THE INFLUENCE OF AGRICULTURAL SOIL AMENDMENTS ON THE DIVERSITY OF SOIL MICRO-ORGANISMS AND DISEASE INCIDENCE

by

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Table of contents

CHAPTER TWO
AN *IN VITRO* MICROCOSM STUDY ON THE EFFECT OF AGRICULTURAL AMENDMENTS ON SOIL MICROBES

CHAPTER THREE
EFFECT OF AGRICULTURAL MANAGEMENT PRACTICES AND HERBICIDE APPLICATION ON SOIL MICROBIAL PROPERTIES IN THE FIELD

CHAPTER FOUR
THE EFFECT OF SOIL AMENDMENTS ON DISEASE SUPPRESSIVENESS TO THE PATHOGEN, *FUSARIUM OXSPORUM*

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PREFACE

The productivity of crops is enhanced by the application of various soil amendments and cultural practices which, despite influencing yield, can also influence soil microorganisms, both positively and negatively. Overlooking the negative consequences of these effects can lead to a reduction in crop production due to sub-optimal soils and disease. The present study investigated the effects of soil amendments and certain cultural practices on the functional diversity and the community structure of soil microbes and their possible influence on disease severity. This study was conducted over a period of three years in soil microcosms and in the field. The dissertation consists of four chapters, each chapter is an independent unit and redundancy between the chapters is therefore unavoidable.

Chapter one is a literature review covering the importance of microorganisms on the health and quality of soil. The dynamics of microorganisms and the roles they play in soil nutrient cycling and other positive effects towards crop plants are reviewed. Recorded effects of agricultural amendments and cultural practices on the functional diversity and community structure of microorganisms and possible outcomes due to these changes are also discussed.

Chapter two utilises soil microcosms to investigate the effects of fourteen amendments comprising inorganic fertilizers, organic manure composts, herbicides, fungicides, cover crops and a liming agent on soil microbes. Changes in microbial populations is analysed both qualitatively and quantitatively using various biochemical and molecular techniques.

Chapter three investigates changes in microbial properties of field soils subjected to compaction, different cropping systems and herbicide (Roundup) application. Microbial activity and diversity are assessed by the same qualitative and quantitative biochemical and molecular techniques used in chapter two.

Chapter four investigates the effects soil microbial diversity on the pathogenicity of *Fusarium oxysporum* on sorghum seedlings in the glasshouse. Microcosm soils generated in the trials discussed in chapter two were used and inoculum made from two isolates of *F. oxysporum* was introduced into each amended/treated soil. Differences in the germination and vegetative growth of sorghum seedlings were assessed and compared to a control treatment comprising vermiculite.

Abbreviations

Wk(s) – Week(s)

FDA – Flourescein diacetate

PCR – Polymerase chain reaction

DGGE – Denaturing gradient gel electrophoresis

AWCD – Average well colour development

ANOVA – Analysis of variance

CLPP – Community linked physiological profiles

PCA – Principle component analysis

2,4-D – 2,4-dicholorophenoxyacetic acid

LAN – Lime ammonium nitrate

SP – super phosphate

CHAPTER ONE

THE INFLUENCE OF AGRICULTURAL PRACTICES ON THE MICROBIAL DIVERSITY OF SOIL IN CROPPING SYSTEMS WITH SPECIAL REFERENCE TO SOILBORNE PATHOGENS

1.0 Introduction

The discovery of microorganisms within soil and their importance in recycling organic matter dates back to the 17th century when John Evelyn stated that soil fertility could be maintained by the addition of organic materials (Magdoff and Vanes, 2000). By the 20th century, large farming devices and chemicals for improving the production of crops on a large scale had been developed and the utilization of these resources resulted in drastic increases in land areas under cultivation (Magdoff and Vanes, 2000). Although agricultural chemicals and machinery increased crop productivity exponentially, the intensive application of these agricultural management tools has also had many negative effects on agricultural soils (Doran and Zeiss, 2000).

Agricultural practices have resulted in considerable capital degradation in terms of: erosion of the top soil; increased nitrogen concentration within soil; and the build-up of various chemicals in the soil and groundwater (Doran and Zeiss, 2000). Agricultural practices negatively affect many soil organisms that are known to play key roles in the maintenance of soil fertility (Doran and Zeiss, 2000; Bin-Ru *et al.*, 2005; Zeng, Yao and Yu, 2006; Brussaard, de Ruiter and Brown, 2007). A reduction in soil quality on farms is therefore the greatest challenge facing food security worldwide in this century (Doran, 2002; Diosma *et al.*, 2005).

Soil quality was described by the Soil Science Society of America (1997) as, "the capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation" (Doran and Zeiss, 2000).

Another similar term i.e. "soil health" is defined as the, "continued capacity of soil to function as a vital living system, within ecosystem and land use boundaries, to sustain biological productivity, promote the quality of air and water, and maintain plant, animal and

human health" (Avidano *et al.*, 2005). These are the most referenced definitions of soil quality/soil health. The Natural Resources Conservation Services of the United States Department of Agriculture, however, summarized both these definitions as: "soil quality is how well soil does what we want it to do" (Janvier *et al*., 2006).

Soil quality has been said to be related to the direct influence of soil on the productivity of crop plants (Doran and Zeiss, 2000). Recently, however, it is seen as the capacity of soil to sustain biological productivity and environmental quality (Bell and Raczkowski, 2007; Brussaard *et al.*, 2007). High quality soil depends on a number of combined factors that enable it to enhance both crop yield and quality (Magdoff and Vanes, 2000). These factors include, *inter alia*, the amount and type of soil nutrients; the drainage status of the soil; the incidence of soilborne pathogens; the microbial content of soil; the chemical composition of soil; and the recovering ability of the soil after compaction or flooding.

This review will briefly outline the nature of the various soil biota and their important role in maintaining soil fertility and determining crop yield and productivity. Specific attention will be given to the effect that different agricultural management practices have on soil dynamics in terms of their positive and negative impact on temporal and spatial interactions among soil biota and their abiotic environment.

2.0 Dynamics of soil organisms

Sustainability of an agroecosystem can be achieved by enhancing the functional biodiversity within it (Altieri, 1999). Soil organisms serve as key components required for the functioning of soil and the maintenance of soil quality. Soil organisms are broadly differentiated into macro-, meso- and microfauna, all of which are important in maintaining soil quality. The contributions of soil organisms towards soil health can be divided into several functional roles. These roles include several biogeochemical cycles, nutrient cycling,

organic matter decomposition and the detoxification of pollutants (Kirk *et al.* 2004; Acosta-Martinez *et al.*, 2007; Shishido *et al.*, 2008).

2.1 Macro-, meso- and microfauna

Meso- and macro-fauna play important roles in maintaining processes directly or indirectly related to nutrient cycling in the soil. They play a direct role in the decomposition of organic matter while they indirectly regulate various microorganisms (Shishido *et al.*, 2008). Micropredators such as mites help to regulate the microorganism community structure in soil by preying on fungi (Giller *et al.*, 1996). Earthworms, mites and springtails directly influence the abundance and diversity of fungi and bacteria in the soil (Cole *et al.*, 2005). Earthworms in turn affect nematode and protozoa populations which indirectly affects bacteria-mediated nutrient cycling in soil (Tao *et al.*, 2008). Earthworms are also one of the most important organisms responsible for increasing the nutrient composition in soil by degrading organic matter and transforming soil nitrogen and carbon (Fragoso et al., 1996; Tao *et al.*, 2008).

A major group of microfauna are the protozoans which play essential roles in soil ecology by feeding on fungi, bacteria and smaller protozoa. They also serve as a food source for organisms belonging to the meso- and macrofauna (Griffiths *et al.*, 2005). The abundance of protozoan communities in soil directly affects the biomass and the diversity of bacterial communities (Clarholm, 2005), which in turn affects their ability to mineralize carbon and nitrogen in soil (Foissner, 1999). The predation of protozoans on bacteria and other microorganisms reduces nutrient competition and improves the nitrogen uptake ability of plants thereby improving plant growth (Bonkowski, 2001). Nitrogen produced from predation on bacteria is immediately ready for plant uptake and may lead to an (30-80%) increase in plant production in a protozoan rich agroecosystem (Bonkowski and Schaefer,

1996; Bonkowski, 2001). Protozoan grazing on bacteria may also cause an increase of nitrifying bacteria in soil, which are responsible for increases in root biomass of plant seedlings (Bonkowski, 2001). Protozoa also have the ability to enhance the production of plant hormones in the rhizosphere which mineralize nutrients (Forge *et al.*, 2002). Bonkowski and Brandt (2002) showed that the addition of amoebal culture fluid to pea seedlings increases seedling biomass due to hormonal effects.

2.2 Microbes

Soil fungi and bacteria are vitally important for nutrient cycling in soil, but may either cause plant diseases or alternatively, protecting plants against pathogens (Liu, Glenn and Buckley, 2008). An important benefit of microbial diversity in soil is that it provides crops with increased resistance to environmental stress and other external abiotic disturbances (Bucher and Lanyon, 2004; Bin-Ru *et al.*, 2005; Brussaard *et al.*, 2007). Microorganisms in soil are often used as bio-indicators of soil quality since they are extremely sensitive to agricultural management practices and are thus widely used in assessing soil health (Bossio *et al.*, 2005; Epelde *et al.*, 2008). Monitoring of bacterial and fungal populations or communities in soil provides a more reliable assay than single parameter analyses such as enzymatic or chemical activity (Avidano *et al.*, 2005).

2.2.1 Bacteria

Soil bacteria are the most metabolic significant group of microorganisms in soil. They are the primary decomposers of organic material and supply the nutrients necessary to enhance plant growth (Bin-Ru *et al.*, 2005). They are also responsible for the transformation of NO_3 and NO_2, which are unavailable to plants, into nitrogen which plants can absorb (Gunapala and Scow, 1997). They are also responsible for more specific transformations of

nutrients in the soil: the mineralization and solubilization of phosphorus, the oxidation, the reduction and the precipitation of iron and the transformation of inorganic sulphur within soil (Beare *et al.*, 1996). Bacteria may also benefit plants directly by forming symbiotic relationships with the roots of plants, e.g. the relationship between nitrogen-fixing bacteria and legumes (Kahindi *et al.*, 1996).

Bacteria are found to be most abundant just below the soil surface, since nutrients and plant compounds are most abundant in the rhizosphere (Xu *et al.*, 2008). Their numbers increase rapidly below this layer of soil to approximately 50 mm deep and then slowly decline as organic carbon decreases. Rhizosphere bacteria provide numerous benefits in terms of buffering plants against pathogen attacks, the solubilization of inorganic nutrients and the production of metabolites promoting plant growth (Höflich, Wiehe and Hecht-Buchholz, 1995; Kozdrój, Trevors and van Elsas, 2004). They also produce various toxic compounds which can control the activity of plant pathogens. For example, resveratrol has been shown to successfully suppress the appearance of grey mould on grapevines (Paul *et al.*, 1998). The phytotoxic substances produced by some soil bacteria can be also used to suppress plant growth and can therefore be used to control weeds (Harris and Stahlman, 1995). The ability of bacteria to reproduce exponentially can also be used to suppress plant pathogens by competing with them for nutrient sources and living space (Kobayashi, Guglielmoni and Clarke, 1995).

2.2.2 Fungi

Fungi are considered to be dominant amongst soil organisms, not only in terms of processes but also in biomass. The biomass of fungi can exceed that of all other soil organisms combined (Thorn, 1997). Fungi serve as the major decomposers in soil (Beare *et al.*, 1996). Saprophytic fungi, such as *Penicillium spp.* and *Cladosporium spp.* directly affect

the carbon cycling ability and degradation of organic material in soil which increase the amount of nutrients available for plants (Deacon *et al.*, 2005). Many fungi and oomycetes are also very destructive plant pathogens (Thorn, 1997; Maor and Shirasu, 2005), and pathogens such as *Fusarium spp.*, *Phytophthora spp.*, *Pythium spp.* and *Rhizoctonia solani* J.G. Kühn, *Verticillium spp.* can lead to severe crop losses if not controlled (Hamel *et al.*, 2005; Chandanie, Kubota, and Hyakumachi, 2008; García *et al.*, 2010).

Other major roles of soil fungi include their involvement in beneficial and detrimental symbioses with plant roots. Mycorrhizal relationships between fungi and roots directly aid nutrient absorption and may also protect plants against pathogenic fungi (Mathimaran *et al.*, 2006). Non-pathogenic fungi may have the ability to decrease the incidence and the activity of their pathogenic forms by indirectly serving as biocontrol agents, if they colonize plant roots before the pathogen (Larkin, Hopkins and Martin, 1993). Free soil living fungi such as *Trichoderma spp.* can form a symbiotic relationship with tomato plants, which suppresses the pathogen, *Sclerotium rolfsii* Sacc. (Liu *et al.*, 2008). Some fungi can also parasitize, or serve as predators, on plant-parasitic nematodes and thereby successfully reduce their severity (Siddiqui and Mahmood, 1996). An important role of soil fungi is the connection they provide between virtually all soil-organisms at all trophic levels, which directly affects the functioning of the soil ecosystem (Thorn, 1997).

3.0 The effect of agricultural chemicals on soil organisms

Soil biota are significantly influenced by their abiotic environment. Agricultural practices that include the application of pesticides and inorganic nutrients which affect the physical, biological and chemical properties of the soil (Magdoff and Vanes 2000) can have major ecological implications (Avidano *et al.*, 2005; Eisenhauer *et al.*, 2009). The addition of biological agents may also lead to the inhibition of beneficial microorganisms which can

ultimately lead to a significant reduction in soil fertility by interfering with the ecological balance of the soil (Ros *et al.*, 2006).

3.1 Fertilizers

Inorganic inputs such as nitrogen, phosphorus, boron and potassium play an important role in enhancing the production of crop plants by serving as essential elements required for plant growth and the maintenance of soil fertility (Kong *et al.*, 2008b). Apart from their direct benefits to crops, these chemical amendments also affect microorganisms within the soil. Although inorganic fertilizers may influence the crop production positively, applying too much can result in negative effects on the target crop. Overuse of chemical fertilizers in soil with high fertility can decrease the diversity of microorganism communities within the soil (Zong-pei *et al.*, 2007) which leads to the deterioration of biological and biochemical properties. The application of inorganic fertilizers can also selectively increase microorganism populations in soil. For example, the application of P and N fertilizers can significantly increase microbial biomass and activity in soil (Benizri and Amiaud, 2005; Gu *et al.*, 2009). The application of K and P fertilizers impacts on the community structure of specific microbial groups by increasing bacterial populations which utilize K and P, thus leading to a decrease in other microorganisms due to competition (Thirukkumaran and Parkinson, 2000; Zhang, Wang and Yao, 2006; Zhang *et al.*, 2007). Höflich *et al*. (1999) showed stimulation in the growth of legumes, gramineae and crucifers by the application of high amounts of N fertilizers in both pot trials and long-term field experiments.

The addition of micronutrients such as boron (B) combined with excessive soil salinity can also alter microbial diversity within soil. The application of high amounts of boron to soil significantly decreases the functional diversity and the richness of microbes in the rhizosphere (Nelson and Mele, 2006). Changes in the NaCl content in soil leads to a change

in the moisture content and pH of soil, which in turn can significantly alter microbial diversity (Nelson and Mele, 2006). Other than the direct change in physical status of the soil, NaCl can also alter the permeability of plant roots thus increasing osmotic pressure which will directly affect soil microbial communities (Wichern, Wichern and Joergensen, 2006).

3.2 Fungicides

The application of pesticides, heavy metals and sludge amendments to soil can result in the reduction of soil microbial biomass, diversity and activity (Crecchio *et al.*, 2006). Many studies have demonstrated that microorganism communities in soil are significantly affected by the addition of fungicides (Demanou *et al.*, 2006; Gopal *et al.*, 2006; Niemi *et al.*, 2008; Eisenhauer *et al.*, 2009; Lupwayi *et al.*, 2009; Zhang *et al.*, 2009a). Fungicides affect non-target soil microorganisms such as non-pathogenic fungi and microbes that are beneficial to the productivity of crops (Bjørlund *et al.*, 1999; Chen and Edwards, 2001; Bending, Rodríguez-Cruz and Lincoln, 2007; Niemi *et al.*, 2008). For example, high application rates of captan and chlorothalonil have been shown to cause shifts in soil nitrogen dynamics and destroy microorganism communities leading to a loss of soil fertility (Chen, Edwards and Subler, 2001). Wakelin *et al.* (2007b) showed that application of the fungicide tolclofos-methyl to maize seeds reduces the number of microorganisms competing with *Fusarium oxysporum* Schltdl. and *F. verticillioides* Nirenberg thus increasing the possibility of disease. Ferreira *et al.* (2009) used PCR-DGGE fingerprinting to show that application of the fungicides tebuconazole and mancozeb to crop plants, significantly affected the community structure of soil microorganisms.

3.3 Insecticides

Pesticides which target insects and arthropods that exist within and above soil may also alter non-target soil microorganisms. The application of pesticides such as λ-Cyhalothrin (Lupwayi *et al.*, 2009), azadirachtin (Gopal *et al.*, 2006) and nemacur (Abramovich and Steinberger, 2006) cause changes to bacterial community structures in soil at a functional level that leads to a decline in soil fertility. Applications of organophosphate and chlorinated hydrocarbon based insecticides are found to directly kill non-target organisms in soil (Das and Mukherjee, 1999). This leads to increases of available N and P in soil. Fungi are generally more resistant to insecticides. The increase in N and P following insecticide addition can lead to shifts in fungal diversity due to the increased food source (Das and Mukherjee, 1999; Vig *et al.*, 2008). Biodegradable residues which remain in soil can serve as a food source for certain microorganisms (Robertson *et al.*, 1998; Gómez *et al*., 1999; Cycoń, Wójcik and Piotrowska-Seget, 2009) which favours certain microorganisms that can lead to a change in the microorganism community.

3.4 Herbicides

Herbicides such as 2,4-dicholorophenoxy-acetic acid (2,4-D) can lead to an increase in microorganisms which degrade the herbicide; a phenomenon that can significantly affect microorganism community structure in soil (Chinalia and Killham, 2006; Zhang *et al.*, 2009b). Glyphosate has been reported to either increase or decrease populations of soil microorganisms (Araújo, Monteiro and Abarkeli, 2003; Lupwayi *et al.*, 2008; Mijangos *et al.*, 2009).

Direct toxic effects of herbicides on soil microorganisms have also been observed immediately after application. Glyphosate, 2,4-D and metsulfuron methyl are found to have direct toxic effects on microorganisms (Zabaloy, Garland and Gómez, 2008). Exposure to

low concentrations of herbicide over a long period of time can also influence microbial communities in soil. The *in situ* application of phenoxy acid herbicides over 216 days stresses microorganisms and thus changes their functional diversity (de Lipthay *et al.*, 2002). Herbicide induced effects on soil microorganisms can also result in changes to N and P activity in the soil (Perucci *et al.*, 1999).

4.0 The effect of biotic factors on soil organisms

Larger microbial communities develop in soils that have been treated with composts, mulches, manures, compared to those that have been supplemented with mineral nutrients (Gunapala and Scow, 1997). Different types of cover crops for example soybean, maize, legumes, have a positive effect on enhancing soil microbial functions and enhancing soil microbial biomass (Han *et al*., 2007). Phytotoxic substances produced by certain cover crops can also have inhibitory effects on seed germination and growth of other plants, a phenomenon referred to as allelopathy. For example, extracts from black mustard have shown a reduction in germination and seedling weight of wild oats (Turk and Tawaha, 2002).

4.1 Organic amendments

The amount of organic matter in soil directly influences soil microorganisms by affecting their biological activity (Rotenberg *et al.*, 2007). One of the major factors in agriculturally induced soil degradation is mineralization of organic matter (Crecchio *et al.*, 2006) which influences the physical, chemical and biological properties of the soil. Organic amendments are well known for their ability to improve soil conditions and this serves as a source of carbon and nutrients, favouring microbial diversity (Gomez *et al.* 2005). Organic amendments indirectly stimulate the biogeochemical cycles in soil and increase certain minerals that are essential for the growth of plants (Ros *et al.*, 2006). Organic amendments

also increase organic matter within the soil, change the structure of the soil and alter several physical, chemical and biological properties (Odlare, Pell and Svensson, 2007). They alter the functional diversity of microorganism communities in soil. Microorganisms which utilize applied organic amendments will out-compete other soil biota; this alters not only the microorganism communities in but also soil fertility as a whole (Geisseler *et al.*, 2010).

4.1.1 Composts

Compost is well known for its ability to enhance plant growth, improve soil quality, enhance microorganism activity and suppress plant pathogens (Abawi and Widmer, 2000; Perez-Piqueres *et al.*, 2005; Ros *et al.*, 2006). Significant changes in microorganism community structure have been demonstrated following the application of different kinds of composts (Perez-Piqueres *et al.*, 2005; Carrera *et al.*, 2007; Nayak, Babu and Adhya, 2007), animal manure (Gomez *et al.*, 2005; Lazcano, Gomez-Brandon and Dominguez, 2008; Takeda *et al.*, 2009) and other organic amendments (Renella *et al.*, 2007; Rotenberg *et al.*, 2007; Tejada *et al.*, 2007). Diosma *et al.* (2005) showed that a substantial increase in organic N in soil leads to an increase in microorganisms which metabolize carboxylic acids. These microorganisms out-compete others due to high N availability thereby altering the functional diversity of other microorganisms and ultimately soil fertility. The long term application of compost in rice paddies significantly increased carbon utilizing soil microorganisms judging by a concomitant increase in enzymatic activity (Nayak *et al.*, 2007). Comparisons between the application of long term organic and inorganic fertilizers showed clear differences in terms of community diversity of the associated microorganisms (Nayak *et al.*, 2007).

4.1.2 Mulches

Organic and inorganic mulches play an important role in the management of several

crops. Mulch consisting of hay and recycled paper waste applied as raw mulch to a high density crop production system was shown to significantly increase the yield of perennial crops (Forge *et al.*, 2002). Mulching suppresses weeds, reduce water loss and increase the availability of nutrients (Forge *et al.*, 2002). Mulches are also used to prevent soil erosion and leaching of fertilizers (Moreno and Moreno, 2008). Mulches in the form of organic waste/residues can improve N and P content, soil structure and water holding capacity of soil (Odlare *et al.*, 2007). Inorganic mulches may vary from plastic to polyethylene mulches and can reduce water loss, weeds and the development of dormant plant diseases (Moreno and Moreno, 2008).

Significant changes in microbial structure, microbial functional diversity and microbial activities due to the application of organic and inorganic mulches have been widely demonstrated (Wakelin *et al.*, 2006; Odlare *et al.*, 2007; Rabary *et al.*, 2007; Huang, Xu and Chen, 2008; Moreno and Moreno, 2008; Seo *et al.*, 2008; Zibilske and Makus, 2009). Forge *et al.* (2002) showed that the use of organic mulches for perennial crops may have profound effects on the protozoa, bacterial and bacterivorous nematode populations within the soil, in addition to an enhancement of the soil's nutrient cycling ability. Organic mulches also have the ability to reduce plant diseases. For example, the application of organic compost mulch enhanced *Pseudomonas* and *Pantoea spp.* populations in the rhizosphere of cucumber (*Cucumis sativus* L. Straight Light) which may serve as biological control agents reducing soilborne diseases (Tiquia *et al.*, 2002).

4.2 Cover Crops

The biomass and diversity of soil microorganisms is significantly influenced by the physical, chemical and biological characteristics of the rhizosphere (Qu and Wang, 2007). Root exudates produced by plants consists of carbohydrates, carboxylic acids and amino

acids and are one of the most important factors affecting microbial activity and diversity within the soil (Benizri and Amiaud, 2005). Phenolic acids present within root exudates are often allelochemicals that may have an effect on microbial activity and diversity (Qu and Wang, 2007). Alteration of the microbial community due to these root exudates is therefore likely to influence soil fertility.

The ability of certain cover crops to alter soil microbial communities has been demonstrated in numerous studies (Behera and Sahani, 2001; Han *et al.*, 2007; Yang *et al.*, 2007; Riglietti, Ruggiero and Crecchio, 2008). Qu and Wang (2007) showed that monocropping of soybean (*Glycine max* L.) results in the build-up of plant allelochemicals within the soil compared to soils undergoing rotational cropping systems. Phenolic compounds produced by certain plants can significantly affect the activity and biomass of microbial organisms within the soil. Benizri and Amiaud (2005) showed that the functional diversity of microorganisms within two different grassland soils is directly influenced by the composition of the rhizodeposits in the respective plant community.

Cover crops that are incorporated as "green manure" are used mainly to reduce soil erosion but can also improve soil quality while increasing soil organic matter and enhancing soil microbial activity thereby reducing the incidence of plant diseases (Tejada *et al.*, 2007). For example, the incorporation of white clover (*Trifolium repens* L.) significantly suppressed root rot caused by *Fusarium spp.* on snap bean (*Phaseolus vulgaris* L.) plants (Abawi and Widmer, 2000). Long-term application of green manure has also been shown to have profound effects by enhancing soil nutrient levels, soil enzyme activities and soil microbial communities (Elfstrand, Hedlund and Mårtensson, 2006).

4.2.1 Allelochemicals

Allelochemicals are produced by plants as secondary products and have a suppressing

effect on other plant species and microorganisms (Qu and Wang, 2007). Several allelochemicals have profound effects on weeds and have therefore been developed into commercial herbicides (Vyvyan, 2001). The advantages of allelochemicals are that they are partially water-soluble, environmentally friendly and more likely to exhibit bioactivity at low concentrations (Vyvyan, 2001).

Allelochemicals in the form of herbicides or pesticides can have both positive and negative effects on soil microorganisms (Weiner and Weiner, 2001; Luo, Angelo and Coyne, 2006; Kato-Noguchi, Ino and Ota, 2007; Ruiyu *et al.*, 2007; Yang *et al.*, 2007; Kong *et al.*, 2008a; Watkins, Nicol and Shaw, 2009). Gopal *et al.* (2006) showed that a high dosage of the allelopathic chemical, azadirachtin, which is effective against a wide range of important insect pests, causes a counterproductive effect on soil fertility. On the other hand, allelopathic compounds may also stimulate the release of root exudates and restrict the growth of several soilborne pathogens while promoting beneficial soil microbes (Sturtz and Christie, 2003; Ruiyu *et al.*, 2007).

4.3 Biological control agents

Although chemicals are the primary means for reducing the incidence of pathogens, they have been shown to degrade the physical, chemical and biological properties of soil (Abawi and Widmer, 2000). An alternative option is to use beneficial microorganisms to promote the growth of crops and provide protection against plant pathogens. Biological control agents reduce plant diseases by mechanisms such as antibiosis, competition, induction of resistance, and the production of extracellular cell wall-degrading enzymes (Zhang *et al*., 2008; Susi *et al.*, 2011). Biological control has been shown to be effective against soilborne pathogens both *in vitro* and *in vivo*. Paul *et al*. (1998) showed a successful *in vitro* suppression of grey mould on grapevine (*Vitis vinifera* L.) caused by the pathogen *Botrytis cinerea* Pers. by using

a *Bacillus* strain which induces the production of phytoalexins by the crop plant. Ji *et al.* (2005) used a strain of *Pseudomonas fluorescens* in conjugation with two plant growth-promoting rhizobacteria to elicit systemic resistance to bacterial spot and speck on tomatoes (*Solanum lycopersicum* L.). Zhang *et al.* (2008) used a combination of biological control agents and organic fertilizers and showed a decrease in *Fusarium* wilt disease of cucumbers (*Cucumis sativus* L.). Certain organic amendments are also considered a form of biological control. By benefitting microorganisms that compete with pathogens and plant-parasitic nematodes, amendments such as plant products and organic manures which contain antagonistic organisms can thus reduce the incidence and severity of diseases (Akhtar and Malik, 1999).

Other advantages of using biological control agents as a method for controlling soilborne pathogens are, that they cause minimal effects on non-target microorganisms and the population of the biological control agent reduces over time (Winding, Binnerup and Pritchard, 2003). For example, the application of *Trichoderma atroviride* P. Karst. which is used to control the fungus *Armillaria mellea* (Vahl) P. Kumm. indicated no long term effects on other soil microorganism communities and neither was microbial diversity affected by the biological control agent (Savazinni, Longa and Pertot, 2009). The application of *Beauveria brongniartii* (Sacc.) Petch, which is used to control European cockchafer, exhibited very little influence on the non-target microbial communities in the soil (Schwarzenbach, Enkerli and Widmer, 2009).

