

Assessment of the biofumigation effect of Canola (*Brassica napus*) on soil microbial community function and structure

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Never accept failure, no matter how often it visits you.

Keep on going. Never give up. Never.

— Dr. Michael Smurfit —

Moet nooit ophou droom nie.

“No dream, no direction.”

— Tony Factor —

Don't be afraid to take big steps.

You can't cross a chasm in two small jumps.

— David Lloyd George —

Table of Contents

Acknowledgements.....	iii
Preface.....	v
Summary.....	vi
Opsomming.....	viii
List of Abbreviations.....	x
List of Figures.....	xiii
List of Tables.....	xvi
Chapter 1: Introduction.....	1
1.1: Soil microbial communities and their role in the soil ecosystem.....	1
1.2: Problem statement.....	3
1.3: Aim and objectives.....	4
1.4: Outline of dissertation chapters.....	4
Chapter 2: Literature Review.....	6
2.1: The soil environment and its properties.....	6
2.2: Microorganisms in the soil environment.....	7
2.2.1: The role of microorganisms in the soil ecosystem.....	7
2.2.2: Factors influencing the soil microbial community.....	8
2.2.3: Methods used to study soil microbial communities.....	10
2.2.3.1: Functional diversity techniques.....	11
2.2.3.2: Structural diversity techniques.....	17
2.2.3.3: Molecular diversity techniques.....	22
2.3: Sunflower cultivation in South African agriculture.....	23
2.4: <i>Sclerotinia sclerotiorum</i> as a major sunflower pathogen.....	24
2.5: Biofumigation with <i>Brassica</i> species.....	29
2.6: Chlorophyll a fluorescence as a plant vitality indicator.....	32

Table of Contents

Chapter 3:	Materials and Methods.....	37
3.1:	Experimental layout.....	37
3.2:	Sampling procedure.....	38
3.3:	Soil physical and chemical analyses.....	39
3.4:	Dehydrogenase activity.....	40
3.5:	Community level physiological profiles (CLPPs).....	41
3.6:	Phospholipid fatty acid (PLFA) analysis.....	41
3.7:	Plant fluorescence measurements.....	43
3.8:	Statistical analyses.....	43
Chapter 4:	Results and Discussion.....	45
4.1:	Soil physical and chemical properties.....	45
4.2:	Dehydrogenase activity.....	48
4.3:	Community level physiological profiles (CLPPs).....	50
4.4:	Phospholipid fatty acid (PLFA) analysis.....	57
4.5:	Chlorophyll a fluorescence measurements.....	70
Chapter 5:	Conclusions and Recommendations.....	80
5.1:	General conclusions.....	80
5.2:	Recommendations for future studies.....	83
References.....		85

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Preface

The experimental work done and discussed in this dissertation for the degree Master of Science in Environmental Sciences (M.Sc.Env.) was carried out in the School of Environmental Sciences and Development, North-West University, Potchefstroom Campus, Potchefstroom, South Africa. This study was conducted full-time during the period of January 2011 to April 2012, under the supervision of Dr. Sarina Claassens and co-supervision of Mrs. Misha de Beer.

The research done and presented in this dissertation signifies original work undertaken by the author and has not been submitted for degree purposes to any other university, before. Appropriate acknowledgements in the text have been made, where the use of work conducted by other researchers have been included.

The language and referencing style used in this dissertation are in accordance with the requirements of the journal *Plant and Soil*.

The opinions, findings, conclusions and recommendations expressed in this dissertation are those of the author and therefore the NRF does not accept any liability in regard thereto.

Clarissa Potgieter

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Summary

Assessment of the biofumigation effect of Canola (*Brassica napus*) on soil microbial community function and structure

Sunflower cultivation in South Africa is threatened to a large extent by the fungal parasite, *Sclerotinia sclerotiorum*, which causes extensive head rot and crop losses of up to 100%. This is a major problem for commercial farmers since it leads to a lower farm income, as the use of fungicides minimises crop damage, but increases production costs and can lead to several environmental problems. Therefore, an alternative is needed which can still control crop diseases, without harbouring health and environmental risks. Due to their biofumigation potential, *Brassica* plant species incorporated into the soil as green manures can be applied as alternatives for chemical pesticides. These plants produce glucosinolates that are hydrolysed upon tissue disruption by the enzyme, myrosinase, into active products for example isothiocyanates. Since isothiocyanates are highly toxic, it can be used instead of conventional pesticides for the inhibition of soil-borne pathogens. However, little is known about the effect of such biofumigants on the natural soil microbial communities required to maintain soil functions.

A greenhouse experiment was conducted to assess the influence of canola (*Brassica napus*) green manure on soil microbial community function and structure. The study consisted of 32 pots containing four treatments of eight replicates each. The treatments included 1) only sunflowers in soil (control), 2) sunflowers in soil incorporated with canola green manure; 3) sunflowers in soil incorporated with canola and inoculated with *S. sclerotiorum* and 4) sunflowers in soil inoculated with *S. sclerotiorum*. The experiment was conducted for 120 days.

From the soil physico-chemical properties conducted before the treatments were applied and after experiment completion, it was evident that the initial stimulating effect of canola manure on the soil carbon, total nitrogen and organic carbon content was not long-lasting. The overall microbial activity assessed with dehydrogenase assays and Biolog® Ecoplates, varied in relation to plant growth cycles, as root secretions differed. Multivariate analysis of the substrate utilisation patterns, distinguished among the treatments. Utilisation profiles illustrated that although different members of microorganisms were active in the various treatments, similar trends could still be observed. All four treatments showed similar diversity profiles after 120 days. Phospholipid fatty acid results indicated a significant increase in microbial biomass for all four treatments over time. The microbial community

structure differed to a lesser extent between treatments, but changed over time within each treatment. Community function varied according to the changing structure. Fatty acid stress ratios for all treatments showed significantly lower stress levels just after manure amendments, as the added organic matter might have stimulated microbial growth. Chlorophyll a fluorescence measurements showed shifts occurring in the photosynthetic efficiency of the sunflowers among the treatments. *S. sclerotiorum* had a suppressive effect on photosystem II functionality leading to lower electron transport and ATP production. Canola green manure amendments had a slight negative effect on sunflower vitality.

Overall the results obtained from this study suggest that incorporation of canola green manure into the soil has an effect on soil microbial community function and structure. Nonetheless, this biofumigation effect is short-lived and microbial communities returned to their initial compositions after the disturbance. The methods applied during this investigation indicated a possible suppressive effect of the canola manure on *S. sclerotiorum*.

Key words:

Biofumigation, Brassica napus, Chlorophyll a fluorescence, Microbial community, Sclerotinia sclerotiorum.

Opsomming

Assessering van die bioberokingseffek van Canola (*Brassica napus*) op grondmikrobiële gemeenskapfunksie en struktuur

Die verbouing van sonneblom in Suid-Afrika word tot 'n groot mate bedreig deur die fungus, *Sclerotinia sclerotiorum*, wat kopvrot en gewasverliese van tot 100% veroorsaak. Dit hou groot probleme in vir kommersiële boere, aangesien dit lei tot 'n laer inkomste, siende dat die gebruik van chemiese gifstowwe insetkoste verhoog en kan lei tot verskeie omgewingsprobleme. Daarom is 'n alternatiewe middel nodig wat gewassiektes kan beheer, sonder om gesondheids- en omgewingsrisiko's tot gevolg te hê. Weens hul bioberokingspotensiaal, kan *Brassica* plantspesies as groenvoer in die grond ingewerk word en as alternatief vir chemiese plaagdoders dien. Hierdie plante produseer glukosinolate, wat gehidroliseer word deur die ensiem mirosinase na aktiewe produkte soos byvoorbeeld isotiosianate. Aangesien isotiosianate hoogs toksies is, kan dit in plaas van konvensionele plaagdoders vir die inhibering van grondgedraagde patogene gebruik word. Daar is egter min inligting oor die effek van sulke bioberokers op die natuurlike grondmikrobiële gemeenskappe betrokke by die handhawing van grondprosesse.

'n Glashuis eksperiment is uitgevoer om die invloed van canola (*Brassica napus*) groenvoer op die grondmikrobiële gemeenskapsfunksie, biomassa en struktuur te bepaal. Die studie het bestaan uit 32 plastiek potte, wat vier behandelings met agt herhalings elk, insluit. Die behandelings sluit in: 1) slegs sonneblom in grond (kontrole), 2) sonneblom in grond behandel met canola groenvoer, 3) sonneblom in grond behandel met canola groenvoer en geïnokuleer met *S. sclerotiorum* asook 4) sonneblom in grond geïnokuleer met *S. sclerotiorum*. Die eksperiment het 120 dae geduur.

Dit is duidelik uit die grond fisies-chemiese resultate, geanaliseer voor die behandelings toegepas is en na eksperiment voltooi is, dat die aanvanklike stimulerende effek van canola groenvoer op die koolstof, totale stikstof en organiese koolstof inhoud, van die grond, korte duur was. Oor die algemeen is gevind dat die mikrobiële aktiwiteit (bepaal deur middel van dehidrogenase analyses) gevarieer het volgens plantgroeisiklusse weens die verskil in wortelsekresies. Onderskeid is getref tussen die verskillende behandelings op grond van hul substraatverbruikpatrone, soos verkry vanaf Biolog® Ecoplates-analises. Hierdie patrone het aangedui dat ongeag daarvan dat verskillende groepe mikroörganismes aktief is in die verskeie behandelings, soortgelyke tendense waargeneem is. Al vier behandelings het soortgelyke diversiteitsprofile getoon na 120 dae. Fosfolipiedvetsuur

resultate het 'n statisties betekenisvolle toename in mikrobiëse biomassa getoon, vir al vier behandelings oor tyd. Die mikrobiëse gemeenskapstruktuur het nie groot verskille tussen die verskeie behandelings gehad nie, maar het wel verander in elke individuele behandeling. Gemeenskapsfunksie het verander volgens die struktuur. Soos aangedui deur die lae fosfolipiedvetsuur stresverhoudings, stimuleer die bygevoegde organiese materiaal mikrobiëse groei. Veranderinge is waargeneem, met behulp van chlorofilfluoresensie metings, in die fotosintese doeltreffendheid van die sonneblomme in die verskillende behandelings. *S. sclerotiorum* het 'n onderdrukkende effek op fotosisteen II funksionaliteit getoon, wat dus lei tot verswakte elektronoordrag en laer ATP produksie. Die byvoeging van canola groenvoer het 'n effense negatiewe invloed op sonneblom lewenskragtigheid gehad.

Uit hierdie studie word waargeneem dat die inwerk van canola groenvoer in die grond, 'n variërende effek op die grondmikrobiëse gemeenskapsfunksie en -struktuur het. Nietemin, hierdie bioberokingseffek is van korte duur en mikrobiëse gemeenskappe keer terug na hul oorspronklike samestellings na die versteuring. Die metodes gebruik tydens hierdie studie het 'n moontlike onderdrukkingseffek van die groenvoer op *S. sclerotiorum* aangedui.

Sleutelterme:

Bioberoking, Brassica napus, Chlorofilfluoresensie, Mikrobiëse gemeenskap, Sclerotinia sclerotiorum.

List of Abbreviations

ABS	absorbance of photon by antenna pigment molecules
ABS/CS	phenomenological energy fluxes at each cross section for absorption
ABS/RC	specific energy fluxes at the reaction centres for absorption
Amino	amino acids
ANOVA	analysis of variance
ATP	adenosine 5'-triphosphate
AWCD	average well colour development
Bmonos	branched monounsaturated fatty acids
Bsat	base saturation
Carbox_a	carboxylic acids
Car_hyd	carbohydrates
CEC	cation exchange capacity
CLPP	community level physiological profile
C: N	carbon to nitrogen
CS	cross section
DGGE	denaturing gradient gel electrophoresis
Dhg	dehydrogenase
DI ₀ /CS	phenomenological energy fluxes at each cross section for dissipation
DI ₀ /RC	specific energy fluxes at the reaction centre for dissipation
DNA	deoxyribonucleic acid
EC	electrical conductivity
EDTA	ethylenediamine-tetra-acetic acid
ET	electron transport
ET ₀ /CS	phenomenological energy fluxes at each cross section for electron transport
ET ₀ /RC	specific energy fluxes at the reaction centre for electron transport
FAMES	fatty acid methyl esters
F: B	fungal to bacterial
FDA	fluorescent diacetate
FE	fumigation-extraction

List of Abbreviations

FI	fumigation-incubation
GC-MS	gas chromatography-mass spectrometry
GSLs	glucosinolates
HPLC	high performance liquid chromatography
INF	iodonitrotetrazolium violet-formazan
INT	iodonitrotetrazolium chloride
ITCs	isothiocyanates
K _{EC}	conversion factor
MBSats	mid-branched saturated fatty acids
mol%	mole percentage
Monos	monounsaturated fatty acids
mRNA	messenger ribonucleic acid
NADP ⁺	nicotinamide adenine dinucleotide phosphate
Nsats	normal saturated fatty acids
OD	optical density
PCA	Principal Component Analysis
PCR	polymerase chain reaction
PDA	potato dextrose agar
PEA	Plant Efficiency Analyser
PHA	poly β -hydroxyalkanoic acid
ϕE_0	quantum yield for electron transport
ϕP_0	maximum quantum yield for primary photochemistry
ϕR_0	efficiency with which an electron is transferred to reduce acceptors at the PS I acceptor side
Phospho	phosphorylated compounds
PI	performance index
PITC	2-phenylethyl isothiocyanates
PLFA	phospholipid fatty acid
Polys	polymers
Polys	polyunsaturated fatty acids
PS I	photosystem one
PS II	photosystem two

List of Abbreviations

ψE_0	efficiency with which an electron can be moved into the electron transport chain further than Q_A^- by a trapped exciton
Q_A	primary quinone acceptor
RC	reaction centre
RDA	Redundancy Analysis
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT-PCR	real-time polymerase chain reaction
S	treatment with only sunflowers in soil (control)
sat: unsat	saturated to unsaturated
SC	treatment with sunflowers in soil incorporated with canola green manure
SCI	treatment with sunflowers in soil incorporated with canola green manure and inoculated with <i>S. sclerotiorum</i>
SDS	sodium dodecyl sulphate
SEM	standard error of mean
SI	treatment with sunflowers in soil inoculated with <i>S. sclerotiorum</i>
SIM	selective inhibition method
SIR	substrate induced respiration
TBsats	terminally branched saturated fatty acids
TGGE	temperature gradient gel electrophoresis
THAM	Tris (hydroxy-methyl)-aminomethane buffer
TSFAME	total soil fatty acid methyl ester
TR	trapping of energy by the reaction centre
TR_0/CS	phenomenological energy fluxes at each cross section for trapping
TR_0/RC	specific energy fluxes at the reaction centre for trapping
TTC	tri-phenyl-tetrazolium chloride
Tukey's HSD	Tukey's Honest Significant Difference

List of Figures

Figure 2.1: An example of a logarithmic time-scale plot of the chlorophyll a fluorescence transients O-JIP over 50 μ s to 1 s. The initial fluorescence value is F_0 at 50 μ s, F_J is at 2 ms, F_I at 30 ms and the maximum fluorescence value is $F_P=F_M$ at 500 ms (Oukarroum et al. 2007).	34
Figure 4.1: Overall dehydrogenase activity of the different treatments at the various sampling periods.	49
Figure 4.2: Subplots of a Principal Component Analysis (PCA) ordination diagram illustrating the relationship between the carbon substrate group utilisation of the different treatments over time. Subplots display treatments a) S and SC, b) S and SI, c) S and SCI. Each sampling period is indicated by the amount of days after sowing the sunflowers followed by the name of the treatment. The eigenvalues for the first two ordination axes were 0.654 and 0.135, respectively. These two axes accounted for 78.8% of the total observed variance. The substrates were classified as: Amines; H_2O – water; Amino – amino acids; Polys – polymers; Carbox_a – carboxylic acids; Esters; Car_hyd – carbohydrates; Phospho – phosphorylated compounds.	53
Figure 4.3: Substrate group utilisation of the different treatments at days 0, 35, 77 and 119 where a) carboxylic acids, b) esters, c) amines and d) carbohydrates are illustrated. Statistically significant differences are indicated by alphabetic letters ($p < 0.05$). The same letters indicate no significant differences. Capital letters indicate statistically significant differences for each treatment over time, whereas the lowercase letters in brackets indicate significant differences between the different treatments at that specific time. Key to abbreviations: OD – optical density.	56
Figure 4.4: Estimated viable biomass of the different treatments at the various sampling periods after sowing the sunflowers.	57
Figure 4.5: Microbial community structure based on the mole% fraction of the major phospholipid fatty acid groups of treatments a) S, b) SC, c) SI and d) SCI for the duration of the experiment.	64
Figure 4.6: Fungal to bacterial ratio of the different treatments at the various sampling periods after sowing the sunflowers. Key to abbreviations: F: B – fungal: bacterial.	65
Figure 4.7: PLFA ratios of the different treatments at the various sampling periods after sowing which represents a) the <i>trans: cis</i> ratio; b) the saturated: unsaturated ratio; c) iso: anteiso ratio and d) Gram-positive: Total PLFAs ratio of the PLFA patterns. Key to abbreviations: sat: unsat – saturated: unsaturated.	66

Figure 4.8: Subplots of a Redundancy Analysis (RDA) ordination diagram illustrating the relationship between the dehydrogenase activity, carbon substrate group utilisation and major phospholipid fatty acid groups of the different treatments over time. Subplots display treatments a) S and SC, b) S and SI, c) S and SCI. The eigenvalues for the first two ordination axes were 0.087 and 0.057, respectively. These two axes accounted for 87.1% of the total observed variance for the species and environmental variables. The substrates were classified as: Amines; H₂O – water; Amino – amino acids; Polys – polymers; Carbox_a – carboxylic acids; Esters; Car_hyd – carbohydrates; Phospho – phosphorylated compounds. Key to abbreviations: Dhg – dehydrogenase; NSats – normal saturated; MBSats – mid-branched saturated; TBSats – terminally branched saturated; Monos – branched monounsaturated; Polys – polyunsaturated.

.....69

Figure 4.9: Performance index total (PI_{Total}) for the duration of the experiment. Only the data points of every three weeks were displayed. All values represent results obtained from 48 replicates.

.....71

Figure 4.10: Reduction of end electron acceptors at the electron acceptor side of PS I over the 119 days. Only the data points of every three weeks were displayed. All values represent results obtained from 48 replicates.

.....72

Figure 4.11: The quantum yield or biochemical efficiency $\psi E_0/(1-\psi E_0) = ET_0/(TR_0-ET_0)$, that describes the potential of an electron to be transferred further than the quinone electron acceptor of PS II (Q_A) into the electron transport chain, for each treatment expressed as a percentage of the control S. Data points of every three weeks were displayed.

.....74

Figure 4.12: OJIP parameters illustrating the functional and structural performances of the sunflower plants on day 35 after sowing the sunflowers. All the parameter values were expressed relative to the control treatment S. The parameters displayed include: k_p – photochemical de-excitation rate constant; PI_{ABS} – performance index for energy conservation from absorbed photons to the reduction of intersystem electron carriers; k_p+k_n – total de-excitation rate; PI_{Total} – performance index for energy conservation from absorbed photons to the reduction of end PS I electron carriers; k_n – nonphotochemical de-excitation rate constant; specific energy fluxes at the reaction centres for absorption (ABS/RC), trapping (TR₀/RC), electron carriers (EC/RC), electron transport (ET/RC), reduction of end electron acceptors (RE/RC); flux ratios or yields include the maximum quantum yield for primary photochemistry ($\phi P_0 = TR_0/ABS$), the efficiency with which an electron can be moved into the electron transport chain further than Q_A⁻ by a trapped exciton ($\psi E_0 = ET_0/TR_0$), the quantum yield for electron transport ($\phi E_0 = ET_0/ABS$), the efficiency of intersystem electron carriers to reduce end electron acceptors ($RE/ET = \delta_{R_0}$) and the efficiency with which an electron is transferred to reduce acceptors at the PS I acceptor side (RE) ($\phi R_0 = RE/ABS$); phenomenological energy fluxes at each cross section (CS) for absorption (ABS/CS), trapping (TR/CS), electron transport (ET/CS) and reduction of end electron acceptors (RE/CS). The amount of active RCs per CS can also be calculated as RC/CS.

.....75

Figure 4.13: OJIP parameters illustrating the functional and structural performances of the sunflower plants on day 119 after sowing the sunflowers. All the parameter values were expressed relative to the control treatment S. The parameters displayed include: k_p – photochemical de-excitation rate constant; PI_{ABS} – performance index for energy conservation from absorbed photons to the reduction of intersystem electron carriers; k_p+k_n – total de-excitation rate; PI_{Total} – performance index for energy conservation from absorbed photons to the reduction of end PS I electron carriers; k_n – nonphotochemical de-excitation rate constant; specific energy fluxes at the reaction centres for absorption (ABS/RC), trapping (TR_0/RC), electron carriers (EC/RC), electron transport (ET/RC), reduction of end electron acceptors (RE/RC); flux ratios or yields include the maximum quantum yield for primary photochemistry ($\phi P_0 = TR_0/ABS$), the efficiency with which an electron can be moved into the electron transport chain further than Q_A^- by a trapped exciton ($\psi E_0 = ET_0/TR_0$), the quantum yield for electron transport ($\phi E_0 = ET_0/ABS$), the efficiency of intersystem electron carriers to reduce end electron acceptors ($RE/ET = \delta_{R_0}$) and the efficiency with which an electron is transferred to reduce acceptors at the PS I acceptor side (RE) ($\phi R_0 = RE/ABS$); phenomenological energy fluxes at each cross section (CS) for absorption (ABS/CS), trapping (TR/CS), electron transport (ET/CS) and reduction of end electron acceptors (RE/CS). The amount of active RCs per CS can also be calculated as RC/CS.

.....76

Figure 4.14: Average fast phase chlorophyll a fluorescence measured on day 56 after sowing the sunflowers was expressed relative to the control, therefore $\Delta V = V_{Treatment} - V_{Control}$. The normalisations were done between a) F_0 (30 μs) and F_P (300 ms) indicating $\Delta V_{OP} = (F_t - F_0)/(F_P - F_0)$; b) F_0 and F_J (3 ms) indicating $\Delta V_{OJ} = (F_t - F_0)/(F_J - F_0)$, also known as the K-band; c) F_J and F_P indicating $\Delta V_{JP} = (F_t - F_J)/(F_P - F_J)$, also known as the I-band (Strasser et al. 2004).

.....78

List of Tables

Table 2.1:	Classification of carbon substrates in Biolog® Ecoplates (Garland and Mills 1991).	17
Table 2.2:	Characteristic phospholipid fatty acids found in specific microbial groups (Bohme et al. 2005; Frostegard and Baath 1996; Pinkart et al. 2002).	21
Table 2.3:	A summary of all the biophysical parameters calculated from the O-JIP test (Tsimilli-Michael et al. 2000; Yusuf et al. 2010).	35
Table 4.1:	Soil physical and chemical characteristics of soil samples tested.	46
Table 4.2:	Dehydrogenase activity (INF µg/g/2h) of all four treatments at the various sampling periods after sowing the sunflowers.	48
Table 4.3:	Average well colour development calculated on carbon substrate utilisation in the Biolog® Ecoplates for each treatment over time.	50
Table 4.4:	Shannon-Weaver Index calculated from Ecoplate optical density values for the different treatments at the various sampling periods.	52
Table 4.5:	Phospholipid fatty acid (PLFA) composition and ratios of the control treatment S over time.	58
Table 4.6:	Phospholipid fatty acid (PLFA) composition and ratios of the treatment SC over time.	59
Table 4.7:	Phospholipid fatty acid (PLFA) composition and ratios of the treatment SI over time.	60
Table 4.8:	Phospholipid fatty acid (PLFA) composition and ratios of the treatment SCI over time.	61

Introduction

1.1. Soil microbial communities and their role in the soil ecosystem

The Soil Survey Manual of the United States Department of Agriculture (1951) defines soil as: "...the collection of natural bodies occupying portions of the earth's surface that support plants and that have properties due to the integrated effects of climate and living matter acting upon parent material, as conditioned by relief, over periods of time."

Soil is one of the earth's most valuable natural resources, since it cannot be renewed by human hand and suffers continuous degradation through human intervention. Soil is used in agriculture for food cultivation, in infrastructure for road and building construction, in recreation for leisure activities and can function as a filtration system for groundwater. It consists of several components including minerals, organic matter, water, air and living organisms (Reynolds 1971). For soil to function as a living ecosystem, it is vital to maintain the soil quality and soil health. Soil quality was defined by the Soil Science Society of America Ad Hoc Committee on Soil Quality (S-581) in 1997 (Karlen et al. 1997) as the potential of a soil to function in various management practices, to maintain plant and animal productivity as well as water and air quality and uphold human infrastructure. Soil health, on the other hand, is the capacity of a soil to withstand disease and limit pathogen outbreaks (Janvier et al. 2007). Soil microorganisms play a major role in the maintenance of soil quality and soil health as well as in the functioning of soil ecosystems.

Soil microorganisms include fungi, bacteria, archaea and protozoa. Microbial communities determine organic matter decomposition rates and the decomposition products in the soil (Bossio et al. 2006). They help with the cycling of carbon and nitrogen in the soil (Grayston et al. 1998), metabolise pesticides and other soil contaminants (Torstensson 1980) and form soil aggregates through the secretion of binding proteins (Six et al. 1998). Several of these microorganisms can also serve as plant pathogens and cause severe crop yield losses in agriculture. Consequently, knowledge about the state of the soil microbial communities is essential for preservation of soil fertility and the long-term cultivation of crops. These soil microbial communities can be studied through the assessment of the microbial biomass, community structure and functional diversity. These community assessments can be used as potential indicators of soil quality disturbances, which may provide an early warning of deteriorating soil fertility (Pankhurst et al. 1995).

A good indicator requires several criteria, including: 1) the measured component must be found in all portions of the soil biomass in known concentrations every time; 2) the component must only be present in viable organisms; 3) it must be quantitatively extractable

from the soil and 4) there must be an accurate and precise technique for estimating the component concentration in soil samples (Jenkinson and Ladd 1981). Several components of individual microorganisms or microbial communities can be used as indicators. The first example is the use of phospholipid fatty acids (PLFAs). Phospholipid fatty acids are essential components of cell membranes (Peacock et al. 2001). The concentrations of PLFAs can give an estimate of the microbial biomass and community structure. After cell death, phospholipids are degraded by phospholipases which, in addition to the fact that they do not occur in storage compounds, show that they are efficient measures of viable biomass (Peacock et al. 2001). Phospholipid fatty acid analysis can be applied to compost (Rogers et al. 1994; Tunlid et al. 1985), water sediments, agricultural soils (Zelles et al. 1991), estuarine sediments (Guckert et al. 1985), decaying leaves and sewage (Findlay 2004). It can accurately indicate changes in microbial community composition due to starvation, exposure to toxicity and limited nutrient availability (Pinkart et al. 2002).

The enzymes present in microorganisms can also be used to indicate certain functions of the microbial community. One example of such an enzyme is dehydrogenase. Dehydrogenase is present in all living microorganisms and is involved in the biological oxidation of organic matter in the soil (Bremner and Tabatabai 1973). Therefore, the dehydrogenase activity can be measured as an indication of the functionality of the metabolically active soil microbial community (Skujins 1976) and may provide information on soil quality.

According to Garland and Mills (1991) substrate utilisation patterns can also be used to assess the metabolic functional diversity of soil microorganisms. These patterns, also referred to as community level physiological profiles (CLPPs), can be obtained through the use of Biolog® plates (Biolog® Inc., Hayward, USA). These plates consist of several wells with carbon substrates needed for microbial growth and tetrazolium violet redox dye (Guckert et al. 1996). As the microorganisms utilise the carbon substrates, the tetrazolium dye changes colour and the colour development can be measured spectrophotometrically (Kelly and Tate 1998). The CLPPs can indicate similarities between microbial populations from different environments and differences in the utilisation patterns may be linked to different active members of the microbial community. The rate of colour development is indicative of the metabolic activity of bacterial cells and the diversity of colour development indicates the microbial diversity (Garland 1997). Community level physiological profiles can be applied to polluted environments (Yao et al. 2000), different vegetation areas (Grayston et al. 1998) and forest management regions (Pietikäinen et al. 2000). The monitoring of agricultural soils with CLPPs has been shown to provide early warning signs of pesticide contamination, which reduces the microbial functional diversity in the soil (Floch et al. 2011).

Since certain soil microorganisms serve as plant pathogens and reduce crop yields in agricultural sectors, farmers are forced to increase pesticide applications. Sunflower cultivation in South Africa is threatened mainly by the fungal parasite, *Sclerotinia sclerotiorum*, which causes extensive head rot and crop losses of up to 100% (Van Wyk and Viljoen 2002). This is a major problem for commercial farmers since it leads to a lower farm income. These infections increase with a higher plant density, high nitrogen fertiliser applications and high rainfall. The use of fungicides and chemical fumigants can minimise the crop damages, but will drastically increase the production costs (Van Wyk and Viljoen 2002). Studies have shown that artificial fumigants can lead to several environmental problems, due to the high toxicity of the various chemicals and their slow degradation rate. These problems can include animal deaths (Newman 2010), the accumulation of the toxic compounds in biota causing neural dysfunction (Newman 2010), groundwater pollution (Newman 2010) and the destruction of the whole microbial community in the soil required to maintain important soil functions. Therefore, an alternative is needed which can still control crop diseases, without harbouring health and environmental risks. The rotation with green manure crops as potential biofumigants is widely explored (Wang et al. 2009).

1.2. Problem statement

Mustard, canola, broccoli and cabbage are cruciferous crop plants of the *Brassica* species. *Brassica* plants show signs of allelopathy towards other plants, making them “poor companion plants”. Allelopathy is when one plant secretes biochemical compounds into the surrounding environment, which influence other plants positively or negatively (Rice 1984). These plants produce glucosinolates (GSLs) that are hydrolysed by the endogenous enzyme, myrosinase, into active products including isothiocyanates (ITCs), thiocyanates and nitriles (Yulianti et al. 2007) upon tissue disruption (Mithen 2001). Due to the toxicity of ITCs, it can be used instead of conventional pesticides for the inhibition of soil-borne pathogens (Smith and Kirkegaard 2002). However, little is known about the effect of such biofumigants on the natural soil microbial communities.

According to Angus et al. (1994) biofumigation is the process in which soil-borne pests are suppressed by the incorporation of glucosinolate-containing plant materials as green manures into the soil. Several studies have found the reduction of soil pathogens such as *Rhizoctonia solani* (Yulianti et al. 2007), *Verticillium dahliae* (Chung et al. 2003) and *Fusarium oxysporum* (Gerik 2005) through the incorporation of *Brassica* green manure into the soil. Unfortunately, very few studies have been done on the destructive sunflower pathogen, *Sclerotinia sclerotiorum*. This fungal parasite induces plant necrosis, resulting in the total degradation of the sunflower head (Van Wyk and Viljoen 2002). Infected sunflowers appear shredded with only the vascular tissues present, making the successful

harvesting of the crop impossible. Various agricultural fields are abandoned to weeds or other non-preferred crops, due to the unsuccessful control of this parasite and lack of knowledge regarding control practices.

In this study the biofumigation effect of canola (*Brassica napus*) as a green manure on the sunflower pathogen *Sclerotinia sclerotiorum* was assessed. Furthermore, changes occurring in the indigenous soil microbial community were monitored by the analyses of microbial community function and structure.

1.3. Aim and objectives

The aim of the current study was to determine the effect of canola (*Brassica napus*) on the sunflower pathogen *Sclerotinia sclerotiorum* and on the native soil microbial community.

Specific objectives included:

- The characterisation of soil microbial activity by means of assays of the enzyme dehydrogenase.
- The characterisation of CLPPs of the native soil microbial communities by means of Biolog® Ecoplate analyses.
- The characterisation of the soil microbial community structure and biomass by analysis of PLFAs.
- The assessment of the plant vitality of sunflower plants through the analysis of fast phase chlorophyll a fluorescence kinetics and changes determined by the O-JIP test.
- The comparison of all the above parameters between inoculated and uninoculated soil treatments.
- Statistical analysis of the data to determine the correlation between the different treatments in terms of their structural and functional properties for the duration of the experiment.

1.4. Outline of dissertation chapters

Chapter 1 provides an introduction to the study, showing the motivation and rationale of the study. It includes the problem statement, aim, specific objectives and the outline of the dissertation chapters.

Chapter 2 contains the overall literature review of the study. It describes the importance of soil as a microbial habitat, the significance of soil microbial communities and the methods used to study these communities. Sunflower cultivation in South African agriculture threatened by the sunflower pathogen *Sclerotinia sclerotiorum*, biofumigation with *Brassica*

plant species as green manures and the measurement of plant vitality through chlorophyll a fluorescence is also discussed.

Chapter 3 describes the experimental layout as well as the materials and methods applied in the study. This includes the sampling procedure, analyses of the soil physical and chemical properties, dehydrogenase activity, CLPPs, PLFAs and chlorophyll a fluorescence patterns. The statistical analyses performed, are also discussed.

Chapter 4 includes the results obtained for each treatment in terms of structural and functional characteristics, as well as a general discussion of all the results.

Chapter 5 provides conclusions of this study and recommendations for future studies.

References for all the chapters are provided at the end of the dissertation.

Literature Review

2.1. The soil environment and its properties

Soil is one of the most important elements contributing to life on earth. It is fundamental in agriculture, infrastructure, forestry, the functioning of biota and forms an essential part of the water cycle. It acts as an essential growth medium for all plants; anchors plant roots and harbours important nutrients for continuous plant growth (Janvier et al. 2007). For soil to maintain ecological processes, it has to have distinct physical and chemical properties that determine the soil quality (Schlesinger et al. 1996). Soil quality can be defined as the ability of a soil to support biological productivity, to uphold environmental quality and to enhance plant and animal welfare (Doran and Parkin 1994). The physical properties of soil are for example soil texture, structure, water content and availability, and the availability of residual organic matter (Hillel 1980). Chemical properties include soil acidity, ion exchange capacity and mineralogy. These properties determine the structure and activity of soil microorganisms (Chapelle 1993; Doblas et al. 2009; Whitford 1996). The biological soil crust, which contains fungi, mosses, soil algae, lichens and cyanobacteria, is very important in functions such as water infiltration, water-holding capacity, runoff production, soil moisture content, the prevention of soil erosion, production of organic carbon and nitrogen and the holder of food webs in dry climates (Belnap and Lange 2001; West 1990; Zaady et al. 2010). The biological soil crust's degree of successional maturity influences the structure of soil microbiota (Bates and García-Pichel 2009).

Soil structure is the distribution of sand, silt, clay particles and organic matter into aggregates (Six et al. 2002). Soil aggregates consist of micro-aggregates that are adhered by roots, fungal hyphae and plants or microbially secreted mucilage to form macro-aggregates. Soil texture indicates the fineness of soil, whether it is coarse, gritty or smooth (Hillel 1980). This means that small, medium and large particles can be present in soil. Different soil types consist of different particle sizes, pH, porosity and aeration, as well as organic matter and have been found to influence microbial communities (Buyer et al. 1999). Sand has the largest particles and this decreases its ability to retain water and nutrients. Therefore, a sand environment will most likely not have such a high microbial population as a clay soil. Clay has the smallest particles. These particles adsorb water molecules to their surfaces and become hydrated. When dehydrated, the particles shrink and form cracks in the soil (Hillel 1980). It has a very high porosity and a huge amount of micro- and macropores. These pores are suitable channels and adsorption surfaces for different

microorganisms. Due to clay's high porosity and consistency, it drains slowly and can hold water longer than sand. This results in the soil being constantly saturated with water, which creates anaerobic conditions for microbial communities (Bossio et al. 2006). These conditions give rise to slower nutrient cycling and decomposition rates (Baker et al. 2001; Bossio et al. 2006). Thus, clay contains more organic carbon than sand (Böhme et al. 2005). Clay minerals can protect microbes against faunal predation (Muller and Hoper 2004) and starvation (Kieft et al. 1997), has less water availability fluctuations than sandy soils (Muller and Hoper 2004) and warms slowly in spring which generates a stable climatic environment for microbes in soil. Clay consists of secondary minerals such as kaolinite, smectite and gibbsite (Hillel 1980). The ion exchange is much higher in secondary minerals than primary minerals. This affects the mobility of ions in the soil, which can impact the transport of pollutants and the availability of plant and microbial nutrients (Hillel 1980). This implies that soil with high clay content can sustain a larger amount of microorganisms (Chodak et al. 2009). Residual organic matter availability in soil is important for microbial activity, because it is used by most microbes as an energy source (Kieft et al. 1993; 1997). The organic carbon substances are the products of decaying materials in the soil. The water content and availability in soil affect the type of microbes, the number of microorganisms and their growth-patterns (Bossio and Scow 1998; Schlesinger et al. 1996). Soil water content uses osmosis and nutrient regulation to control the microbial activity in the soil (Killham 1994). An inconsistent moisture content in dry areas decreases microbial abundance, since soil moisture is needed for nutrient diffusion and bacterial mobility (Kieft et al. 1997). Soils with continuous water availability are densely vegetated, have more oxygen from plant-driven gas exchanges and a higher amount of nutrients derived from rhizosphere secretions (Cordova-Kreylos et al. 2006).

2.2. Microorganisms in the soil environment

2.2.1. The role of microorganisms in the soil ecosystem

Microorganisms in soil include fungi, bacteria, archaea and protozoa. Soil microorganisms play an important role in the biogeochemical cycling of nitrogen, carbon, sulphur and phosphorus which are needed for plant nutrition (Ben-David et al. 2004; Grayston et al. 1998; Zelles 1999), they secrete extracellular polysaccharides needed in soil aggregate formation (Six et al. 1998; Tabuchi et al. 2008; Winding et al. 2005), degrade pesticides (Torstensson 1980) and can control the availability of heavy metals (Stolz and Oremland 1999). They also preserve energy and nutrients in their biomass (Jenkinson and Ladd 1981), perform enzymatic processes, can be plant pathogens, change plant features, fix nitrogen (Tabuchi et al. 2008), decompose complex organic matter (Tabuchi et al. 2008;

Zelles 1999), degrade organic pollutants (Zhang et al. 2008) and maintain soil fertility and quality (Zelles 1999). They can be used as indicators of soil contamination, restoration or deterioration (Pankhurst et al. 1995).

2.2.2. Factors influencing the soil microbial community

Several factors, other than the soil properties, can influence the structure, size, diversity and biomass of the soil microbial community. Such factors include fertilisers (Bossio et al. 1998), pesticides, soil cover, cultivation techniques, tillage practices (Staley 1999), crop type (Grayston et al. 1998), crop rotation (McGill et al. 1986), different plant species (Bossio et al. 2006; Esperschütz et al. 2007), deforestation (Moore-Kucera and Dick 2008b), clear-cutting and harvesting methods (Moore-Kucera and Dick 2008b), temperature fluctuations, ultra-violet radiation, osmotic pressure (Zhang et al. 2008), soil depth (Peacock et al. 2001) and heavy metal contamination (Frostegård et al. 1993).

The effect of the addition of fertilisers on the microbial community composition has been investigated (Baumann et al. 2009). It was found that adding inorganic nitrogen to a microbial community led to a stable community structure earlier in its growth phase than a community without inorganic nitrogen. Other studies found that organic compost increased the total microbial biomass more than artificial fertilisers (Esperschütz et al. 2007). This is because it provides organic substrates directly to the microbes. Unfortunately, the increasing use of fertilisers and pesticides may increase nitrate leaching into underground water tables (Galloway et al. 2004). Anthropogenic activities such as excessive tillage and cultivation practices lead to soil carbon loss which increases atmospheric carbon dioxide and contributes to greenhouse gas accumulation (Smith 2004). Tillage practices may affect several physical properties of soil such as bulk density (Wander et al. 1998), pore size distribution (Hermawan and Cameron 1993), water holding capacity (Trojan and Linden 1998), moisture content (Azooz and Arshad 1995) and aggregation (Chan and Mead 1988). Virgin grassland (no-till areas) soil moisture, organic matter, carbon to nitrogen ratio, water holding capacity, microbial biomass and total carbon, nitrogen and sulphur are much higher than in cultivation areas with extensive tillage techniques (McKinley et al. 2005). Permanent vegetation cover was found to lead to a higher organic carbon content which causes the microbial community structure to change and biomass to increase (Zelles et al. 1995). Plants help to stabilise slopes, support soil physical and chemical properties and contribute to microbial resources (Seastone-Moynahan et al. 2002).

Different plant species have an influence on the composition of soil microbial communities, since plants secrete different root exudates into the rhizosphere (Smalla et al. 2001). Plant root exudates include carbohydrates, sugars, organic acids, amino acids, hormones, vitamins and dead cells (Gardenas et al. 2010). Soil microbial communities

change and acclimatise according to the plant growth cycles (Xu et al. 2009). This is related to the changing soil temperature, soil moisture and quality of secreted root exudates with the altering growth stages. Several studies have shown that certain microbial communities can be linked to specific plant species (Bach et al. 2008; Brodie et al. 2002; Myers et al. 2001). For example, it was found that fungal (18:2 ω 6,9) and protozoan (20:4) fatty acid markers increased near trees which support the fact that fungi, Gram-negative bacteria and protozoa are associated with roots (Bach et al. 2008). The upper soil horizons with a high organic material content are usually richer in bacteria than lower horizons because of the amino acids, sugars and organic compounds that roots secrete (Unger et al. 2009). Thus, soil microbiota decrease with soil depth (Peacock et al. 2001).

Soil microorganisms are important in forests where local litter break down and nutrient mineralisation is necessary for sustainable tree growth (Moore-Kucera and Dick 2008a). The microorganisms control the division of carbon between storage products and the formation of carbon dioxide (Moore-Kucera and Dick 2008a). As the influence of carbon dioxide on the structure and function of microorganisms in grassland were examined, it was found that higher carbon dioxide concentrations lead to changes in soil microbial structures and increasing soil microbial biomass (Drissner et al. 2007). This is likely due to more root litter production and faster rhizo-deposition (Drissner et al. 2007). Deforestation, clear-cutting and harvesting methods can change the soil microclimate, cause soil compaction, alter the litter layers, decrease the amount of organic matter, change root growth, influence nutrient cycles, alter the temperature and moisture availability and impact the vegetation of the forest floor. The bacterial and fungal communities in the soil change in response to these environmental shifts (Moore-Kucera and Dick 2008b). This is because the organic nutrients required for microbial growth are less, as a result of litter declines and no more root turnovers. Fungi are filamentous and degrade lignin. The compaction due to clear-cutting is therefore a major disturbance for fungal growth. The decrease in bacteria may be due to the low fungal activity, as fungal decomposition products provide organic carbon. After clear-cutting, very few old and mature trees are left. This diminishing task destroys several root systems associated with mycorrhizal fungi and also bacteria aiding in this symbiotic relationship. Furthermore, after clear-cutting, the soil biodiversity is affected by the changes in soil temperature, nutrient availability and water content (Moore-Kucera and Dick 2008b).

The complexity of soil biodiversity ensures the sustainability of important functions as the environment changes (Bengtsson 1997). Soils with a higher biodiversity are more resistant and resilient to disturbances (Griffiths et al. 2001). Soil stability includes resilience (the ability of the ecosystem to recover after a disturbance) and resistance (the potential of the ecosystem to withstand a disturbance) (McNaughton 1994). Since microbial communities play an important role in the functioning of the soil ecosystem, they are

responsible for the stability of the soil. High population densities, wide distribution and optimum growth rates can ensure the resistance of a microbial community to an environmental disturbance. The microbial community has a high resilience if it can quickly recover from a disturbance through gene mutations or physiological flexibility (Allison and Martiny 2008).

Andrews and Harris (1986) suggested a concept that classifies microorganisms according to their ecological preferences. This concept implies that microorganisms can be divided into r and K-strategists according to their growth rates and substrate utilisation. Fast growing organisms form visible colonies within 24 hours after nutrient amendments. These organisms are called r-strategists. In contrast, K-strategists grow much slower and form colonies later (Kozdroj and Van Elsas 2000). Fast-growing r-strategists possess enzymes of low substrate affinity, whereas slow-growing K-strategists have enzymes with a high substrate affinity (Blagodatskaya et al. 2009). The r-strategists are dominant in environments rich in easily degradable substrates. They are usually present on young roots and in the rhizosphere (Sarathchandra et al. 1997), which is known as the soil zone nearest to and influenced by plant roots (Nehl et al. 1996). K-strategists dominate in nutrient-poor areas low in microbial density. They have a diversity of metabolic pathways enabling them to catabolise complex insoluble organic compounds (Blagodatskaya et al. 2007). K-strategists use more energy for extracellular enzyme production and defence mechanisms against predation, than for growth (Fontaine et al. 2003). Unstable environments are dominated by r-strategists, whereas K-strategists dominate stable environments (Pianka 1970).

Several methods exist for the characterisation of soil microbial communities based on their ecological attributes such as functional diversity, substrate utilisation and community structure.

