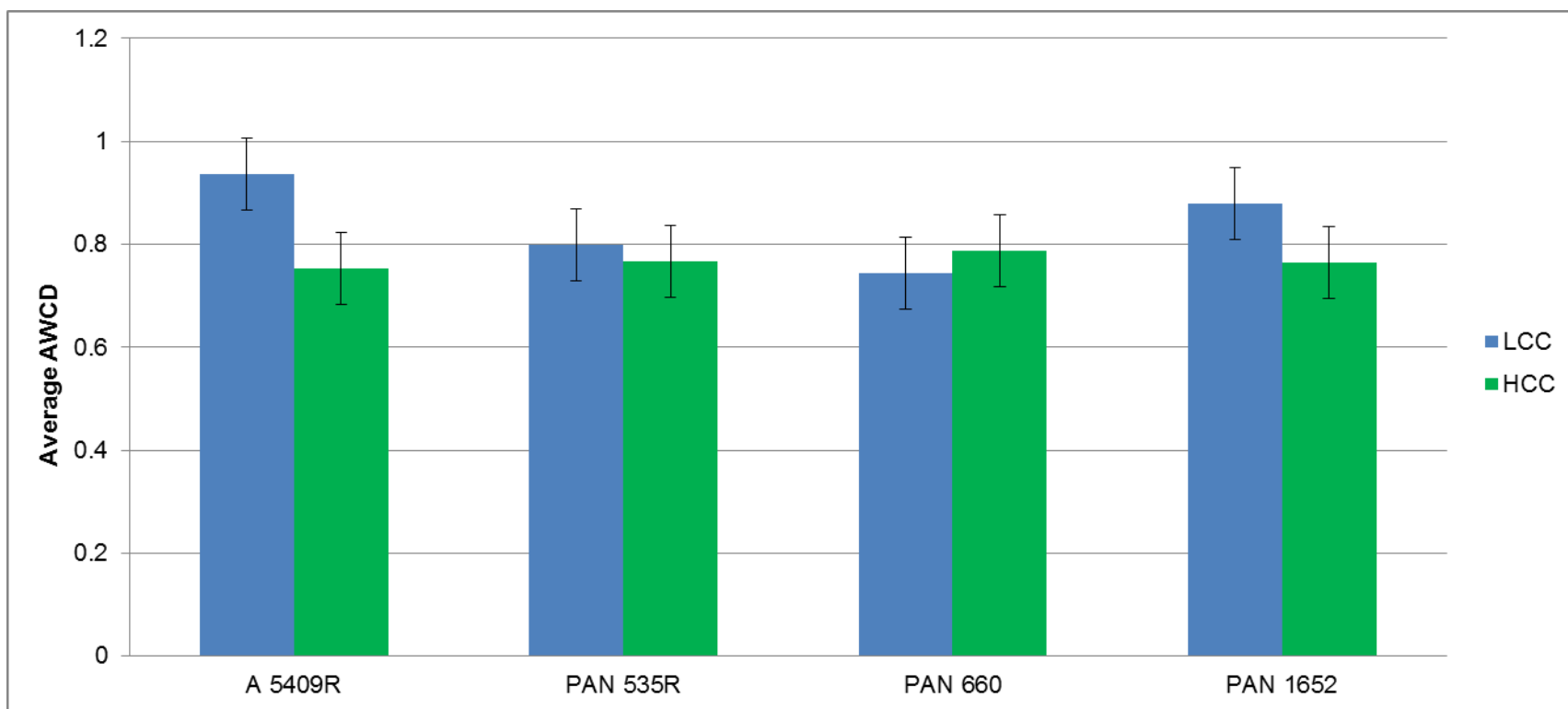
Figure 5.1. Variation in average well colour development (AWCD) of the soil bacterial community in the rhizosphere of glyphosate resistant (GR) and non-resistant soybean. (LCC represents low clay content soil while HCC represents high clay content soil; A 5409 and PAN 535R are GR while PAN 660 and PAN 1652 are non-GR).

Fisher's LSD (P<0.05): Soil = 0.07

Figure 5.2. Average ergosterol concentrations as determined for the rhizosphere soil of the respective cultivars. (LCC represents low clay content soil while HCC represents high clay content soil; A 5409 and PAN 535R are GR while PAN 660 and PAN 1652 are non-GR).

No significant differences were observed.

Figure 5.3. Microbial activity, as determined by fluorescein diacetate (FDA) hydrolysis for the rhizosphere soil of the respective soybean cultivars (LCC represents low clay content soil while HCC represents high clay content soil; A 5409 and PAN 535R are GR while PAN 660 and PAN 1652 are non-GR).
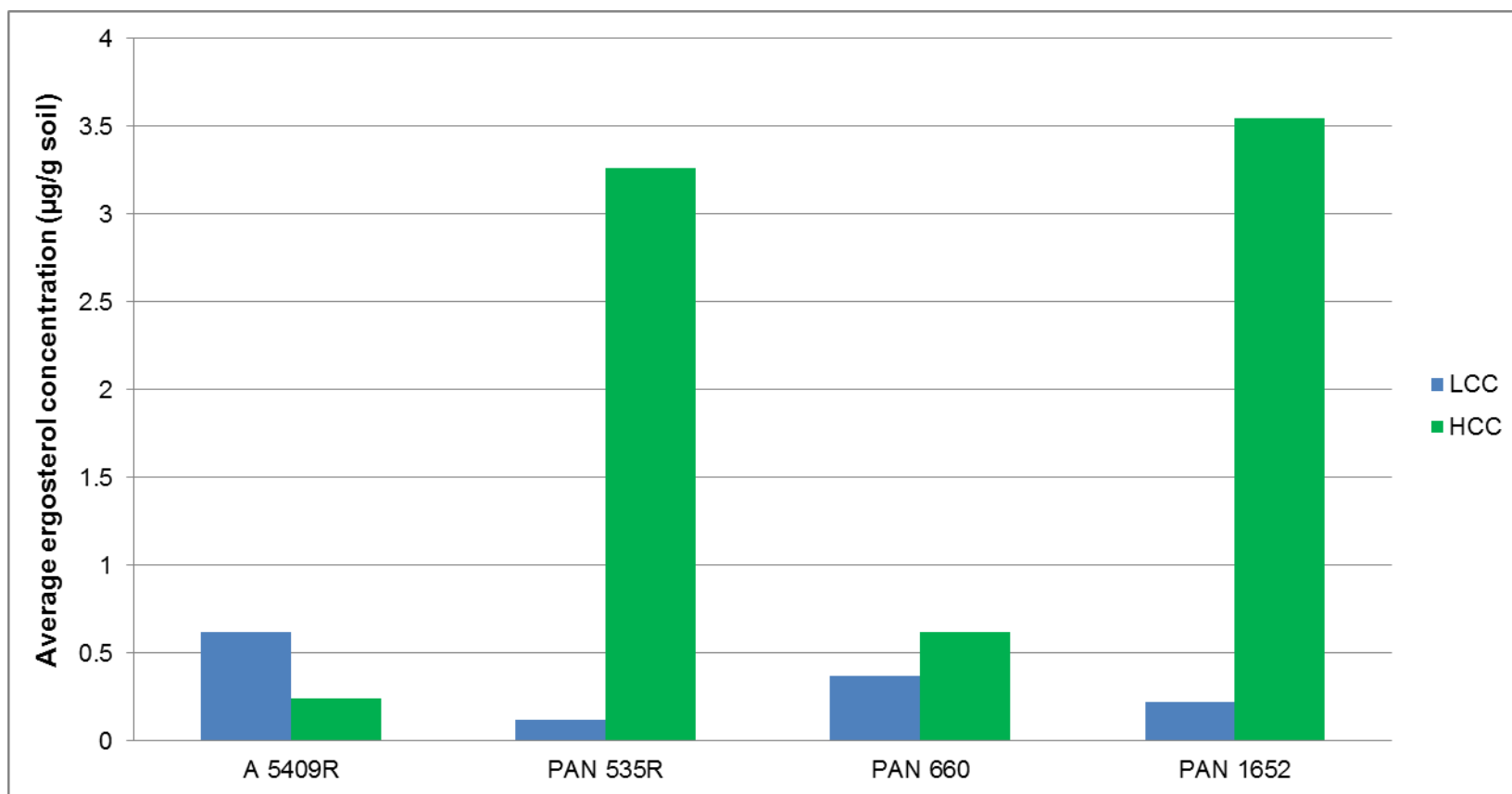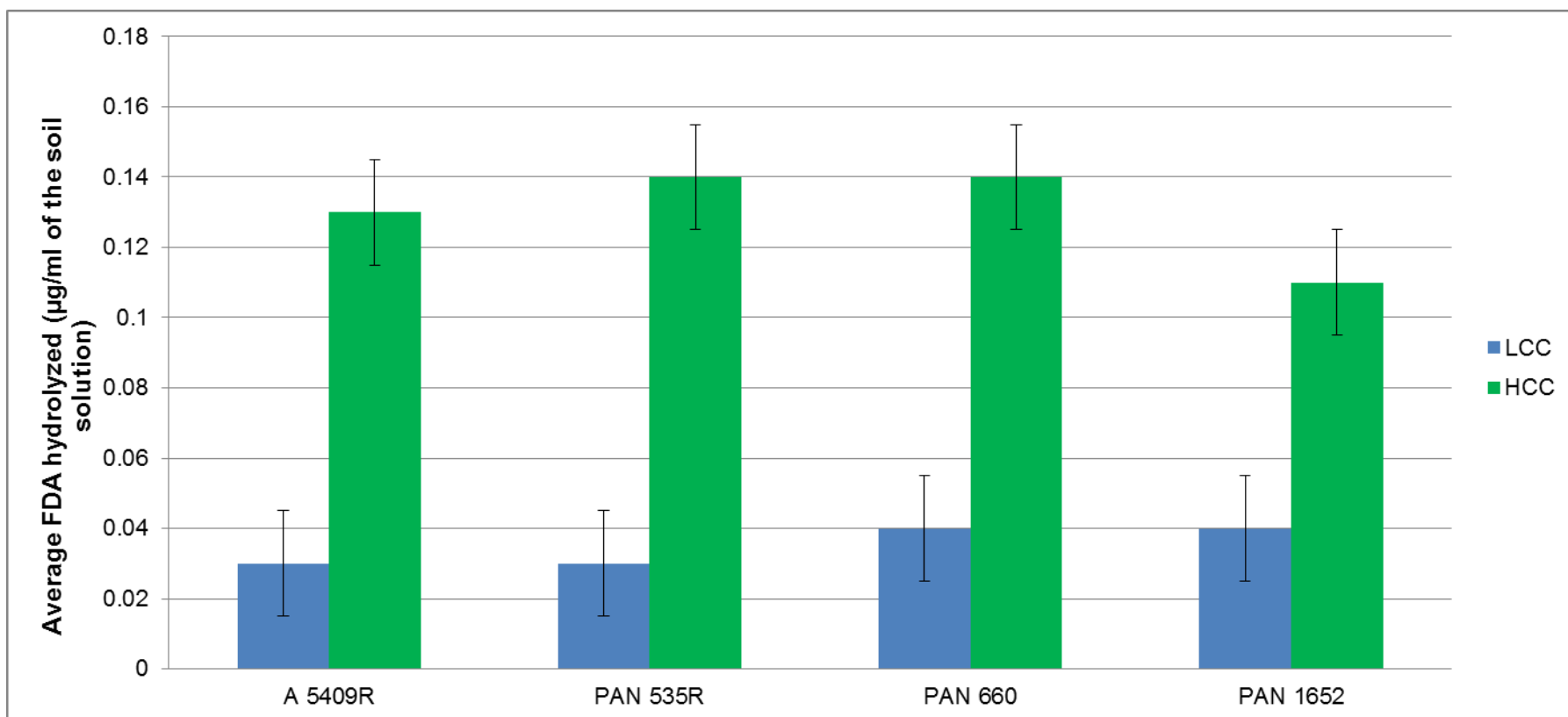
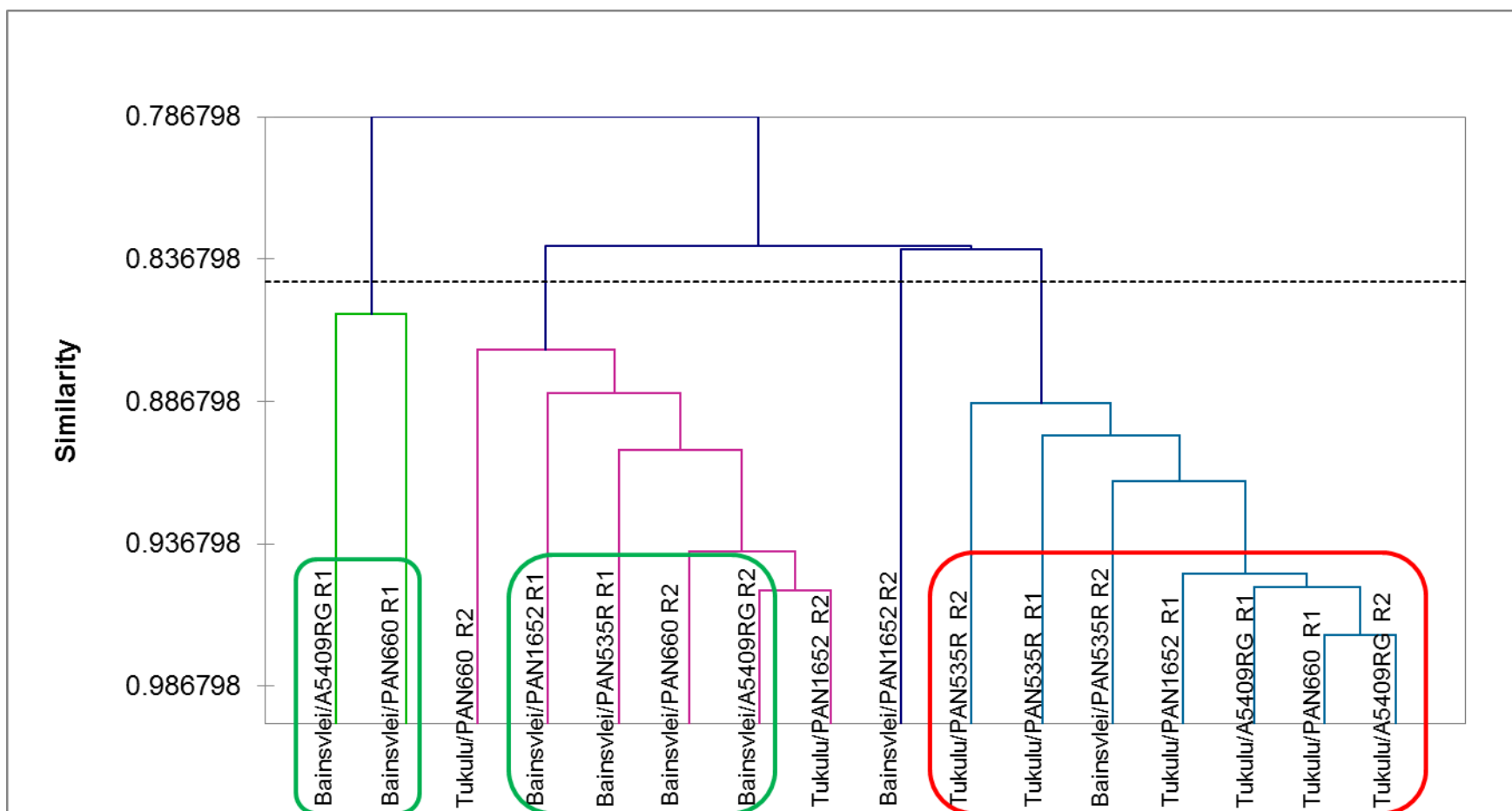*Fisher's LSD (P<0.05): Soil = 0.015*

Figure 5.4. Cluster analysis for the BiologEcoPlate™ data for soybean rhizosphere soil. (Bainsvlei represents low clay content soil while Tukulu represents the high clay content soil. R1 and R2 represent trial 1 and trial 2 respectively. A 5409RG and PAN 535R represents the GR cultivars while PAN 660 and PAN 1652 represents the conventional cultivars).