5.0 Cultural practices

Conventional farming systems such as tillage, monocropping, soil fumigation and solarization can eventually lead to the deterioration of biological, physical and chemical properties of soil. This is mainly due to the continuous removal of nutrients and the use of

insecticides, pesticides and other agricultural chemicals which deplete soil fertility by reducing population of beneficial soil microbiota (Zong-pei *et al.*, 2007). The control of soilborne diseases is often performed by using fumigants and solarization, which can impact further on microorganism communities. Other practices such as crop rotation can have beneficial effects on soil fertility and the judicious integration of cultural practices is therefore crucial for achieving sustainable soil fertility (Masri and Ryan, 2005).

5.1 Tillage vs. zero tillage

Tillage practices impact negatively on soil structure, soil organic matter content and soil moisture; factors which directly influence microbial diversity in soil (Rahman, Chan, and Heenan, 2006). Intensive tillage may reduce the C content by up to 50% (Acosta-Martinez *et al.*, 2007). Tillage also results in physical relocation of food sources of microorganisms, impacting directly on the distribution and diversity of macro-, meso- and microorganisms in soil (Simmons and Coleman, 2008). Comparisons between the effect of traditional tillage and conservation tillage on microorganism activity and biomass shows increased storage of organic matter and enhanced microorganism biomass in the latter (Melero *et al*., 2009).

Numerous experiments have demonstrated the effects of reduced tillage or zero tillage on the composition of microorganism communities in soil (Govaerts *et al.*, 2005; Adl, Coleman and Read, 2006; Manns, Maxwell and Emery, 2007; Miura *et al.*, 2007; Nicolardot *et al.*, 2007). According to Diosma *et al.* (2005), reduced tillage leads to greater soil microbial diversity than in soils in surrounding grassland areas. Jin *et al.* (2009) showed that different tillage practices produce consistent differences in enzyme activities in soil. Soil microbial diversity is also influenced by combining residue management practices. For example, the combination of zero tillage and residue retention significantly increases microbial biomass (Govaerts *et al.*, 2007). Microbial fatty acids are significantly influenced

by different conventional tillage practices. This is mostly due to the effects of tillage on nutrient concentration and soil moisture (Simmons and Coleman, 2008).

Zero-tillage and fallowing are often included in rotation systems instead of bare-soil fallow treatments. This preserves soil fertility, reduces soil erosion and accumulates soil water (Drijber *et al.*, 2000). The effect of bare-soil fallow treatments in rotation programs has been shown to significantly reduce soil carbon. Asuming-Brempong *et al.* (2008) showed that soil carbon sequestration directly relates to the composition of the microbial populations. As carbon sequestration decreased so too did the soil bacterial, fungal and prokaryote populations.

5.2 Monocropping

Monocropping often leads to the increased incidence of pathogens, weeds, pests and ultimately degradation and loss of soil fertility. Monocropping and the repeated use of resistant cultivars, crop rotation and soil fumigation can also lead to a reduction in crop production (Qu and Wang, 2007). This may be due to an imbalance in the physical and chemical properties of the soil, the allelopathic effects of root exudates and residues of crop plants and weeds, and the build-up of pests and pathogens. Monocropping favours the development of soilborne pathogens such as *F. oxysporum*, which causes wilt diseases on a wide variety of crop plants (Zhang *et al*., 2008).

Various kinds of organic compounds are released by different crops. This is a vital factor influencing the functional diversity of microorganisms in the soil (Olsson and Alström, 2000). Monocropping may therefore favour the development of specific soil microorganisms. Long-term monocropping may lead to a depletion of organic C and N in soil and directly affect soil microorganism communities, both quantitatively and qualitatively. An 11-year-long study on monoculture wheat showed a significant decrease in soil organic C

and a clear decrease in microbial biomass (Chen *et al.*, 2009).

5.3 Crop rotation

Crop rotation affects the biological and biochemical properties of soil by changing the C content (Zong-pei *et al.*, 2007). A change in C content, combined with a change in soil temperature and soil moisture dynamics due to crop rotation, will consequently lead to shifts in microbial populations (Bucher and Lanyon, 2004; Bossio *et al.*, 2005; Asuming-Brempong *et al.*, 2008; Kulmatiski and Beard, 2008). Mathimaran *et al.* (2006) showed that crop rotation has significant effects on arbuscular mycorrhizal communities in soil. Compared to crop rotation, land-use intensification can lead to decreased microbial diversity. Shen *et al.*, 2007 showed an increase of disease attributed to *F. oxysporum* in soil without rotation compared to soil undergoing rice/wheat rotation.

A successful crop rotation programme may have the same positive effects on functional diversity of soil organisms as zero tillage. The chemical and physical properties of soil under rotation cropping are of a much higher quality than under conventional tillage and higher yields can therefore be achieved (Govaerts *et al.*, 2007). Zero tillage, plant residue retention and crop rotation in combination can result in higher microbial biomass and more micro-flora activity than in soil under conventional tillage (Govaerts *et al.*, 2005).

5.4 Solarization

The technique involves covering soil with clear plastic for periods of time to allow heat build-up. It produces effects similar to that of a chemically fumigated soil by reducing the incidence of pests and pathogens (Hasing, Motsenbocker and Monlezun, 2003; Tamiette and Valentino, 2005). It can increase crop yield and by heating the soil, not only removes pathogens in the soil but other beneficial microorganisms as well. This leads to rapid re-

colonization by fast growing microorganisms and leads to a change in the microbial diversity of the soil (Culman *et al.*, 2006; Scopa *et al.*, 2009).

Solarization increases soil temperature which induces changes in the physical and chemical properties of the soil, soil structure, water potential and soil pH (Gelsomino and Cacco, 2005). Microorganisms are also very sensitive to physical changes of soil, e.g. surrounding temperature and moisture level, and there is a significant compositional shift of bacterial communities within soil that has been solarized (Gelsomino and Cacco, 2005).

Solarization is often used in combination with other techniques to further benefit crop yield. By combining soil solarization with the application of *Bacillus subtilis* Ehrenberg (Gasoni *et al*., 2007) and *Trichoderma harzianum* Rifai, disease incidence was reportedly reduced and crop yield of table beat (*Beta vulgaris* L.) improved (Porras, Barrau and Romero, 2006). Oka, Shapira and Fine (2007) combined solarization with composts in an attempt to control root-knot nematodes and showed that it was significantly more effective than using compost alone.

5.5 pH

Soil microorganisms are significantly influenced by soil pH (Yao, Bowman and Shi, 2006). Wakelin *et al*. (2007a) showed that changes in pH affect microbial diversity in agricultural soil ecosystems as well as microbial enzyme activities. The latter are affected more by a change in pH than changes in organic material (Acosta-Martinez *et al.* 2007). Changes in pH can also affect the community structure of the soil microbial community. Pietri and Brookes (2008) showed that a change in soil pH within a single soil type has significant effects on soil microbial biomass and activity. The application of liming agents (Bucher and Lanyon, 2004; Kjøller and Clemmensen, 2009) and other amendments which alter soil pH (Yuan and Li, 2007) can drastically change the diversity of microorganisms in

soil. Application of chemical nutrients that change soil pH can also result in changes in the microbial community structure of that soil (Zong-pei *et al.* 2007). Cang *et al.* (2006) reported negative effects of high pH introduced by NaClO on the functional diversity of the microbial organisms in soil.

6.0 Conclusions

Although the presence of soil microorganisms was discovered in the 17th century, they have only recently been considered of significant importance to agricultural systems. Their presence is vital to maintain regulatory functions in soil that determine soil fertility and quality. Many biotic and abiotic factors however, have a direct and indirect impact on their functional and structural diversity and biomass in soil. These factors influence their interactions with plants both above and below ground. These interactions occur both temporally and spatially and can be either beneficial to plant health and yield, or result in disease-associated losses.

Agricultural management practices, such as tillage and various soil amendments, which are used to enhance crop yields, can influence the chemical and physical environment of soil resulting in direct impacts on microorganisms. Although the positive effects of these practices can be seen immediately, the negative effects may cause more damage to the soil ecosystem in the long run. Negative effects of management techniques which physically remove organisms from the soil i.e. solarization or tillage not only result in reduced plant growth but may also reduce the functional abilities of the soil. The negative effects of chemical amendments which remove specific organisms from soil i.e. herbicides, pesticides or fungicides may lead to opportunistic pathogens or weeds developing due to vacant niches and increased food sources.

A combination of management practices and soil amendments should therefore be

considered. Combined management systems not only remove unwanted organisms but also enhance microbial diversity in order to prevent the outbreak of disease. A successful combination of management practices and amendments will encourage the recovery of functional systems in soil and also improve the potential yield of crops. Future investigations and comparisons of various combinations and their effects on soil microorganisms is therefore of the utmost importance.

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CHAPTER TWO

AN *IN VITRO* MICROCOSM STUDY ON THE EFFECT OF AGRICULTURAL AMENDMENTS ON SOIL MICROBES

ABSTRACT

The effect of 14 soil amendments and treatments containing inorganic fertilizers, organic manure composts, herbicides, fungicides, a liming agent and cover crops were investigated for their effect on soil microbes. Soil amendments and treatments were applied over a total period of 12 months and microbial activity was measured at 3-month-intervals. Microbial activity and community profiles were determined by means of Biolog Eco™ plates and fluorescein diacetate hydrolysis (FDA). Distinctive changes in microbial populations were recorded as a result of the various soil treatments. Eco™ plate analysis showed that at the end of the 12-month-period overall carbon utilization was highest in soil treated with Roundup and 2,4-dicholorophenoxyacetic acid (2,4-D), and lowest in soil amended with chicken manure compost. FDA analysis however indicated that highest microbial activity at the end of the 12-month-period was in 2,4-D treated soil and the lowest in Roundup treated soil. DGGE analysis showed distinct changes in microbial diversity, as a result of the soil amendments compared to the control treatments.

Introduction

Agricultural soil amendments are used in almost all agricultural practices today. Their impact ranges from enhancing crop yield to providing protection against potential pests and pathogens (Carrera *et al.*, 2007; Lupwayi *et al.,* 2009). Soil amendments, however, also influence microbial organisms present in the agroecosystem. Since microbes are an extremely important constituent in soil because they mediate several ecosystem processes such as decomposition, nutrient transformation, and organic matter formation in soil, anything that can negatively impact on them is of concern to growers (Wang *et al.,* 2008). Soil microbes are easily disturbed by changes to their surrounding environment. Changes in C and N availability, carbon content, pH, soil moisture and chemical content of the soil may lead to changes in the diversity and structure of microorganisms in soil and influence the functional cycles mediated by them (Perucci *et al.,* 1999; Zhong *et al.,* 2009).

The application of amendments such as chemical fertilizers can affect the physical, chemical and biological properties of soil. Studies have shown that changes in the available N and organic matter in soil due to chemical fertilizer application leads to exponential growth of microbial organisms due to the abundant food source (Zhong, Cai and Zhang, 2007). However, the application of chemical fertilizers may also have a negative impact on community structure or biomass of soil microbial organisms. A comparison of the effects of chemical fertilizer on microbial organisms in soil showed that sole applications of N fertilizer resulted in a significantly lower microbial biomass and reduced diversity when compared to a balanced mixture of chemical fertilizers and manure composts (Gu *et al.,* 2009). Different chemical fertilizers also have a different effect on the microbial communities in soil. For example, the separate application of N, P and K fertilizers resulted in the development of significantly different microbial communities (Zhong *et al.,* 2007; Wang *et al.,* 2008).

The application of organic compost is known to increase microbial activity, biomass

and diversity in soil. Long-term application of manure has been shown to have a profound effect on these parameters (Zhong *et al.,* 2009). Zhang and Fang (2006) showed a significant increase in microbial biomass, organic C and N in deep ploughed soil treated with manure compost compared to soil treated with chemical fertilizers. Several composts have also been shown to have a suppressive effect on soil-borne pathogens, due to antagonistic organisms present in the compost (Perez-Piqueres *et al.,* 2005).

Cover crops are an alternative source of organic matter in soil and are also known to prevent soil erosion and enhance the physical, chemical and biological properties of soil (Tejada *et al.,* 2007). "Green manuring", or the application of cover crop residues, is therefore a substitute for organic compost amendment (Elfstrand, Hedlund and Mårtensson, 2006). The other benefit of cover crops is their potential biofumigation effect. Several reports have shown successful suppressive effects of cover crop residues and extracts on both pathogens and weeds (Kato-Noguchi *et al.,* 2002; Turk and Tawaha, 2002; Xuan *et al.,* 2004; Yulianti, Sivasithamparam and Turner, 2005).

Pesticides (fungicides, insecticides and herbicides) improve crop yields by controlling specific pests, weeds and pathogens, but they are also known for their inhibitory effects on non-target organisms in the agroecosystem (Ferreira *et al.,* 2009). The removal of non-target microbial organisms may impact negatively on the microbial mediated functional cycles in soil, ultimately influencing soil quality and fertility (Ferreira *et al.,* 2009). Post-emergence herbicides such as glyphosate and 2,4-dichlorophenoxyacetic acid (2,4-D) may remain in soil and become a food source for certain microbial organisms thus causing changes in the microbial community structure which can impact negatively on soil fertility (Zabaloy, Garland and Gómez, 2008). A change in vegetation due to herbicide application can sometimes lead to changes in rhizosphere exudates which can also ultimately impact on microbial activity and enzyme activity in soil (Turk and Tawaha, 2002).

The application of liming agents and other pH altering amendments significantly impacts microbial diversity, activity, biomass as well as microbial community structure in soil (Chagnon *et al.,* 2000). Comparisons between the effects of pH, fungicides, organic matter and fertilizers, showed that pH plays a more important role in influencing the community composition of microbial organisms (Blagodatskaya and Anderson, 1998; Fernández-Calviño *et al.,* 2010). The application of liming agents significantly changes the community composition of microbial organisms. Changes in pH apparently cause shifts in the fungal/bacterial ratio and gram-positive: gram-negative ratio in soil (Pietri and Brookes, 2008).

The use of soil microcosms to investigate the effects of soil amendments on soil ecological processes has recently replaced traditional single organism tests (Bogomolov *et al.,* 1996). Advantages of using soil microcosms are, an easily controllable environment, low costs and the easily reproducible results they offer. Microcosms are thus a far better method for studying soil microbes than using field trials (Chen and Edwards, 2001). Several ecological studies investigating the effects of fungicide and herbicide amendments on microbial organisms in soil by using microcosms have provided conclusive proof of changes in microbial activity, biomass and diversity (Burrows and Edwards 2001; Chen, Edwards and Subler, 2001a).

The objectives of the present study were to investigate the short-term effects of several soil amendments on the diversity of microbes in soil microcosms. The study investigated the positive or negative effects of each amendment on the quantity and diversity of the organisms utilizing various biochemical and molecular methods.

Materials and Methods

Experimental design

Soil preparation and greenhouse trial design: Loam soil, rich in organic material, was collected at 5 cm depth from a well vegetated area at Krugersdrift dam near Bloemfontein (28˚53'5.64"S, 25˚57'33.48"E), South Africa. The soil was sieved and homogenized and placed in 5L plastic pots to within 3 cm from the top. Five replicate pots were used for each treatment and the experiment was designed as a randomized complete block. Four individual trials, each 12 wks in duration were conducted over a total period of 48 wks. Each trial entailed the application of 10 soil amendments, 2 cover crop treatments and 2 control treatments. At the termination of each 12-wk-trial, above- and below-ground plant parts were destructively removed from the soil and the soils from 5 replicate pots per treatment were homogenised before commencing with the next trial. Two (duplicate) experiments were run concurrently.

Treatments: A total of 10 soil amendments, 2 cover crop treatments and two control treatments were applied to the soil microcosms (Table 1). Ryegrass seeds *(Lolium perenne)* were sown in all pots (2g/pot) to serve as a cover crop. The rye grass seeds were allowed to grow for 2 wks before commencing with the application of the soil amendments. Amendments were applied to the soil surface of the microcosms every 4 wks over a total period of 12 wks. The two cover crop treatments (mustard, nemat) were planted at the same time as the ryegrass cover crop. Two control treatments, a positive and a negative control, were prepared. The positive control was sown with ryegrass seeds (2g/pot) while in the negative control, the soil was left bare for the duration of the 48 wk experiment. Water was applied to each pot (300 ml) three times a week and the temperature of the glasshouse was kept constant throughout the day and night at ±21˚C.

Soil sampling and storage: Soil samples were collected at the end of each 12-wk-trial,

one week after the final application of the respective amendment in relevant treatments. Soil was collected destructively from the middle of each pot in a positive treatment and mixed thoroughly to form a bulk sample. The samples were immediately stored at 4˚C, and used for Biolog Eco™ plate and FDA analysis. Sub-samples were stored at -80˚C for DNA extraction.

Qualitative analyses

Biolog Eco™ plate

The use of Biolog Eco™ plates (Biolog™, USA) is a soil essay technique adapted from Palmroth *et al.* (2004) and Akmal *et al.* (2005). The 14 bulk soil samples from respective treatments were placed in the proportion of 2g soil to 200 ml of deionized distilled water in 250 ml Erlenmeyer flasks. The flasks were then shaken for 30 min on a rotary shaker at 90 rpm, and 95 ul of each suspension was pipetted into each of the 96 wells in the Biolog Eco™ plate. The Eco™ plates were then incubated at 25 ºC for 6 days and evaluated at 595 nm with an absorbance microplate reader (ELx808™, Biotek). Data from both concurrent experiments were averaged to produce a single set of data and then analyzed by determining the average well colour development (AWCD) and carbon utilization (Garland and Mills, 1991; Cai *et al.*, 2010). Principle component analysis (PCA) and cluster analysis were conducted to determine possible relationships among various treatments. A GLM-ANOVA was conducted to determine significant changes in community linked physiological profiles (CLPP) between treatments. PCA and cluster analyses were conducted using XLSTAT (Version 2011.4.03, Addinsoft®). The GLM-ANOVA was conducted with NCSS 2000 (BMDP Statistical Software Inc.).

PCR-DGGE (Polymerase chain reaction-denaturing gradient gel electrophoresis)

DNA Extraction: Extraction of DNA from soil was done using the PowerSoil™ DNA Isolation Kit. DNA was extracted from 0.25 g of soil and the genomic DNA samples were checked by running them on a 1 % agrose gel stained with ethidium bromide with UV illumination. The concentration of the respective DNA samples was determined by using a Nano Drop spectrophotometer (3300 fluorospectrometer, Thermo Scientific) before conducting the 16S rDNA Polymerase Chain Reaction (PCR).

16S and DGGE rDNA PCR: The 16S rDNA PCR amplification of the extracted DNA was performed by using the standard protocol (SOP_29) developed by the Metagenomics Platform laboratory of the University of the Free State, Bloemfontein. The protocol makes use of the 16S primers 27F and 1492R which are bacteria specific (Lane *et al.*, 1991). The 16S PCR mixture consists of 10 uM of each primer set, 10 uM of dNTPs, 1-50 ng of genomic DNA and 0.125 ul Taq DNA polymerase (SuperTherm) with a final volume of 50 ul. The 16S PCR was performed with an initial denaturing step of 95 ˚C for 2 min followed by 30 cycles of amplification. The cycles ran at 95 ˚C for 30 s, 52 ˚C for 45 s, 72 ˚C for 1 min and a final extension step of 72 ˚C for 10 min. The final products of the PCR were checked by running them on a 1 % agrose gel stained with ethidium bromide with UV illumination. Gel stabs were then performed on the 16S rDNA PCR products to collect templates for further analysis. Amplification was performed by using the same PCR program as the 16S PCR with a forward primer, 341F-GC and reverse primer, 517R (Muyzer *et al.*, 1997).

DGGE analyses: DGGE analyses was performed in the Metagenomics Platform laboratory (Department of Microbiology, University of the Free State) using a DCode™ Universal Mutation Detection System (Biorad). The DGGE PCR amplifications were verified on a 1 % agarose gel electrophoresis 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 the polyacrylamide gel (Tezenva *et al.*,

2008). The analysis was carried out according to the standard procedure developed in the Metagenomics Platform laboratory (SOP_19b). The results were viewed by staining with ethidium bromide and visualized under UV transillumination. The banding profile of each sample was then quantified and compared by using Quantity One® 1-D analysis imaging software (Biorad Molecular Imager Gel Doc™ XR System) to determine relatedness (SOP_66) (Green, Leigh and Neufeld, 2009). PCA and cluster analysis was conducted to determine possible relationships among treatments. PCA and cluster analysis was conducted with XLSTAT (Version 2011.4.03, Addinsoft®).

Quantitative analyses

FDA (Flourescein diacetate) analysis

This procedure was adapted and modified from Schnürer and Roswell (1982). A stock solution was first prepared by dissolving FDA in acetone at a concentration of 2mg/ml and stored at -20 ºC. A buffer solution of 60 mM sodium phosphate solution was prepared, the buffer solution were adjusted to pH 7.6 and stored at 4 ºC. Soil samples of 0.5 g were added to 20 ml of the buffer and followed by 0.2 ml of the FDA solution. The mixture was then placed in a rotary shaker and agitated at 90 rpm for 1 h. After shaking, the soil solutions were placed in 2ml eppendorf tubes on ice to stop the FDA from being hydrolyzed further. The eppendorf tubes were then centrifuged at 6000 rpm to remove excess debris. The supernatants were then measured at 490 nm using a spectrophotometer (CARY Bio 100 UV-Visible Sectrophotometer). A GLM-ANOVA was conducted to determine significant changes of microbial enzyme activity between treatment and control samples using the statistical program NCSS 2000 (BMDP Statistical Software Inc.).

Results

Biolog Eco™ plate results

Biolog Eco™ plate readings for the two concurrent experiments were very similar. A high correlation (r^2 = 0.9707) was found between mean readings for the 31 carbon sources between the two experiments, and the data for the two experiments were therefore combined.

Average well colour development (AWCD) analysis: The AWCD value represents total metabolic activity of soil bacterial community utilization of the 31 carbon sources in the Biolog Eco™ plate. The AWCD in all treatments generally increased with an increase in planting time up to 9 months and then gradually decreased to 12 months except for two treatments: lime and mustard cover crop. At the end of 12 months, the average AWCD value over two trials was highest in the herbicide treatments (Figure 1), and lowest in the chicken manure compost and the bare soil control treatment. There was a significant difference ($P < 0.05$) between the AWCD value of the 3N:1P:5K fertilizer treatment, the herbicides (Roundup, 2,4D) and Nemat compared to the negative control (bare soil treatment) (LSD = 26.3353). No significant differences ($P > 0.05$) were found between the AWCD values of any of the amended soils and the positive control (rye grass treatment).

Carbon group utilization analysis: The 31 carbon sources in the Biolog Eco™ plate were separated into 6 carbon groups: 10 carboxylic acids, 6 amino acids, 2 amines, 2 phosphate-carbons, 7 carbohydrates and 4 complex carbon sources. The highest utilized carbon source was the complex carbon sources and the lowest utilized group, the phosphate-carbons (Figure 2). The same utilization pattern was seen over the entire 12 month planting period. At the end of 12 months, a significant difference ($P < 0.05$) was found in the utilization of phosphate-carbon (LSD = 0.3942), amino acid (LSD = 0.3325), amines (LSD = 0.5529) and carboxylic acid (LSD = 0.3075) carbon groups between treatments. Similarly, chemical fertilizers, sheep manure compost, and both positive and negative controls had a significantly higher utilization ratio ($P < 0.05$) of amino acid and amine carbon groups than

did chicken manure compost. However no significant differences ($P > 0.05$) were evident between the positive control, the fertilizer amendments (NPK, LAN, super phosphate) and the sheep manure compost. No significant differences ($P > 0.05$) were observed between the two fungicide treatments (tilt, benomyl), herbicide treatments and the positive control.

PCA and cluster analyses: Cluster analysis showed clear changes in microbial diversity as affected by soil amendments (Figure 3). Cluster analyses differentiated the soil samples into 3 clusters and clearly indicated that the negative control, lime and the covercrop treatments effectively altered the microbial composition in the treated soils compared to the untreated positive control (Figure 4). Four main groups of treatments could be distinguished and the results showed that the negative control, LAN, chicken manure and mustard cover crop treatments were most effective in changing microbial diversity (Figure 4).

Microbial activity assessed by FDA

Microbial enzyme activity: The FDA readings between the two experiments were very similar. A high correlation ($r^2 = 0.9753$) was found between the means of the three replicates between the two experiments, and data were therefore combined.

Microbial activity generally increased for most treatments except for Roundup and the two cover crop treatments (mustard and Nemat). A general increase in microbial activity was seen up to 6 months followed by a decrease to 12 months. This was observed in all treatments as well as both control treatments with the exception of both cover crops and sheep manure compost treated soil (Figure 5). The cover crop treatments consistently showed decreased microbial activity from 3 months up to 12 months, while the sheep manure compost showed increased enzyme activity up to 9 months and then a decrease at 12 months. At the end of 12 months, the highest microbial activity for both trials was observed for2,4-D while the lowest was Roundup and the negative control (Figure 5). A significant difference

($P < 0.05$) was evident between all the treatments and the negative control (LSD = 0.3952). However no significant differences ($P < 0.05$) were evident between any of the treatments and the positive control.

Microbial diversity assessed by PCR-DGGE

Both PCA and cluster analysis showed distinct changes in microbial diversity in soil following soil amendments (Figures 7, 8, 9 and 10). The PCA plot of experiment 1 showed little resemblance to that of experiment 2, although several similarities were observed between the two experiments. Firstly, the negative control showed a consistent positive relationship with the Roundup treatment. Secondly, the inorganic fertilizers (LAN, NPK, SP) were closely related, and thirdly, the Nemat treatment had no relationship with the other treatments (Figures 7 and 8). Differences observed between experiment 1 and 2 were: firstly, the positive control, which showed no relationship with the other treatments in the PCA plot of experiment 2 but a close relationships with the negative control, Roundup, inorganic fertilizers and manure composts in experiment 1 (Figures 7 and 8). Secondly, the lime treatment had no relationship with all other treatments in experiment 1 but a close relationship to NPK fertilizer, chicken manure, 2,4-D and tilt treatment in experiment 2. Thirdly, the tilt treatment showed no relationship with other treatments in the PCA plot of experiment 1 but showed a closed relationship with lime, chicken manure, NPK fertilizer and 2,4-D in experiment 2 (Figures 7 and 8). The treatments that showed the most distinct changes in microbial diversity compared to both control treatments in the PCA plot were the lime, tilt, 2,4 D and nemat treatments in experiment 1 and LAN, super phosphate fertilizer (SP), lime and chicken manure treatments in experiment 2 (Figures 7 and 8).

Discussion

In the present study, short-term changes in microbial activity, diversity and community structure of 14 agricultural treatments in loam soil were assessed in microcosms. Results indicated distinctive changes mediated by all treatments in terms of microbial metabolic activity as assessed by carbon group utilization patterns of the Biolog Eco™ plates. No significant increase/decrease in metabolic activity was found by comparing the positive control to the other treatments. This may be due to the short-term nature of this experiment or limitations of Biolog Eco™ plate analysis. Dong *et al.* (2008) discussed several limitations of the Eco™ plate analysis compared to PCR-DGGE analysis and PLFA analysis and indicated that Eco™ plate analysis is very sensitive to microbial density and cannot therefore represent soil metabolic diversity. Eco™ plate data indicated a higher level of microbial metabolic activity in herbicides and NPK fertilizer treatment but reduced metabolic activity in lime, LAN fertilizer, chicken manure and the negative control treatment. The increase in microbial metabolic diversity by the application of NPK fertilizer may be related to available N and P which can indirectly affect microbial populations in soil (Zhong *et al.*, 2009). Combinations of mineral fertilizer and other organic inputs such as manure composts and straw incorporation have been shown to have profound effects on soil microbial properties (Juan *et al.*, 2008). Our results are also consistent with Zhong *et al.* (2007), who observed increases in microbial functional diversity following the application of NPK fertilizer compared to non-fertilized soil. The increase in metabolic diversity following herbicide application may be due to residual chemicals which may serve as nutrients that enhance microbial growth (Mijangos *et al.*, 2009). This observation is consistent with the findings of Zabaloy *et al.* (2008), who reported increases in microbial functional diversity after long-term application of 2,4-D and Roundup.

The decrease in microbial metabolic diversity observed in lime treated soil may be caused by a change in soil pH. Changes in soil pH have been found to significantly affect

soil microbial community and microbial biomass in several studies (Pietri and Brookes, 2008; Rousk, Brookes and Bååth, 2009; Fernández-Calviño *et al.*, 2010). Changes in soil pH directly affect soil fungal-bacterial ratios and may lead to significant changes in microbial functional systems in soil (Blagodatskaya and Anderson, 1998). Clivot *et al.* (2011) reported decreases in bacterial diversity of acidobacteria and gram-positive taxa, which are dominant in soil bacterial communities, of limed soil compared to non-limed soil. The decrease in metabolic activity observed in the LAN and chicken manure treatment may be due to a high nitrogen input into soil, causing a shift of microbial communities towards nitrifying bacteria and a reduction in other microbial organisms. Zhong, Cai and Zhang (2007), reported a significant increase in nitrifying bacterial populations in soil treated with N fertilizer compared to other fertilizers. Our findings are also consistent with Wang *et al.* (2008) who observed decreases in metabolic diversity in chemical fertilizer treated soil compared to untreated soil when using Biolog Eco™ plates.

