NOTE TO USERS

FATTY ACID BIOMARKER ANALYSIS TO CHARACTERIZE SOIL MICROBIAL COMMUNITIES IN SOYBEAN AGROECOSYSTEMS WITH SCLEROTINIA STEM ROT DISEASE

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SOIL MICROBIAL COMMUNITIES IN SOYBEAN AGROECOSYSTEMS AFFECTED BY SCLEROTINIA

ABSTRACT

Soybean (*Glycine max* (L.) Merr.) is one the major crops produced worldwide. However, soybean is susceptible to many diseases. Sclerotinia stem rot (SSR) disease caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is considered one of the most important fungal diseases of soybean. It can be controlled by chemicals (e.g. fungicides), by breeding cultivars with disease resistance and by cultural control (e.g. increasing the width between rows, reducing plant populations). A promising and complementary method of controlling SSR disease in the field is the application of biological control agents. Biological control agents introduced in a soil environment will interact with other soil food web organisms, as do the pathogenic organism and infected plants, which may change the genetic and functional diversity in soil microbial communities. Profiling these changes may lead to an improved understanding of the interactions between these players (biological control agents, pathogens, soil biota and plants) in the biological control phenomenom, permiting us to exploit naturally-occurring ecological relationships and develop more sustainable approaches to control soybean diseases. Fatty acid biomarkers analysis was used to profile microbial communities in soils. Two laboratory studies were conducted to evaluate the methods used for extraction and profiling the fatty acid biomarkers from soil samples with a range of soil properties (clay content, organic matter content). The first study investigated the best solvent mixture for recovering fatty acid biomarkers from soil using an automated pressurized solvent extraction (PSE) system. Solvent mixtures containing chloroform and methanol were more efficient at extracting fatty acids from agricultural soils than hexane:2-propanol and acetone. The second study presented an exploratory pyrolysis-mass spectrometry technique to rapidly fingerprint soil lipids extracted from different agroecosystems. Pyrolysis-mass spectrometry discriminated among soils and crop production systems in the same way as the fatty acid profiling. I also report on the efficicacy of biological control agents to control Sclerotinia stem rot disease in soybean. A two-year study was conducted in soybean fields under conventional or no tillage to determine whether *Trichoderma virens* (SoilGard™) and arbuscular mycorrhizal fungi (a mixture of *Glomus intraradices* and *G. mosseae*), used alone or in combination, could reduce sclerotinia stem rot (SSR) disease incidence. Generally, SSR disease indicators, as well as the soybean yield, were not affected significantly by the biological control treatments. I then studied whether changes in microbial community composition were related to the inoculation of the biological control agents and the

disease incidence in soybean fields. Inoculation of biological control agents changes the expression of many soil fatty acids during both years of the trial. Also, in the plots with severely diseased plants, fatty acids biomarkers of gram positive and actinomycetes bacteria were significantly greater than in plots with healthy plants. I conclude that further improvement in laboratory techniques and procedures will permit researchers to efficiently extract and characterize soil lipids, providing new insight into soil organic matter dynamics and soil microbial ecology. Further study will be needed to verify the efficacy and optimize the application method, dose and timing of biocontrol agents to provide protection against SSR disease in soybean fields.

RESUME

Le soya (*Glycine max* (L.) Merr.) est une des cultures les plus importantes dans le monde entier. Cependant, le soya est sensible à beaucoup de maladies. Le pourridié sclérotique causé par *Sclerotinia sclerotiorum* (Lib.) de Bary est consideré comme une des maladies fongiques les plus importantes du soya. Cette maladie peut être contrôlée par des produits chimiques (par exemple, des fongicides), par des croisements de cultivars résistants à la maladie et par des pratiques culturales (par exemple, en augmentant la largeur entre les rangs, en réduisant la densité des semis). L'utilisation d'agents de lutte biologique est une méthode prometteuse et complémentaire à ces moyens pour contrôler le pourridié sclérotique. Les agents de lutte biologique introduits dans un sol interagiront avec d'autres organismes du sol, de même qu'avec l'agent pathogène et les plantes infectées. Cette interaction pourrait changer la diversité génétique et fonctionnelle des communautés microbiennes du sol. La caractérisation de ces changements pourrait amener à comprendre les interactions entre les différents éléments impliqués (les agents de lutte biologique, les agents pathogènes, les composants vivants du sol et les plantes) dans le phénomène du contrôle biologique. Cette caractérisation permet d'exploiter les relations écologiques naturelles et de développer des approches plus durables pour contrôler cette maladie du soya. L'analyse des acides gras biomarqueurs a été employée pour caractériser les communautés microbiennes dans les sols. Dans un premier temps, deux études de laboratoire ont été entreprises pour évaluer les méthodes employées pour l'extraction et la caractérisation des acides gras biomarqueurs à partir d'échantillons de sol présentant une gamme de propriétés spécifiques (par exemple, quantités d'argile et de matière organique). La première étude évaluait le meilleur solvant organique pour extraire les acides gras biomarqueurs du sol au moyen d'un extracteur à solvant pressurisé. Les mélanges de solvants contenant le chloroforme et le méthanol étaient plus efficaces pour extraire les acides gras à partir des sols agricoles que les solvants organiques tels que l'hexane:2-propanol et l'acétone. La deuxième étude présentait une technique exploratoire de pyrolyse couplée à la spectrométrie de masse qui permet de prendre plus rapidement les empreintes lipidiques de sols provenant de différents agroécosystèmes. La pyrolyse couplée à la spectrométrie de masse permet de différencier les sols et les systèmes de production de culture de la même façon qu'en utilisant la technique de caractérisation par les acides gras biomarqueurs. Je présente aussi une étude sur l'efficacité des agents de lutte biologique contre le pourridié sclérotique du soya. Dans un deuxième