2.2.3. Methods used to study soil microbial communities

Morphological features cannot be used to classify and identify microorganisms (Muyzer 1999). They are small and lack characteristic external attributes. There is a significant correlation between biodiversity of soil microorganisms, the interactions of functional groups and soil quality (Bengtsson 1998). Microorganisms seldom occur in isolation in soil. Microbial communities interact with each other, leading to changes in soil microbial community structures and biomass. Thus, it is more meaningful to study the activity of functional groups and species in soil ecosystems and how the community structure and viable microbial biomass changes over time. Methods used to study soil microbial communities can be divided into techniques used to determine functional diversity, structural diversity and molecular diversity.

2.2.3.1. Functional diversity techniques

Functional diversity techniques include direct plate counts, enzymatic activity, biomass methods, respiration and community level physiological profiling.

According to Pinkart et al. (2002) microbial biomass has traditionally been estimated by **viable direct counts**. This may have been sufficient for monocultures, but is not accurate for environmental samples where the sample only represents 0.1-10% of the whole community. Plate counts are fast, inexpensive (Kirk et al. 2004) and can distinguish between different microbial groups (Frostegård and Bååth 1996). Unfortunately, traditional culturing techniques cannot detect unculturable organisms (White et al. 1997), are unable to differentiate between viable and dead cells or soil granules (Jenkinson and Ladd 1981) and benefit fast growing species (Kirk et al. 2004). Pinkart et al. (2002) emphasises that direct microscopic counts measure abundance and not biomass.

In **fluorescence microscopic methods**, dyes are used to stain bacteria or fungi and their numbers are counted under a microscope (Joergensen and Wichern 2008). Proteins and nucleic acids are stained with fluorescein isothiocyanate, ethidium bromide or acridine orange. Fluorescent diacetate (FDA) stains metabolically active fungal hyphae (Bloem et al. 1995). Fluorescent diacetate is a specific fluorochrome which is taken up by metabolically active cells and hydrolysed by proteases, esterases or lipases into fluorescein (Jensen et al. 1998). Fluorescein can be measured with a spectrophotometer at a wavelength of 490 nm (Adam and Duncan 2001). Unfortunately, not all the stains distinguish between living and dead cells and some stains degrade easily under unfavourable conditions (Nannipieri et al. 2003).

Certain biomass methods make use of the functionality of microbiota in a microbial community. The catalisation of all metabolic processes in soil by enzymes is a major reason why the measurement of **enzymatic activity** can be used as an index for microbial metabolic diversity (Nannipieri et al. 2002) and as an early indicator of soil degradation and stress (Chaer et al. 2009). Enzymes control the turnover of organic matter and nutrients in the soil (Klose et al. 2006), degrade toxic substances (Wang et al. 2007) and stabilise soil aggregates (Dick et al. 1994). The soil enzyme levels vary among diverse soil types because of different quantities of organic material, microbial colonisation and biological reaction rates. These enzymes originate from viable or non-living microorganisms, plant roots and secretions, as well as soil animals (Makoi and Ndakidemi 2008). Examples of such soil enzymes include amylase, arylsulphatase, β -glucosidase, cellulase, chitinase, dehydrogenase, phosphatase, protease and urease (Makoi and Ndakidemi 2008).

Amylase is a starch hydrolysing enzyme that breaks starch substances down to glucose, oligosaccharides or maltose (Thoma et al. 1971). It is mainly produced by plants.

Several factors can affect the functioning of amylase in the soil especially vegetation type, soil type and management practices (Pancholy and Rice 1973). Arylsulphatase enzymes are involved in the hydrolysis of aromatic sulphate esters ($R-O-SO_3^-$) into phenols ($R-OH$) and sulphate (SO_4^{2-}) or sulphate sulphur (SO_4-S) (Tabatabai 1994). Bacteria release these enzymes when available sulphur becomes limited (McGill and Colle 1981).

β -glucosidase is responsible for the biodegradation of β -glucosides into glucose (Martinez and Tabatabai 1997). The enzyme originates from living microbial cells and can adsorb to clay or humic substances (Skujins 1976). β -glucosidase can act as a soil quality indicator, provide evidence of historical biological activity and indicate the impact of management practices on soils (Ndiaye et al. 2000).

Cellulase is the enzyme that facilitates the hydrolysis of polysaccharides known as cellulose to glucose, cellobiose and other oligosaccharides (Deng and Tabatabai 1994). It is very important, since it degrades one of the most recalcitrant polymers in nature. Cellulase is mainly synthesised in plants and some fungi and bacteria. Fungicides, soil pH, temperature, water content and aerobic soil conditions all influence the cellulase activity in agricultural soils (Deng and Tabatabai 1994).

Chitinase forms part of the structural component of fungal cell walls to prevent pathogen invasion (Chet and Henis 1975) and facilitates the degradation of chitin. Plants secrete chitinase as a defence mechanism against microbial or pathogenic infections. Shapira et al. (1989) demonstrated chitinase's potential to control soil-borne pathogens such as *Sclerotium rolfsii* and *Rhizoctonia solani*.

Phosphatase is an important enzyme in the phosphorous cycle. It is involved in the hydrolysis of esters and phosphoric acid anhydrides (Speir and Ross 1978). When there is a phosphorous deficiency in the surrounding soil, acid phosphatase secretion in plant roots is stimulated to increase the availability of phosphate in soil. Therefore, it helps the plant to handle phosphorous limitations (Ndakidemi 2006).

The mineralisation of nitrogen is mainly facilitated by the soil enzyme protease, which is usually observed as a carbohydrate complex in the soil. Protease activity is influenced by the concentration of soil humic acids (Makoi and Ndakidemi 2008; Nannipieri et al. 1996). The enzyme urease catalyses the hydrolysis of urea (fertiliser) into ammonia (NH_3) and carbon dioxide (CO_2). During this reaction the soil pH becomes alkaline (rises) and the highly volatile NH_3 is lost into the atmosphere (Simpson and Freney 1988). This leads to rapid nitrogen losses. Soil urease is synthesised by plants and microbes and occur as intra- and extracellular enzymes in the soil. Various factors impact urease activity including soil organic matter content, soil depth, heavy metals, temperatures and cropping history (Makoi and Ndakidemi 2008; Yang et al. 2006).

Dehydrogenase is an enzyme existing in intact viable cells, without functioning extracellularly in the soil (Gao et al. 2010). It plays a role in the oxidation of organic matter by electron transport reactions (Kandeler et al. 1996). Given that these reactions occur during microbial respiration processes, the dehydrogenase activity can provide insight into the soil fertility. This enzyme can also indicate changes in the soil environment due to pesticide applications (Floch et al. 2011), management techniques, pollution (García and Hernández 1997) and serve as a direct indicator of the soil microbial activity as it only occurs in viable cells (Nannipieri et al. 1996). Enzymatic activity is linked to the soil nutritional status within agricultural (Klose et al. 1999), polluted (Nannipieri 1994) and pesticide treated environments (Klose and Ajwa 2004). The application of microbial fertilisers was found to stimulate dehydrogenase activity in the soil rhizosphere (Shengnan et al. 2011). This can be linked to the increased microbial activity. Tiwari et al. (1989) showed that the dehydrogenase activity was higher in flooded than in non-flooded soil. This was due to a lower redox potential. The oxygen in the soil is exhausted after flooding, resulting in changes in the microbial communities from aerobic to anaerobic conditions (Makoi and Ndakidemi 2008). Therefore, dehydrogenase activity can provide an indication of the microbial oxidative activity in soil (Trevors 1984).

Microbial biomass is an estimate of the amount of bio-elements stored in a microbial population and is therefore an essential indicator of soil fertility (Beck et al. 1997). There are various methods to determine the soil microbial biomass including **chloroform (CHCl₃) fumigation-incubation (FI)** (Jenkinson and Powlson 1976), **CHCl₃ fumigation-extraction (FE)** (Vance et al. 1987) and substrate-induced respiration (SIR) (Anderson and Domsch 1978). According to Jenkinson (1988) the CHCl₃ FI method is only appropriate for aerated soils with a pH higher than 4.8 and low organic carbon content. In contrast, SIR and CHCl₃ FE methods are less dependent on the soil conditions (Vance et al. 1987). In the FI method, moist soil samples are fumigated with chloroform for 24 hours and then incubated with NaOH. The microbial biomass carbon is calculated by determining the difference between CO₂ evolved from fumigated soil and CO₂ evolved from non-fumigated soil (Beck et al. 1997). In the FE method, moist soil samples are extracted with potassium sulphate (K₂SO₄) and fumigated with CHCl₃. The microbial biomass carbon (C) is then calculated by dividing the difference between the organic C extracted from the fumigated soil and from the non-fumigated soil with a K_{EC} factor of 0.45 (Joergensen 1996). The recolonising microbial population degrades the fumigated (killed) microorganisms in a soil sample after CHCl₃ fumigation (Brookes et al. 1985). Various studies used a chloroform FE method to estimate the soil microbial biomass-nitrogen and carbon (Amato and Ladd 1988; Yao et al. 2000). The samples were fumigated with chloroform for 24 hours after which the extracted nitrogen and carbon were colorimetrically identified at 570 nm or through gas chromatography. This

method measures the total microbial biomass directly, but does not give information on the structure of the microbial community (Peacock et al. 2001).

Soil respiration is the process in which organic compounds are oxidised by the uptake of oxygen and the release of CO₂. Anderson and Domsch (1978) reported the main factors influencing the respiration rate of a soil as the physiological status of the microbial community, the age distribution of the microbial community and the carbon availability in the soil. Total respiration measured can indicate the complex metabolic activities occurring in heterogeneous soil microbial communities (Anderson and Domsch 1975). The **substrate-induced respiration (SIR)** method is simple, rapid and affordable and can be used to determine the microbial biomass C in soil samples. It is based on the stimulated response of microbes to the addition of an easily degradable carbon substrate, such as glucose. The production rate of the respiratory CO₂, excreted during the stimulated metabolic reaction is a measure of the metabolically active microbial biomass (Bailey et al. 2002; Van de Werf and Verstraete 1987). For successful determination, two requirements should be met namely 1) the soil must be saturated with water and 2) the optimal amount of glucose added for significant microbial growth must be determined (Bailey et al. 2002). Moist soil is incubated with glucose at 25°C for two hours. The amount of CO₂ generated is measured by gas chromatography, titration (Cheng and Coleman 1989) or with an infrared gas analyser (Sparling 1995). The SIR and the microbial biomass carbon are calculated with an equation from Anderson and Domsch (1978). The SIR response of microbes over 0-6 hours reflects the initial soil microbial community before any growth on added substrates occurred (Anderson and Domsch 1973; 1978). The SIR method can be used to study the effects of tillage (Adams and Laughlin 1981), pesticides (Anderson et al. 1981), heavy metals (Brookes and McGrath 1984) and deforestation (Ayanaba et al. 1976) on the microbial biomass. Advantages of the SIR method include that it is inexpensive and requires only substrates and a CO₂ efflux estimator. Unfortunately, this method requires the optimisation of the soil water content, the glucose concentrations and the incubation period (West and Sparling 1986). Other limitations include the preference of glucose metabolising organisms above other organisms to estimate the soil microbial biomass (Smith et al. 1985) and the inhibiting effect of soil texture and organic matter content on the SIR method (Lin and Brookes 1999).

Bacteria and fungi are both important in the decomposition of organic matter in soil. The **substrate-induced respiration inhibition (SIRIN) or selective inhibition method (SIM)** is a modification of the SIR method, where specific antibiotics are used to selectively inhibit bacterial or fungal respiration (Anderson and Domsch 1975). The addition of a fungicide such as cycloheximide or captan (Beare et al. 1990) suppresses fungal respiration, making it possible to calculate the bacterial contribution to soil respiration. The opposite is

true when bactericides such as streptomycin sulphate, oxytetracycline (Beare et al. 1990), bronopol (Bailey et al. 2003) and chloroamphenicol (Nakamoto and Wakahara 2004) are added. These antibiotics inhibit protein synthesis; therefore soil respiration is not completely inhibited. The glucose-metabolising systems are still able to function normally. Glucose is added as a substrate to stimulate CO₂ production during soil respiration. After incubation, the contribution of bacteria and fungi to the soil respiration can be determined by comparing the antibiotic-treated soil respiration with the untreated soil respiration. The CO₂ efflux can be measured with gas chromatography, titration or with an infrared gas analyser. The fungal-to-bacterial activity ratio is estimated as respiration inhibition caused by the fungicide divided by the inhibition caused by the bactericide. F: B ratios have been found to be 0.54 to 3.04 for agricultural soils (Beare et al. 1992), 13.5 for restored prairie soil (Bailey et al. 2002) and 18.2 for acidic beech soil (Blagodatskaya and Anderson 1998). The SIR/SIM methods have been used in arable (Anderson and Domsch 1975), grassland (West 1986), rhizosphere soils and in plant matter (Beare et al. 1990). Several disadvantages of the SIM method exist. This method favours fast-growing organisms that metabolise glucose rapidly (Bååth and Anderson 2003). Antibiotics can be adsorbed in soil to humus substances or utilised by autochthonous microbes as a carbon source (Bailey et al. 2003). The applied selective inhibitors kill competitive organisms leading to the abnormal growth of the remaining organisms (Parkinson et al. 1971). Preliminary experiments are needed to determine the optimal inhibitor concentrations before selectivity problems occur.

Biolog plates are one of the techniques frequently used to assess community level physiological profiles (Garland and Mills 1991). Biolog plates measure the functional diversity of a microbial community through their assimilation of different carbon substrates (Stefanowicz 2006). The carbon utilisation pattern is referred to as the CLPP. Differences in the utilisation patterns can be linked to different active members of the microbial community. There are various CLPP plate types available. GN2 and GP2 plates are used to identify pure cultures of Gram-negative and Gram-positive bacteria respectively, where each plate contains 95 carbon substrates (Konopka et al. 1998). Ecoplates are developed for the characterisation of environmental samples. They contain 31 unique carbon substrates in triplicate as shown in Table 2.1, to account for the sample variability (Guckert et al. 1996). SFN₂ and SFP₂ plates are used to assess fungal profiles. These plates have the same substrates as GN2 and GP2 plates, but without tetrazolium dye (fungi cannot reduce it) and with added antibiotics to suppress bacterial contamination (Buyer et al. 2001). These substrates are for example amino acids, carbohydrates and carboxylic acids (Stefanowicz 2006). Each well contains colourless tetrazolium dye. A suspension of the soil is made and incubated in the plate wells. When the microorganisms utilise the substrates, the dye is changed to a violet formazan and the colour development is measured

spectrophotometrically (Kelly and Tate 1998). The Biolog method and CLPPs have several advantages. The methods are fast, reproducible, inexpensive, produce large amounts of data, can compare the functionality of microbial communities, have replicates to ensure the reliability of the inoculation, the similarities between microbial populations from different environments can be estimated, the rate of colour development indicates the metabolic activity of bacterial cells and the diversity of colour development indicates the microbial diversity (Garland 1997; Kirk et al. 2004; Zak et al. 1994). The Biolog plates can also be modified to represent real soil conditions with relevant carbon sources.

A disadvantage of Biolog plates is that they are selective and cater mainly for fast-growing bacteria that can grow at high substrate concentrations (Kirk et al. 2004; Smalla et al. 1998). In Biolog wells, the organisms are spatially more limited than on agar plates and certain organisms are not able to come in direct contact with the substrates. Thus, the substrate response pattern relies more on the competitive ability of the microorganisms in the soil suspension than on the relative proportions of each organism initially (Wünsche et al. 1995). Other disadvantages, also applicable to Ecoplates, include that the carbon substrates in the wells are present in high concentrations and are not always found in soil (Campbell et al. 1997), not all bacteria are capable of reducing tetrazolium dye (Winding et al. 1994), the tri-phenyl-tetrazolium chloride (TTC) indicator dye may be toxic to certain species (Winding et al. 1994; Yao et al. 2000) and the pH of the medium is buffered at 6.5 (Yao et al. 2000). In the study of Yao et al. (2000) the impact of different pH levels on the CLPP method was determined and it showed that this method can be used to assess pollution-induced stress on soil communities. The incubation of inoculated Ecoplates over a few days is needed for colour development due to growth on carbon substrates. When organisms utilise the substrates in the wells, it means that they can adapt to artificial conditions (Degens 1999).

Community level physiological profiles and the Biolog method have been applied to indicate changes in the diversity of microbial communities in oil contaminated soils (Wünsche et al. 1995), different vegetation areas (Grayston et al. 1998), rhizosphere soils (Garland 1996; Winding 1994), sewage sludge (Banerjee et al. 1997; Fritze et al. 2000), genetically-modified plant materials (Donegan et al. 1995), compost (Mondini and Insam 2003), heavy metal contaminated soils (Bååth et al. 1998; Ellis et al. 2001; Fritze et al. 2000), alkaline soils (Pankhurst et al. 2001), straw residues amended with biocontrol agents (Pfender et al. 1996) and forest management sites (Pietikäinen et al. 2000).

Table 2.1: Classification of carbon substrates in Biolog® Ecoplates (Garland and Mills 1991).

Carbohydrates		Amino acids	
N-acetyl-D-glucosamine	$C_8H_{15}NO_6$	L-Arginine	$C_6H_{14}N_4O_2$
β -methyl-D-glucoside	$C_7H_{14}O_6$	L-Asparagine	$C_4H_8N_2O_3$
D-Mannitol	$C_6H_{14}O_6$	L-Phenylalanine	$C_9H_{11}NO_2$
D-Xylose	$C_5H_{10}O_5$	L-Serine	$C_3H_7NO_3$
i-Erythritol	$C_4H_{10}O_4$	L-Threonine	$C_4H_9NO_3$
D-Cellobiose	$C_{12}H_{22}O_{11}$	Glycyl-L-glutamic acid	$C_7H_{12}N_2O_5$
α -D-lactose	$C_{12}H_{22}O_{11}$		
Carboxylic acids		Polymers	
D-Galactonic-acid- γ -Lactone	$C_6H_{10}O_6$	Tween 40	
D-Galacturonic acid	$C_6H_{10}O_7$	Tween 80	
2-Hydroxy Benzoic acid	$C_7H_6O_3$	α -Cyclodextrin	$C_{36}H_{60}O_{30}$
4-Hydroxy Benzoic acid	$C_7H_6O_3$	Glycogen	$(C_6H_{10}O_5)_n$
γ -Hydroxybutyric acid	$C_4H_8O_3$	Amines	
α -Ketobutyric acid	$C_4H_6O_3$	Phenylethylamine	$C_8H_{11}N$
D-Glucosaminic acid	$C_6H_{13}NO_6$	Putrescine	$C_4H_{12}N_2$
Itaconic acid	$C_5H_6O_4$	Esters	
D-Malic acid	$C_4H_6O_5$	Pyruvic acid methyl ester	$C_4H_8O_3$
Phosphorylated compounds			
Glucose-1-Phosphate	$C_6H_{13}O_9P$		
D, L- α -Glycerol Phosphate	$C_3H_9O_6P$		

2.2.3.2. Structural diversity techniques

Structural diversity techniques include the analyses of fatty acid methyl esters (FAMES), ergosterol and PLFAs.

Lipids and nucleic acids, because of their cellular richness and chemical variation, are usually used to analyse microbial communities (Drenovsky et al. 2004). **Total soil fatty acid methyl ester (TSFAME)** methods extract lipids from living or dead microbial and animal cells, plant tissues, cellular storage compounds and cell membranes. In the

TSFAME method sodium hydroxide (NaOH) is added to the samples. After that, the cells are lysed in a water bath for 30 minutes to set the fatty acids free. The fatty acids are then converted to FAMES and extracted from an aqueous phase into an organic phase. Gas chromatography is used to quantify the FAMES (Drenovsky et al. 2004). The TSFAME method is fast, requires little soil and extracts more total fatty acids compared to other methods, because it includes lipids from membranes, decomposing material and storage compounds. It is also influenced by external factors (Kirk et al. 2004) and it is difficult to interpret the profiles (Drenovsky et al. 2004). It is therefore better to use this method when sites and treatments are compared, when time is limited and only small soil samples are available (Drenovsky et al. 2004).

Fungal biomass is a good indicator of soil fertility and for monitoring environmental pollution (Montgomery et al. 2000). Barajas-Aceves et al. (2002) found that the ergosterol content can be used to estimate the fungal biomass in soil. **Ergosterol** is an essential sterol in the phospholipid bilayer of every fungal cell membrane. It increases membrane microviscosity (Peacock and Goosey 1989), mediates membrane permeability (Weete 1989) and contributes to cell wall synthesis (Hart and Brookes 1996). Although ergosterol is exclusive to fungi, very small quantities can be found in some protozoa, cyanobacteria and algae. Bacteria, archaea and plants contain no ergosterol in their cell membranes (Cheng et al. 2008). For ergosterol extraction, methanol is added to moist soil and the samples are ultrasaponified in an ice-water bath. Hexane is added to the samples and left overnight to separate the two phases. The hexane layer is transferred and analysed with high performance liquid chromatography (HPLC) (Barajas-Aceves et al. 2002). Advantages of ergosterol extraction include the high reproducibility and extraction efficiency of the method, the fact that ergosterol is specific for fungi and the substance can be exactly defined by chromatography (Newell et al. 1987). It can also indicate the production of secondary metabolites, for example mycotoxins (Czaczek et al. 2002). Various studies have shown that ergosterol does not degrade rapidly after cell-death. Samples can therefore be stored for long periods at -80°C without experiencing ergosterol losses (Gessner et al. 1991). Several disadvantages exist. The method is slow, requires large sample sizes and reagent volumes and includes complex treatment steps such as saponification or sonication (Grant and West 1986). Ergosterol concentrations may be influenced by the adsorption of the sterol to soil particles and the protection of the fungal cell membrane by soil components (Montgomery et al. 2000). Ergosterol content can be used to study fungal dominance in various environments including soil (Bååth and Anderson 2003; Grant and West 1986), house dust (Axelsson et al. 1995), decomposing plant material (Newell et al. 1987), mycorrhizae (Frey et al. 1992), aquatic ecosystems (Gessner and Chauvet 1993), pesticide treated soil (Hart and Brookes 1996) and building materials (Reeslev et al. 2003).

Phospholipid fatty acid analysis is a culture-independent (Zhang et al. 2008), biochemical method that is used to indicate structural changes in a microbial community (Findlay 1996; Moore-Kucera and Dick 2008b; Pinkart et al. 2002) and to estimate the viable microbial biomass (Crossman et al. 2006; White et al. 1979; Zelles 1999). Findlay (2004) advised that biomolecules studied in microbial community structure analyses should possess five basic characteristics:

- A universal arrangement for the parent molecule, but a restricted arrangement for specific compounds.
- Appear in constant sections in all cells.
- These sections should not differ with growth or environments.
- Their concentrations should still indicate limited ratios.
- Have a rapid turnover time.

The requirements for cellular constituents to qualify as microbial biomass indicators have been described (Pinkart et al. 2002). They should be universally distributed, have a short lifetime after cell-death and should be consistently formed throughout microbial growth patterns. Phospholipids, which consist of fatty acids, satisfy these requirements (Pinkart et al. 2002; Zelles 1999). Fatty acids may exist freely, as glycerides or may be attached to phospholipids, glycolipids, lipoproteins, lipopolysaccharides and lipoteichoic acid (Zelles 1999). In soil, both cellular and extracellular lipids are present. Cellular lipids, derived from viable or non-viable plant or microbial cells, are used to determine the current status of the microbial community in the soil. In contrast, extracellular lipids are derived from plants or microbes and gives information about the microbial history (Zelles 1999).

Polar lipids, especially phospholipids, are found in all living eukaryotic and bacterial cell membranes. After cell-death, the phospholipids lose their phosphate groups through the degradation by exogenous and endogenous phospholipases into neutral lipid diglycerides which are not found in dead cells (Crossman et al. 2006; Moore-Kucera and Dick 2008b). For example, a microbial community in its active growing phase will have a diglyceride fatty acid / PLFA ratio of $\mu \sim 0$ (White and Ringelberg 1996). It has been reported that the PLFA technique can fingerprint a microbial community (Klamer and Bååth 2004). Phospholipids are not present in storage compounds or in anthropogenic contaminants (Zelles et al. 1992) and are more stable than storage lipids (which differ according to growth conditions and the carbon / nitrogen ratio of the substrate).

Lipid analysis can supply phenotypic information about a community such as growth rate, exposure to toxicity, starvation, nutrient deficiencies, unbalanced growth and anoxic / oxic metabolic conditions (White and McNaughton 1997). In contrast, nucleic acid analysis

gives more structural information (White et al. 1998). Phospholipid fatty acid profiles indicate the physiological status of microorganisms, because microbes change their cell membrane composition in response to environmental stress and pollution (Cordova-Kreylos et al. 2006; Pinkart et al. 2002). These changes may be caused by temperature fluctuations (Pettersson and Bååth 2003), soil depth (Fierer et al. 2003; Peacock et al. 2001), manuring (Frostegård et al. 1997; Peacock et al. 2001), heavy metal contamination (Frostegård et al. 1993) and flooding (Bossio and Scow 1998). The fatty acid changes due to cell stress occur when monounsaturated fatty acids change into cyclopropyl fatty acids and cis-fatty acids transform into trans-fatty acids (Guckert et al. 1986; 1991). Microbes can change their membrane fluidity by altering the fatty acid chain length, the ratio of saturated to unsaturated fatty acids and the ratios of iso- to anteiso-branched fatty acids (Kaneda 1991). Some bacteria, when their essential nutrients are limited, form poly β -hydroxyalkanoic acid. This is a temporary carbon storage compound. PLFA is formed when the essential nutrients are available for the organism to grow and divide (Pinkart et al. 2002).

Certain characteristic fatty acids are found in specific microbial groups as shown in Table 2.2 (Pinkart et al. 2002). By dividing the mole% of the fungal fatty acid marker 18:2 ω 6,9 by the sum of the mole% of the bacterial fatty acid markers (a15:0, i15:0, 15:0, i16:0, 16:1 ω 7c, 16:1 ω 9t, i17:0, a17:0, 17:0, cy17:0, 18:1 ω 7c and cy19:0), a fungal to bacterial (F: B) biomass ratio can be determined. This F: B biomass ratio can be used as a biomarker for soil quality. It can differ in response to soil fertility (Pennanen et al. 1999), nitrogen availability (Leckie et al. 2004), soil pH, soil texture and soil management practices (Lauber et al. 2008). Studies have found that increasing bacterial abundance is linked to increasing fertility, whereas fungal biomass is higher in soil with a lower nutrient content (Pennanen et al. 1999; Wallenstein et al. 2006). According to McGill et al. (1981) the carbon to nitrogen (C: N) ratio of bacterial biomass is calculated to vary between 3 and 6, whereas fungal biomass is within the range of 5 and 15. This suggests that fungi will dominate when nitrogen is limited, but bacteria will dominate when nitrogen is sufficient (Hu et al. 2001) or applied as a fertiliser (De Vries et al. 2006). Consequently, an ecosystem with a higher fungal biomass, indicative of less nitrogen fertilisation, will have lower nitrogen leaching problems. A study was performed to determine if fungal-dominated soils are more efficient at using carbon than soils with lower F: B biomass ratios (Thiet et al. 2006). They used a carbon-labelled glucose substrate to indicate the relative portions used for biomass and respiration. It was concluded that soil ecosystems dominated by fungal communities (high F: B ratios) sequester more carbon into biomass and metabolite production than systems with bacterial dominance (low F: B ratios).

Table 2.2: Characteristic phospholipid fatty acids found in specific microbial groups (Böhme et al. 2005; Frostegård and Bååth 1996; Pinkart et al. 2002).

Structural characteristics of fatty acids	Fatty acids	Specific microbial groups
Straight chain saturated	14:0, 15:0, i15:0, a15:0, i16:0, 16:0, i17:0, a17:0, 17:0, cy17:0, 16:1 ω 7c, 16:1 ω 9t, 18:0, 18:1 ω 7, cy19:0	All bacteria
Monounsaturated	16:1 ω 7c, 16:1 ω 7t, 16:1 ω t, cy17:0, 18:1 ω 7, 18:1 ω 7c, 18:1 ω 9c, cy19:0	Gram-negative bacteria
Terminally branched saturated	a15:0, i15:0, i16:0, i17:0, a17:0	Gram-positive bacteria
Mid-chain branched saturated	10Me16:0, 10Me17:0, 10Me18:0	Actinomycetes
Polyunsaturated	18:2 ω 6,9	Fungi
Cyclopropyl / long chain saturated	20:0, 22:0, 24:0	Eukaryotes

For PLFA analysis, lipids are extracted from lyophilised soil samples with a single-phase chloroform-methanol-phosphate buffer. Fractionation in a silicic acid column separates phospholipids from neutral lipids and glycolipids (Moore-Kucera and Dick 2008b; Wu et al. 2009). The phospholipids undergo alkaline methanolysis to form FAMES. These FAMES are dissolved in hexane and analysed with gas chromatography (Moore-Kucera and Dick 2008b; Wu et al. 2009).

Phospholipid fatty acid analysis has several advantages. It is quick, reproducible, easy, culture-independent (Zhang et al. 2008), can analyse a large number of samples, can distinguish between large functional groups within microbial communities (Joergensen and Wichern 2008; Zelles 1999), fingerprint a community's composition, detect fatty acids in low concentrations in the soil, extract fungal and bacterial spore lipids better than other methods (McNaughton et al. 1997) and can identify the organisms that cause microbial community changes (Zelles 1999).

Unfortunately, PLFA analysis cannot show changes in the populations of individual

species (Zelles 1999) and it requires extensive solvent extraction and product concentration (Pinkart et al. 2002).

2.2.3.3. Molecular diversity techniques

The analysis of genomic deoxyribonucleic acid (DNA) can fingerprint a microbial community, be used to clone new genes and provide information on gene expression and regulation. When analysing DNA, the first step is the removal of the DNA from the cell by lysing the cell membrane. Various chemicals are added to the cell suspensions such as EDTA, NaCl and SDS to break the cell membranes and release the DNA in the solution (Nocker et al. 2007). Thereafter, the DNA is purified and precipitated with ammonium acetate and ethanol. The purity is measured spectrophotometrically at 260 nm and 280 nm, since the DNA and RNA ring structures absorb at abovementioned wavelengths, respectively. For DNA the purity ratio is $A_{260} / A_{280} \sim 1.8$ and for RNA $A_{260} / A_{280} \sim 2.0$ (Nocker et al. 2007).

Several molecular-based techniques exist that can be used to investigate the DNA extracted from a cell, for example polymerase chain reaction (PCR), Real-Time (RT) PCR, denaturing (DGGE) and temperature gradient gel electrophoresis (TGGE), DNA microarrays and hybridisation. These are only a few of the vast number of methods developed for molecular research.

Polymerase chain reaction is a straightforward process in which DNA is amplified. The PCR process consists of three main steps: i) denaturation, ii) annealing, and iii) elongation. During denaturation, double-stranded DNA is treated with heat to separate the strands. The addition of specific oligonucleotide primers that anneal to the target DNA ensures amplification of only the target sequence. Deoxyribonucleic acid polymerase is added to catalyse the elongation of the annealing primers. These steps are repeated several times to make sure enough DNA are produced. Polymerase chain reaction is a versatile method and can be modified to clone rare genes, be used to study mRNA expression and insert certain characteristics of an organism into another organism (Lo and Chan 2006). Polymerase chain reaction products can be analysed with various techniques. Agarose gel electrophoresis with added ethidium bromide as a staining agent separates the products according to their size ranges.

During **RT-PCR**, a fluorescent probe attached to the oligonucleotide primer can provide an indication of the amount of newly synthesised DNA (Heid et al. 1996). These probes (fluorophores) bind covalently to the oligonucleotide primer or may even bind to double stranded DNA. As the PCR process progresses, these probes change fluorescence and are quantified by the thermocycler (Pryor and Wittwer 2008).

Denaturing gradient gel electrophoresis separates double-stranded DNA sequences based on their different melting conditions and their mobility in the

electrophoresis gel. This implies that different bacterial populations are indicated by different bands in the gel. These bands can be excised and sequenced to identify specific microbial groups. Denaturing gradient gel electrophoresis consists of three steps namely i) the extraction of DNA from samples, ii) the amplification of the target DNA through PCR and iii) the separation of the PCR products with DGGE. The electrophoresis gels contain acrylamide, urea and formamide. These gels are stained with ethidium bromide or SYBR Green to visualise the bands (Zhang 2008). Denaturing gradient gel electrophoresis can be applied to study complex communities, monitor microbial community changes in environmental samples, compare DNA extraction methods and analyse cultures used for enrichment purposes. It provides the direct visualisation of microbial diversity in the form of bands. Unfortunately, this method has numerous shortcomings. The primers used during the PCR process already select for a specific microbial group, thus decreasing the diversity potential. Unfortunately, DGGE is liable to the same biases as PCR for example, the formation of primer dimers and unwanted products, contamination and unsuccessful amplification (Nocker et al. 2007).

Deoxyribonucleic acid **microarrays** provide an indication of the differences between gene expression in various cells. Gene expression is the ability of a gene to code for a protein, which gives an organism a specific characteristic. During this technique, DNA is attached to a glass slide and left to hybridise or bind with complementary labelled DNA fragments. The level of hybridisation is analysed according to colour allocations, where red represents more hybridisation and green, less hybridisation. This method is used for tumour investigations, drug development, mutation detection and the identification of bacterial pathogens during epidemic outbreaks (Uttamchandani et al. 2004). It can be applied to study the genes involved in human cancers, especially for early detection (Trevino et al. 2007). Environmental applications of microarrays have been conducted on samples from oil fields (Voordouw et al. 1992), terrestrial soils (Shen et al. 1998) and salt marsh sediments (Bagwell and Lovell 2000).

2.3. Sunflower cultivation in South African agriculture

Agriculture is an essential part of the South African economy. Companies and individual entities involved in agricultural practices purchase important goods such as implements, fertilisers and chemical products from manufacturers while providing raw materials for industrial use. Agricultural practices provide employment in rural areas and play an important role in foreign exchange. The gross income for South Africa from commercial agricultural products for 2010 was R 128 587 million (Department of Agriculture, Forestry and Fisheries 2010), which is 0.4% lower than the R 129 071 million in 2009. This was due to lower production levels or lower prices of sunflower seed and maize. Sunflower parasites

such as *Sclerotinia sclerotiorum* can also cause major crop losses, reducing the overall farm income.

Sunflower (*Helianthus annuus* L.) is the third major crop cultivated in South Africa. According to area estimates conducted by the Department of Agriculture, Forestry and Fisheries (2010) 575 200 ha of sunflower seeds were planted for the 2010/11 season, which is 44.6% higher than the 397 700 ha planted in the 2009/10 season. Ninety-seven percent of sunflower is used for oil production, only 1% for seed purposes and approximately 2% for other products such as birdseeds and oil cake for livestock farming. Sunflower oil is manufactured and marketed as refined oil for domestic and industrial cooking and baking purposes. It is also found in margarine and other consumer products. Oil cake is used as a protein ingredient of balanced animal feed (Department of Agriculture, Forestry and Fisheries 2010). Due to the current concern about the extensive depletion of petroleum resources in South Africa, studies on alternative sources have been conducted (Seiler 2007). It was found that sunflower can be used for bio-ethanol production.

Sunflower can be used in the clean-up process, phytoremediation (Schwitzguébel et al. 2002) which is a process that involves the extraction, absorption and detoxification of chemical pollutants from the ecosystem (soil, water and air) by plants (Gavrilescu et al. 2009). In a study conducted by Phytotech after the Chernobyl Ukraine nuclear disaster, sunflower was grown on rafts in ponds contaminated with radioactive ^{137}Cs and ^{90}Sr . After 4-8 weeks there was a dramatic decrease in these radionuclide levels (Glass 1999). They also found that sunflower can reduce uranium concentrations by 95% within 24 hours (Ramaswami et al. 2001).

In South Africa, sunflower can be planted in November and December in the eastern parts of the country and till mid January in the western parts. Sunflower grows well in arid conditions and can tolerate drought better than other crops. They are able to extract water and nutrients from soil depths far beyond the reach of maize and sorghum (Zegada-Lizarazu and Monti 2010). For optimal yield and production efficiency, sunflower diseases should be controlled.

2.4. *Sclerotinia sclerotiorum* as a major sunflower pathogen

Several pathogenic fungi are important parasites of sunflower and cause threatening diseases. Such fungi are for example *Sclerotium rolfsii* (stem rot), *Verticillium dahliae* (*Verticillium* wilt) and *Sclerotinia sclerotiorum* (White rot or head rot).

Sclerotium rolfsii infects both roots and stems. Wilting and stunting occurs and leads to plant death. Infected plants develop a necrotic lesion with a dark brown border at the soil surface. Infected roots and stems form white mycelial strands or sclerotia that become dark brown (Van Wyk and Viljoen 2002).

Verticillium dahliae causes *Verticillium* wilt; it is described as leaf mottle (interveinal chlorosis). *Verticillium dahliae* enters the plant through the roots and colonises the vascular tissues. Visual signs of infection include defoliation, wilting, stunting and necrosis (Sanogo 2007). *Verticillium dahliae* reduces photosynthesis and transpiration through defoliation and the degradation of chlorophyll. The pathogen persists in soil for several years by forming multi-celled microsclerotia which act as resting bodies (Wilhelm 1955). It limits the production yield of crops and high yield losses may occur.

Sclerotinia sclerotiorum (Lib) de Bary is an international damaging fungal pathogen which causes serious yield and quality losses in over 400 plant species (Bom and Boland 2000; Purdy 1979). *S. sclerotiorum* threatens crops such as soybean, sunflower, peanut, chickpea, canola, dry bean, dry pea and various vegetables (Boland and Hall 1994). Crop losses of 0-100% have been reported. It survives as sclerotia in the soil or as mycelia in plants. Infected plants show unique symptoms. Leaves have water-soaked lesions that broaden quickly by moving down the petiole into the stem, girdling the plant organ (Bolton et al. 2006; Purdy 1979). Infected stems usually possess dark lesions near the soil surface; the stem bark dies off, leaving a white mycelium spreading up the stalk, forming sclerotia on the plant surface as it develops (Purdy 1979). In early stages of development, a plant may not appear affected. However, as the disease progresses, plant leaves become yellow, wilt and die. Sunflower heads wilt and show signs of fungal growth between the seeds (Van Wyk and Viljoen 2002). The head tissues degrade completely, leaving only the vascular tissue and a few parenchyma cells (Purdy 1979).

Purdy (1979) described the life cycle of *S. sclerotiorum*. Sclerotia germinate to form hyphae (white to tan, hyaline, septate, branched and multinucleate) that invade dead organic matter and create a mycelium necessary for mycelial infection (Bolton et al. 2006). Sclerotia can also form apothecia which, when ripe, blow ascospores into air currents. These ascospores attach themselves to plant parts with the help of excreted mucilage gel. They germinate and form infectious mycelia. Mycelia produce sclerotia on plant parts, in fruit cavities or directly in the soil, completing the cycle. The cuticle of the host plant is penetrated and the inner tissues degraded by secreted enzymes. *S. sclerotiorum* secretes pectinases that hydrolyse the pectin in the plant cell wall, helping with penetration and colonisation of the plant host. The degraded pectin serves as a carbon source for the fungus (Bolton et al. 2006). Proteases, cellulases and gluco-amylases have been found in *S. sclerotiorum* infected tissues (Lumsden 1979). The fungus also secretes oxalic acid that has several functions:

- The secretion of oxalic acid lowers the pH to $\pm$ 4-5, which is optimal for various cell wall degrading enzymes (Maxwell and Lumsden 1970; Yang et al. 2007).

- Oxalic acid reacts with cell wall calcium ions (Ca^{2+}) bound to pectins, disrupting the cell wall and making the hydrolysis of pectases possible by polygalacturonase (Bateman and Beer 1965).
- It suppresses the early warning defence response termed the oxidative burst (Cessna et al. 2000).
- It induces stomatal opening and inhibits closure, leading to foliar wilting (Guimaraes and Stotz 2004).
- The oxalic acid may be toxic to the plant.

Several studies have demonstrated that *S. sclerotiorum* mutants unable to synthesise oxalic acid are less pathogenic than the wild types (Dickman and Mitra 1992; Godoy et al. 1990). Therefore, oxalic acid is important for fungal virulence. Considerable advances in the expression of oxalate-degrading enzymes, such as oxalate oxidase that transforms oxalic acid into H_2O_2 and CO_2 , into crops can improve the resistance of sunflower (Burke and Riesenbergs 2003) and soybean (*Glycine max*) (Donaldson et al. 2001) to *Sclerotinia* diseases (Hu et al. 2003). Unfortunately, there is always the possibility of horizontal gene transfer or the escape of transgenes into wild plants, resulting in more aggressive wild types (Bolton et al. 2006).

Sclerotia, the over-wintering bodies of the fungus, are hyphal aggregates which can germinate and produce apothecia which release ascospores in spring or summer (Lu 2003). The production of sclerotia occurs in the flowering and seeding organs of the plant – that is why sclerotia have been found in harvest samples (Bolton et al. 2006). Sclerotia are formed when the mycelial growth experiences nutrient limitations. Sclerotia consist of various compounds including β -glucan, chitin, glycogen, trehalase, mannitol, glucose and fructose (Le Tourneau 1979). The presence of macronutrients such as phosphorous (P), magnesium (Mg), potassium (K) and sulphur (S) are essential for sclerotia formation and growth. A sclerotium is a multi-hyphal structure consisting of a thick-walled coloured rind, a thin-walled cortex and a pale central medulla (Liang et al. 2010). The sclerotium develops according to three characteristic stages: 1) initiation of small formations through hyphal aggregation; 2) development through increasing size as well as the secretion of liquid droplets and 3) maturation by melanin deposition in peripheral rind cells followed by internal stabilisation (Liang et al. 2010). Huang (1982) described three types of *S. sclerotiorum* sclerotia. Normal black sclerotia are black with a smooth surface. Abnormal black sclerotia, found in samples from infected sunflower heads, are wrinkled with a broken rind and a brown medulla. Tan sclerotia lack melanin, are brown and form white apothecia, in contrast with the normally

melanised black sclerotia that produce brown apothecia. Melanin is a dark pigment essential for fungal persistence, pathogenicity and provides protection against ultra-violet radiation and temperature extremes (Bell and Wheeler 1986; Huang 1982; 1983). The survival of *S. sclerotiorum* sclerotia is dependent on several environmental factors including temperature, moisture, aeration (Liang et al. 2010), pH, light and nutrients (Chet and Henis 1975). It was found that light increases the amount of sclerotia produced, whereas darkness increases the sclerotium dry weight. *S. sclerotiorum* prefers growth temperatures between 0 - 30°C with an optimum pH range of 2.5 - 9.

The sclerotia can germinate myceliogenically or carpogenically depending on the environmental field conditions (Huang et al. 2006). Carpogenic germination forms apothecia that release ascospores into the air, resulting in the infection of above-ground plant tissues, causing diseases such as head rot of sunflower (Huang 1983), white bean mold (Boland and Hall 1987) and blossom blight of alfalfa (*Medicago sativa* L.) (Huang et al. 2000). Carpogenic germination of *S. sclerotiorum* sclerotia are dependent on the geographic origin of the cultures, the temperature at which sclerotia formation took place as well as the temperature at which the parent inoculum was produced (Huang and Kozub 1989; 1993). Teo and Morrall (1985) found that carpogenic germination of the sclerotia was the highest at a soil water content equal to field capacity (-0.11 to -0.4 bar). This is where the soil contains sufficient moisture and oxygen for microbial growth.

Myceliogenic germination, on the other hand, produces mycelia which infect underground tissues leading to stem rot, plant wilting and root rot. Myceliogenic germination requires high humidity (Huang et al. 1998) and occurs when exogenous nutrients are available (Huang 1985). This type of germination is related to the level of black melanin pigment deposited in the rind cell walls (Huang 1985). The black sclerotia germinated very slowly compared to the others. Brown and light-brown sclerotia developed larger colonies than the black sclerotia. The percentage of fungal contaminants was much higher in the light-brown sclerotia than the black sclerotia. Therefore, the dark melanin pigments in the sclerotial rind contribute to myceliogenic germination control (dormancy / resting period) and resistance to invasive microorganisms. The black sclerotia remain intact in soil until the protective rind is broken or degraded by microorganisms.

Adams and Ayers (1979) emphasised that about 90% of the life cycle of *S. sclerotiorum* is spent in soil as sclerotia. *Sclerotinia* diseases can spread in several ways. Direct contact between infected plants and healthy plants can initiate infections (Huang and Hoes 1980), ascospores can be blown by wind currents, sclerotia or mycelium can adhere to farm machinery, seedlings, animal coats or humans and be spread to other fields (Bardin and Huang 2001). The incorporation of manures consisting of infected plant tissues on fields, may initiate the infestation of uncontaminated fields (Adams and Ayers 1979).