Microbial activity as determined by the rate of hydrolysed flourescein diacetate was higher in all treatments including the positive control, but with the exception of the negative control, Roundup and both covercrop treatments (mustard and Nemat). An increase in microbial activity was recorded in all fertilizer and manure compost treated soil. This was expected since an increase in plant biomass can increase microbial biomass due to the rhizosphere effect (Hao *et al.*, 2007; Zhong *et al.*, 2009). Plants release several compounds in the root zone which can directly influence microbial populations in that area, sugars and amino acids that can be utilized by microbial organisms. The rhizosphere effect increases microbial biomass and results in the development of an enhanced microbial population in the root zone (Sturz and Christie, 2003). Marschner *et al.* (2001) showed rapid increases in rhizosphere bacterial communities around root zones and related this trend to organic acids, sugars and other compounds within root exudates that attracts opportunistic bacteria. The

increase in microbial activity observed in fungicide treated soil was unexpected since reports of benomyl application indicate a short-term decrease in microbial activity (Smith, Hartnett and Rice, 1999; Chen, Edwards and Subler, 2001a). This phenomenon may however be explained by the ability of microorganisms to utilize certain chemical residues and fungal remains following the application of fungicides. Broad spectrum fungicides can directly affect soil fungal-bacterial ratios. Chen, Edwards and Subler (2001b) reported an increase in bacterial activity after the application of benomyl which they related to reduced fungal populations and abundant fungal remains and fungicide residues in soil.

Microbial activity which decreased in the negative control and Roundup treated soil is possibly due to the absence of ryegrass. The lack of the plant mediated rhizosphere effect causes significant changes to the physical and chemical properties of soil. Guenet *et al.* (2011) reported a decrease in microbial biomass in long-term fallow soil compared to soil which has been under a wheat monoculture. This decrease is possibly caused by the lack of secondary plant products and sugars available within the root exudates. Marschner *et al.* (2001) reported a decrease in bacterial diversity at the root tip compared to older root zones, and related this effect to the accumulation of opportunistic bacterial species due to the root exudates. The decrease in microbial activity observed in the mustard and Nemat cover crop treatments was expected since *Brassica* plants are well known for their allelopathic effects on microorganisms. Larkin and Griffin (2006) reported the inhibition of several plant pathogens following the incorporation of several *Brassica* crops which included rapeseed, radish, turnip, yellow mustard and Indian mustard. Brassicas also affect soil pH and carbon content in soil which may indirectly influence soil microbial activity. Kim, Owens and Kwon (2009) showed distinct increases in pH and carbon content in soil planted with Indian mustard compared to fallow soil.

Relationships between the bacterial community structures of amended/treated soil

were assessed by means of Biolog Eco™ plate and PCR-DGGE fingerprints. Biolog data showed very similar patterns for the herbicides, fertilizer (NPK) and the positive control. Bacterial communities in the negative control, cover crops and lime treated soil were distinctively different from that of the positive control. The close relationship between fertilizer (NPK) and herbicide treatment is possibly the result of an increase in bacterial enhancing nutrients in the soil medium. Changes in soil chemical properties have been shown in several studies to significantly affect soil bacterial composition and diversity (Marschner, Kandeler and Marschner, 2002; Stark *et al.*, 2007). The distinct differences observed between the negative control, cover crops and lime treatments from the other treatments can also possibly be explained by changes in soil chemical properties and lack of the rhizosphere effect. Loranger-Merciris *et al.* (2006), using Biolog Eco™ plate analysis, found similar changes in bacterial community structure in soil under different plant species and related this change to differences in root exudates.

PCR-DGGE data showed that soil bacterial communities were most affected by 2,4-D, lime, Nemat and fungicide treatments. Bacterial species which could utilize residues from the herbicide and fungicide treatments flourished and replaced bacterial species that could not (Rai, 1991). Changes in microbial diversity could possibly be caused by similar factors affecting microbial activity. Reduced fungal populations, an abundance of fungal remains and residues of fungicides not only increase microbial activity but also shift microbial diversity in favour of organisms capable of utilizing these compounds (Chen, Edwards and Subler, 2001b). Changes in microbial diversity observed in cover crop treatments, could be related to allelochemicals and secondary plant compounds. Allelochemicals present in the root exudates of certain plants not only negatively affect soil microbial communities by direct toxic effects, but also contain compounds such as flavones which are capable of influencing microbial community structure (Kong *et al.*, 2008). Other plant compounds such as essential

oils and plant phenolics present in plant material could also change the microbial diversity in soil. In a review, Inderjit and Weiner (2001) discussed the effects of plant compounds and allelochemicals on soil microbial organisms and showed several cases of changes in soil microbial properties due to the incorporation of these plant compounds.

Microbial community structure as reflected by PCR-DGGE and Biolog Eco™ plate results was inconsistent. This was anticipated since each analytical technique focuses on different aspects when determining the relationships of microbial community in soil. Similar differences between the results of Eco™ plates and PCR-DGGE analysis were also observed by Wang *et al.* (2008), who indicated that it was because Biolog Eco™ plates reflected C utilization by fast growing bacteria only, while PCR-DGGE shows changes of the entire bacterial community structure. Inconsistency between Biolog and PCR-DGGE analysis were also found by Dong *et al.* (2008), who related the difference to the sensitivity of Biolog technique to inoculum density and its inability to detect unculturable bacteria.

The results of the present study identified several major factors that possibly can influence soil microbial organisms. Changes in nutrient sources and pH, the presence of allelopathic substances and the presence of plant cover seem to be the major factors influencing soil microbial activity and diversity. This may ultimately influence soil fertility, disease suppression and crop yield. Further research to standardise the techniques used to evaluate soil microbial populations both quantitatively and qualitatively is therefore required. Clarification of the effects of soil amendments on soil microbes could assist farmers to monitor and asses management practices that lead to significantly increased crop yields.

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Table 1. Treatments and application rates of soil amendments used in microcosm trials.

Treatments	**Application rates**
Lime	4.5Tonne/ha
Fertilizers	
3N:1P:5K	30g/m²
Lime Ammonium Nitrate (LAN)	30g/m²
Superphosphate	15g/m²
Manure compost	
Chicken manure compost	65g/m²
Dried Sheep manure	65g/m²
Herbicide	
Round up (glyphosate)	1L/15m²
2,4-D (2,4-dicholorophenoxyacetic acid)	0.95kg/ha
Fungicide	
Benomyl	51mg/kg
Propiconazole (Tilt)	0.25mg/kg
Cover crops	
Mustard (*Brassica juncea*)	2g/pot
Nemat *(Eruca sativa)*	2g/pot
Control treatment	
Positive treatment, Ryegrass, (*Lolium perenne*)	2g/pot
Negative treatment	fallow

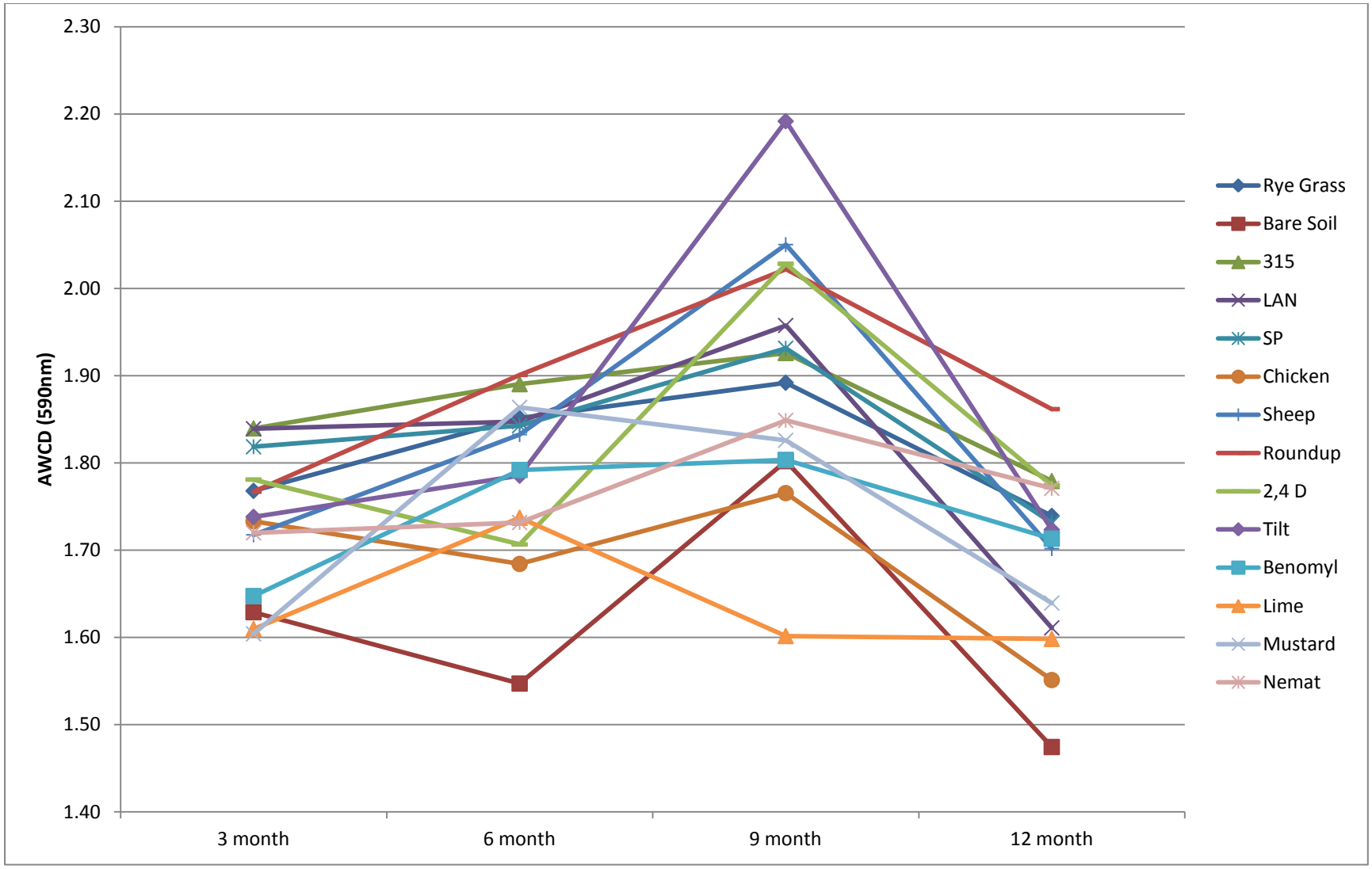

Figure 1. Average AWCD of Biolog Eco™ plate data of two trials affected by 14 treatments over a 3 to 12 month planting period.

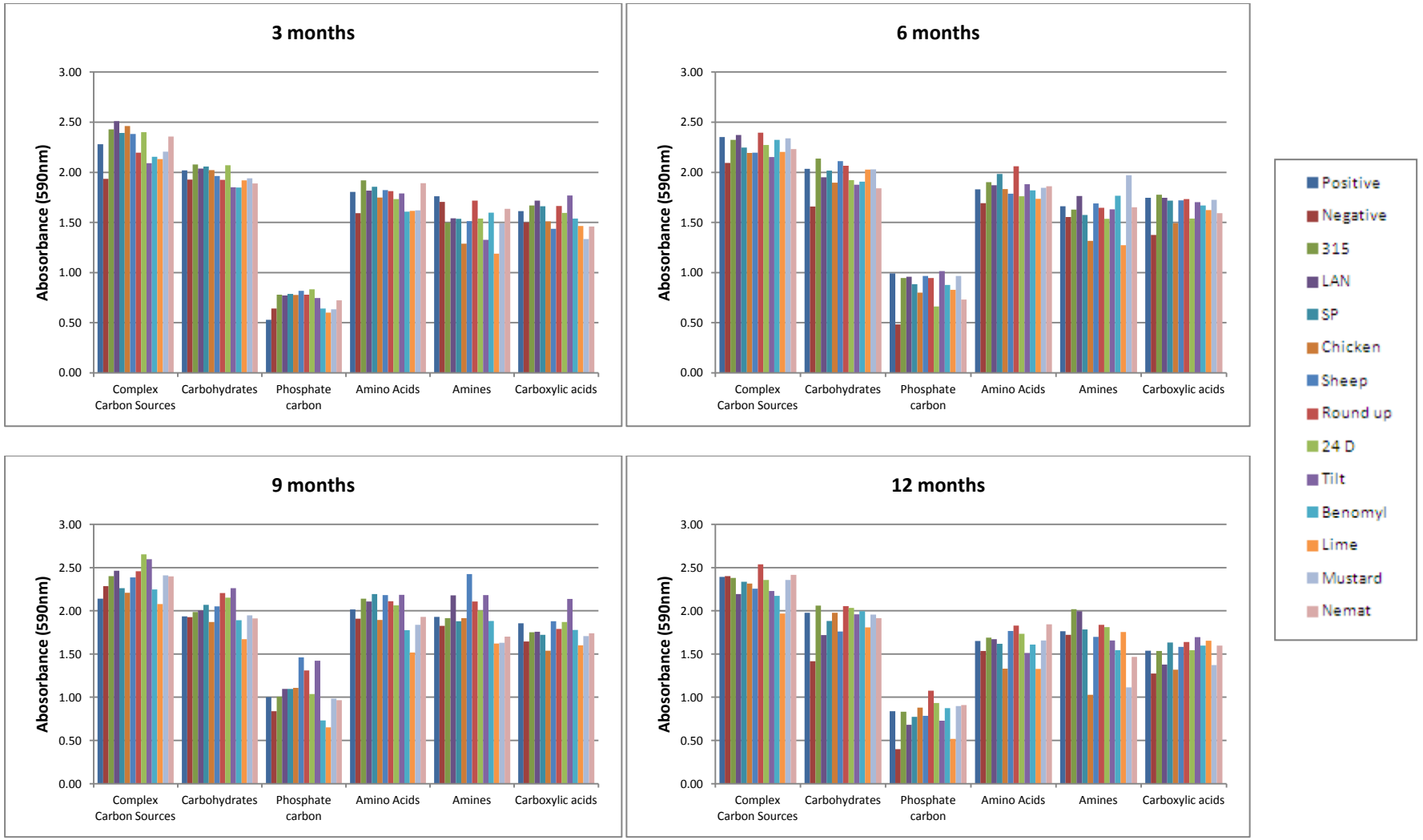

Figure 2. Average utilization of six carbon source groups in Biolog Eco™ plate data of two trials affected by 14 treatments over a 3 month to 12 month planting period.

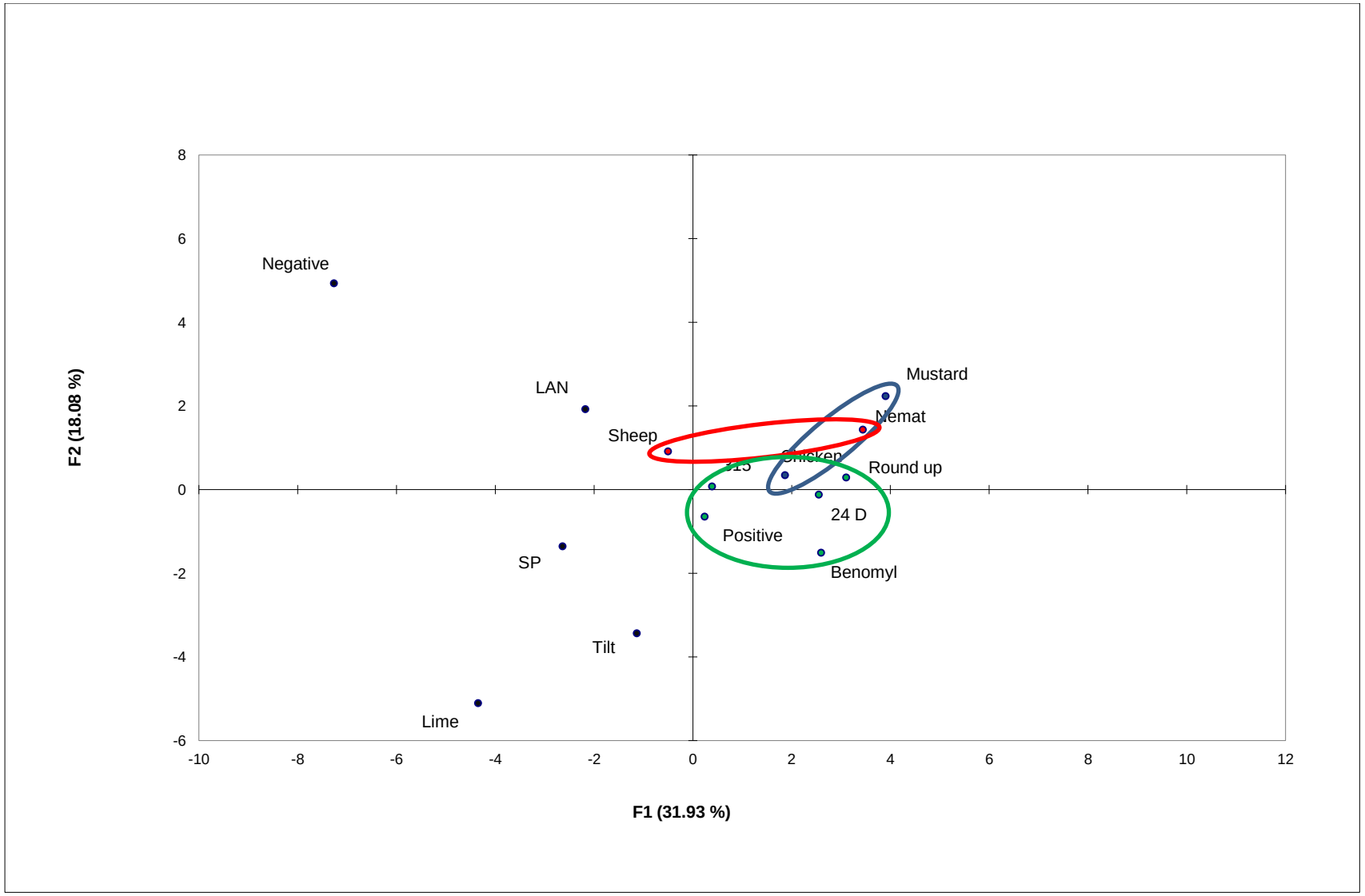

Figure 3. PCA plot of average Biolog Eco™ plate data of two trials affected by 14 treatments at the end of 12 months.

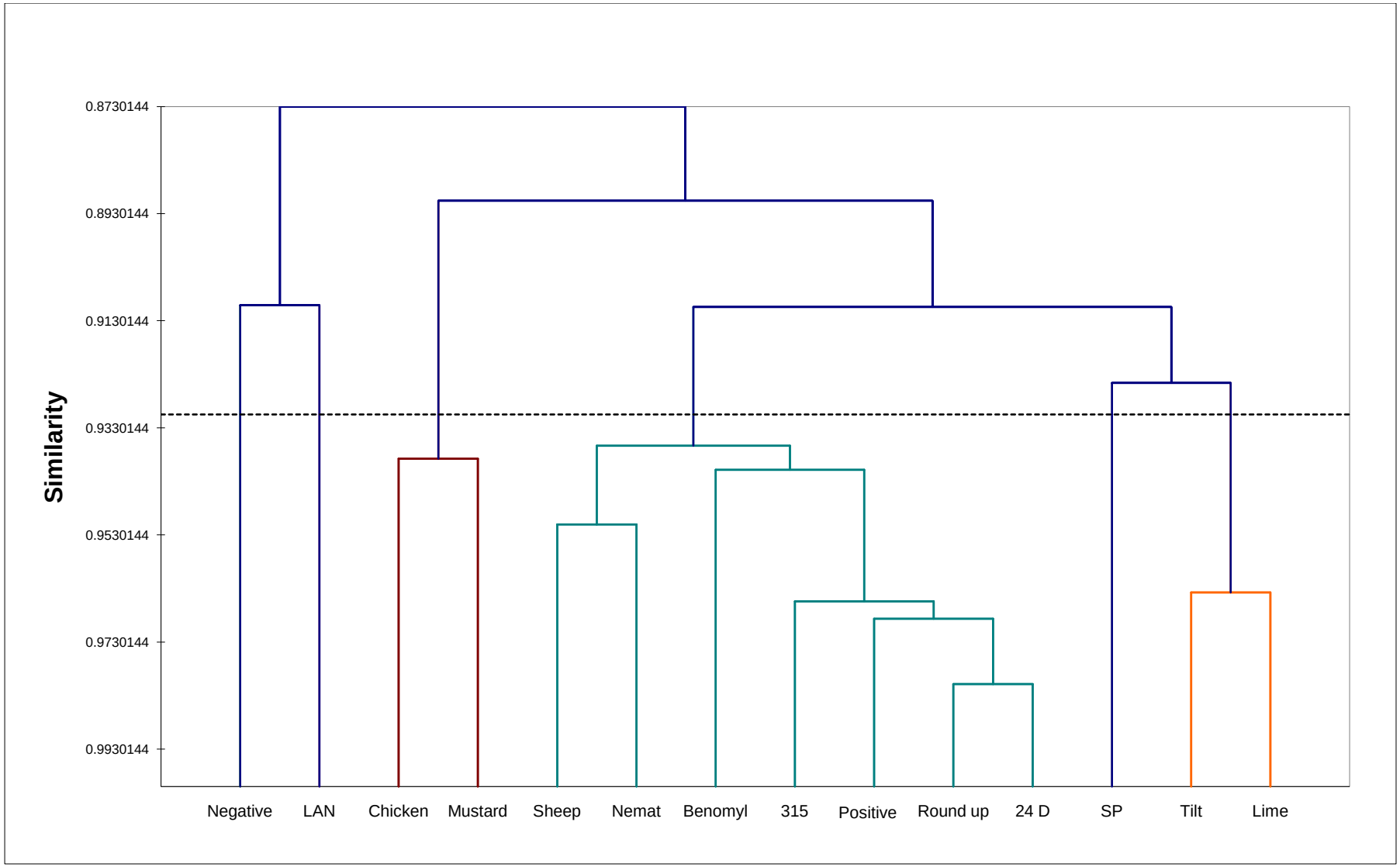

Figure 4. Dendogram of average Biolog Eco™ plate data of two trials affected by 14 treatments at the end of the 12 months.

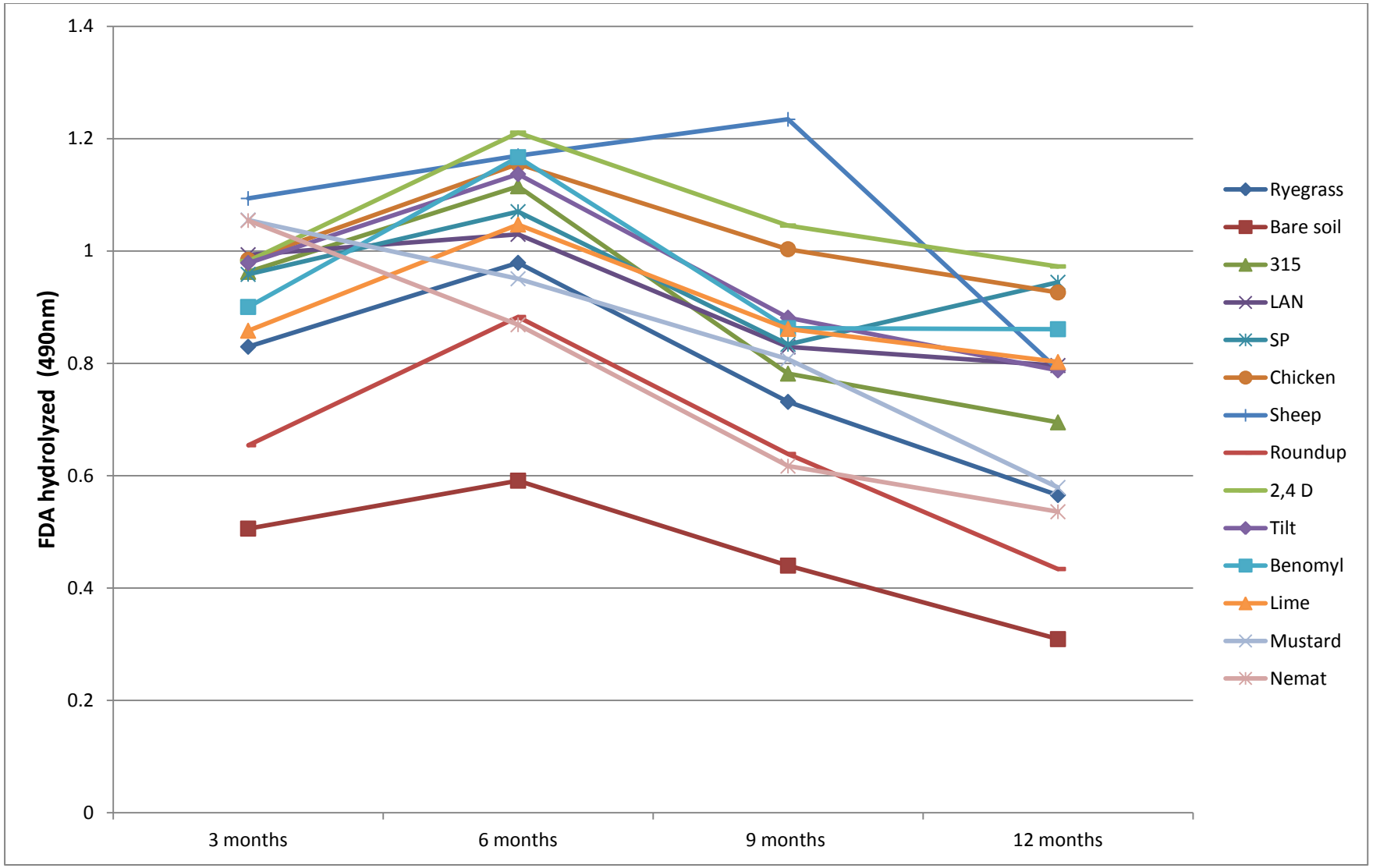

Figure 5. Average FDA hydrolyses of two trials affected by 14 treatments over a 3-12 month planting period.

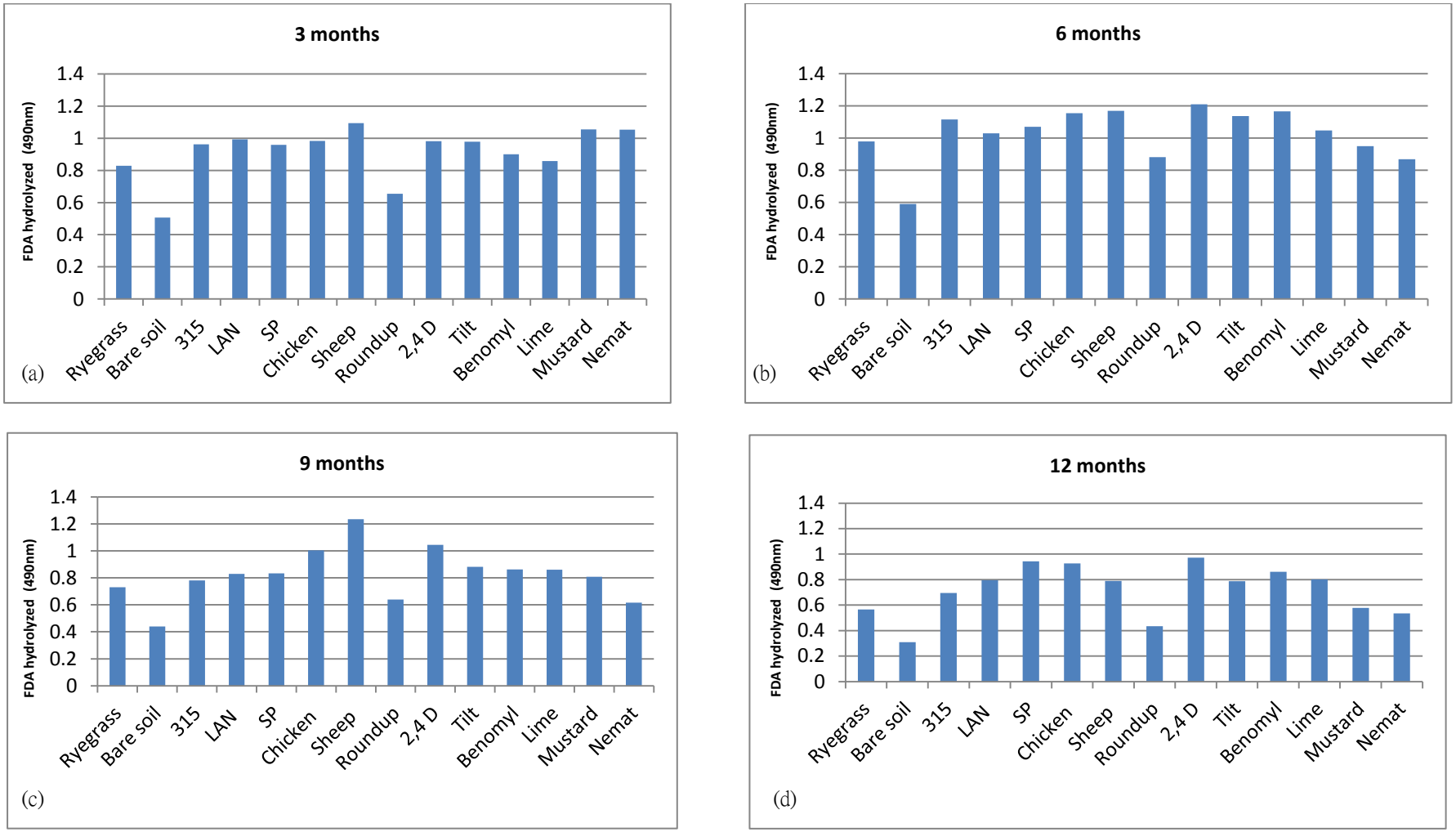

Figure 6. Average FDA hydrolyses of two trials affected by 14 treatments over a 3-12 month planting period.