temps, une étude de deux ans a été effectuée dans les champs de soya en labour conventionnel ou en semis direct afin de déterminer si le *Trichoderma virens* (SoilGard™) et les champignons mycorhiziens à arbuscules (un mélange de *Glomus intraradices* et *G. mosseae*), utilisés seuls ou combinés ensemble, pourraient réduire l'incidence du pourridié sclérotique du soya. Généralement, les indicateurs du pourridié sclérotique du soya, de même que le rendement en graines de soya, n'ont pas été significativement affectés par les traitements de lutte biologique. Par la suite, j'ai étudié si des changements dans la composition des communautés microbiennes étaient reliés à l'inoculation des agents de contrôle biologique et à l'incidence de maladie dans les champs de soya. L'inoculation d'agents de lutte biologique changeait les niveaux de plusieurs types d'acides gras durant les deux années de l'expérience. De plus, dans les parcelles très affectées par la maladie, les niveaux des acides gras biomarqueurs des bactéries Gram+ et d'actinomycètes étaient significativement plus élevés que dans les parcelles avec des plantes saines. En conclusion, je pense que des améliorations dans les techniques de laboratoire et les méthodologies vont permettre aux chercheurs d'extraire et de caractériser plus efficacement les lipides dans les sols, ouvrant de nouvelles perspectives dans l'étude de la dynamique de la matière organique et de l'écologie microbienne du sol. D'autres études seront nécessaires afin de vérifier l'efficacité et d'optimiser la méthode, le dosage et le temps d'inoculation des agents de lutte biologique pour pouvoir protéger les champs de soya contre le pourridié sclérotique.

PREFACE

This Ph.D. thesis is composed of five chapters. The first chapter is a review of the literature regarding the ecology of the soybean disease Sclerotinia stem rot, its biological control by soil microorganisms, and fatty acid biomarker analysis as a method to evaluate the dynamics of indigenous and introduced microorganisms in soil systems. Chapters 2 to 5 will be submitted for publication in scientific journals. Since fatty acid profiling is widely used in soil science for characterizing organic matter and microbial communities, we performed two laboratory experiments to verify the utility of this technique for soils with a broad range of characteristics (clay content, organic matter content) known to affect microbial survival and activity. Chapter 2 investigates the best solvent mixture for recovering fatty acid biomarkers from soil using an automated pressurized solvent extraction system. This chapter was submitted to the Soil Science Society of America Journal (R. Jeannotte, C. Hamel, S. Jabaji-Hare and J.K. Whalen). Chapter 3 presents a study using pyrolysis mass spectrometry to rapidly fingerprint soil lipids as a complementary tool to gas chromatography-flame ionization detection in the characterization of soil lipids and will be submitted to the Journal of Analytical and Applied Pyrolysis (R. Jeannotte, C. Hamel, S. Jabaji-Hare and J.K. Whalen). Chapters 4 and 5 are based on a two year field trial. This field trial was to test biological agents in their capacity to control Sclerotinia stem rot disease in soybean crops. Chapter 4 presents the results of the assessment of BCAs on crop characteristics and disease incidence. This chapter will be submitted for publication in the journal Phytoprotection (R. Jeannotte, C. Hamel, R. Hogue, J.K. Whalen and S. Jabaji-Hare). Chapter 5 investigates the microbial changes related to the inoculation of the biological control agents and the disease in soybean fields. This chapter will be submitted for publication in the journal Plant & Soil (R. Jeannotte, C. Hamel, R. Hogue, J.K. Whalen and S. Jabaji-Hare). A comprehensive conclusion reviews the major aspects of this research and suggests areas for further investigations. Finally, the contributions of this Ph.D. thesis to science are summarized and explicited after the general conclusions of the thesis.