Through several field studies conducted, it can be concluded that approximately 0-10 sclerotia are present per kilogram of natural soil in contaminated areas. *Sclerotinia* species can increase their sclerotial inoculum density by forming secondary sclerotia in the soil even without a host, or by generating sclerotia on their hosts. Weeds can also serve as hosts for this fungus. Reports have shown that sclerotia survive for at least 3-8 years in soil. The highest number of ascospores is distributed within a few metres of the source. The number of ascospores decreases as the distance from the source increases. A disease originates from inoculum produced within the field (Wegulo et al. 2000).

Thus, different control treatments are needed for the different sclerotial germination types. Foliar treatments can protect the above-ground tissues, whereas soil treatments can reduce under-ground tissue infections (Bardin and Huang 2001). Various studies have shown that additions of slow-release nitrogen fertilisers to soil inhibit the germination of *S. sclerotiorum* sclerotia (Huang et al. 2006). The amendment of soil with S-H mixture (compound containing urea, oyster shell powder, potassium nitrate, calcium superphosphate and mineral ash) reduced the viability of *S. sclerotiorum* sclerotia by 80% (Huang and Sun 1991).

Various control techniques have been developed namely sclerotium parasites, protectant chemicals, chemicals that kill or inhibit sclerotia, crop rotations, irrigation and resistant or tolerant crop cultivars (Steadman 1979). Seeds can be treated to prevent *Sclerotinia* foot rot of sunflowers. Synthetic fungicides such as methyl bromide, formaldehyde or cyanamide may be utilised to inhibit sclerotial germination and ascospores formation. However, the crop canopy density may affect the precision of fungicidal applications. Various biocontrol agents have been developed to inhibit *Sclerotinia* diseases. *Coniothyrium minitans* is a mycoparasite well known for its effective reduction in *Sclerotinia* wilt of sunflowers and parasitisation of sclerotia in soil (Huang 1980). It parasitises the sclerotia by the secretion of exo-enzymes, such as chitinase and β -glucanase, which weakens the sclerotial rind for easier entry. Huang (1977) indicated the ability of *C. minitans* to invade sclerotia even inside the infected host plant, for example sunflower roots and stems. Unfortunately, the influence of environmental factors such as temperature fluctuations can be fatal for these biocontrol agents (Hannusch and Boland 1996).

Cultural methods used to decrease *Sclerotinia* diseases include increased row spacing and plant spacing within rows, continued monoculture over several years (Huang and Kozub 1991), high irrigation to increase sclerotial decomposition and burying sclerotia to avoid germination (Bardin and Huang 2001). Monoculture agriculture has several disadvantages including the physical degradation of soil, increased reliance on chemicals for pest and weed control, excessive nutrient leaching and a loss of biodiversity (Zegada-Lizarazu and Monti 2010). In contrast, crop rotation improves soil structure, permeability

and soil water infiltration, and reduces soil erosion. It decreases the occurrence of plant diseases, especially those requiring a host present for survival. Pathogens with a wide range of hosts or survival structures (sclerotia of *S. sclerotiorum* or *Rhizoctonia solani*) may not be successfully inhibited (Janvier et al. 2007). It enhances soil fertility, reduces the application of synthetic fertilisers and increases biodiversity. Yet, it still requires more agricultural skills or management as well as a wide range of farming equipment. During conservation tillage, crop materials are left in the soil to degrade by itself. This organic matter source increases microbial biomass and activity (Pankhurst et al. 2002) together with the higher moisture in the soil surface. However, due to the lack of tillage, soil pathogens survive easier under conventional tillage practices (Janvier et al. 2007). Tillage disturbs the soil habitat, exposes organisms to the risk of predation, fragments fungal hyphae networks leading to the loss of cell contents and reduces fungal sporulation by exposing the propagules to higher temperatures at the soil surface (Roger-Estrade et al. 2010). Deep ploughing may control diseases, since it removes sclerotia from the aerated soil surface. Moore (1949) found that flooding a field for 23-45 days destroyed the sclerotia of *S. sclerotiorum* almost completely. However, this is not always possible in non-irrigated areas. High temperatures of near 35°C for three weeks or more was also found to reduce sclerotia survival (Adams 1975).

2.5. Biofumigation with *Brassica* species

Fumigation is critical in agriculture; it is used to control plant diseases for maintaining crop yields and quality. Several fumigants are widely applied, for example methyl bromide and chloropicrin are used to control fungal pests (Lazzeri et al. 2003), whereas Larkin and Griffin (2007) used the chemical, metham sodium, to inhibit *Verticillium*. However, Omirou et al. (2010) demonstrated that metham sodium for example suppresses microbial activity, decreases microbial biomass and leads to structural changes in the microbial community. These artificial fumigants are highly volatile and non-specific, which can lead to environmental pollution and the destruction of the whole microbial community in the soil. The use of fumigants also increases farming input costs. Due to the current demand for food safety, the use of chemical pesticides is reduced. Nematicides are highly toxic to humans, contaminate groundwater and can be absorbed by plants (Oka 2010). Therefore, an alternative is needed that can still control crop diseases without harbouring health and environmental risks. The rotation with green manure crops as potential biofumigants has been widely explored (Wang et al. 2009).

Biofumigation is the process in which soil-borne pests are suppressed by the incorporation of glucosinolate-containing plant materials as green manures into the soil (Angus et al. 1994). Biofumigation is said to control harmful soil pathogens including

Fusarium, *Sclerotinia*, *Verticillium* and *Pyrenochaeta* (Omirou et al. 2010). The biofumigation process involves the planting of glucosinolate-containing crops in a crop rotation with the crushing and ploughing of the crop back into the soil as a green manure, usually during the flowering stage (when GSL concentration is the highest in the tissues). Incorporation takes place at a depth of 10-20 cm (Bellostas et al. 2007). The GSL products are primarily released after crushing and bruising the plant material (Motisi et al. 2010). The incorporation of green manures into the soil can also contribute to the improvement of soil texture, soil infiltration and nutrient values (Cherr et al. 2006). The added plant materials can aid the native microbial community with competition, parasitism, antagonism and predation against the soil-borne pathogens (Raaijmakers et al. 2009).

Plants of the family Brassicaceae contain GSLs that are hydrolysed into various products upon tissue degradation. Crop plants of the *Brassica* species include cabbage (*Brassica oleracea*), broccoli, radish (*Raphanus sativus*), canola (*Brassica napus*), cauliflower, mustard (*Brassica juncea*) and white mustard (*Sinapsis alba*) (Larkin and Griffin 2007; Wang et al. 2009). These GSLs are hydrolysed by the enzyme myrosinase (thioglucoside glucohydrolase E.C.3.2.3.1) (Brown and Morra 1997; Kirkegaard et al. 2000; Matthiessen and Kirkegaard 2006) into ITCs, thiocyanates, nitriles, epithionitriles, oxazolidinethiones (Eberlein et al. 1998; Fahey et al. 2001; Lacey 2000) and volatile sulphurous compounds such as methanethiol, methyl sulphide and dimethyl disulphide (Wang et al. 2009). These products, especially ITCs, are highly toxic to various microorganisms and provide the potential of use as biofumigants (Fahey et al. 2001; Yulianti et al. 2007). Isothiocyanates react with sulph-hydryl groups, amine groups and the disulphide bonds of proteins causing the degradation of enzymes and the inhibition of microbial growth (Brown and Morra 1997). Isothiocyanates are effective against bacteria, fungi, weeds and nematodes such as *Meloidogyne incognita* and *Tylenchulus semipenetrans* (Lazzeri et al. 2004; Zasada and Ferris 2003). Glucosinolates are stored in cell vacuoles (Rausch and Wachter 2005), whereas myrosinase is accumulated in myrosin cells (Hoglund et al. 1991). Various factors influence the successful hydrolysis of GSLs to ITCs including the soil physical and chemical properties (Morra and Kirkegaard 2002), water availability, temperature fluctuations, the GSL content (Matthiessen et al. 2004) and the amount of tissue disruption by freezing, drying or maceration to increase the contact between the enzyme and the GSLs (Morra and Kirkegaard 2002).

Morra and Kirkegaard (2002) found that freezing the green manure tissues before incorporation, increased the GSL release by 13 and 25% in comparison with coarsely-chopped materials. Increasing the soil moisture content aids the GSL hydrolysis process and prevents the loss of volatile ITCs. This implies a higher ITC concentration released into the soil. It was also found that most of the ITCs will be released within four days after tissue

incorporation, depending on the soil temperature, water content and the physical and chemical properties (Zasada and Ferris 2004). The ITC concentration in the soil decreases rapidly within a few days due to microbial degradation. It has been reported that after 1-2 weeks no ITC could be detected (Gimsing and Kirkegaard 2006). Soil type can also influence the degradation rate of ITCs (Gimsing et al. 2006). Degradation is much faster in clayey than sandy soil. Clayey soil contains more nutrients, have more microbial populations and stabilise cellular enzymes through the adsorption to clay particles.

Different GSLs are defined by the variations in their organic side chains in the molecular structure (Mithen 2001). The structure of GSLs (β -D-thioglucosides) consists of a sulphonated oxime group, a β -thioglucose group and a side chain. The side chain can vary between aliphatic, aromatic or indole categories (Omirou et al. 2010). Aliphatic ITCs are methyl and 2-propenyl ITCs, whereas the aromatic ITCs include benzyl and 2-phenylethyl ITCs (PITC). Matthiessen and Shackleton (2005) demonstrated that the ITCs derived from aliphatic GSLs (allyl-ITCs) are more active in the soil than those from aromatic GSLs (2-phenylethyl-ITCs). It was found that the green manures of *Brassicas* with more short-chain aliphatic ITCs are more efficient in pest suppression and those with long-chain aromatic ITCs have a low biofumigation capacity. It was concluded that aliphatic GSLs degrade much easier than aromatic GSLs. Aliphatic ITCs are characteristic of shoots and the aromatic ITCs are mostly found in roots (Kirkegaard and Sarwar 1998). 2-propenyl ITC is poisonous to *Verticillium dahliae*, *Helminthosporium solani*, *Sclerotium rolfsii*, *Sclerotinia sclerotiorum* and *Phytophthora capsici* (Chung et al. 2003).

In a study performed by Friberg et al. (2009) the incorporation of mustard (*Brassica juncea*) as a green manure decreased the inoculum density of *Rhizoctonia solani* in the soil. High concentrations of GSL hydrolysis are needed to successfully suppress pathogen growth. *R. solani* produce pseudosclerotia or thick-walled hyphae as survival bodies. These survival structures are less susceptible to GSL hydrolysis products than young hyphae, restricting the biocidal potential of green manure to inhibit fungal pathogens (Yulianti et al. 2006). It has been demonstrated that fungal suppression was higher in treatments where both mustard roots and shoots were used rather than separately (Snapp et al. 2007).

Bacteria and actinomycetes are more resistant to toxicants than fungi and oomycetes (Kreutzer 1963). In the study of Smith and Kirkegaard (2002) the growth sensitivity of fungi, bacteria and oomycetes to 2-phenylethyl ITC were tested, since it is the product of the main root GSL of canola (Kirkegaard et al. 2000). It was found that the sensitivity of fungi and oomycetes are higher for aromatic ITCs than for aliphatic ITCs. Unfortunately, GSLs may leach through the subsoil by macropore transport or by secretion from plant roots into non-targeted areas and limit their activity in soil. It was found that ITCs may reduce nitrogen (N) mineralisation from crop residues and inhibit nitrifying bacterial communities (Bending and

Lincoln 2000). The long-term use of ITCs may directly suppress soil diseases or indirectly cause changes in the indigenous soil microbial community (Smith and Kirkegaard 2002).

Canola (*Brassica napus*) suppresses soil-borne pathogens when it is ploughed into the soil as a green manure crop (Gardiner et al. 1999). It contains several types of GSLs including but-3-enyl, benzyl, phenethyl and 2-hydroxy-but-3-enyl (Bjerg and Sørensen 1987). Canola roots contain mostly the GSL gluconasturtin, whereas the shoots have more aliphatic GSLs (Sarwar and Kirkegaard 1998). Gluconasturtin is degraded to PITC which has a low volatility, but is toxic in the liquid state (Sarwar et al. 1998). It is the dominant GSL when both roots and shoots of canola are incorporated (Gardiner et al. 1999). Canola green manures are effective biofumigants against black scurf caused by *R. solani* (Larkin and Griffin 2007; Larkin and Honeycutt 2006). Canola is used in crop rotations to improve the yields of later crops such as wheat. Wheat yield increases of approximately 25% have been detected in canola-rotation practices. Chan and Heenan (1996) conducted a study to determine the impact of crops grown, on the soil structure and physical properties. They found that canola made soil more porous; the canola soil had the highest dissolved carbon and cation concentration (K + sodium - Na) in comparison with barley, lupin and pea. They emphasised the positive effect canola has on the soil structure. Canola's tap-root system deeply penetrates the soil, enhancing water infiltration while decreasing soil erosion and water runoff. Canola increases the soil pH from 4.8 to 5.3-6.2 in the rhizosphere, resulting in higher Fe/Al phosphate solubility (Wang et al. 2007). It is very important to remember that although the pathogens are not killed completely by the biofumigation, they may be weakened so that they cannot survive the environmental conditions and the microbial competition in the soil. Plants can also be influenced by the volatile ITCs in the soil. Therefore, it is essential to monitor the plant vitality of crops grown in biofumigated soil.

2.6. Chlorophyll a fluorescence as a plant vitality indicator

The sunlight that a plant's antenna pigments absorb can be used in three ways: it can be utilised in photosynthetic processes, dissipated as heat energy or emitted as chlorophyll a fluorescence (light energy) (Maxwell and Johnson 2000).

During photosynthesis, light energy is absorbed by the antenna pigments P700 and P680 in the photosystems I and II, respectively. The excitation energy is transferred to an electron acceptor (plastoquinone Q_A) leading to the reduction of $NADP^+$, the oxidation of water to oxygen and the production of ATP (Stirbet and Govindjee 2011). Plastoquinone Q_A accepts an electron from photosystem II and transfers it to the electron carrier Q_B . As long as Q_A holds on to an electron, the reaction centre (RC) is closed, since it cannot accept other electrons. This is when chlorophyll a fluorescence is emitted. Therefore, closed reaction centres (Q_A is fully reduced) result in lower photosynthetic efficiency and thus, a

higher fluorescence yield (Maxwell and Johnson 2000). Stress factors cause certain RCs to become inactive and incapable of Q_A reduction. These RCs are known as “silent” RCs (Strasser et al. 2000).

Several environmental factors can cause plant stress including drought (Marsal and Girona 1997), chemical soil pollution (Percival et al. 1998), water excess, soil compaction (Day et al. 2000) and nutrient deprivation (Morales et al. 2000). The chlorophyll a fluorescence measurements can provide insight into the efficiency of PS II and the functionality of the photosynthetic apparatus (Krause and Weis 1991). The measurement of the chlorophyll a fluorescence can be an indicator of plant stress as a result of herbicide applications (Yusuf et al. 2010), salinity (Misra et al. 2001) or mineral deficiencies (Lichtenthaler and Miede 1997). Usually under stress conditions, photosystem II function is reduced, leading to an increase in total chlorophyll a fluorescence.

All oxygenic photosynthetic material exhibits a range of fluorescence rise phases, known as the Kautsky effect (Briantais et al. 1986), during the first second (s) of illumination of pre-darkened leaves with continuous light. These phases range from the initial (F_0) to the maximum (F_M) fluorescence values. Strasser et al. (1995) named these phases O, J, I, P (Figure 2.1) from the slower to the faster part of the transient when it is plotted on a logarithmic time-scale from 50 μ s to 1 s. The initial fluorescence levels are O (0 ms) and J (2 ms), I (30 ms) is the intermediate level and the peak level is P (500 ms - 1s). The O-J phase is linked to the photochemical reduction of the primary quinone acceptor Q_A in PS II reaction centres to O_A^- and is sensitive to light intensity and temperature (Strasser et al. 1995). The JI-phase indicates the reduction of the inter-system electron carriers, whereas the IP-phase represents the reduction of PS I electron acceptors (Yusuf et al. 2010).

Several biophysical parameters can be derived from the O-JIP test to describe the PS II energy flow and function (Tsimilli-Michael et al. 2000). F_0 is the state when all RCs are open and F_M is the state when all RCs are closed. F_P is where the maximum fluorescence intensity takes place. ABS is the absorbance of a photon by the antenna pigment molecules. A portion of the absorbed energy is trapped (TR) by the RC and the rest is lost as heat or fluorescence. The trapped energy is converted to redox energy by electron transport (ET) to Q_A and Q_B (Tsimilli-Michael et al. 2000). As shown in Table 2.3 various biophysical parameters at time zero (onset of fluorescence induction) can be calculated (Tsimilli-Michael et al. 2000). Several energy fluxes can be estimated from these parameters, namely:

- The specific energy fluxes at the reaction centres for absorption (ABS/RC), trapping (TR₀/RC), dissipation (DI₀/RC) and electron transport (ET₀/RC).

- The flux ratios or yields include the maximum quantum yield for primary photochemistry ($\phi P_0 = TR_0/ABS = F_0/F_M$), the efficiency with which an electron can be moved into the electron transport chain further than Q_A^- by a trapped exciton ($\psi E_0 = ET_0/TR_0$), the efficiency with which an electron is transferred to reduce acceptors at the PS I acceptor side (RE) ($\phi R_0 = RE_0/ABS_0$) and the quantum yield for electron transport ($\phi E_0 = ET_0/ABS = \phi P_0 / \psi_0$).
- The phenomenological energy fluxes at each cross section (CS) for absorption (ABS/CS), trapping (TR_0/CS), dissipation (DI_0/CS) and electron transport (ET_0/CS). The amount of active RCs per CS can also be calculated as RC/CS .

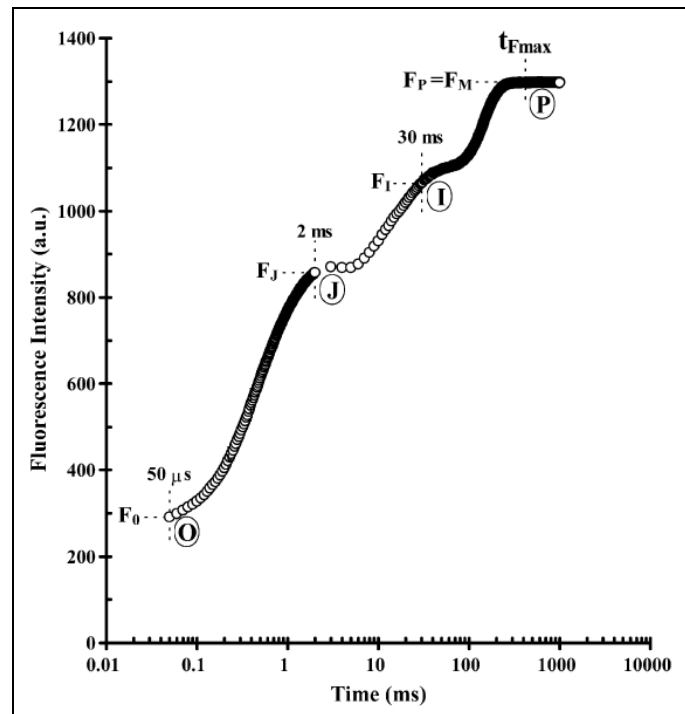


Figure 2.1: An example of a logarithmic time-scale plot of the chlorophyll a fluorescence transients O-JIP over $50 \mu s$ to 1 s. The initial fluorescence value is F_0 at $50 \mu s$, F_J is at $2 ms$, F_I at $30 ms$ and the maximum fluorescence value is $F_P = F_M$ at $500 ms$ (Oukarroum et al. 2007).

Table 2.3: A summary of all the biophysical parameters calculated from the O-JIP test (Tsimilli-Michael et al. 2000; Yusuf et al. 2010).

Extracted data from the O-JIP test	
F_0	Fluorescence intensity at 50 μ s
F_{300}	Fluorescence intensity at 300 μ s
F_J	Fluorescence intensity at 2ms (J-step)
F_I	Fluorescence intensity at 30ms (I-step)
F_M	Maximum fluorescence intensity
V_J	$(F_J - F_0) / (F_M - F_0)$
$M_0 = (\Delta F / \Delta t)_0 / (F_M - F_0)$	$4 (F_{300} - F_0) / (F_M - F_0)$
Specific energy ratios per reaction centre	
ABS / RC	$M_0 (1/V_J) (1/\phi P_0)$
TP ₀ / RC	$M_0 (1/V_J)$
DI ₀ / RC	$(ABS/RC) - (TR_0/RC)$
ET ₀ / RC	$M_0 (1/V_J) \psi_0$
Quantum yields or efficiencies	
$\phi P_0 = TR_0/ABS$	$[1 - (F_0/F_M)]$
$\Psi_0 = ET_0/TR_0$	$1 - V_J$
$\phi E_0 = ET_0/ABS$	$[1 - (F_0/F_M)] \psi_0$
$\phi R_0 = RE_0/ABS_0$	$(1 - V_I) / (1 - V_J)$
$\Psi E_0 = ET_0/TR_0$	$1 - V_J$
Phenomenological energy fluxes	
ABS/CS	$ABS/CS_M = F_M$
TR ₀ /CS	$\phi P_0 (ABS/CS)$
DI ₀ /CS	$(ABS/CS) - (TR_0/CS)$
ET ₀ /CS	$\phi P_0 \psi_0 (ABS/CS)$
Reaction centre density = RC/CS	$\phi P_0 (V_J/M_0) (ABS/CS)$

A performance index (PI_{Total}) can be calculated to describe the plant vitality. The PI_{Total} gives an idea of the potential energy conservation as intersystem electron acceptors are reduced. The PI_{Total} consists of four equations:

- $$PI_{Total} = \frac{\gamma RC}{1-\gamma RC} \cdot \frac{\varphi P_o}{1-\varphi P_o} \cdot \frac{\psi E_o}{1-\psi E_o} \cdot \frac{\delta R_o}{1-\delta R_o}$$

where $\gamma RC/(1-\gamma RC) = (RC/ABS)$ gives the chlorophyll concentration per reaction centre as light energy is absorbed per reaction centre; $\varphi P_o/(1-\varphi P_o) = TR_o/ABS$ indicates the trapping quantum efficiency; $\psi E_o/(1-\psi E_o) = ET_o/(TR_o-ET_o)$ provides the conversion efficiency of the excited energy to electron transport and $\delta R_o/(1-\delta R_o) = RE_o/ABS_o$ refers to the involvement of the reduced end electron acceptors in transference (Scheepers 2011; Strasser and Tsimilli-Michael 2001).

The O-JIP test is reliable, non-invasive, inexpensive and provides a considerable amount of information. The portable PEA (Plant Efficiency Analyser) instrument used for the O-JIP test can be used by non-specialised people and the measuring time is only a few seconds (Hermans et al. 2003). This method can be used to supplement quality assessment surveys, monitor the quality of harvest yields, indicate new genotypes in biotechnological studies and provide early warning signs of stress on plants and environmental degradation (Strasser et al. 2004). Unfortunately, it can only handle a limited number of environmental stress factors (Tsimilli-Michael et al. 2000).

Materials and Methods

3.1. Experimental layout

A greenhouse experiment was conducted at the North-West University, Potchefstroom, South Africa. The coordinates of the greenhouse are 26°41'358"S, 27°05'437"E with 1356 m above sea level. A randomised block design was used to place 32 plastic pots (30 cm) in the greenhouse with a photoperiod of 15 h light and 9 h dark. The temperature fluctuated between 26°C during the day and 20°C at night. The pots were filled with a mixture of potting soil and playpen sand (Culterra [Pty] Ltd., South Africa) at a 3:1 soil to sand ratio and equipped with glass fibre wicks at different levels in the soil to ensure continuous moisture and homogeneous wetting of the soil. Beneath each pot a water bucket was situated that served as a water reservoir, in which glass fibre wicks dangled (Boll et al. 1992). As the soil environment became dry, the glass fibre wicks absorbed water, from the continuously filled water bucket, into the surrounding soil. This ensured a soil moisture content of approximately 60% (Boll et al. 1992). Prior to the inoculation of the soil with the sunflower pathogen, the greenhouse was treated with a fungicide to ensure a non-fungal environment. The 32 pots consisted of four treatments of eight replicates each. The treatments included:

- Sunflowers in soil only,
- Sunflowers in soil inoculated with *Sclerotinia sclerotiorum*,
- Sunflowers in soil incorporated with canola (*Brassica napus*) green manure,
- Sunflowers in soil incorporated with canola green manure and inoculated with *S. sclerotiorum*.

Sclerotinia sclerotiorum (ATCC 10939) culture was obtained from the Agricultural Research Council's Plant Protection Research Institute (ARC-PPRI), Pretoria, South Africa. These cultures were maintained on potato dextrose agar (PDA) with added streptomycin sulphate to prevent bacterial contamination (Bom and Boland 2000). The inoculum was prepared by transferring four agar plugs aseptically from a 7-14 day old PDA culture of *S. sclerotiorum* to petri dishes filled with 20-30 g of sterile wet oats (Rodriguez et al. 1999). The oats was prepared by adding 3 ml distilled water per 5 g of oats and autoclaving for 15 min at 121°C on two consecutive days (Larkin and Griffin 2007). The inoculated oats was incubated at

26°C for eight days and then air dried for 48 h in sterile dessicators. The viability of the inoculum was confirmed by plating the oats on PDA plates. The inoculum was incorporated into the soil at a rate of 12 g kg⁻¹ soil and was allowed to acclimatise for 24 h (Larkin and Griffin 2007) in the soil before the canola was applied. Soil treatments without inoculum received sterile oats at the same rate.

The canola (*Brassica napus* L.) cultivar AV Garnet from Agricol (Pty) Ltd., Potchefstroom, South Africa was planted in a field plot of 4 x 4m. After 80 days of growth, during the early flowering stage (McCully et al. 2008), the canola (the whole plant - roots, shoots and leaves) was removed and frozen until use. According to Sarwar and Kirkegaard (1998) the GSL concentrations are the highest during this stage. On the day of incorporation, 24 h after *S. sclerotiorum* inoculation (Larkin and Griffin 2007), the canola tissues were cut into 5 x 20 mm pieces and bruised with a meat mallet to ensure maximum GSL release (Yulianti et al. 2007). Approximately 15 g kg⁻¹ soil of the green manure was applied to the specific treatments and incorporated into the soil at a depth of 10-20 cm (Omirou et al. 2010). Soil treatments without added canola green manure were also mixed at a depth of 10-20 cm to disrupt the soil environment. The pots were watered regularly to ensure sufficient soil moisture for ITC release.

Sunflower seeds of the cultivar PANNAR 7351 (PANNAR SEED [Pty] Ltd., Greytown, South Africa) were sown two weeks after canola incorporation. Three seeds were sown in each pot at a depth of 2-3 cm. Seedlings emerged after three days and were watered twice a day for ±12 days. After 12 days, the two less vigorous seedlings were removed from each pot and from then on the remaining seedlings received water once a day for 21 days. After 21 days, the plant roots were old enough to absorb water through the glass fibre wicks. A solution of commercially available fertiliser (Dr. Fisher's Multifeed 19:8:16[43] Classic, Plaaskem [Pty] Ltd., Whitfield) was applied to each plant every third day for the remainder of the experiment. This sunflower cultivar reaches a physiological maturity stage in ±115 days. Therefore, the experiment was conducted for 120 days.

3.2. Sampling procedure

Soil samples were obtained aseptically at a depth of 0-15 cm from each pot (Claassens et al. 2006). Sampling of the soil for the microbiological analyses took place on the day of sunflower sowing (day 0), 21 days after sowing and from then on every two weeks for the duration of the experiment. All microbiological analyses were conducted on four replicates per treatment. Each replicate (composite) consisted of two individual samples. The soil samples for the dehydrogenase activity and community level physiological profiling were kept cool on ice and stored at 4°C until use. Soil samples for the PLFA were kept on ice,

frozen at -80°C overnight and lyophilised immediately after sampling. All soil samples were mixed thoroughly, homogenised and passed through a 2 mm sieve before being analysed (Alef and Nannipieri 1995). Soil physical and chemical analyses were done on the soil before the different treatments were applied and after the experiment was completed.

The fluorescence measurements were done weekly from day 35 onwards on four intact sunflower plants per treatment, where 12 readings were performed per plant.

3.3. Soil physical and chemical analyses

The physical and chemical analyses of the soil were performed by an independent laboratory, Eco Analytica (North-West University, Potchefstroom), according to standard techniques.

The water-soluble basic cation fraction (calcium - Ca, Mg, Na and K) and micro-elements present (iron - Fe, manganese - Mn, copper - Cu and zinc - Zn) was determined by a 1:2 (v/v) water extraction method (Peech 1965) and quantified using atomic absorption spectrometry with a Spectr. AA-250 (Varian, Australia). Acetylene-air was used for determining the basic cations (Ramirez-Munoz 1968).

The extraction of exchangeable cations (Ca, Mg, Na and K) is done with ammonium acetate for the utilisation in fertilisation recommendations in the agricultural sector. The exchangeable cation concentrations were determined by the replacement of the cations with ammonium ions after the addition of excess ammonium acetate solution to the soil samples at a temperature of 20°C (Schollenberger and Simon 1945). The replaced ions were analysed with a Spectr. AA-250 (Varian, Australia) and gave an indication of the nutrient content of the soil (Thomas 1982). The exchangeable-ion status of the soil samples was used to calculate the percentage base saturation, which expresses the content of exchangeable bases as a percentage of the cation exchange capacity (CEC) measured at pH 7.0 or 8.2 (Claassens 2007). The equation is as follows:

$$Bsat = \frac{Xb}{CEC} \times 100\%$$

Where Bsat = Base saturation

Xb = sum of exchangeable bases (Ca, Mg, K and Na)

CEC = Cation exchange capacity

The anions (chloride - Cl, nitrate - NO₃, nitrite - NO₂, phosphate - PO₄ and sulphate - SO₄) were extracted with a 1:2 water extraction procedure (Sonnevelt and Van den Ende 1971) and quantified using ion chromatography (Metrohm 761 Compact IC, Switzerland).

Ammonium (NH_4) concentrations were measured by means of the ammonia-selective electrode method (Banwart et al. 1972). The boron (B) concentration was estimated spectrophotometrically at a wavelength of 420 nm (John et al. 1975).

Electrical conductivity (EC) of a saturated extract is an indication of the total soluble salts in the soil (Bower and Wilcox 1965). The soil samples were saturated with distilled water and the EC measured with a conductivity meter (Radiometer PHM 80, Copenhagen). The pH of the soil was determined as described by McLean (1982) with distilled water (pH [H_2O]) and potassium chloride (pH [KCl]) by means of a calibrated pH meter (Radiometer PHM 80, Copenhagen).

A P-Bray 1 analysis was conducted to quantify the P concentration according to Bray and Kurtz (1945). P-Bray 1 solution was added to each soil sample, filtrated and the P concentration measured with a Technicon Auto analyser (OI Analytical, College Station, Texas, USA).

The quantification of the particle size distribution and sand - silt - clay fraction of the soil were conducted by means of a hydro-method where the soil samples are sieved, treated with hydrogen peroxide and heated in a sand bath for 4 h. After cooling, Calgon is added to each sample and the soil suspension tested for particle size with a Thermo Scientific ERTCO® Soil Hydrometer, ASTM 152H (Cole-Parmer, Illinois) (ASTM 1985).

The organic C was estimated by the loss on ignition as the soil sample was heated at 650°C for 6 h in a Platinum crucible (Jackson 1958).

The total C and total N of the soil were quantified by means of a LECO TruSpec CN analyser (LECO Corp., St. Joseph, Mich.). The analysis consisted of three phases: the soil sample was purged of any atmospheric gases, combusted and flushed in a hot furnace at 950°C with oxygen, and analysed (Allison et al. 1965). The values were expressed in parts per million (ppm) or as a weight percentage.

3.4. Dehydrogenase activity

The enzyme dehydrogenase is active in biological oxidation reactions taking place in all microorganisms (Taylor et al. 2002). Therefore, it is an indication of microbial activity in the soil (Taylor et al. 2002). Applying the method of Von Mersi and Schinner (1991) described in Alef and Nannipieri (1995), 1 g of field moist soil was incubated at 40°C for 2 h in the dark with 1.5 ml Tris (hydroxy-methyl)-aminomethane buffer (THAM) and 2 ml iodonitrotetrazolium chloride (INT) (5 mg ml^{-1} in 2% v/v N,N-dimethylformamide). Sterilised 1 g soil samples (autoclaved at 121°C for 20 min) served as controls. To end the reaction, 10 ml of N, N-dimethylformamide/ethanol (1:1 v/v) extraction solution was added to each

sample, followed by shaking the samples at 20 min intervals for 1 h. The soil suspensions were filtered through Whatman no. 2 filter paper and the filtrate (iodonitrotetrazolium violet-formazan [INF] product) absorbances measured spectrophotometrically at 464 nm.

A calibration curve was set up by mixing 0, 1, 2 and 5 ml of INF solution with 13.5 ml of extraction solution, respectively. The calibration concentrations were 0, 100, 200 and 500 µg INF per test. The dehydrogenase activity was expressed as µg INF g⁻¹ dry weight 2h⁻¹ and calculated by the equation (Claassens 2007):

$$INF (\mu g g^{-1} dwt 2h^{-1}) = \frac{S_1 - S_0}{dwt}$$

Where S_1 = INF (µg) of the test

S_0 = INF (µg) of the control

dwt = dry weight of 1 g moist soil.

3.5. Community level physiological profiles (CLPPs)

The CLPPs were assessed by the use of Biolog® Ecoplates (Garland and Mills 1991) to measure the functional diversity of the microbial community through their assimilation of different carbon substrates (Garland and Mills 1991) in the plate wells. Each Ecoplate contains 31 carbon substrates in triplicate including a control (water) as shown in Table 2.1 in the Literature Review, to account for the sample variability (Guckert et al. 1996).

Soil dilutions were made by shaking ±10 g of moist soil in 90 ml of sterile distilled water at 25°C for 1 h at 200 rpm in a shaking incubator. After settling for 1 h, a 1 ml aliquot of the supernatant was added to 2 ml sterile distilled water. 0.1 ml of that solution was added to 9.9 ml sterile water, mixed, followed by 2 ml pipetted into 18 ml sterile distilled water. The final 1:3000 dilution was used to inoculate the plate (Campbell et al. 1997). The Biolog® Ecoplates (Biolog® Inc., Hayward, USA) were inoculated with 150 µl of soil dilution. The reduction of the tetrazolium dye was detected with a Powerwave Microplate Spectrophotometer (BioTek Instruments, Winooski, VT) at a wavelength of 590 nm every day at 8:00 and 15:00, until the threshold absorbance or average well colour development (AWCD) reached 0.25-0.3 (Winding et al. 1994).

3.6. Phospholipid fatty acid (PLFA) analysis

The total viable microbial biomass was indicated by the total amount of phospholipid fatty acids (White et al. 1979) and the microbial community structure was assessed according to the concentrations (mole %) of specific fatty acids (McKinley et al. 2005).

The glassware used in the lipid analysis was washed with phosphate-free soap, rinsed five times with warm, distilled and nano-pure water and baked in a muffle furnace at 450°C for 4 hours.

A modified Bligh and Dyer (1959) method was used to extract lipids from the ± 5 g lyophilised soil samples with a one-phase chloroform-methanol-phosphate buffer (White et al. 1979). The phases separated overnight. The chloroform, containing the lipids, was at the bottom (Findlay 2004).

The lipids were dissolved in chloroform and transferred to silicic acid columns where they were separated into phospholipids, glycolipids and neutral lipids (Guckert et al. 1985; Zelles 1999). The phospholipids underwent alkaline methanolysis to form fatty acid methyl esters (FAMES) (Guckert et al. 1985; McKinley et al. 2005). Fatty acid methyl esters were dissolved in hexane and analysed using an Agilent GC-MS system comprising of a 6890 series II gas chromatograph coupled to a 5973 mass selective detector. Injector temperature was set at 230°C with MS quadrupole. The source and transfer line temperatures were set at 150°C, 230°C and 180°C respectively. Separation was achieved on a 60 m Equity 1 column with a 250 μ m diameter and 1.00 μ m film thickness. The carrier gas used was helium applied at a constant flow of 25 cm/sec. 1 μ l of the sample was injected via an Agilent 7683 auto-injector in splitless mode while the oven temperature was programmed to start at 60°C for 2 minutes, ramped at 10°C / minute to 150°C and then increased at 3°C / minute to 320°C. For an internal standard, methyl nonadecanone (19:0) was used.

The fatty acid nomenclature follows the form A: B ω C. “A” is the total number of carbon atoms, “B” is the number of double bonds and “C” is the position of the first double bond from the aliphatic (methyl) end. The suffixes “c” and “t” indicate *cis* and *trans* geometry. All double bonds are supposedly *cis*. Anteiso- and iso-branching are indicated by the prefixes “a” and “i”, “OH” and “Me” refer to a hydroxyl group and a methyl group, respectively and the preceding number indicates the position from the carboxyl end of the molecule. “cy” shows a cyclopropyl fatty acid and “br” refers to unknown branching (Bossio et al. 2006; Guckert et al. 1985; Tabuchi et al. 2008; Zaady et al. 2010; Zelles 1999).

The sum of the PLFAs i15:0, a15:0, 15:0, i16:0, 16:1 ω 9, 16:1 ω t, i17:0, a17:0, cy17:0, 17:0, 18:1 ω 7 and cy19:0 was used to estimate bacterial biomass. Fungal biomass was indicated by the 18:2 ω 6,9 PLFA (Frostegard and Baath 1996). Gram-positive bacteria were associated with the PLFAs i15:0, a15:0, i16:0, i17:0 and a17:0 (O’Leary and Wilkinson 1988) whereas Gram-negative bacteria were marked by cy17:0, cy19:0 and 18:1 ω 7 (Zelles 1997). 10Me17:0 and 10Me18:0 indicated Actinomycetes biomass (Lechevalier 1977). (i15:0 +

i17:0 / a15:0 + a17:0) was used to determine the ratio of iso- to anteiso branched PLFAs (McKinley et al. 2005).

3.7. Plant fluorescence measurements

Chlorophyll a fluorescence emitted by photosystem II during photosynthesis can be a useful indicator of photosynthetic changes, plant functional efficiency (Misra et al. 2001) and be used to determine a performance index (Strasser et al. 1995) indicative of plant vitality.

Each week the sunflower plants were dark-adapted for 1 h, to ensure that Q_A is fully oxidised and all the RCs are open, before the non-intrusive chlorophyll a fluorescence measurements (O-JIP) were conducted on attached leaves with a Handy-PEA® chlorophyll fluorometer (Plant Efficiency Analyser, Hansatech Instruments, King's Lynn, Norfolk, UK). The leaves reached a dark adapted state due to one hour of darkness, following the light period and were illuminated at a wavelength of 650 nm from an LED light source (Hermans et al. 2003). Six light-emitting diodes excited continuous light ($3200 \mu\text{mol}/\text{m}^2\text{s}$) on a 5 mm diameter leaf surface for a period of 300 ms. All the JIP-parameters were directly calculated and processed with Biolyzer HP3 (R. M. Rodriguez, Bioenergetics Laboratory, University of Geneva, Switzerland).

3.8. Statistical analyses

Parametric and nonparametric statistical analyses were performed on all the data sets obtained by using Statistica 10.0 (StatSoft, Inc., Tulsa, Oklahoma, USA, 2012). The Shapiro-Wilk's test was used to test for normality. In the case of the data being parametric (distributed normally), a one-way breakdown ANOVA was performed followed by the use of Tukey's Honest Significant Difference (HSD) test to determine statistical significant differences between the various treatment samples ($p < 0.05$). In the case of nonparametric data, the Kruskal-Wallis ANOVA and the Median test were applied to determine statistical significant differences between the samples. If no difference was found between the parametric and nonparametric analyses, the parametric analyses were used to discuss the relevant statistics (Claassens 2007).

Multivariate ordination analyses such as Principal Component Analysis (PCA) and Redundancy Analysis (RDA) using CANOCO (Canoco for Windows Version 4.5, Biometris-Plant Research International, Wageningen, The Netherlands) were conducted on the data to identify the correlations between the various soil treatments and the soil microbial structure, function, enzymatic activity and plant fluorescence measurements.

For the CLPP measurements, the standardised patterns were obtained by subtracting the absorbance of the control (water) well from the substrate wells (Garland and

Mills 1991). The standardised optical density was estimated by dividing the absorbance of each well by the average absorbance of the whole plate. Any negative values were converted to zero (Habig 2003). An AWCD value of 0.25 absorbance was used as reference point for the multivariate analysis (Winding 1994). Principal Component Analysis was performed on the Biolog data to investigate the comparison of the various treatments by means of the carbon utilisation patterns of the microbial communities. Statistical analyses were also conducted on the different carbon substrate groups to distinguish between the functional diversity of the microorganisms in the soil treatments (Habig 2003).

The Shannon Weaver substrate diversity index (H') was estimated by using the amount of utilised substrates (Lupwayi et al. 2001) as an indication of the substrate richness and diversity of the extent of specific substrate utilisation as a marker for substrate evenness. The equation is as follows:

$$H' = -\sum [P_i \ln (P_i)]$$

Where P_i = proportional optical density value of each well on substrate i
 Σ = the number of substrates on a plate

Results and Discussion

Throughout this chapter, the four treatments will be referred to as: S – only sunflowers in soil (control); SC – sunflowers in soil incorporated with canola green manure; SCI – sunflowers in soil incorporated with canola green manure and inoculated with *S. sclerotiorum*; SI – sunflowers in soil inoculated with *S. sclerotiorum*.

4.1. Soil physical and chemical properties

The soil physical and chemical analyses were conducted by an independent laboratory according to the standard procedures described in the Materials and Methods. Soil samples were tested before the different treatments were applied and after the experiment was completed for each treatment. Thus, only the physical and chemical results after the completion of the experiment (119 days) will be discussed, unless stated otherwise.

From the results shown in Table 4.1 it is clear that the concentrations of the macro-elements Ca, Mg, K and Na differed significantly between the treatments (Tukey's HSD test – $p < 0.05$). De Beer et al. (2004) cited norm value concentrations for Ca, Mg, K and Na as 1.8, 0.6, 0.8 and <1.0 , respectively. The Mg and Na concentrations were drastically elevated for all four treatments, with treatment SCI having the highest values of 6.36 and 7.25 (mmol/l), respectively. When the exchangeable cation ratios were estimated, it became evident that all the treatments deviated from the norm percentage ratios of, Ca 65: Mg 25: K 8: Na 2 (De Beer et al. 2004). The ratios for each treatment were S – 47: 46: 1: 6; SC – 49: 42: 2: 7; SCI – 45: 42: 3: 9 and SI – 52: 43: 1: 5. These results indicated that there were severe Ca and K deficiencies in the soils after the experiment, with highly elevated Mg and Na percentages in all four treatments. These trends had the highest occurrence in treatment SCI.

The norm concentrations of SO_4 , NO_3 , NH_4 , Cl and HCO_3 are 0.9, 2.1, 0.04, <1.0 and 0.8 respectively (De Beer et al. 2004). It is clear that the SO_4 concentration was significantly elevated for all the treatments with SCI having the highest value (10.92 mmol/l). The NO_3 , NH_4 and Cl concentrations were also very high for treatment SCI. The micro-elemental concentrations of the elements Fe, Mn, Cu, Zn and B should correspond with the norm concentrations of 10.0, >1.0 , 1.0, 1.0 and 10.0 respectively (De Beer et al. 2004). The Fe concentration was very high in all the soils. Furthermore, the Zn and B concentrations were significantly higher in all the treatments after the completion of the experiment, than before the different treatments were applied.

Table 4.1: Soil physical and chemical characteristics of soil samples tested.