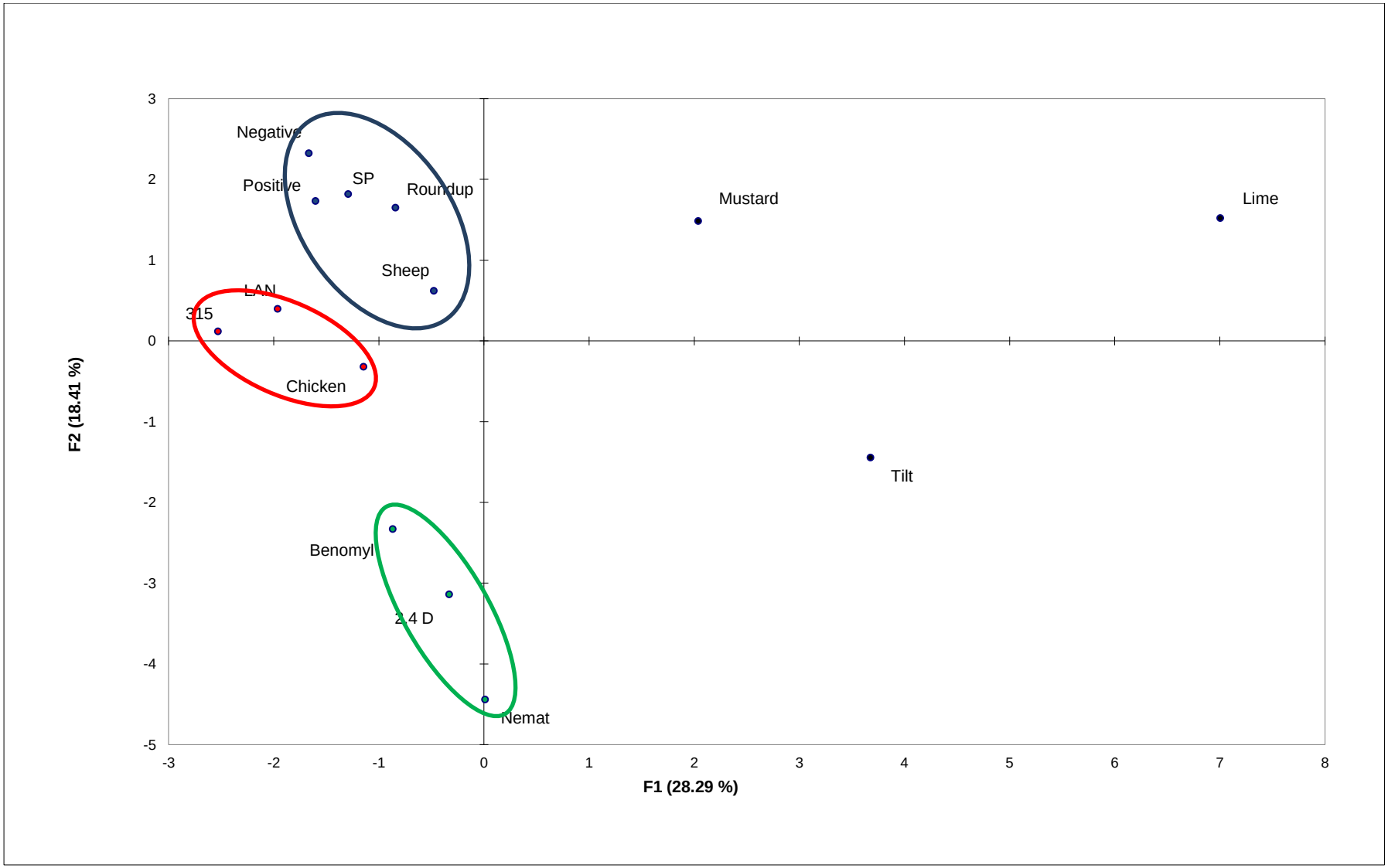

Figure 7. PCA plot of DGGE banding affected by 14 treatments at the end of the 12 months of experiment 1.

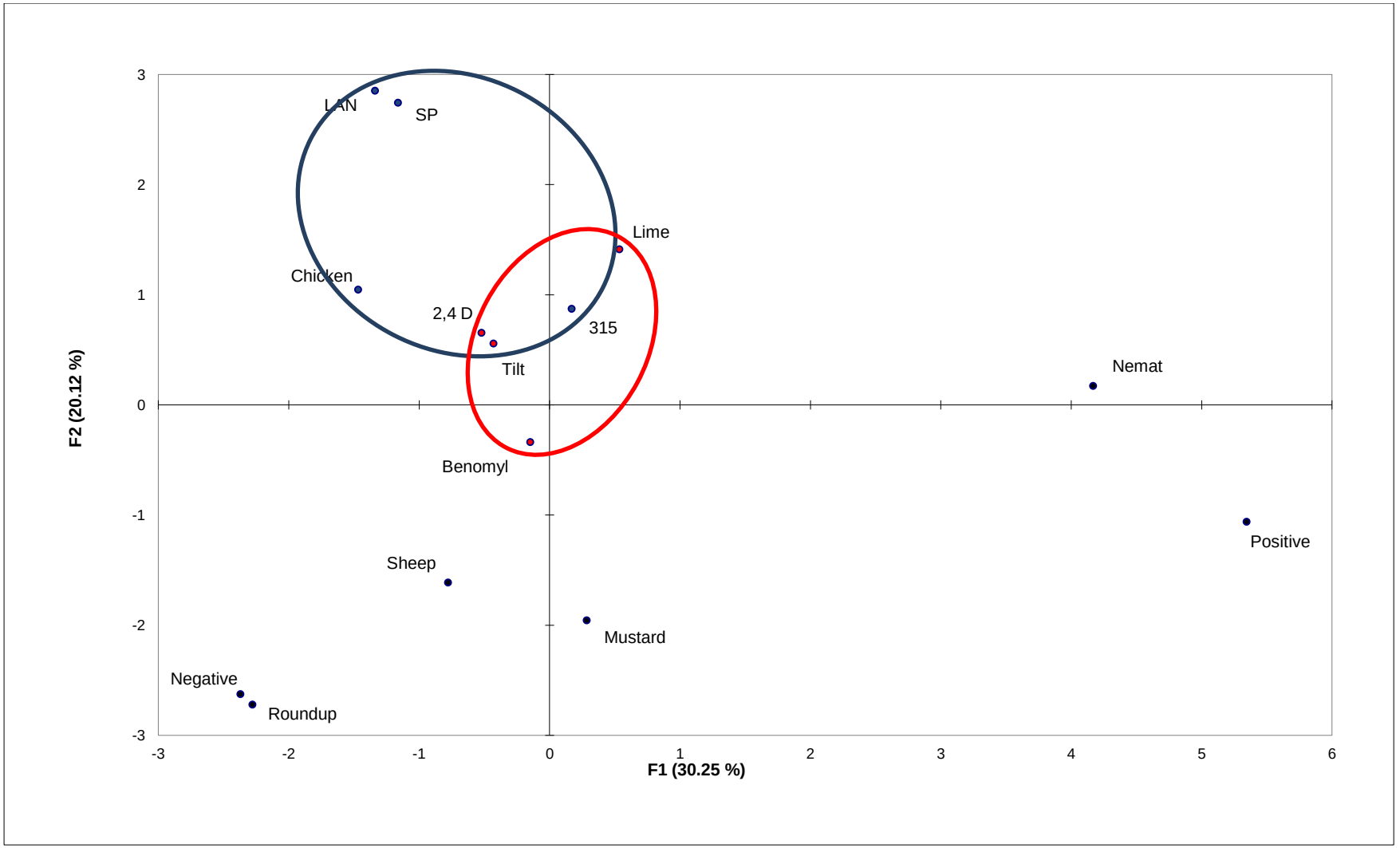

Figure 8. PCA plot of DGGE banding affected by 14 treatments at the end of the 12 months of experiment 2.

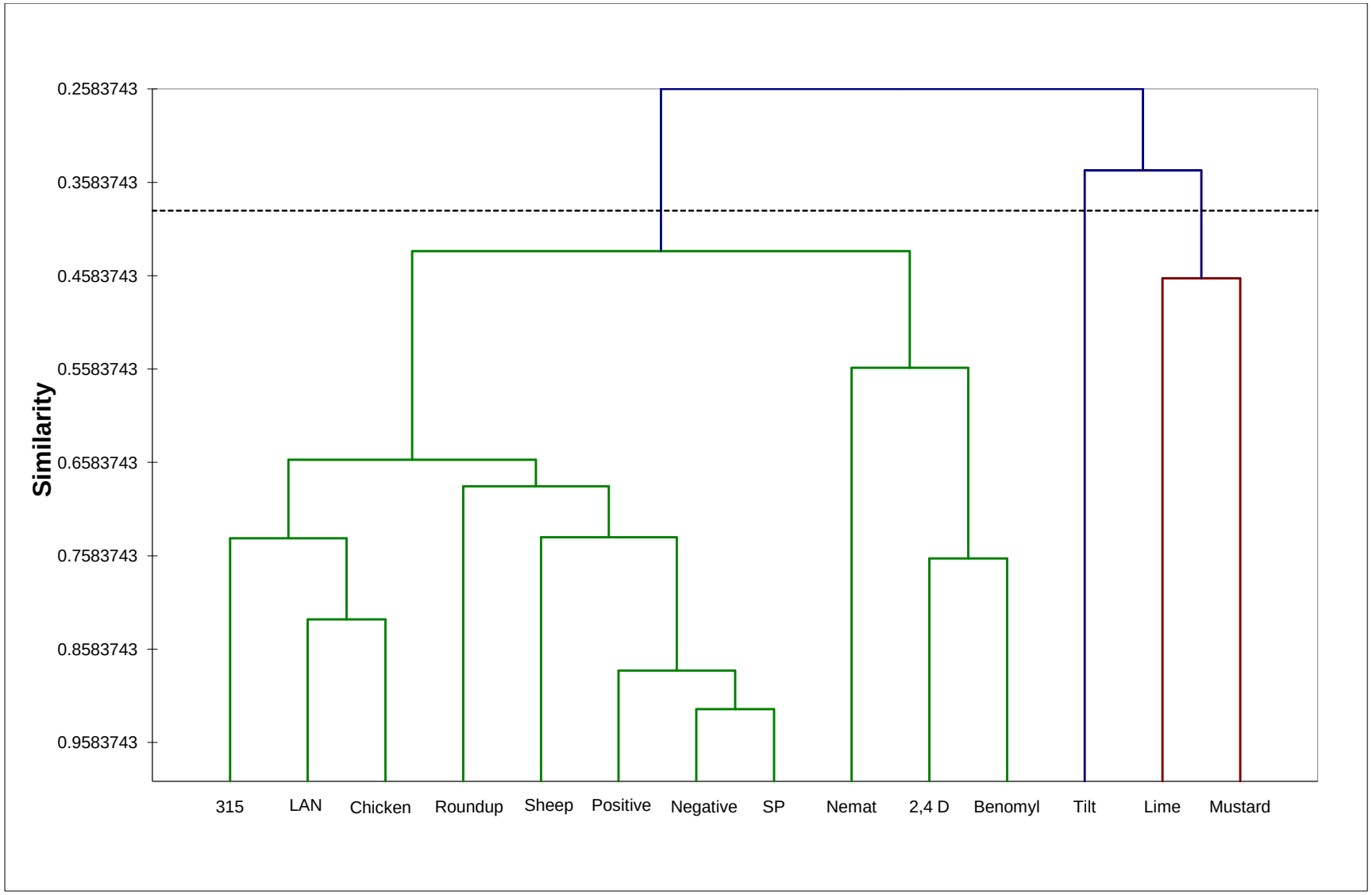

Figure 9. Dendogram of DGGE banding affected by 14 treatments at the end of the 12 months of experiment 1.

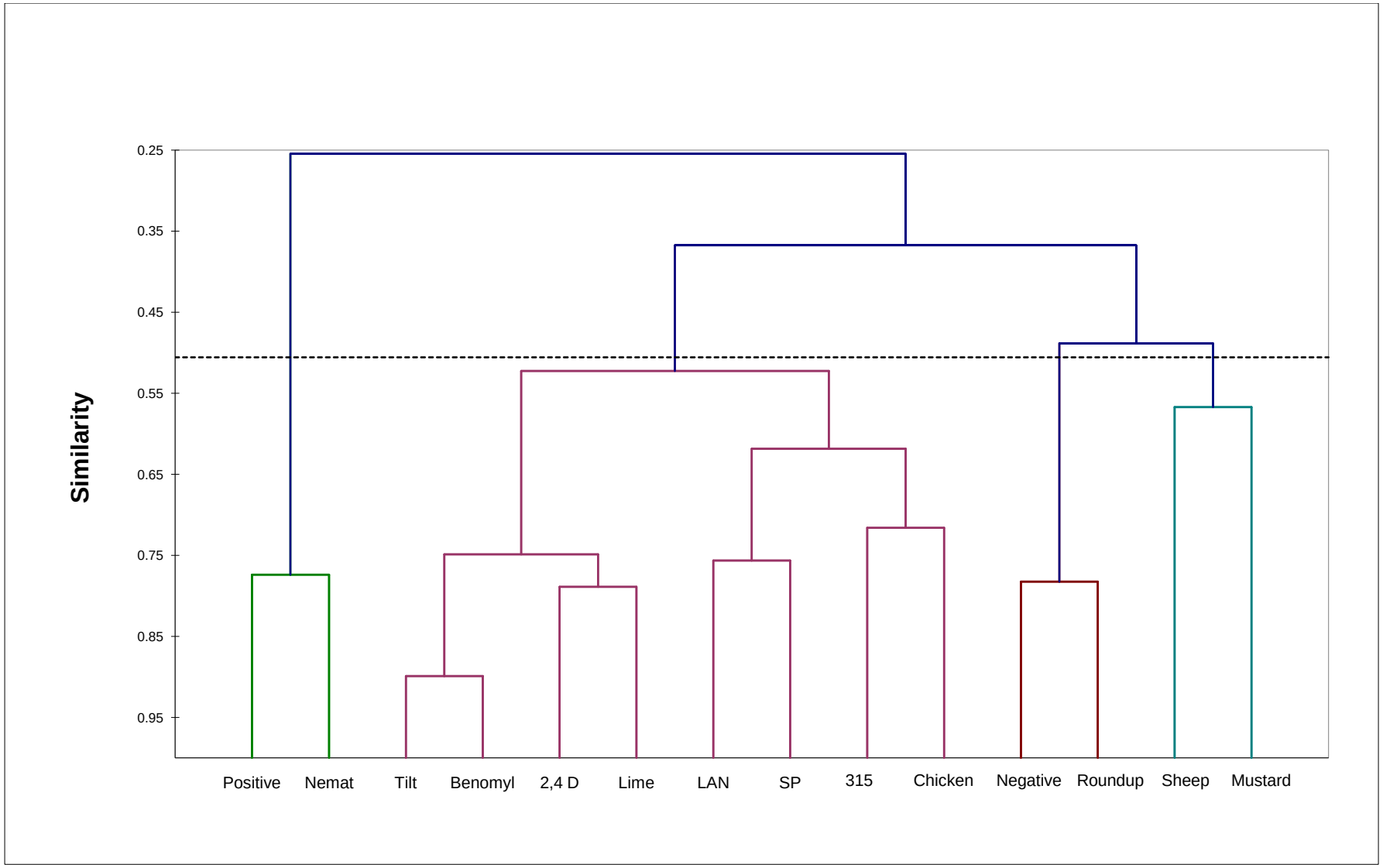

Figure 10. Dendogram of DGGE banding affected by 14 treatments at the end of the 12 months of experiment 2.

CHAPTER THREE

EFFECT OF AGRICULTURAL MANAGEMENT PRACTICES AND HERBICIDE APPLICATION ON SOIL MICROBIAL PROPERTIES IN THE FIELD

ABSTRACT

Changes in microbial properties of soils affected by compaction, different cropping systems and herbicide application were investigated. Soil samples collected from a farm near Wesselsbron (27˚51`S, 26˚22`E) in the Free State were analysed by determining their inherent microbial activity, microbial biomass and microbial diversity. Microbial activity and diversity were determined by means of Biolog Eco™ plate, denaturing gradient gel electrophoresis (DGGE), fluorescein diacetate hydrolysis (FDA), and active C content. Changes in enzyme activity, microbial biomass and microbial diversity were found in all samples compared to control samples. Most microbial activity occurred in soil undergoing crop rotation and previously treated with Roundup. Microbial biomass was highest in soil undergoing crop rotation while least microbial activity occurred in compacted soil to which Roundup had been applied. The lowest microbial biomass was measured in the soil under compaction. Changes in microbial diversity were observed in all soil samples compared to the controls. These results indicate that soil compaction changes in cropping systems and herbicide application can influence soil microbial diversity.

Introduction

Microbes are a vital component of soil due to the crucial role they play in nutrient cycling, toxin removal, decomposition and soil structure (Zhong *et al.*, 2009; Blagodatsky *et al*., 2010; Yao and Shi, 2010; Garcia-Pausas and Paterson, 2011). Due to their sensitivity towards changes in the environment, microbial organisms are often used as an indicator of soil quality and fertility (Bending *et al.*, 2004; Epelde *et al.*, 2008). Changes in the microbial biomass, functional diversity and structure of soil microbial organisms often serve as parameters indicating changes in structure, fertility, quality and microbial metabolism of soil (Bending *et al.*, 2004; Zhang and Wang, 2006).

In the past, studies concerning the effects of management practices on soil biological properties mainly focused on specific soil organisms, microbial biomass and activity. Recently, more attention has been devoted to investigating microbial diversity over a broader range. Agricultural management practices influence the physical, chemical and biological properties of soil, which impact directly on the habitat of soil microbes. Various tillage practices for example have a significant impact on microbial populations by changing the physical structure of soil. Jin *et al.* (2009) investigated in the effect of different tillage practices on the enzyme activity in soil and demonstrated an increase in microbial biomass and yield in soils with no-tillage and mulching compared to conventional tillage and reduced tillage. Microbial diversity is also affected by tillage practices. Diosma *et al.* (2005) compared the effect of conventional tillage and reduced tillage on microbial diversity and found higher microbial diversity in soil undergoing reduced tillage.

Soil compaction is an almost unavoidable factor that effects the physical structure of soil due to the wide use of mechanized farming equipments in conventional agriculture systems. Soil compaction influences soil porosity, water content and respiration, which reduces microbial activity in soil and shifts the microbial community from aerobic to

anaerobic (Whalley, Dumitru and Dexter, 1994; Jensen *et al.*, 1996). In severe cases of soil compaction, the functional ability and enzyme activities mediated by microbial organisms can be greatly decreased (Jordan, Ponder and Hubbard, 2002).

Variation in crop species because of crop rotation, intercropping and cover crops can also alter the physical and chemical properties of soil. This in turn will lead to changes in the functional diversity of microbial communities under different cropping systems (Wang *et al.*, 2006). Gil *et al.* (2010) showed that bacterial and fungal diversity increases in soil undergoing crop rotation compared to soil undergoing tillage treatments. Increases in microbial and enzyme activity also has been observed in multi-cropping and crop rotation systems compared to monocropping systems (Dodor and Tabatabai, 2003; Wang, Wang and Huang, 2007; Chen, Chen and Marschner, 2008).

Agricultural soil amendments have been shown to severely affect soil microbial organisms within an agroecosystem. Applications of chemical fertilizer and organic manures changes the nutrient and pH status in soil, which in turn influences the functional diversity and biomass of microbial organisms (Han, Kemmitt and Brookes, 2007; Wakelin *et al.*, 2007). While both inorganic and organic fertilizers are known to increase microbial biomass due to N and C input into soil, they impact differently on microbial diversity. Wang *et al.* (2008) found no significant changes in microbial diversity with the application of organic manure, but a decrease when chemical fertilizers were applied.

The non-target effect of fungicides, herbicides and pesticides on microbial organisms has always been a topic of interest (Lupwayi *et al.*, 2009). Long-term fungicide and herbicide inputs may result in a reduction of soil processes mediated by microbial organisms. For example the application of Cu fungicides, Captan and benomyl results in a decrease in nutrient cycling, nitrogen dynamics and enzyme activities in soil due to the direct toxic effect of these chemicals on microbes (Chen and Edwards, 2001; Wang, Zhou and Cang, 2009).

Similar non-target effects are reported for the herbicides, glyphosate and 2,4-D, where changes in both microbial activity and community structure were found in herbicide treated soil compared to no herbicide treatment (Zabaloy, Garland and Gómez, 2008; Mijangos *et al*., 2009)

The present study investigated the effect of various agricultural practices on the microbial diversity of an agricultural field belonging to a eutrophic sandy textured Clovelly and Avalon soil forms (Schoeman, 1989). The effects of crop rotation and monocropping, glyphosate application, and soil compaction were the three focus areas of the study. Microbial activity and diversity was evaluated by using various biochemical and molecular techniques.

Materials and Methods

Sample collection

The study was conducted on a farm near Wesselsbron (27˚51`S, 26˚22`E), in the Free State region of South Africa in February, 2010. Wesselsbron experiences continental climate (hot and rainy in summer, cold and dry in winter) with a mean midday to night-time temperature of 18˚C/0˚C in June and 30˚C/15˚C in January. Wesselsbron receives a mean precipitation of about 71mm in January and mean precipitation of about 0mm in July and belongs to the G2 climate zone (Schoeman, 1989; Wesselsbron climate)

Soil samples were collected in a maize cropping system during the growing season from nine sub-sites characterized by different agricultural management practices or herbicide applications (Table 1). Two soil samples at 10 cm and 40 cm from uncultivated grass veld nearby were collected as control samples. Soil was collected from the root zone area at 10 cm depths for all samples with a soil auger except the compacted sample. The compacted soil sample was collected between crop rows adjacent to the M/G samples within ± 3 m

radius at a 10 cm depth. Four replicates of each sample were collected, and replicates were homogenized to create a bulk sample which was then stored at 5˚C prior to analysis. Sub-samples were stored at -80˚C for DNA extraction.

Qualitative analyses

Community linked physiological profile (CLPP): The procedure makes use of Biolog Eco™ plates (Biolog™, USA) and was adapted from Palmroth *et al.* (2004) and Akmal *et al.* (2005). The eleven soil samples (Table 1) were cub-divided into lots of 2 g and placed in flasks containing 200 ml of deionized distilled water. The flasks were shaken for 30 min on a rotary shaker at 90 rpm, and 95 ul of each suspension was pipetted into each of the 96 wells in the Biolog Eco™ plate. The Eco™ plates were then incubated at 25 ºC for 6 days and then evaluated at 595 nm with an absorbance microplate reader (ELx808™, Biotek). The data were first analyzed by determining average well colour development (AWCD) and carbon group utilization. This AWCD value represents the overall metabolic activity of soil bacterial community utilization of 31 carbon sources in the Biolog Eco™ plate. Principle component analysis (PCA) and cluster analysis was then performed on the data to determine which factors affected microbial diversity compared to the control samples. The PCA and cluster analyses were done by using the statistical program XLSTAT (Version 2011.4.03, Addinsoft®).

PCR-DGGE (Polymerase chain reaction-denaturing gradient gel electrophoresis): Extraction of DNA from soil was done by using the PowerSoil™ DNA Isolation Kit. DNA was extracted from 0.25 g of soil and the genomic DNA samples were checked by running them on a 1 % agrose gel stained with ethidium bromide with UV illumination. The concentration of the respective DNA samples was determined by using a Nano Drop

spectrophotometer (3300 fluorospectrometer, Thermo Scientific) before conducting the 16S rDNA Polymerase Chain Reaction (PCR).

Following DNA extraction, the 16S rDNA PCR amplification of the extracted DNA was performed by using the standard protocol (SOP_29) developed by the Metagenomics Platform laboratory of the University of the Free State, Bloemfontein. The protocol makes use of the 16S primers 27F and 1492R which are bacteria specific (Lane *et al.*, 1991). The 16S PCR mixture consist of 10 uM of each primer set, 10 uM of dNTPs, 1-50 ng of genomic DNA and 0.125ul Taq DNA polymerase (SuperTherm) with the final reaction mixture volume of 50 ul. The 16S PCR was performed with an initial denaturing step of 95 ˚C for 2 min followed by 30 cycles of amplification. The cycles ran at 95 ˚C for 30 seconds, 52 ˚C for 45 seconds, 72 ˚C for 1 min and a final extension step of 72 ˚C for 10 min. The final products of the PCR were checked by running them on a 1 % agrose gel stained with ethidium bromide with UV illumination. Gel stabs were then performed on the 16S rDNA PCR products to collect templates for further analysis. Amplification was performed by using the same PCR program as the 16S PCR with a forward primer, 341F-GC and reverse primer, 517R (Muyzer *et al.*, 1997).

DGGE analysis was performed in the laboratories of the Metagenomics Platform (Department of Microbial, Biochemical and Food Biotechnology, University of the Free State) using a DCode™ Universal Mutation Detection System (Biorad). The DGGE PCR amplifications were verified on a 1 % agarose gel electrophoresis 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 the polyacrylamide gel (Tezenva *et al.*, 2008). The analysis was carried out according to the standard procedure developed in the Metagenomics Platform laboratory (SOP_19b). The results were viewed by staining with ethidium bromide and visualized under UV

transillumination. The banding profile of each sample was then quantified and compared by using Quantity One® 1-D analysis imaging software (Biorad Molecular Imager Gel Doc™ XR System) to determine relatedness (SOP_66) (Green, Leigh and Neufeld, 2009). PCA and cluster analysis was conducted to determine possible relationships among treatments. The PCA and cluster analysis was conducted with the statistical program XLSTAT (Version 2011.4.03, Addinsoft®).

Quantitative analyses

FDA (Flourescein diacetate) analysis: This procedure was adapted and modified from Schnürer and Roswell (1982). A stock solution was first prepared by dissolving FDA in acetone at a concentration of 2 mg/ml and stored at -20 ºC. A buffer solution of 60 mM sodium phosphate solution was prepared, the buffer solution adjusted to pH 7.6 and stored at 4 ºC. Soil samples of 0.5 g were added to 20 ml of the buffer followed by 0.2 ml of the FDA solution. The mixture was then placed in a rotary shaker at 90 rpm for an hour. After shaking, the soil solutions were placed in 2 ml eppendorf tubes on ice to stop the FDA from being hydrolyzed further. The eppendorf tubes were then centrifuged at 6000 rpm to remove excess debris. Supernatants were then analyzed in cuvettes (1 ml) at 490 nm using a spectrophotometer (CARY Bio 100 UV-Visible Sectrophotometer).

Active soil carbon (C) content: This technique was adapted and modified from Weil *et al.*, (2003). Active soil C content was determined by permanganate oxidation, which represents microbial biomass and respiration in soil. A stock solution of 0.2 M $KMnO_4$ was first prepared by using 0.1 M $CaCl_2$ solution as a buffer and stored in a dark bottle away from sunlight at room temperature. Soil samples were first dried and 2.5 g were added to 20 ml of 0.02 M $KMnO_4$. The soil suspension was then shaken at 120 rpm for 2 min with a rotary

shaker and rested for 8min. After the soil settled, 0.5 ml of the supernatant was placed in 49.5 ml of water to dilute the colour of the $KMnO_4$. The diluted solutions were then measured with a spectrophotometer (CARY Bio 100 UV-Visible Sectrophotometer) at 550 nm.