Figure 5.5. Cluster analysis of DGGE data for trial 1. (Paradys represents high clay content (HCC) soil and Bains the low clay content (LCC) soil; PAN660 and PAN1652 represents the two conventional cultivars used; PANA5409R and PAN535R represents glyphosate resistant cultivars used).
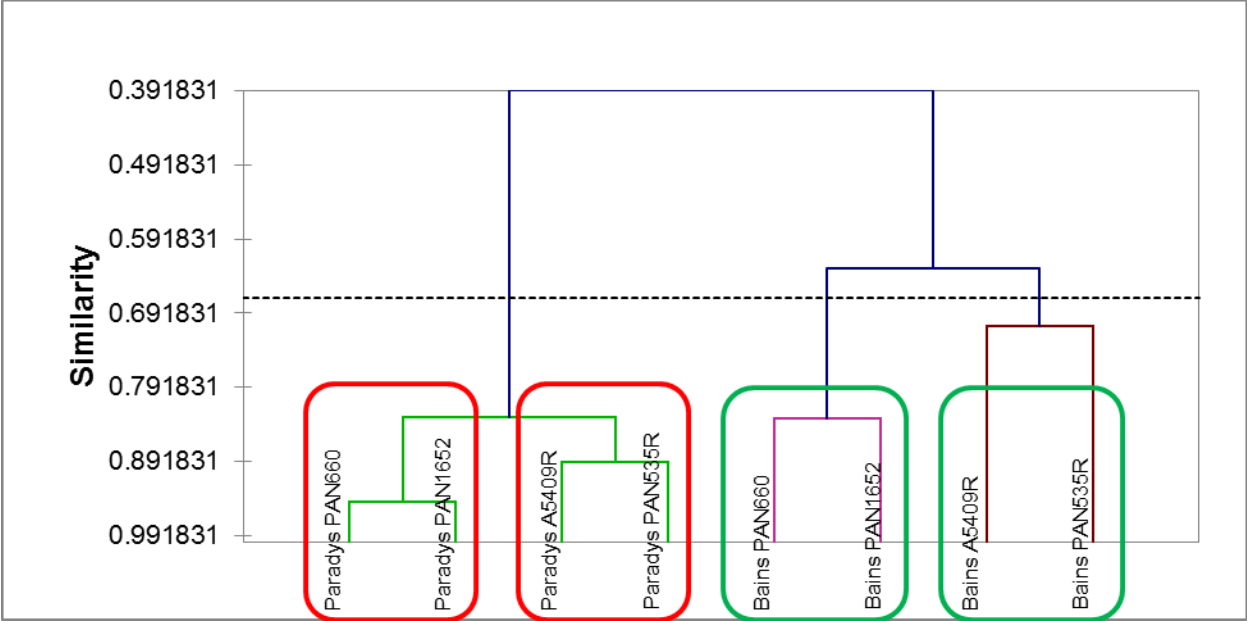
Figure 5.6. Cluster analysis of DGGE data for trial 2. (Paradys represents high clay content (HCC) soil and Bains the low clay content (LCC) soil PAN660 and PAN1652 represents the two conventional cultivars used; PANA5409R and PAN535R represents glyphosate resistant cultivars used).