Properties	Treatments				
	Before	After S	After SC	After SCI	After SI
Macro-elements (mmol/l)					
Ca	0.44 ± 0.03 a	0.90 ± 0.27 ab	1.22 ± 0.14 b	3.44 ± 0.17 c	0.60 ± 0.20 ab
Mg	0.46 ± 0.03 b	3.06 ± 0.18 a	3.32 ± 0.16 a	6.36 ± 0.33 d	1.72 ± 0.46 c
K	1.14 ± 0.02 b	0.51 ± 0.09 a	0.97 ± 0.10 b	2.13 ± 0.19 c	0.20 ± 0.07 a
Na	0.36 ± 0.01 b	5.16 ± 0.51 a	4.39 ± 0.17 a	7.25 ± 0.30 d	2.86 ± 0.47 c
PO ₄	<0.01 ± 0.00 d	0.08 ± 0.02 ab	0.10 ± 0.02 b	0.39 ± 0.02 c	0.02 ± 0.01 a
SO ₄	1.09 ± 0.01 b	5.53 ± 0.41 a	5.10 ± 0.01 a	10.92 ± 0.40 d	3.12 ± 0.75 c
NO ₃	0.07 ± 0.02 a	0.95 ± 0.17 a	2.56 ± 0.57 b	3.84 ± 0.35 c	0.34 ± 0.23 a
NH ₄	0.05 ± 0.02 a	0.03 ± 0.02 a	0.11 ± 0.03 a	0.62 ± 0.05 b	0.02 ± 0.02 a
Cl	0.65 ± 0.06 a	1.00 ± 0.21 ab	1.15 ± 0.13 b	1.77 ± 0.12 c	0.81 ± 0.06 ab
HCO ₃	0.52 ± 0.17 ab	0.33 ± 0.04 a	0.38 ± 0.10 a	0.78 ± 0.02 b	0.37 ± 0.08 a
Micro-elements (μmol/l)					
Fe	20.85 ± 1.84 c	39.99 ± 1.83 ab	36.67 ± 1.64 a	46.29 ± 2.38 b	38.59 ± 3.41 ab
Mn	2.07 ± 0.30 a	1.22 ± 0.47 a	1.13 ± 0.31 a	8.96 ± 2.11 b	1.52 ± 0.40 a
Cu	0.11 ± 0.06 a	0.36 ± 0.12 ab	0.33 ± 0.07 ab	0.85 ± 0.06 c	0.52 ± 0.06 b
Zn	0.33 ± 0.10 b	4.66 ± 0.57 a	5.69 ± 0.27 a	23.32 ± 1.90 c	2.79 ± 1.03 ab
B	<1.00 ± 0.00 a	67.03 ± 4.07 a	74.19 ± 3.44 a	292.79 ± 18.17 c	29.45 ± 10.77 b
Nutrient Status (mg/kg)					
Ca	1916.67 ± 60.17 b	2214.83 ± 39.29 c	2510.50 ± 119.68 a	2919.50 ± 67.18 d	2583.67 ± 18.21 a
Mg	967.33 ± 18.73 b	1305.83 ± 20.18 a	1287.17 ± 18.92 a	1643.00 ± 38.39 c	1274.67 ± 72.36 a
K	444.83 ± 8.84 c	102.50 ± 7.07 ab	211.00 ± 41.95 b	380.50 ± 46.01 c	67.83 ± 14.62 a
Na	111.17 ± 23.34 b	332.33 ± 45.22 a	405.67 ± 80.00 a	671.50 ± 36.70 c	257.33 ± 42.95 ab
P	27.06 ± 2.13 a	86.93 ± 6.82 b	100.15 ± 17.67 b	220.05 ± 1.95 c	30.92 ± 7.15 a
Exchangeable cations (cmol(+)/kg)					
Ca	9.56 ± 0.30 b	11.05 ± 0.02 c	12.53 ± 0.60 a	14.57 ± 0.34 d	12.89 ± 0.09 a
Mg	7.96 ± 0.15 b	10.75 ± 0.17 a	10.59 ± 0.16 a	13.52 ± 0.32 c	10.49 ± 0.60 a
K	1.14 ± 0.02 c	0.26 ± 0.02 ab	0.54 ± 0.11 b	0.98 ± 0.12 c	0.17 ± 0.04 a
Na	0.48 ± 0.10 b	1.44 ± 0.20 a	1.76 ± 0.35 a	2.92 ± 0.16 c	1.12 ± 0.19 ab
Chemical properties					
pH	6.70 ± 0.31 b	6.16 ± 0.06 ab	6.09 ± 0.12 a	6.30 ± 0.07 ab	6.49 ± 0.06 ab
pH(H ₂ O)	6.08 ± 0.07 b	5.57 ± 0.02 a	5.70 ± 0.01 a	5.72 ± 0.03 a	6.16 ± 0.15 b
pH(KCl)	5.43 ± 0.05 b	5.99 ± 0.02 a	6.12 ± 0.02 a	6.05 ± 0.04 a	6.02 ± 0.27 a
EC (mS/cm)	0.34 ± 0.01 a	1.37 ± 0.12 b	1.46 ± 0.08 b	2.97 ± 0.13 c	0.78 ± 0.18 a
P-Bray I (ppm)	27.06 ± 2.13 a	86.93 ± 6.82 b	100.15 ± 17.67 b	220.05 ± 1.98 c	30.92 ± 7.15 a
Total C (%)	17.50 ± 3.14 a	10.31 ± 1.88 a	10.74 ± 1.34 a	10.73 ± 0.32 a	20.15 ± 4.77 a
Total N (%)	0.43 ± 0.33 a	0.15 ± 0.02 a	0.20 ± 0.04 a	0.25 ± 0.03 a	0.29 ± 0.08 a
Organic C (%)	7.84 ± 0.56 b	6.53 ± 0.36 a	6.86 ± 0.19 ab	7.28 ± 0.23 ab	6.82 ± 0.20 ab
CEC (cmol(+)/kg)	34.47 ± 2.05 b	19.01 ± 0.37 a	19.50 ± 0.43 a	22.22 ± 0.15 a	19.37 ± 1.27 a
Base saturation (%)	55.93 ± 4.70 c	123.71 ± 2.47 a	130.55 ± 6.06 ab	143.96 ± 1.41 b	127.85 ± 5.40 ab

¹All values ± SEM represent results obtained from triplicates (n=3).

²Statistically significant differences are indicated by alphabetic letters (p < 0.05). The same letters indicate no significant differences.

³EC – electrical conductivity; ppm – parts per million; CEC – cation exchange capacity.

The electrical conductivity (EC) was significantly higher in the S, SC and SCI treatments at the end of the experiment, compared to the norm concentration range between 0.48 and 0.8 mS/cm. The P concentration was sufficient for treatments S, SC and SCI, with values in close proximity to the 100 ppm norm value. Treatment SCI had a significantly higher P concentration than the other treatments, whereas treatment SI showed a significantly lower P

concentration with a value of 30.92 ppm. The percentages of total C, total N and organic C were lower for all the treatments after the completion of the experiment than at the beginning, except for treatment SI which had elevated levels of total C. The cation exchange capacity (CEC) was significantly lower in all the soil treatments. The base saturation percentage was higher than 100% for all the soils.

It should be noted that the macro-elements (mmol/l) and micro-elements ($\mu\text{mol/l}$) were analysed with a 1: 2 water extraction method. Information for this method provides a good indication of the available nutrients for plant absorption (Peech 1965). Since the commercial fertiliser applied to all the treatments contained high amounts of Mg, the elevated Mg concentrations could be ascribed to fertiliser applications. However, the fertiliser applied contained no Na; therefore the Na elevated in all four treatments might be as a result of the water used in the irrigation system. As the pots represent a closed system with poor soil drainage, the accumulated Na was not dispersed efficiently (Astera 2007). Treatment SCI had almost twice as high Mg, Na and P concentrations than the other treatments, which may be related to the possible inhibition of *S. sclerotiorum* development by the green manure, as these elements are used by the fungal pathogen for sclerotia formation and growth (Le Tourneau 1979).

The increased concentrations of the salts (SO_4 , NO_3 , NH_4 , and Cl) might also be attributed to the limited soil drainage which caused the salt accumulation in the soil (Brady 1974). The increased amount of Zn, B and Fe in the soil might have been as a result of the high concentrations of these elements in the commercial fertiliser applied to the treatments. Since the soil pH values were higher than 5.5, these micro-elements were not soluble for plant absorption (Black 1968). The high EC values could be associated with the high macro-elemental concentrations, as EC is a measure of the total salt content in the soil. The lower total C, total N and organic C percentages were in contrast with various other studies that found higher soil C, total N and organic C in the soil after green manure incorporation (Kirchner et al. 1993; N'Dayegamiye and Tran 2001). It might have been possible that during the experiment the contents of these parameters were high, but were gradually depleted by microbial degradation processes (Bernard et al. 2012).

The CEC is the number of exchange positions on a soil particle available for cations to adsorb to. This provides an indication of the number of cations free for transfer between particles and plants. The base saturation percentage is the amount of exchange positions occupied by these exchangeable cations (Black 1968). The reduced CEC could be linked to the lower organic matter present at the end of the experiment, given that organic material (humus / green manure) usually have higher CEC values than sand. In general, the CEC decreases with decreasing pH (Herselman 2007), which was also evident in this study. The elevated base saturation percentage suggested that the soils had no more adsorption

capacity left for the added elemental nutrients at the end of the experiment. Hence, at the end of the experiment all the added elements were unavailable to the plants and leached out of the soil.

It can be concluded that, except for the possible inhibition of *S. sclerotiorum* by the canola green manure leading to higher macronutrient concentrations in treatment SCI, no explicit effect of the various treatments on the soil characteristics was observed. The commercial fertiliser used in this study might have contributed to the elevated concentrations of Fe, Zn and B, which are toxic to plants at high levels. However, the pH ensured the normal development of the plants since these metals are unavailable for plants at pH levels higher than 5.5. Overall, no lasting effect of the canola green manure amendments on the soil C, total N and organic C content was found at the end of the study.

4.2. Dehydrogenase activity

During this study the activity of the soil enzyme dehydrogenase was quantified as a measure of the soil microbial activity. Dehydrogenase is only found in viable cells without occurring extracellularly in the soil. Hence, it can be employed as a sensitive indicator of the potential microbial activity in the soil (Nannipieri et al. 1990). The average dehydrogenase activity for the different treatments at the various days after sowing the sunflowers is summarised in Table 4.2. Tukey's HSD test performed on the data, showed no statistically significant differences ($p > 0.05$) among the treatments at the different sampling periods or for the specific treatments over time.

Table 4.2: Dehydrogenase activity (INF $\mu\text{g/g/2h}$) of all four treatments at the various sampling periods after sowing the sunflowers.

Days after sowing	Treatments			
	S	SC	SCI	SI
0	265.65 $\pm$ 94.77 A(a)	336.76 $\pm$ 81.57 A(a)	273.05 $\pm$ 79.21 A(a)	250.27 $\pm$ 44.83 A(a)
21	188.27 $\pm$ 11.92 A(a)	203.72 $\pm$ 17.96 A(a)	192.23 $\pm$ 43.37 A(a)	178.44 $\pm$ 20.66 A(a)
35	181.22 $\pm$ 16.02 A(a)	261.85 $\pm$ 43.08 A(a)	235.01 $\pm$ 33.34 A(a)	158.55 $\pm$ 24.65 A(a)
49	190.54 $\pm$ 30.24 A(a)	173.92 $\pm$ 25.46 A(a)	158.1 $\pm$ 14.58 A(a)	207.14 $\pm$ 51.44 A(a)
63	159.95 $\pm$ 8.53 A(a)	169.04 $\pm$ 3.49 A(a)	166.14 $\pm$ 38.96 A(a)	149.29 $\pm$ 11.58 A(a)
77	178.24 $\pm$ 23.13 A(a)	152.08 $\pm$ 15.36 A(a)	123.63 $\pm$ 14.98 A(a)	151.92 $\pm$ 35.37 A(a)
91	223.98 $\pm$ 27.49 A(a)	195.28 $\pm$ 38.46 A(a)	153.44 $\pm$ 20.40 A(a)	149.60 $\pm$ 33.83 A(a)
105	210.76 $\pm$ 16.45 A(a)	334.94 $\pm$ 54.37 A(a)	207.62 $\pm$ 43.91 A(a)	238.06 $\pm$ 44.01 A(a)
119	155.81 $\pm$ 46.43 A(a)	252.18 $\pm$ 52.42 A(a)	164.77 $\pm$ 28.01 A(a)	120.38 $\pm$ 16.44 A(a)

¹All values $\pm$ SEM represent results obtained from treatment replicates ($n = 4$).

² Statistically significant differences are indicated by alphabetic letters ($p < 0.05$). The same letters indicate no significant differences.

³Capital letters indicate statistically significant differences for each treatment over time, whereas the lowercase letters in brackets indicate significant differences between the different treatments at that specific time.

⁴INF: iodonitroterazolium chloride-formazan.

Although no statistically significant differences could be found, the overall dehydrogenase activity displayed in Figure 4.1 indicated changes in the activities of the treatments over time and variations between treatments. In general, the average dehydrogenase activity of all the treatments was higher on the day of sowing the sunflowers (Day 0) than at the end of the experiment on Day 119. The SC treatment showed the highest dehydrogenase activity overall, with a maximum value of 336.76 INF $\mu\text{g.g}^{-1}\text{2h}^{-1}$. Treatment SI had on average the lowest activity compared to the other treatments.

All the treatments received oats at the same incorporation rate (12 g. kg^{-1}) at the beginning of the experiment (Larkin and Griffin 2007). The treatments without *S. sclerotiorum* inoculum (treatments S and SC) received sterile oats, whereas the inoculated treatments (treatments SCI and SI) received inoculated oats. Microorganisms change their membrane functioning as a response to their surrounding environment. This causes variations in their soil enzyme activities. Therefore, after an initial increase in dehydrogenase activity due to the additional oats as an extra nutrient source, the activity could have been inhibited by the gradual degradation of the added nutrients, the adaptation of the community to the amendments or the adsorption of the nutrients to soil particles (Floch et al. 2011).

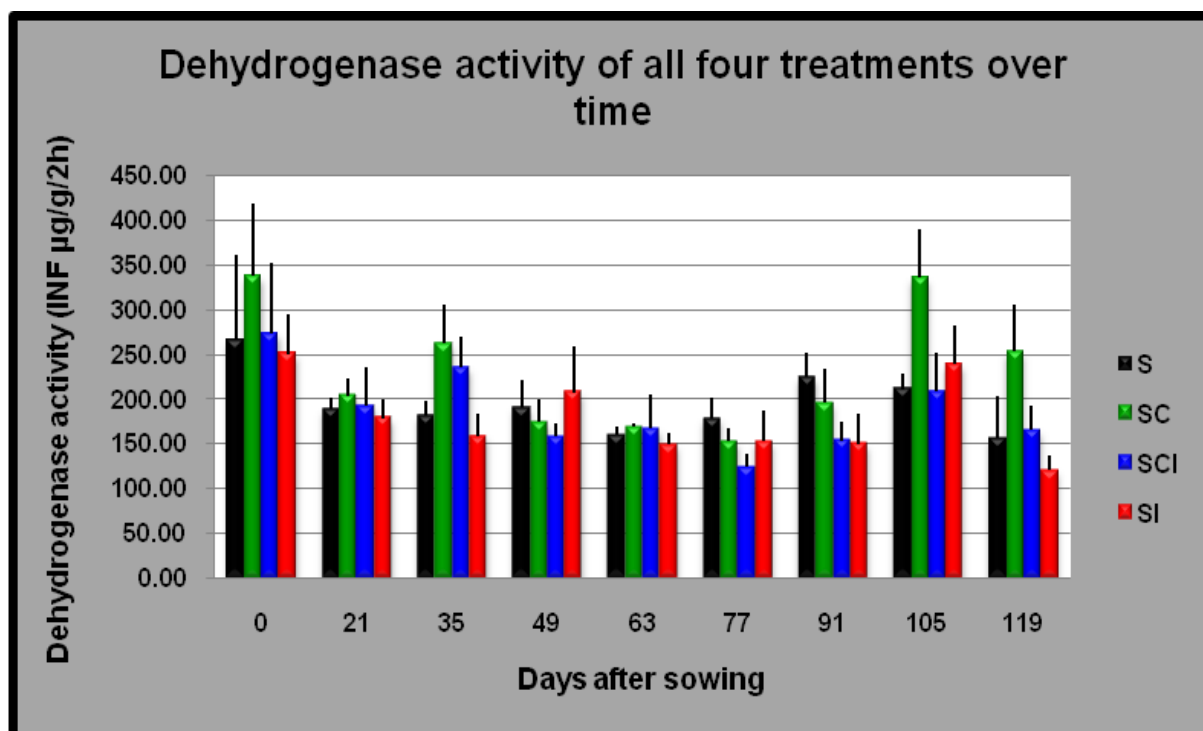


Figure 4.1: Overall dehydrogenase activity of the different treatments at the various sampling periods.

The high activity of treatment SC could be due to the fact that the addition of canola green manure enhances the soil organic matter content, leading to more organic C present in the soil ecosystem. Dehydrogenase activity is directly associated with the organic C content of

the soil (N'Dayegamiye and Tran 2001; Wiggins and Kinkel 2005). However, no significant enhancement of the soil organic C or total C was observed in the soil physical and chemical results. The low dehydrogenase activity of treatment SI might indicate the possible inhibition of *S. sclerotiorum* occurring in the soil. Floch et al. (2011) stated that if enzyme activities decrease, inhibition occurs, but if it increases the enzyme activities are stimulated. The addition of the inoculated oats might have enhanced the abundance of various soil microbial populations leading to competition, parasitism or predation in the soil environment (Lupwayi et al. 1998; Mazzola et al. 2001). These populations could have contained antibiotic producing Streptomyces, which might have suppressed *S. sclerotiorum* activity (Wiggins and Kinkel 2005).

From the results obtained, it is evident that the incorporation of canola green manure into the soil, as an additional temporary carbon source, had a stimulating effect on the dehydrogenase activity. Although fluctuations occurred, a gradual decline in the average dehydrogenase activity for each treatment was observed. This might be as a result of changing root secretions during the different sunflower growth stages (Xu et al. 2009). Inoculation of the soil with *S. sclerotiorum* had a negative effect on the dehydrogenase activity, possibly due to the stimulation (by oats amendments) of antibiotic producing bacteria that inhibited fungal growth.

4.3. Community level physiological profiles (CLPPs)

In this study the characterisation of the soil microbial diversity as CLPPs, through the use of Biolog® Ecoplates was employed. The AWCD of all the utilised carbon substrates in the plates was estimated as an average optical density value across all wells, for each treatment at the different sampling periods (Bernard et al. 2012).

Table 4.3: Average well colour development calculated on carbon substrate utilisation in the Biolog® Ecoplates for each treatment over time.

Days after sowing	Treatments			
	S	SC	SCI	SI
0	0.58 ± 0.06 CD(a)	0.68 ± 0.07 B(a)	0.45 ± 0.05 AD(a)	0.53 ± 0.06 A(a)
21	0.43 ± 0.05 ABC(ab)	0.32 ± 0.04 A(b)	0.54 ± 0.06 AB(a)	0.54 ± 0.06 A(a)
35	0.39 ± 0.05 ABC(a)	0.39 ± 0.04 A(a)	0.57 ± 0.06 AB(ab)	0.61 ± 0.07 A(b)
49	0.29 ± 0.04 A(a)	0.47 ± 0.05 AB(a)	0.77 ± 0.09 BC(b)	0.51 ± 0.06 A(a)
63	0.31 ± 0.04 A(b)	0.53 ± 0.06 AB(a)	0.58 ± 0.06 ABC(a)	0.57 ± 0.06 A(a)
77	0.35 ± 0.04 AB(a)	0.36 ± 0.05 A(a)	0.48 ± 0.05 AD(ab)	0.56 ± 0.07 A(b)
91	0.70 ± 0.08 D(a)	0.53 ± 0.07 AB(a)	0.58 ± 0.07 ABC(a)	0.49 ± 0.06 A(a)
105	0.54 ± 0.06 BCD(a)	0.55 ± 0.07 AB(a)	0.87 ± 0.09 C(b)	0.66 ± 0.07 A(ab)
119	0.26 ± 0.04 A(a)	0.35 ± 0.05 A(ab)	0.25 ± 0.04 D(a)	0.48 ± 0.06 A(b)

¹All values ± SEM represent results obtained from treatment replicates (n = 48).

²Statistically significant differences are indicated by alphabetic letters (p < 0.05). The same letters indicate no significant differences.

³Capital letters indicate statistically significant differences for each treatment over time, whereas the lowercase letters in brackets indicate significant differences between the different treatments at that specific time.

Statistically significant differences ($p < 0.05$) were found in the AWCD between treatments S (control), SC and SCI over time (indicated by capital letters), as can be seen in Table 4.3. Although the AWCD for the abovementioned treatments fluctuated significantly over time and between the treatments, the average AWCD was significantly lower at the end of the experiment. From day 21 onwards, statistically significant differences occurred between the different treatments at the specific sampling periods (letters in brackets). For the majority of the sampling periods, treatments SCI and SI had higher AWCD values compared to the control or treatment SC.

The significantly lower AWCD of all four treatments at the end of the experiment correlates with the lower dehydrogenase activity observed. The AWCD is indicative of the oxidative potential of soil microorganisms growing in the plate wells (Farnet et al. 2008). Changes in the AWCD imply that a variety of carbon sources have been used and that different organisms are active (Shengnan et al. 2011). The fluctuations apparent over time might be ascribed to the possible changes in root exudate secretions as the sunflowers progress in their growth stages (Schneider and Miller 1981; Xu et al. 2009). The results obtained for treatments SCI and SI are in contrast with the dehydrogenase activity results, indicating lower values for these two treatments. The AWCD results therefore suggest that different microorganisms were active in the various treatments, based upon the various carbon substrates utilised.

Diversity indices such as the Shannon-Weaver index can be used as a biodiversity measurement. In this study the Shannon-Weaver index was calculated as an average value, from the OD values for each treatment at the various sampling stages. The Shannon-Weaver substrate diversity index for the different treatments over time is displayed in Table 4.4. The index values for the various treatments over time ranged from 2.48 to 3.01. The diversity indices for treatments S and SC showed significant differences over time (indicated by capital letters), whereas the treatments SCI and SI showed no statistically significant differences, except on day 119. On days 63 and 77 statistically significant differences occurred between the various treatments.

The Shannon-Weaver index provides an indication of the overall species richness and evenness based on the assortment of carbon sources utilised in the Ecoplate wells (Farnet et al. 2008). Species richness refers to the amount of substrates assimilated, whereas species evenness describes the diverse range of specific substrates digested (Shannon and Weaver 1949). The high index values for the treatments indicated that a wide range of carbon substrates were utilised throughout the experiment, signifying high substrate diversity (Sharma et al. 1998). The significant changes between the treatments on days 63 and 77 could be associated with the plant growth stages. During this period the transition of the sunflowers from flower bud stage to early flowering phase took place (Schneider and

Miller 1981). Soil microbial communities change and acclimatise according to plant growth cycles (Xu et al. 2009). This can be related to the changing quality of secreted root exudates with the altering growth stages. It is evident that when the Shannon-Weaver index is applied, no clear distinction could be made between the different treatments based on the incorporation of canola green manure into the soil or the inoculation of the soil with *S. sclerotiorum*.

Table 4.4: Shannon-Weaver Index calculated from Ecoplate optical density values for the different treatments at the various sampling periods.

Days after sowing	Treatments			
	S	SC	SCI	SI
0	2.91 ± 0.07 A(a)	3.01 ± 0.05 B(a)	2.85 ± 0.08 A(a)	2.80 ± 0.08 A(a)
21	2.94 ± 0.03 A(a)	2.76 ± 0.05 AB(a)	2.85 ± 0.07 A(a)	2.85 ± 0.06 A(a)
35	2.77 ± 0.05 AB(a)	2.73 ± 0.06 A(a)	2.80 ± 0.06 A(a)	2.80 ± 0.08 A(a)
49	2.72 ± 0.06 AB(a)	2.73 ± 0.06 A(a)	2.86 ± 0.07 A(a)	2.82 ± 0.06 A(a)
63	2.78 ± 0.04 A(a)	2.85 ± 0.05 AB(ab)	2.98 ± 0.03 A(b)	2.82 ± 0.06 A(ab)
77	2.70 ± 0.05 AB(a)	2.64 ± 0.08 A(a)	2.80 ± 0.06 A(ab)	2.92 ± 0.05 A(b)
91	2.88 ± 0.06 A(a)	2.75 ± 0.07 AB(a)	2.83 ± 0.09 A(a)	2.79 ± 0.05 A(a)
105	2.85 ± 0.06 A(a)	2.76 ± 0.06 AB(a)	2.91 ± 0.07 A(a)	2.83 ± 0.07 A(a)
119	2.52 ± 0.09 B(a)	2.68 ± 0.08 A(a)	2.48 ± 0.09 B(a)	2.73 ± 0.06 A(a)

¹All values ± SEM represent results obtained from treatment replicates (n = 48).

²Statistically significant differences are indicated by alphabetic letters (p < 0.05). The same letters indicate no significant differences.

³Capital letters indicate statistically significant differences for each treatment over time, whereas the lowercase letters in brackets indicate significant differences between the different treatments at that specific time.

According to Lepš and Šmilauer (2003) ordination methods can be used to point out continuous change in community composition, variations in vegetation site patterns and gradients occurring over time. Ordination methods classify groups of internally homogenous objects into distinct groups. After classification, the homogenous species have the same ecological behaviour and distribution (Lepš and Šmilauer 1999). Principal component analysis reduces the dimensionality of the data set without losing the variation. The variance is illustrated by principal components (PCs) or axes, where the first two components indicate the highest variance (Yeung and Ruzzo 2000). In an RDA, species and sample variables are correlated with environmental variables creating a tri-plot. In the abovementioned diagrams, samples are represented by points, whereas species and environmental variables are represented by vectors (arrows). Dissimilarities are associated with the distance between the samples; the closer samples are to each other or to a vector, the higher the similarity between them. The linear correlation coefficients among the species can be indicated by the angles between the species vectors. Species vectors pointing in the same direction have a large positive correlation, whereas vectors pointing in opposite directions indicate large negative correlations.

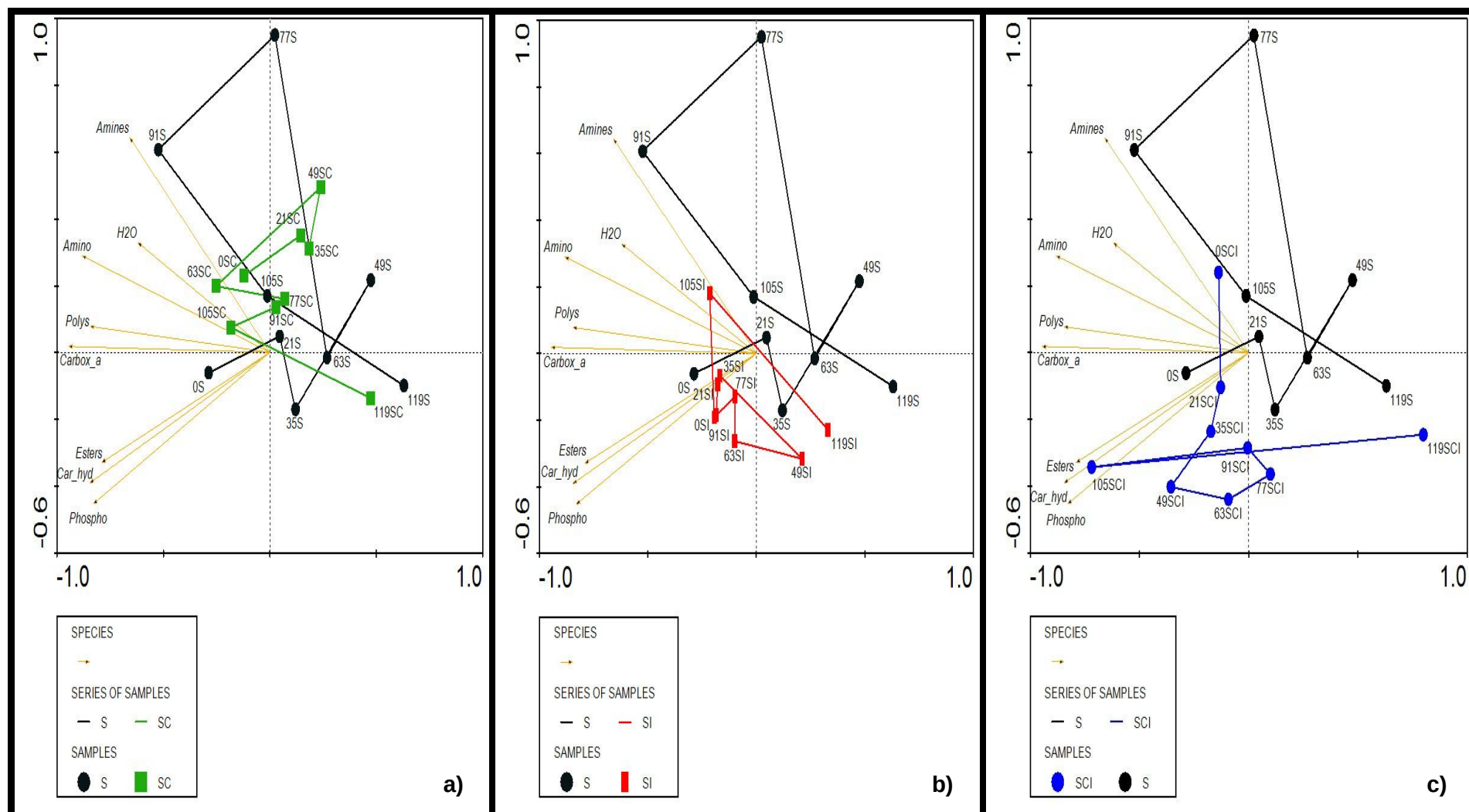


Figure 4.2: Subplots of a Principal Component Analysis (PCA) ordination diagram illustrating the relationship between the carbon substrate group utilisation of the different treatments over time. Subplots display treatments a) S and SC, b) S and SI, c) S and SCI. Each sampling period is indicated by the amount of days after sowing the sunflowers followed by the name of the treatment. The eigenvalues for the first two ordination axes were 0.654 and 0.135, respectively. These two axes accounted for 78.8% of the total observed variance. The substrates were classified as: Amines; H₂O – water; Amino – amino acids; Polys – polymers; Carbox_a – carboxylic acids; Esters; Car_hyd – carbohydrates; Phospho – phosphorylated compounds.

If a species vector points in the same direction as a vector of an environmental variable, the species values are positively correlated with the environmental variable. The direction of a species vector is related to the increase in species abundance. The axes of an ordination diagram distinguish between different ecological gradients within the samples. An eigenvalue higher than 0.3 is indicative of a strong gradient (Ter Braak and Verdonschot 1995).

A PCA was performed on the Biolog® Ecoplate carbon substrate group utilisation data to illustrate the relationship between the various treatments over the 119 days after sowing the sunflowers. Due to the overcrowded display of the main plot (not shown), subplots were drawn comparing the substrate utilisation profiles of each treatment over time with the control (Figure 4.2). This implies that all the subplots possess the same eigenvalues, seeing as they are only visual variations selected from the main multivariate ordination diagram.

Figure 4.2a illustrates the substrate guild utilisation patterns over time of the treatment SC compared to the control treatment S. Treatment SC showed a similar trend over time to the control S, except for distinct differences on days 77 and 91. The control S showed a positive correlation with amines on day 91. At the end of the experiment, on day 119, both treatments grouped in the same quadrant of the first ordination axis. The utilisation profile of treatment SI also changed in the same manner as the control S and treatment SC, as could be seen in Figure 4.2b. Treatment SI showed a similar endpoint on day 119 to SC and S. It was apparent that treatment SCI (Figure 4.2c) displayed different trends than the other treatments, from days 0 to 63, but from day 77 onwards a similar tendency could be observed. On day 119, treatment SCI grouped in the same quadrant as the other treatments S, SC and SI.

Based on the results obtained from the PCA ordination plots, it is evident that although different modifications took place within each treatment's soil microbial community, a similar utilisation trend could still be observed over time for the treatments S, SC and SI. The possible inhibition of the fungal pathogen by the canola green manure in treatment SCI might have led to the abnormal fluctuations during days 0 to 63. This corresponds with the soil physical and chemical results for treatment SCI. Even though the substrate utilisation patterns of the four treatments differed to a great extent, the treatments showed a high correlation at the end of the experiment on day 119. This feature might be ascribed to the resilience of the microbial communities to return to a similar functional composition after disturbances (Floch et al. 2011).

Specific substrate utilisation profiles in the four treatments at different sampling periods for certain substrate groups with high relevance can be seen in Figure 4.3. Tukey's HSD test

indicated a statistically significant decline in the carboxylic acid utilisation (Figure 4.3a) for each treatment over time (indicated by capital letters) and among the various treatments at certain sampling periods (in brackets).

Statistically significant differences could also be observed for the ester utilisation (Figure 4.3b), amine utilisation (Figure 4.3c) and carbohydrate utilisation (Figure 4.3d) over time for individual treatments and between treatments at specific sampling periods. In comparison to the other substrate groups, amine utilisation had overall much lower OD values. This may be explained by the complexity of the substrates classified as amines and the reduced potential of soil microbial communities to metabolise these carbon sources (Bernard et al. 2012). However, control S was characterised by a higher utilisation of amines on day 91, which correlates with the slightly higher dehydrogenase activity, AWCD and substrate diversity for this treatment on day 91. All four treatments had no significant differences in carboxylic acid and ester utilisation on days 0 and 77. No significant differences in the utilisation of carboxylic acids and carbohydrates were found for treatments SCI and SI on day 35. These utilisation trends signify that although different treatments were applied, the soil microbial communities still functioned in the same manner. In the majority of treatments the substrate utilisation of all the substrates was lower at the end of the experiment, which could be seen from the negative correlation with the various substrate groups. It should be remembered that an ordination examines the variance among different samples relative to each other (Kent and Coker 1992). In a PCA bi-plot, a carbon substrate with a poor correlation shows that its utilisation was not distinctive among the microbial communities in the samples. Therefore, it was not significant in distinguishing between them. These lower end values are parallel to the lower substrate diversity, AWCD and dehydrogenase activity found on day 119. This might be as a result of the gradual degradation of the added nutrients by microbial communities or the acclimatisation of the communities to the changed environment (Floch et al. 2011). The development of the sunflowers could possibly have contributed to the changing microbial diversity, since different root exudates secreted during plant growth phases function as nutrient sources for microbial growth (Bolton et al. 1993; Campbell et al. 1997).

It can be concluded, that the incorporation of the soil with canola green manure or the inoculation with *S. sclerotiorum* did have an effect on the substrate utilisation profiles of the microbial communities. However, it seemed as if the microbial communities still functioned at similar rates or through similar processes. In the end this effect was not long-lasting and the soil microbial communities returned to their original functionality positions.

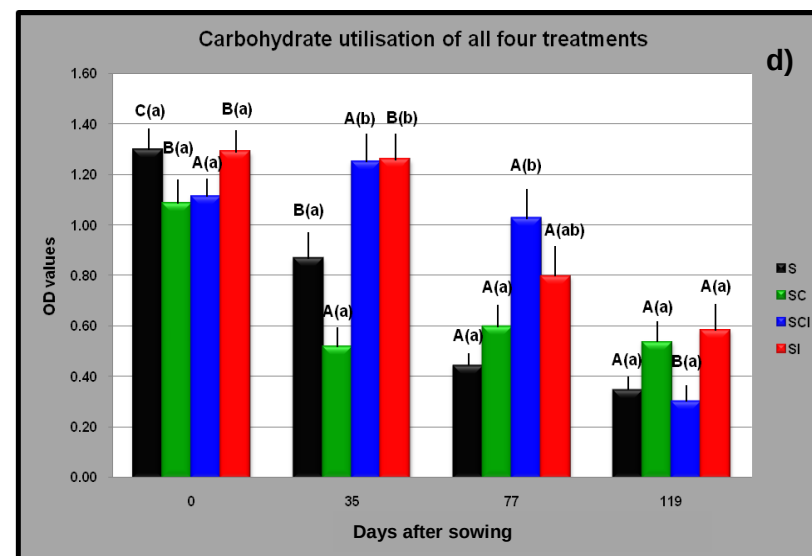
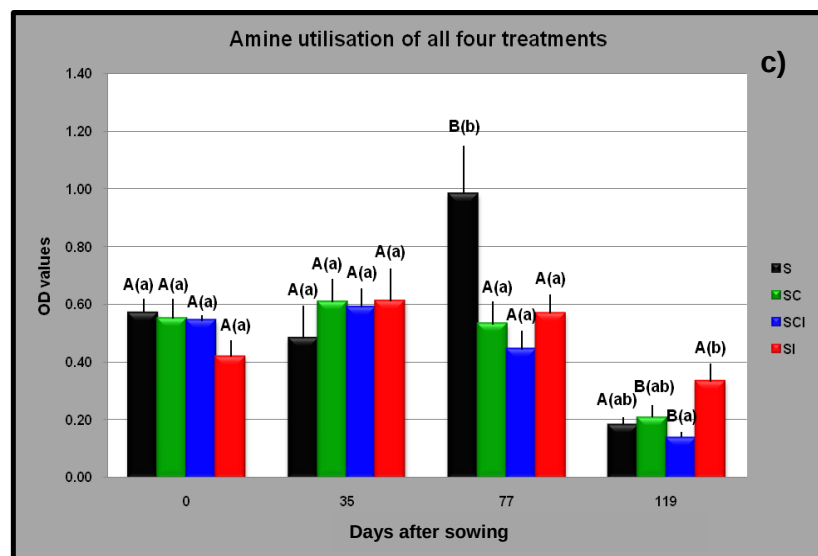
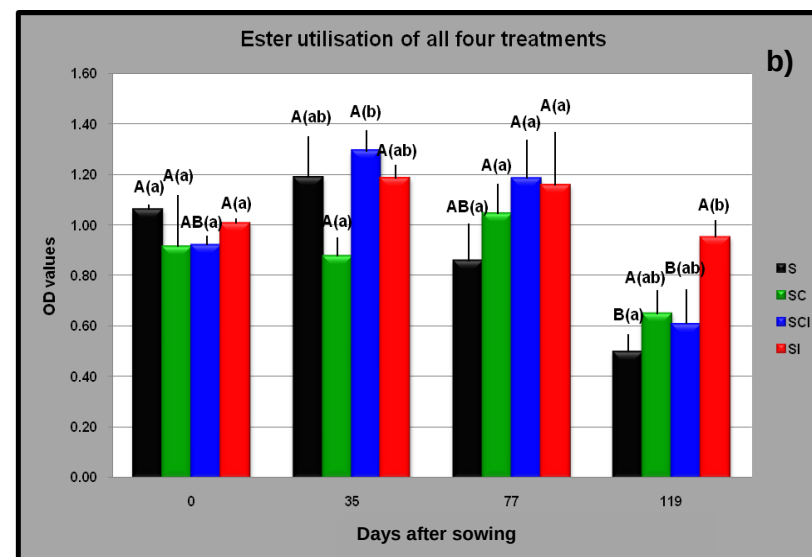
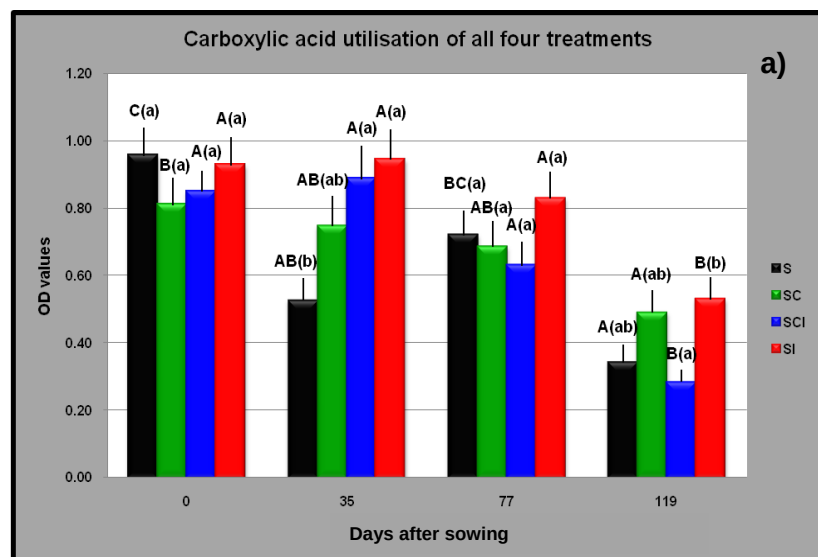


Figure 4.3: Substrate group utilisation of the different treatments at days 0, 35, 77 and 119 where a) carboxylic acids, b) esters, c) amines and d) carbohydrates are illustrated. Statistically significant differences are indicated by alphabetic letters ($p < 0.05$). The same letters indicate no significant differences. Capital letters indicate statistically significant differences for each treatment over time, whereas the lowercase letters in brackets indicate significant differences between the different treatments at that specific time. Key to abbreviations: OD – optical density.

4.4. Phospholipid fatty acid (PLFA) analysis

The estimated viable microbial biomass, microbial community structure based on the mole percentage (mol%) fraction of the concentrations of major PLFA structural groups and the stress ratios derived from PLFA data (with statistically significant differences) for the different treatments over time are summarised in Tables 4.5 to 4.8.

The total viable microbial biomass (Figure 4.4) was indicated by the total amount of PLFAs (White et al. 1979). The microbial biomass for the control S ranged from 21481.96 to 130834.75 pmol per gram soil dry weight (Table 4.5), for treatment SC it ranged between 27768.09 and 105635.51 pmol per gram soil dry weight (Table 4.6), treatment SI showed biomass abundances between 22588.11 and 102369.27 pmol per gram soil dry weight (Table 4.7) and for treatment SCI it ranged from 22890.83 to 108441.72 pmol per gram soil dry weight (Table 4.8). Overall, the biomass increased significantly for all the treatments for the duration of the experiment (indicated by capital letters), (Figure 4.4 and Tables 4.5 to 4.8).

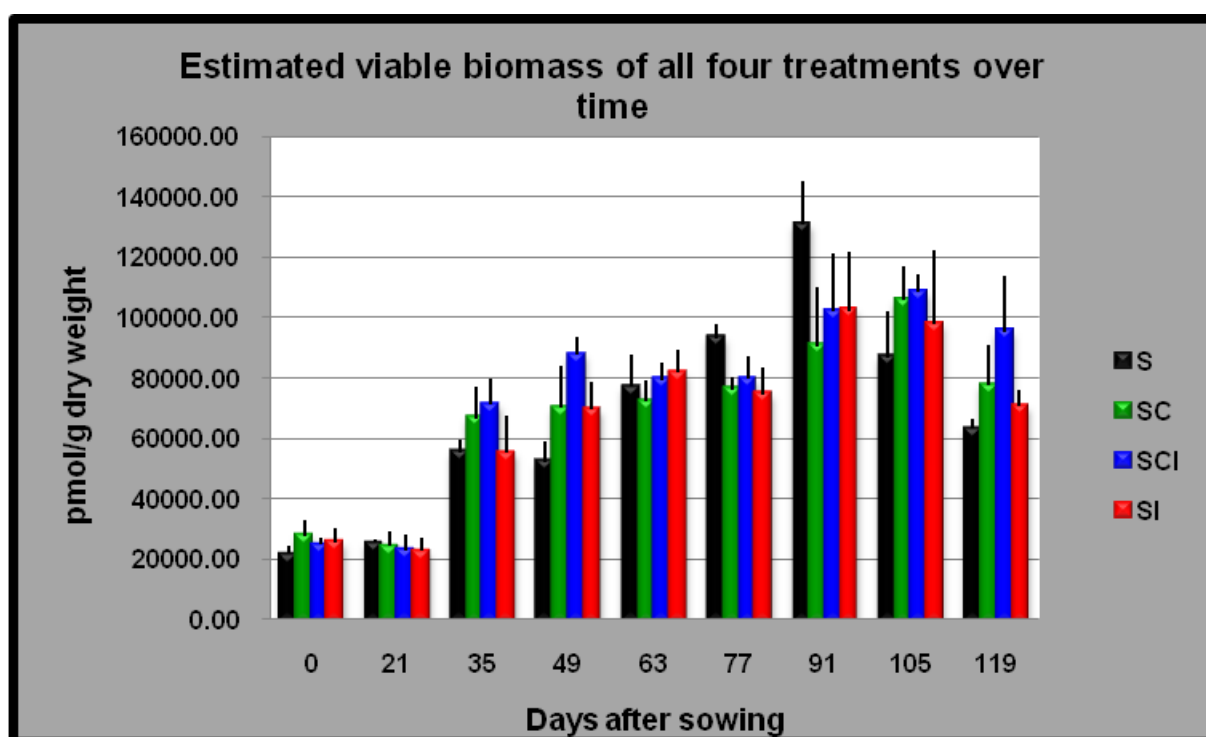


Figure 4.4: Estimated viable biomass of the different treatments at the various sampling periods after sowing the sunflowers.

While very few significant differences were found among the different treatments at the various sampling days, the treatments can still be distinguished from one another.

Table 4.5: Phospholipid fatty acid (PLFA) composition and ratios of the control treatment S over time.