Results

Qualitative analyses

CLPP: Average well colour development (AWCD): No clear differences could be discerned between samples (Figures 1 and 9). The highest AWCD value in soil treated with Roundup was soil treated with Roundup 3 month prior sampling (W/M Roundup), and the lowest AWCD in soil treated with Roundup ± 1 month prior sampling (M/Roundup) (Figure 1). The highest AWCD value in soils undergoing rotation and compaction was the control B, and the lowest was M/Fallow (Figure 9). There were no significant differences between the control samples (Control A and Control B) and treated soils.

CLPP: Carbon source utilization: Carbon source utilization reflected clear differences between management practices on the farm (Figures 2 and 10). The most utilized carbon source group was the amino acids and the least utilized group was phosphate carbon. Differences in carbon group utilization were seen in soils treated with Roundup (Figure 2), with consistent decreases in both phosphate carbons, the amines evident (M/Roundup, W/M Roundup) compared to the non treated soil (M/No Roundup, W/M No Roundup). Carboxylic acid utilization was higher for both Roundup treated soils compared to soils not treated with Roundup.

Differences in carbon group utilization were also seen in soil undergoing rotation and compaction (Figure 10). Carbon group utilization was found to be higher in all carbon groups of the maize monocrop treatment (M/Mono) compared to the maize-fallow soil

(M/Fallow) with the exception of the amine carbon group. Differences are also evident between both control samples (Control A and Control B) and several other treatments in all 6 carbon group utilization patterns.

CLPP: PCA and dendogram cluster analysis: PCA plots of the Biolog data showed distinctive differences in soil microbial diversity between treatments (Figures 3 and 11). The PCA plot of soil treated with Roundup indicated that controls A and B had no relationship with the other four treatments (Figure 3). A close relationship was evident between M/Roundup and M/No Roundup, which were undergoing the same rotation. In the PCA plots of soil treated with Roundup, M/Roundup was found to be the major factor affecting soil microbial diversity (Figure 3). A high similarity was also found between both control samples and the W/M Roundup sample. Cluster analysis also showed that microbial diversity varied significantly between treatments (Figure 4). No relationships in microbial diversity were seen between both M/Roundup and W/M Roundup treated soils with the M/No Roundup and W/M No Roundup soils (Figure 4).

The PCA plots of soils from different rotations and compacted soils indicated that Control A had no relationship with the other treatments, while Control B was found to be related to M/G Compaction and G/M samples (Figure 11). No relationship in microbial diversity was seen between M/Fallow and M/Mono soil samples. In the cluster analysis of soil undergoing rotation and compaction M/Mono was found to be the major factor affecting soil microbial diversity (Figure 12). A high similarity was also found between the samples M/G and M/G Compaction.

Microbial diversity assessed by PCR-DGGE: Statistical analysis showed distinct differences in microbial diversity as affected by the different management practices. Control samples (Control A and Control B) were not related to any treatments (Figures 5 and 13). In

the PCA plot of soils treated with Roundup, no clear groups can be identified (Figure 5). Soil applied with Roundup 3 months prior to sampling (W/M Roundup) and soil without Roundup (W/M No Roundup) showed a distinct difference in microbial diversity. However the soil treated with Roundup ± 1 month before sampling (M/Roundup) and soil without Roundup (M/No Roundup) showed a close relationship between their microbial diversity. Cluster analysis on Roundup treated soils showed no relationship between both control samples (Control A and Control B) and the treated samples except soil undergoing wheat/maize rotation (W/M No Roundup) (Figure 6). Soil treated with Roundup 3 months prior sampling (W/M Roundup) showed no relationship compared to soil without Roundup undergoing the same rotation (W/M No Roundup).

In the PCA plot of soils undergoing crop rotation and compaction, a cluster which contains all soils planted with maize and not undergoing rotation (M/Mono, M/Fallow) as well as compacted soil (M/G Compaction) was evident (Figure 13). Compaction was also found to affect microbial diversity. Soil without compaction (M/G) had no relationship in terms of microbial diversity with the compacted soil (M/G Compaction). Results of the cluster analysis on soils undergoing crop rotation and compaction showed that all treatments had no relationship with both control samples (Control A and Control B) (Figure 14). A compaction effect between compacted soil (M/G Compaction) and the adjacent within crop row soil (M/G) was also observed; the microbial diversity of the two samples showed no relationship with each other.

Quantitative Analyses

Microbial enzyme activity assessed by FDA: Soil tests using FDA hydrolysis showed distinct differences in soil treatments compared to both control samples (Figures 7 and 15). Lower enzyme activity was observed in Roundup treated samples compared to Control A,

except for W/M Roundup and W/M No Roundup samples (Figure 7). The highest enzyme activity was W/M Roundup and the lowest M/Roundup and Control B. A lower enzyme activity was found in soil that was applied with Roundup just before sampling (M/Roundup) compared to soil with no Roundup (M/No Roundup). However, a higher enzyme activity was found in soil treated with Roundup 3 months before sampling (W/M Roundup) compared to soil with no Roundup (W/M No Roundup).

Enzyme activity in soils undergoing crop rotation and compaction was highest in soil undergoing maize/groundnut rotation (M/G) and the lowest in compacted soil (M/G Compaction) and Control B (Figure 15). Enzyme activity was higher in soil planted with maize undergoing maize/groundnut rotation (M/G) compared to soil planted with groundnut undergoing a groundnut/maize rotation program (G/M). Greater enzyme activity was also observed in soil undergoing a 3-year maize monocrop system (M/Mono) compared to soil following a maize/fallow rotation (M/Fallow). Compaction was as an important factor influencing enzyme activity. In-between row soil samples (M/G Compaction) displayed a distinctively lower enzyme activity than within the crop rows (M/G).

Microbial biomass assessed by measuring biologically active soil carbon: Microbial biomass of soil samples revealed distinct differences between treatments. Fluctuations in microbial biomass were observed in control samples compared to most of the other samples (Figures 8 and 16). Microbial biomass was highly affected by the application of Roundup (Figure 8). The sample with the highest microbial biomass was W/M No Roundup and the lowest was W/M Roundup. Sample M/Roundup, where Roundup was applied ± 1 month before sampling, showed no difference in active C content compared to soil without Roundup (M/No Roundup).

Microbial biomass was also was influenced by compaction (Figure 16). In soils undergoing crop rotation and compaction, the highest microbial biomass was in

maize/groundnut rotation (M/G) and the lowest in compacted soil which was collected from the crop row adjacent to the M/G sample (M/G Compaction). Crop type and fallow soils also influenced microbial biomass (Figure 16). Microbial biomass was lower in soil planted with groundnut (G/M) than soil planted with maize (M/G). Microbial biomass was also lower in the maize monocrop treatment (M/Mono) than the soil collected from a maize/fallow rotation treatment (M/Fallow).

Discussion

In this study, our objective was to identify the effect of soil management practices and herbicide application on the microbial status of soil in a maize cropping system. Results indicated changes in microbial diversity, biomass and activity as affected by rotation, soil compaction and the application of Roundup. Variation in the status of soil microbial organisms can be associated with the physical and chemical changes due to soil management protocols (Diosma *et al.*, 2005; Jin *et al.*, 2009). Similar studies showed that these changes may ultimately lead to reduced soil fertility and crop production. Li *et al.* (2008) closely related microbial biomass with the soil organic carbon and soil total nitrogen and suggested that microbial biomass may be used as an indicator of soil fertility. Similarly, García-Orenes *et al.* (2010) related microbial biomass to active carbon to study the effects of certain agricultural management systems on soil and indicated a decrease in soil quality following the application of herbicides.

The effect of glyphosate on microbes in soil has always been a topic of considerable interest, since glyphosate can either increase or decrease microbial populations in soil (Araújo, Monteiro and Abarkeli, 2003; Mijangos *et al.*, 2009). The present study showed clear differences following 1 month pre-application of glyphosate (M/Roundup) compared to 3 month pre-application (W/M Roundup). Enzyme activity (FDA) and microbial biomass

(Active C) were lower in soils treated with Roundup. This is inconsistent with studies showing glyphosate to enhance microbial activity by serving as a nutrient source (Araújo, Monteiro and Abarkeli, 2003). It is possible that the lower enzyme activity observed is caused by the removal of certain functional groups of microbes due to a non-target effect of the herbicide (Mijangos *et al.*, 2009). Our results are however consistent with the findings of Lupwayi *et al.* (2009) who showed no significant differences in both microbial activity and microbial biomass following the application of glyphosate and 2,4-D herbicides to soil but instead changes in microbial diversity were observed.

Microbial diversity and community structure assessed by Biolog Eco™ plate and PCR-DGGE data on the other hand showed some relationship between immediate Roundup application and no Roundup. This difference in microbial diversity in Roundup treated soil may be also caused by the non-target effect of the herbicide. By using similar techniques, studies have shown that glyphosate has the effect of removing certain groups of microbial organisms from soil (Ratcliff, Busse and Shestak, 2006; Zabaloy, Garland and Gómez, 2008). By using Biolog Eco™ plates and PCR-DGGE, Mijangos *et al.* (2009) showed that the application of glyphosate significantly changed the community structure of soil microbial organisms.

In the present study, compaction seems to play an important role in affecting microbial biomass, enzyme activity and microbial community structure. Both microbial biomass and enzyme activity were lower in compacted soils compared to the adjacent maize/groundnut planted soil. Slight changes in carbon utilization patterns were also observed between compacted and non-compacted samples. This difference in the biological status of soil can be associated with changes in its physical structure due to compaction. Compacted soil decreases habitable micro-pores and the macro-pores in soil, which in turn affects the aeration status of soil and inhibits aerobic microbial organisms (Jensen *et al.*,

1996). A reduction in soil porosity also influences soil water infiltration and soil water content, which may indirectly influence microbial organisms (Pengthamkeerati *et al.*, 2005). Our results correspond with the findings of Li, Allen, and Wollum II (2003), who reported a decreased microbial C-to-N ratio in soil undergoing compaction, indicating a decrease in the total soil bacterial population.

Microbial diversity as assessed by Biolog Eco™ plate and PCR-DGGE, showed contradicting results in terms of compaction. The relationship between carbon utilization patterns of both sets of soil samples was expected since samples were collected adjacent to each other. However the community structure depicted by DGGE fingerprints of the compacted soil seems to have no relationship with that of the adjacent field soil. Soil compaction has previously been shown to influence C/N ratio in soil and decrease N mineralization (Breland and Hansen, 1995; Nevens and Reheul, 2002; Li, Allen, and Wollum II, 2003). The variation observed in microbial diversity could therefore be associated with a lack of available C/N in the compacted soil in addition to a lack of plant cover on the compacted area. Similar results are reported by Pengthamkeerati, Motavalli and Kremer (2011), who indicated variation of microbial functional diversity in soil under severe compaction.

The present study indicated that changes in cropping systems can distinctively affect soil microbial activity, biomass and diversity. Soil biological properties affected by maize-groundnut crop rotation, maize/fallow rotation and maize monocropping were compared. Crop rotation in general had a higher enzyme activity and microbial biomass than the maize/fallow rotation and maize monocrop treatments. This corresponds to the findings of several other authors (Seiter, Ingham, and William, 1998; Dodor and Tabatabai, 2003; Gil *et al.*, 2010), where increases in the microbial status of soil undergoing crop rotation were compared to monocropping systems. Higher microbial activity observed in soil undergoing

crop rotation could be caused by specific compounds produced by roots of the crop in the rhizosphere and an increase in soil organic matter which serves as additional food sources that enhance microbe development (Gil *et al.*, 2010). Lower microbial activity and biomass in soil undergoing monocropping and fallow rotation systems compared to crop rotation systems are well known (Chen, Chen and Marschner, 2008; González-Chávez *et al.*, 2009). Monocropping systems are known to degrade soil physical and chemical properties, which can directly affect soil microbial status (Lal, 1997; Nevens and Reheul, 2002; Chen *et al.*, 2009). Acosta-Martínez, Mikha and Vigil (2007) also showed a negative effect of wheat-fallow rotation systems on microbial community structure and biomass during the fallow period of the rotation system.

Although no relationships were found between treatments in terms of CLPP of the soil, PCR-DGGE showed a close relationship between the maize/fallow and maize/monocrop treatment. Differences in carbon utilization patterns between the cropping systems was expected since crop rotation has been found to improve soil organic matter, while monocrop systems deplete soil organic matter (Masri and Ryan, 2005). Soil undergoing crop rotation contains enhanced nutrients and other plant residues from the alternate crop, which positively affects microbial community structure (Moore, Klose and Tabatabai, 1999). Fallow soils and monocrop systems have a negative impact on microbial community and diversity due to reduced nutrient cycling compared to crop rotation systems (Acosta-Martínez, Mikha and Vigil, 2007). The present study is consistent with the findings of Chen, Chen and Marschner (2008), who observed changes in microbial community and microbial biomass in soil undergoing legume rotation compared to soil undergoing a monocrop system.

In conclusion, microbial community structure was clearly affected by soil management practices and herbicide application in the present study. Although many trends observed are not clear due to the single point nature of the field sampling technique, the effect

of practices such as the application of glyphosate herbicide, soil compaction and monocropping are distinct. To fully understand these dynamics in soil microbial status, further studies on individual factors should be conducted in the field and in microcosms. The information acquired will hopefully add to the knowledge base of the effect of anthropogenic interference on soil ecosystems.

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Table 1. Soil samples collected from soils near Wesselsbron

Sample code	**Sample details**
Roundup treatments	
M/Roundup	Maize, Roundup applied within ± 1 month
W/M Roundup	Wheat on maize rotation, Roundup applied ± 3 months prior to sampling
M/No Roundup	Maize, no Roundup applied for previous 5 years
W/M No Roundup	Wheat on Maize rotation
Rotation and compaction	
M/G	Maize on groundnut rotation
M/G Compaction	Maize on groundnut rotation, in between row (compaction)
G/M	Groundnut on maize rotation
M/Fallow	Maize fallow rotation (3 rotations)
M/Mono	3yr Maize monocrop
Control treatments	
Control A	Veld sample (10-15cm)
Control B	Veld sample (40 cm)

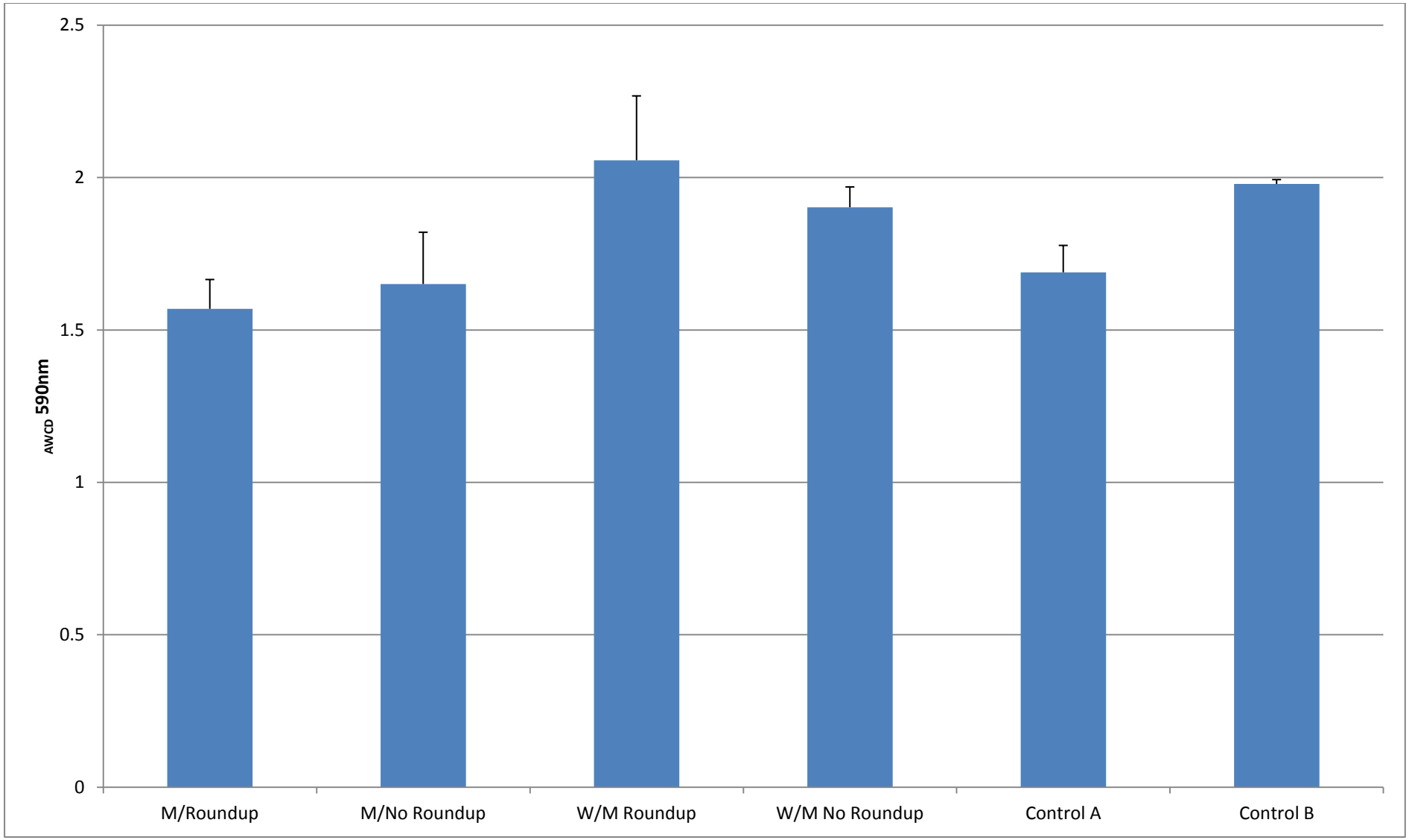

Figure 1. Biolog Eco™ plate data (AWCD) comparing Roundup Vs no-Roundup treatments.

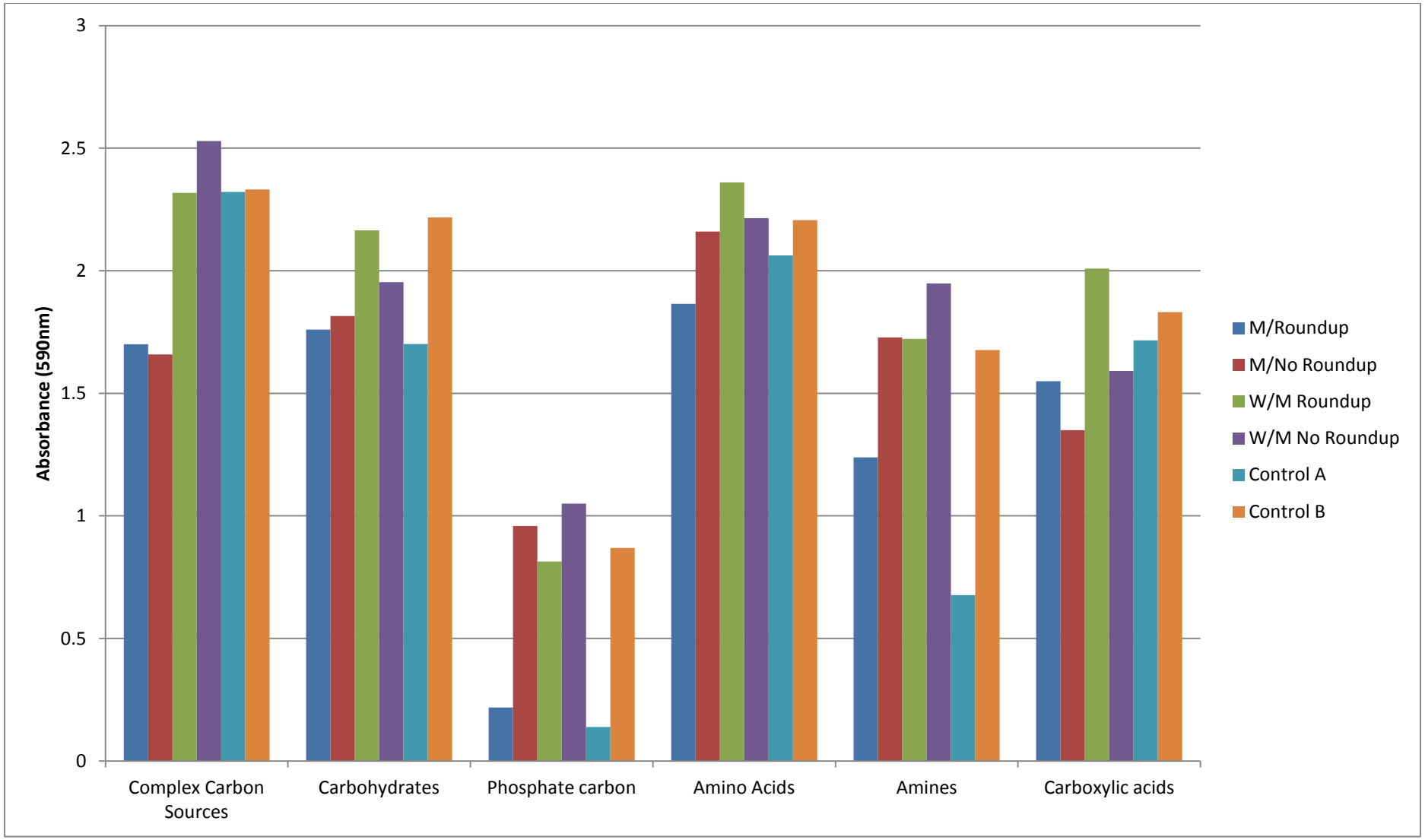

Figure 2. Biolog Eco™ plate data (carbon group utilization) comparing Roundup Vs no-Roundup treatments.

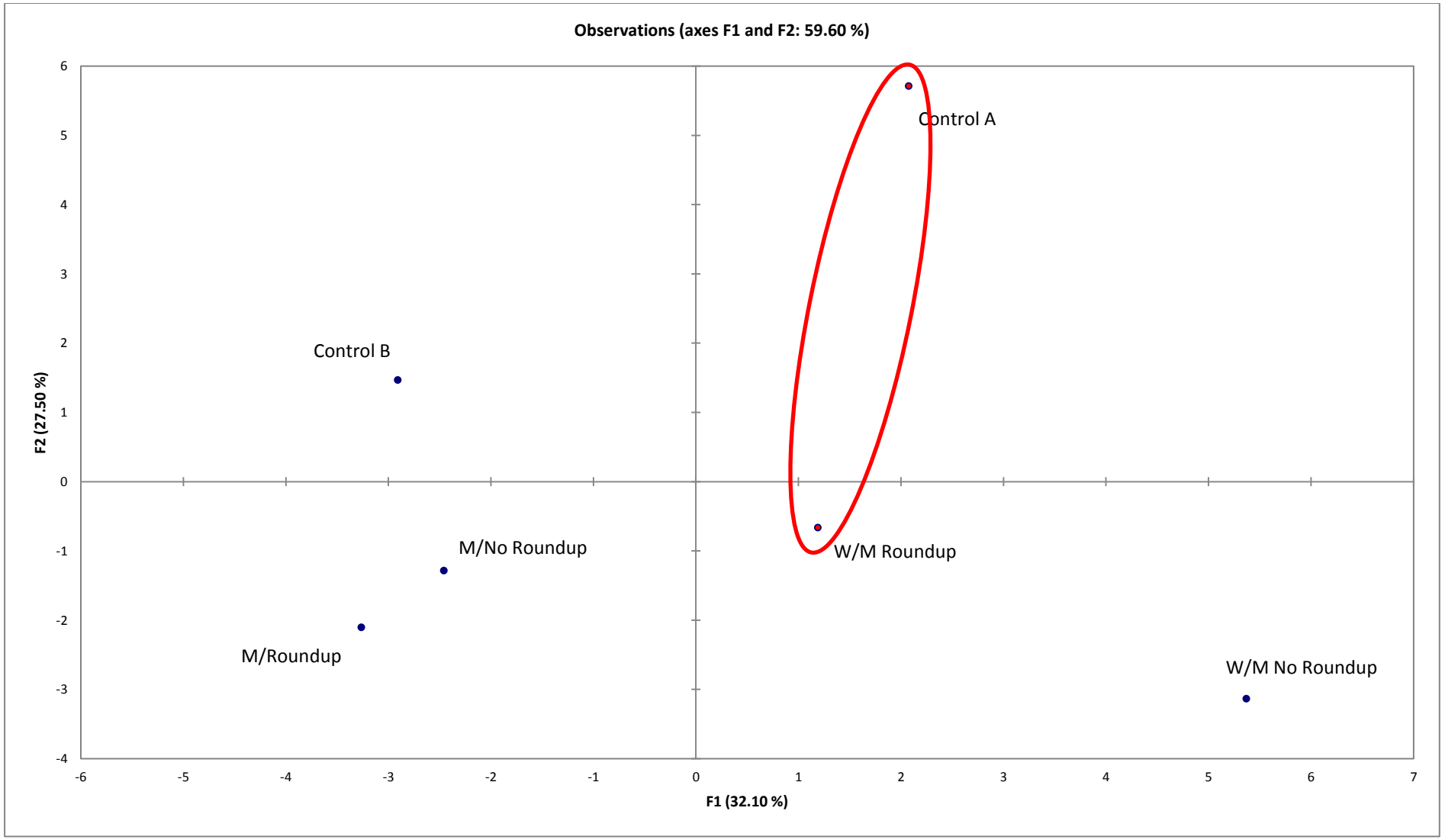

Figure 3. Biolog Eco™ plate data (PCA) comparing Roundup Vs no-Roundup treatments.

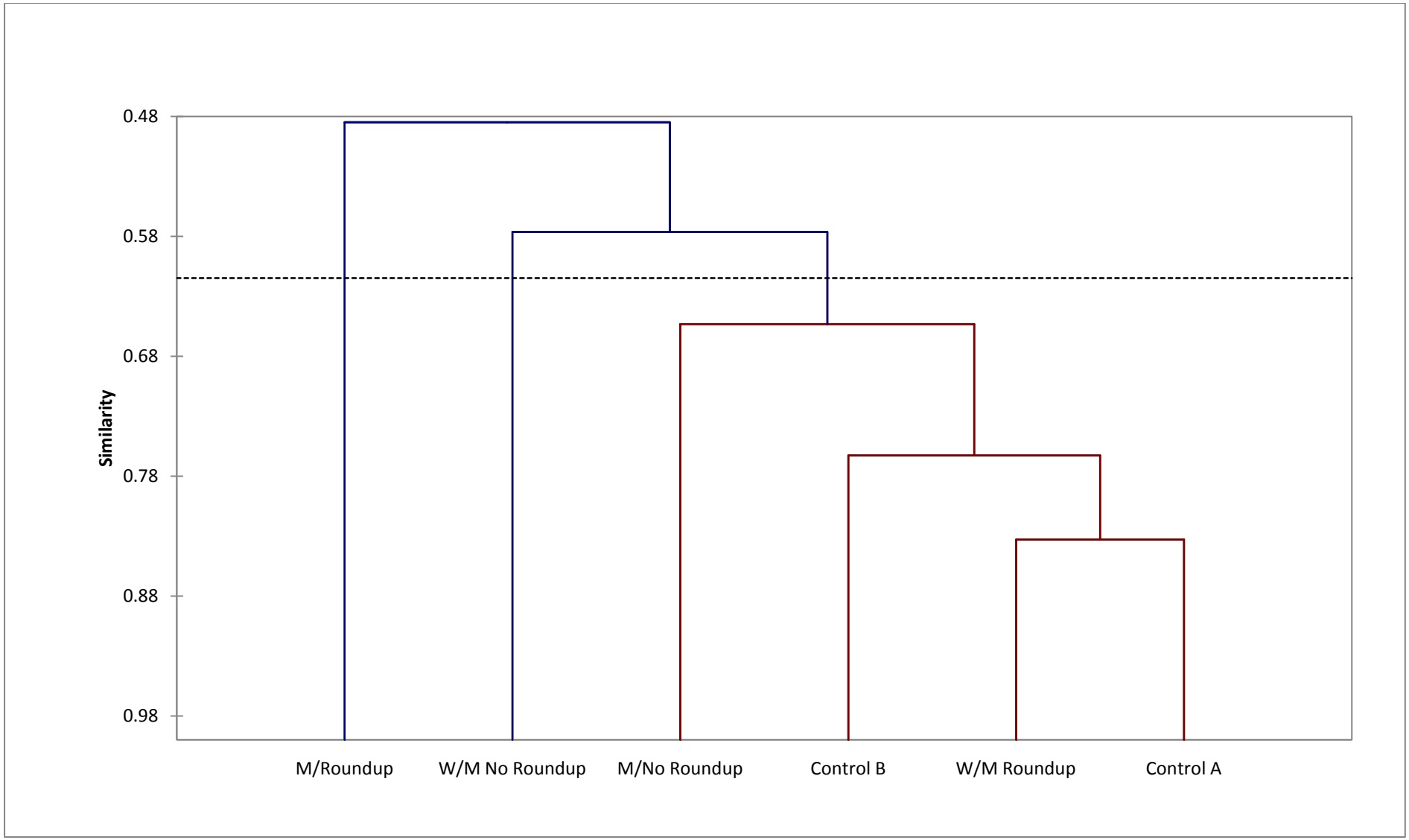

Figure 4. Biolog Eco™ plate data (dendogram) comparing Roundup Vs no-Roundup treatments.