Figure 5.7. Cluster analysis for T-RFLP data for trial 1. (Paradys represents high clay content (HCC) soil and Bains the low clay content (LCC) soil; PAN660 and PAN1652 represents the two conventional cultivars used; PANA5409R and PAN535R represents glyphosate resistant cultivars used).
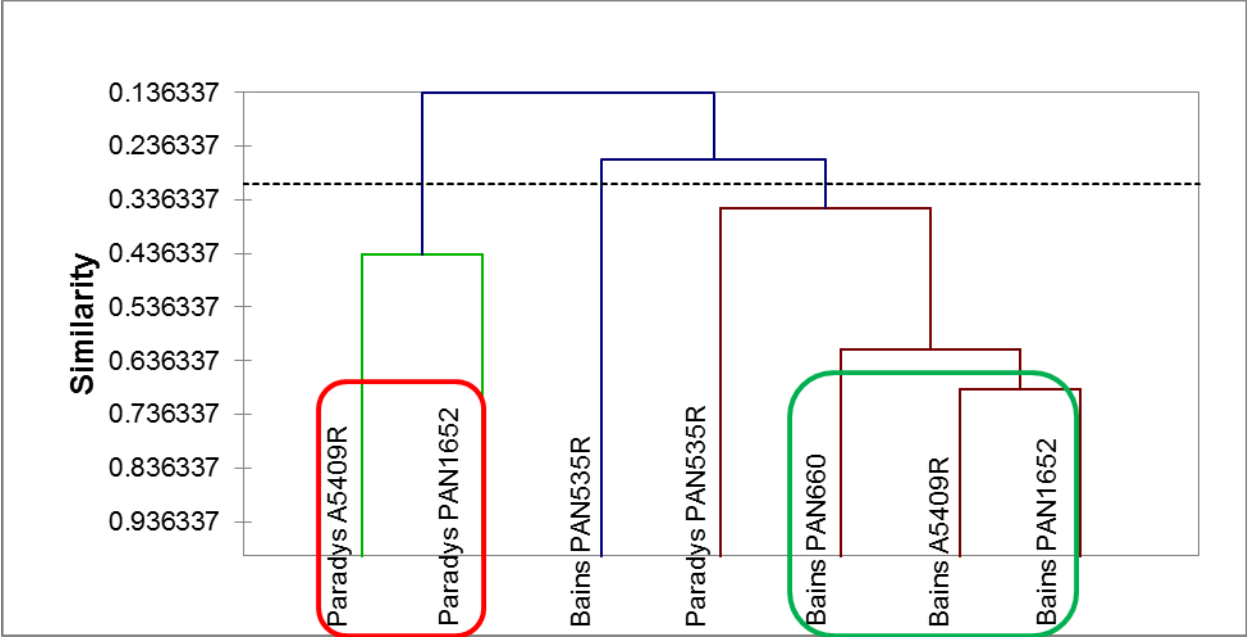
Figure 5.8. Cluster analysis for T-RFLP data for trial 2. (Paradys represents high clay content (HCC) soil and Bains the low clay content (LCC) soil; PAN660 and PAN1652 represents the two conventional cultivars used; PANA5409R and PAN535R represents glyphosate resistant cultivars used).
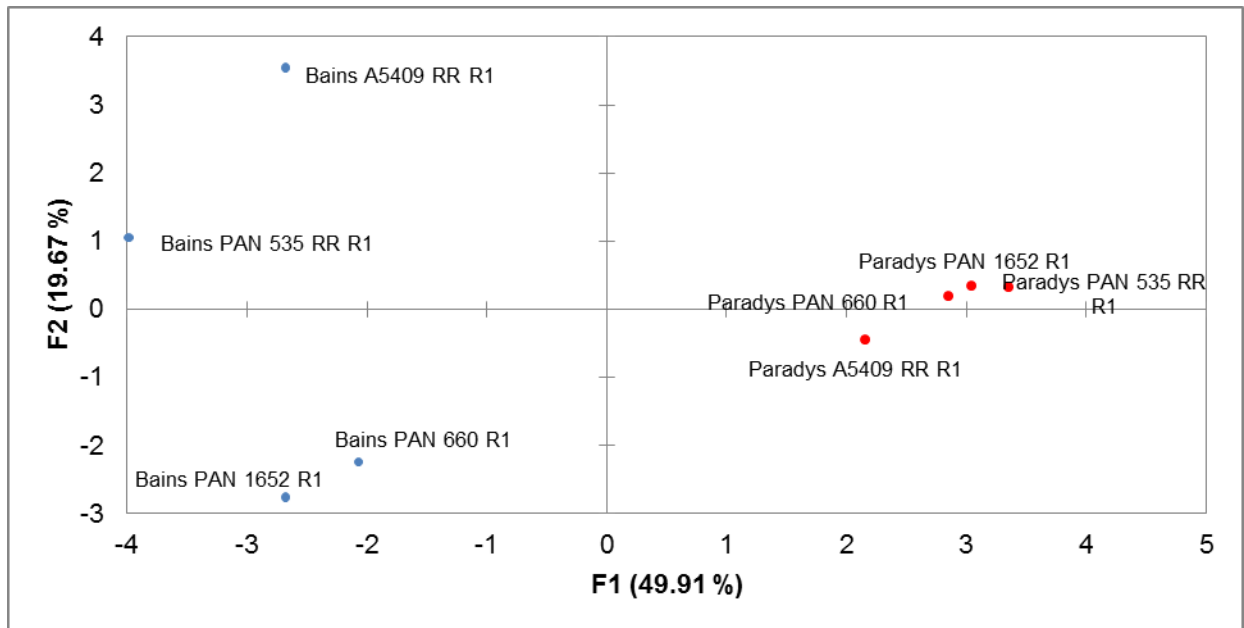
Figure 5.9. Principle component analysis of DGGE data for trial 1. (Paradys represents high clay content (HCC) soil and Bains the low clay content (LCC) soil; PAN660 and PAN1652 represents the two conventional cultivars used; PANA5409R and PAN535R represents glyphosate resistant cultivars used).
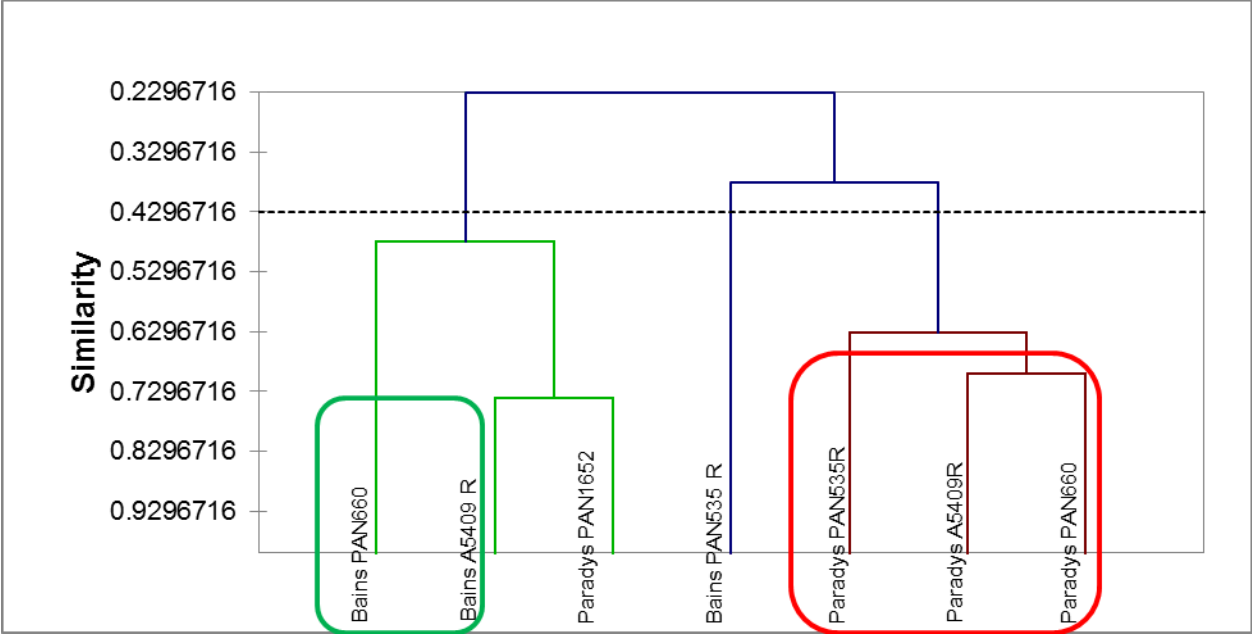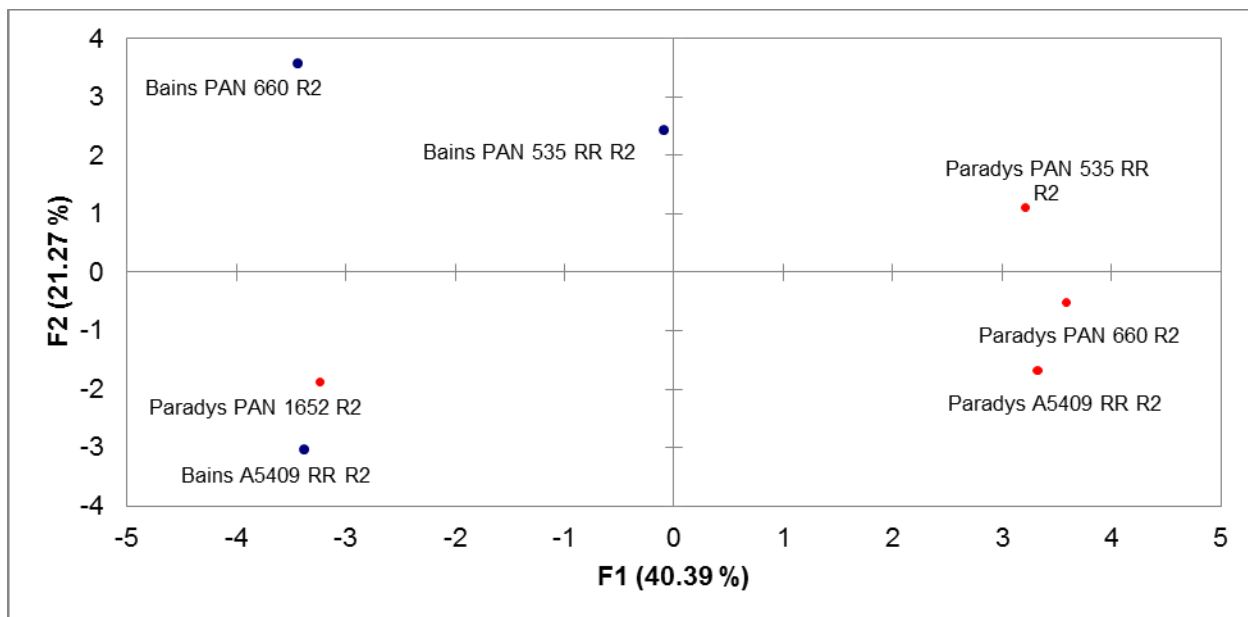
Figure 5.10. Principle component analyses of DGGE data for trial 2. (Paradys represents high clay content (HCC) soil and Bains the low clay content (LCC) soil; PAN660 and PAN1652 represents the two conventional cultivars used; PANA5409R and PAN535R represents glyphosate resistant cultivars used).
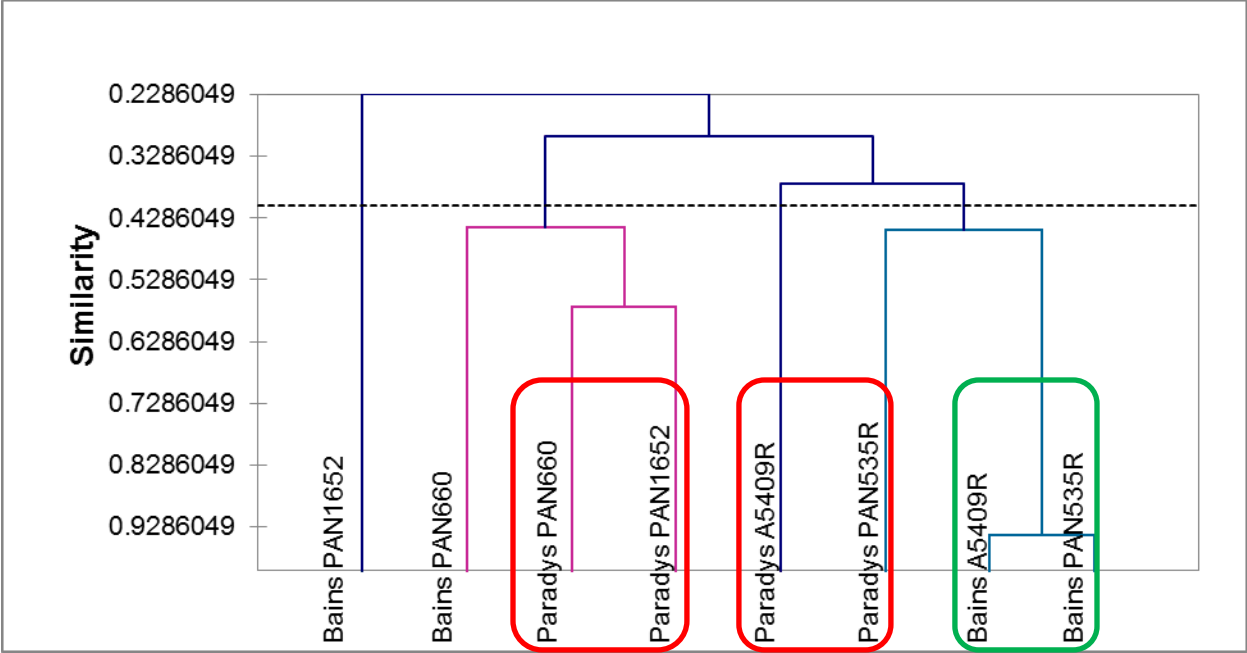
Figure 5.11. Principle component analysis of T-RFLP data for trial 1. (Paradys represents high clay content (HCC) soil and Bains the low clay content (LCC) soil; PAN660 and PAN1652 represents the two conventional cultivars used; PANA5409R and PAN535R represents glyphosate resistant cultivars used).
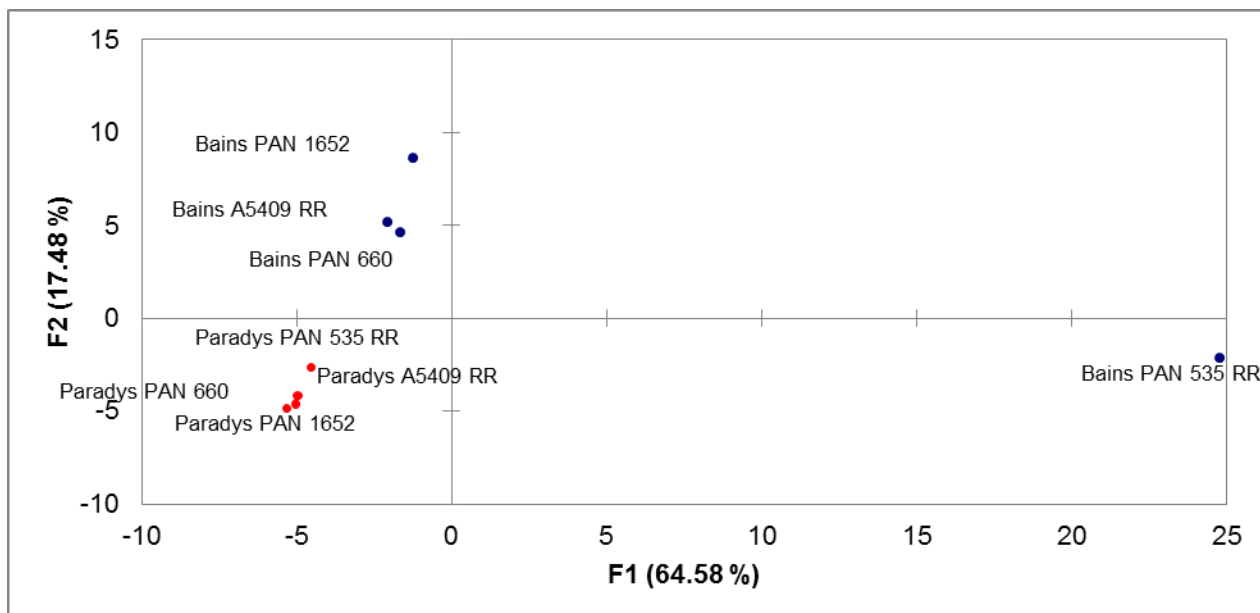
Figure 5.12. Principle component analysis of T-RFLP data for trial 2. (Paradys represents high clay content (HCC) soil and Bains the low clay content (LCC) soil; PAN660 and PAN1652 represents the two conventional cultivars used; PANA5409R and PAN535R represents glyphosate resistant cultivars used).
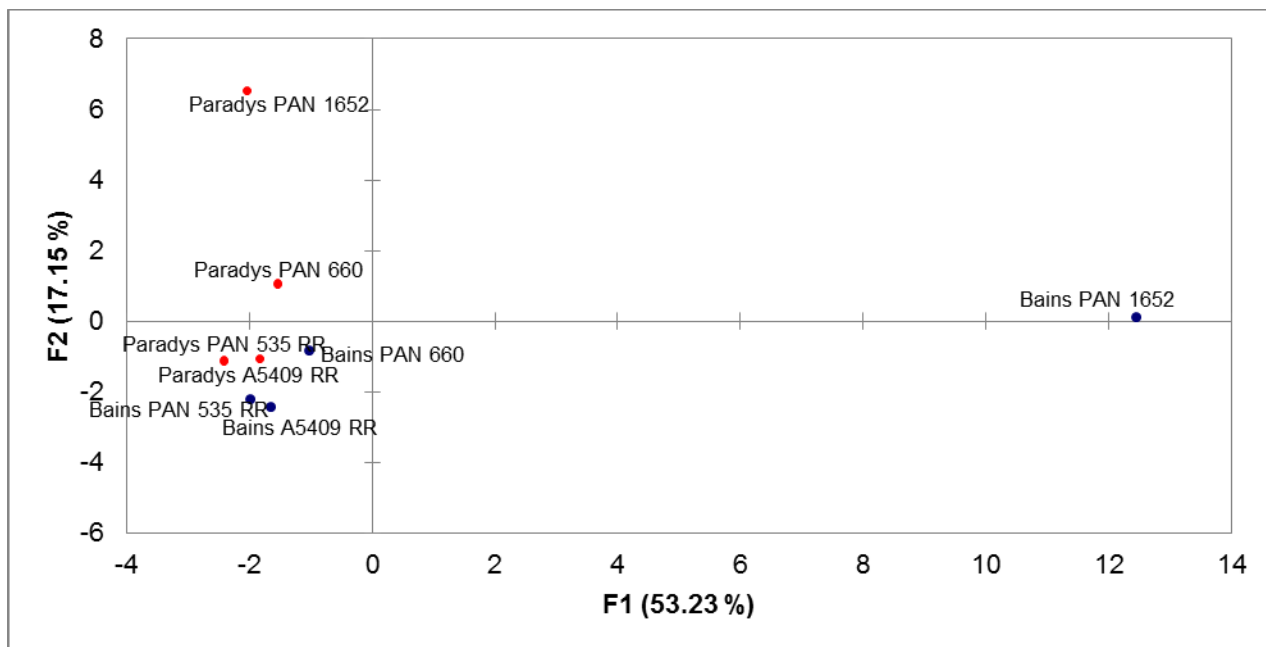
CHAPTER 6

The effect of genetically modified maize on disease incidence as influenced by glyphosate application

ABSTRACT

The effect of genetically modified (GM) maize, as well as glyphosate application, on root disease caused by *Fusarium verticilloides* (Sacc.) Nirenberhg was evaluated in two soils differing in clay content. Disease incidence was evaluated by determining root dry mass, as well as observing disease symptoms on leaves and roots by means of a disease rating scale. *Bacillus thuringiensis* (BT) as well as glyphosate resistant (GR) maize hybrids from two near-isogenic families was evaluated. Soil clay content had a significant effect on the amount of ergosterol extracted from infected roots. *Fusarium verticillioides* did not enhance root disease plants treated with glyphosate more than those not treated. The reduced ergosterol content indicated that glyphosate did however have a distinct fungicidal effect on fungi associated with maize roots. Results also suggested that glyphosate had a negative effect on root dry mass.

Keywords: *GR maize, BT maize, Glyphosate, Disease incidence.*

INTRODUCTION

The introduction of genetically modified (GM) crops into agricultural ecosystems has raised questions regarding their ecological impact on the soil ecosystem (Icoz & Stotzky, 2008). Concerns are mostly centered on the risks incurred when GM plants are grown in agro-ecosystems. The risks include invasiveness or dispersal of the GM plant into the native ecosystem and can have an indirect impact on crop diversity, gene flow through pollen transfer or through horizontal gene transfer with associated micro-organisms (Icoz & Stotzky, 2008). The development of resistance in target organisms, and non-target effects on native flora and fauna including effects on the biodiversity of beneficial and antagonistic micro-organisms in soil is also relevant (Nielsen *et al.*, 2001; Eastham & Sweet, 2002).

Glyphosate is the most extensively used herbicide in the history of agriculture (Neumann *et al.,* 2006; Johal & Huber, 2009). It is a highly effective broad-spectrum, non-selective, post-emergence, systematic herbicide which is used to effectively control weeds in crop production (Araujo, Monteiro & Abarkeli, 2003; Arregui *et al.*, 2003; Johal & Huber, 2009). Glyphosate resistance (GR) has been sold commercially in many crops since 1996 and is currently, throughout the world, the major weed-management system employed in soybeans, maize, cotton and canola (Johnson *et al.*, 2009).

Glyphosate is rapidly translocated to, and accumulates in, metabolic sink tissues, reproductive tissues and roots, rendering these tissues vulnerable to infection by pathogens (Pline-Srnic, 2005). Thus, the increasing use of glyphosate resistant crops (aka Round-up Ready) has increased concerns regarding its potential environmental impact (Haney, Senseman & Hons, 2002). Although Duke & Cerdeira (2007) state that

no agriculturally significant effect of glyphosate on soil micro-organisms has been documented, it has been reported to influence soil microbial activity (Kremer, *et al.*, 2005; Johal & Huber, 2009).

Increased fungal populations that develop under glyphosate treated GR crops can adversely affect plant growth and biological processes in the soil and rhizosphere (Kremer *et al.*, 2005). The consequences of enhanced root colonization of GR crops include the development of root disease by increased numbers of parasitic and opportunistic pathogens in the rhizosphere, competition for nutrients, or the enrichment of specific micro-organisms that are either detrimental or beneficial for crop growth (Kremer, *et al.*, 2005).

Negative side effects of glyphosate on non-target organisms are generally considered marginal, due to rapid microbial degradation and immobilization in soils (Neumann *et al.*, 2006). According to Wibawu, *et al.* (2009) the use of glyphosate at recommended field rate does not result in phytotoxic residues in the soil, thus causing no adverse effects to plant growth and development. However, Pline-Srnic (2005) expressed concern among growers about glyphosate and GR crops that include perceptions of increased sensitivity to disease and environmental stress.

The effects of glyphosate on non-target organisms can be both direct and indirect. It can have extensive unintended effects on nutrient efficiency and disease severity (Johal & Huber, 2009). A significant increase in disease severity associated with the wide spread application of glyphosate can be the result of direct glyphosate-induced weakening of plant defences, increased pathogen populations and enhanced virulence (Johal & Huber, 2009). Indirect effects due to the composition of root

exudates of glyphosate resistant crops which alter rhizosphere populations have also been reported (Duke & Cerdeira, 2007). Indirect effects of glyphosate on disease predisposition result from, a) immobilization of specific micronutrients involved in disease resistance, b) reduced growth and vigour of the plant from accumulation of glyphosate in meristematic root, shoot, and reproductive tissues, c) altered physiological efficiency, or d) modification of soil microflora affecting the availability of nutrients involved in physiological disease resistance (Johal & Huber, 2009).