	Days after sowing								
S	0	21	35	49	63	77	91	105	119
Viable microbial biomass (pmol.g ⁻¹ soil dry weight)	21481.96 ± 2631.13 B(a)	25293.91 ± 1126.74 B(a)	55353.70 ± 3961.01 AB(a)	52273.51 ± 6753.22 AB(a)	76911.34 ± 10738.87 AC(a)	93302.04 ± 4571.00 C(a)	130834.75 ± 14718.11 D(a)	86901.59 ± 15241.38 AC(a)	63004.93 ± 3437.65 AC(a)
PLFA groups (mol%)									
Normal saturated	46.22 ± 1.75 C(a)	38.45 ± 0.65 BD(a)	30.96 ± 0.08 A(a)	30.75 ± 0.25 A(a)	31.25 ± 0.28 A(a)	32.77 ± 1.09 A(a)	41.20 ± 2.51 BC(a)	40.56 ± 1.72 BC(b)	33.85 ± 0.30 AD(ab)
Mid-chain branched saturated	4.43 ± 0.22 E(a)	4.23 ± 0.09 E(a)	2.04 ± 0.13 D(a)	1.51 ± 0.20 BD(a)	1.19 ± 0.17 AB(a)	0.94 ± 0.10 AC(a)	0.65 ± 0.07 C(a)	1.01 ± 0.10 ABC(a)	1.42 ± 0.09 AB(a)
Terminally branched saturated	29.55 ± 2.48 D(a)	33.97 ± 1.47 D(a)	22.28 ± 0.89 C(a)	16.04 ± 2.13 BC(a)	13.14 ± 1.39 AB(a)	12.29 ± 1.14 AB(a)	8.40 ± 0.76 A(a)	10.13 ± 0.61 A(a)	14.05 ± 0.44 AB(a)
Monounsaturated	16.44 ± 0.82 A(a)	18.75 ± 0.98 A(a)	29.49 ± 0.88 A(a)	26.94 ± 0.35 A(a)	24.07 ± 1.36 A(a)	30.62 ± 9.05 A(a)	36.34 ± 11.36 A(a)	20.50 ± 1.23 A(a)	24.52 ± 0.88 A(a)
Polyunsaturated	1.93 ± 0.21 B(a)	3.13 ± 0.15 B(a)	14.47 ± 1.55 AB(a)	24.07 ± 2.66 A(a)	29.78 ± 2.83 A(a)	22.87 ± 8.77 A(a)	13.02 ± 9.55 AB(a)	27.30 ± 2.02 A(a)	25.39 ± 1.20 A(a)
PLFA ratios									
Fungal / Bacterial ⁴	0.01 ± 0.00 A(a)	0.03 ± 0.01 A(a)	0.40 ± 0.06 AB(a)	0.85 ± 0.17 AB(a)	1.28 ± 0.22 B(a)	1.46 ± 0.13 AB(a)	1.93 ± 0.05 AB(a)	1.45 ± 0.15 B(a)	1.07 ± 0.09 AB(a)
Saturated / Unsaturated ⁵	3.29 ± 0.13 CD(a)	2.44 ± 0.15 ACD(a)	1.15 ± 0.04 B(a)	1.42 ± 0.06 AB(a)	1.73 ± 0.18 AB(a)	2.26 ± 0.32 AC(a)	3.49 ± 0.58 D(a)	2.94 ± 0.24 CD(b)	1.86 ± 0.10 AB(a)
trans / cis ⁶	0.45 ± 0.04 B(a)	0.08 ± 0.02 AB(a)	0.07 ± 0.01 AB(a)	0.09 ± 0.04 AB(a)	0.06 ± 0.02 A(a)	0.14 ± 0.04 AB(a)	0.27 ± 0.09 AB(a)	0.16 ± 0.05 AB(a)	0.07 ± 0.01 AB(a)
Gram (+) / Total PLFAs ⁷	0.26 ± 0.02 D(a)	0.30 ± 0.01 D(a)	0.19 ± 0.01 C(a)	0.15 ± 0.02 BC(a)	0.12 ± 0.01 AB(a)	0.11 ± 0.01 AB(a)	0.08 ± 0.01 A(a)	0.09 ± 0.01 A(a)	0.13 ± 0.00 AB(a)
Iso / Anteiso ⁸	3.00 ± 0.19 CD(a)	3.20 ± 0.17 D(a)	2.82 ± 0.04 BCD(a)	2.44 ± 0.08 AB(a)	2.46 ± 0.12 ABC(a)	2.22 ± 0.11 A(a)	2.10 ± 0.08 A(a)	2.39 ± 0.07 AB(a)	2.31 ± 0.20 AB(a)

¹All values ± SEM represent results obtained from treatment replicates (n = 4).

²Statistically significant differences are indicated by alphabetic letters (p < 0.05). The same letters indicate no significant differences.

³Capital letters indicate statistically significant differences for each treatment over time, whereas the lowercase letters in brackets indicate significant differences between the different treatments at that specific time.

⁴Fungi: 18:2ω6, 9; Bacteria: Sum of i15:0, a15:0, 15:0, i16:0, 16:1ω9, 16:1ωt, i17:0, a17:0, cy17:0, 17:0, 18:1ω7 and cy19:0 (Frostegård and Bååth 1996).

⁵Straight chain saturated: Monounsaturated PLFAs (Pinkart et al. 2002).

⁶16:1ω7t: 16:1ω7c (McKinley et al. 2005).

⁷Gram-positive PLFA indicators (i15:0, a15:0, i16:0, 10me16:0 and 17:0) to total PLFAs (McKinley et al. 2005).

⁸Iso: Anteiso branched PLFAs (i15:0 + i17:0 / a15:0 + a17:0) (McKinley et al. 2005).

Table 4.6: Phospholipid fatty acid (PLFA) composition and ratios of the treatment SC over time.

SC	Days after sowing								
	0	21	35	49	63	77	91	105	119
Viable microbial biomass (pmol.g ⁻¹ soil dry weight)	27768.09 ± 5346.29 BC(a)	24268.83 ± 4935.00 C(a)	66635.35 ± 10302.60 ABC(a)	69937.62 ± 13896.58 AB(ab)	72235.30 ± 6813.89 AB(a)	76178.68 ± 4051.16 A(a)	90535.03 ± 19473.83 A(a)	105635.51 ± 11665.64 A(a)	77656.76 ± 13108.76 A(a)
PLFA groups (mol%)									
Normal saturated	47.95 ± 0.96 C(a)	42.03 ± 0.91 B(a)	31.13 ± 0.48 A(a)	30.89 ± 0.29 A(a)	31.60 ± 0.43 A(a)	31.48 ± 0.52 A(a)	40.55 ± 1.50 B(a)	39.57 ± 0.81 B(ab)	32.43 ± 0.23 A(a)
Mid-chain branched saturated	3.65 ± 0.36 B(a)	4.33 ± 0.32 B(a)	1.65 ± 0.18 A(a)	1.33 ± 0.18 A(a)	1.26 ± 0.09 A(a)	1.00 ± 0.11 A(a)	0.96 ± 0.15 A(a)	0.95 ± 0.12 A(a)	1.17 ± 0.21 A(a)
Terminally branched saturated	27.83 ± 1.80 C(a)	30.15 ± 2.45 C(a)	20.09 ± 0.74 B(a)	13.94 ± 0.98 AB(a)	14.47 ± 1.71 AB(a)	11.71 ± 1.57 A(a)	11.37 ± 0.33 A(b)	10.96 ± 1.14 A(a)	13.53 ± 1.57 A(a)
Monounsaturated	17.67 ± 0.41 A(a)	18.61 ± 1.30 A(a)	30.31 ± 1.22 A(a)	25.18 ± 1.10 A(a)	24.98 ± 1.41 A(a)	23.54 ± 1.33 A(a)	28.02 ± 6.90 A(a)	28.14 ± 7.71 A(a)	24.61 ± 1.99 A(a)
Polyunsaturated	1.74 ± 0.10 B(a)	3.39 ± 0.43 B(a)	16.13 ± 1.69 AB(a)	28.04 ± 2.53 A(a)	27.08 ± 2.61 A(a)	31.74 ± 2.51 A(a)	18.61 ± 7.25 AB(a)	19.94 ± 8.23 A(a)	27.70 ± 3.33 A(a)
PLFA ratios									
Fungal / Bacterial ⁴	0.01 ± 0.00 B(a)	0.03 ± 0.00 BC(a)	0.45 ± 0.08 ABC(a)	1.10 ± 0.18 AC(a)	1.13 ± 0.22 A(a)	1.52 ± 0.26 A(a)	1.16 ± 0.23 ABC(a)	1.42 ± 0.38 ABC(a)	1.15 ± 0.30 A(a)
Saturated / Unsaturated ⁵	3.08 ± 0.07 C(a)	2.65 ± 0.19 CD(a)	1.15 ± 0.08 A(a)	1.63 ± 0.13 AB(a)	1.69 ± 0.15 AB(a)	1.91 ± 0.23 BD(a)	2.66 ± 0.19 C(a)	2.71 ± 0.24 C(ab)	1.77 ± 0.24 AB(a)
trans / cis ⁶	0.46 ± 0.07 B(a)	0.13 ± 0.04 A(a)	0.13 ± 0.02 A(a)	0.09 ± 0.01 A(a)	0.07 ± 0.01 A(a)	0.07 ± 0.02 A(a)	0.08 ± 0.02 A(b)	0.07 ± 0.02 A(a)	0.09 ± 0.03 A(a)
Gram (+) / Total PLFAs ⁷	0.24 ± 0.01 C(a)	0.26 ± 0.02 C(a)	0.18 ± 0.01 B(a)	0.13 ± 0.01 AB(a)	0.13 ± 0.01 AB(a)	0.11 ± 0.01 A(a)	0.10 ± 0.00 A(b)	0.10 ± 0.01 A(a)	0.13 ± 0.01 AB(a)
Iso / Anteiso ⁸	2.72 ± 0.13 AB(a)	3.37 ± 0.14 C(a)	2.81 ± 0.09 BC(a)	2.50 ± 0.13 AB(a)	2.62 ± 0.13 AB(a)	2.25 ± 0.06 AB(a)	2.23 ± 0.18 A(a)	2.22 ± 0.09 A(a)	2.21 ± 0.24 A(a)

¹All values ± SEM represent results obtained from treatment replicates (n = 4).

²Statistically significant differences are indicated by alphabetic letters (p < 0.05). The same letters indicate no significant differences.

³Capital letters indicate statistically significant differences for each treatment over time, whereas the lowercase letters in brackets indicate significant differences between the different treatments at that specific time.

⁴Fungi: 18:2ω6, 9; Bacteria: Sum of i15:0, a15:0, 15:0, i16:0, 16:1ω9, 16:1ωt, i17:0, a17:0, cy17:0, 17:0, 18:1ω7 and cy19:0 (Frostegård and Bååth 1996).

⁵Straight chain saturated: Monounsaturated PLFAs (Pinkart et al. 2002).

⁶16:1ω7t: 16:1ω7c (McKinley et al. 2005).

⁷Gram-positive PLFA indicators (i15:0, a15:0, i16:0, 10me16:0 and 17:0) to total PLFAs (McKinley et al. 2005).

⁸Iso: Anteiso branched PLFAs (i15:0 + i17:0 / a15:0 + a17:0) (McKinley et al. 2005).

Table 4.7: Phospholipid fatty acid (PLFA) composition and ratios of the treatment SI over time.

SI	Days after sowing								
	0	21	35	49	63	77	91	105	119
Viable microbial biomass (pmol.g ⁻¹ soil dry weight)	25591.76 ± 4518.26 BC(a)	22588.11 ± 4709.93 B(a)	55244.91 ± 12343.81 ABC(a)	69413.85 ± 9176.29 ABC(ab)	81775.88 ± 7741.78 A(a)	74598.00 ± 9144.16 AC(a)	102369.27 ± 19345.53 A(a)	97833.62 ± 24557.60 A(a)	70589.24 ± 5706.66 ABC(a)
PLFA groups (mol%)									
Normal saturated	46.04 ± 2.41 D(a)	40.29 ± 1.14 BC(a)	30.79 ± 0.63 A(a)	30.65 ± 0.14 A(a)	31.76 ± 0.49 A(a)	32.12 ± 0.42 A(a)	40.97 ± 1.36 CD(a)	36.05 ± 2.02 AB(a)	33.57 ± 0.47 A(ab)
Mid-chain branched saturated	3.72 ± 0.15 C(a)	3.92 ± 0.47 C(a)	1.77 ± 0.14 B(a)	1.24 ± 0.07 AB(a)	1.03 ± 0.14 AB(a)	0.98 ± 0.09 AB(a)	0.83 ± 0.15 A(a)	0.79 ± 0.23 AB(a)	1.43 ± 0.26 AB(a)
Terminally branched saturated	30.92 ± 3.81 C(a)	29.23 ± 2.71 C(a)	19.97 ± 1.64 B(a)	15.92 ± 1.01 AB(a)	13.79 ± 1.51 AB(a)	13.61 ± 1.03 AB(a)	9.83 ± 0.66 A(ab)	11.71 ± 2.03 AB(a)	16.37 ± 2.19 AB(a)
Monounsaturated	15.82 ± 1.84 A(a)	20.75 ± 1.76 AB(a)	26.88 ± 1.07 AB(a)	25.78 ± 0.67 AB(a)	25.04 ± 0.84 AB(a)	23.56 ± 1.44 AB(a)	20.67 ± 0.69 AB(a)	34.21 ± 7.40 B(a)	24.81 ± 0.45 AB(a)
Polyunsaturated	1.91 ± 0.17 B(a)	4.00 ± 0.39 B(a)	19.95 ± 2.65 A(a)	25.78 ± 1.33 A(a)	27.84 ± 2.72 A(a)	29.10 ± 2.47 A(a)	27.32 ± 2.32 A(a)	16.77 ± 7.76 A(a)	23.22 ± 2.68 A(a)
PLFA ratios									
Fungal / Bacterial ⁴	0.01 ± 0.00 C(a)	0.04 ± 0.01 CD(a)	0.59 ± 0.15 ACD(a)	0.98 ± 0.10 AB(a)	1.22 ± 0.23 AB(a)	1.25 ± 0.22 AB(a)	1.45 ± 0.24 B(a)	1.10 ± 0.22 ABD(a)	0.86 ± 0.15 AB(a)
Saturated / Unsaturated ⁵	3.32 ± 0.33 E(a)	2.37 ± 0.20 CD(a)	1.26 ± 0.09 A(a)	1.48 ± 0.07 AB(a)	1.65 ± 0.11 ABC(a)	1.77 ± 0.16 ABC(a)	2.75 ± 0.13 DE(a)	2.04 ± 0.26 BCD(a)	1.70 ± 0.04 ABC(a)
trans / cis ⁶	0.55 ± 0.01 B(a)	0.13 ± 0.04 A(a)	0.07 ± 0.01 A(a)	0.09 ± 0.01 A(a)	0.09 ± 0.01 A(a)	0.14 ± 0.02 A(a)	0.30 ± 0.03 A(a)	0.11 ± 0.02 A(a)	0.14 ± 0.02 A(a)
Gram (+) / Total PLFAs ⁷	0.27 ± 0.03 C(a)	0.26 ± 0.02 C(a)	0.18 ± 0.01 B(a)	0.14 ± 0.01 AB(a)	0.12 ± 0.01 AB(a)	0.12 ± 0.01 AB(a)	0.09 ± 0.01 A(ab)	0.12 ± 0.02 AB(a)	0.15 ± 0.02 AB(a)
Iso / Anteiso ⁸	3.19 ± 0.22 CD(a)	3.57 ± 0.18 D(a)	2.91 ± 0.19 BC(a)	2.58 ± 0.15 ABC(a)	2.70 ± 0.07 ABC(a)	2.38 ± 0.12 AB(a)	2.36 ± 0.17 AB(a)	2.21 ± 0.18 A(a)	2.34 ± 0.10 AB(a)

¹All values ± SEM represent results obtained from treatment replicates (n = 4).

²Statistically significant differences are indicated by alphabetic letters (p < 0.05). The same letters indicate no significant differences.

³Capital letters indicate statistically significant differences for each treatment over time, whereas the lowercase letters in brackets indicate significant differences between the different treatments at that specific time.

⁴Fungi: 18:2ω6, 9; Bacteria: Sum of i15:0, a15:0, 15:0, i16:0, 16:1ω9, 16:1ωt, i17:0, a17:0, cy17:0, 17:0, 18:1ω7 and cy19:0 (Frostegård and Bååth 1996).

⁵Straight chain saturated: Monounsaturated PLFAs (Pinkart et al. 2002).

⁶16:1ω7t: 16:1ω7c (McKinley et al. 2005).

⁷Gram-positive PLFA indicators (i15:0, a15:0, i16:0, 10me16:0 and 17:0) to total PLFAs (McKinley et al. 2005).

⁸Iso: Anteiso branched PLFAs (i15:0 + i17:0 / a15:0 + a17:0) (McKinley et al. 2005).

Table 4.8: Phospholipid fatty acid (PLFA) composition and ratios of the treatment SCI over time.

	Days after sowing								
SCI	0	21	35	49	63	77	91	105	119
Viable microbial biomass (pmol.g ⁻¹ soil dry weight)	24777.89 ± 2181.35 B(a)	22890.83 ± 5062.82 B(a)	71166.95 ± 8634.03 A(a)	87711.60 ± 5989.15 A(b)	79367.13 ± 5835.60 A(a)	79582.83 ± 7435.98 A(a)	101966.78 ± 19321.18 A(a)	108441.72 ± 5831.68 A(a)	95437.44 ± 18209.99 A(a)
PLFA groups (mol%)									
Normal saturated	43.47 ± 1.64 C(a)	39.36 ± 1.07 B(a)	30.86 ± 0.37 A(a)	30.98 ± 0.32 A(a)	31.35 ± 0.04 A(a)	31.24 ± 0.14 A(a)	41.52 ± 1.24 BC(a)	39.13 ± 0.47 B(ab)	33.76 ± 0.54 A(b)
Mid-chain branched saturated	3.60 ± 0.29 B(a)	3.71 ± 0.38 B(a)	1.66 ± 0.19 A(a)	1.31 ± 0.18 A(a)	1.05 ± 0.14 A(a)	1.00 ± 0.07 A(a)	1.00 ± 0.13 A(a)	0.87 ± 0.15 A(a)	1.23 ± 0.23 A(a)
Terminally branched saturated	34.40 ± 2.19 C(a)	31.62 ± 2.05 C(a)	19.84 ± 2.01 B(a)	16.77 ± 1.55 AB(a)	13.92 ± 1.07 AB(a)	13.53 ± 0.91 AB(a)	10.58 ± 1.08 A(ab)	12.47 ± 1.63 A(a)	16.27 ± 2.08 AB(a)
Monounsaturated	15.07 ± 1.04 D(a)	19.66 ± 1.12 B(a)	28.08 ± 0.60 A(a)	26.02 ± 0.57 A(a)	25.56 ± 0.64 A(a)	26.19 ± 0.76 A(a)	20.90 ± 0.78 B(a)	21.43 ± 0.92 BC(a)	25.31 ± 1.87 AC(a)
Polyunsaturated	2.00 ± 0.23 C(a)	3.93 ± 0.41 C(a)	18.93 ± 2.65 A(a)	24.10 ± 2.10 AB(a)	27.52 ± 1.57 B(a)	27.39 ± 1.71 AB(a)	25.59 ± 2.45 AB(a)	25.62 ± 2.57 AB(a)	22.84 ± 3.24 AB(a)
PLFA ratios									
Fungal / Bacterial ⁴	0.01 ± 0.00 C(a)	0.05 ± 0.01 C(a)	0.59 ± 0.13 AC(a)	0.87 ± 0.13 AB(a)	1.14 ± 0.12 AB(a)	1.13 ± 0.11 AB(a)	1.30 ± 0.24 B(a)	1.25 ± 0.25 AB(a)	0.82 ± 0.22 AB(a)
Saturated / Unsaturated ⁵	3.43 ± 0.26 C(a)	2.32 ± 0.16 B(a)	1.24 ± 0.03 A(a)	1.46 ± 0.08 A(a)	1.55 ± 0.05 A(a)	1.49 ± 0.04 A(a)	2.63 ± 0.13 B(a)	2.54 ± 0.13 B(ab)	1.69 ± 0.10 A(a)
trans / cis ⁶	0.41 ± 0.06 B(a)	0.12 ± 0.02 A(a)	0.14 ± 0.04 A(a)	0.12 ± 0.02 A(a)	0.09 ± 0.02 A(a)	0.15 ± 0.04 A(a)	0.15 ± 0.04 A(ab)	0.18 ± 0.05 A(a)	0.15 ± 0.09 A(a)
Gram (+) / Total PLFAs ⁷	0.29 ± 0.02 C(a)	0.27 ± 0.02 C(a)	0.18 ± 0.02 B(a)	0.15 ± 0.01 AB(a)	0.12 ± 0.01 AB(a)	0.12 ± 0.01 AB(a)	0.10 ± 0.01 A(ab)	0.11 ± 0.01 A(a)	0.15 ± 0.02 AB(a)
Iso / Anteiso ⁸	3.38 ± 0.29 BC(a)	3.45 ± 0.25 C(a)	2.72 ± 0.19 AB(a)	2.47 ± 0.11 A(a)	2.48 ± 0.10 A(a)	2.41 ± 0.11 A(a)	2.28 ± 0.14 A(a)	2.17 ± 0.08 A(a)	2.17 ± 0.17 A(a)

¹All values ± SEM represent results obtained from treatment replicates (n = 4).

²Statistically significant differences are indicated by alphabetic letters (p < 0.05). The same letters indicate no significant differences.

³Capital letters indicate statistically significant differences for each treatment over time, whereas the lowercase letters in brackets indicate significant differences between the different treatments at that specific time.

⁴Fungi: 18:2ω6, 9; Bacteria: Sum of i15:0, a15:0, 15:0, i16:0, 16:1ω9, 16:1ωt, i17:0, a17:0, cy17:0, 17:0, 18:1ω7 and cy19:0 (Frostegård and Bååth 1996).

⁵Straight chain saturated: Monounsaturated PLFAs (Pinkart et al. 2002).

⁶16:1ω7t: 16:1ω7c (McKinley et al. 2005).

⁷Gram-positive PLFA indicators (i15:0, a15:0, i16:0, 10me16:0 and 17:0) to total PLFAs (McKinley et al. 2005).

⁸Iso: Anteiso branched PLFAs (i15:0 + i17:0 / a15:0 + a17:0) (McKinley et al. 2005).

Figure 4.4 and Tables 4.5 to 4.8 show that treatments SC and SCI had on average the highest biomass abundance compared to the control S and treatment SI.

The increasing biomass could be ascribed to the adaptation of the soil microbial communities to the additional amendments (oats or green manure) which lead to the stimulation of microbial growth (Floch et al. 2011). According to Smalla et al. (2001), the root exudates secreted in the soil rhizosphere can influence the microbial growth patterns. The rising biomass trend might also reflect the stimulation of microbial biomass due to the different root exudates secreted by the flowering plants, which function as carbon nutrients (Xu et al. 2009). The substrate utilisation profiles also indicated this trend. The results for treatments SC and SCI are in accordance with other studies confirming that green manure applications enhance microbial biomass, since it provides added available nutrients (Elfstrand et al. 2007; Lachmund and Manz 2005).

The microbial community structure based on the mol% fraction of the various PLFA groups for the different treatments over time is displayed in Figure 4.5. The concentrations of the specific PLFA groups for the treatments S, SC, SI and SCI are summarised in Tables 4.5 to 4.8, respectively. Based on these results it is evident that the concentration of mid-branched saturated fatty acids (MBsats – indicative of Actinomycetes) (Frostegard and Baath 1996) and terminally branched saturated fatty acids (TBsats – indicative of Gram-positive bacteria) (Frostegård and Bååth 1996) significantly decreased, on average, for each treatment over time. However, TBsats showed a slight increase in all the treatments on day 119. In general, the concentration of monounsaturated fatty acids (Monos – representative of Gram-negative bacteria) (Peacock et al. 2001) significantly increased over time. The polyunsaturated fatty acids (Polys), indicative of fungi, displayed an overall increase in the fungal concentration for the duration of the experiment in all the treatments. Treatments SI and SCI showed on average higher fractions of Polys than the control S and treatment SC. Only a few statistically significant differences were observed among the treatments at specific sampling periods. Normal saturated fatty acids (Nsats) are found in all organisms (Zelles 1999) and fluctuated over time for each treatment.

It could be noted that the concentration of Actinomycetes (MBsats) and Gram-positive bacteria (TBsats) significantly decreased for each treatment over time, whereas the concentration of Gram-negative bacteria (Monos) increased over time. Actinomycetes possess a filamentous structure and a high metabolic activity (Potts 1994). According to Waldrop et al. (2000), Gram-positive bacteria grow slowly and can metabolise a wider range of substrates than Gram-negative bacteria. Gram-positive bacteria are considered to be K-strategists. K-strategists rely upon physiological adaptations to the environment for survival. They tend to grow slowly, endure nutrient deficiencies and represent an established

microbial community (Atlas and Bartha 1998). Gram-negative bacteria are considered to be r-strategists. r-Strategists have a high reproductive rate, grow at low population densities and represent the first colonisers of a habitat (Atlas and Bartha 1998). Hence, Gram-positive bacteria tolerate environmental stress better than Gram-negative bacteria.

Based on the increased concentration of Gram-negative bacteria, it is evident that the microbial communities in all the treatments shifted towards a more active community characterised by r-strategist ecology. This might be due to the incorporation of additional carbon sources (green manure or oats) into the soil environment. However, the rise in TBsats (indicative of Gram-positive bacteria) at the end of the experiment might signify the shift of the microbial communities to K-strategist ecology, as the communities adapted to the modified environment. In view of the fact that the composition showed high concentrations of Gram-positive bacteria on day 0 and 21, it could be hypothesised that the soil microbial communities displayed resilience to return to their original composition after the disturbance. Results from the CLPPs also showed signs of resilience. The higher fungal concentration in treatments SCI and SI may be attributed to the addition of *S. sclerotiorum* inoculum into the soil.

It can be concluded that the addition of an additional carbon source stimulated the microbial growth, without leading to permanent shifts in the microbial composition. The soil microbial communities showed signs of resilience to return to their original structure after the disturbances. Inoculation of the soil with *S. sclerotiorum* enhanced the fungal concentration in the soil.

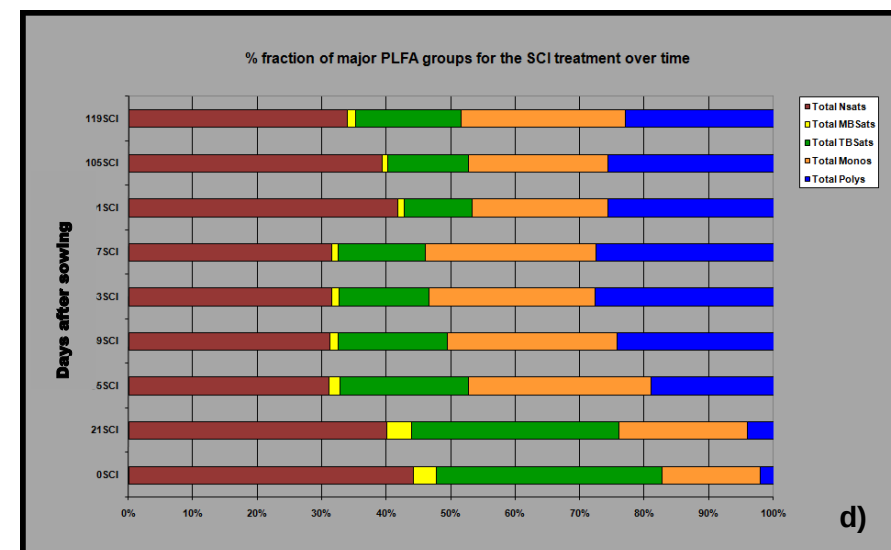
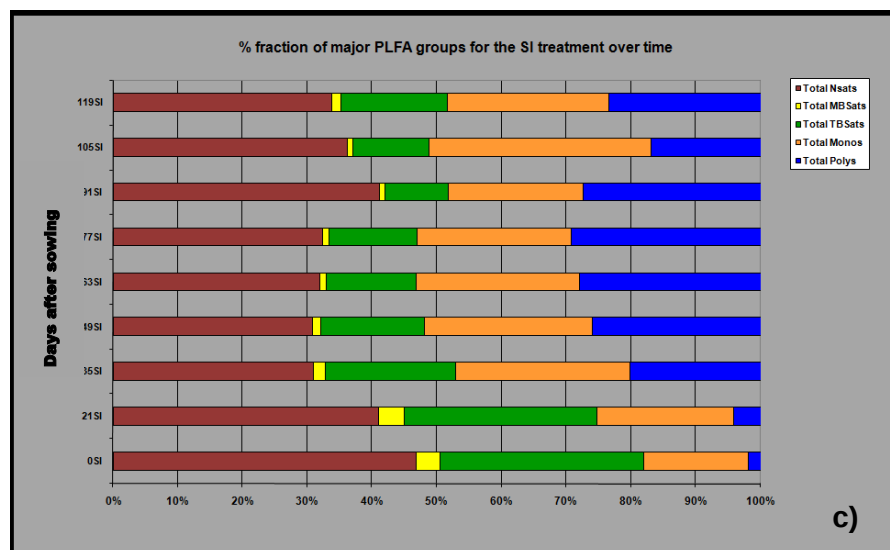
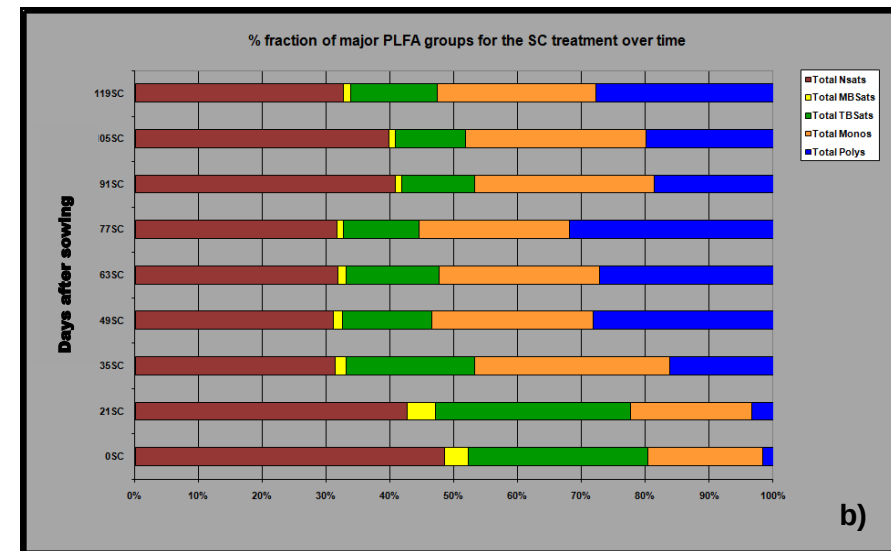
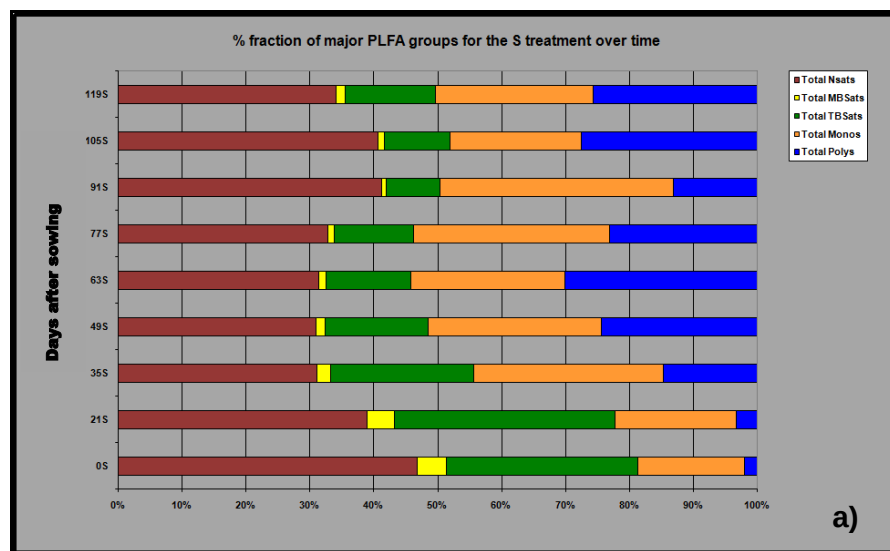


Figure 4.5: Microbial community structure based on the mol% fraction of the major phospholipid fatty acid groups of treatments a) S, b) SC, c) SI and d) SCI for the duration of the experiment.

By dividing the mole% of the fungal fatty acid marker 18:2 ω 6, 9 by the sum of the mole% of the bacterial fatty acid markers (a15:0, i15:0, 15:0, i16:0, 16:1 ω 7c, 16:1 ω 9t, i17:0, a17:0, 17:0, cy17:0, 18:1 ω 7c and cy19:0), a fungal to bacterial (F: B) biomass ratio can be determined. Throughout the experiment, significant increases in the F: B ratio could be observed for all the treatments over time (Tables 4.5 to 4.8 and Figure 4.6). No significant differences were found among the different treatments at each sampling period.

The F: B biomass ratio can be used as a biomarker for soil quality (Frostegård et al. 1996). An increase in the F: B ratio indicates the rise in fungal concentrations in the soil microbial community (Frostegård and Bååth 1996). Fungi decompose cellulose, which has high carbon content and thus a high C: N ratio. Fungal cell walls consist of chitin and melanin that require high carbon amounts. Therefore, cellulose is an ideal food source for fungi. Bacteria need more nitrogen for growth (Wichern and Hafeel 2004). Hendrix et al. (1986) stated that fluctuations in the F: B ratios are linked to soil processes such as nutrient cycling, decomposition and carbon sequestration. The added *S. sclerotiorum* inoculum could lead to elevated F: B ratios, as the fungal component increases. The changes observed in the treatments over time could be as a result of the degradation of the added oats at the beginning of the experiment. This is in collaboration with the high fungal concentrations observed in the PLFA structural groups. On average, the F: B ratio of treatment SCI was lower than in treatment SI, which might indicate the inhibition of the fungal pathogen by canola biofumigation.

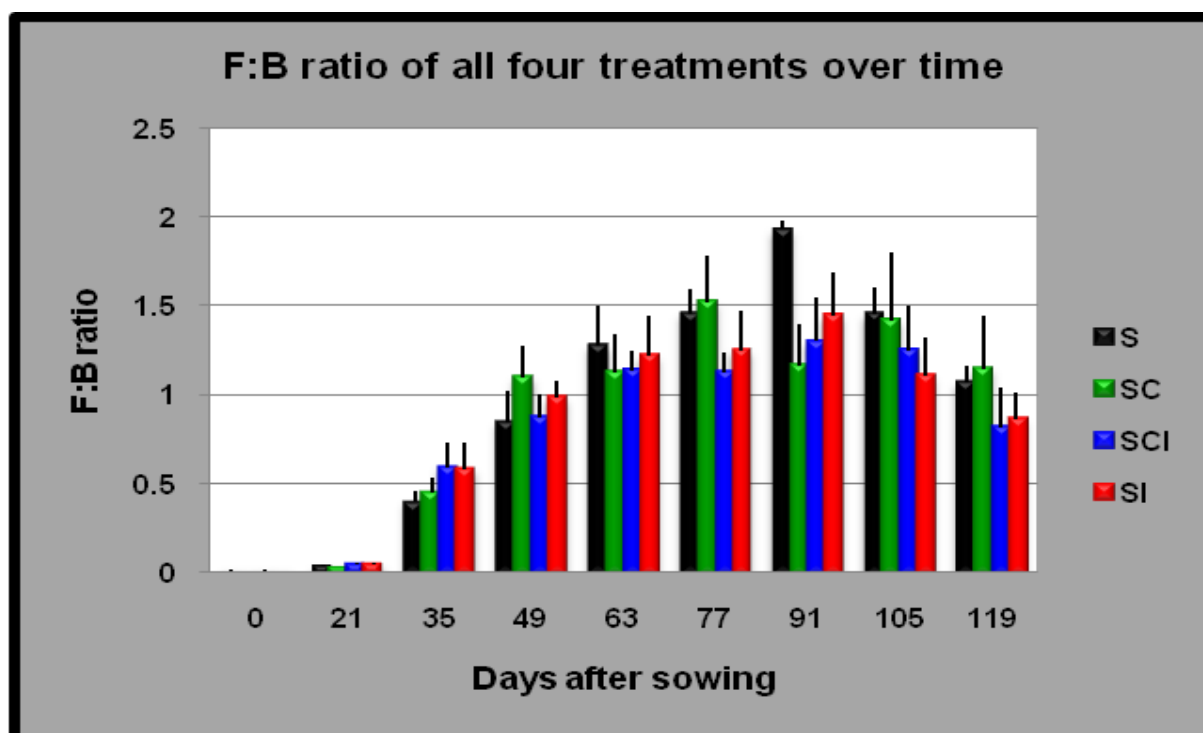


Figure 4.6: Fungal to bacterial ratio of the different treatments at the various sampling periods after sowing the sunflowers. Key to abbreviations: F: B – fungal: bacterial.

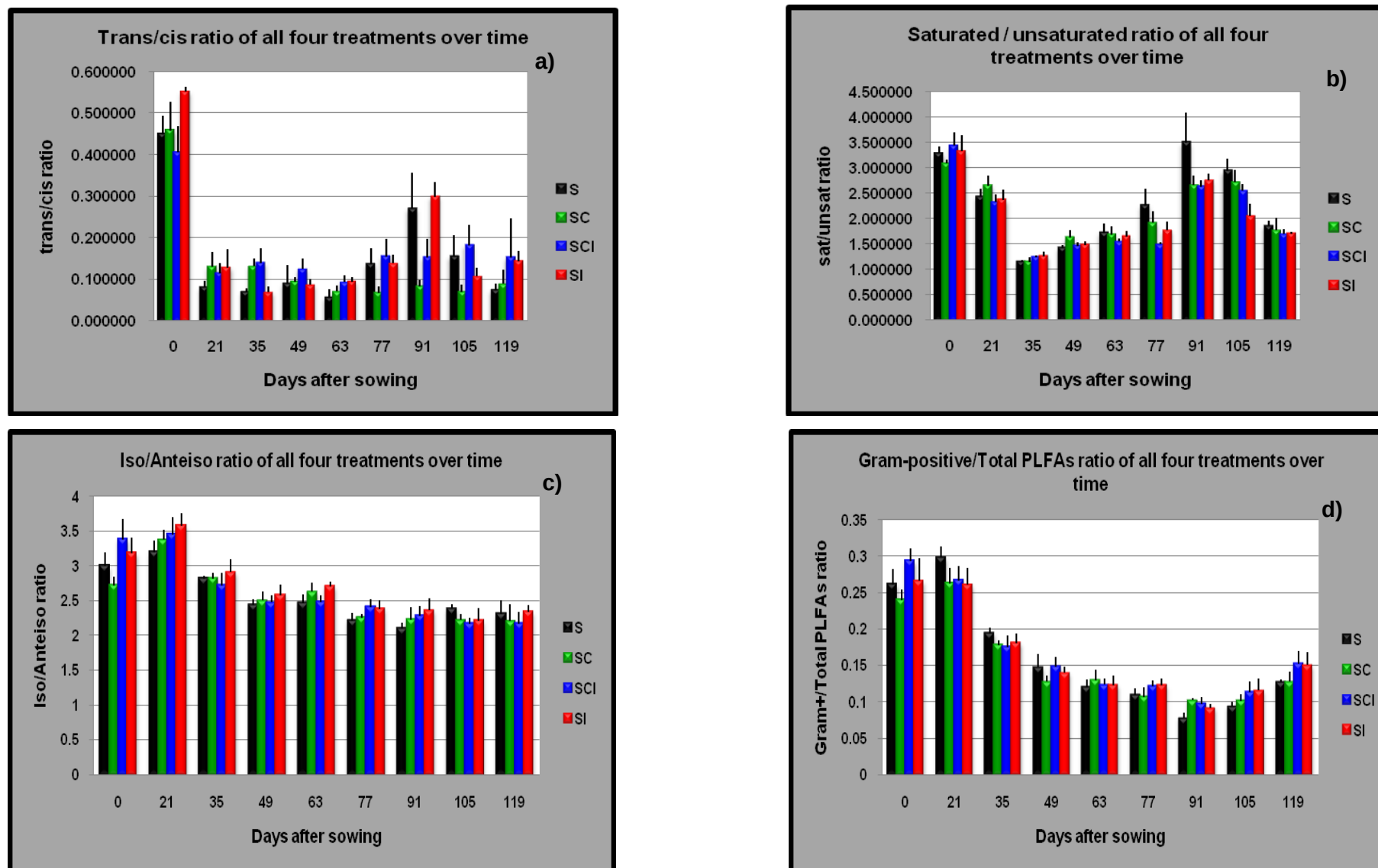


Figure 4.7: PLFA ratios of the different treatments at the various sampling periods after sowing which represents a) the *trans*: *cis* ratio; b) the saturated: unsaturated ratio; c) iso: anteiso ratio and d) Gram-positive: Total PLFAs ratio of the PLFA patterns. Key to abbreviations: sat: unsat – saturated: unsaturated.

The *trans/cis* ratio of the monounsaturated PLFAs 16:1 ω 7 and 18:1 ω 7 can indicate stress conditions such as anaerobic conditions, nutrient limitations and temperature fluctuations (Fang et al. 2006; Guckert et al. 1986). A *trans/cis* ratio higher than 0.1 indicates starvation. For healthy, non-stressed communities, this value is lower than 0.05 (Heipieper et al. 1992). All of the treatments had very high *trans/cis* ratios on the day of sowing the sunflowers (Figure 4.7a) and these values were not significantly different from each other (Tables 4.5 to 4.8). Lower *trans/cis* ratios were found for all four treatments from day 21 to day 63, with a few significant differences over time. From day 77 onwards, the ratio increased for all the treatments, signifying a higher stress level, except for treatment SC.

The modification of the volatile *cis*-monoenoic fatty acids to their cyclic-derivatives and the formation of *trans*-monoenoic PLFAs help bacteria maintain membrane functionality during nutrient deficiencies (Kieft et al. 1994). The soil microbial communities might have experienced stress conditions on day 0, due to the disturbance of the soil environment as the amendments were applied and the soil prepared for the experimental layout. The lower *trans/cis* ratios during days 21 and 63 could be explained by the stimulated growth rate of the microbes as they utilise the carbon sources in the soil. From day 77 onwards, the *trans/cis* ratio of treatment SC remained < 0.1, thus illustrating that the incorporation of canola green manure into the soil can contribute to the stability of the microbial communities under modified conditions (Kieft et al. 1994).

In Figure 4.7b and in the Tables 4.5 to 4.8 the saturated: monounsaturated PLFA ratio indicated a significantly lower nutrient stress level during days 35 and 77, with a significant rise in the ratio between days 91 and 105 for all the treatments. The lower stress level during days 35 and 77 is in correlation with the low *trans/cis* ratio, confirming the stimulated microbial growth. It seems as if the microorganisms experienced stress during the flowering stage of the sunflowers ($\pm$ 77 – 105 days after sowing the sunflowers), as could also be observed from the fluctuations occurring in the PLFA structural groups. This substantiates the changing effect plant growth cycles, due to varying root secretions, has on the soil microbial communities (Xu et al. 2009).

An increase in the iso to anteiso PLFAs is associated with nutritional stress or changes occurring in the microbial community (McKinley et al. 2005). Although no significant differences could be found among the treatments at the various sampling periods (Figure 4.7c and Tables 4.5 to 4.8), the ratios significantly declined for each treatment over time. This implies lower nutritional stress experienced by the microbes. Therefore, the stress observed from the other ratios are not caused by nutrient deprivation, but may possibly be caused by the allelopathic potential of the sunflowers (Khanh et al. 2005). Phenolic compounds and terpenoids such as heliaspirones, heliannuols and helibisabonols are present as allelochemicals (Macias et al. 2003) in these plants. When these phenolics are

present in the soil, they lead to a high inhibitory potential (Khaliq et al. 2011).

In this study, the ratio of Gram-positive to total PLFAs is estimated with the sum of the biomarkers i15:0, a15:0, i16:0, 10me16:0 and 17:0 against the total PLFAs (McKinley et al. 2005). No significant differences were observed among the different treatments at each sampling period (Figure 4.7d and Tables 4.5 to 4.8). The ratio significantly decreased for all the treatments over time from day 35 onwards (lower Gram-positive concentrations) with an increase in the ratio (higher Gram-positive concentrations) on day 119. As already stated, Gram-positive bacteria are considered to be K-strategists. They grow slowly and can metabolise a wide range of substrates. They are thought to be representative of a more established microbial community (Atlas and Bartha 1998). The soil microbial community initially showed signs of K-strategist ecology, as the ratio of Gram-positive to total PLFAs were high on day 0. A decrease in this ratio from day 35 onwards might indicate other members of the community becoming active as the soil amendments were degraded. This could be related to the diverse substrate utilisation patterns found. At the end of the experiment the increased ratio represents the stimulation of Gram-positive bacteria, indicating the acclimatisation to the changed environment leading to a higher stability and a return to K-strategist dominance. Correlations could be made with the PLFA group results, as well as with the similar compositions observed in the CLPPs on day 119. Again it is clear that the soil amendments did not permanently modify the soil microbial communities, they were able to return to similar compositions after the disturbance due to a high resilience.

An RDA was conducted on the dehydrogenase activity, Biolog® Ecoplate carbon substrate group utilisation data and PLFAs to illustrate the correlation between the functional diversity or activity and community structure, of the different treatments over time (Figure 4.8). Due to the overcrowded display of the main plot (not shown), subplots were drawn comparing the relationship of each treatment over time with the control. This implies that all the subplots possess the same eigenvalues, seeing as they are only visual variations selected from the main multivariate ordination diagram.