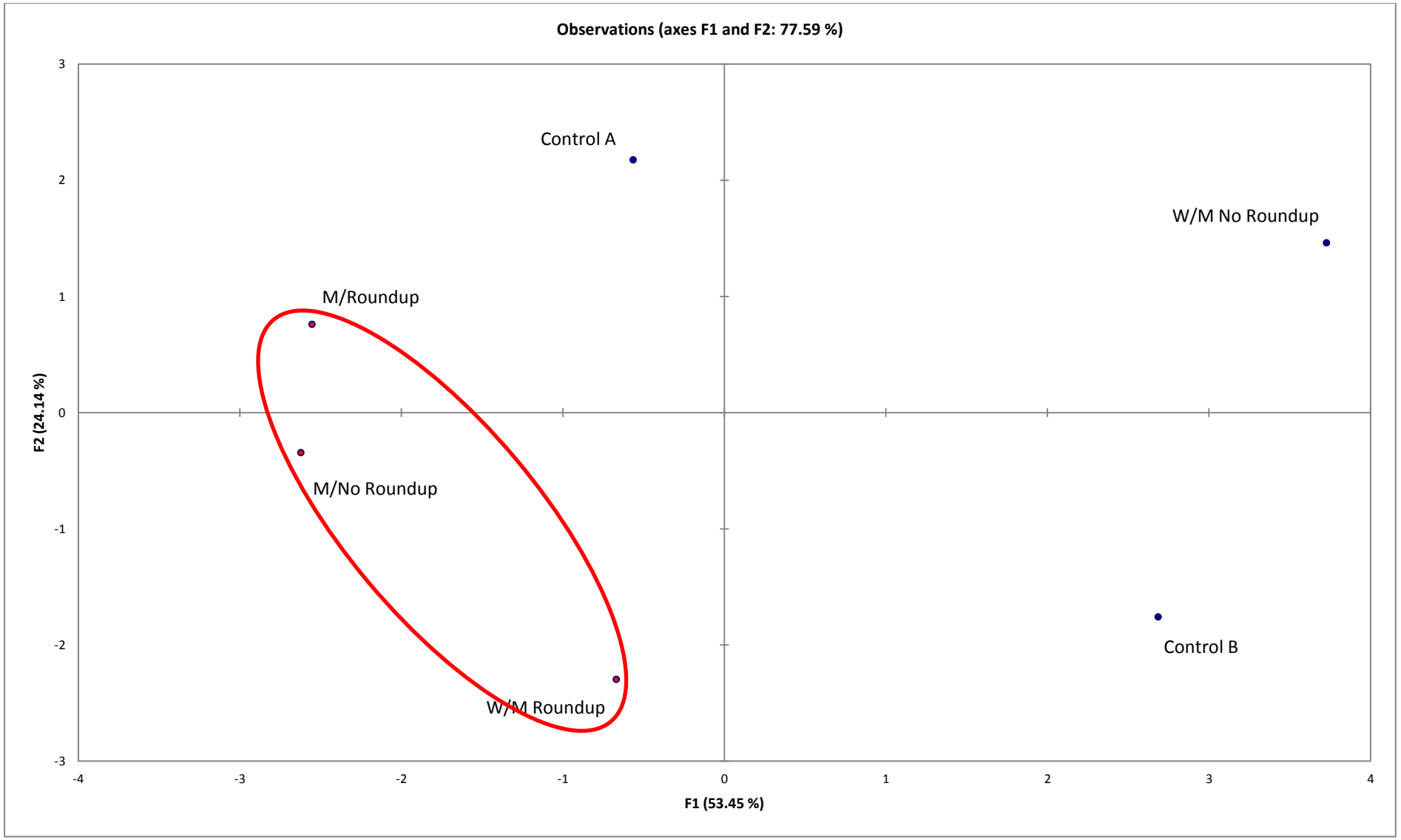

Figure 5. PCR-DGGE data (PCA) comparing Roundup Vs no-Roundup treatments.

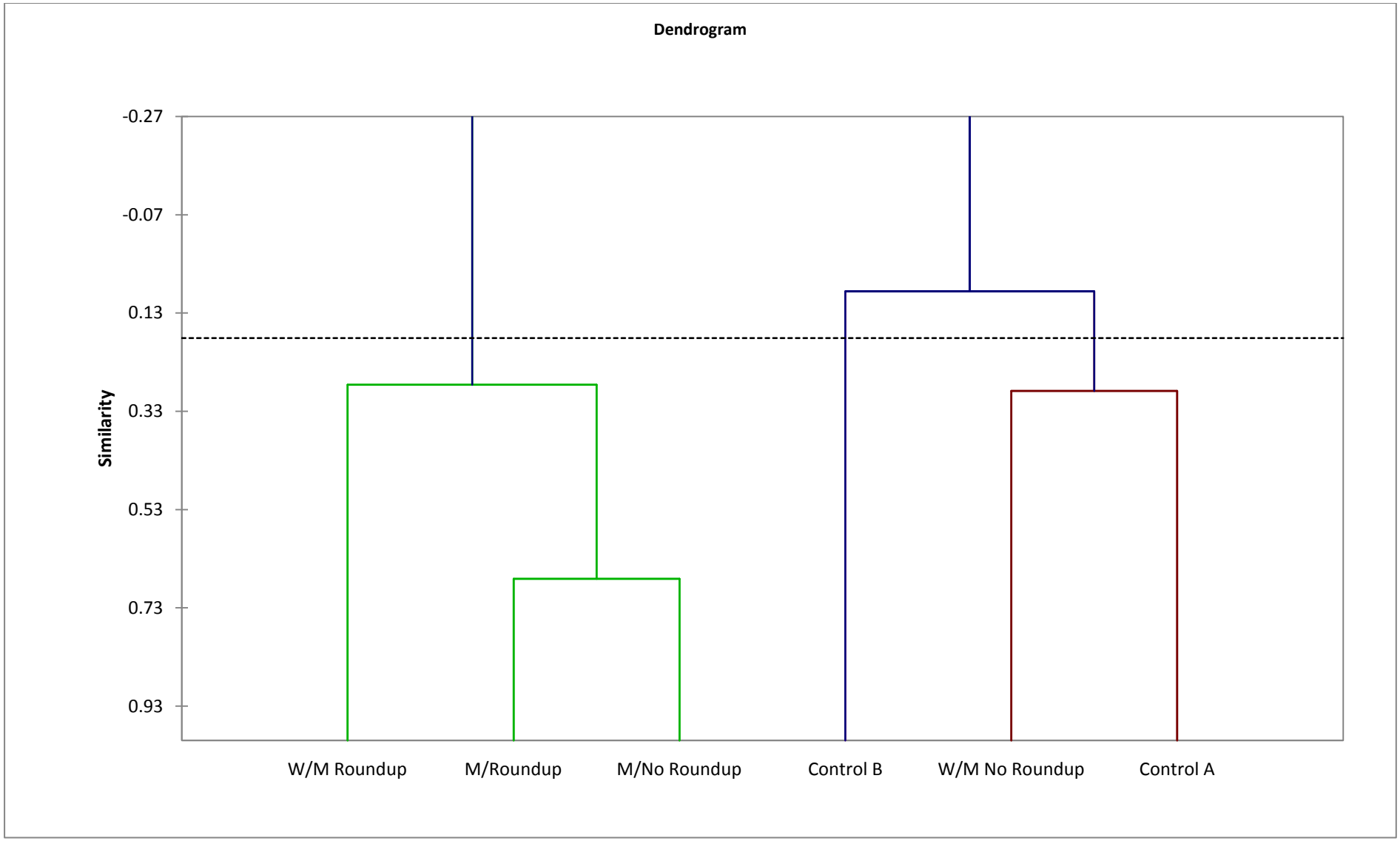

Figure 6. PCR-DGGE data (dendogram) comparing Roundup Vs no-Roundup treatments.

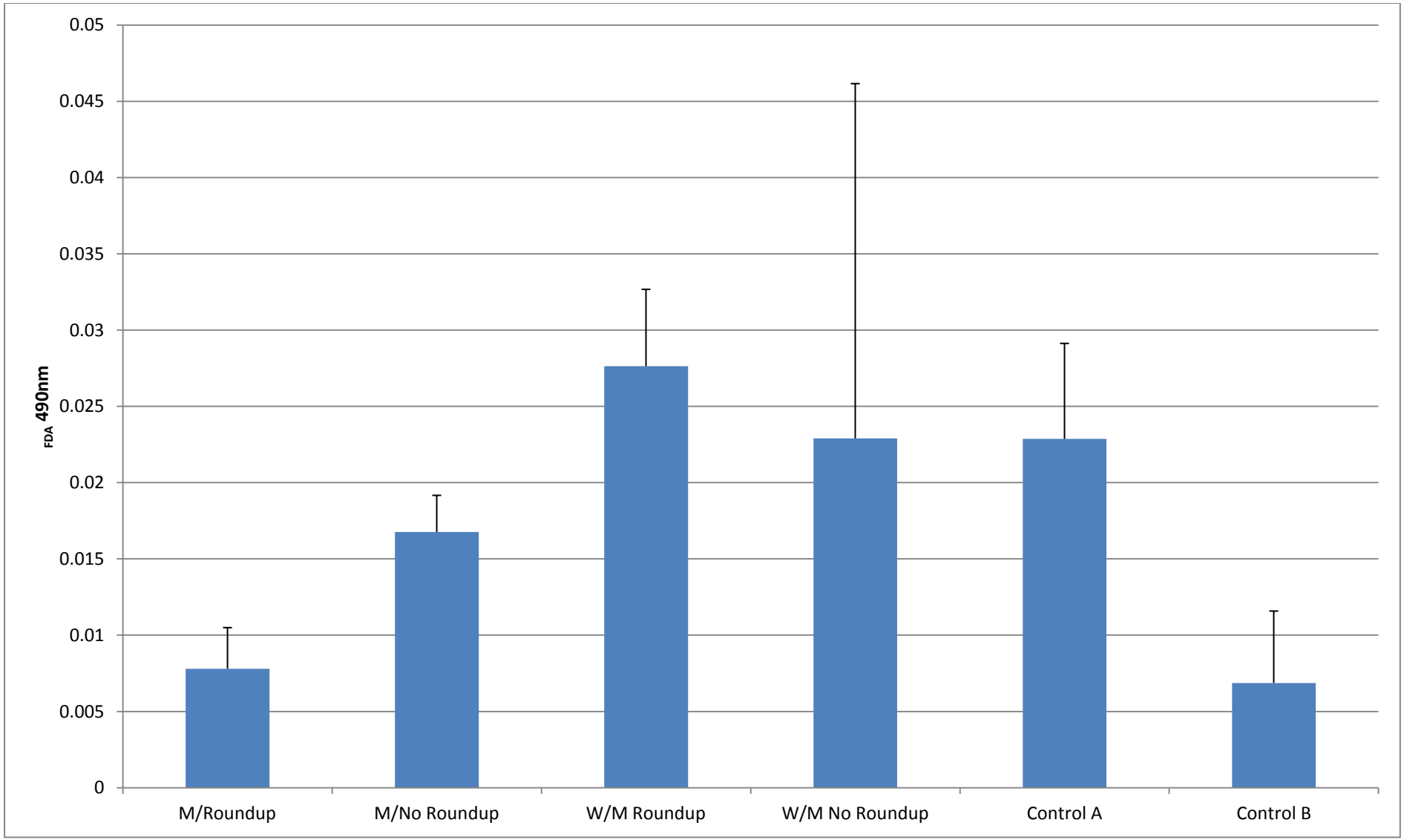

Figure 7. FDA data comparing Roundup Vs no-Roundup treatments.

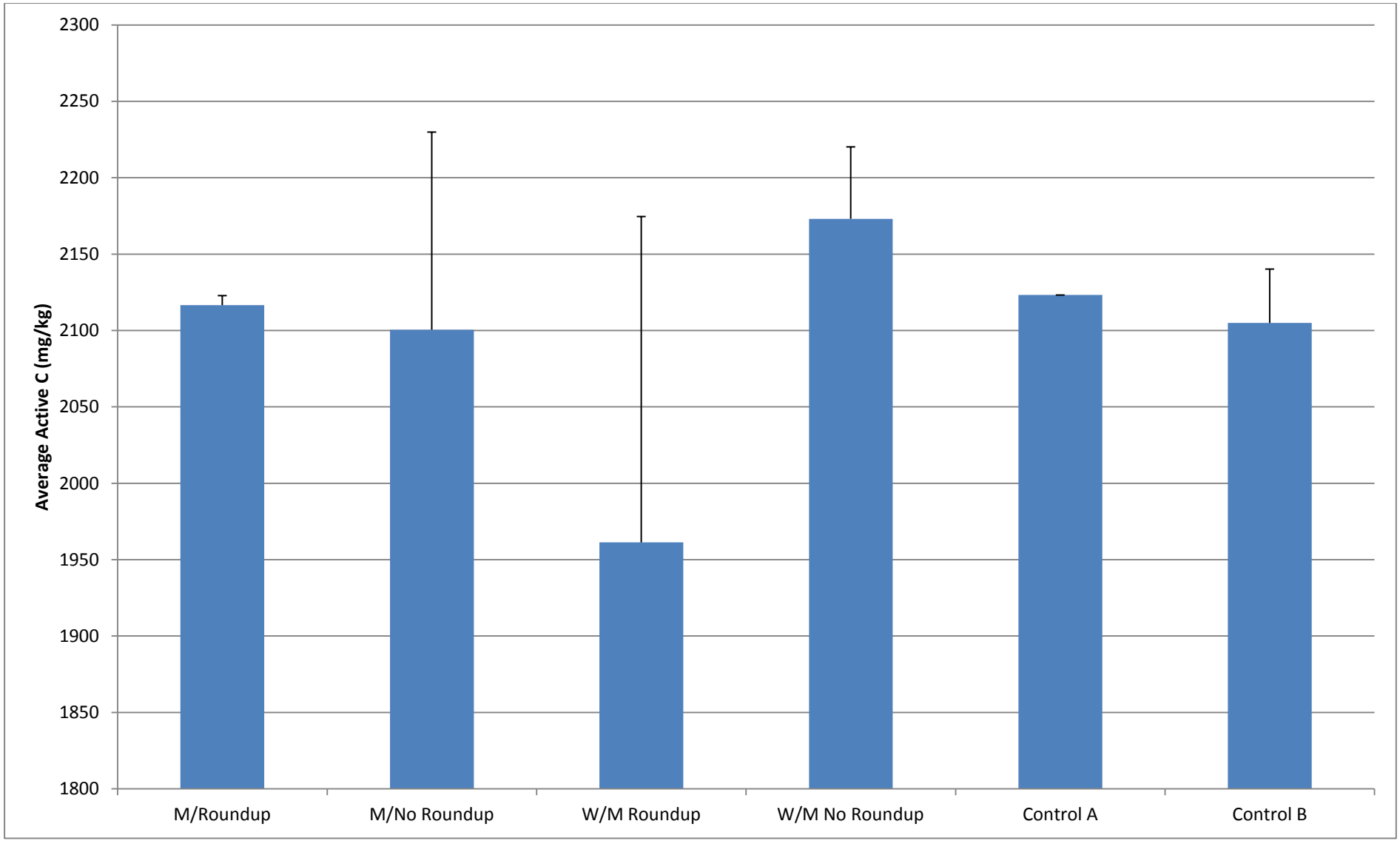

Figure 8. Active C data comparing Roundup Vs no-Roundup treatments.

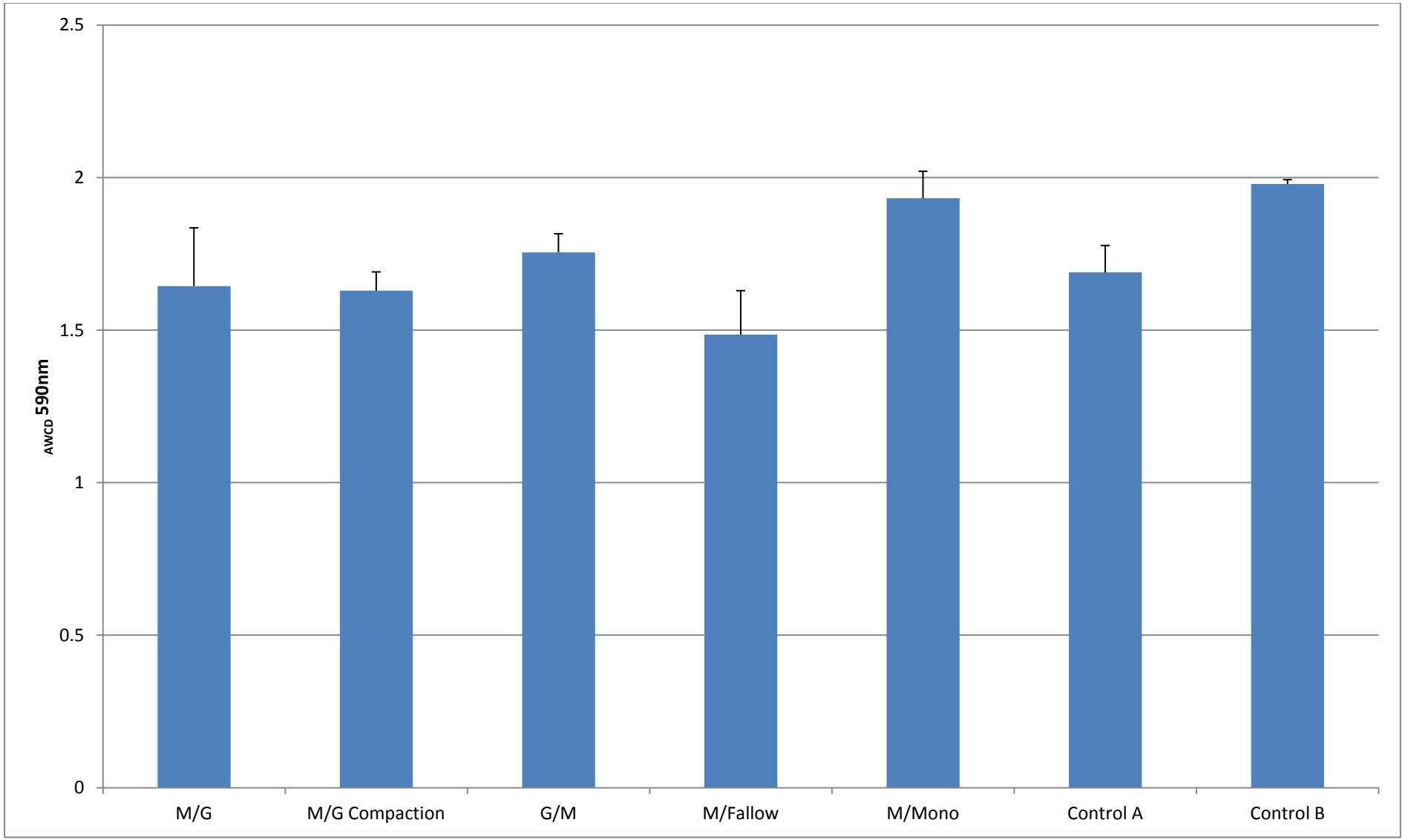

Figure 9. Biolog Eco™ plate data (AWCD) comparing crop rotation, fallow and compaction.

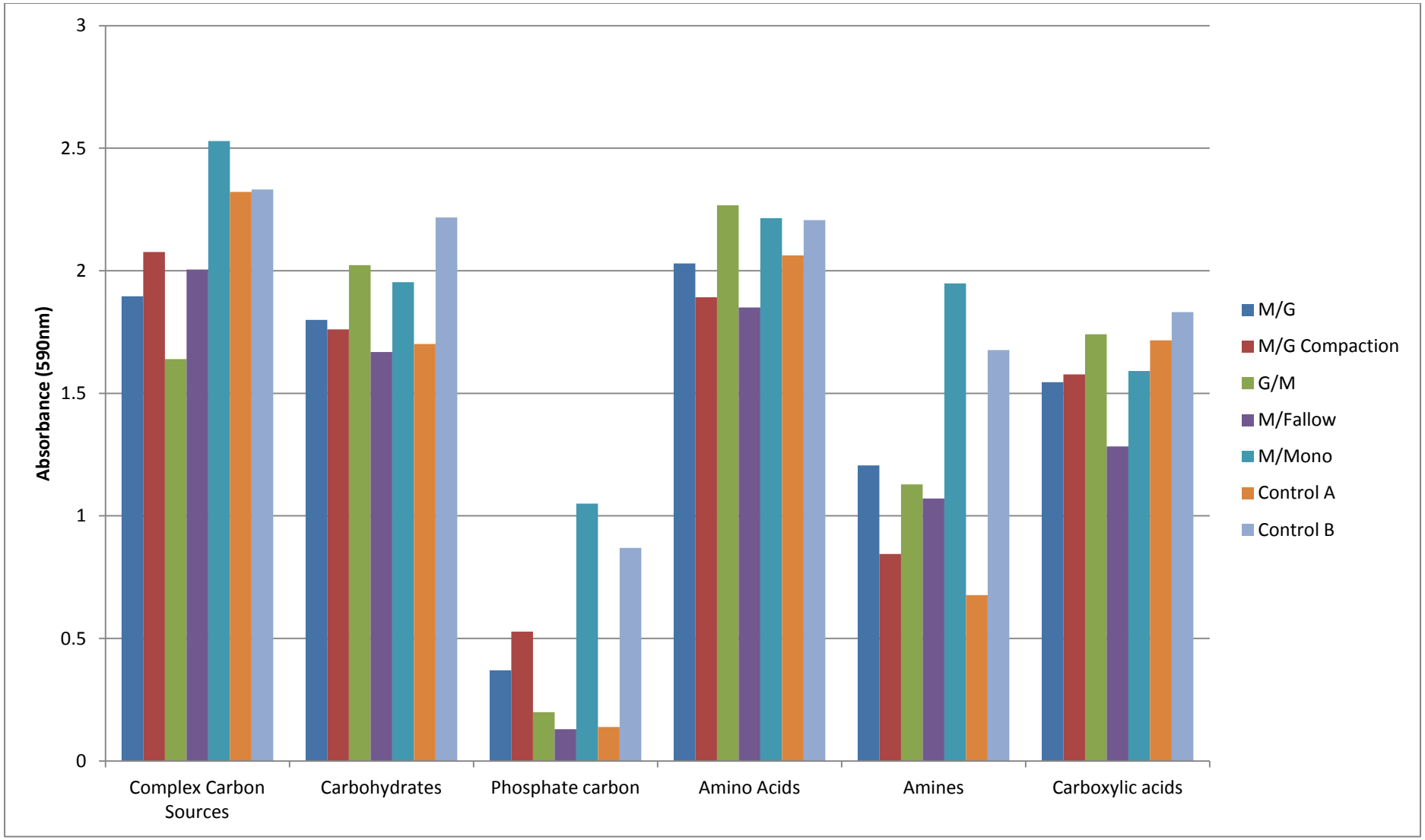

Figure 10. Biolog Eco™ plate data (carbon group utilization) comparing crop rotation, fallow and compaction.

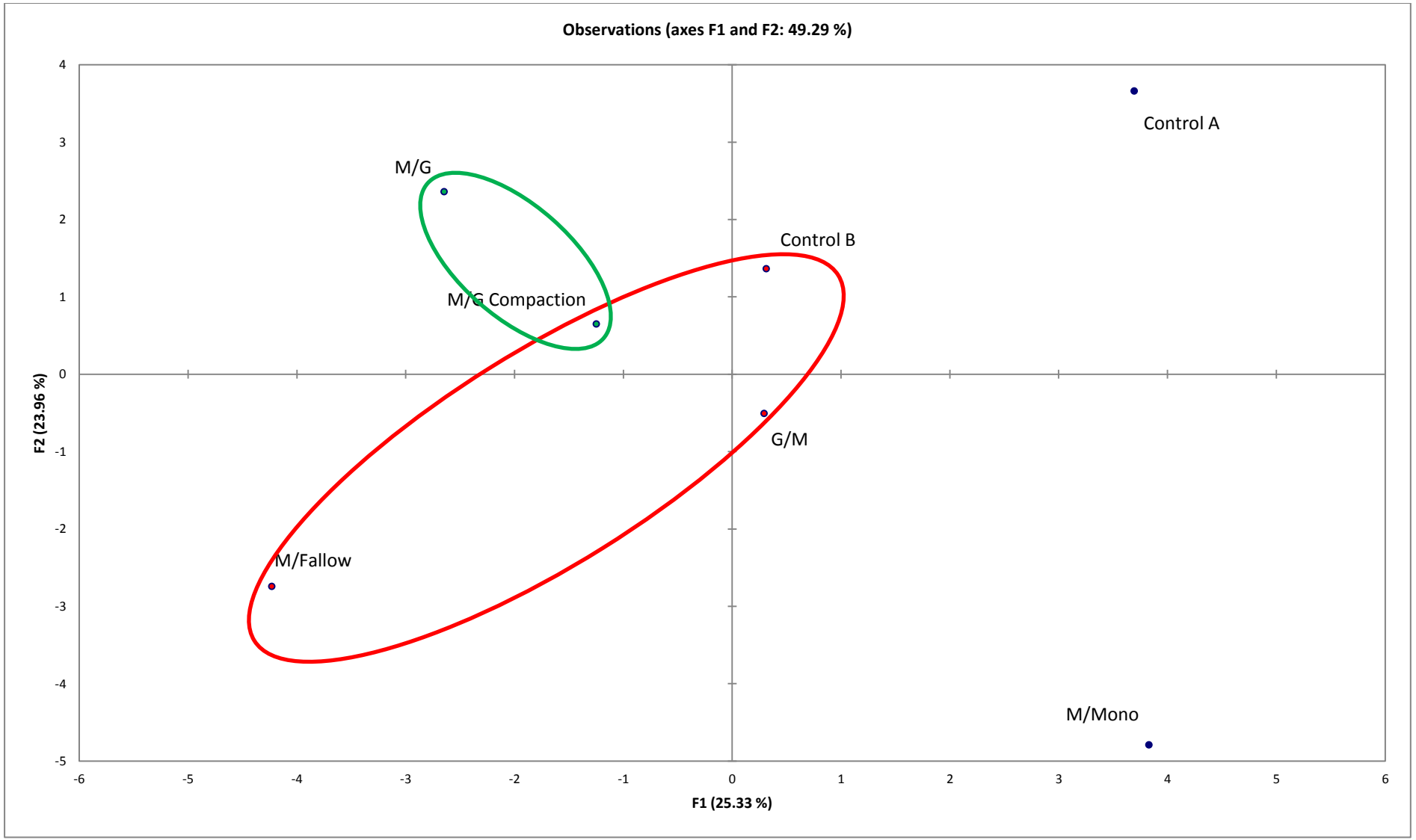

Figure 11. Biolog Eco™ plate data (PCA) comparing crop rotation, fallow and compaction.