Insect-resistant maize has also been developed by transferring the insecticidal Cry proteins from the bacterium *Bacillus thuringiensis* (BT) into various crops to reduce injury to the crops by pests (Icoz & Stotzky, 2008). The current BT maize hybrids are targeted to larval pests collectively classified as "corn borers". The European corn borer, *Ostrinianubilalis* Hübner, is the most damaging insect attacking maize globally (Icoz & Stotzky, 2008). The Cry proteins from transgenic maize can also affect rhizosphere microbial communities directly or indirectly (Buyer & Blackwood, 2004). In general, assessment of the non-target effects of the BT protein toxins indicates that there is a low hazard level to most groups of non-target organisms (Clark, Phillips & Coats, 2005). Fungi however, appear to be most affected by Cry proteins (Icoz & Stotzky, 2008). The roots of BT maize are less colonized by mycorrhizae than their non-BT near-isogenic counterparts (Turrin *et al.*, 2004; Castaldini *et al.*, 2005). This may potentially lead to the BT maize not only losing an important symbiont, which contributes to the plants nutrition, but plants may be more susceptible to insect pests (Icoz & Stotzky, 2008).

In the present study, the effect of the BT trait and that of the GR trait in maize, as well as the effect of glyphosate application, on *Fusarium verticillioides* (Sacc.) Nirenberg

incidence and severity was investigated. It was hypothesized that firstly, BT and GR maize will have greater disease incidence than non- BT and non-GR maizen and secondly, that glyphosate application to GR maize will increase *F. verticilloides* incidence and consequently lead to reduced biomass.

MATERIALS AND METHODS

Soil sampling.   Soil was collected from an agricultural experimental farm, Kenilworth near Bloemfontein, South Africa (-29.020397 S; 26.145308 E). The soil had a 10 year history of annual tilling, and when sampling, maize was being cultivated on the field. The soil is categorized as Kenilworth-Bainsvlei ecotype and an example of a fine sandy loam soil in a semi-arid area (Soil Classification Working Group, 1991), and will henceforth be referred to as low clay content (LCC) soil. A second soil sample was from a second experimental farm, Paradys near Bloemfontein (-29.222950 S; 26.210747 E). This soil is categorized as Tukulu according to the Soil classification working group (1991), and will be referred to as high clay content (HCC) soil. In both instances, bulk soil samples were collected from the top 5 cm of the surface. Chemical and textural analyses were conducted for both soils (Table 6.1 & 6.2).

Inoculum preparation and soil infestation.   A *F. verticillioides* isolate was obtained from the culture collection maintained by the Plant Protection Research Institute (PPRI) of the Agricultural Research Council in Pretoria. The isolate was plated onto a 90-mm-diameter Petri-dish containing potato dextrose agar (PDA). A potato dextrose broth (PDB) was prepared as described by Chen & Wang (2005). Mycelial

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plugs (n=3) from a 3 day old culture maintained on PDA, were placed into 200 ml of PDB, in an Ehrlenmeyer flask, and agitated with a shaker for 3 days at room temperature. The mycelium infested broth was homogenized for 15-20 secs using a Heidolph Silent crusher M.

Sorghum seeds were suspended in water and left over night. Approximately 50 ml of the homogenized broth was then poured over 300 g of sorghum and kept at 23 °C for one week, shaking the bottle daily to distribute the inoculum evenly. After one week, the air-dried sorghum was ground to a coarse powder. The top layer of soil in each 2000 cm^3 planting bag was infested with 40 g of inoculum powder before planting maize seeds.

Trial layout and planting. Eight maize hybrids from two near-isogenic families were used, PAN 4P-116, PAN 4P-316Bt, PAN 4P-516 R (GR), PAN 4P-716Bt&R (GR), PAN 6P-110, PAN 6Q-308Bt, PAN 6Q-508 R (GR), and PAN 6Q-708Bt&R (GR). Four to five seeds were planted in each planting bag, containing respective soils mentioned previously. After germination plants were thinned to three per bag.

Each trial was a complete randomized design with three replicates per treatment. Two experiments were conducted. In the first experimet, BT and non-BT, and GR and non-GR resistant hybrids were compared to determine if the BT and GR genes had an effect on disease development. The second entailed GR hybrids treated with or without (control treatment) glyphosate to determine the effect of glyphosate itself on the disease incidence and severity. Glyphosate was applied as Roundup[®] at a recommended field application rate 4.7 L ha^{-1} at the V2 growth stage. Plants were maintained in the

glasshouse at 24°C day temperature and 19°C night temperature. Plants were watered daily using filtered municipal water to eliminate chlorine. Plants were fertilized weekly using Multifeed® Classic (19: 8: 16; N: P: K) in accordance to the manufacturer's instructions. Plants were sampled 56 days after planting. Soil was removed from the roots by gently washing it off with municipal water to ensure minimum damage to the root system. After plants were removed from soil, soil samples were taken and after a series of dilutions were made the soil suspension was plated onto PDA to re-isolate *F. verticilloides*. Isolations were also made from roots and plated onto PDA. In both cases, the isolations were incubated at 24 °C in a Labcon growth chamber.

Disease severity rating. A rating scale (Table 6.3) as described by Reid *et al* (2002) with modifications was used to rate disease severity on leaves and roots.

Ergosterol assessment. Ergosterol was extracted from the maize roots as described by Jambunathan, Kherdekar & Vaidya (1991), with minor modifications, to evaluate fungal infection of maize roots. The roots were ground to a fine powder in liquid nitrogen. The total amount of powder available for each sample was determined and added to 50 ml of methanol in a 50 ml capped centrifuge tubed and shaken on a Multi Reax shaker for 40 min at 1600 rpm. After the mixture was allowed to settle, 25 ml of the supernatant was added to 3 g of KOH in a clean 50 ml capped centrifuge tube. The mixture was again shaken for 5 min at 1600 rpm or until the KOH was dissolved. Hexane (10 ml) was then added and the mixture was incubated at 75 °C for 30 min in a water bath. After incubation, the mixture was allowed to cool down to room temperature after which the hexane layer which accumulated at the top of the mixture was transferred to a clean 50 ml capped centrifuge tube. An additional 10 ml of hexane was

added and mixed well with the mixture left in the original centrifuge tube which was incubated in the water bath. The hexane layer was again removed and added to the previously removed aliquot. The last two steps were repeated. The hexane extract was then evaporated in a hot water bath connected to an air pump to speed up the evaporation process. The residue left after evaporation was dissolved in 5 ml methanol and filtered through a 0.45 µm millipore filter The filtrate was analyzed at an emission wavelength of 282 nm using a Perkin Elmer high performance liquid chromatograph (HPLC) fitted with a Phenomenex C18.5 µm 4.6 x 150 mm analytical column.

Statistical analysis. Ergosterol concentrations were calculated and data from both trials were pooled and subjected to analysis of variance (ANOVA) and, when F values were significant ($P<0.05$), means were separated using Fisher's protected least significant difference (LSD) test. Data were analysed using NCSS (Hintze, 2001). A GLM ANOVA was conducted for each technique and means were compared using Fisher's least significance difference (LSD) at $P < 0.05$.

RESULTS

Soil analysis. Chemical and textural characteristics indicated clear differences between HCC and LCC soils. The HCC soil consistently had higher K, Fe, Cu, N and C levels than the LCC soil, while LCC soil consistently had higher levels of Ca, Mg, Na, Zn and P than the HCC soil. The HCC soil had a lower pH and higher organic matter content than LCC soil (Tables 6.1 & 6.2).

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Treatments without glyphosate application: *Fusarium verticillioides* was successfully re-isolated from infested soil as well as roots. No difference in terms of disease symptoms were observed between the leaves and roots of all eight cultivars under conditions of this test. No significant differences or significant interactions (P<0.05) were observed in terms of root dry biomass (Table 6.5). Both isogenic families did however show higher root dry biomass values in the LCC (Bainsvlei) soil than for the HCC (Tukulu) soil, except in the case of the GR and BT stack genes hybrids, i.e. PAN 4P-716BR and PAN 6Q-708BR. In the case of PAN 4P-716BR and PAN 6Q708BR the HCC soil showed higher root dry biomass (Figure 6.1). Ergosterol concentrations of the roots differed significantly for those cultivated in HCC versus LCC soils (P<0.05), as well as between different hybrids (Table 6.7). Ergosterol content was overall significantly lower in roots of plants cultivated in LCC soil than those cultivated in the HCC soil (Figure 6.3).

Treatments with glyphosate application: No difference was observed in terms of disease symptoms between leaves and roots of all GR maize hybrids, with and without glyphosate application. The interaction between soil and glyphosate treatment was significant (P<0.05) under conditions of this test (Table 6.6). Hybrids in general, as well as the hybrids within their isogenic lines, reacted differently in terms of root dry biomass with and without glyphosate application (Figure 6.2).

The interactions between soil type and glyphosate treatment as well as that between cultivar and glyphosate treatment was significant (P<0.05) (Table 6.8) in terms of root ergosterol concentrations. The two isolines reacted differently to glyphosate application in terms of root ergosterol concentrations. Hybrids in isoline 4P reacted

differently to glyphosate application. PAN 4P-516R glyphosate application led to a lower ergosterol concentration for roots cultivated in HCC soil compared to those cultivated in the LCC soil where root ergosterol concentration was higher with glyphosate application. For PAN 4P-716BR, glyphosate application yielded higher ergosterol concentration in both soils (Figure 6.4). For isoline 2, 6Q, glyphosate application lead to lower root ergosterol concentrations for hybrids cultivated in both soils.

DISCUSSION

In the present study, no differences were observed in terms of *F. verticillioides* disease symptoms, root dry mass and ergsoterol concentraion between BT and GR hybrids compared to conventional hybrids. The level of disease observed was not sufficient to draw a conclusion regarding the hypothesis.

Glyphosate application to GR hybrids mostly resulted in a decrease in root ergosterol concentrations, in contrast to the findings of Sanogo, Yang & Scherm (2000), Kremer *et al.* (2005) and Kremer and Means (2009) who observed an increase in fungal root colonization with glyphosate application to GR crops. The application of glyphosate resulted in a decrease in root ergosterol concentration for both cultivars of isoline 2 (6Q) cultivated in both soils, indicating that glyphosate might have a fungicidal effect on root fungi. As a broad-spectrum mineral chelator, glyphosate also might inhibit ergosterol synthesis similar to EPSPS and numerous other enzymes. These findings are consistent with the results obtained in chapters 2 and 7, where glyphosate had an inhibitory effect on fungal isolates *in vitro* and root fungi respectively. Glyphosate application to GR hybrids however mostly led to a decrease in root dry biomass,

consistent to the findings of Sanogo *et al.* (2000) on other crops. The accumulation of glyphosate in root tips also could restrict their growth to reduce root dry biomass.

All eight hybrids evaluated are marketed as having a medium to poor tolerance to low pH soils (Pannar, 2011). Thus the lower root dry biomass in the HCC soil may be attributed to a difference in pH between the HCC and LCC soil with HCC having a much lower pH than LCC and, consequently, greater absorption of glyphosate in the soil (Fahrenhorst *et al.,* 2009).

Results obtained from the present study suggest that the BT and GR traits in maize does not influence *F. verticilloides* disease incidence under the conditions of this experiment, however glyphosate application has a distinct fungicidal effect on fungi associated with maize roots. This could in turn lead to a decrease in beneficial fungal species, such as mycorhizzae, thus leaving the plant more susceptible to attack by pathogenic soil fungi. Furthermore, glyphosate application reduced root dry biomass, suggesting a definite negative effect of glyphosate on plant physiology. Results suggest that soil chemical and physical properties play a major role in the manner in which maize hybrids react to glyphosate applications and pathogenic fungi.

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Table 6.1. Soil chemical characteristics.

Soil type	pH	Ca (mg/kg)	K (mg/kg)	Mg (mg/kg)	Na (mg/kg)	Zn (mg/kg)	Fe (mg/kg)	Cu (mg/kg)	P (mg/kg)	N (mg/kg)	C (mg/kg)
Bainsvlei (LCC)	6.02	474	120	76	44	1.78	16.36	1	19.76	359.2	1390
Tukulu (HCC)	4.98	334	168	66	28	1.44	110.6	1.44	15.03	492.3	2600

Table 6.2. Soil textural characteristics.

Soil type	Organic matter (OM) %	Slit %			Sand %		
		Coarse	Fine	Clay	Coarse	Medium	Fine
Bainsvlei (LCC)	0.232	3.33	3.33	3.33	0.67	8	81.17
Tukulu (HCC)	0.399	1.670	4.000	9.330	2.070	6.470	73.770

Table 6.3. Disease severity scale as described by Reid *et al* (2002) with minor modifications.

Severity rating	Severity %	Symptoms (Leaves)	Symptoms (Roots)
1	0	No visible symptoms	No visible symptoms
2	1-3	Leaves showing minor symptoms	Roots showing minor symptoms (roots tips with lesions)
3	4-10	Individual leaves with necrotic/ yellowing symptoms	Root lesions
4	11-25	Individual leaves with necrotic/ yellowing symptoms	Root lesions
5	26-50	Individual leaves with necrotic/ yellowing symptoms	Root lesions
6	51-75	Plant with wilt symptoms	Root lesions
7	>75	Plant with wilt symptoms	Root lesions

Table 6.4. ANOVA as calculated for root dry biomass for treatments without glyphosate application.

Source Term	DF	Sum of Squares	Mean Square	F-ratio	Probability level	Power (Alpha = 0.05)
A: Soil	1	16.75427	16.75427	0.12	0.729987	0.062682
B: Hybrid	7	661.2902	94.47002	0.69	0.676401	0.214767
AB	7	580.5202	82.93146	0.61	0.740620	0.190910
C:Reps	5	397.6256	79.52512	0.58	0.711762	0.167722
ABC	35	2490.104	71.14582	0.52	0.948471	0.233156
S	17	2313.397	136.0822			
Total (Adjusted)	72	6961.908				
Total	73					

*Term significant at alpha = 0.05

Table 6.5. ANOVA as calculated for root dry biomass for treatments with glyphosate application.

Source Term	DF	Sum of Squares	Mean Square	F-ratio	Probability level	Power (Alpha = 0.05)
A: Soil	1	463.371	463.371	5.23	0.026547*	0.611103
B: Gly Tr	1	56.89945	56.89945	0.64	0.426740	0.123189
AB	1	574.7502	574.7502	6.49	0.014049*	0.704386
C: Hybrid	3	665.672	221.8907	2.50	0.069974	0.585508
AC	3	138.2902	46.09674	0.52	0.670258	0.148571
BC	3	301.2195	100.4065	1.13	0.344776	0.286585
ABC	3	378.3567	126.1189	1.42	0.247115	0.354070
D: Reps	5	53.59451	10.7189	0.12	0.987094	0.074992
ABCD	15	1697.354	113.1569	1.28	0.252093	0.684208
S	49	4340.604	88.58376			
Total (Adjusted)	84	8642.235				
Total	85					

*Term significant at alpha = 0.05

Table 6.6. ANOVA as calculated for ergosterol concentrations for treatments without glyphosate application.