The eigenvalues for the first two ordination axes were 0.087 and 0.057, respectively. These two axes accounted for 78.7% of the total observed variance. The species-environment relation was explained by the cumulative percentage variance for the four axes of 52.5, 87.1, 95.1 and 98.3%, respectively. The first axis correlated strongly with biomass ($r^2=0.4254$), whereas the second axis correlated with Monos ($r^2=0.6639$) and Polys ($r^2=-0.5644$). The effect of the environmental variables on the first canonical axis was not significant ($p=0.1140$), whereas the overall effect was significant ($p=0.01$).

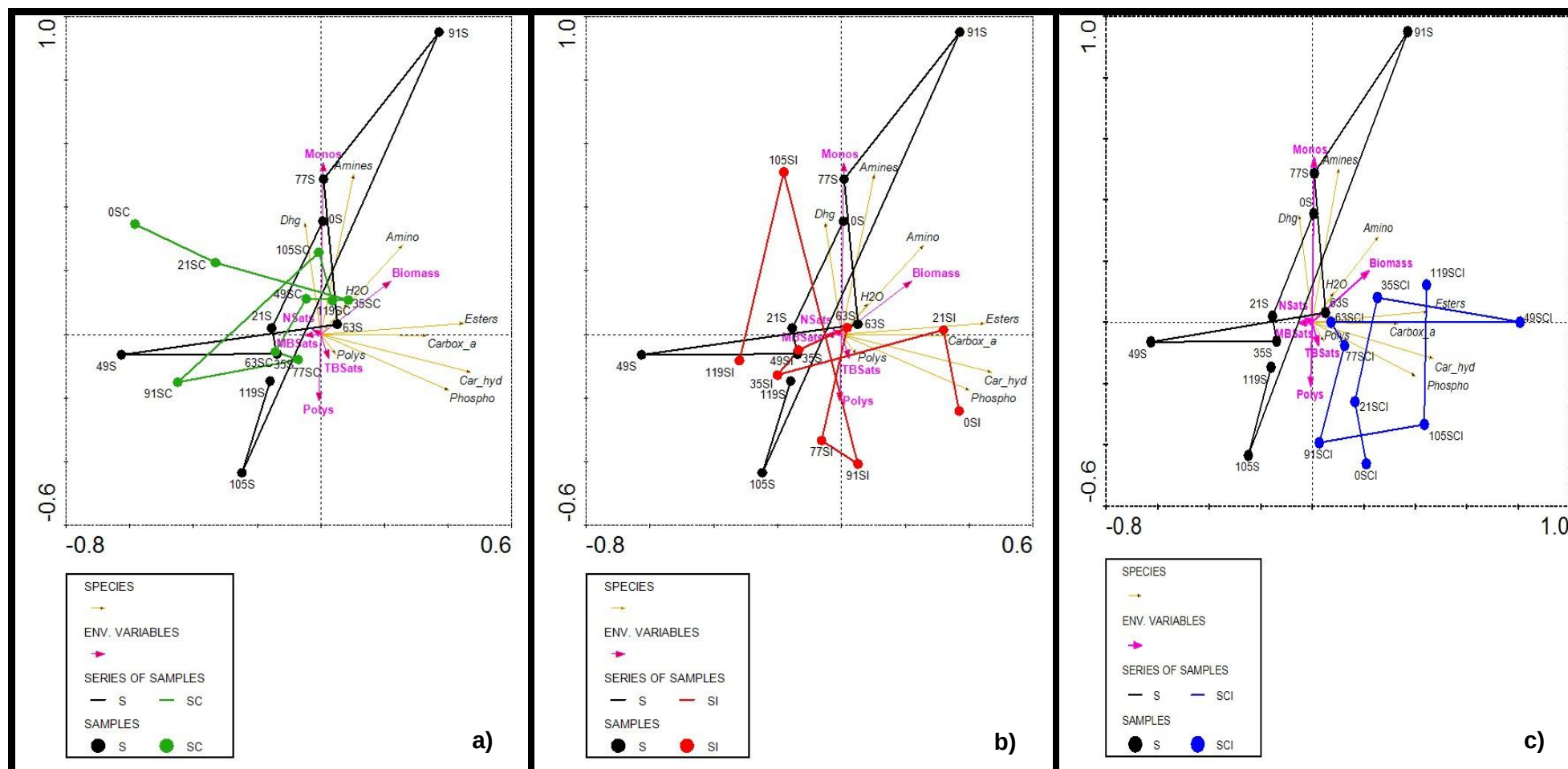


Figure 4.8: Subplots of a Redundancy Analysis (RDA) ordination diagram illustrating the relationship between the dehydrogenase activity, carbon substrate group utilisation and major phospholipid fatty acid groups of the different treatments over time. Subplots display treatments a) S and SC, b) S and SI, c) S and SCI. Each sampling period is indicated by the amount of days after sowing the sunflowers followed by the name of the treatment. The eigenvalues for the first two ordination axes were 0.087 and 0.057, respectively. These two axes accounted for 87.1% of the total observed variance for the species and environmental variables. The substrates were classified as: Amines; H₂O – water; Amino – amino acids; Polys – polymers; Carbox_a – carboxylic acids; Esters; Car_hyd – carbohydrates; Phospho – phosphorylated compounds. Key to abbreviations: Dhg – dehydrogenase; NSats – normal saturated; MBSats – mid-branched saturated; TBSats – terminally branched saturated; Monos – branched monounsaturated; Polys – polyunsaturated.

The RDA ordination diagrams (Figure 4.8a to 4.8c) illustrated a high association between the functional diversity and community structure of the soil microbial communities as the total observed variance explained 87.1% of these correlations. This implied that as the microbial composition changed, the functional diversity varied accordingly. A clear distinction could be made among the specific treatments. All four treatments showed varying fluctuations over time (Figures 4.8a to 4.8c). Treatment SCI displayed the least variance (Figure 4.8c), as the clustering remained mostly in the second quadrant, which is in accordance with the gradual change in microbial community structure (Figure 4.5d). This might be due to the possible inhibition of the inoculum by the canola green manure. The soil physico-chemical results (Table 4.1) and substrate utilisation patterns (Figure 4.2c) also indicated a possible inhibition occurring. It is evident that in all four treatments a drastic change occurred during days 91 to 105 occurred, which might be as a result of the changing root exudates secreted during the plant flowering stages (Xu et al. 2009). This was also observed in the previous results (Figure 4.1, 4.2, 4.4 and Tables 4.3, 4.4). Treatments SI (Figure 4.8b) and SCI (Figure 4.8c) showed higher correlations with Polys on days 77 and 91 than the control S and treatment SC, which may be ascribed to the addition of *S. sclerotiorum* to the soil. From the RDA, it seems as if Gram-negative bacteria, represented by Monos, utilises amines, whereas Gram-positive bacteria (indicated by TBsats) and fungi (Polys) correlated with the polymers, phosphorylated compounds and carbohydrates. Other investigations also found changes in the substrate utilisation as a result of structural changes (Dong et al. 2008; Grayston et al. 2004).

It may be concluded that the treatment of the soil with canola green manure or *S. sclerotiorum* led to changes in the soil microbial community structure, which resulted in functional variations. However, these changes did not have a long-lasting effect as similar compositions could be observed at the end of the experiment.

4.5. Chlorophyll a fluorescence measurements

The chlorophyll a fluorescence measurements can provide insight into the efficiency of PS II and the functionality of the photosynthetic apparatus. During this study, chlorophyll a fluorescence measurements were conducted weekly from day 35 (after sowing) onwards. Only the data collected every three weeks were displayed, unless stated otherwise. From various data sets gathered during these measurements, a performance index (PI_{Total}) of the sunflowers was estimated. PI_{Total} is related to the generation of photosynthetic metabolites and is therefore an indication of the overall photosynthetic potential, vitality, growth and stress responses of the plant (Strasser and Tsimilli-Michael 2001). This PI_{Total} is a sensitive parameter of the JIP test, as it consists of four quantum efficiencies (Scheepers 2011; Strasser and Tsimilli-Michael 2001; Yusuf et al. 2010):

- $\gamma RC/(1-\gamma RC) = (RC/ABS)$ gives the chlorophyll concentration per reaction centre as light energy is absorbed per reaction centre;
- $\phi P_0/(1-\phi P_0) = TR_0/ABS$ indicates the trapping quantum efficiency;
- $\psi E_0/(1-\psi E_0) = ET_0/(TR_0-ET_0)$ provides the conversion efficiency of the excited energy to electron transport and;
- $\delta R_0/(1-\delta R_0) = RE_0/ABS_0$ refers to the involvement of the reduced end electron acceptors in transference.

Thus, several changes occurring in the OJIP kinetics would be conveyed in the PI_{Total} .

The PI_{Total} of the sunflowers of each treatment, for the 119 days after sowing the sunflowers, is represented in Figure 4.9.

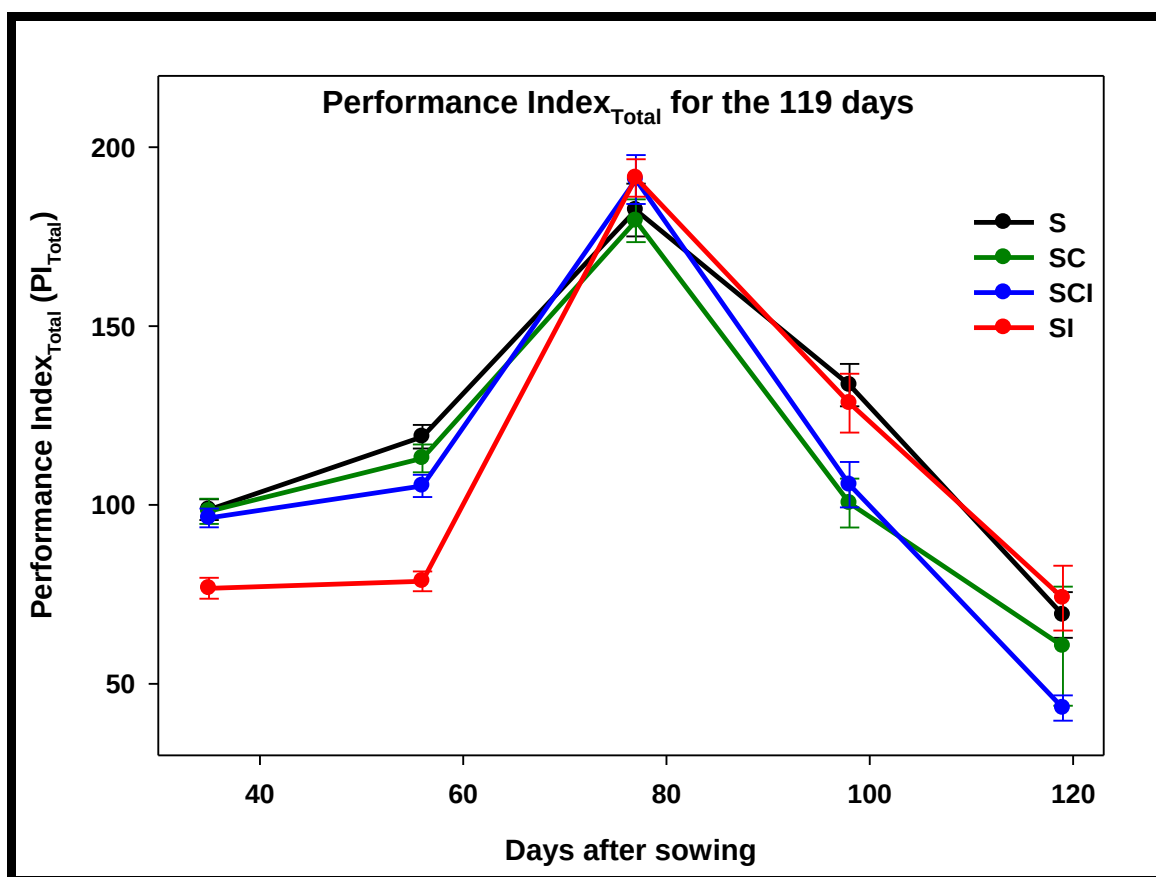


Figure 4.9: Performance index total (PI_{Total}) for the duration of the experiment. Only the data points of every three weeks were displayed. All values represent results obtained from 48 replicates.

From Figure 4.9 it is evident that the sunflowers showed normal growth and development trends for the duration of the experiment, as an increased photosynthetic efficiency was observed during the active vegetative stages (days 35 to 77) and a lower efficiency after

flowering (from days 77 onwards) (Schneider and Miller 1981). As the PI_{Total} increased, higher energy conservation was expressed by the plants (Yusuf et al. 2010). This could be seen from days 35 to 77, during which the sunflowers experienced active growth. The highest PI_{Total} was measured on day 77 after sowing, which may be associated with the high plant vitality or photosynthetic capacity of the sunflowers before flowering stage. Thereafter, the plants showed a decline in energy conservation (PI_{Total}) from days 84 until 119, as the plants physiologically matured and seed development reduced photosynthesis. Treatment SI showed significantly lower PI_{Total} levels from day 35 compared to the control (treatment S), but the PI_{Total} increased from day 77 onwards. This implied that the inoculation of the soil with *S. sclerotiorum* only had a suppressive effect on the sunflowers during the vegetative growth stage. The incorporation of canola green manure showed no enhanced effect on the performance index of the sunflowers, with similar trends to treatment SCI. Changes observed in the PI_{Total} may be linked to the decrease in the reduction of the end electron acceptors at the PS I electron acceptor side, expressed as $[\delta R_0/(1-\delta R_0)] = RE_0/ABS_0$ (Strasser et al. 2010). This quantum efficiency is illustrated in Figure 4.10.

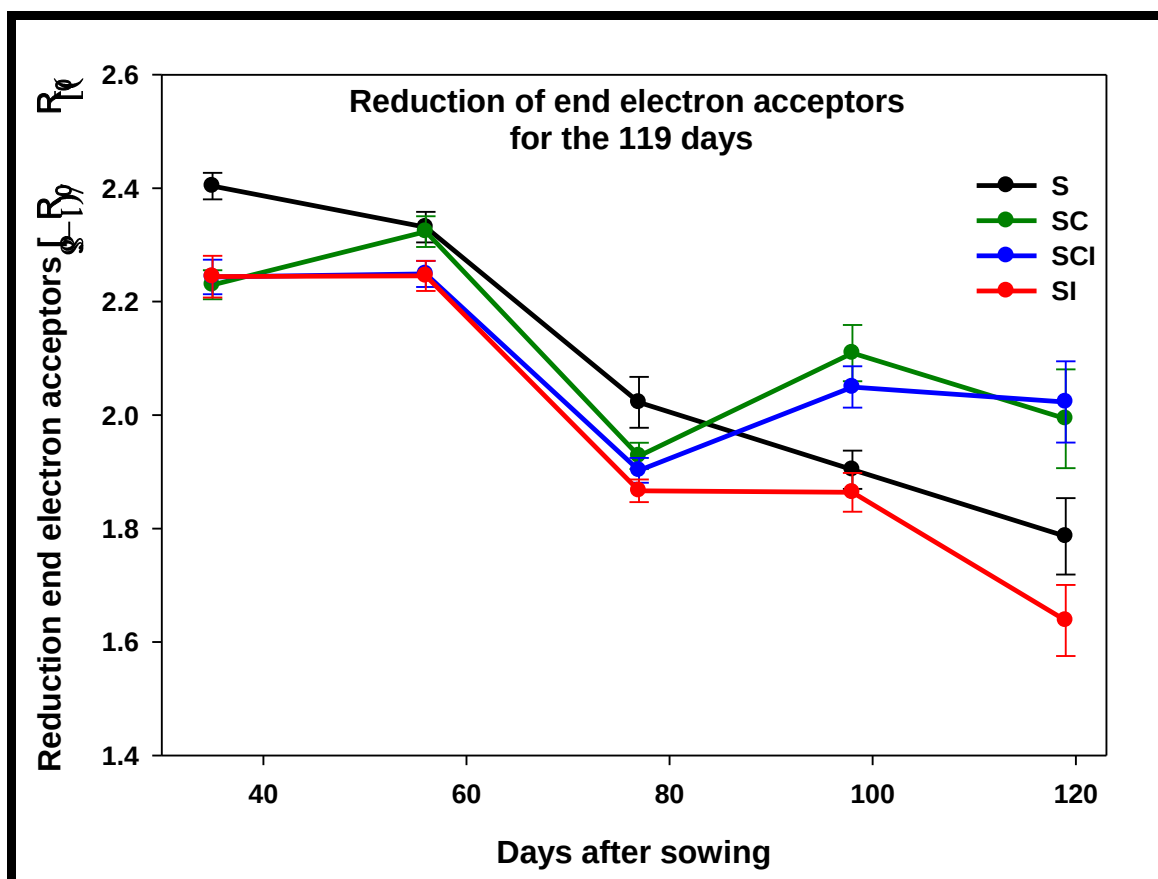


Figure 4.10: Reduction of end electron acceptors at the electron acceptor side of PS I over the 119 days. Only the data points of every three weeks were displayed. All values represent results obtained from 48 replicates.

Of the four phases (O, J, I, P) exhibited in a fluorescence curve (see Figure 2.1 in Literature Review), the I-P phase (3 – 300 ms) is associated with the reduction of end electron acceptors, such as ferredoxin and NADP^+ , at the electron acceptor side of PS I (Chen et al. 2011). Due to the natural ageing process of the plants, the enzyme ferredoxin- NADP^+ -reductase was inactivated and the reduction of end electron acceptors decreased. This led to the accumulation of electrons at the acceptor side of PS I, which caused structural and functional damage as well as decreased plant vitality (Schansker et al. 2005). It is clear that treatment SI had a significantly lower rate of reduction of end electron acceptors throughout the study, compared to the control treatment S (Figure 4.10) and the treatments SC and SCI. Thus, the inoculum might have had a negative effect on the electron transfer of PS I.

Treatment SC showed a higher rate of reduction than the treatments SCI and SI, but was still lower than the control for days 35 to 84. From day 84 onwards, the two treatments SC and SCI illustrated higher reduction rates than the control. This might be ascribed to the addition of canola to the soil that enhanced soil organic nutrients and decreased the effect of the inoculum on the plants.

The quantum efficiency $\psi E_0/(1-\psi E_0) = ET_0/(TR_0-ET_0)$, that describes the potential of an electron to be transferred further than the quinone electron acceptor of PS II (Q_A) into the electron transport chain (Yusuf et al. 2010) forms part of the PI_{Total} and showed alterations over the 119 days. Figure 4.11 illustrated the abovementioned quantum efficiency of each treatment as a percentage value relative to the control S.

It is evident that treatments SC and SCI showed slightly higher efficiencies on days 35 and 56, whereas SI had similar or lower values compared to the control. Higher quantum yields lead to the stimulation of the electron transport reaction, whereas a lower $\psi E_0/(1-\psi E_0) = ET_0/(TR_0-ET_0)$ value could be linked to the reduced rate of the Q_A reduction (Bussotti et al. 2011). This implied that the *S. sclerotiorum* inoculation of the SI soil had a suppressive effect on the sunflowers in their early active growth phase, as could also be seen from the PI_{Total} (Figure 4.9). On day 77 it was found that all three treatments had significantly higher values than the control S. This could be directly related to the high PI_{Total} , since the performance index is in linear correlation with the electron transport parameters (Chen et al. 2011). For treatment SI the efficiency remained significantly higher than the control for the last sampling periods, but SC and SCI displayed a drastic decline in quantum yield. This decline for treatments SC and SCI might be associated with the blockage of the electron flow further than Q_A^- into the electron transport chain, as a natural ageing process (Chen et al. 2011).

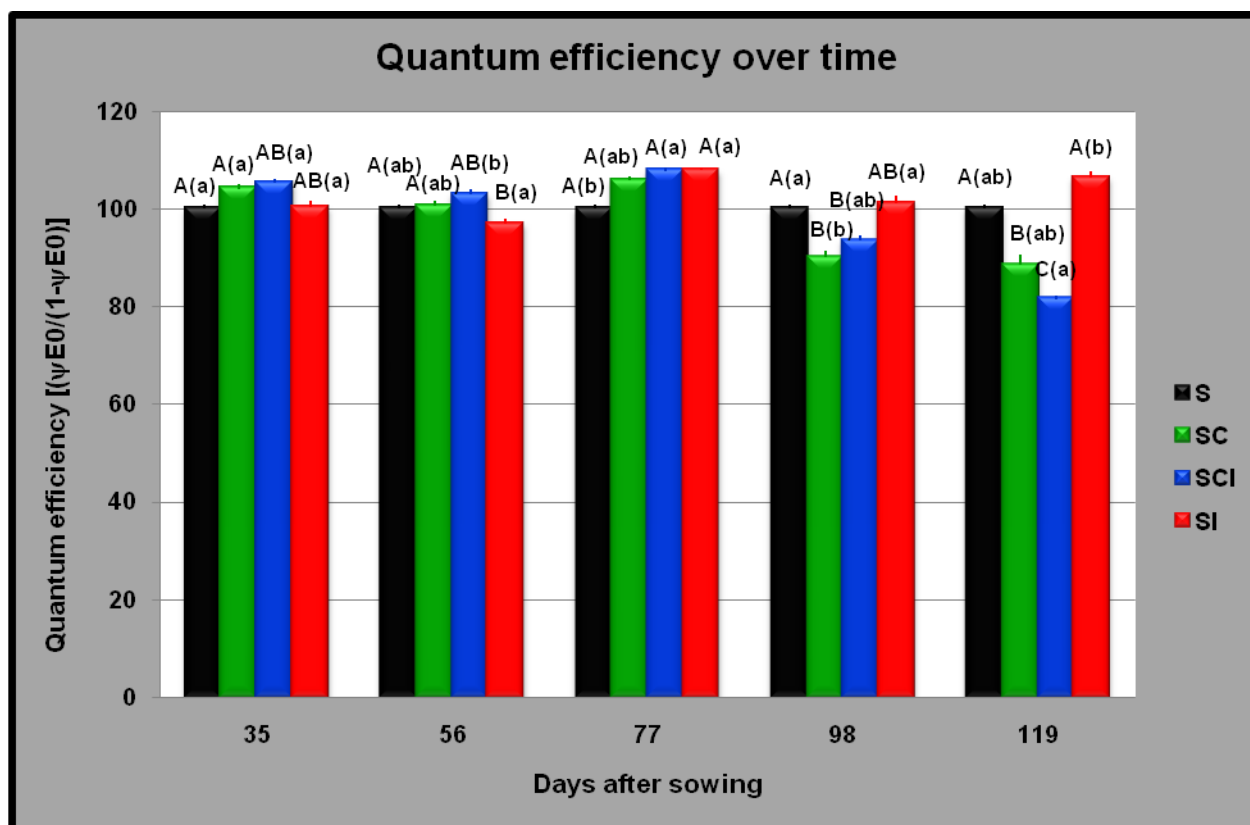


Figure 4.11: The quantum yield or biochemical efficiency $\psi E_0/(1-\psi E_0) = ET_0/(TR_0-ET_0)$, that describes the potential of an electron to be transferred further than the quinone electron acceptor of PS II (Q_A) into the electron transport chain, for each treatment expressed as a percentage of the control S. Data points of every three weeks were displayed. Capital letters indicate statistically significant differences for each treatment over time ($p < 0.05$), whereas the lowercase letters in brackets indicate significant differences between the treatments at that specific time.

Multiparametric radar plots were plotted to illustrate the OJIP parameters based on the structural and functional performances of the sunflower plants. All the parameter values were normalised by using the control treatment S as the reference, therefore S values represent 1.0.

Treatments SC and SCI showed similar trends to the control S on day 35. The results for 35 days after sowing depicted in Figure 4.12 indicated a drastic decrease in the PI_{Total} and PI_{ABS} of treatment SI compared to the control S and treatments SC and SCI. This can be ascribed to the slightly lower maximum quantum yield for primary photochemistry ($\phi P_0 = TR_0/ABS$), efficiency with which an electron can be moved into the electron transport chain further than Q_A^- by a trapped exciton ($\psi E_0 = ET_0/TR_0$), decreased reduction of end electron acceptors by intersystem electron carriers ($RE/ET = \delta_{R_0}$) and efficiency with which an electron is transferred to reduce acceptors at the PS I acceptor side ($\phi R_0 = RE/ABS$). The electron transport chain is blocked further than Q_A , which resulted in the accumulation of Q_A^- and the closure of RCs of PS II (Chen et al. 2011). It might be possible that the pathogen *S. sclerotiorum* secreted compounds hampering the activity of the Q_B site, leading to electron

transport inhibition. The decrease in primary photochemistry may be associated with the changes in k_p and k_n values as alterations occurred in the absorbance and trapping of photons by reaction centres (Yusuf et al. 2010). The changes in the absorbance and trapping could be observed in the increased parameters ABS/RC, TR_0 /RC, ABS/CS and TR/CS. These parameters signified a decrease in the amount of active RCs due to inactivation, which led to the excessive absorption and trapping of photons without sufficient use thereafter (Yusuf et al. 2010).

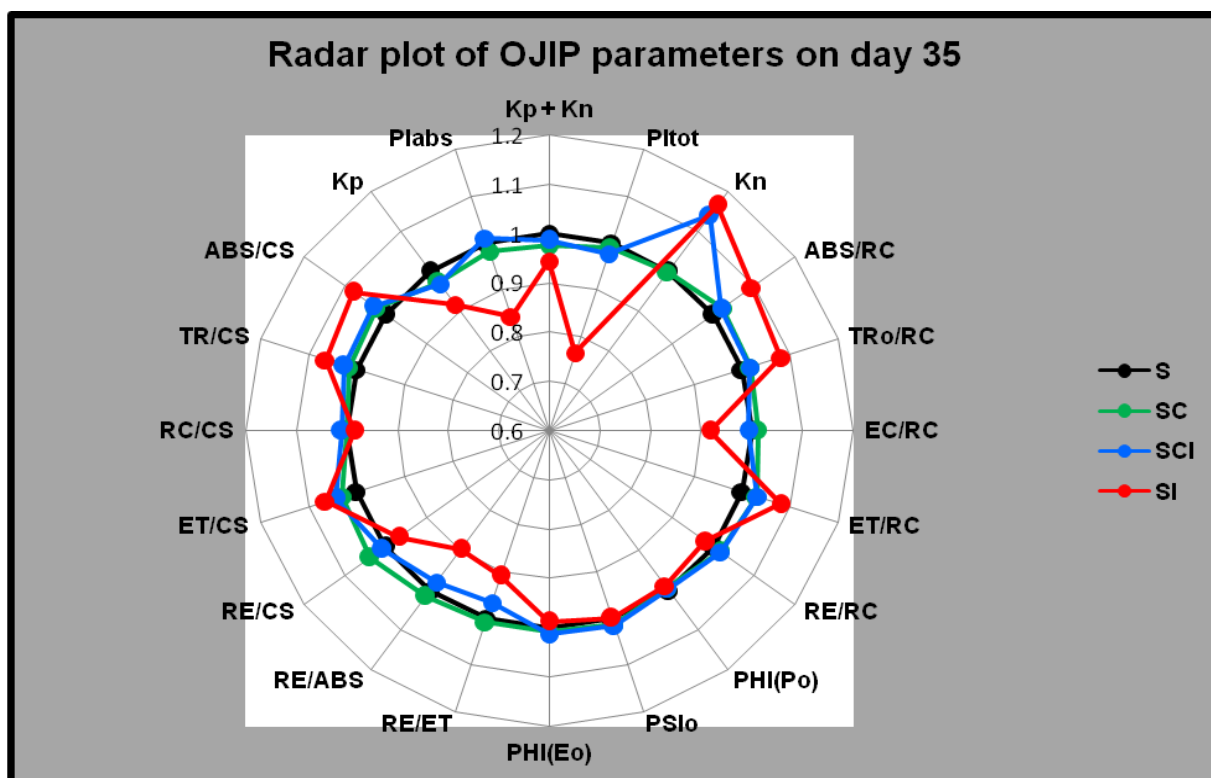


Figure 4.12: OJIP parameters illustrating the functional and structural performances of the sunflower plants on day 35 after sowing the sunflowers. All the parameter values were expressed relative to the control treatment S. The parameters displayed include: k_p – photochemical de-excitation rate constant; PI_{ABS} – performance index for energy conservation from absorbed photons to the reduction of intersystem electron carriers; k_p+k_n – total de-excitation rate; PI_{Total} – performance index for energy conservation from absorbed photons to the reduction of end PS I electron carriers; k_n – nonphotochemical de-excitation rate constant; specific energy fluxes at the reaction centres for absorption (ABS/RC), trapping (TR_0 /RC), electron carriers (EC/RC), electron transport (ET/RC), reduction of end electron acceptors (RE/RC); flux ratios or yields include the maximum quantum yield for primary photochemistry ($\phi P_0 = TR_0/ABS$), the efficiency with which an electron can be moved into the electron transport chain further than Q_A^- by a trapped exciton ($\psi E_0 = ET_0/TR_0$), the quantum yield for electron transport ($\phi E_0 = ET_0/ABS$), the efficiency of intersystem electron carriers to reduce end electron acceptors ($RE/ET = \delta_{R_0}$) and the efficiency with which an electron is transferred to reduce acceptors at the PS I acceptor side (RE) ($\phi R_0 = RE/ABS$); phenomenological energy fluxes at each cross section (CS) for absorption (ABS/CS), trapping (TR/CS), electron transport (ET/CS) and reduction of end electron acceptors (RE/CS). The amount of active RCs per CS can also be calculated as RC/CS.

At the end of the study, on day 119, there were dramatic changes apparent in the treatments SC and SCI (Figure 4.13). Overall treatment SI showed similar trends to the control S,

except for the slightly higher electron carriers per reaction centre (EC/RC) parameter. The PI_{Total} and PI_{ABS} of both SC and SCI were significantly decreased. Since the PI_{Total} consists of four quantum efficiencies, this could be as a result of the decline in these flux ratios or yields such as the maximum quantum yield for primary photochemistry ($\phi P_0 = TR_0/ABS$), the efficiency with which an electron can be moved into the electron transport chain further than Q_A^- by a trapped exciton ($\psi E_0 = ET_0/TR_0$), the quantum yield for electron transport ($\phi E_0 = ET_0/ABS$) and the efficiency of intersystem electron carriers to reduce end electron acceptors ($RE/ET = \delta_{R0}$) (Yusuf et al. 2010).

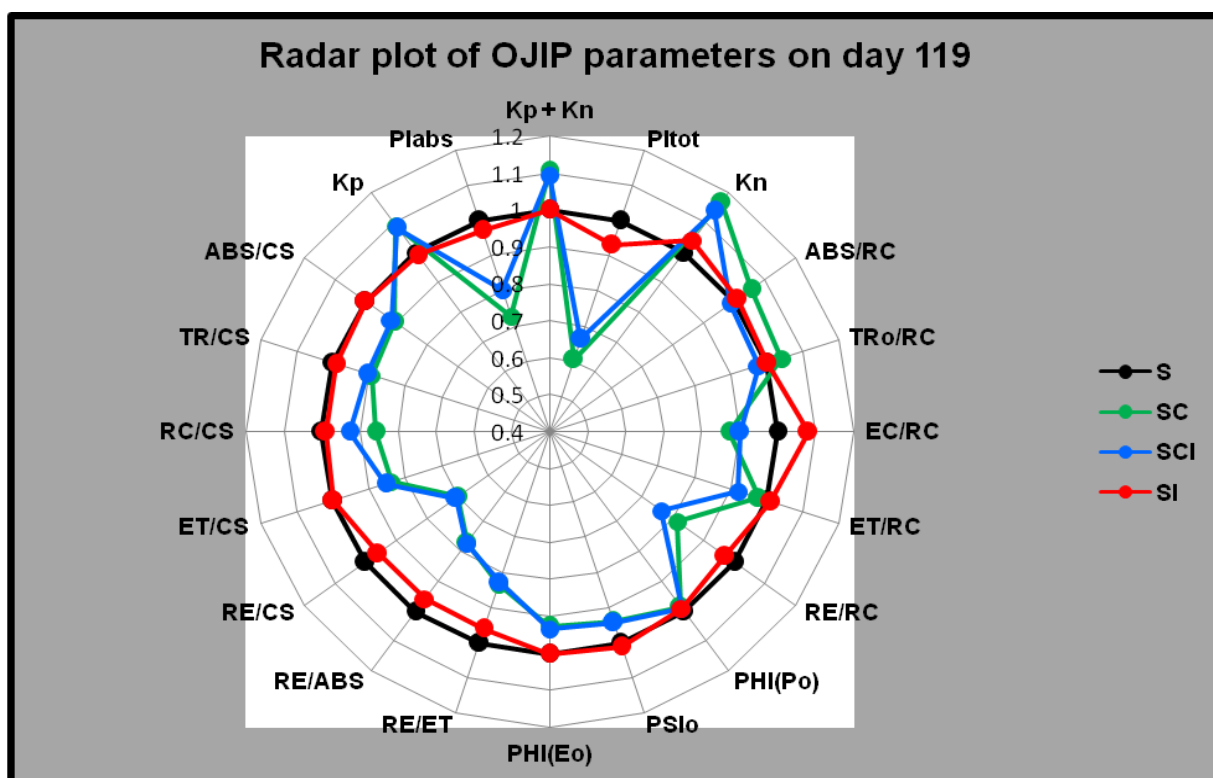


Figure 4.13: OJIP parameters illustrating the functional and structural performances of the sunflower plants on day 119 after sowing the sunflowers. All the parameter values were expressed relative to the control treatment S. The parameters displayed include: k_p – photochemical de-excitation rate constant; PI_{ABS} – performance index for energy conservation from absorbed photons to the reduction of intersystem electron carriers; k_p+k_n – total de-excitation rate; PI_{Total} – performance index for energy conservation from absorbed photons to the reduction of end PS I electron carriers; k_n – nonphotochemical de-excitation rate constant; specific energy fluxes at the reaction centres for absorption (ABS/RC), trapping (TR_0/RC), electron carriers (EC/RC), electron transport (ET/RC), reduction of end electron acceptors (RE/RC); flux ratios or yields include the maximum quantum yield for primary photochemistry ($\phi P_0 = TR_0/ABS$), the efficiency with which an electron can be moved into the electron transport chain further than Q_A^- by a trapped exciton ($\psi E_0 = ET_0/TR_0$), the quantum yield for electron transport ($\phi E_0 = ET_0/ABS$), the efficiency of intersystem electron carriers to reduce end electron acceptors ($RE/ET = \delta_{R0}$) and the efficiency with which an electron is transferred to reduce acceptors at the PS I acceptor side (RE) ($\phi R_0 = RE/ABS$); phenomenological energy fluxes at each cross section (CS) for absorption (ABS/CS), trapping (TR/CS), electron transport (ET/CS) and reduction of end electron acceptors (RE/CS). The amount of active RCs per CS can also be calculated as RC/CS.

Declines were observed for both these treatments in all the phenomenological fluxes per cross section and the specific energy fluxes at the reaction centres, except for absorbance (ABS/RC) and trapping per reaction centre (TR₀/RC). As mentioned above, these two parameters provide an indication of the number of active RCs. Therefore, an increase is associated with the transformation of RCs to non-Q_A-reducing centres or silent centres (Yusuf et al. 2010). Thus, the electron transport chain was uncoupled (seen from $\psi E_0 = ET_0/TR_0$) leading to the build up of Q_A⁻, fewer electrons transferred to the end acceptors (seen from $\phi E_0 = ET_0/ABS$) and the lower reduction of end electron acceptors to form energy (seen from $\delta_{R_0} = RE_0/ET_0$). This implied the closure of reaction centres and in the end the inhibition of plant vitality (PI_{Total}). Based on these results it is evident that the incorporation of the soil with canola green manure might have had an inhibitory effect on plant vitality after flowering.

Differences in the variable fluorescence transients of the different treatments were displayed as normalisations at various time intervals. After normalisation the different treatment values were expressed as values relative to the control S ($\Delta V = V_{\text{Treatment}} - V_{\text{Control}}$). In Figure 4.14a the overall fluorescence rise, containing the typical O, J, I and P peaks, can be observed. This rise has a similar trend to an expected fluorescence rise (Figure 2.1 in Literature Review). When normalisations of the data at F₀ (30 μ s) and F_P = F_M (300 ms) were done [$\Delta V_{OP} = (F_t - F_0)/(F_P - F_0)$] with a gain factor of 10, various curves became noticeable that were not as pronounced in the original fluorescence rise (Strasser et al. 2004).

The normalisation between steps O (30 μ s) and J (3 ms) to generate $\Delta V_{OJ} = (F_t - F_0)/(F_J - F_0)$ leads to the visualisation of the ΔV_K -band (0.3 ms), illustrated in Figure 4.14b. The ΔV_K -band is related to the functionality of the oxygen evolving complex of photosynthesis as well as the relationship between the electrons given by the donor side of PS II and the electrons leaving the RCs at the acceptor side (Oukarroum et al. 2007; Strasser 1997). The positive ΔV_K -band observed for the treatments SC, SCI and SI was a result of higher fluorescence, due to the blockage of the oxygen evolving complex which led to the accumulation of electron carriers at the RC. Consequently, an imbalance occurred between the electrons donated and electrons required for transport. Thus, the disruption of the electron transfer at the RC signified the lower potential for electron transport and the reduction of the energy carriers NADP⁺ and ferredoxin (Strasser et al. 2004). Treatment SI had the highest fluorescence, whereas SC and SCI had lower fluorescence compared to the control. It seems the incorporation of canola green manure into the soil had a negative effect on the functionality of the oxygen evolving complex, but prevented total reduction of the activity of the oxygen evolving complex by the fungal pathogen (treatment SCI).

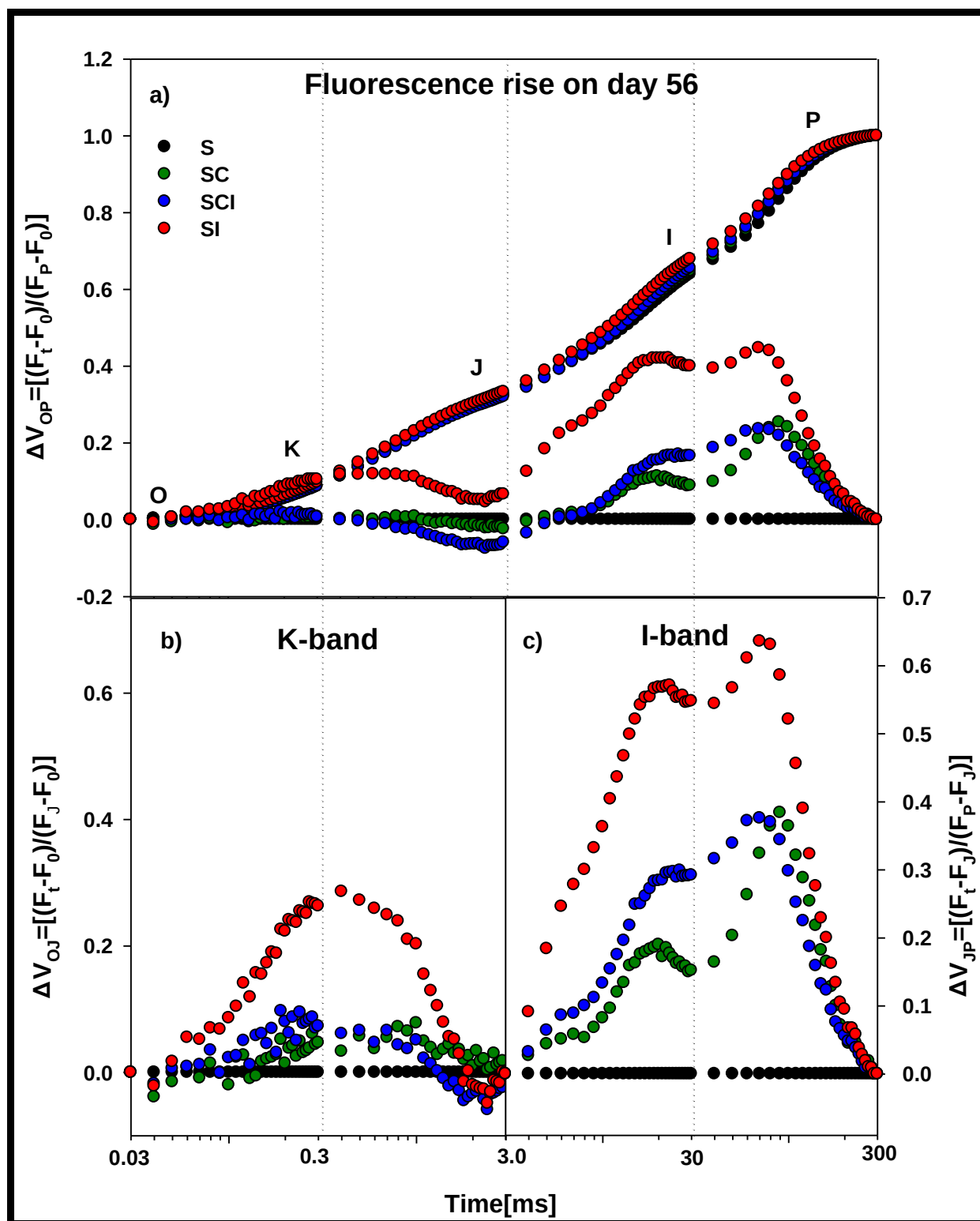


Figure 4.14: Average fast phase chlorophyll a fluorescence measured on day 56 after sowing the sunflowers was expressed relative to the control, therefore $\Delta V = V_{\text{Treatment}} - V_{\text{Control}}$. The normalisations were done between a) F_0 (30 μ s) and F_P (300 ms) indicating $\Delta V_{OP} = (F_t - F_0)/(F_P - F_0)$; b) F_0 and F_J (3 ms) indicating $\Delta V_{OJ} = (F_t - F_0)/(F_J - F_0)$, also known as the K-band; c) F_J and F_P indicating $\Delta V_{JP} = (F_t - F_J)/(F_P - F_J)$, also known as the I-band (Strasser et al. 2004).

When the data was normalised between steps J (3 ms) and P (peak 300 ms) to generate $\Delta V_{JP} = (F_t - F_J)/(F_P - F_J)$, a ΔV_I -band became visible as displayed in Figure 4.14c. The positively increased ΔV_I -band of all the treatments compared to the control, revealed elevated fluorescence as the enzyme ferredoxin-NADP⁺-reductase was inactive and therefore no reduction of the end electron acceptors took place at the electron acceptor side of PS I (Schansker et al. 2005). From the results it is evident that the addition of canola green manure in treatment SCI ensured a lower fluorescence yield than for treatment SI, which received no green manure amendments.

Overall the fluorescence parameters indicated a suppressive effect by the fungal pathogen *S. sclerotiorum* on the photosynthetic efficiency of the sunflower plants. However, canola amendments showed possible inhibitory effects on the influence of the fungal pathogen on the plant photosynthetic potential. The addition of canola green manure into the soil had a slight negative impact on the plant vitality of the sunflowers at the end of the study, due to the uncoupling of the electron transport chain and the lower reduction of end electron acceptors. Therefore, the degradation products (for example isothiocyanates) of the canola green manures might have been harmful to the sunflowers during their seed development stage (after flowering).

Conclusions and Recommendations

5.1. General conclusions

In this research study, the biofumigation effect of Canola (*Brassica napus*) as a green manure on the soil microbial community function and structure was assessed. A greenhouse experiment was conducted for 120 days to measure the influence of the canola on the soil microbial community after sowing sunflowers. Seeing as *Sclerotinia sclerotiorum* is a sunflower pathogen without a successful control agent, it was used to inoculate certain treatments to investigate the possible inhibitory effect of the canola green manure on the pathogen. For the microbiological analyses, statistically significant differences were observed among the different treatments and for each treatment over time.

From the soil physical and chemical characteristics, it is apparent that the possible inhibition of the *S. sclerotiorum* inoculum by the canola green manure amendments had a significant effect on the soil properties (compared to the other treatments). This pathogen is known for its high P and Mg absorption from the soil environment (Liang et al. 2010). Therefore, the accumulation of these macronutrients in the soil signified the reduced growth of this pathogen. The commercial fertiliser used in this study might have contributed to the drastically elevated micro-elemental levels. These elements, namely Fe, Zn and B, can be highly toxic to plants at pH levels lower than 5.5. These micro-elements held no threat for the sunflower plants, since the soil pH of the different treatments ranged between 6 and 7. The increased Na concentrations found in the soils could be a result of the water used for irrigation, but this should be confirmed by water analysis. The increased organic C, total C and total N in the soil due to manure amendments, at the beginning of the study, did not have a long-term effect after the 119 days. This could be attributed to the gradual degradation of the added organic matter by soil microorganisms.

Results obtained from the assays of dehydrogenase activity illustrated that the addition of canola green manure might have contributed to the soil organic carbon content, which led to enhanced microbial activity. Seeing as these results are not substantiated by the soil physico-chemical data, it might have been possible that during the experiment the contents of these parameters were high, but were gradually depleted by microbial degradation reactions after 119 days. In general, even though fluctuations occurred, a gradual decrease in the dehydrogenase activity for all the treatments was observed. This might be due to changes in plant root secretions (Xu et al. 2009), the adaptation of the microbial communities to the amendments or the gradual degradation of the added

substrates (Floch et al. 2011). These amendments might have stimulated the growth of antibiotic producing bacteria that could reduce *S. sclerotiorum* outbreaks. Streptomycetes are an example of such gram-positive antibiotic producing bacterial populations in soil (Anderson and Wellington 2001). The lower microbial activity suggested competition occurring between the pathogen and the native microbial populations.