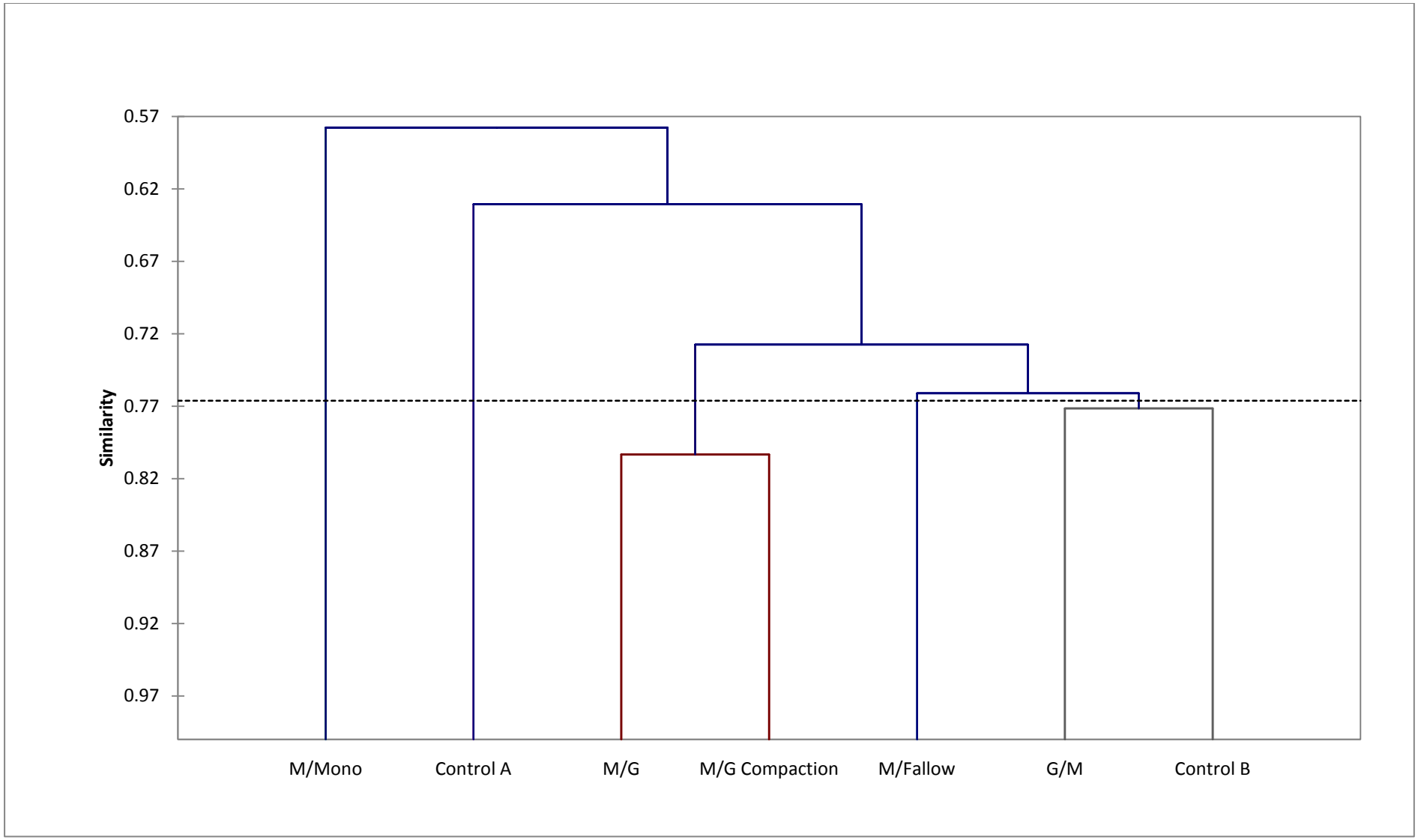

Figure 12. Biolog Eco™ plate data (dendogram) comparing crop rotation, fallow and compaction.

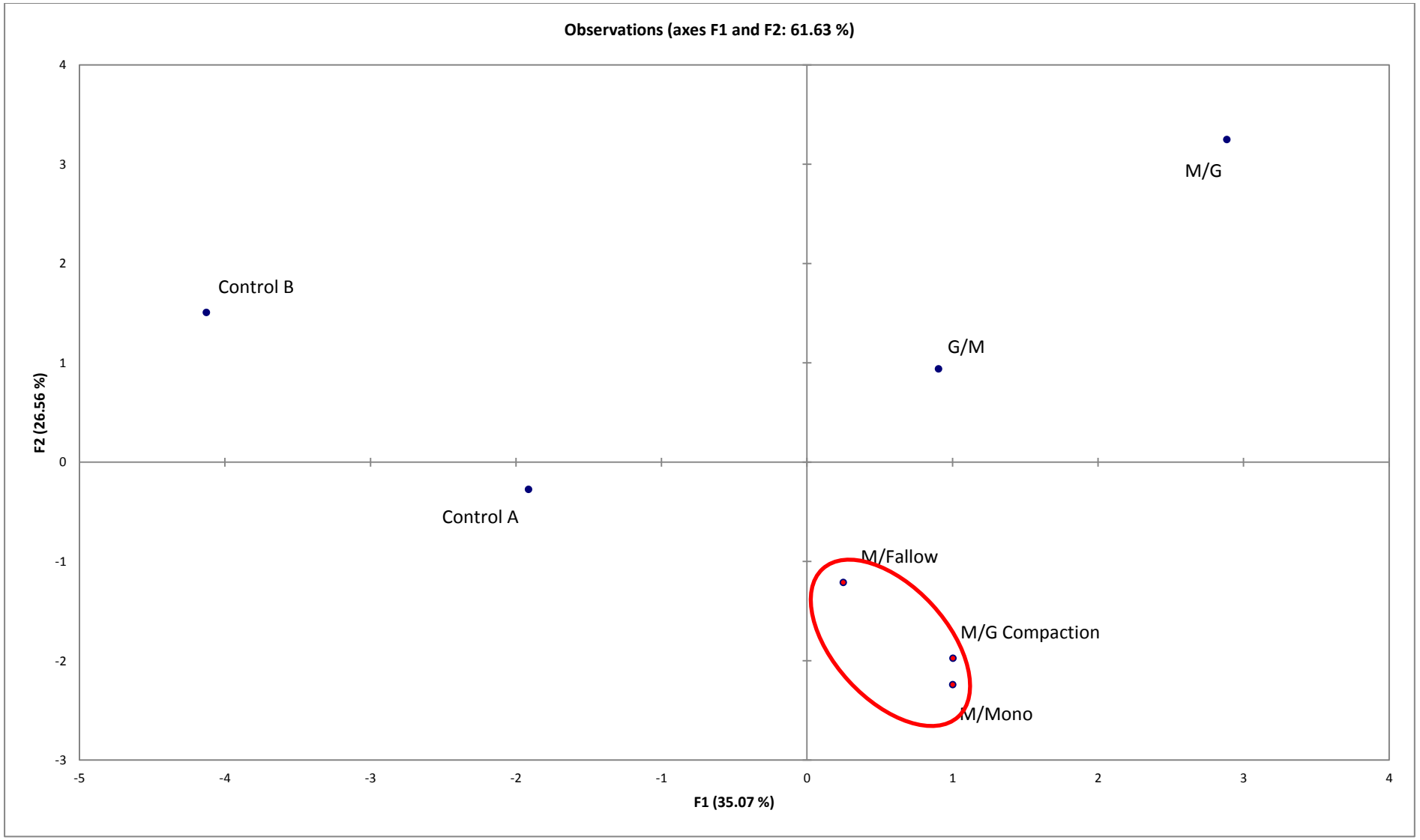

Figure 13. PCR-DGGE data (PCA) comparing crop rotation, fallow and compaction.

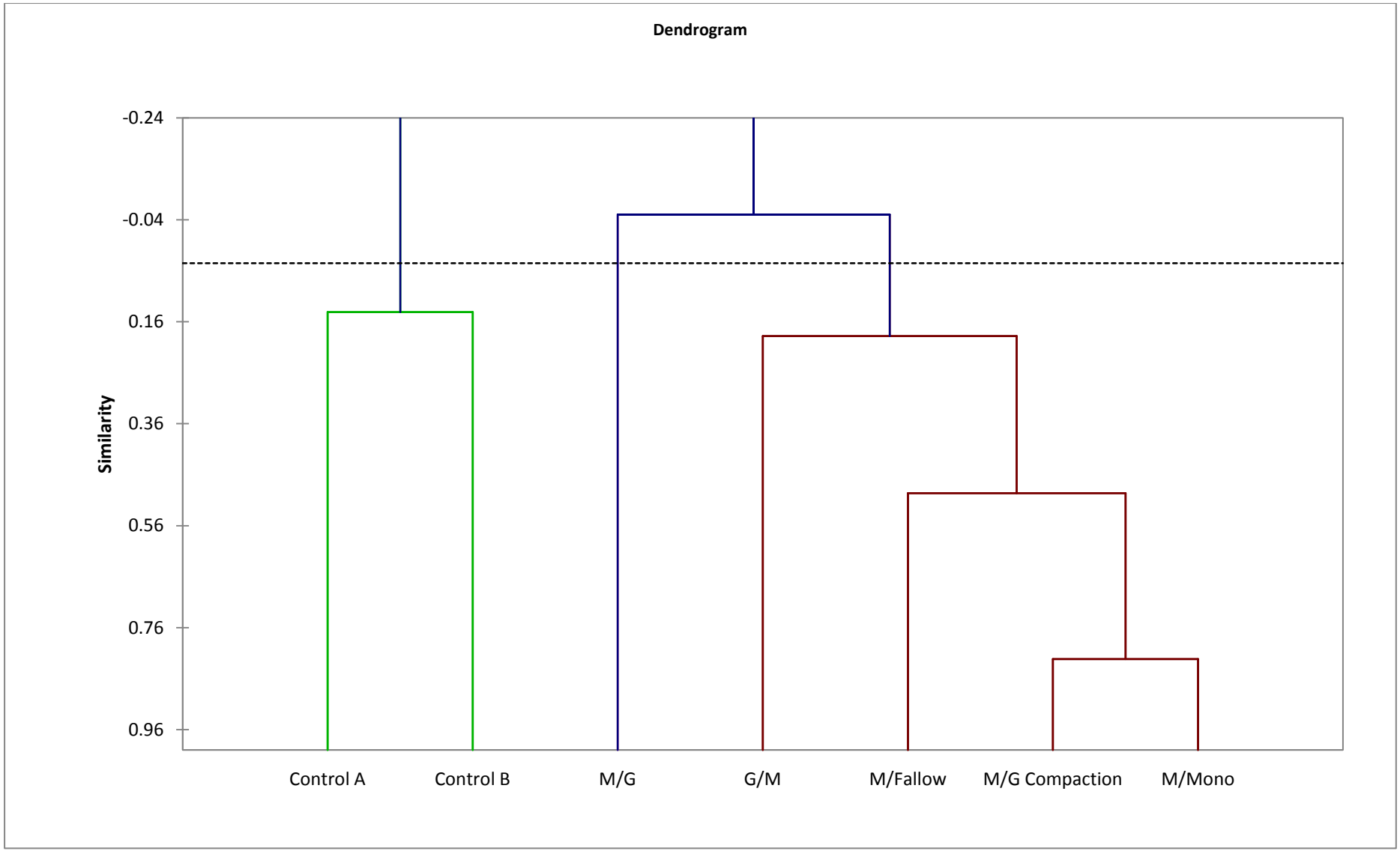

Figure 14. PCR-DGGE data (dendogram) comparing crop rotation, fallow and compaction.

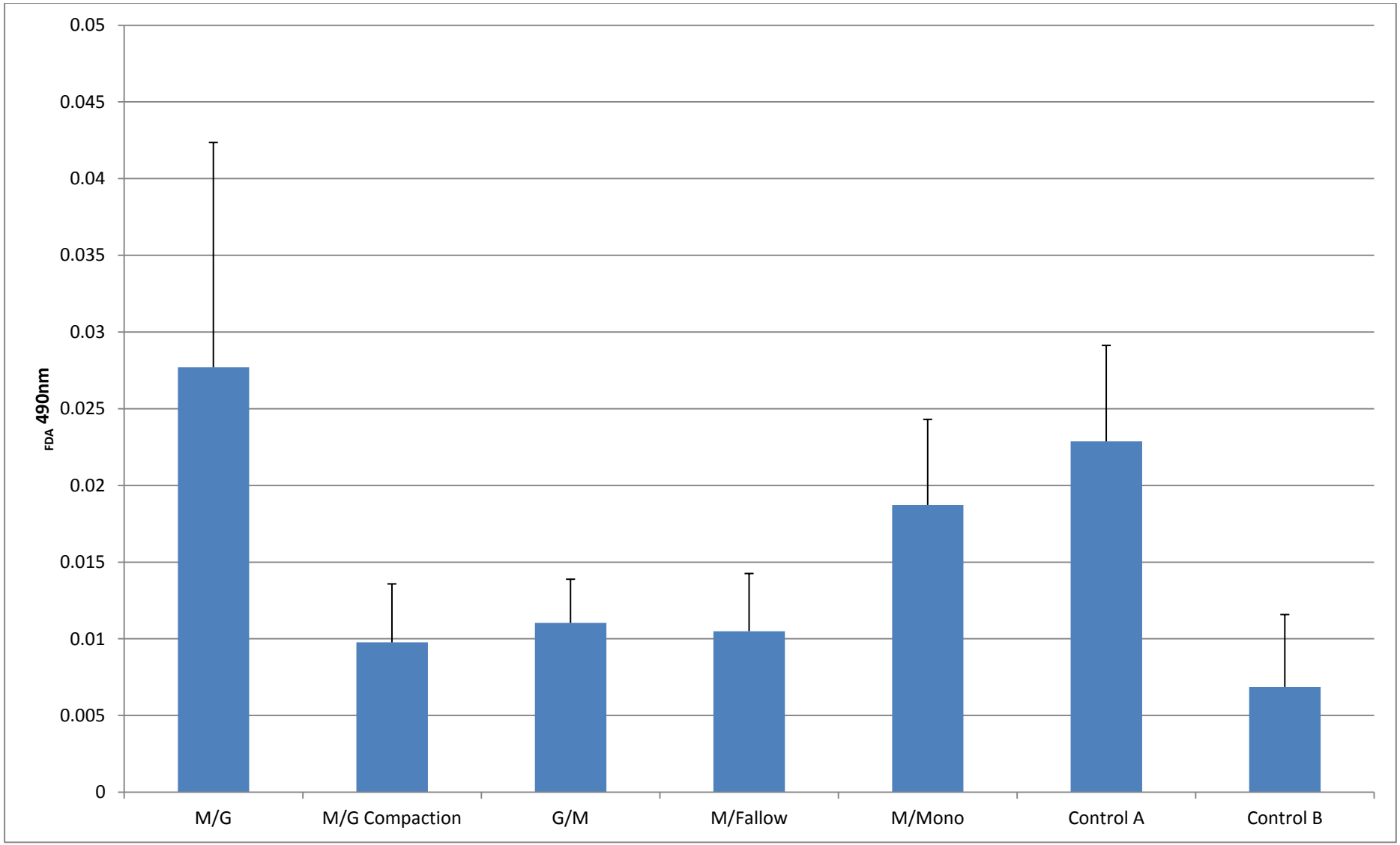

Figure 15. FDA data comparing crop rotation, fallow and compaction.

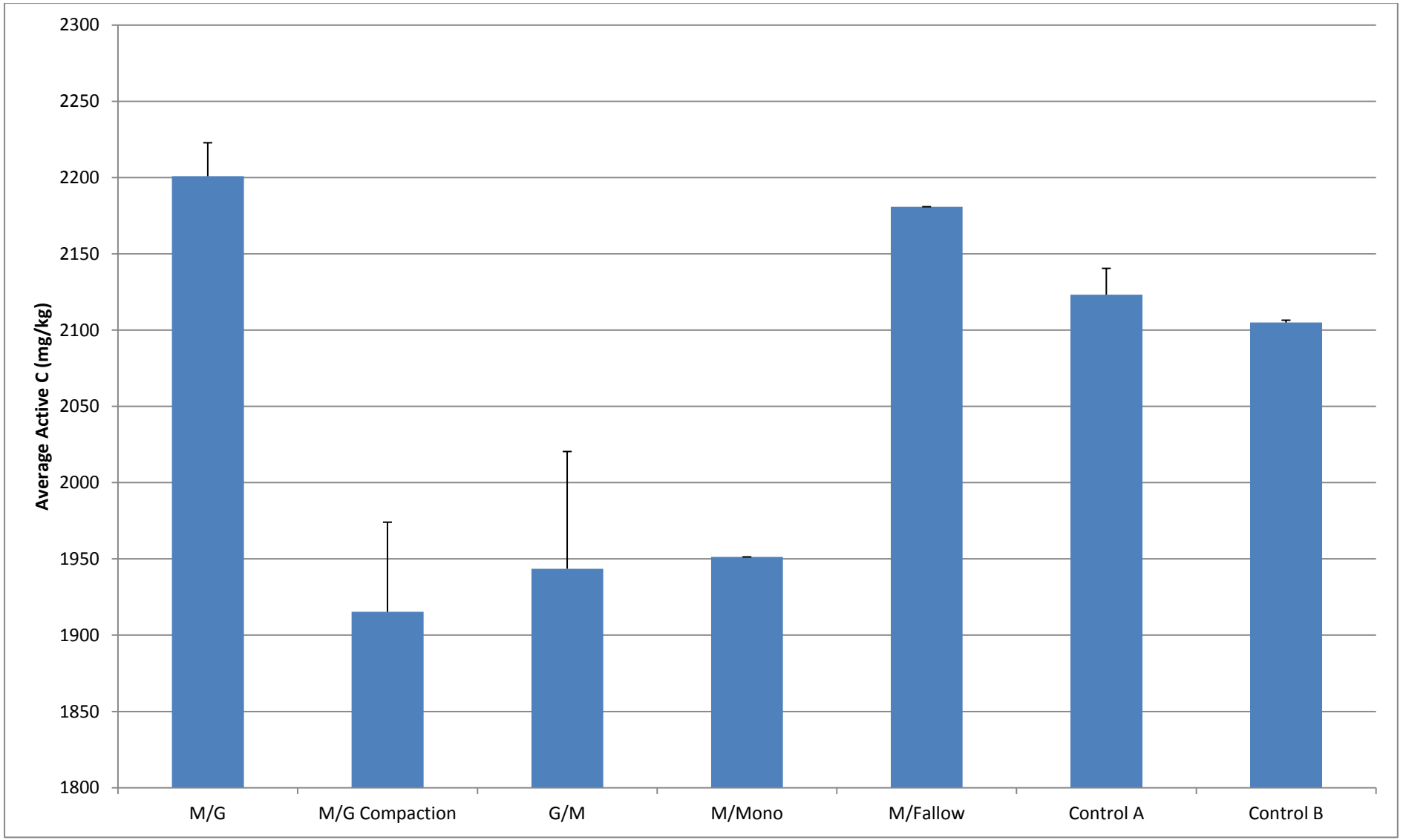

Figure 16. Active C data comparing crop rotation, fallow and compaction.

CHAPTER FOUR

THE EFFECT OF SOIL AMENDMENTS ON DISEASE SUPPRESSIVENESS TO THE PATHOGEN, *FUSARIUM OXYSPORUM*

ABSTRACT

The effect of fourteen soil treatments on the pathogenicity of *Fusarium oxysporum* on sorghum cultivar PAN 8420 was determined in the glasshouse. Two isolates of *F. oxysporum* previously isolated from diseased sorghum roots were obtained from the culture collection of the Department of Plant Sciences at the University of the Free State. Significant differences in the germination of sorghum seeds and the vegetative growth of sorghum seedling were observed in plants grown in several previously treated soils compared to a vermiculite control treatment. Germination in certain treatments incorporating inorganic fertilizers and herbicides was significantly higher than in the same treatments without the pathogen. Vegetative growth of seedlings in super phosphate (SP) fertilizer, benomyl fungicide, mustard (*B. juncea*) and lime treated soil was significantly greater than in same treatments without the pathogen.

Introduction

Soilborne pathogens increase in population size under monoculture cropping and cause between 40-80% yield losses if not controlled (Choi *et al.*, 2006). Grain crops such as sorghum, maize and wheat suffer great losses each year due to soilborne pathogens. Several of these soilborne pathogens are found in the Genus *Fusarium*, which can cause severe root rot symptoms on grain crops (Idris, Labuschagne and Korsten, 2006). *Fusarium oxysporum* Schlecht is one of these pathogens, it causes crown and root rot symptoms on sorghum crops and greatly reduce their yield (Idris *et al.*, 2006). Traditional control methods for soilborne pathogens are mainly via solarisation and fumigation, but since the worldwide ban on methyl bromide in 2005, alternative control methods had to be found (Horinouchi *et al.*, 2007). Although direct methods such as solarization, biological control agents and applications of fungicides have all shown promising effects in controlling soilborne pathogens (Pavlou and Vakalounakis, 2004; Choi *et al.*, 2006; Horinouchi *et al.*, 2007), there has been less focus on the use of microbes to control soilborne pathogens.

Microbes can inhibit the development of plant pathogens by means of direct or indirect interactions with host plants and with the pathogen themselves. Direct interference can result from the production of antibiotics and enzymes as well as predatory effects (Wehner *et al.*, 2009; García *et al.*, 2010). Indirect interference can result from competitive interactions with pathogens due to the utilization of common C/N resources (Wehner *et al.*, 2009). Microbes are easily affected by the changes in physical and chemical structure of soil (Lupwayi *et al.*, 2009) and many agricultural amendments have been shown to affect organic matter, moisture, oxygen content and pH in the soil (Chinalia and Killham, 2006; Bending, Rodríguez-Cruz, and Lincoln, 2007; Kong *et al.*, 2008; Li *et al.*, 2008). These physical and chemical changes can thus enhance or reduce the ability of soil microbes to suppress soilborne pathogens.

Certain agricultural practices may improve the biological properties of soil and suppress

plant pathogens. For example, an increase in soil organic matter resulting from the application of animal manure composts, inorganic fertilizers and cover crop residues can provide an abundant food source for soil microbes. This will lead to an increase in total soil microbial biomass and changes in the function and structure of the microbial community (de Lipthay *et al.*, 2002; Forge, Bittman and, Kowalenko, 2004; Chinalia and Killham, 2006; Ratcliff, Busse and Shestak, 2006; Tejada *et al.*, 2007; Gu *et al.*, 2009). The application of liming agents reduces soil acidification and changes the bacterial/fungal ratio in soil, which in turn increases microbial biomass and activity in soil that suppresses soil pathogens (Blagodatskaya and Anderson, 1998; Fuentes *et al.*, 2005). Numerous antagonistic organisms present inside composts have also successfully been used to suppress soilborne pathogens in both laboratory and field experiments (Perez-Piqueres *et al.*, 2005; Choi *et al.*, 2006).

In contrast to the positive effects discussed above, negative effects of agricultural soil amendments that decrease microbial diversity and activity in soil and lead to an increase in disease incidence, have been shown in numerous studies (Smith, Hartnett and Rice, 1999; Chen, Edwards and Subler, 2001; Zabaloy, Garland and Gómez, 2008). For example, the sole application of N rich fertilizers, without supplementary organic matter, decreases soil C content, which in turn decreases microbial biomass and activity (Marschner, Kandeler and Marschner, 2002). Non-target effects of fungicides and pesticides have been shown to not only decrease total microbial biomass in soil but also result in changes to the functional diversity of soil microbes (Sigler and Turco, 2002; Ferreira *et al.*, 2009).

The objective of the present study was to determine the possible suppressive or enhancing effects of cover crops, fertilizer, manure compost, fungicides, herbicides, and lime amended soils in microcosms on the soilborne pathogen *Fusarium oxysporum*.

Materials and methods

Inoculum preparation: Two isolates of *F. oxysporum* (F.o.1 and F.o.2) were selected as pathogens in the present study. Both isolates were isolated from diseased sorghum roots and previously shown to be pathogenic towards sorghum plants in the glasshouse although F.o.1 was less pathogenic than F.o.2. Inoculum preparation entailed the use of oat seeds (100g), soaked in 100 ml distilled water for 24 hours before excess water was decanted and seeds were autoclaved for 25 min at 121 ºC for two consecutive days. Agar plugs from the mycelial colony of each isolate, grown on potato dextrose agar (PDA), were added to bottles containing oat seeds, after which the bottles were incubated in the dark at 25 ºC for 2 weeks. Inoculum was removed from the bottles, oven dried at 30 ºC for 72 hours, ground into a fine powder and stored in sterile plastic containers at 4 ºC.

Soil preparation: Soil was obtained from a previous microcosm trial (see Chapter 2), where the soil had been subjected to 14 treatments (Table 1) over a period of 12 months. Various amendments had been applied every month and a cover crop destructively removed when soil was analysed every 3 months. The same batches of soil were then replanted with the same cover crop and subjected to the same treatment. At the conclusion of the 12-month-long microcosm trial, the cover crop was destructively removed from the microcosms, whereupon the soil was homogenized into 14 separate bulk lots. Soil from each treatment was placed into 18 cm diameter pots. Vermiculite was used as a positive control treatment for the experiment.

Greenhouse inoculation: Each batch of soil was subjected to three treatments: a control treatment (no pathogen) and two treatments with isolates F.o.1 and F.o.2. Inoculum powder, equal to 3% of the total dry soil mass per pot was added to the respective pots prior to the planting of sorghum seeds. Each treatment was replicated in four separate pots and 15 sorghum seeds were sown in each pot. Sorghum cultivar PAN 8420 was used for the

experiment due to its known susceptibility to *F. oxysporum* (van Rooyen, 2012). The experiment was carried out in the glasshouse with temperature controlled at ±21˚C. Pots were watered 3X a week and seedlings were grown for 6 weeks before the experiment was terminated.

Assessment and data analysis: Soil was carefully removed from the roots of individual sorghum plants and leaf length, root length, mesocotyl length, root rot, mesocotyl rot and fresh weight was determined for each plant. A GLM-ANOVA was performed on the data by using the statistical program NCSS 2000 (BMDP Statistical Software Inc.).

Results

Disease assessment - germination: Infected sorghum seedlings showed symptoms of root rot and mesocotyl rot visible as a pink to purple discolouration on the primary and secondary roots. Germination percentage of sorghum seeds was affected by both isolates of *F. oxysporum* with the mean germination across all 14 treated soils for isolate F.o.1 = 65% and F.o.2 = 51.7%. Mean germination across all treated soils without *F. oxysporum* (i.e. controls) was 75% and significantly greater ($P < 0.05$) than treatments with the pathogen.

Several soils from various microcosm treatments significantly affected the germination of sorghum seeds without the presence of *F. oxysporum* (Figure 1). A significant decrease ($P < 0.05$) in germination was observed in soil previously planted with ryegrass *(Lolium perenne)* compared to fallow soil and soil treated with Roundup, Nemat *(Eruca sativa)* and mustard (*Brassica juncea*) (Figure 1). Soil planted with ryegrass and treated with LAN, had a significantly lower ($P< 0.05$) germination percentage compared to the other microcosm soils (Figure 1). The highest germination percentage was in fallow soil and soil treated with Roundup, mustard (*B. juncea*) and Nemat *(E. sativa)*. These treatments supported significantly higher ($P< 0.05$) germination rate than the treatments: ryegrass,

315(NPK), LAN and super phosphate (SP), sheep manure, Propiconazole (tilt) and lime (Figure 1).

The pathogenicity of *F. oxysporum* on sorghum seed was also significantly affected by microcosm soils. A significantly higher ($P < 0.05$) germination was observed for ryegrass and LAN fertilizer microcosm soils inoculated with F.o.1 and F.o.2 compared to the same soil without *F. oxysporum* (Figure 1). Significantly lower ($P< 0.05$) germination was observed in lime and mustard (*B. juncea*) microcosm soils inoculated with F.o.1 and F.o.2 compared to the same soils without *F. oxysporum* (Figure 1). Similarly, significantly lower ($P< 0.05$) germination was observed in Roundup and benomyl microcosm soils inoculated with F.o.2 compared to the same soil inoculated with F.o.1 and without *F. oxysporum* (Figure 1).

Disease assessment - vegetative growth: No significant ($P > 0.05$) difference was found in root rot, mesocotyl rot and root length for inoculated plants compared to non-inoculated plants. Significant differences ($P < 0.05$) were however found in leaf length, mesocotyl length and plant fresh weight for inoculated plants compared to non-inoculated plants.

Leaf length was significantly affected by different microcosm soils. Sorghum plants grown in microcosm soils treated with inorganic fertilizers (NPK, LAN, SP), organic manures (chicken, sheep), ryegrass, fallow, 2,4-D and Nemat *(E. sativa)* but without *F. oxysporum* had a significantly greater ($P < 0.05$) leaf length than plants grown in vermiculite without *F. oxysporum* inoculum (Figure 2). The longest mean leaf length was recorded in SP treated soil and the shortest in the vermiculite control. Significant differences ($P < 0.05$) in leaf length, as affected by *F. oxysporum,* were also observed (Figure 2). Plants had significantly longer leaf lengths ($P < 0.05$) for F.o.1 and F.o.2 in all treatments than plants grown in vermiculite without the pathogen (Figure 2). Sorghum plants grown in the mustard (*B. juncea*) microcosm soil and inoculated with F.o.1, and limed soil inoculated with F.o.2,

had significantly longer ($P < 0.05$) leaf lengths than plants grown in the same soil without *F. oxysporum* (Figure 2).

Mean mesocotyl length was not significantly different ($P > 0.05$) between plants grown in microcosm soil compared to those grown only in vermiculite (Figure 3). However significant differences in mesocotyl length ($P < 0.05$) were observed when *F. oxysporum* was added (Figure 6). Sorghum plants grown in mustard microcosm soil had significantly longer mesocotyl lengths ($P < 0.05$) when inoculated with both isolates of *F. oxysporum* compared to non-inoculated soil.