Source Term	DF	Sum of Squares	Mean Square	F-ratio	Probability level	Power (Alpha = 0.05)
A: Soil	1	5289493	5289493	52.90	0.000000*	1.000000
B: Hybrid	7	1691007	241572.5	2.42	0.046636*	0.759344
AB	7	658263.8	94037.69	0.94	0.492476	0.325544
C:Reps	5	1909877	381975.4	3.82	0.009562*	0.881311
ABC	35	4334358	123838.8	1.24	0.285430	0.722270
S	27	2699830	99993.72			
Total (Adjusted)	82	1.55454E+07				
Total	83					

*Term significant at alpha = 0.05

Table 6.7. ANOVA as calculated for ergosterol concentrations for treatments with glyphosate application.

| Source Term	DF	Sum of Squares	Mean Square	F-ratio	Probability level	Power (Alpha = 0.05)
A: Soil	1	1653093	1653093	23.07	0.000015*	0.996994
B: Gly Tr	1	700544.6	700544.6	9.78	0.002971*	0.865410
AB	1	43342.63	43342.63	0.60	0.440462	0.118818
C:Hybrid	3	51552.5	17184.17	0.24	0.868150	0.092196
AC	3	1083828	361275.8	5.04	0.004007*	0.895150
BC	3	709391.6	236463.9	3.30	0.027903*	0.718702
ABC	3	29665.56	9888.52	0.14	0.936827	0.073501
D: Reps	5	173020.9	34604.18	0.48	0.787290	0.167365
ABCD	15	1166419	77761.23	1.09	0.393629	0.592950
S	49	3511110	71655.3			
Total (Adjusted)	84	8952167				
Total	85					

*Term significant at alpha = 0.05
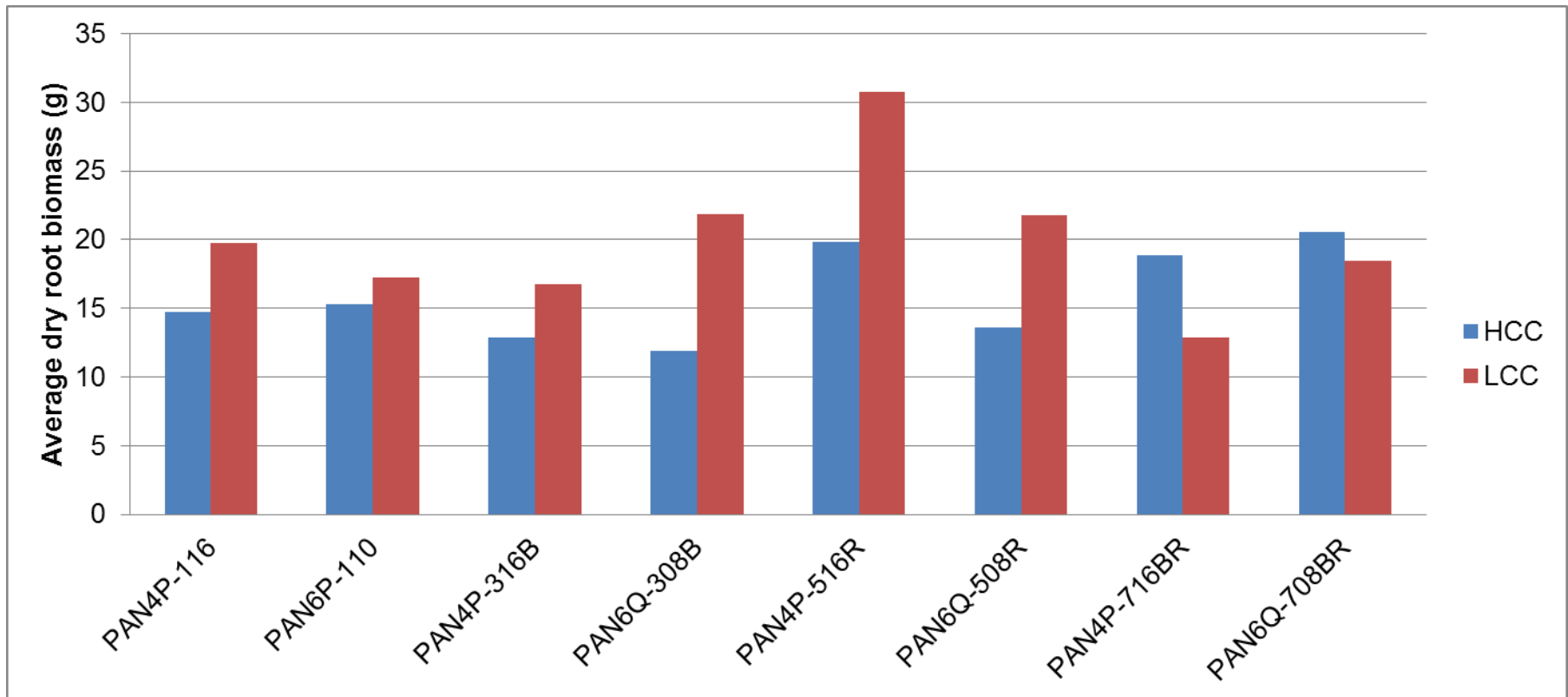
Figure 6.1. The effect of the GR gene on dry root biomass in two different soil types, one with high clay content (HCC) and the other with low clay content (LCC). (PAN4P-516R, PAN4P-716BR, PAN6Q-508R and PAN6Q-708BR impart the GR gene while PAN4P-116, PAN4P-316B, PAN6P-110 and PAN6P-308B are non-GR).

No statistical differences were observed between treatments.
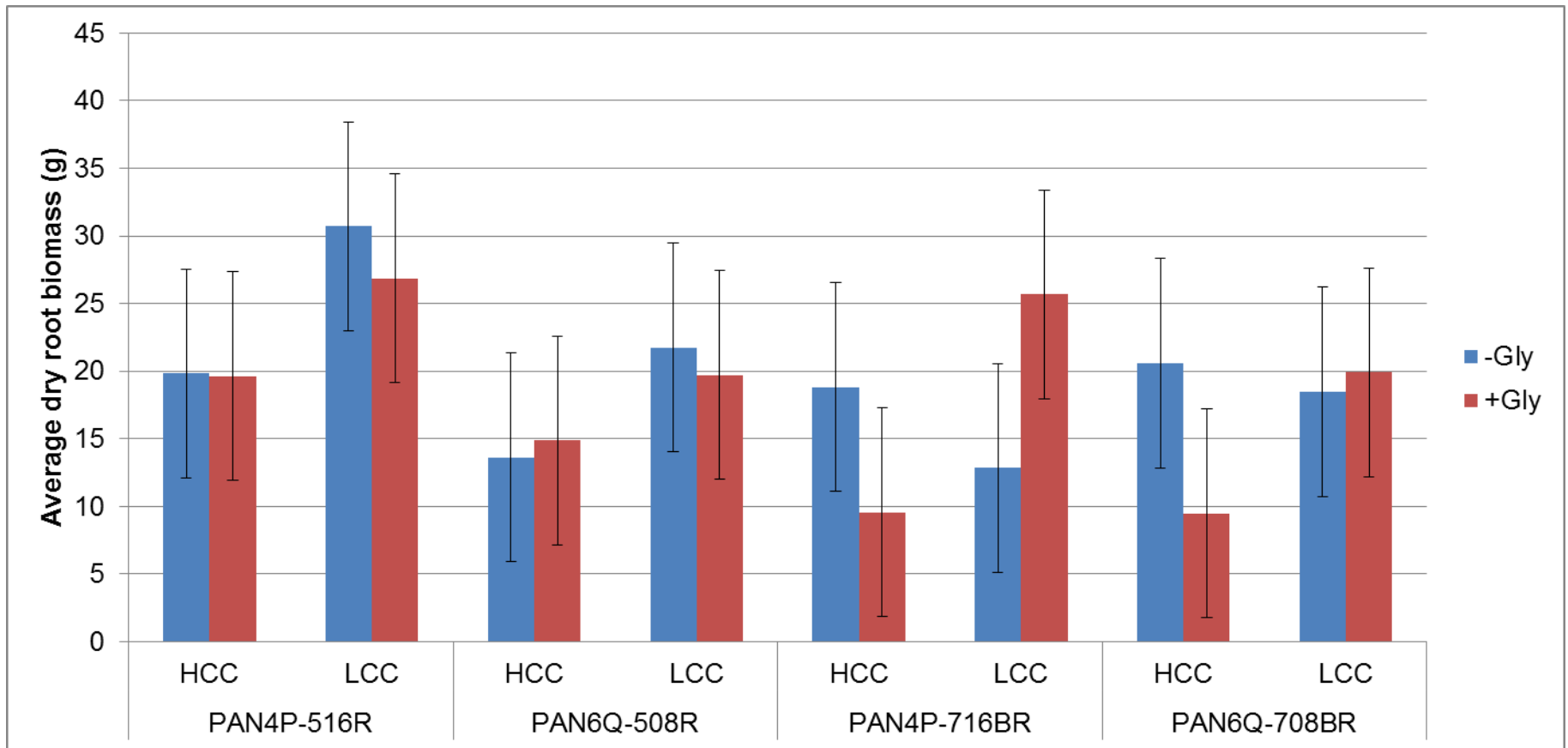
Figure 6.2. The effect of the glyphosate on dry root biomass on GR maize in two different soil types, one with high clay content (HCC) and the other with low clay content (LCC).

Fisher's LSD (P<0.05): Soil = 3.86

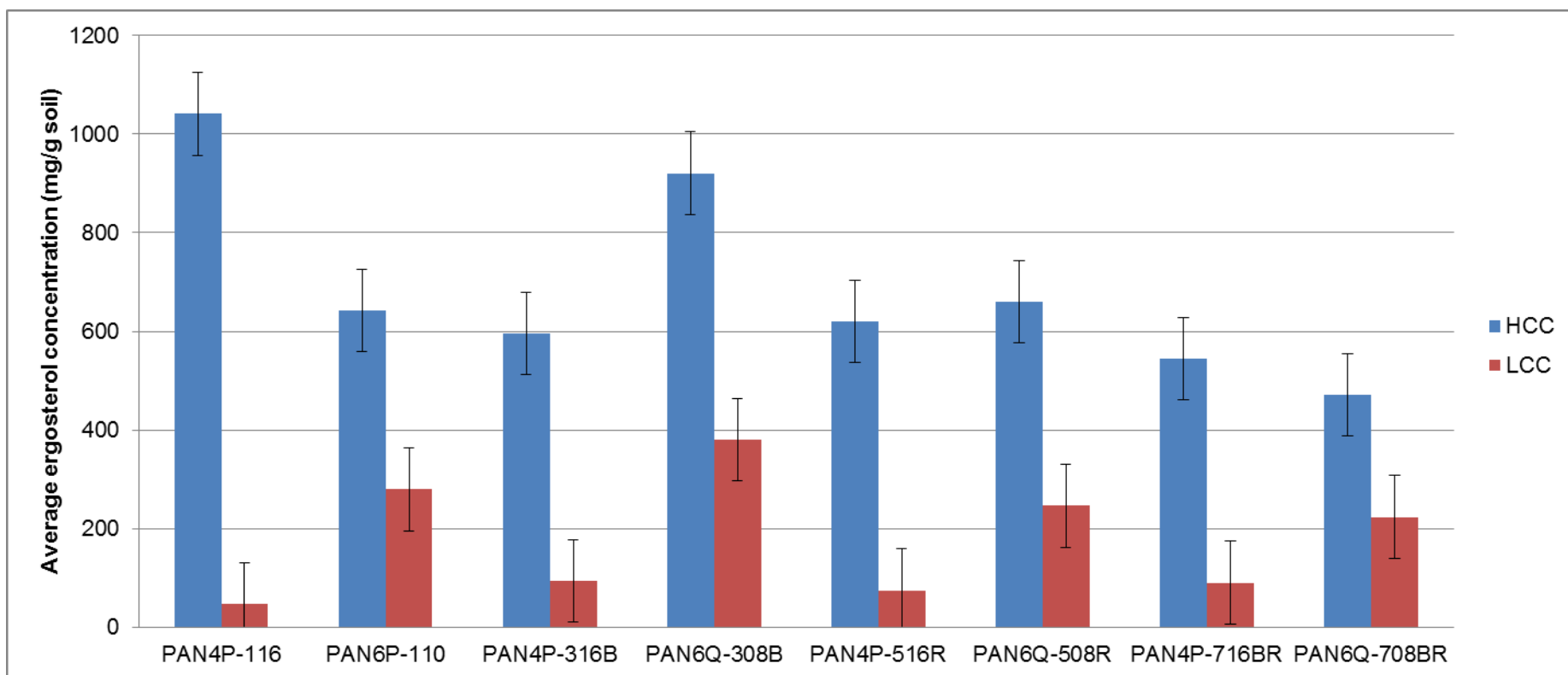**Figure 6.3.** The effect of the GR gene in maize on ergosterol concentration in two different soil types. HCC refers to high clay content soil while LCC refers to low clay content soil. (PAN4P-516R, PAN4P-716BR, PAN6Q-508R and PAN6Q-708BR imparts the GR gene while PAN4P-116, PAN4P-316B, PAN6P-110 and PAN6P-308B are non-GR).