Based on the results acquired from the AWCD and Shannon-Weaver substrate diversity index, it is obvious that the microbial activity and function decreased over time for each treatment. All the treatments showed signs of high functional diversity, with changes arising during the different sunflower growth stages. This can be ascribed to the changing quality of the root exudates secreted by the sunflower plants (Xu et al. 2009). Results obtained from the multivariate analysis (PCA) of the substrate group utilisation profiles demonstrate that the addition of green manure or fungal inoculum gave rise to diverse changes in substrate utilisation. However, it seemed as if the microbial communities still functioned at similar rates or through similar processes represented by the similar trends. The soil microbial communities showed a high resilience at the end of the experiment, as they returned to similar compositions after the experimental disturbance.

It can be concluded from the PLFA analysis that the added green manure increased the viable microbial biomass, due to the additional organic carbon available to the microbial communities. The different root exudates secreted by the sunflowers might have had a stimulating effect on the biomass. In all the treatments, the concentration of TBsats, indicative of Gram-positive bacteria, decreased and the concentration of Monos, representative of Gram-negative bacteria, increased. Gram-positive bacteria are considered to be K-strategists and represent a more established community than the Gram-negative bacteria, which are considered to be r-strategists. At the end of the experiment, there was a marked increase in the Gram-positive bacteria which implies the return of the soil microbial community to a more stable community. Again it is evident that the soil microbial community has a high resilience. It is clear that the soil microbial community composition changed over time, without permanent shifts occurring. The soil microbial community structure showed no distinct differences between the various treatments. The stress ratios derived from the fatty acid profiles signify an overall lower stress level experienced by the microbes, before flowering. This may be ascribed to high quality root exudates secreted by the sunflowers. However, these stress levels increased at the end of the study (after flowering), which may not be linked to nutritional stress but possibly the allelopathic potential of the sunflower plant itself. Several studies have demonstrated that sunflower plants can inhibit weed germination and other subsequent crops (Bashir et al. 2011; Macias et al. 2005). This allelopathic mechanism may influence soil microbial communities negatively. The addition of canola

green manure ensured microbial stability under these stressful conditions, possibly because of the over stimulation into a more resistant microbial community, unaffected by environmental disturbances. The incorporation of the inoculated soil with green manure did have a decreasing effect on the F: B ratio, implying the inhibition of the fungal pathogen *S. sclerotiorum*. A direct correlation was found between microbial community function and structure. As the microbial composition changed, the functional diversity varied accordingly, since the utilisation of specific substrate groups could be associated with certain structural groups (Figure 4.8a to 4.8c and Figure 4.5a to 4.5d in Results and Discussion). In all the treatments a drastic variation during plant flowering stages occurred, which might be as a result of the changing root exudates secreted during these phases (Xu et al. 2009). This was also observed in the previous results (Figure 4.1, 4.2, 4.4 and Tables 4.3, 4.4). It was evident that treatment of the soil with canola green manure or *S. sclerotiorum* led to changes in the soil microbial community structure, which resulted in functional variations. Yet, these changes did not have a long-lasting effect as similar compositions could still be observed at the end of the experiment.

From the chlorophyll a fluorescence measurements, it was observed that the sunflowers showed normal growth and development throughout the experiment. The inoculation of the soil with the fungal pathogen *S. sclerotiorum* inhibited the sunflower plant vitality or performance indexes during vegetative growth. This inhibition was caused by the disruption of the oxygen evolving complex in photosystem II, which resulted in the accumulation of electron carriers at the reaction centres. Fewer electrons were available for transfer through the electron transport chain to the photosystem I. The reduction of end electron acceptors such as NADP^+ and ferredoxin were also decreased, thus resulting in lower ATP formation. The incorporation of canola showed a slight negative influence on the photosynthetic potential of the sunflowers after flowering, but it seemed as if the addition of the canola manure ensured a possible protective barrier for plant vitality against *S. sclerotiorum*.

The final conclusions from this study is that the incorporation of the soil with canola green manure enhances the indigenous soil microbial communities, leading to changes in the microbial function and activity, without causing permanent alterations in the soil microbial community structure. The soil microbial communities showed more significant changes over time than among the different treatments. No distinct suppressive effect on the soil ecosystem could be observed for the sunflower pathogen, *S. sclerotiorum*, based on the microbiological methods applied. Conversely, *S. sclerotiorum* showed an inhibitory effect on sunflower performance, as determined by fluorescence transients. According to the results obtained from this study, the incorporation of canola (*Brassica napus*) green manure into the

soil has an effect on the soil microbial community function and structure. Nonetheless, this biofumigation effect is short-lived and microbial communities can return to their initial compositions after the disturbance. It seems as if canola does have a possible suppressive effect on the sunflower pathogen, *S. sclerotiorum*.

5.2. Recommendations for future studies

From the conclusions drawn from this investigation, several recommendations are proposed. Before conducting further studies in the greenhouse, samples of the water should be analysed for physical and chemical characteristics to ensure elemental suitability. The application of Hoagland's solution should rather be used as fertiliser in greenhouse trials, than the commercial fertiliser used in this study, as extremely high heavy metal concentrations was observed.

The use of molecular-based techniques should be considered to quantify the amount of fungal pathogen at the beginning and the end of the experiment. This will shed some light on whether the green manure suppresses and reduces the *S. sclerotiorum* abundance in the soil environment. Real-Time PCR will be the most appropriate molecular method to apply. During this method, a fluorescent probe attaches to the oligonucleotide primer, specific for the fungal pathogen, and changes fluorescence as the PCR process progresses. The amount of newly synthesised DNA is quantified with a thermocycler that measures the number of fluorescent probes (Pryor and Wittwer 2008). This indicates the amount of DNA present, specific for *S. sclerotiorum*. Isotope labelled substrates can be combined with PLFA analysis and rRNA analyses to characterise the metabolically active part of the microbial community (Frostegård et al. 2010).

Seeing as *S. sclerotiorum* degrades plant inner tissues with secreted enzymes, plants may suffer from water stress as the vascular tissues (required for water conduction) are damaged. The estimation of the leaf water potential, by means of a Scholander pressure chamber, can provide an indication of the potential water stream in xylem tissues (Scholander et al. 1964). The leaf water potential is directly related to the negative pressure in the vascular tissues. As pressure is applied to a cut off leaf placed in the chamber, the water in the xylem is forced to the surface (Scheepers 2011). If a higher pressure is needed for surface excretion, the xylem water potential is low and the plants experience water stress. Thus, it is hypothesised that when *S. sclerotiorum* invasion is severe, xylem tissues would be damaged and therefore lower leaf water potential would be observed.

Future research is needed on the specific biological and environmental factors leading to *Sclerotinia* outbreaks under natural environmental conditions. These knowledge gaps restrict the ability to predict future disease outbreaks and to develop effective control

strategies. Factors promoting the generation of functional sclerotia should also be investigated (Grogan 1979). The mycoparasite, *Coniothyrium minitans*, is known for its parasitising effect of sclerotia by the secretion of enzymes that weakens the sclerotial rind for easier entry (Huang 1977). The application of *C. minitans* as a biological control spraying agent on infected plants should be studied. The shelf-life of such a biological agent ought to be determined.

It would be wise to conduct this study under natural field conditions, where multiple manure amendments have been applied. The variability due to seasonal changes, management practices and pesticide or fertiliser applications should be taken into account in a commercial agricultural sector. Such a research output would provide a broad overview of the long-term impacts of biofumigants on the native soil microbial communities present in an agricultural soil, as well as the effects on subsequent crops in crop rotations and production yields. Repeated monitoring of the soil physical and chemical properties should take place to ensure a continuous assessment of the organic amendment impacts on the soil characteristics.

Alternative biofumigants, especially mustard species, should be tested for their biofumigation potential against *S. sclerotiorum*. Mustard species, for example Indian mustard (*Brassica juncea*) or White mustard (*Sinapsis alba*) are known to have the highest glucosinolate content of all the biofumigant crops (Morra and Kirkegaard 2002). The optimal incorporation rate of each biofumigant crop must be estimated, as well as the optimal incorporation soil depth, soil moisture and tissue disruption technique needed for the best glucosinolate release and isothiocyanate formation. Knowledge about the optimal age or growth phases of each biofumigant crop, during which the glucosinolate concentrations are at their highest, as well as the appropriate seasonal cycle to plant these crops are still limited. The possibility remains that these isothiocyanates can be extracted from soil and applied as commercial spray agents on crop plants. Studies on the abovementioned topics can provide farmers with the necessary information to maintain a healthy soil environment, by reducing pesticide applications, while still controlling soil-borne pests.

References

The referencing style used in this dissertation is in accordance with the requirements of the journal *Plant and Soil*.

- Adam G, Duncan H (2001) Development of a sensitive and rapid method for the measurement of total microbial activity using fluorescein diacetate (FDA) in a range of soils. *Soil Biol Biochem* 33:943-951
- Adams PB (1975) Factors affecting survival of *Sclerotinia sclerotiorum* in soil. *Plant Dis* 59:599-603
- Adams PB, Ayers WA (1979) Ecology of *Sclerotinia* species. *Phytopathology* 69(8):896-899
- Adams TMcM, Laughlin RJ (1981) The effects of agronomy on the carbon and nitrogen contained in the soil biomass. *J Agr Sci (Cambridge)* 97:319-327
- Alef K, Nannipieri P (1995) *Methods in Applied Soil Microbiology and Biochemistry*. (eds.) Academic Press, London, 576p
- Allison LE, Bollen WB, Moodie CD (1965) Total carbon. In: Black CA et al (ed) *Methods of soil analysis, Part 2*. Agronomy 9:1346-1366. Am. Soc. of Agron., Inc., Madison, Wis
- Allison SD, Martiny JBH (2008) Resistance, resilience, and redundancy in microbial communities. *PNAS* 105(1):11512-11519
- Amato M, Ladd JN (1988) Assay for microbial biomass based on ninhydrin-reactive nitrogen in extracts of fumigated soils. *Soil Biol Biochem* 20:107-114
- American Society for Testing and Materials (1985) Standard test method for particle-size analysis of soils. *Annual Book of ASTM Standards*. American Society for Testing and Materials, Philadelphia, pp 422-463
- Anderson AS, Wellington EMH (2001) The taxonomy of *Streptomyces* and related genera. *Int J Syst Evol Microbiol* 51:797-814
- Anderson JPE, Armstrong RA, Smith SN (1981) Methods to evaluate pesticide damage to the biomass of the soil microflora. *Soil Biol Biochem* 13:149-153
- Anderson JPE, Domsch KH (1973) Quantification of bacterial and fungal contributions to soil respiration. *Arch Microbiol* 93:113-127
- Anderson JPE, Domsch KH (1975) Measurement of bacterial and fungal contributions to respiration of selected agricultural and forest soils. *Can J Microbiol* 21:314-322
- Anderson JPE, Domsch KH (1978) A physiological method for the quantitative measurement of microbial biomass in soils. *Soil Biol Biochem* 10:215-221
- Andrews JH, Harris RF (1986) r and K-selection and microbial ecology. In: Marshall KC (ed) *Advances in Microbial Ecology*. Plenum Press, New York, pp 99-144
- Angus JF, Gardner PA, Kirkegaard JA, Desmarchelier JM (1994) Biofumigation: isothiocyanates released from *Brassica* roots inhibit the growth of the take-all fungus. *Plant Soil* 162:107-112
- Astera M (2007) Cation exchange capacity in soils, simplified. *Soil Minerals.com*. http://www.soilminerals.com/Cation_Exchange_Simplified.htm. Accessed 5 April 2012
- Atlas RM, Bartha R (1998) *Microbial Ecology* 4th edn. Menlo Park, California: Benjamin/Cummings Science Publishing
- Axelsson BO, Saraf A, Larsson L (1995) Determination of ergosterol in organic dust by gas chromatography-mass spectrometry. *J Chromatogr B* 666:77-84
- Ayanaba AA, Tuckwell SB, Jenkinson DS (1976) The effects of clearing and cropping on the organic reserves and biomass of tropical forest soils. *Soil Biol Biochem* 8:519-525
- Azooz RH, Arshad MA (1995) Tillage effects on thermal conductivity under long-term no tillage and conventional tillage systems. *Can J Soil Sci* 76:143-152

- Bååth E, Anderson TH (2003) Comparison of soil fungal/bacterial ratios in a pH gradient using physiological and PLFA-based techniques. *Soil Biol Biochem* 35:955-963
- Bååth E, Diaz-Ravina M, Frostegård Å, Campbell CD (1998) Effect of metal-rich sludge amendments on the soil microbial community. *Appl Environ Microbiol* 64:238-245
- Bach LH, Frostegård Å, Ohlson M (2008) Variation in soil microbial communities across a boreal spruce forest landscape. *Can J Forest Res* 38:1504-1516
- Bagwell CE, Lovell CR (2000) Persistence of selected *Spartina alterniflora* rhizoplane diazotrophs exposed to natural and manipulated environmental variability. *Appl Environ Microbiol* 66:4625-4633
- Bailey VL, Smith JL, Bolton JR (2002) Fungal-to-bacterial ratios in soils investigated for enhanced C sequestration. *Soil Biol Biochem* 34:997-1007
- Bailey VL, Smith JL, Bolton H (2003) Novel antibiotics as inhibitors for the selective respiratory inhibition method of measuring fungal: bacterial ratios in soil. *Biol Fert Soils* 38:154-160
- Baker III TT, Lockaby BG, Conner WH, Meier CE, Stanturf JA, Burke MK (2001) Leaf litter decomposition and nutrient dynamics in four southern forested floodplain communities. *Soil Sci Soc Am J* 65:1334-1347
- Banerjee MR, Burton DL, Depoe S (1997) Impact of sewage sludge application on soil biological characteristics. *Agr Ecosyst Environ* 66:241
- Banwart WL, Brember JM, Tabatabai MA (1972) Determination of ammonium in soil extracts and water samples by an ammonia electrode. *Commun Soil Sci Plan* 3:449
- Barajas-Aceves M, Hassan M, Tinoco R, Vazquez-Duhalt R (2002) Effects of pollutants on the ergosterol content as indicator of fungal biomass. *J Microbiol Meth* 50:227-236
- Bardin SD, Huang HC (2001) Research on biology and control of *Sclerotinia* diseases in Canada. *Can J Plant Pathol* 23:88-98
- Bashir U, Javaid A, Bajwa R (2011) Comparative tolerance of different rice varieties to sunflower phytotoxicity. *J Med Plants Res* 5(26):6243-6245
- Bateman DF, Beer SV (1965) Simultaneous production and synergistic action of oxalic acid and polygalacturonase during pathogenesis of *Sclerotium rolfsii*. *Phytopathology* 55:204-211
- Bates ST, García-Pichel F (2009) A culture-independent study of free-living fungi in biological soil crusts of the Colorado Plateau: their diversity and relative contribution to microbial biomass. *Environ Microbiol* 11:56-67
- Baumann K, Marschner P, Smernik RJ, Baldock JA (2009) Residue chemistry and microbial community structure during decomposition of eucalypt, wheat and vetch residues. *Soil Biol Biochem* 41:1966-1975
- Beare MH, Neely CL, Coleman DC, Hargrove WL (1990) A substrate-induced respiration (SIR) method for measurement of fungal and bacterial biomass on plant residues. *Soil Biol Biochem* 22:585-594
- Beare MH, Parmelee RW, Hendrix PF, Cheng W, Coleman DC, Crossley Jr. DA (1992) Microbial and faunal interactions and effects on litter nitrogen and decomposition in agroecosystems. *Ecol Monogr* 62:569-591
- Beck T, Joergensen RG, Kandeler E, Makeschin F, Nuss E, Oberholzer HR, Scheu S (1997) An inter-laboratory comparison of ten different ways of measuring soil microbial biomass C. *Soil Biol Biochem* 29(7):1023-1032
- Bellostas N, Sørensen JC, Sørensen H (2007) Profiling glucosinolates in vegetative and reproductive tissues of four *Brassica* species of the U-triangle for their biofumigation potential. *J Sci Food Agric* 87:1586-1594
- Bell AA, Wheeler MH (1986) Biosynthesis and functions of fungal melanins. *Ann Rev Phytopathol* 24:411-451
- Belnap J, Lange OL (2001) Biological Soil Crusts: structure, function and management. In: *Ecological Studies Series*, vol 150. Springer-Verlag, Berlin

- Ben-David EA, Holden PJ, Stone DJM, Harch BD, Foster LJ (2004) The use of phospholipid fatty acid analysis to measure impact of acid rock drainage on microbial communities in sediments. *Microb Ecol* 48:300-315
- Bending GD, Lincoln SD (2000) Inhibition of soil nitrifying bacteria communities and their activities by glucosinolates hydrolysis products. *Soil Biol Biochem* 32:1261-1269
- Bengtsson J (1997) What kind of diversity? Species richness, keystone species or functional groups? In: Wolters V (ed) Functional implications of biodiversity in soil. Ecosystem research report no. 24. Office for Official Publications of the European Communities, Luxembourg, pp 59-66
- Bengtsson J (1998) Which species? What kind of diversity? Which ecosystem function? Some problems in studies of relations between biodiversity and ecosystem function. *Appl Soil Ecol* 10:191-199
- Bernard E, Larkin RP, Tavantzis S, Erich MS, Alyokhin A, Sewell G, Lannan A, Gross SD (2012) Compost, rapeseed rotation, and biocontrol agents significantly impact soil microbial communities in organic and conventional potato production systems. *Appl Soil Ecol* 52:29-41
- Bjerg B, Sørensen H (1987) Quantitative analysis of glucosinolates in oilseed rape based on HPLC of desulfoglucosinolates and HPLC of intact glucosinolates. In: Wathelet JP (ed) Glucosinolates in Rapeseeds: Analytical Aspects. Lancaster Martinus Nijhoff, Boston, MA, USA, pp 125-150
- Black CA (1968) Soil-Plant relationships, 2nd end. Wiley, New York, pp 274-353
- Blagodatskaya EV, Anderson T-H (1998) Interactive effects of pH and substrate quality on the fungal-to-bacterial ratio and QCO₂ of microbial communities in forest soils. *Soil Biol Biochem* 30:1269-1274
- Blagodatskaya EV, Blagodatsky SA, Anderson T-H, Kuzyakov Y (2007) Priming effects in Chernozem induced by glucose and N in relation to microbial growth strategies. *Appl Soil Ecol* 37:95-105
- Blagodatskaya EV, Blagodatsky SA, Anderson T-H, Kuzyakov Y (2009) Contrasting effects of glucose, living roots and maize straw on microbial growth kinetics and substrate availability in soil. *Eur J Soil Sci* 60:186-197
- Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37:911-917
- Bloem J, Bolhuis PR, Veninga MR, Wieringa J (1995) Microscopic methods for counting bacteria and fungi in soil. In: Alef K and Nannipieri P (ed) Methods in Applied Soil Microbiology and Biochemistry. Academic Press, San Diego, pp 162-172
- Böhme L, Langer U, Böhme F (2005) Microbial biomass, enzyme activities and microbial community structure in two European long-term field experiments. *Agr Ecosyst Environ* 109:141-152
- Boland GJ, Hall R (1987) Epidemiology of white mold of white bean in Ontario. *Can J Plant Pathol* 9:218-224
- Boland GJ, Hall R (1994) Index of plant hosts of *Sclerotinia sclerotiorum*. *Can J Plant Pathol* 16:93-108
- Boll J, Steenhuis TS, Selker JS (1992) Fiberglass wicks for sampling of water and solutes in the vadose zone. *Soil Sci Soc Am J* 56:701-707
- Bolton HJ, Fredrikson JK, Elliot LF (1993) Microbiology of the rhizosphere. In: Metting Jr FB (ed) Soil Microbial Ecology. Marcel Dekker, Inc., New York, pp 27-63
- Bolton MD, Thomma BPHJ, Nelson BD (2006) *Sclerotinia sclerotiorum* (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen. *Mol Plant Pathol* 7(1):1-16
- Bom M, Boland GJ (2000) Evaluation of disease forecasting variables for *Sclerotinia* stem rot (*Sclerotinia sclerotiorum*) of canola. *Can J Plant Sci* 80:889-898
- Bossio DA, Fleck JA, Scow KM, Fujii R (2006) Alteration of soil microbial communities and water quality in restored wetlands. *Soil Biol Biochem* 38:1223-1233
- Bossio DA, Scow KM (1998) Impacts of carbon and flooding on soil microbial communities: phospholipid fatty acid profiles and substrate utilization patterns. *Microb Ecol* 35:265-278
- Bossio DA, Scow KM, Gunapala N, Graham KJ (1998) Determinants of soil microbial communities: effects of agricultural management, season and soil type on phospholipid fatty acid profiles. *Microb Ecol* 36:1-12

- Bower CA, Wilcox LV (1965) Soluble salts. In: Black CA et al (ed) Methods of soil analysis. Agronomy 9:933-951. Am. Soc. of Agron., Inc., Madison, Wis
- Brady NC (1974) The nature and properties of soils, 8th end. Macmillan Publishing Co. Inc., New York
- Bray RH, Kurtz LT (1945) Determination of total, organic and available forms of phosphorus in soils. Soil Sci 59:39-45
- Bremner JM, Tabatabai MA (1973) Nitrogen-15 enrichment of soils and soil-derived nitrate. J Environ Qual 2:363-365
- Briantais JM, Vernotte C, Krause GH, Weiss E (1986) Chlorophyll a fluorescence of higher plants: Chloroplasts and leaves. In: Govindjee, Ames J and Fork DC (ed) Light Emission by Plants and Bacteria. Academic Press, New York, pp 539-583
- Brodie E, Edwards S, Clipson N (2002) Bacterial community dynamics across a floristic gradient in a temperate upland grassland ecosystem. Microb Ecol 44:260-270
- Brookes PC, Landman A, Pruden G, Jenkinson DS (1985) Chloroform fumigation and the release of soil nitrogen: a rapid direct extraction method to measure microbial biomass nitrogen in soil. Soil Biol Biochem 17(6):837-842
- Brookes PC, McGrath SP (1984) Effects of metal toxicity on the size of the soil microbial biomass. J Soil Sci 35:341-346
- Brown PD, Morra MJ (1997) Control of soilborne plant pests using glucosinolate-containing plants. Adv Agron 61:167-231
- Burke JM, Riesenberger LH (2003) Fitness effect of transgenic disease resistance in sunflowers. Science 300:1250
- Bussotti F, Desotgiu R, Cascio C, Pollastrini M, Gravano E, Gerosa G, Marzuoli R, Nali C, Lorenzini G, Salvatori E, Manes F, Schaub M, Strasser RJ (2011) Ozone stress in woody plants assessed with chlorophyll a fluorescence. A critical reassessment of existing data. Environ Exp Bot 73:19-30
- Buyer JS, Roberts DP, Millner P, Russek-Cohen E (2001) Analysis of fungal communities by sole carbon source utilization profiles. J Microbiol Meth 45:53-60
- Buyer JS, Roberts DP, Russek-Cohen E (1999) Microbial community structure and function in the spermosphere as affected by soil and seed type. Can J Microbiol 45:138-144
- Campbell CD, Grayston SJ, Hirst DJ (1997) Use of rhizosphere carbon sources in sole carbon source tests to discriminate soil microbial communities. J Microbiol Meth 30:33-41
- Cessna SG, Sears VE, Dickman MB, Low PS (2000) Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. Plant Cell 12:2191-2200
- Chaer GM, Myrold DD, Bottomley PJ (2009) A soil quality index based on the equilibrium between soil organic matter and biochemical properties of undisturbed coniferous forest soils of the Pacific Northwest. Soil Biol Biochem 41:822-830
- Chan KY, Heenan DP (1996) The influence of crop rotation on soil structure and soil physical properties under conventional tillage. Soil Till Res 37:113-125
- Chan KY, Mead JA (1988) Surface physical properties of a sandy soil under tillage practices. Aust J Soil Res 26:549-559
- Chapelle F (1993) Ground-water microbiology and geochemistry. Wiley, New York, 424p
- Chen S, Zhou F, Yin C, Strasser RJ, Yang C, Qiang S (2011) Application of fast chlorophyll a fluorescence kinetics to probe action target of 3-acetyl-5-isopropyltetramic acid. Environ Exp Bot 71:269-279
- Cheng W, Coleman DC (1989) A simple method for measuring CO₂ in a continuous air-flow system: modifications to the substrate-induced respiration technique. Soil Biol Biochem 21:385-388
- Cheng JYW, Lau APS, Fang M (2008) Assessment of the atmospheric fungal prevalence through field ergosterol measurement I – Determination of the specific ergosterol content in common ambient fungal spores and yeast cells. Atmos Environ 42:5526-5533
- Cherr CM, Scholberg JMS, McSorley R (2006) Green manure approaches to crop production: A synthesis. Agron J 98:302-319
- Chet I, Henis Y (1975) Sclerotial morphogenesis in Fungi. Ann Rev Phytopathol 13:169-192

- Chodak M, Pietrzykowski M, Niklinska M (2009) Development of microbial properties in a chronosequence of sandy mine soils. *Appl Soil Ecol* 41:259-268
- Chung WC, Huang JW, Huang HC, Jen JF (2003) Control, by Brassica seed pomace *Pythium ultimum* combined with *Pseudomonas boreopolis*, of damping-off of watermelon caused by *Pythium* sp. *Can J Plant Pathol* 25:285-294
- Claassens S (2007) Measuring rehabilitation success of coal mining disturbed areas: A spatial and temporal investigation into the use of soil microbial properties as assessment criteria. Thesis: Ph. D. Env. Sci. School of Environmental Sciences and Development. North-West University, Potchefstroom, South Africa, 103p
- Claassens S, Riedel KJ, Van Rensburg L, Bezuidenhout JJ, Jansen van Rensburg PJ (2006) Microbial community function and structure on coal mine discard under rehabilitation. *S Afr J Plant Soil* 23(2):105-112
- Cordova-Kreylos AL, Cao Y, Green PG, Hwang H, Kuivila KM, Lamontagne MG, Van De Werfhorst LC, Holden PA, Scow KM (2006) Diversity, composition, and geographical distribution of microbial communities in California salt marsh sediments. *Appl Environ Microbiol* 72(5):3357-3366
- Crossman ZM, Wang ZP, Ineson P, Evershed RP (2006) Investigation of the effect of ammonium sulphate on populations of ambient methane oxidising bacteria by ^{13}C -labelling and GC / C / IRMS analysis of phospholipid fatty acids. *Soil Biol Biochem* 38:983-990
- Czaczyk K, Trojanowska K, Stachowiak B (2002) Inhibition of ergosterol biosynthesis in fungal plant pathogens by *Bacillus* sp. *Pol J Environ Stud* 11(5):593-597
- Day SD, Seiler JR, Persaud N (2000) A comparison of root growth dynamics of silver maple and flowering dogwood in compacted soil at differing soil water contents. *Tree Physiol* 20(4):257-263
- De Beer M, Van Rensburg L, Claassens S, Jansen van Rensburg PJ (2004) Assessment of the herbicide induced plant physiological damage to *Cynodon dactylon*. Technical report for ESKOM, North-West University, Potchefstroom Campus
- Degens BP (1999) Catabolic response profiles differ between microorganisms grown in soils. *Soil Biol Biochem* 31:475-477
- Deng SP, Tabatabai MA (1994) Cellulase activity of soils. *Soil Biol Biochem* 26:1347-1354
- De Vries FT, Hoffland E, Van Eekeren N, Brussaard L, Bloem J (2006) Fungal/bacterial ratios in grasslands with contrasting nitrogen management. *Soil Biol Biochem* 38:2092-2103
- Dick RP, Sandor JA, Eash NS (1994) Soil enzyme activities after 1500 years of terrace agriculture in the Colca Valley, Peru. *Agr Ecosyst Environ* 50:123-131
- Dickman MB, Mitra A (1992) *Arabidopsis thaliana* as model for studying *Sclerotinia sclerotiorum* pathogenesis. *Physiol Mol Plant Pathol* 41:255-263
- Doblas ME, Sánchez FP, Gonzalez AM (2009) Different microhabitats affect soil macroinvertebrate assemblages in a Mediterranean arid ecosystem. *Appl Soil Ecol* 41:329-335
- Donaldson PA, Anderson T, Lane BG, Davidson AL, Simmonds DH (2001) Soybean plants expressing an active oligomeric oxalate oxidase from the wheat *gf-2.8* (germin) gene are resistant to the oxalate-secreting pathogen *Sclerotinia sclerotiorum*. *Physiol Mol Plant Pathol* 59:297-307
- Donegan KK, Palm CL, Fieland VL, Porteous LA, Ganio LM, Schaller DL, Bucaro LQ, Seidler RJ (1995) Changes in levels, species and DNA fingerprints of soil microorganisms associated with cotton expressing the *Bacillus thuringiensis* var. *Kurstaki* endotoxin. *Appl Soil Ecol* 2:111-124
- Dong X, Huai-Ying Y, De Yong G, Chang-Yong (2008) Soil microbial community structure in diverse land use systems: A comparative study using Biolog, DGGE, and PLFA analyses. *Pedosphere* 18(5):653-663
- Doran JW, Parkin TB (1994) Defining and assessing soil quality. In: Doran JW, Coleman DC, Bezdicek DF and Stewart BA (eds) *Defining soil quality for a sustainable environment*, vol 35. SSSA Special Publications, Madison, WI, USA, pp 3-21

- Drenovsky RE, Elliott GN, Graham KJ, Scow KM (2004) Comparison of phospholipid fatty acid (PLFA) and total soil fatty acid methyl esters (TSFAME) for characterizing soil microbial communities. *Soil Biol Biochem* 36:1793-1800
- Drissner D, Blum H, Tscherko D, Kandeler E (2007) Nine years of enriched CO₂ changes the function and structural diversity of soil microorganisms in a grassland. *Eur J Soil Sci* 58:260-269
- Eberlein CV, Morra MJ, Guttieri MJ, Brown PD, Brown J (1998) Glucosinolate production by five field-grown *Brassica napus* cultivars used as green manures. *Weed Technol* 12:712-718
- Elfstrand S, Hedlund K, Mårtensson A (2007) Soil enzyme activities, microbial community composition and function after 47 years of continuous green manuring. *Appl Soil Ecol* 35:610-621
- Ellis RJ, Neish B, Trett MW, Best JG, Weightman AJ, Morgan P, Fry JC (2001) Comparison of microbial and meiofaunal community analyses for determining impact of heavy metal contamination. *J Microbiol Meth* 45:171-185
- Esperschütz J, Gattinger A, Mäder P, Schlöter M, Fliebbach A (2007) Response of soil microbial biomass and community structures to conventional and organic farming systems under identical crop rotations. *FEMS Microbiol Ecol* 61:26-37
- Fahey JW, Zalcman AT, Talalay P (2001) The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* 56:5-51
- Fang J, Uhle M, Billmark K, Bartlett DH, Kato C (2006) Fractionation of carbon isotopes in biosynthesis of fatty acids by a piezophilic bacterium *Moritella japonica* strain DSK1. *Geochim Cosmochim Acta* 70:1753-1760
- Farnet AM, Prudent P, Cigna M, Gros R (2008) Soil microbial activities in a constructed soil reed-bed under cheese-dairy farm effluents. *Bioresour Technol* 99:6198-6206
- Fierer N, Schimel JP, Holden PA (2003) Variations in microbial community composition through two soil depth profiles. *Soil Biol Biochem* 35:167-176
- Findlay RH (1996) The use of phospholipid fatty acids to determine microbial community structure. In: Akkermans ADL, Van Elsas JD and De Bruijn F (ed) *Molecular microbial ecology manual*. Kluwer, Dordrecht 4, pp 1-17
- Findlay RH (2004) Determination of microbial community structure using phospholipid fatty acid profiles. *Molecular Microbial Ecology Manual* 2(4.08):983-1004
- Floch C, Chevremont A, Joanico K, Capowiez Y, Criquet S (2011) Indicators of pesticide contamination: Soil enzyme compared to functional diversity of bacterial communities via Biolog® Ecoplates. *Eur J Soil Biol* 47:256-263
- Fontaine S, Mariotti A, Abbadie L (2003) The priming effect of organic matter: a question of microbial competition? *Soil Biol Biochem* 35:837-843
- Frey B, Buser H-R, Schuepp H (1992) Identification of ergosterol in vesicular-arbuscular mycorrhizae. *Biol Fert Soils* 13:229-234
- Friberg H, Edel-Hermann V, Faivre C, Gautheron N, Fayolle L, Faloya V, Montfort F, Steinberg C (2009) Cause and duration of mustard incorporation effects on soil-borne plant pathogenic fungi. *Soil Biol Biochem* 41:2075-2084
- Fritze H, Pietikäinen J, Pennanen T (2000) Distribution of microbial biomass and phospholipid fatty acids in Podzol profiles under coniferous forest. *Eur J Soil Sci* 51:565-573
- Frostegård Å, Bååth E (1996) The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. *Biol Fert Soils* 22:59-65
- Frostegård Å, Petersen SO, Bååth A, Nielsen TH (1997) Dynamics of a microbial community associated with manure hot spots as revealed by phospholipid fatty acid analysis. *Appl Environ Microbiol* 63:2224-2231
- Frostegård Å, Tunlid A, Bååth E (1993) Phospholipid fatty acid comparison, biomass and activity of microbial communities from two soil types experimentally exposed to different heavy metals. *Appl Environ Microbiol* 59:3605-3617
- Frostegård Å, Tunlid A, Bååth E (1996) Changes in Microbial Community Structure during long-term incubation in two soils experimentally contaminated with metals. *Soil Biol Biochem* 28(1):55-63

- Frostegård Å, Tunlid A, Bååth E (2010) Use and misuse of PLFA measurements in soils. *Soil Biol Biochem*: 1-5
- Galloway JN, Dentener FJ, Capone DG, Boyer EW, Howarth RW, Seitzinger SP, Asner GP, Cleveland CC, Green PA, Holland EA, Karl DM, Michaels AF, Porter JH, Townsend AR, Vorosmarty CJ (2004) Nitrogen cycles: past, present and future. *Biogeochemistry* 70:153-226
- Gao Y, Zhou P, Mao L, Zhi Y, Zhang C, Shi W (2010) Effects of plant species coexistence on soil enzyme activities and soil microbial community structure under Cd and Pb combined pollution. *J Environ Sci* 22(7):1040-1048
- García C, Hernández T (1997) Biological and biochemical indicators in derelict soils subject to erosion. *Soil Biol Biochem* 29:171-177
- Gardenas AI, Agren GI, Bird JA, Clarholm M, Hallin S, Ineson P, Katterer T, Knicker H, Nilsson SI, Nasholm T, Ogle S, Paustian K, Persson T, Stendahl J (2010) Knowledge gaps in soil carbon and nitrogen interactions – From molecular to global scale. *Soil Biol Biochem*: 1-16
- Gardiner J, Morra MJ, Eberlein CV, Brown PD, Borek V (1999) Allelochemicals released in soil following incorporation of rapeseed (*Brassica napus*) green manures. *J Agr Food Chem* 47:3837-3842
- Garland JL (1996) Patterns of potential C source utilization by rhizosphere communities. *Soil Biol Biochem* 28:223-230
- Garland JL (1997) Analysis and interpretation of community-level physiological profiles in microbial ecology. *FEMS Microb Ecol* 24:289-300
- Garland JL, Mills AL (1991) Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Appl Environ Microbiol* 57:2351-2359
- Gavrilescu M, Pavel LV, Cretescu I (2009) Review: Characterization and remediation of soils contaminated with uranium. *J Hazard Mater* 163:475-510
- Gerik JS (2005) Evaluation of soil fumigants applied by drip irrigation for *Liatris* production. *Plant Dis* 89:883-887
- Gessner MO, Bauchrowitz MA, Escautier M (1991) Extraction and quantification of ergosterol as a measure of fungal biomass in leaf litter. *Microb Ecol* 22:285-291
- Gessner MO, Chauvet E (1993) Ergosterol-to-biomass conversion factors for aquatic hyphomycetes. *Appl Environ Microbiol* 59:502-507
- Gimsing AL, Kirkegaard JA (2006) Glucosinolate and isothiocyanates concentration in soil following incorporation of *Brassica* biofumigants. *Soil Biol Biochem* 38:2255-2264
- Gimsing AL, Sørensen JC, Tovgaard L, Jørgensen AMF, Hansen HCB (2006) Degradation kinetics of glucosinolates in soil. *Environ Toxicol Chem* 25(8):2038-2044
- Glass DJ (1999) US and international markets for phytoremediation 1999-2000. D, Glass Associates, Inc, Needham, MA, USA.
- Godoy G, Steadman J, Dickman M, Dam R (1990) Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *S.sclerotiorum* on *Phaseolus vulgaris*. *Physiol Mol Plant Pathol* 37:179-191
- Grant WD, West AWJ (1986) Measurements of ergosterol, diamino pimelic acid and glucosamine in soil. Evaluation as indicators of microbial biomass. *J Microbiol Meth* 6:47-53
- Grayston SJ, Campbell CD, Bardgett RD, Mawdsley JL, Clegg CD, Ritz K, Griffiths BS, Rodwell JS, Edwards SJ, Davies WJ, Elston DJ, Millard P (2004) Assessing shifts in microbial community structure across a range of grasslands of differing management intensity using CLPP, PLFA and community DNA techniques. *Appl Soil Ecol* 25:63-84
- Grayston SJ, Wang S, Campbell CD, Edwards AC (1998) Selective influence of plant species on microbial diversity in the rhizosphere. *Soil Biol Biochem* 30:369-378
- Griffiths BS, Ritz K, Wheatley R, Kuan HL, Boag B, Christensen S, Ekelund F, Sørensen SJ, Muller S, Bloem J (2001) An examination of the biodiversity-ecosystem function relationship in arable soil microbial communities. *Soil Biol Biochem* 33:1713-1722

- Grogan RG (1979) *Sclerotinia* species: Summary and comments on needed research. *Phytopathology* 69(8):908-910
- Guckert JB, Antworth CP, Nichols PD, White DC (1985) Phospholipid, ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. *FEMS Microbiol Ecol* 31:147-158
- Guckert JB, Carr GJ, Johnson TD, Hamm BG, Davidson DH, Kumagai Y (1996) Community analysis by Biolog: curve integration for statistical analysis of activated sludge microbial habitats. *J Microbiol Meth* 27:183-197
- Guckert JB, Hood MA, White DC (1986) Phospholipid ester-linked fatty acid profile changes during nutrient deprivation of *Vibrio cholerae* – increases in the trans cis ratio and proportions of cyclopropyl fatty acids. *Appl Environ Microbiol* 52:794-801
- Guckert JB, Ringelberg DB, White DC, Hanson RS, Bratina BJ (1991) Membrane fatty acids as phenotypic markers in the polyphasic taxonomy of methylotrophs within the Proteobacteria. *J Gen Microbiol* 137:2631-2641
- Guimaraes RL, Stotz HU (2004) Oxalate production by *Sclerotinia sclerotiorum* deregulates guard cells during infection. *Plant Physiology Preview*: 1-9
- Habig JH (2003) Soilborne disease suppressiveness / conduciveness: analysis of microbial community dynamics. Dissertation: M.Sc. Env. Sci. School of Environmental Sciences and Development. North-West University, Potchefstroom, South Africa, 158p
- Hannusch DJ, Boland GJ (1996) Influence of air temperature and relative humidity on biological control of white mold of bean (*Sclerotinia sclerotiorum*). *Phytopathology* 86:156-162
- Hart MR, Brookes PC (1996) Effects of two ergosterol-inhibiting fungicides on soil ergosterol and microbial biomass. *Soil Biol Biochem* 28(7):885-892
- Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. *Genome Res* 6:986-994
- Heipieper HJ, Dissenbach R, Keweloch H (1992) Conversion of *cis* unsaturated fatty acids to *trans*, a possible mechanism for the protection of phenol-degrading *Pseudomonas putida* P8 from substrate toxicity. *Appl Environ Microbiol* 58:1847-1852
- Hendrix PF, Parmelee RW, Crossley DA, Coleman DC, Odum EP, Groffman PM (1986) Detritus food webs in conventional and no-tillage agroecosystems. *BioScience* 36:374-380
- Hermans C, Smeyers M, Rodríguez RM, Eyletters M, Strasser RJ, Delhaye J (2003) Quality assessment of urban trees: A comparative study of physiological characterisation, airborne imaging and on site fluorescence monitoring by the OJIP-test. *J Plant Physiol* 160:81-90
- Hermawan B, Cameron KC (1993) Structural changes in a silt loam under long-term conventional or minimum tillage. *Soil Till Res* 26:139-150
- Herselman JE (2007) The concentration of selected trace metals in South African soils. Dissertation: Doctor of Philosophy, Department of Soil Science. University of Stellenbosch, Stellenbosch, South Africa, 58p
- Hillel D (1980) *Applications of Soil Physics*. Academic Press, New York, 385p
- Hoglund A-S, Lenman M, Falk A, Rask L (1991) Distribution of myrosinase in rapeseed tissues. *Plant Physiol* 95:213-221
- Hu X, Bidney DL, Yalpani N, Duvick JP, Crasta O, Folkerts O, Lu G (2003) Overexpression of a gene encoding hydrogen peroxide-generating oxalate oxidase evokes defense responses in sunflower. *Plant Physiol* 133:170-181
- Hu S, Chapin III FS, Firestone MK, Field CB, Chiariello NR (2001) Nitrogen limitation in a grassland under elevated CO₂. *Nature* 409:188-191
- Huang HC (1977) Importance of *Coniothyrium minitans* in survival of sclerotia of *Sclerotinia sclerotiorum* in wilted sunflower. *Can J Bot* 55:289-295
- Huang HC (1980) Control of *Sclerotinia* wilt of sunflower by hyperparasites. *Can J Plant Pathol* 2:26-32
- Huang HC (1982) Morphologically abnormal sclerotia of *Sclerotinia sclerotiorum*. *Can J Microbiol* 28:87-91

- Huang HC (1983) Sclerotinia wilt and head rot of sunflower. Canadex 632.145. Agriculture Canada
- Huang HC (1985) Factors affecting myceliogenic germination of sclerotia of *Sclerotinia sclerotiorum*. Phytopathology 75(4):433-437
- Huang HC, Acharya SN, Erickson RS (2000) Etiology of alfalfa blossom blight caused by *Sclerotinia sclerotiorum* and *Botrytis cinerea*. Plant Pathol Bull 9:11-16
- Huang HC, Erickson RS, Phillippe LM, Mueller CA, Sun SK, Huang JW (2006) Control of apothecia of *Sclerotinia sclerotiorum* by soil amendment with S-H mixture or Perlka® in bean, canol and wheat fields. Soil Biol Biochem 38:1348-1352
- Huang HC, Hoes JA (1980) Importance of plant spacing and sclerotial position to development of Sclerotinia wilt of sunflower. Plant Dis 64:81-84
- Huang HC, Kokko EG, Erickson RS, Hynes RK (1998) Infection of canola pollen by *Sclerotinia sclerotiorum*. Plant Pathol Bull 7:71-77
- Huang HC, Kozub GC (1989) A simple method for production of apothecia from sclerotia of *Sclerotinia sclerotiorum*. Plant Prot Bull 31:333-345
- Huang HC, Kozub GC (1991) Monocropping to sunflower and decline of Sclerotinia wilt. Bot Bull Acad Sin (Taipei) 32:163-170
- Huang HC, Kozub GC (1993) Influence of inoculum production temperature on carpogenic germination of sclerotia of *Sclerotinia sclerotiorum*. Can J Microbiol 39:548-550
- Huang HC, Sun SK (1991) Effects of S-H mixture or Perlka™ on carpogenic germination and survival of sclerotia of *Sclerotinia sclerotiorum*. Soil Biol Biochem 23:809-813
- Jackson ML (1958) Soil chemical analysis. Prentice-Hall Inc., Englewood Cliffs, New York, pp 87-110
- Janvier C, Villeneuve F, Alabouvette C, Edel-Hermann V, Mateille T, Steinberg C (2007) Soil health through soil disease suppression: Which strategy from descriptors to indicators? Soil Biol Biochem 39:1-23
- Jenkinson DS (1988) Determination of microbial biomass carbon and nitrogen in soil. In: Wilson JR (ed) Advances in Nitrogen Cycling in Agricultural Ecosystems. CAB International, Wallingford, pp 368-386
- Jenkinson DS, Ladd JN (1981) Microbial biomass in soil: measurement and turnover. In: Paul EA and Ladd JN (ed) Soil Biochemistry, vol. 5. Dekker, New York, pp 415-471
- Jenkinson DS, Powlson DS (1976) The effects of biocidal treatments on metabolism in soil: V. A method for measuring soil biomass. Soil Biol Biochem 8:209-213
- Jensen C, Neumeister-Kemp H, Lysek G (1998) Fluorescence microscopy for the observation of Nematophagous fungi inside soil. Mycologist 12(3):107-111
- Joergensen RG (1996) The fumigation-extraction method to estimate soil microbial biomass: calibration of the k_{ec} value. Soil Biol Biochem 28:25-31
- Joergensen RG, Wichern F (2008) Quantitative assessment of the fungal contribution to microbial tissue in soil. Soil Biol Biochem 40:2977-2991
- John MK, Chuah HH, Neufeld JH (1975) Application of improved azomethine-H method to the determination of boron in soils and plants. Anal Lett 8:559-568
- Kandeler E, Kampichler C, Horak O (1996) Influence of heavy metals on the functional diversity of soil microbial communities. Biol Fert Soils 23:299-306
- Kaneda T (1991) Iso- and anteiso-fatty acids in bacteria: Biosynthesis, function and taxonomic significance. Microbiol Rev 55:288-302
- Karlen DL, Mausbach MJ, Doran JW, Cline RG, Harris RF, Schuman GE (1997) Soil quality: a concept, definition, and framework for evaluation. Soil Sci Soc Am J 61:4-10
- Kelly JJ, Tate RL (1998) Use of BIOLOG for the analysis of microbial communities from zinc-contaminated soils. J Environ Qual 27:600-608
- Kent M, Coker P (1992) Vegetation description and analysis. A practical approach. John Wiley & Sons, Great Britain
- Khaliq A, Matloob A, Cheema ZA, Farooq M (2011) Allelopathic activity of crop residue incorporation alone or mixed against rice and its associated grass weed jungle rice (*Echinochloa colona* [L.] Link). Chilean J Agricultural Research 71(3):418-423