Mean fresh weight of plants was significantly different ($P < 0.05$) between sorghum plants grown in the various microcosm soils compared to the vermiculite control (Figure 4). Plants grown in microcosm soils with NPK, LAN fertilizer, and two organic composts (chicken and sheep manure) weighed significantly more ($P < 0.05$) than plants grown in vermiculite (Figure 4). A significant difference in fresh weight ($P < 0.05$) was observed in soil inoculated with *F. oxysporum* compared to non-inoculated soils (Figure 4). Sorghum plants grown in limed microcosm soil had significantly greater fresh weight ($P < 0.05$) in soil inoculated with both isolates of *F. oxysporum* compared to non-inoculated soil. Plants grown in benomyl treated microcosm soil inoculated with F.o.1 and mustard (*B. juncea*) soil inoculated with F.o.2, weighed significantly more ($P < 0.05$) than plants grown in the same microcosm soils without *F. oxysporum* (Figure 4).

Discussion

Agricultural amendments have often been shown to significantly influence biological and chemical properties of soil (Abawi and Widmer, 2000; Weil and Magdoff, 2004; Rotenberg *et al.*, 2007). Changes in biological properties can play an important role in disease suppressiveness by influencing microorganisms capable of suppressing soilborne

pathogens (Suárez-Estrella *et al.*, 2006). Studies have shown that successful inhibition of pathogens in soil is mediated more by biological functions than chemical functions (Reuveni *et al.*, 2001; Choi *et al.*, 2006). In the present study, the pathogenicity of *F. oxysporum* in soils previously treated with specific amendments was tested in the glasshouse on sorghum seedlings. Significant changes in germination and vegetative growth of seedlings inoculated with *F. oxysporum* were recorded.

The suppressiveness of *F. oxysporum* pathogenicity observed in the present study by inorganic fertilizer and manure is consistent with previous findings by other workers (Bateman and Coşkun, 1995; Cotxarrera *et al.*, 2001; Suárez-Estrella *et al.*, 2006; Melero-Vara *et al.*, 2011). Increased microorganism populations that result from fertilizer and manure amendments can effectively compete with pathogens for food resources (Thermorshuizen *et al.*, 2006). An additional factor that could be responsible for disease suppressiveness is the presence of antagonistic microorganisms in composts. Such organisms suppress soilborne pathogens by indirectly competing with the pathogen for food sources or directly as predators (Postma, Montanari and van den Boogert, 2003; Oka, 2009). Choi *et al.* (2006) and Reuveni *et al.* (2001) reported successful suppression by compost of *F. oxysporum* and attributed this to the presence of antagonistic organisms.

Broad spectrum fungicides can cause changes in the fungal-bacterial ratios in soil and directly affect nutrient cycling (Smith, Hartnett and Rice, 1999). A reduction in fungal populations due to the effect of fungicides can also result in an increase in bacterial populations (Chen *et al.*, 2001). Hanks (1996) showed successful control of *F. oxysporum* induced basal rot in daffodils (*Narcissus spp.*) by the application of several fungicides which boosted bacterial populations. Chen *et al.* (2001) also indicated changes in the fungal-bacterial ratio in soil treated with the same fungicides, and suggested that increased bacterial populations are responsible for the suppressive effect on *F. oxysporum*.

The suppression of soilborne diseases by plant mulches has been shown in several previous studies (Tegegne, Pretorius and Swart, 2007; Hashem *et al.*, 2010). The incorporation of plant materials could either promote the growth of microbial antagonists or release allelopathic compounds which directly affect the growth of soilborne pathogens (Pavlou and Vakalounikis, 2004). *Brassica* crops have been shown to suppress a wide spectrum of soilborne pathogens (Schreiner and Koide, 1993). This suppressiveness is caused by volatile compounds (glucosinolates) released from the decomposing plant materials which have antifungal properties (Larkin and Griffin, 2006). *Brassica* spp. may however, in some cases favour soilborne pathogens and promote disease development. Njoroge, Riley and Keinath (2007) reported inconsistent results following the incorporation of *Brassica* spp. into soil. For example, *F. oxysporum* and *Pythium* spp. populations were significantly higher in soil incorporated with broccoli (*Brassica oleracea*) compared to control plots (Blok *et al.*, 2000). Our results are consistent with these studies since only one isolate of *F. oxysporum* (F.o. 2) was to some extent suppressed in soil previously planted with Nemat, while no suppressive effect was seen in the soil inoculated with the other isolate (F.o. 1).

Results of the present study indicated significant changes in the aggressiveness of the soilborne pathogen *F. oxysporum* by organically and inorganically amended soils. Further research should be conducted to determine of the effectiveness of these amendments on other soilborne pathogens and investigate the possibility of manipulating the effect in field studies. This may provide cheaper alternative cultural control methods of soilborne diseases.

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Table 1. Treatments and application rates of soil amendments used in microcosm tests.

Treatments	**Application rates**
Lime	4.5 Tonne/ha
Fertilizers	
3N:1P:5K	30g/m²
Lime Ammonium Nitrate (LAN)	30g/m²
Super phosphate (SP)	15g/m²
Manure compost	
Chicken manure compost	65g/m²
Dried Sheep manure	65g/m²
Herbicide	
Round up (glyphosate)	1L/15m²
2,4-D (2,4-dicholorophenoxyacetic acid)	0.95kg/ha
Fungicide	
Benomyl	51mg/kg
Propiconazole (Tilt)	0.25mg/kg
Cover crops	
Mustard (*Brassica juncea*)	2g/pot
Nemat *(Eruca sativa)*	2g/pot
Control treatments	
Ryegrass *(Lolium perenne)*	2g/pot
Bare soil (Fallow)	No covercrop/amendment

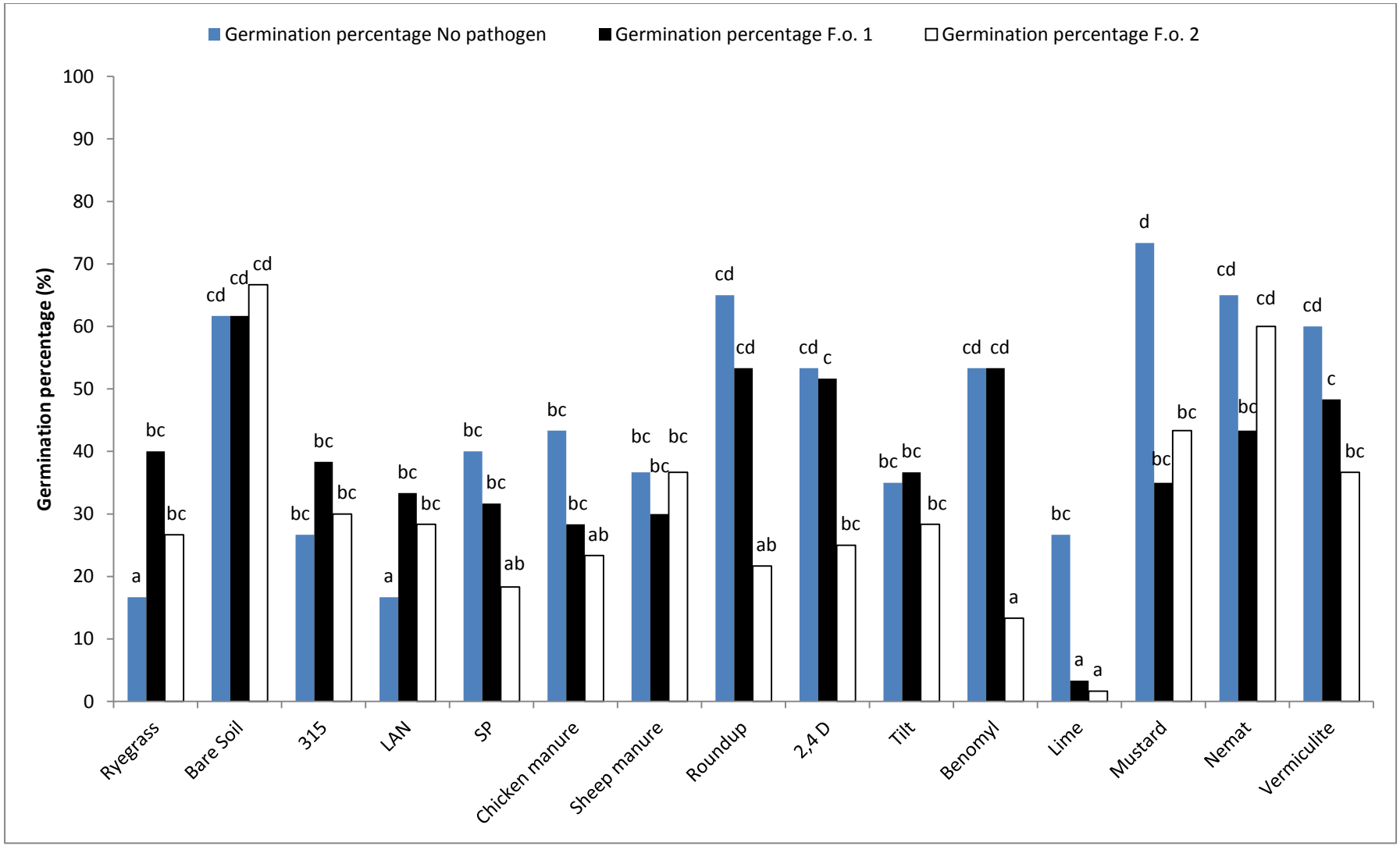

Figure 1. Percentage germination of sorghum seedlings challenged by 2 isolates of *F. oxysporum*. Bars with the same lowercase letter/s are not significantly different (P>0.05) (LSD = 3.497).

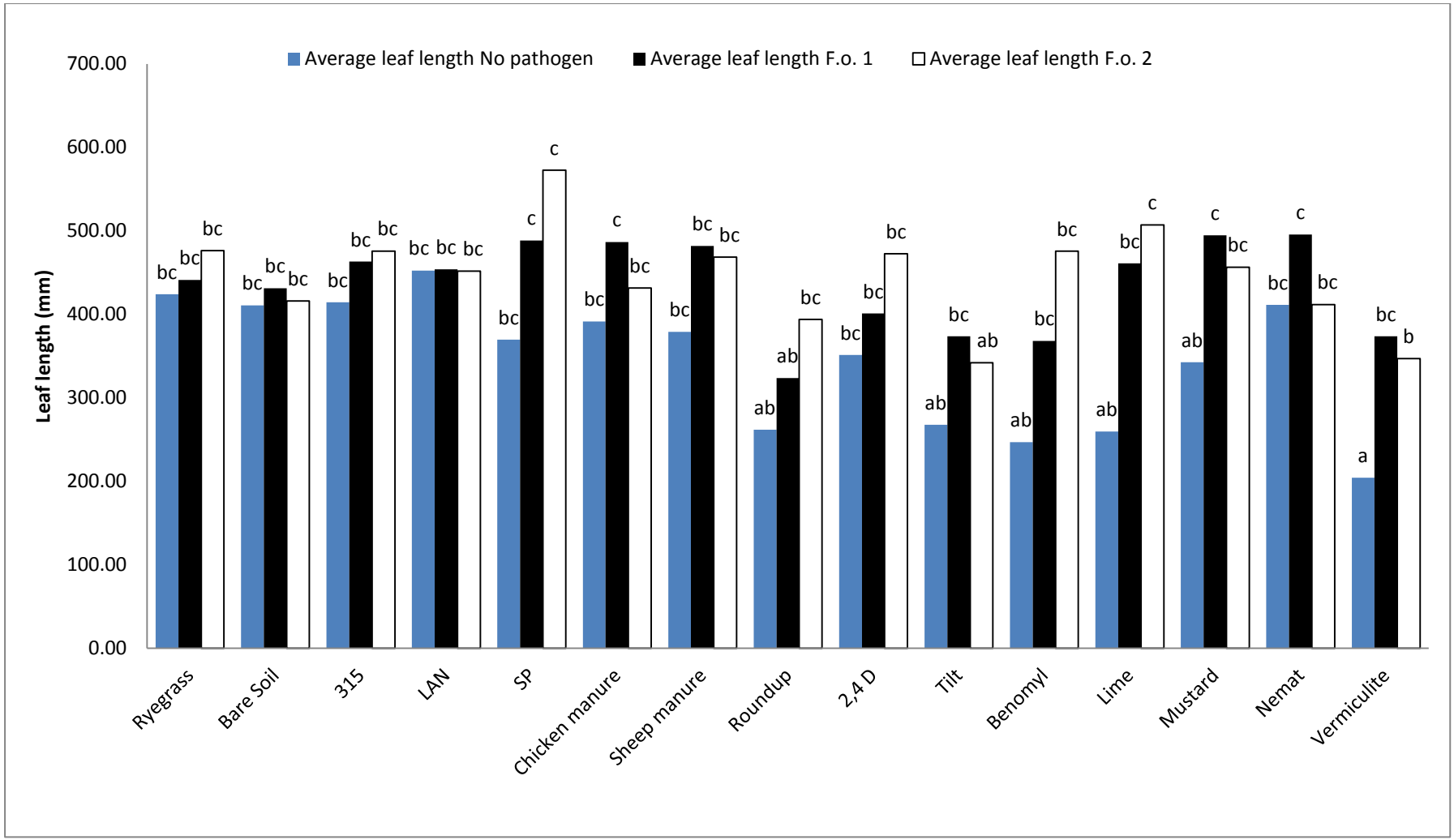

Figure 2. Average leaf length of sorghum seedlings challenged by 2 isolates of *F. oxysporum*. Bars with the same lowercase letter/s are not significantly different (P>0.05) (LSD = 139.02).

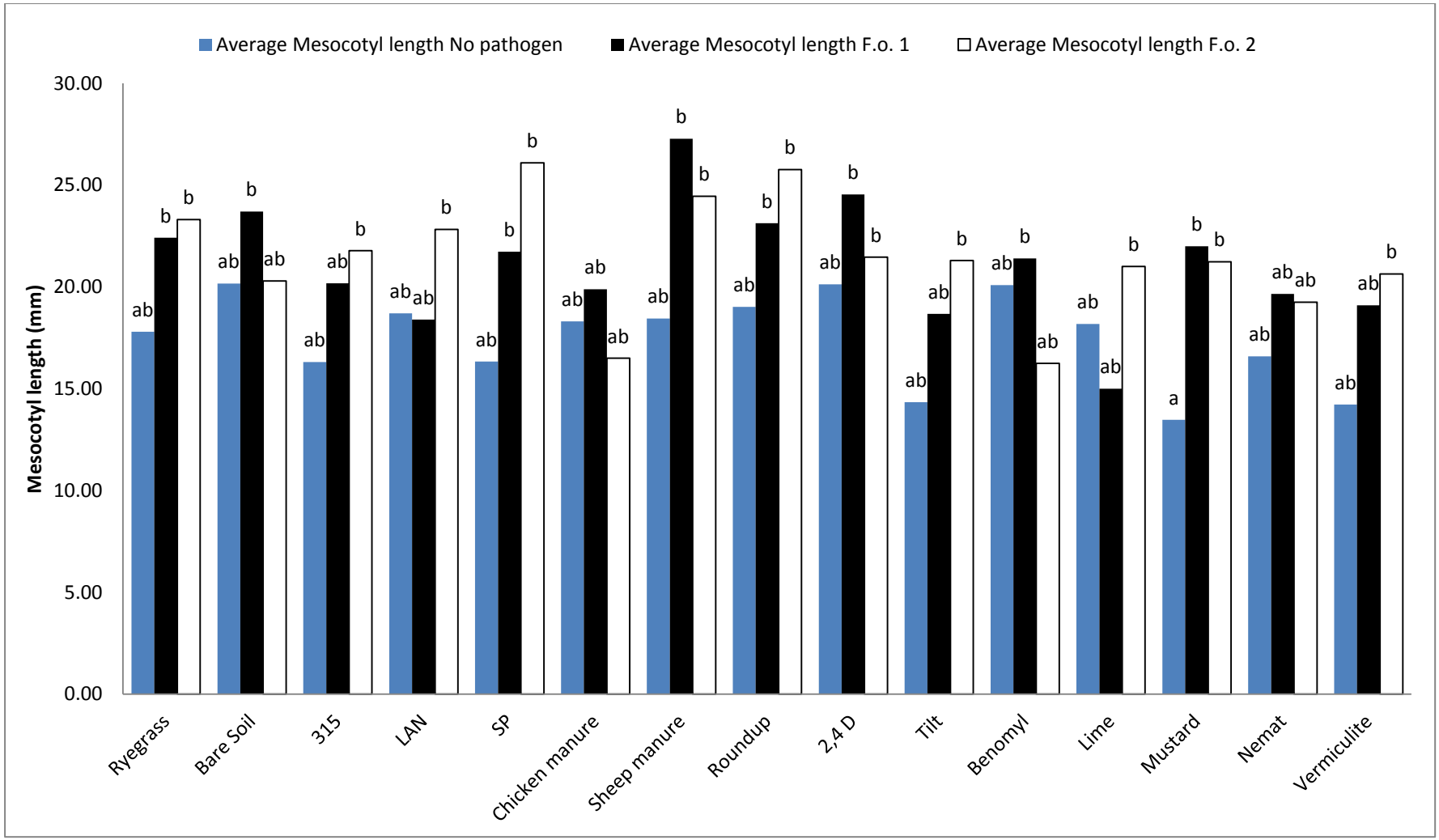

Figure 3. Average mesocotyl length of sorghum seedlings challenged by 2 isolates of *F. oxysporum*. Bars with the same lowercase letter/s are not significantly different (P>0.05) (LSD = 6.956).

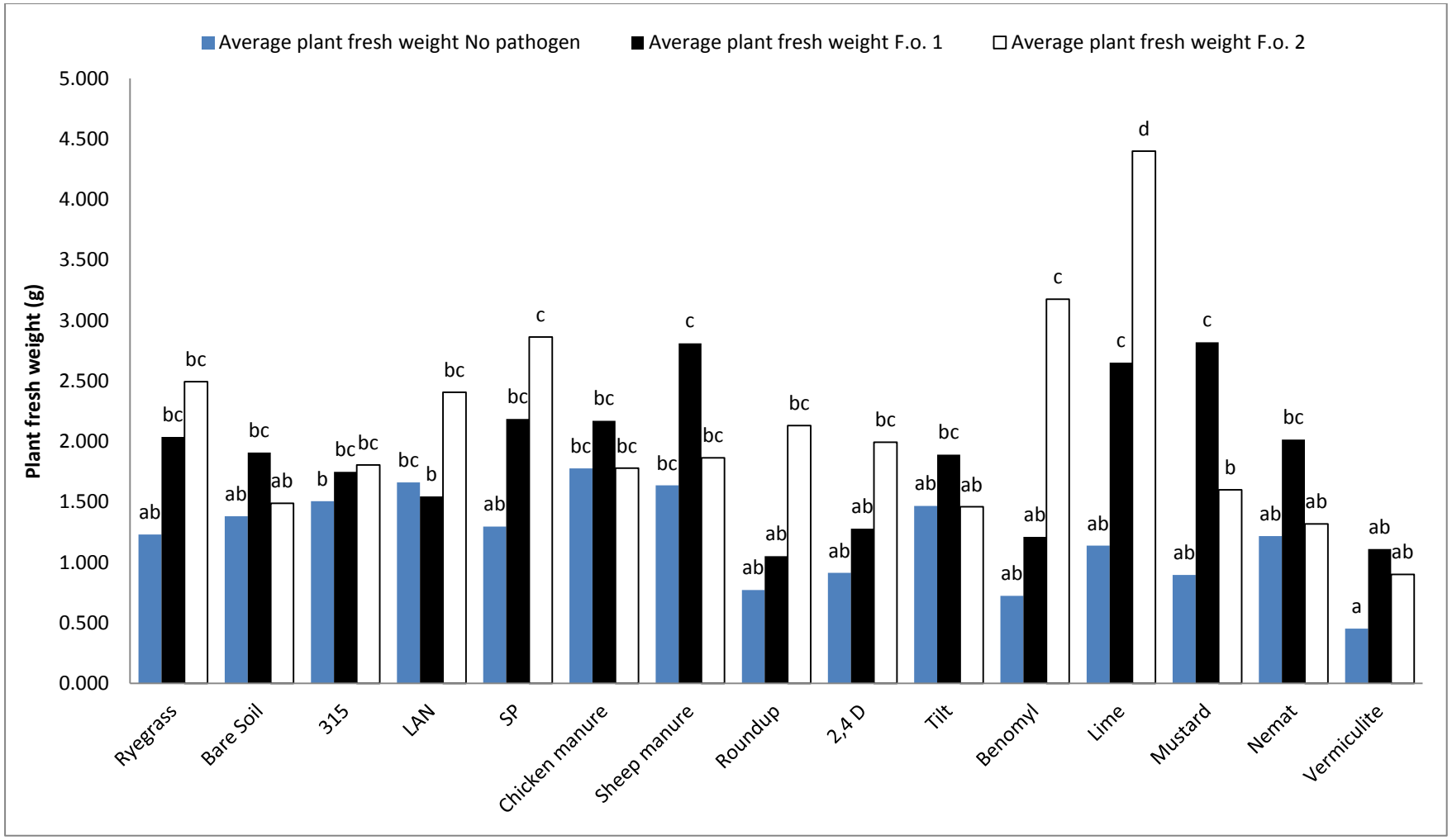

Figure 4. Average plant fresh weight of sorghum seedlings challenged by 2 isolates of *F. oxysporum*. Bars with the same lowercase letter/s are not significantly different (P>0.05) (LSD = 1.038).

SUMMARY

Soil amendments and agricultural practices that are utilised to enhance crop production, can impact, either directly or indirectly, on soil microorganisms which are responsible for a whole range of processes that maintain soil health. The main aim of the present investigation was to determine the effects of these amendments and cultural practices on microbial diversity, activity and community structure. The potential impacts of these practices on the pathogenicity of soilborne pathogens such as *F. oxysporum*, were also evaluated.

Trials conducted in soil microcosms investigated the effects of 10 soil amendments and 2 cover crops on microbial organisms. Qualitative analysis of microbial diversity, using Biolog Eco™Plates and PCR-DGGE, showed distinct differences in treated soils, compared to control treatments. Fertilizer amendments, herbicides, fungicides, cover crops and lime were all found to have a noteworthy impact on soil microbial diversity. A major factor influencing microbial diversity was a cover crop, viz. Nemat *(Eruca sativa)*. Quantitative analysis of microbial activity using fluoroscein diacetate analysis (FDA) also showed distinct differences in treated soils compared to the controls. All treatments, except Roundup and both cover crop treatments increased microbial activity in soil compared to the control treatments. The highest microbial activity was observed in soil treated with the herbicide 2,4-D, and the lowest in the negative control treatment and soil treated with Roundup.

A field study investigated the effects of Roundup, crop rotation and soil compaction on soil microbial populations. Qualitative analysis of maize fields treated with Roundup showed distinct differences between soils treated at various periods prior to analysis, and those not treated at any time. Qualitative analysis depicting microbial activity and biomass also showed distinct differences between Roundup soil and no-Roundup soil. Microbial activity seems to be immediately inhibited by the application of Roundup while microbial

biomass was found to be negatively affected in the long-term.

Qualitative analysis of soils subjected to rotation and compaction, also showed noteworthy differences in the microbial diversity of treated compared to non-treated soils. Both compaction and rotation was found to be a major factor influencing microbial biomass. Quantitative analysis of soils subjected to rotation and compaction, also showed distinct differences in microbial activity and biomass of the treated soils compared to the controls. Compacted soil had a lower microbial biomass and activity than non-compacted soil.

Significant differences in sorghum seed germination and vegetative growth was found between microcosm soils compared to a vermiculite control treatment. Microcosm soil treated with rye grass, LAN, Roundup and mustard, significantly increased sorghum seedling germination and reduced the pathogenicity of *F. oxysporum*. Limed microcosm soil significantly reduced seedling germination while super phosphate (SP), benomyl, lime and mustard microcosm soil significantly improved the vegetative growth of sorghum seedlings by reducing the pathogenic effect of *F. oxysporum*.

Results from the present study confirm the potential impact of soil amendments and cultural practices on soil microorganisms and the effects they may have towards soilborne diseases. This information may provide insights on improving soil health while helping to increase crop production by means of alternative cultural methods to control soilborne pathogens.

OPSOMMING

Grondtoevoegings en landboupraktyke wat verhoodge gewasproduksie ten doel het, kan ‘n direkte of indirekte impak uitoefen op grondmikro-organismes wat verantwoordelik is vir ‘n hele reeks prosesse wat grondgesondheid bepaal. Die vernaamste doel van die ondersoek was om die uitwerking van hierdie grondtoevoegings en bewerkingspraktyke op mikrobiese-diversiteit, -aktiwiteit en –gemeenskapstrukture in grond te bepaal. Die potensieële impak wat hierdie praktyke op die patogenisiteit van grondgedraagde patogene soos *Fusarium oxysporum* het, is ook geëvalueer.

Proewe wat in ‘n mikro-omgewing uitgevoer is het die effek van 10 grondtoevoegings en 2 dekgewasse op mikrobiese organismes ondersoek. Kwalitatiewe analise van mikrobiese- diversiteit, deur gebruik te maak van “Eco™Plates” en “PCR-DGGE”, het duidelike verskille tussen behandelde grond en kontrole-behandelings getoon. Kunsmis-, onkruiddoder-, swamdoder-, kalktoedienings en dekgewasse het almal ‘n noemenswaardige impak op mikrobiese-diversiteit in grond getoon. ‘n Belangrike faktor wat mikrobiese-diversiteit beïnvloed het was die dekgewas Nemat *(Eruca sativa)*. ‘n Kwantitatiewe analise van mikrobiese aktiwiteit gemeet deur middle van fluoresien-diasetaat analise (FDA), het duidelike verskille tussen behandelde grond en kontrole-behandelings getoon. Alle behandelings, behalwe Roundup en beide die dekgewasbehandelings, het mikrobiese aktiwiteit in die grond verhoog in vergelyking met die kontrole-behandelings. Die hoogste mikrobiese aktiwiteit was waargeneem in grond wat met die onkruiddoder 2,4-D behandel is, en die laagste in die negatiewe kontrole-behandeling en in grond wat met Roundup behandel is.

‘n Veldproef het die invloed van Roundup, wisselbou en grondkompaksie op mikrobiese populasies in die grond ondersoek. ‘n Kwalitatiewe analise van mielielande wat met Roundup behandel is, het beduidende verskille tussen grond wat voorheen behandel is en

dié wat glad nie behandel is nie, getoon. ‘n Kwalitatiewe analise wat mikrobiese aktiwiteit en biomassa weerspieël, het ook duidelike verskille tussen Roundup-behandelde en -onbehandelde grond getoon. Mikrobiese aktiwiteit blyk onmiddelik deur Roundup toediening beïnvloed te word, terwyl mikrobiese biomassa slegs in die langtermyn negatief beïnvloed te word.

‘n Kwalitatiewe analise van grond wat aan wisselbou en kompaktering onderwerp was, het ook noemenswaardige verskille in die mikrobiese-diversiteit van behandelde en nie-behandelde grond getoon. Beide kompaksie en wisselbou het getoon belangrike faktore te wees wat mikrobiese biomassa beïnvloed. ‘n Kwantitatiewe analise van grond wat aan kompaksie en wisselbou onderwerp was, het ook duidelike verskille in mikrobiese aktiwiteit en biomassa tussen behandelde en nie-behandelde grond getoon. Gekompakteerde grond het ‘n beduidende laer mikrobiese-biomassa en -aktiwiteit as nie gekompakteerde grond getoon.

Beduidende verskille in die ontkieming van sorgumsaad, en in vegetatiewe groei is gevind tussen grond uit voorafbehandelde mikro-omgewings en vermikuliet-kontroles. Grond wat met roggras, KAN, Roundup en mosterdsaad behandel is, het saailingontkieming beduidend laat toeneem en die patogenisiteit van *F. oxysporum* verminder. Kalkbehandelde grond het saailingontkieming beduidend laat afneem terwyl grond uit voorafbehandelde mikro-omgewing wat met superfosfaat (SP), benomyl, kalk en mosterdsaad behandel is, die vegetatiewe groei van sorghumsaailinge verbeter het deur die patogeniese effek van *F. oxysporum* te veminder.

Resultate van hierdie ondersoek het die potensiele impak van grondtoevoegings en bewerkingspraktyke op grond-mikroorganismes en die positiewe invloed daarvan op grondgedraagde patogene bevestig. Dié bevindinge kan insiggewend wees ten opsigte van verbeterde grondgesondheid en kan bydra tot verhoogde gewasproduksie en alternatiewe bewerkingsmetodes om die beheer van grondgedraagde patogene te bewerkstellig.