Fisher's LSD (P<0.05): Maize hybrid = 83.77 (indicated above); Soil = 132.45
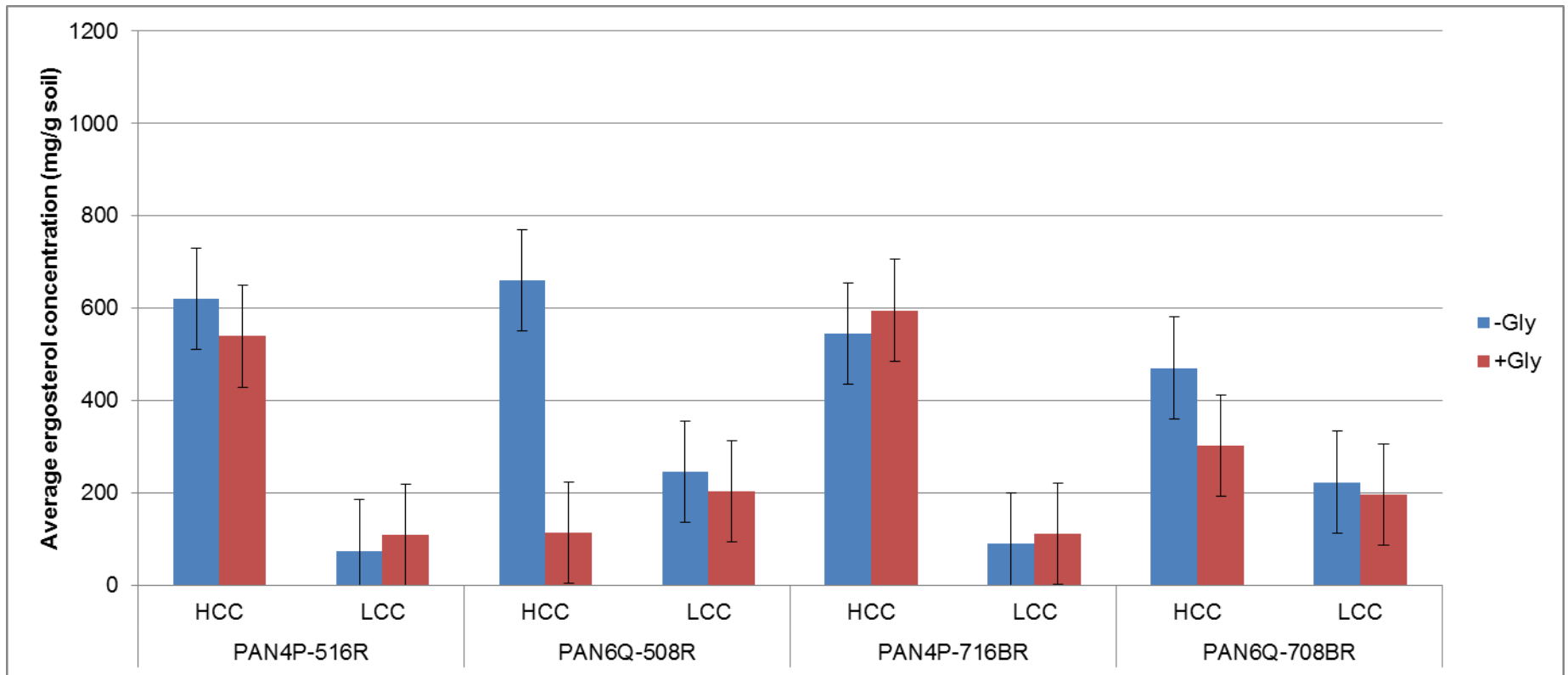
Figure 6.4. The effect of the glyphosate on ergosterol concentration of GR maize in two different soil types. (HCC refers to high clay content soil, and LCC to low clay content soil).

Fisher's LSD (P<0.05): Glyphosate treatment = 109.883 (indicated above); Soil = 109.883

CHAPTER 7

The effect of glyphosate resistant soybeans on *Sclerotinia* severity as influenced by glyphosate application

ABSTRACT

The effect of glyphosate resistant (GR) soybeans and glyphosate application on the pathogenicity of *Sclerotinia sclerotiorum* was investigated in glasshouse pot trials. Four soybean lines (A 5409R, PAN 535R, PAN 660 and PAN 1652) were assessed in two respective soils differing in clay content. Root ergosterol concentration and root dry mass were evaluated. No visual symptoms of disease were observed on leaves or roots. Leaves treated with glyphosate showed signs of herbicide damage. Root dry mass showed a significant interaction between cultivar and glyphosate treatment. Ergosterol concentrations in roots revealed differences between the roots of soybeans grown in the two types of soil.

Keywords: GR soybean; glyphosate; disease incidence.

INTRODUCTION

Glyphosate is a broad spectrum herbicide with limited residual soil activity. It is 'detoxified' in soil by chelation, but not necessarily degraded; and can then be desorbed later again to be active (Bott *et al.,* 2008, 2011). The herbicide is quickly absorbed and readily translocated in the phloem and is thus particularly effective against perennial weeds. Glyphosate kills plants by inhibiting the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSP synthase). The EPSP synthase catalyses a critical step in the synthesis of the three aromatic amino acids, tryptophan, phenylalanine and tyrosine, and predispose plants to soilborne pathogens which constitute the herbicidal mode of action (Johal & Rahe, 1984, 1988, 1990; Lévesque & Rahe, 1992; Johal & Huber, 2009; Schafer *et al.,* 2009, 2010). The EPSP synthase pathway is found only in plants and micro-organisms (Hopkins & Hüner, 2004).

Glyphosate resistant (GR) soybean was the first transgenic crop species available for large-scale cultivation. Launched in 1996, it introduced a new, simpler weed management program in row crops (Dill, 2005). However, the addition of the gene that imparts herbicide tolerance can alter certain physiological processes in plants (Gordon, 2007). For example, changes in the composition of soybean root exudates can influence many soil and plant related defence responses. Studies have shown that glyphosate application can alter plant physiology through decreased phytoalexin production, thus rendering plants more susceptible to pathogens (Holliday & Keen, 1982; Keen, Holliday & Yoshikawa, 1982; Sharon, Amsellem & Gressel, 1992). Glyphosate reportedly decreases the plant's defences so that even moderately virulent strains of pathogenic bacteria and fungi are rendered pathogenic (Holliday & Keen,

1982; Keen *et al.*, 1982; Sharon *et al.*, 1992). It is also possible that glyphosate can enhance the virulence of certain pathogens by suppressing the elicited appearance of non-phytoalexin phenolics which could inhibit the production of precursors for other defense pathways, such as lignin biosynthesis (Sharon *et al.*, 1992).

With increased glyphosate application, parameters such as photosynthesis, water absorption, and biomass production decrease drastically (Zobiole *et al.*, 2010). Thus, GR soybeans that have high transpiration rates due to glyphosate application will require more water than usual (Zobiole *et al.*, 2010).

The fungal pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary causes stem rot or white mould and is one of the most devastating and cosmopolitan soil-borne plant pathogens. It infects more than 500 species of plants worldwide, including field crops, fruit crops, ornamentals, trees, shrubs and numerous weeds (Saharan & Mehta, 2008) The white mould pathogen infects many broadleaf plants, but highly susceptible crops are: soybean, dry beans, snap bean, lima bean, sunflowers, canola, carrots, and cabbage (Malenčič *et al.*, 2010).

In the present study, the reaction of GR soybeans, and the effect of glyphosate application on *S. sclerotiorum* incidence and severity was investigated. The primary objective was to determine if GR soybean lines have less disease resistance than non-GR lines. A second objective was to determine if glyphosate application to GR soybeans leads to reduced plant biomass.

MATERIALS AND METHODS

Soil collection. Two contrasting batches of bulk soil were collected for pot trials. Firstly, a soil categorized as Kenilworth-Bainsvlei ecotype and an example of a fine sandy loam soil in a semi-arid area (Soil Classification Working Group, 1991) was collected from an agricultural experimental farm, Kenilworth near Bloemfontein (-29.020397 S; 26.145308 E). The soil has a 10 year history of annual tilling, and during sampling, non-GM maize was being cultivated on the field. It will henceforth be referred to as low clay content (LCC) soil (Table 7.1). A second batch of soil was collected from the experimental farm, Paradys (-29.222950 S; 26.210747 E), and is catergorized as Tukulu according to the Soil Classification Working Group (1991). It will henceforth be referred to as high clay content (HCC) soil. In both instances, bulk soil was collected from the top 5 cm of the soil surface. Chemical and textural analysis was conducted for both soils (Table 7.1 & 7.2) by the Department Climate-, Crop-, and Soil Sciences at the University of the Free State.

Inoculum preparation and soil infestation. A *S. sclerotiorum* isolate (originally isolated from an *S. sclerotiorum* infested sunflower flowering head) was obtained from the culture collection of the Department Plant Sciences at the University of the Free State, Bloemfontein.   The isolate was plated onto a 90-mm-diameter Petri-dish containing potato dextrose agar (PDA). A potato dextrose broth (PDB) was prepared as described by Chen & Wang (2005). Three-day-old mycelial plugs (n = 3), maintained on PDA, were placed into 200ml of PDB in a Erlenmeyer flask, and agitated on a rotary shaker for 3 days at room temperature.   The mycelium infested broth was then

homogenized for 15-20 seconds using a Heidolph Silentcrusher M to make sure the mycelium was evenly distributed in the suspension.

Sorghum seeds were suspended in water and left overnight. Approximately 50 ml of the homogenized broth was then poured over 300g of sorghum contained in a 1L bottle and kept at 23°C for one week. The bottle was shaken by hand daily to distribute the inoculum evenly. After one week incubation at 23ºC, the seeds were air-dried at room temperature for 3 days, and ground to a coarse powder. The inoculum powder (40g) was incorporated into the top layer of soil in each 2000 cm^3 planting bag, before soybean seeds were planted.

Trial layout. Four soybean cultivars were used, A5409 R (GR), PAN 535R (GR), PAN 660 and PAN 1652. Cultivars were not of isogenic origin, as isogenic soybean lines could not be obtained. In the first experiment five seeds from each cultivar, and treated with commercial *Bradyrhizobium japonicum*, were planted in each soil type. After germination when plants were approximately 10 cm in height they were thinned to three plants per bag. The experiment was a completely randomized design with four replicates per treatment and repeated twice. No glyphosate was applied to plants in this experiment. The objective was to compare GR and non-tolerant cultivars to determine if the GR gene had an effect on disease development. A second similar experiment entailed treating glyphosate resistant cultivars with glyphosate to determine the effect of glyphosate on disease, caused by *S. slerotiourum,* incidence and severity. Control treatments were not treated with glyphosate. Plants were maintained in the glasshouse at 24°C day temperature and 19°C night temperature. Plants were watered

daily using filtered municipal water to eliminate possible chlorine pollution. Plants were fertilized weekly using Multifeed® Classic (19: 8: 16; N: P: K).

In the second experiment only the two GR cultivars were planted as described above. The cultivars were planted in two sets per soil type. One set was treated with glyphosate, applied as Roundup®, at recommended field application rate at growth stage V4 (Lee, Penner & Hammerschmidt, 2003; Zobiole *et al.*, 2010), while the other set received no Roundup® treatment.

Assessment. Plants were sampled 56 days after planting, at the R1 reproductive stage (beginning of bloom) which was 32 days after glyphosate application. Soil was removed from roots by gently washing them with fresh tap water to ensure minimum damage to the root system. Cleaned roots were ground to a fine powder with liquid nitrogen after which the whole root sample was weighed. A rating scale (Table 6.3) as described by Reid *et al* (2002) with modifications was used to rate disease severity on leaves and roots. After plants were removed from soil, a series of soil dilution plates were made on PDA in order to re-isolate *S. sclerotiorum*. Isolations were also made from roots and plated onto PDA in 90 mm Petri-dishes that were incubated at 24 °C in a Labcon growth chamber.

Ergosterol assessment. Ergosterol was extracted from soybean roots as described by Jambunathan, Kherdekar & Vaidya (1991), with modifications, to evaluate fungal infection of the roots. Roots were ground to a fine powder in liquid nitrogen. The total amount of powder available for each sample was determined and added to 50 ml of methanol in a 50 ml capped centrifuge tube and shaken on a Multi Reax shaker for

40 min at 1600 rpm. After the mixture was allowed to settle, 25 ml of the supernatant was added to 3 g of KOH in a clean 50 ml capped centrifuge tube. The mixture was again shaken for 5 min at 1600 rpm or until the KOH was dissolved. Ten ml of hexane was then added and the mixture was incubated at 75 °C for 30 min in a water bath. After incubation, the mixture was allowed to cool down to room temperature after which the hexane layer which accumulated at the top of the mixture was transferred to a clean 50 ml capped centrifuge tube. Ten millilitres of hexane was added and mixed well with the mixture left in the original centrifuge tube which was incubated in the water bath. The hexane layer was again removed and added to the previously removed aliquot. The last two steps were repeated. The hexane extract was then evaporated in a hot water bath connected to an air pump to speed up the evaporation process. The residue left after evaporation was dissolved in 5 ml methanol and filtered through a 0.45 µm millipore filter The filtrate was analyzed at an emission wavelength of 282 nm using a Perkin Elmer high performance liquid chromatograph (HPLC) fitted with a Phenomenex C18.5 µm 4.6 x 150 mm analytical column.

Statistical analysis. Ergosterol concentrations were calculated and data from both trials were pooled and subjected to analysis of variance (ANOVA) and, when F values were significant ($P<0.05$), means were separated using Fisher's protected least significant difference (LSD) test to determine significance of treatment. Data were analysed with NCSS (Hintze, 2001), and a GLM-ANOVA was conducted on data from assessments made. Means were compared using Fisher's LSD.

RESULTS

Soil analysis. Chemical and textural characteristics indicated clear differences between HCC and LCC soils. The HCC soil consistently had higher K, Fe, Cu, N and C levels than the LCC soil, while LCC soil consistently had higher levels of Ca, Mg, Na, Zn and P than the HCC soil. High clay content soil had a lower pH and higher organic matter content than LCC soil (Tables 7.1 & 7.2).

Experiment 1. *S. sclerotiorum* was successfully re-isolated from contaminated soils, but not from soybean roots. Thus, no visual differences, between cultivars, in terms of disease symptoms caused by *S. sclerotiorum* were observed on leaves and roots. Symptoms varied between no visual symptoms to a few leaves showing signs of yellowing, irrespective of cultivar inoculum or soil type.

There was a significant difference in root dry biomass ($P<0.05$) between cultivars (Table 7.3). For cultivars, A 5409R, PAN 535R and PAN 660, mean dry root mass was less for plants cultivated in the HCC soil than those cultivated in the LCC soil (Figure 7.1). However, for PAN 1652, mean root dry mass was less in the LCC soil than in HCC soil.

Root ergosterol concentrations differed significantly in terms of the soil it was cultivated in ($P<0.05$) (Table 7.5). Roots of plants grown in the LCC soil had significantly lower ergosterol concentrations than those grown in HCC soil (Figure 7.3). There was, however, no significant ($P<0.05$) difference in terms of ergosterol concentration between GR and non-GR soybean cultivars.