- Khanh TD, Chung MI, Xuan TD, Twata S (2005) The exploitation of crop allelopathy in sustainable agricultural production. *J Agron Crop Sci* 191:172-184
- Kieft TL, Amy PS, Brockman FJ, Fredrickson JK, Björnstad BN, Rosacker LL (1993) Microbial abundance and activities in relation to water potential in the vadose zones of arid and semiarid sites. *Microb Ecol* 26:59-78
- Kieft TL, Ringelberg DB, White DC (1994) Changes in ester-linked phospholipid fatty acid profiles of subsurface bacteria during starvation and desiccation in a porous medium. *Appl Environ Microbiol* 60:3292-3299
- Kieft TL, Wilch E, O'Connor K, Ringelberg DB, White DC (1997) Survival and phospholipid fatty acid profiles of surface and subsurface bacteria in natural sediment microcosms. *Appl Environ Microbiol* 63(4):1531-1542
- Killham K (1994) *Soil ecology*. Cambridge Univ. Press, Melbourne, 242p
- Kirchner MJ, Wollum II AG, King LD (1993) Soil microbial populations and activities in reduced chemical input agroecosystems soils. *Soil Soc Am J* 57:1289-1295
- Kirk JL, Beaudette LA, Hart M, Moutoglis P, Klironomos JN, Lee H, Trevors JT (2004) Methods of studying soil microbial diversity. *J Microbiol Meth* 58:169-188
- Kirkegaard JA, Sarwar M (1998) Biofumigation potential of brassicas. I. Variation in glucosinolates profiles of diverse field-grown brassicas. *Plant Soil* 201:71-89
- Kirkegaard JA, Sarwar M, Wong PTW, Mead A, Howe G, Newell M (2000) Field studies on the fumigation of take-all by *Brassica* break crops. *Aust J Agr Res* 51:445-456
- Klamer M, Bååth E (2004) Estimation of conversion factors for fungal biomass determination in compost using ergosterol and PLFA 18:2 ω 6, 9. *Soil Biol Biochem* 36:57-65
- Klose S, Acosta-Martinez V, Ajwa HA (2006) Microbial community composition and enzyme activities in a sandy loam soil after fumigation with methyl bromide or alternative biocides. *Soil Biol Biochem* 38:1243-1254
- Klose S, Ajwa HA (2004) Enzyme activities and microbial biomass in agricultural soils fumigated with methyl bromide alternatives. *Soil Biol Biochem* 36:1625-1635
- Klose S, Moore JM, Tabatabai MA (1999) Arylsulfatase activity of the microbial biomass in soils as affected by cropping systems. *Biol Fert Soils* 29:46-54
- Konopka A, Oliver L, Turco R (1998) The use of carbon substrate utilization patterns in environmental and ecological microbiology. *Microb Ecol* 35:103-115
- Kozdroj J, Van Elsas JD (2000) Response of the bacterial community to root exudates in soil polluted with heavy metals assessed by molecular and cultural approaches. *Soil Biol Biochem* 32:1405-1417
- Krause GH, Weis E (1991) Chlorophyll fluorescence and photosynthesis: The basics. *Ann Rev Plant Physiol Plant Mol Biol* 42:313-349
- Kreutzer WA (1963) Selective toxicity of chemicals to soil microorganisms. *Ann Rev Phytopathol* 1:101-126
- Lacey MJ (2000) *Studies on common Scab of potato*. Doctor of Philosophy, University of Tasmania, Hobart
- Lachmund C, Manz W (2005) Substrate utilization profiles of natural microbial communities for the characterization of river sediments with different contamination levels. Workshop "Functional and genetic characterization of microbial communities: Sole-carbon-source test." Abstracts book, Lisbon University Faculty of Sciences, Lisbon
- Larkin RP, Griffin TS (2007) Control of soilborne potato diseases using *Brassica* green manures. *Crop Prot* 26:1067-1077
- Larkin RP, Honeycutt CW (2006) Effects of different 3-yr cropping systems on soil microbial communities and soilborne disease of potato. *Phytopathology* 96:68-79
- Lauber CL, Strickland MS, Bradford MA, Fierer N (2008) The influence of soil properties on the structure of bacterial and fungal communities across land-use types. *Soil Biol Biochem* 40:2407-2415
- Lazzeri L, Baruzzi G, Malaguti L, Antoniaci L (2003) Replacing methyl bromide in annual strawberry production with glucosinolates-containing green manure crops. *Pest Manag Sci* 59:983-990

- Lazzeri L, Leoni O, Manici LM (2004) Biocidal plant dried pellets for biofumigation. *Ind Crop Prod* 20:59-65
- Lechevalier MP (1977) Lipids in bacterial taxonomy – a taxonomists view. *Crit Rev Microbiol* 5:109-210
- Leckie SE, Prescott CE, Grayston SJ, Neufeld JD, Mohn WW (2004) Characterization of humus microbial communities in adjacent forest types that differ in nitrogen availability. *Microb Ecol* 48:29-40
- Lepš J, Šmilauer P (1999) Multivariate analysis of ecological data. Faculty of Biological Sciences, University of South Bohemia Ceske Budejovice, 110p
- Lepš J, Šmilauer P (2003) Multivariate analysis of ecological data using CANOCO. Cambridge University Press, 15p
- Le Tourneau D (1979) Morphology, cytology, and physiology of *Sclerotinia* species in culture. *Phytopathology* 69(8):887-890
- Liang V, Rahman MH, Strelkov SE, Kav NNV (2010) Developmentally induced changes in the sclerotial proteome of *Sclerotinia sclerotiorum*. *Fungal Biology* 114:619-627
- Lichtenthaler HK, Miehe JA (1997) Fluorescence imaging as a diagnostic tool for plant stress. *Trends Plant Sci* 2:316-320
- Lin Q, Brookes PC (1999) An evaluation of the substrate-induced respiration method. *Soil Biol Biochem* 31:1969-1983
- Lo YMD, Chan KCA (2006) Introduction to the Polymerase Chain Reaction. In: Lo et al (ed) *Clinical Applications of PCR*. Humana Press, Totowa, New Jersey, 200p
- Lu G (2003) Minireview: Engineering *Sclerotinia sclerotiorum* resistance in oilseed crops. *Afr J Biotechnol* 2(12):509-516
- Lumsden RD (1979) Histology and physiology of pathogenesis in plant disease caused by *Sclerotinia* species. *Phytopathology* 69:890-896
- Lupwayi NZ, Arshad MA, Rice WA, Clayton GW (2001) Bacterial diversity in water-stable aggregates of soils under conventional and zero tillage management. *Appl Soil Ecol* 16:251-261
- Lupwayi NZ, Rice WA, Clayton GW (1998) Soil microbial diversity and community structure under wheat as influenced by tillage and crop rotation. *Soil Biol Biochem* 30:1733-1741
- Macias FA, David C, Molinillo JMG, David M, Varela RM, Torres A (2003) Synthesis of heliannane skeletons. Facile preparation of (–)-heliannuol D. *Tetrahedron* 59:1679-1683
- Macias FA, Galindo JLG, Torres A, Varcla RM, Marin D, Molinillo JM (2005) Proceedings of 4th World Congress on Allelopathy, August 2005, Wagga Wagga, Australia
- Makoi JHJR, Ndakidemi PA (2008) Review: Selected soil enzymes: Examples of their potential roles in the ecosystem. *Afr J Biotechnol* 7(3):181-191
- Marsal J, Girona J (1997) Effects of water stress cycles on turgor maintenance processes in pear leaves (*Pyrus communis*). *Tree Physiol* 17:327-333
- Martinez CE, Tabatabai MA (1997) Decomposition of biotechnology by-products in soils. *J Environ Qual* 26:625-632
- Matthiessen JN, Kirkegaard JA (2006) Biofumigation and enhanced biodegradation: opportunity and challenge in soilborne pest and disease management. *CRC Crit Rev Plant Sci* 25:235-265
- Matthiessen JN, Shackleton MA (2005) Biofumigation: environmental impacts on the biological activity of diverse pure and plant-derived isothiocyanates. *Pest Manag Sci* 61:1043-1051
- Matthiessen JN, Warton B, Shackleton MA (2004) The importance of plant maceration and water addition in achieving high *Brassica*-derived isothiocyanates levels in soil. *Agroindustria* 3:277-280
- Maxwell K, Johnson GN (2000) Review article: Chlorophyll fluorescence – a practical guide. *J Exp Bot* 51(345):659-668
- Maxwell M, Lumsden R (1970) Oxalic acid production by *S.sclerotiorum* in infected bean and in culture. *Phytopathology* 60:1395-1398

- Mazzola M, Granatstein DM, Elfving DC, Mullinix K (2001) Suppression of specific apple root pathogens by *Brassica napus* seed meal amendment regardless of glucosinolate content. *Phytopathology* 91:673-679
- McCully ME, Miller C, Sprague SJ, Huang CX, Kirkegaard JA (2008) Distribution of glucosinolates and sulphur-rich cells in roots of field-grown canola (*Brassica napus*). *New Phytol* 180:193-205
- McGill WB, Cannon KR, Robertson A, Cook FD (1986) Dynamics of soil microbial biomass and water-soluble organic C in Breton L after 50 years of cropping to two rotations. *Can J Soil Sci* 66:1-19
- McGill WB, Colle CV (1981) Comparative aspects of cycling of organic C, N, S and P through soil organic matter. *Geoderma* 26:267-286
- McGill WB, Hunt HW, Woodmansee RG, Reuss JO (1981) PHOENIX, a model of the dynamics of carbon and nitrogen in grassland soils. In: Clark FE and Rosswall T (ed) *Terrestrial Nitrogen Cycles*. Ecological Bulletins, Stockholm, Sweden, pp 49-116
- McKinley VL, Peacock AD, White DC (2005) Microbial community PLFA and PHB responses to ecosystem restoration in tallgrass prairie soils. *Soil Biol Biochem* 37:1946-1958
- McLean EO (1982) Soil pH and lime requirements. In: Page AL (ed) *Methods of soil analysis*. Part 2. Am. Soc. Agron. Madison, Wis, 199p
- McNaughton SJ (1994) Biodiversity and function of grazing ecosystems. In: Schulze ED and Mooney HA (ed) *Biodiversity and Ecosystem Function*. Springer-Verlag, London, pp 361-383
- McNaughton SJ, Jenkins TL, Wimpee MH, Cormier MR, White DC (1997) Rapid extraction of lipid biomarkers from pure culture and environmental samples using pressurized accelerated hot solvent extraction. *J Microbiol Meth* 31:19-27
- Misra AN, Srivastava A, Strasser RJ (2001) Utilization of fast chlorophyll a fluorescence technique in assessing the salt / ion sensitivity of mung bean and Brassica seedlings. *J Plant Physiol* 158:1173-1181
- Mithen RF (2001) Glucosinolates and their degradation products. *Adv Bot Res* 35:213-262
- Mondini C, Insam H (2003) Community level physiological profiling as a tool to evaluate compost maturity: a kinetic approach. *Eur J Soil Biol* 39:141
- Montgomery HJ, Monreal CM, Young JC, Seifert KA (2000) Determination of soil fungal biomass from soil ergosterol analyses. *Soil Biol Biochem* 32:1207-1217
- Moore WD (1949) Flooding as a means of destroying sclerotia of *Sclerotinia sclerotiorum*. *Phytopathology* 39:920-927
- Moore-Kucera J, Dick RP (2008a) Application of ¹³C-labeled litter and root materials for in situ decomposition studies using phospholipid fatty acids. *Soil Biol Biochem* 40:2485-2493
- Moore-Kucera J, Dick RP (2008b) PLFA profiling of microbial community structure and seasonal shifts in soils of a Douglas-fir chronosequence. *Microb Ecol* 55:500-511
- Morales F, Belkhouja R, Abadia A, Abadia J (2000) Photosystem II efficiency and mechanisms of energy dissipation in iron-deficient, field-grown pear trees (*Pyrus communis* L.). *Photosynth Res* 63:9-21
- Morra MJ, Kirkegaard JA (2002) Isothiocyanate release from soil-incorporated *Brassica* tissues. *Soil Biol Biochem* 34:1683-1690
- Motisi N, Doré T, Lucas P, Montfort F (2010) Dealing with the variability in biofumigation efficacy through an epidemiological framework. *Soil Biol Biochem* 42:2044-2057
- Muller T, Hoper H (2004) Soil organic matter turnover as a function of the soil clay content: consequences for model applications. *Soil Biol Biochem* 36:877-888
- Muyzer G (1999) DGGE/TGGE a method for identifying genes from natural ecosystems. *Curr Opin Microbiol* 2:317-322
- Myers RT, Zak DR, White DC, Peacock A (2001) Landscape-level patterns of microbial community composition and substrate use in upland forest ecosystems. *Soil Sci Soc Am J* 65:359-367
- Nakamoto T, Wakahara S (2004) Development of substrate induced respiration (SIR) method combined with selective inhibition for estimating fungal and bacterial biomass in humic Andosols. *Plant Prod Sci* 7(1):70-76

- Nannipieri P (1994) The potential use of soil enzymes as indicators of soil productivity, sustainability, and pollution. In: Pankhurst CE, Doube BM, Gupta VVSR and Grace PR (ed) *Soil Biota: Management in Sustainable Farming Systems*. CSIRO Australia, Victoria, pp 238-244
- Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G, Renella G (2003) Microbial diversity and soil functions. *Eur J Soil Sci* 54:655-670
- Nannipieri P, Grego S, Ceccanti B (1990) Ecological significance of biological activity. In: Bollag J-M and Stotzky G (ed) *Soil Biochemistry*, vol. 6. Marcel Dekker, New York, pp 293-355
- Nannipieri P, Kandeler E, Ruggiero P (2002) Enzyme activities and microbial and biochemical processes in soil. In: Burns RG and Dick RP (ed) *Enzymes in the Environment: Activity, Ecology, and Applications*. Marcel Dekker, New York, pp 1-33
- Nannipieri P, Sequi p, Fusi P (1996) Humus and enzyme activity. In: Piccolo A (ed) *Humic Substances in Terrestrial Ecosystems*. Elsevier, New York, pp 293-328
- Ndakidemi PA (2006) Manipulating legume / cereal mixtures to optimize the above and below ground interactions in the traditional African cropping systems. *Afr J Biotechnol* 5(25):2526-2533
- N'Dayegamiye A, Tran TS (2001) Effects of green manures on soil organic matter and wheat yields and N nutrition. *Can J Soil Sci* 81:371-382
- Ndiaye EL, Sandeno JM, McGrath D, Dick RP (2000) Integrative biological indicators for detecting change in soil quality. *Am J Altern Agric* 15:26-36
- Nehl DB, Allen SJ, Brown JF (1996) Deleterious rhizosphere bacteria: an integrating perspective. *Appl Soil Ecol* 5:1-20
- Newman MC (2010) *Fundamentals of Ecotoxicology*, 3rd edn. Taylor & Francis Group, Boca Raton, 541p
- Newell SY, Miller JD, Fallon RD (1987) Ergosterol content of salt-marsh fungi: effect of growth conditions and mycelial age. *Mycologia* 79:688-695
- Nocker A, Burr M, Camper AK (2007) Genotypic microbial community profiling: A critical technical review. *Microb Ecol* 54:276-289
- Oka Y (2010) Mechanisms of nematode suppression by organic soil amendments – A review. *Appl Soil Ecol* 44:101-115
- O'Leary WM, Wilkinson SG (1988) Gram-positive bacteria. In: Rateledge C and Wilkinson SG (ed) *Microbial Lipids*. Academic Press, London, pp 117-185
- Omirou M, Rousidou C, Bekris F, Papadopoulou KK, Menkissoglou-Spiroudi U, Ehaliotis C, Karpouzas DG (2010) The impact of biofumigation and chemical fumigation methods on the structure and function of the soil microbial community. *Microb Ecol* 61(1):201-213
- Oukarroum A, Madidi SE, Schansker G, Strasser RJ (2007) Probing the responses of barley cultivars (*Hordeum vulgare* L.) by chlorophyll a fluorescence OLKJIP under drought stress and re-watering. *Environ Exp Bot* 60:438-446
- Pancholy SK, Rice EL (1973) Soil enzymes in relation to old field succession: amylase, cellulose, invertase, dehydrogenase and urease. *SSSAJ* 37:47-50
- Pankhurst CE, Hawke BG, McDonald HJ, Kirkby CA, Buckerfield JC, Michelson P, O'Brien KA, Gupta VVSR, Doube BM (1995) Evaluation of soil biological properties as potential bioindicators of soil health. *Aus J Exp Agric* 35:1015-1028
- Pankhurst CE, Kirkby CA, Hawke BG, Harch BD (2002) Impact of a change in tillage and crop residue management practice in soil chemical and microbiological properties in a cereal-producing red duplex soil in NSW, Australia. *Biol Fert Soils* 35:189-196
- Pankhurst CE, Yu S, Hawke BG, Harch BD (2001) Capacity of fatty acid profiles and substrate utilization patterns to describe differences in soil microbial communities associated with increased salinity or alkalinity at three locations in South Australia. *Biol Fert Soils* 33:204-217
- Parkinson D, Gray TRG, Williams ST (1971) *IBP handbook No. 19: Methods for studying the ecology of soil microorganisms*. Blackwell Scientific Publ., Oxford
- Peacock GA, Goosey MW (1989) Separation of fungal sterols by normal-phase high-performance liquid chromatography. *J Chromatogr* 469:293-304

- Peacock AD, Mullen MD, Ringelberg DB, Tyler DD, Hedrick DB, Gale PM, White DC (2001) Soil microbial community responses to dairy manure or ammonium nitrate applications. *Soil Biol Biochem* 33:1011-1019
- Peech M (1965) Hydrogen-ion activity. In: Black CA (ed) *Methods of soil analysis. Part 2.* Am. Soc. Agron. Madison, Wis. pp 914-926
- Pennanen T, Liski J, Bååth E, Kitunen V, Uotila J, Westman CJ, Fritze H (1999) Structure of the microbial communities in coniferous forest soils in relation to site fertility and stand development stage. *Microb Ecol* 38:168-179
- Percival GC, Biggs MP, Dixon GR (1998) The influence of sodium chloride and waterlogging stresses on *Alnus Cordata*. *J Arboric* 24(1):19-27
- Pettersson M, Bååth E (2003) Temperature-dependent changes in the soil bacterial community in limed and unlimed soils. *FEMS Microbiol Ecol* 45:13-21
- Pfender WR, Fieland VP, Ganio LM, Seidler RJ (1996) Microbial community structure and activity in wheat straw after inoculation with biological control organisms. *Appl Soil Ecol* 3:69-78
- Pianka ER (1970) On r- and K-selection. *Am Nat* 104:592-597
- Pietikäinen J, Hiukka R, Fritze H (2000) Does short-term heating of forest humus change its properties as a substrate for microbes? *Soil Biol Biochem* 32:277-288
- Pinkart HC, Ringelberg DB, Piceno YM, Macnaughton SJ, White DC (2002) Biochemical approaches to biomass measurement and community structure analysis. In: Hurst CJ, Crawford RL, Knudsen GR, McInerney MJ and Stetzenbach LD (ed) *Manual of Environmental Microbiology*, 2nd edn. American Society for Microbiology Press, Washington DC, pp 101-113
- Potts M (1994) Desiccation tolerance of prokaryotes. *Microbiol Rev* 58:755-805
- Pryor RJ, Wittwer CT (2008) Real-Time polymerase chain reaction and melting curve analysis. In: Lo et al (ed) *Clinical Applications of PCR*, 2nd edn. Humana Press, Totowa, New Jersey, 200p
- Purdy LH (1979) *Sclerotinia sclerotiorum*: History, diseases and symptomatology, host range, geographic distribution and impact. *Phytopathology* 69(8):875-880
- Raaijmakers JM, Paulitz TC, Steinberg C, Alabouvette C, Moënne-Loccoz Y (2009) The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant Soil* 321:341-361
- Ramaswami A, Carr P, Burkhardt M (2001) Plant uptake of uranium: Hydroponic and soil system studies. *International Journal of Phytoremediation* 3:189-201
- Ramirez-Munoz J (1968) Atomic absorption spectroscopy and analysis by atomic absorption flame photometry. New York, Am. Elsevier Publ. Co
- Rausch T, Wachter A (2005) Sulfur metabolism: a versatile platform for launching defence operations. *Trends in Plant Sci* 10:503-509
- Reeslev M, Miller M, Nielsen KF (2003) Quantifying mold biomass on gypsum board: comparison of ergosterol and beta-N-acetylhexosaminidase as mold biomass parameters. *Appl Environ Microbiol* 69:3996-3998
- Reynolds SG (1971) A manual of introductory soil science and simple soil analysis methods. Information Document, No.24. South Pacific Commission, Noumea, New Caledonia, 237p
- Rice EL (1984) Allelopathy. Academic Press, Orlando, FL, 422p
- Rodriguez E, Pickard MA, Vazquez-Duhalt R (1999) Industrial dye decolorization by laccase from Ligninolytic fungi. *Curr Microbiol* 38:27-32
- Roger-Estrade J, Anger C, Bertrand M, Richard G (2010) Tillage and soil ecology: Partners for sustainable agriculture. *Soil Till Res* 111:33-40
- Rogers HH, Runion GB, Krupa SV (1994) Plant responses to atmospheric CO₂ enrichment with emphasis on roots and the rhizosphere. *Environ Pollut* 83:155-189
- Sanogo S (2007) Interactive effects of two soilborne pathogens, *Phytophthora capsici* and *Verticillium dahliae*, on chile pepper. *Phytopathology* 97(1):37-43
- Sarathchandra SU, Burch G, Cox NR (1997) Growth patterns of bacterial communities in the rhizoplane and rhizosphere of white clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.) in long-term pasture. *Appl Soil Ecol* 6:293-299

- Sarwar M, Kirkegaard JA (1998) Biofumigation potential of brassicas. II. Effect of environment and ontogeny on glucosinolate production and implications for screening. *Plant Soil* 201:91-101
- Sarwar M, Kirkegaard JA, Wong PTW, Desmarchelier JM (1998) Biofumigation potential of *Brassicas*. III. In vitro toxicity of isothiocyanates to soil-borne fungal pathogens. *Plant Soil* 210:103-112
- Schansker G, Toth SZ, Strasser RJ (2005) Methylviologen and dibromothymo-quinone treatments of pea leaves reveal the role of photosystem I in the Chl a fluorescence rise OJIP. *Biochim Biophys Acta* 1706:250-261
- Scheepers CCW (2011) Physiological and biochemical constraints on photosynthesis of leguminous plants induced by elevated ozone in open-top-chambers. Dissertation: M.Sc. Plant Physiology. School of Environmental Sciences and Development, North-West University, Potchefstroom, South Africa, 153p
- Schlesinger WH, Raikes JA, Hartley AE, Cross AF (1996) On the spatial pattern of soil nutrients in desert ecosystems. *Ecology* 72:364-374
- Schneider AA, Miller JF (1981) Description of Sunflower growth stages. *Crop Sci* 21:901-903
- Scholander PF, Hammel HT, Hemmingsen EA, Bradstreet ED (1964) Hydrostatic pressure and osmotic potential in leaves of mangroves and some other plants. *P Natl Acad Sci USA* 52:119-125
- Schollenberger CJ, Simon RH (1945) Determination of exchange capacity and exchangeable cations in soil with ammonium acetate. *Soil Sci* 59:13-24
- Schwitzguébel J, Van der Lelie D, Baker A, Glass DJ, Vangronsveld J (2002) Phytoremediation: European and American trends. Successes, obstacles and needs. *J Soil Sediment* 2(2):91-99
- Seastone-Moynahan O, Zabinski CA, Gannon JE (2002) Microbial community structure and carbon utilization diversity in a mine tillage revegetation study. *Restor Ecol* 10:77-87
- Seiler GJ (2007) The potential of wild sunflower species for industrial uses. *Helia* 30(46):175-198
- Shannon CE, Weaver W (1949) The mathematical theory of communication. Urbana, Illinois: University of Illinois
- Shapira R, Ordentlich A, Chet I, Oppenheim AB (1989) Control of plant diseases by chitinase expressed from cloned DNA in *Escherichia coli*. *Phytopathology* 79:1246-1249
- Sharma S, Rangger A, Von Lütow M, Insam H (1998) Functional diversity of soil bacterial communities increases after maize litter amendment. *Eur J Soil Biol* 34:53-60
- Shen Y, Stehmeier LG, Voordouw G (1998) Identification of hydrocarbon-degrading bacteria in soil by reverse sample genome probing. *Appl Environ Microbiol* 64:637-645
- Shengnan C, Jie G, Hua G, Qingjun Q (2011) Effect of microbial fertilizer on microbial activity and microbial community diversity in the rhizosphere of wheat growing on the Loess Plateau. *Afr J Microbiol Res* 5(2):137-143
- Simpson JR, Freney JR (1988) Interacting processes in gaseous nitrogen loss from urea applied to flooded rice fields. In: Pushparajah E et al. (ed) *Proceedings of International Symposium on Urea Technology and Utilization*. Malaysian Society of Soil Science, Kuala Lumpur, pp. 281-290
- Six J, Elliott ET, Paustian K, Doran JW (1998) Aggregation and soil organic matter accumulation in cultivated and native grassland soils. *Soil Sci Soc Am J* 62:1367-1377
- Six J, Feller C, Denef K, Ogle SM, Moraes Sa JC, Albrecht A (2002) Soil organic matter, biota and aggregation in temperate and tropical soils – Effects of no-tillage. *Agronomie* 22(7/8):755-775
- Skujins J (1976) Extracellular enzymes in soil. *Crit Rev Biotechnol* 4:383-421
- Smalla K, Wachtendorf U, Heuer H, Liu WT, Forney L (1998) Analysis of BIOLOG GN substrate utilization patterns by microbial communities. *Appl Environ Microbiol* 64:1220-1225
- Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, Roskot N, Heuer H, Berg G (2001) Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. *Appl Environ Microbiol* 67:4742-4751
- Smith P (2004) Carbon sequestration in croplands: the potential in Europe and the global context. *Eur J Agron* 20:229-236

- Smith BJ, Kirkegaard JA (2002) *In vitro* inhibition of soil microorganisms by 2-phenylethyl isothiocyanate. *Plant Pathol* 51:585-593
- Smith GA, Nickels JS, Kerger BD, Davis JD, Collins SP, Wilson JT, McNabb JF, White DC (1985) Quantitative characterization of microbial biomass and community structure in subsurface material: a prokaryotic consortium responsive to organic contamination. *Can J Microbiol* 32:104-111
- Snapp SS, Date KU, Kirk W, O'Neil K, Kremen A, Bird G (2007) Root, shoot tissues of *Brassica juncea* and *Cereal secale* promote potato health. *Plant Soil* 294:55-72
- Sonnevelt C, Van Den Ende J (1971) Soil analysis by means of a 1:2 volume extract. *Plant Soil* 35:505-516
- South Africa. Department of Agriculture, Forestry and Fisheries (2010) Crops and Markets. Fourth quarter 2010 91(946):1-5
- Sparling GP (1995) The substrate-induced respiration method. In: Alef K and Nannipieri P (ed) *Methods in Applied Soil Microbiology and Biochemistry*. Academic Press, London, pp 397-404
- Speir TW, Ross DJ (1978) Soil phosphatase and sulphatase. In: Burns RG (ed) *Soil Enzymes*. Academic Press, London, UK, pp 197-250
- Staley TE 1999 Soil microbial biomass alterations during the maize silage growing season relative to tillage method. *Soil Sci Soc Am J* 63:1845-1847
- Statsoft, Inc. (2012) STATISTICA (data analysis software system), version 10. www.statsoft.com
- Steadman JR (1979) Control of plant diseases caused by *Sclerotinia* species. *Phytopathology* 69(8):904-907
- Stefanowicz A (2006) The Biolog plates technique as a tool in ecological studies of microbial communities: Review. *Pol J Environ Stud* 15(5):669-676
- Stirbet A, Govindjee (2011) On the relation between the Kautsky effect (chlorophyll a fluorescence induction) and photosystem II: Basics and applications of the OJIP fluorescence transient. *Journal of Photochemistry and Photobiology B: Biology*
- Stolz JF, Oremland RS (1999) Bacterial respiration of arsenic and selenium. *FEMS Microbiol Rev* 23:615-627
- Strasser RJ (1997) Donor side capacity of photosystem II probed by chlorophyll a fluorescence transients. *Photosynth Res* 52:147-155
- Strasser RJ, Srivastava A, Govindjee (1995) Polyphasic chlorophyll a fluorescence transient in plants and cyanobacteria. *Photochem Photobiol* 61:32-42
- Strasser RJ, Srivastava A, Tsimilli-Michael M (2000) The fluorescence transient as a tool to characterize and screen photosynthetic samples. In: Yunus M, Pathre U and Mohanty P (ed) *Probing Photosynthesis: Mechanism, Regulation and Adaptation*. Taylor and Francis, London, UK, pp 443-480
- Strasser RJ, Tsimilli-Michael M (2001) Stress in plants, from daily rhythm to global changes, detected and quantified by the JIP-test. *Chimie Bouvella (SRC)* 75:3321-3326
- Strasser RJ, Tsimilli-Michael M, Qiang S, Goltsev V (2010) Simultaneous *in vivo* recording of prompt and delayed fluorescence and 820-nm reflection changes during drying and after rehydration of the resurrection plant *Haberlea rhodopensis*. *Biochim Biophys* 1797:1313-1326
- Strasser RJ, Tsimilli-Michael M, Srivastava A (2004) Analysis of the chlorophyll a fluorescence transient. In: Papageorgiou GC and Govindjee (ed) *Chlorophyll Fluorescence: A signature of photosynthesis*. Kluwer Academic Publishers. Printed in The Netherlands
- Tabatabai MA (1994) Soil enzymes. In: Weaver RW, Angle JS, Bottomley PS (ed) *Methods of soil analysis, part 2. Microbiological and biochemical properties*. SSSA Book Series No. 5. Soil Sci. Soc. Am. Madison, Wis., pp 775-833
- Tabuchi H, Kato K, Nioh I (2008) Season and soil management affect soil microbial communities estimated using phospholipid fatty acid analysis in a continuous cabbage (*Brassica oleracea* var. *Capitata*) cropping system. *Soil Sci Plant Nutr* 54:369-378
- Taylor JP, Wilson B, Mills MS, Burns RG (2002) Comparison of microbial numbers and enzymatic activities in surface soils and subsoils using various techniques. *Soil Biol Biochem* 34:387-401

- Teo BK, Morrall RAA (1985) Influence of matric potentials on carpogenic germination of sclerotia of *Sclerotinia sclerotiorum*. I. Development of an inclined box technique to observe apothecium production. *Can J Plant Pathol* 7:359-364
- Ter Braak CJF, Verdonschot PFM (1995) Canonical correspondence analysis and related multivariable methods in aquatic ecology. *Aquat Sci* 57:153-298
- Thiet RK, Frey SD, Six J (2006) Do growth yield efficiencies differ between soil microbial communities differing in fungal: bacterial ratios? Reality check and methodological issues. *Soil Biol Biochem* 38:837-844
- Thoma JA, Spradlin JE, Dygert S (1971) Plant and animal amylases. In: Boyer PD (ed) *The Enzymes*, pp 115-189
- Thomas GW (1982) Exchangeable acidity. In: Page AL (ed) *Methods of soil analysis. Part 2*. Am. Soc. Agron. Madison, Wis, pp 159-161
- Tiwari MB, Tiwari BK, Mishra RR (1989) Enzyme activity and carbon dioxide evolution from upland and wetland rice soil under three agricultural practices in hilly regions. *Biol Fert Soils* 7:359-364
- Torstensson L (1980) Role of microorganisms in decomposition. Interaction between Herbicides and the Soil (Hance RJ, eds). London: Academic Press, pp 159-177
- Trevino V, Falciani F, Barrera-Saldana, HA (2007) DNA microarrays: a powerful genomic tool for biomedical and clinical research. *Mol Med* 13:527-541
- Trevors JT (1984) Dehydrogenase activity in soil: A comparison between INT and TTC assay. *Soil Biol Biochem* 16:673-674
- Trojan MD, Linden DR (1998) Macroporosity and hydraulic properties of earthworm-affected soils as influenced by tillage and residue management. *Soil Sci Soc Am J* 62:1687-1692
- Tsimilli-Michael M, Eggenberg P, Biro B, Köves-Pechy K, Vörös I, Strasser RJ (2000) Synergistic and antagonistic effects of arbuscular mycorrhizal fungi and *Azospirillum* and *Rhizobium* nitrogen-fixers on the photosynthetic activity of alfalfa, probed by the polyphasic chlorophyll a fluorescence transient O-J-I-P. *Appl Soil Ecol* 15:169-182
- Tunlid A, Baird BH, Trexler MB, Olsson S, Findlay RH, Odham G, White DC (1985) Determination of phospholipid ester-linked fatty acids and poly β -hydroxybutyrate for the estimation of bacterial biomass and activity in the rhizosphere of the rape plant *Brassica napus* (L.). *Can J Microbiol* 31:1113-1119
- Unger IM, Kennedy AC, Muzika R (2009) Flooding effects on soil microbial communities. *Appl Soil Ecol* 42:1-8
- U.S. Dept. Agric. (1951) *Soil survey manual. Handbook, No.18*
- United States Salinity Laboratory Staff (1969) *Diagnosis and improvement of saline and alkaline soils*. U.S. Laboratory Handbook 60, USDA, Washington
- Uttamchandani M, Neo JL, Ong BNZ, Moochhala S (2008) Applications of microarrays in pathogen detection and biodefence. *Trends Biotechnol* 27(1):53-61
- Vance ED, Brookes PC, Jenkinson DS (1987) An extraction method for measuring soil microbial biomass-C. *Soil Biol Biochem* 19:703-707
- Van de Werf H, Verstraete W (1987) Estimation of active soil microbial biomass by mathematical analysis of respiration curves: calibration of the test procedure. *Soil Biol Biochem* 19:261-265
- Van Wyk PS, Viljoen A (2002) *Sunflower diseases and Pests*. Agricultural Research Council, 58p
- Von Mersi W, Schinner F (1991) An improved and accurate method for determining dehydrogenase activity of soils with iodinitrotetrazolium chloride. *Biol Fert Soils* 11:216-220
- Voordouw G, Voordouw JK, Jack TR, Foght J, Fedorak PM, Westlake DWS (1992) Identification of distinct communities of sulphate-reducing bacteria in oil-fields by reverse sample genome probing. *Appl Environ Microbiol* 58:3542-3552
- Waldrop MP, Balser TC, Firestone MK (2000) Linking microbial community composition to function in a tropical soil. *Soil Biol Biochem* 32:1837-1846
- Wallenstein MD, McNulty S, Fernández IJ, Boggs J, Schlesinger WH (2006) Nitrogen fertilization decreases forest soil fungal and bacterial biomass in three long-term experiments. *Forest Ecol Manag* 222:459-468

- Wander MM, Bidart MG, Aref S (1998) Tillage impacts on depth distribution of total and particulate organic matter in three Illinois soils. *Soil Sci Soc Am J* 62:1704-1711
- Wang D, Marschner P, Solaiman Z, Rengel Z (2007) Belowground interactions between intercropped wheat and *Brassicas* in acidic and alkaline soils. *Soil Biol Biochem* 39:961-971
- Wang D, Rosen C, Kinkel L, Cao A, Tharayil N, Gerik J (2009) Production of methyl sulphide and dimethyl disulfide from soil-incorporated plant materials and implications for controlling soilborne pathogens. *Plant Soil* 324:185-197
- Weete JD (1989) Structure and function of sterols in fungi. *Adv Lipid Res* 23:115-167
- Wegulo SN, Sun P, Martinson CA, Yang XB (2000) Spread of Sclerotinia stem rot of soybean from area and point sources of apothecial inoculum. *Can J Plant Sci* 80:389-402
- West AW (1986) Improvement of the selective inhibition technique to measure eukaryote-prokaryote ratios in soils. *Microbiol Meth* 5:125-138
- West NE (1990) Structure and function of microphytic soil crusts in wildland ecosystems of arid to semi-arid regions. *Adv Ecol Res* 20:179-223
- West AW, Sparling GP (1986) Correlation between four methods to estimate total microbial biomass in stored, air-dried and glucose-amended soils. *Soil Biol Biochem* 18(6):569-576
- White DC, Davis WM, Nickels JS, King JD, Bobbie, RJ (1979) Determination of the sedimentary Microbial Biomass by Extractable Lipid Phosphate. *Oecologia* 40:51-62
- White DC, Flemming CA, Leung KT, Macnaughton SJ (1998) In situ microbial ecology for quantitative appraisal, monitoring and risk assessment of pollution remediation in soils, the subsurface, the rhizosphere and in biofilms. *J Microbiol Meth* 32:93-105
- White DC, Macnaughton SJ (1997) Chemical and molecular approaches for rapid assessment of the biological status of soils. In: Pankhurst CE, Doube BM and Gupta VVSR (ed) *Biological Indicators of Soil Health*. CAB International, pp 371-396
- White DC, Pinkart HC, Ringelberg AB (1997) Biomass measurements: biochemical approaches. In: Hurst CJ, Knudson GR, McInerney MJ, Stetzenbach LD and Walter MV (ed) *Manual of Environmental Microbiology*. ASM Press, DC, pp 91-101
- White DC, Ringelberg DB (1996) Monitoring deep subsurface microbiota for assessment of safe long-term nuclear waste disposal. *Can J Microbiol* 42:375-381
- Whitford WG (1996) The importance of the biodiversity of soil biota in arid ecosystems. *Biodivers Conserv* 5:185-195
- Wichern F, Hafeel K (2004) The fungal-bacterial ratio: Tipping the balance for soil health. *Soil is Alive Newsletter* 3(3), School of Earth and Geographical Sciences, The University of Western Australia
- Wiggins BE, Kinkel LL (2005) Green manures and crop sequences influence potato diseases and pathogen inhibitory activity of indigenous Streptomycetes. *Phytopathology* 95:178-185
- Wilhelm S (1955) Longevity of *Verticillium* wilt fungus in the laboratory and the field. *Phytopathology* 45:180-181
- Winding A (1994) Fingerprinting bacterial soil communities using Biolog microtitre plates. In: Ritz K, Dighton J and Giller KE (ed) *Beyond the biomass*. Wiley-Sayce, London, pp 85-94
- Winding A, Binnerup SJ, Sorensen J (1994) Viability of indigenous soil bacteria assayed by respiratory activity and growth. *Appl Environ Microbiol* 60:2869
- Winding A, Hund-Rinke K, Rutgers M (2005) The use of microorganisms in ecological soil classification and assessment concepts. *Ecotox Environ Safe* 62:230-248
- Wu Y, Ding N, Wang G, Xu J, Wu J, Brookes PC (2009) Effects of different soil weights, storage times and extraction methods on soil phospholipid fatty acid analyses. *Geoderma* 150:171-178
- Wünsche L, Brüggemann L, Babel W (1995) Determination of substrate utilization patterns of soil microbial communities: An approach to assess population changes after hydrocarbon pollution. *FEMS Microbiol Ecol* 17:295-306
- Xu Y, Wang G, Jin J, Liu J, Zhang Q, Liu X (2009) Bacterial communities in soybean rhizosphere in response to soil type, soybean genotype, and their growth stage. *Soil Biol Biochem* 41:919-925

- Yang B, Srivastava S, Deyholos MK, Kav NNV (2007) Transcriptional profiling of canola (*Brassica napus* L.) responses to the fungal pathogen *Sclerotinia sclerotiorum*. *Plant Sci* 173:156-171
- Yang ZX, Liu SQ, Zheng DW, Feng SD (2006) Effects of cadmium, zinc and lead on soil enzyme activities. *J Environ Sci* 18(6):1135-1141
- Yao H, He Z, Wilson MJ, Campbell CD (2000) Microbial biomass and community structure in a sequence of soils with increasing fertility and changing land use. *Microb Ecol* 40:223-237
- Yeung KY, Ruzzo WL (2000) An empirical study on Principle Component Analysis for clustering gene expression data. Department of Computer Science & Engineering, University of Washington, Seattle, WA
- Yulianti T, Sivasithamparam K, Turner DW (2006) Response of different forms of propagules of *Rhizoctonia solani* AG2-1 (ZG-5) exposed to the volatiles produced in soil amended with green manures. *Ann Appl Biol* 148:105-111
- Yulianti T, Sivasithamparam K, Turner DW (2007) Saprophytic and pathogenic behaviour of *R. solani* AG2-1 (ZG-5) in a soil amended with *Diplotaxis tenuifolia* or *Brassica nigra* manures and incubated at different temperatures and soil water content. *Plant Soil* 294:277-289
- Yusuf MA, Kumar D, Rajwanshi R, Strasser RJ, Tsimilli-Michael M, Govindjee, Sarin NB (2010) Overexpression of γ -tocopherol methyl transferase gene in transgenic *Brassica juncea* plants alleviates abiotic stress: Physiological and chlorophyll a fluorescence measurements. *Biochim Biophys Acta* 1797:1428-1438
- Zaady E, Ben-David EA, Sher Y, Tzirkin R, Nejdat A (2010) Inferring biological soil crust successional stage using combined PLFA, DGGE, physical and biophysiological analyses. *Soil Biol Biochem* 42:842-849
- Zak JC, Willig MR, Moorhead DL, Wildman HG (1994) Functional diversity of microbial communities: a quantitative approach. *Soil Biol Biochem* 26:1101-1108
- Zasada IA, Ferris H (2003) Sensitivity of *Meloidogyne javanica* and *Tylenchulus semipenetrans* to isothiocyanates in laboratory assays. *Phytopathology* 93:747-750
- Zasada IA, Ferris H (2004) Nematode suppression with brassicaceous amendments: application based upon glucosinolates profiles. *Soil Biol Biochem* 36:1017-1024
- Zegada-Lizarazu W, Monti A (2010) Energy crops in rotation. A review. *Biomass Bioenerg* :1-14
- Zelles L (1997) Phospholipid fatty acid profiles in selected members of soil microbial communities. *Chemosphere* 35:275-294
- Zelles L (1999) Fatty acid patterns of phospholipids and lipopolysaccharides in the characterization of microbial communities in soil: a review. *Biol Fert Soils* 29:111-129
- Zelles L, Adrian P, Bai QY, Stepper K, Adrian MV, Fischer K, Maier A, Ziegler A (1991) Microbial activity measured in soils stored under different temperature and humidity conditions. *Soil Biol Biochem* 23:955-962
- Zelles L, Bai QY, Beck T, Beese F (1992) Signature fatty acids in phospholipids and lipopolysaccharides as indicators of Microbial Biomass and community structure in agricultural soils. *Soil Biol Biochem* 24(4):317-323
- Zelles L, Bai QY, Rackwitz R, Chadwick D, Beese F (1995) Determination of phospholipid – and lipopolysaccharide-derived fatty acids as an estimate of microbial biomass and community structures in soils. *Biol Fert Soils* 19:115-123
- Zhang Z (2008) DGGE technique and its application in marine environmental microbial diversity study. National Marine Environmental Monitoring Center. [Powerpoint Presentation]. 2008-10-30
- Zhang B, Bai Z, Hoefel D, Tang L, Yang Z, Zhuang G, Yang J, Zhang H (2008) Assessing the impact of the biological agent *Bacillus thuringiensis* on the indigenous microbial community within the pepper plant phyllosphere. *FEMS Microb Lett* 284:102-108