Experiment 2. *S. sclerotiorum* was successfully re-isolated from contaminated soils, but not from soybean roots. Plants treated with glyphosate showed symptoms of dead leaves due to herbicide damage, while those not treated displayed no symtoms. No visual differences were observed on roots in terms of appearance. There was a significant ($P<0.05$) interaction between glyphosate and cultivar in terms of dry root mass (Table 7.4). In all treatments, glyphosate application resulted in a significant ($P<0.05$) decrease in dry root mass compared to no application (Figure 7.2). The interaction between soil characteristics and glyphosate treatment was significant ($P<0.05$) in terms of the ergosterol concentration measured in roots (Table 7.6). A significantly lower ergosterol concentration was observed for the same cultivar and same soil type when comparing glyphosate vs no glyphosate treatments (Figure 7.4).

DISCUSSION

Herbicide application has often been cited as an example of a management practice that effect soilborne plant pathogens and disease development in various cropping systems. It is possible that the activity of herbicides could extend beyond their target organisms and inhibit both spore germination and mycelial growth. They can also reduce the level of phytoalexin production, and interfere with other physiological processes in plants (Sanyal & Shrestha, 2008). In certain instances, glyphosate application can lead to an increase in fungal biomass, and in others, it has an inhibitory or fungicidal effect (Feng *et al.,* 2005).

No effect of glyphosate on *S. sclerotiorum per se* was observed, in both experiments, due to the lack of infection of the sunflower *S. sclerotiorum* on GR and non-GR soybean, irrespective of treatment with glyphosate. This is consistent with previous findings that glyphosate does not increase Sclerotinia stem rot (Lee, Penner & Hammerschmidt, 2000; Lee *et al.,* 2003). However, this phenomenon could also be due to a too early sampling date or avirulence of the sunflower *S. Sclerotiorum* isolate on soybeans. *S. sclerotiorum* symptoms mainly start to appear after flowering, while sampling in the present study took place during the early flowering stage.

All four soybean cultivars used in experiment 1 (and the two GR cultivars used in experiment 2) are well adapted to different environmental conditions, including different soil characteristics (Jarvie, 2012)[2]. In experiment 1, the differing soil characteristics between the HCC and LCC soil did not have a significant ($P<0.05$) effect on root dry mass. These results are consistent with Tesfamariam *et al* (2009) where biomass production of sunflower seedlings was not influenced by the two contrasting soils used in their study, i.e. acidic Arenosol and calcareous loess subsoil. Ergosterol concentrations, however, clearly indicated significant differences ($P<0.05$) between HCC and LCC soil. This clearly indicates that soil characteristics govern which fungal populations will be associated with soybean roots.

In experiment 2, glyphosate had a significant ($P<0.05$) effect on ergosterol concentration in roots and was lower in treated than non-treated plants (Figure 7.4). This is consistent with the inhibitory/ fungicidal effect of glyphosate on *F. solani* f.sp.

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[2] Personel communication, Dr Anthony Jarvie, Pannar SEED (Pty) Ltd.

glycine in vitro (Sanogo *et al.,* 2000) and *Phakopsora pachyrhizi* Syd & P Syd, which causes Asian soybean rust in GR soybeans (Feng *et al.*, 2008). The fungicidal effect of glypohosate *in vitro* was also observed in a previous study (see chapter 2).

Glyphosate application also decreased root biomass of soybean plants. Glyphosate has been shown to affect GR soybean growth and yield (Zablotowicz & Reddy, 2004), or cause injury to GR soybean (Reddy & Zablotowicz, 2003; Reddy, Rimando & Duke, 2004). These results therefore suggest that if glyphosate persists and accumulates in the soil, root growth could be significantly reduced leading to impaired nutrient uptake by crop plants (Huber, 2010), and thus a decrease in yields.

Glyphosate applied at recommended field rate, to GR soybeans, decreased dry root mass significantly ($P<0.05$) compared to the same GR cultivar not treated with glyphosate. This trend occurred in both HCC and LCC soils. Our results are consistent with those of other workers where glyphosate also decreased dry root biomass (Reddy, Hoagland & Zablotowicz, 2000; Sanogo *et al.*, 2000; Bott *et al.*, 2008; Zobiole *et al.*, 2010). This phenomenon can possibly be explained by the toxic and deleterious effect glyphosate has on the bacterial symbiont, *Bradyrhizobium japonicum*, and consequently inhibition of the nitrogen fixation process (Zablotowicz & Reddy, 2004; Duke & Cerdeira, 2007; Zablotowicz & Reddy, 2007). The symbiont pocesses a glyphosate-sensitive enzyme, EPSP synthase, glyphosate thus inhibits or kills the microbe. Furthermore, glyphosate is preferentially translocated to soybean nodules where it accumulates and immobilizes Ni required for ureide synthesis (Zobiole *et al.,* 2010). Applying glyphosate to GR soybeans can thus reduce nodulation, nodule size, and leghemoglobin content of nodules (Duke & Cerdeira, 2007).

The effect of herbicides on plant pathogens and disease development is controversial (Sanyal & Shrestha, 2008). Herbicides have the ability to alter physiological processes in crop plants, which in turn can change their susceptibility to certain plant pathogens (Johal & Huber, 2009). Contrasting results in the literature however suggest that the interactions between disease severity and herbicides are specific to each crop-herbicide combination. It is obvious that various other factors, including crop variety, pathogen species, environmental conditions, and soil characteristics could also influence these interactions. Further research to elucidate the precise nature of these interactions is therefore urgently required. Future research should also include root and rhizosphere soil sampling after the flowering stages of soybean, as *Sclerotinia* symptoms on soybean mainly become visual then. Yield should also be taken into account to determine whether soil characteristics and glyphosate application combined has a significant effect on yield production, as this will be an important consideration to be taken into account by producers.

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Table 7.1. Chemical characteristics for high clay content and low clay content soil.

Soil type	pH	Ca (mg/kg)	K (mg/kg)	Mg (mg/kg)	Na (mg/kg)	Zn (mg/kg)	Fe (mg/kg)	Cu (mg/kg)	P (mg/kg)	N (mg/kg)	C (mg/kg)
Bainsvlei (LCC)	6.02	474	120	76	44	1.78	16.36	1	19.76	359.2	1390
Tukulu (HCC)	4.98	334	168	66	28	1.44	110.6	1.44	15.03	492.3	2600

Table 7.2. Textural properties for high clay content and low clay content soil.

Soil type	Organic matter (OM) %	Slit %			Sand %		
		Coarse	Fine	Clay	Coarse	Medium	Fine
Bainsvlei (LCC)	0.232	3.33	3.33	3.33	0.67	8	81.17
Tukulu (HCC)	0.399	1.670	4.000	9.330	2.070	6.470	73.770

Table 7.3. ANOVA table for dry root biomass for all four cultivars without glyphosate treatment.

Term	DF	Sum of Squares	Mean Square	F-ratio	Probability level	Power (Alpha = 0.05)
A: Soil	1	1.888128	1.888128	0.30	0.587446	0.075291
B: Cultivars	3	79.41402	26.47134	4.19	0.011340*	0.718066
AB	3	36.23558	12.07853	1.91	0.142852	0.372657
C: Replicates	7	52.89391	7.556273	1.20	0.326589	0.389922
S	40	252.4536	6.311341			
Total (Adjusted)	54	447.2435				
Total	55					

*Term significant at alpha = 0.05

Table 7.4. ANOVA table for dry root biomass for the two glyphosate resistant cultivars with and without glyphosate treatment.

Term	DF	Sum of Squares	Mean Square	F-ratio	Probability level	Power (Alpha = 0.05)
A: Soil	1	10.03807	10.03807	2.33	0.134339	0.260813
B:Glyphosate treatment	1	65.57513	65.57513	15.20	0.000327*	0.920186
AB	1	4.054603	4.054603	0.94	0.337641	0.133388
C: Cultivars	1	31.12764	31.12764	7.21	0.010162*	0.640484
AC	1	3.126121	3.126121	0.72	0.399263	0.113869
BC	1	20.18689	20.18689	4.68	0.036011*	0.463509
ABC	1	0.5288968	0.5288968	0.12	0.727919	0.060544
D: Replicates	7	30.58986	4.36998	1.01	0.435540	0.337762
S	44	189.84	4.314546			
Total (Adjusted)	58	377.2492				
Total	59					

*Term significant at alpha = 0.05

Table 7.5. ANOVA table for ergosterol concentrations for all four cultivars without glyphosate treatment.

Term	DF	Sum of Squares	Mean Square	F-ratio	Probability level	Power (Alpha = 0.05)
A: Soil	1	98632.49	98632.49	19.97	0.000263*	0.988492
B: Cultivars	3	31605.51	10535.17	2.13	0.129744	0.458404
AB	3	7696.716	2565.572	0.52	0.674000	0.136148
C: Replicates	7	42916.82	6130.975	1.24	0.329592	0.391730
ABC	21	132351.3	6302.443	1.28	0.298668	0.570624
S	19	93848.06	4939.372			
Total (Adjusted)	54	485171.2				
Total	55					

*Term significant at alpha = 0.05

Table 7.6. ANOVA table for ergosterol concentrations for the two glyphosate resistant cultivars with and without glyphosate treatment.

| Term	DF	Sum of Squares	Mean Square	F-ratio	Probability level	Power (Alpha = 0.05)
A: Soil	1	62840.48	62840.48	15.45	0.000357*	0.969048
B:Glyphosate treatment	1	19016.08	19016.08	4.68	0.037126*	0.558098
AB	1	19189.73	19189.73	4.72	0.036320*	0.561879
C: Cultivars	1	7809.978	7809.978	1.92	0.174110	0.271384
AC	1	5296.712	5296.712	1.30	0.261107	0.199226
BC	1	2481.183	2481.183	0.61	0.439721	0.118520
ABC	1	994.2414	994.2414	0.24	0.623917	0.076982
D: Replicates	7	24491.16	3498.738	0.86	0.546238	0.316703
ABCD	7	25352.43	3621.775	0.89	0.523735	0.327872
S	37	150474.2	4066.87			
Total (Adjusted)	58	334636				
Total	59					

*Term significant at alpha = 0.05
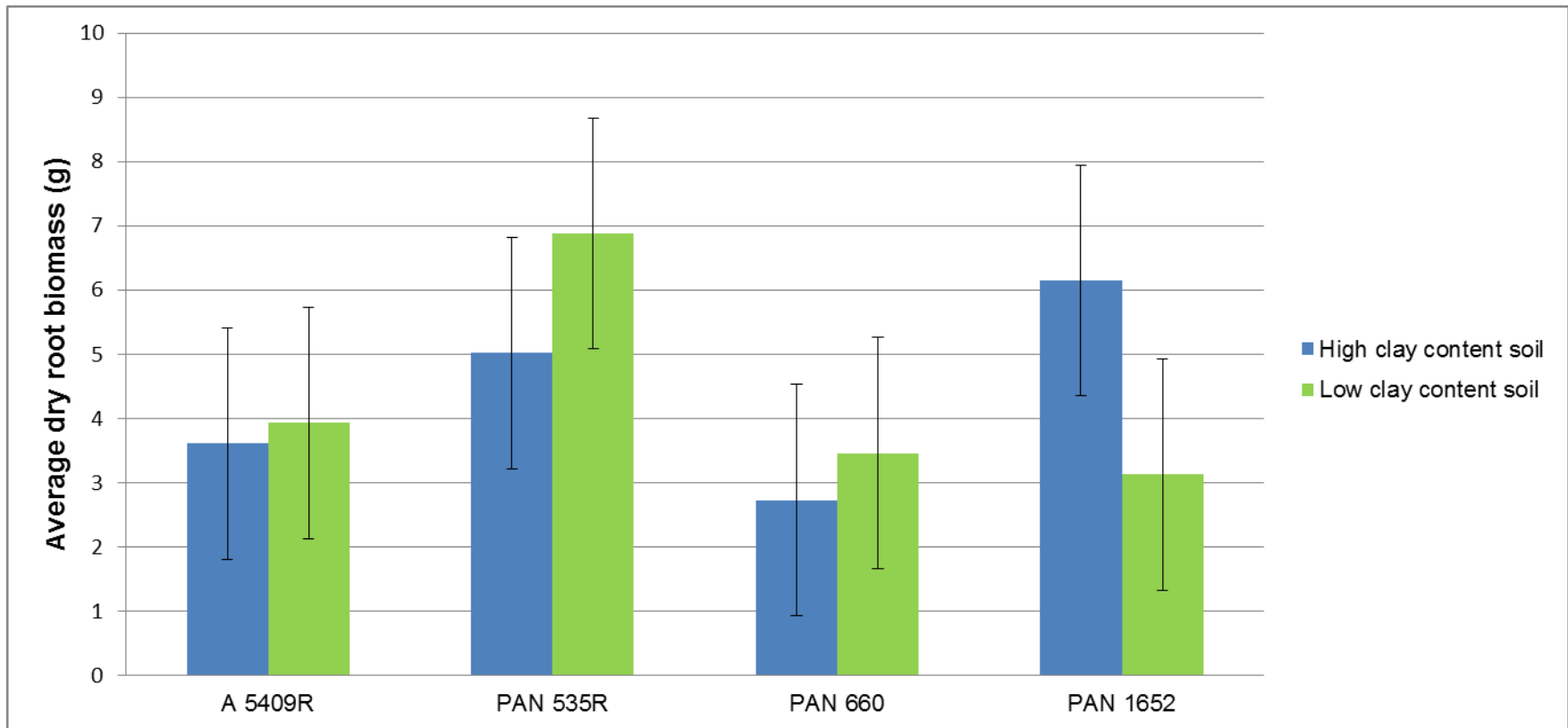
Figure 7.1. The effect of the GR gene on dry root biomass in two different soil types. (A 5409R and PAN 535R imparts the GR gene while PAN 660 and PAN 1652 are non-GR).
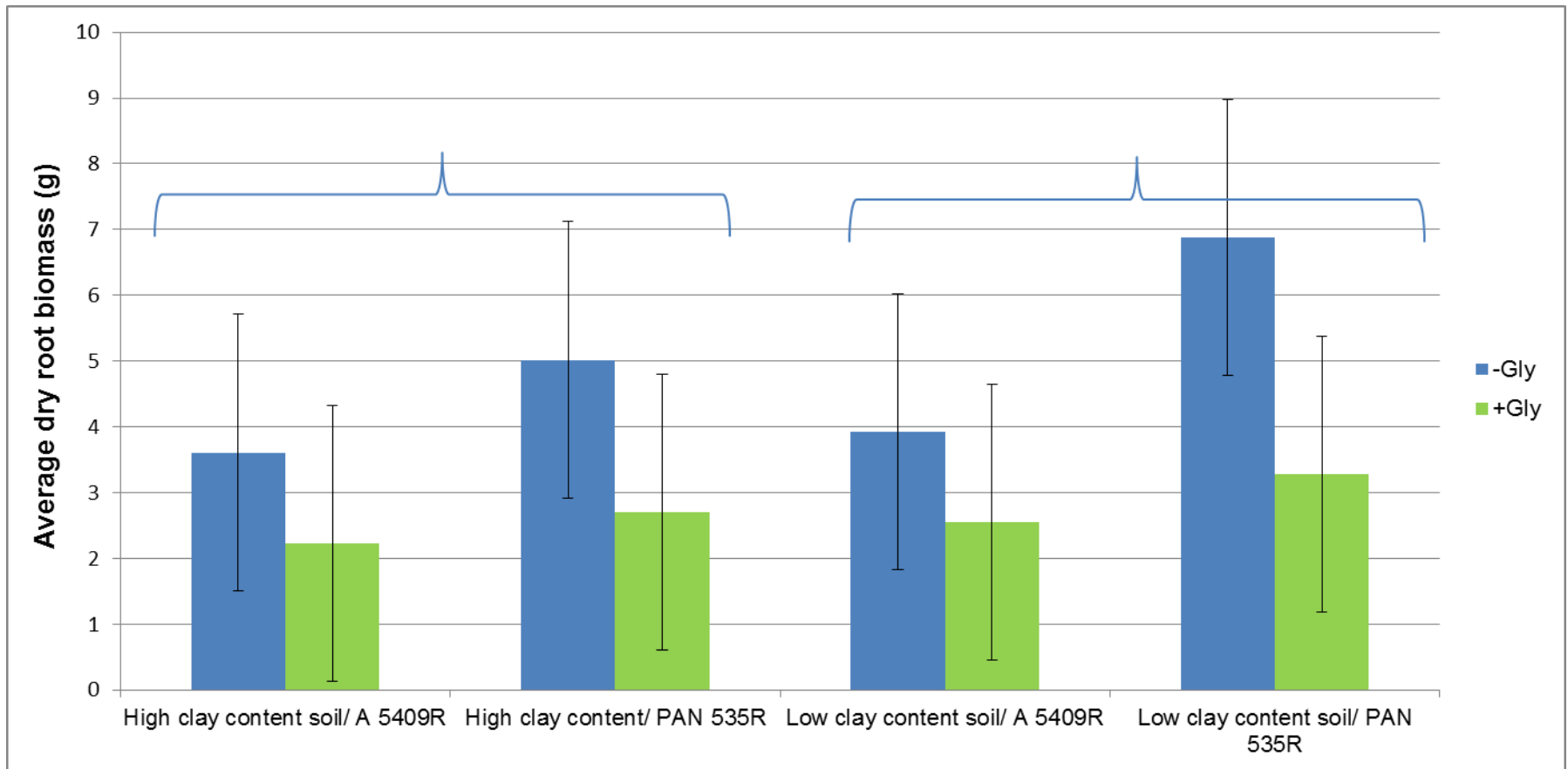
Fisher's LSD (P<0.05): Cultivar = 1.80

Figure 7.2. The effect of the glyphosate on GR maize dry root biomass in two different soil types. (A 5409R and PAN 535R imparts the GR gene while PAN 660 and PAN 1652 are non-GR).

Fisher's LSD (P<0.05): Cultivar = 1.05 (illustrated above); Glyphosate treatment = 1.05; Cultivar x Glyphosate treatment = 2.098
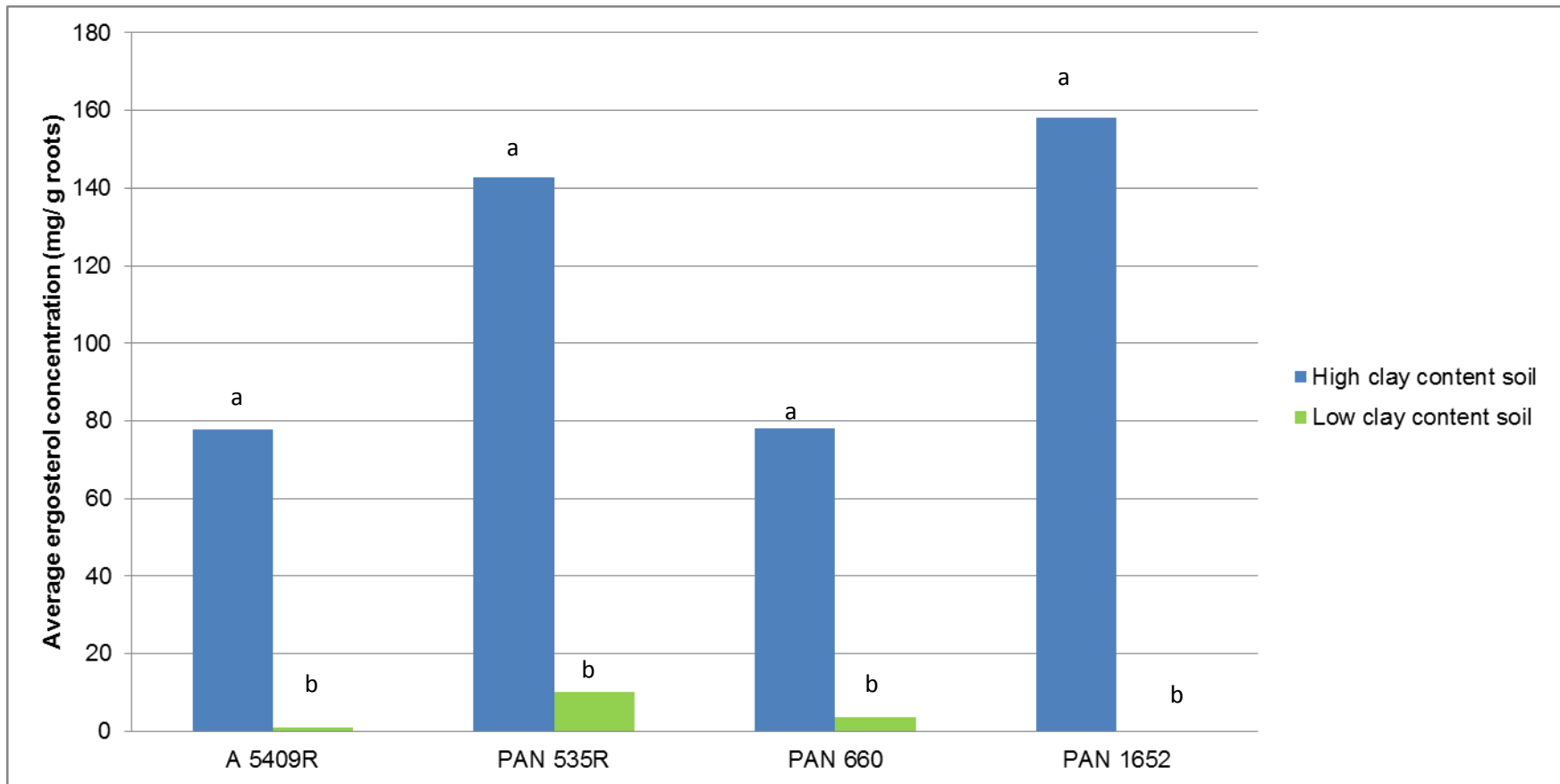
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Figure 7.3. The effect of the GR gene on ergosterol concentration in two different soil types. (A 5409R and PAN 535R imparts the GR gene while PAN 660 and PAN 1652 are non-GR).
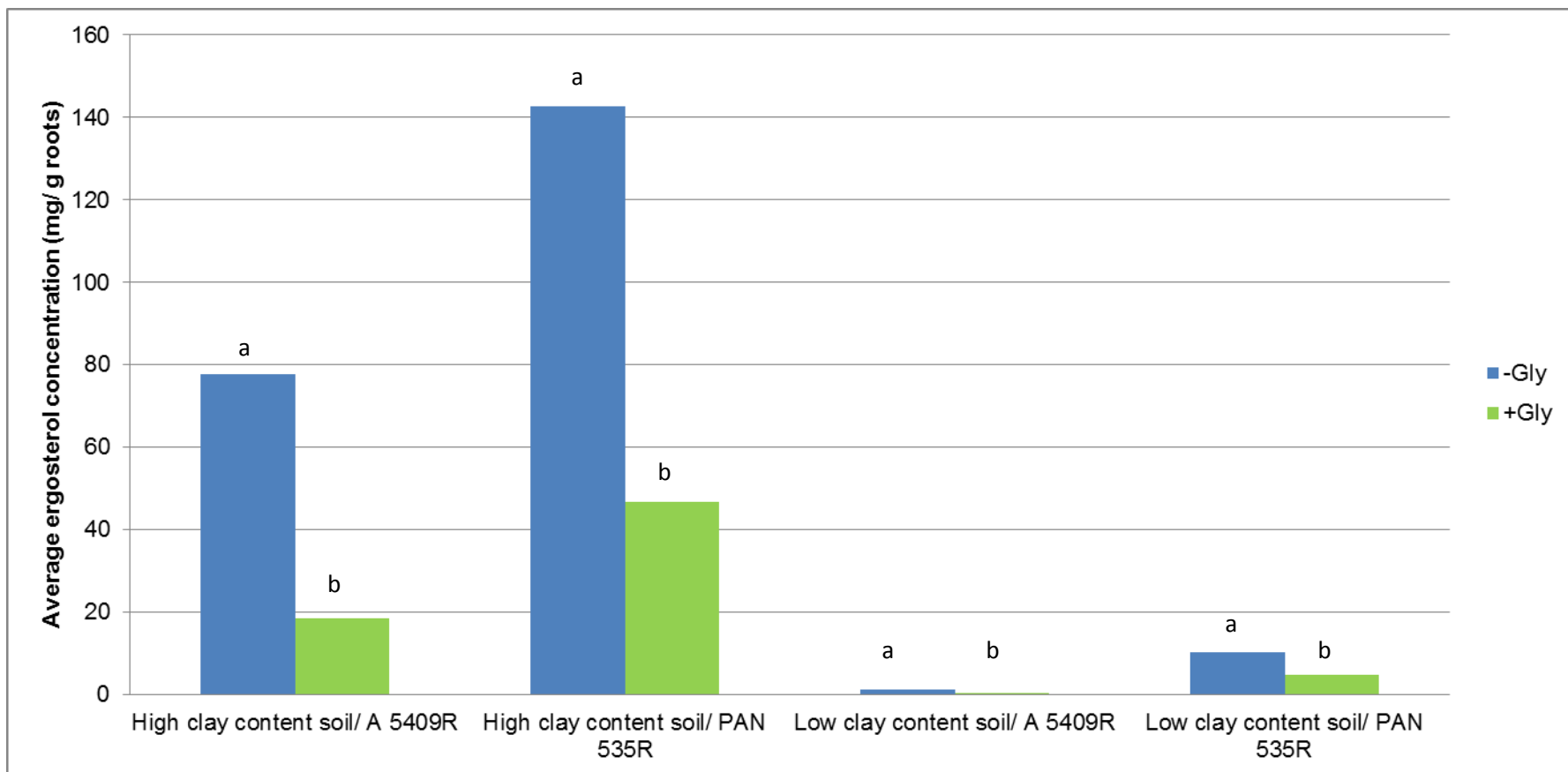
Fisher's LSD (P<0.05): Soil = 36.774

Figure 7.4. The effect of the glyphosate on ergosterol concentration in two different soil types. (A 5409R and PAN 535R imparts the GR gene while PAN 660 and PAN 1652 are non-GR).

Fisher's LSD (P<0.05): Glyphosate treatment = 32.396 (illustrated above); Soil = 32.396; Glyphosate treatment x Soil = 64.79

OPSOMMING

Tydens die studie is gevind dat onkruiddoders, teen aanbevole dosisse aangewend, 'n inhiberende effek op fungi *in vitro* het. Nadat die effek van onkruiddoders op grond mikrobiese en bakterie aktiwiteit in eksperimentele mikrokosms getoets is, is egter gevind dat grond met hoë organiese materiaal inhoud 'n buffer effek op die grondmikrobes het.

Mikrobiese, sowel as bakteriese aktiwiteit in die ryk grond mikrokosms het toegeneem na glifosaat behandeling, wat 'n aanduiding is dat mikrobes die onkruiddoder as bron van koolstof en voeding gebruik het.

Geen noemenswaardige verskille in terme van rhizosfeer bakteriese aktiwiteit tussen glifosaat weerstandbiedende (GW) mielie en sojaboonkultivars in vergelyking met konvensionele kultivars is opgelet nie. Klei- en organiese materiaal konsentrasie in die grond het wel 'n noemenswaardige effek gehad. Grond met hoër klei- en organiese materiaal inhoud het hoër bakteriese aktiwiteit getoon. Hoër ergosterolvlakke is ook geïsoleer uit grond met hoë klei- en organise materiaal inhoud as uit grond met 'n lae klei- en organise materiaal inhoud.

Die droë wortelmassa van GW mielie- en sojaboonkultivars het nie noemenswaardig verskil van konvensionele kultivars nie. Wanneer glifosaat toegedien is, het GW kultivars se droë wortelmassa noemenswaardig meer afgeneem as met GW kultivars wat geen glifosaatbehandeling ontvang het nie. Dieselfde patroon is waargeneem in terme van ergosterol konsentrasie, wat duidelik getoon het dat glifosaat 'n fungisiediese effek op wortelswamme gehad het.

SUMMARY

During the present study it was found that herbicides, applied at the recommended dosage, has an inhibitory effect on fungi *in vitro*. However, when the effect of herbicides on soil microbial and bacteria activity was tested in experimental microcosms, it was found that soil with a high organic matter content had a buffer effect on soil microbes.

Microbial activity increased after glyphosate treatment in rich soil microcosms, indicating tha soil microbes utilized glyphosate as a source of carbon and nutrients or that the toxicity of glyphosate to specific soil microflora and microfauna increased the available nutrient base for increased activity of resistant organisms.

No significant differences were observed in terms of rhizosphere bacterial activity between glyphosate resistant (GR) maize and soybean in comparison to their conventional counterparts. Clay- and organic matter concentration in soil did however have a significant influence. Soil with a high clay- and organic matter content had higher microbial activity than soil with low clay- and organic matter content. Higher ergosterol levels were also isolated from high clay- and organic matter soil than low clay- and organic matter soil.

Dry root biomass of GR maize and soybean kultivars did not differ significantly from their conventional counterparts. However, when glyphosate was applied to GR maize and soybean, dry root biomass decreased significantly compared to GR maize and soybean which received no glyphosate treatment. The same pattern was observed

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in terms of ergosterol concentrations, thus indicating that glyphosate had a physiological

effect on the respective crop roots.