CONTRIBUTION OF AUTHORS

In this thesis, the work done is described using the first person singular form "I" rather than the first person plural form "we" because this is the convention in thesis writing, but the reader should be aware that the work was a team effort. All of the publications coming from the thesis will have multiple authors, to recognize the valuable contributions of team members to the work.

The manuscripts included in this thesis as Chapters 2 to 5 will be submitted for publication in scientific journals; the candidate and his supervisors, Dr. Joann K. Whalen, Dr. Suha Jabaji-Hare and Dr. Chantal Hamel, will appear as co-authors on the four papers. Dr. Richard Hogue, a co-investigator in the field project, contributed in the field work, in doing specific laboratory analyses (detection by PCR analysis of mycorrhizal fungi and *Trichoderma* spp. in soil and root samples as well as the evaluation of the percentage root colonization by mycorrhizal fungi) that will be included in chapters 4 and 5, as well as in editorial revisions of the manuscripts. The candidate was responsible of conducting the research, analyzing the data and preparing the manuscripts. Dr. Suha Jabaji-Hare, Dr. Chantal Hamel, and Dr. Joann K. Whalen provided general guidance and editorial revisions throughout the entire process.

My specific contributions to each chapter were as follows: For chapters 2 and 3, I selected experimental protocols, designed the experiments, analyzed the results and wrote the manuscripts. For chapters 4 and 5, I contributed to the development of the field experiment (experimental design), was responsible for the field work and laboratory analyses, performed the statistical analyses and prepared the manuscripts.

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Doing a Ph.D. is a complete human experience that brings you knowledge, expertise, and humility. Moreover, it helps people around you develop the virtue of patience.

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For all the persons that contribute to deepen my understanding of soil and my everlasting desire to make it healthier.

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LIST OF ABBREVIATIONS

ABBREVIATION	SIGNIFICATION
A	Acetone
AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance
ASE	Accelerated solvent extractor
BCA	Biological control agent
CM	Chloroform:methanol
CMB	Chloroform:methanol:buffer
CT	Conventional tillage
CTAB	Hexadecyltrimethylammonium bromide
CycloFA	Fatty acid with a cyclopropyl ring
DA	Discriminant analysis
DF	Discriminant function
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DS	Dry soil
ECL	Equivalent chain length
EDTA	Ethylenediaminetetraacetic acid
EL-FAME	Ester-linked fatty acid methyl ester
FA	Fatty acid
FAME	Fatty acid methyl ester
G.i.	*Glomus intraradices*
G.m.	*Glomus mosseae*
GC	Gas chromatography
GC-FID	Gas chromatography-flame ionization detector
GC-MS	Gas chromatography- mass spectrometry detector
HCl	Hydrochloric acid
HP	Hexane:2-propanol
HPLC	High pressure liquid chromatography
HYFA	Hydroxy fatty acid
KOH	Potassium hydroxide
LSD	Least significant difference
MAB	Metastable atom bombardment
MDE	Mutation Detection Enhancement
MIS	Microbial identification system
MixedFA	Fatty acid containing more than one chemical feature
MonoUFA	Mono unsaturated chain fatty acid
NaCl	Sodium chloride
NT	No tillage
OM	Organic matter
PC	Principal component
PCA	Principal components analysis
PCR	Polymerase chain reaction

PLFA	Phospholipid fatty acid
PolyUFA	Poly unsaturated chain fatty acid
PSE	Pressurized solvent extraction
PVP	Polyvinyl pyrrolidone
Py-GC-MS	Pyrolysis gas chromatography-mass spectrometry
Py-MAB-TOF-MS	Pyrolysis metastable atom bombardment time-of-flight mass spectrometry
Py-MS	Pyrolysis mass spectrometry
SAFA	Saturated chain fatty acid
Sem	Standard error of the mean
SMB-C	Soil microbial biomass carbon
SSCP	Single-strand conformational polymorphism
SSR	Sclerotinia stem rot
SSR-DSI	Sclerotinia stem rot disease severity index
T.v.	*Trichoderma virens/Trichoderma harzianum.*
TBE	Tris-Borate-EDTA
TL-FAME	Total lipid-fatty acid methyl ester
TotalFA	Total fatty acid
T-RFLP	Terminal restriction fragment length polymorphism
TRI	*Trichoderma virens*
UFA	Unsaturated chain fatty acid
UnspecificFA	Fatty acid not specific to any biological group

INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) is a major food crop produced in many parts of the world. In Canada, soybeans are an important component of human and animal diets, and more than 3 millions tons of soybeans were produced in 2005 alone (Statistics Canada, 2006). However, soybean is susceptible to diseases caused by viruses, bacteria, fungi and nematodes. Sclerotinia stem rot [caused by *Sclerotinia sclerotiorum* (Lib.) de Bary] is a fungal disease that was reported to be the cause of significant yield reductions in Quebec soybean fields during 2000 (Rioux, 2001). Since Sclerotinia stem rot could be devastating many other field crops such as canola, sunflower, pea, and bean, as well as vegetables like carrot, lettuce and cabbage (Purdy, 1979; Boland and Hall, 1994), there is an urgent need to find efficient ways to control this disease. Several options exist for control of Sclerotinia stem rot, including chemical control (e.g. fungicides), disease resistance by cultivar breeding, cultural control (e.g. increasing the width between rows, reducing plant populations) and biological control (Tu, 1997; Zhou and Boland, 1998; Bardin and Huang, 2001). Biological control is a natural function of soil biota that could be stimulated indirectly using complex substrates like composts (Noble, 2005) or directly by the introduction of organisms with specific spectra of activity toward the target pathogen/disease (Zhou and Boland, 1998).

The growing importance of sustainable development in national and international policies, as well as in the public and scientific opinions, is leading us to consider an ecological approach to control crop diseases. Biocontrol strategies, which rely on predator-prey interactions to control pathogens and diseases, are consistent with the development of more sustainable agricultural systems. Soil is the origin of many, if not all, pests and the source of many, if not all, biological agents developed for controlling pathogens responsible for crop diseases. For example, *Sclerotinia sclerotiorum*, the causal agent of Sclerotinia stem rot disease, lives most of its life in the soil as sclerotia and apothecia (Willetts and Wong 1980). Potential biological control agents, such as *Trichoderma virens*, have been isolated from sclerotia (Phillips, 1986b), while *Coniothyrium minitans* was discovered by simply baiting the sclerotia with soil (Whipps et al., 1993).

Although several biocontrol agents for Sclerotinia stem rot have been identified, there is a major gap in our knowledge of how effective these biocontrol agents are in the field. Although biocontrol

agents may act specifically to suppress or eliminate *Sclerotinia sclerotiorum*, they are expected to interact with other organisms in the soil food web. These interactions may be direct (e.g., competitive, synergistic, antagonistic) between the biocontrol agent and indigenous soil microorganisms, or indirect (e.g., a reduction in *Sclerotinia sclerotiorum* may improve soybean growth, thus stimulating more root growth and leading to an increase in microbial activities or a change in microbial communities in the rhizosphere). We must profile and monitor changes in soil microbial communities to better understand the complex interactions between biocontrol agents, the pathogen *Sclerotinia sclerotiorum* and indigenous soil microorganisms as well as the plant; such research will permit us to optimize control strategies for plant diseases based on an ecological approach.

However, quantifying the genetic and functional diversity of soil microbial communities still presents a challenge because we are unable to culture many of these microorganisms. As well, collecting representative samples of soil microorganisms is difficult due to the high spatial and temporal variability of these organisms (Kirk et al., 2004). Yet, progress has been made and soil microbial communities can be described using molecular [e.g., DNA (Deoxyribonucleic acid)-based profiling methods such as DGGE (Denaturing gradient gel electrophoresis), T-RFLP (Terminal restriction fragment length polymorphism)], biochemical (e.g., phospholipid fatty acids, total fatty acids, sterols, etc.) and physiological approaches (e.g., community-level physiological profiling using Biolog microplates). The strengths and limitations of each approach have been reviewed by Kirk et al. (2004), Spiegelman et al. (2004), Leckie (2005) and Liu et al. (2006).

This thesis describes a biochemical approach to evaluate soil microbial communities, based on fatty acid biomarker analysis. This analysis could involve different procedures such as direct saponification-methylation, direct *in situ* transesterification, and lipid extraction-fractionation of lipid classes-transesterification of soil lipids that permits researchers to extract specific fatty acid biomarkers of various microbial groups such as bacteria, actinomycota, saprophytic fungi, mycorrhizal fungi (White and Ringelberg, 1998; Zelles, 1999; Schutter and Dick, 2000). The analysis of fatty acid biomarkers is a quantitative procedure giving insight into the viable biomass, community composition, nutritional/physiological status and metabolic activity. It may be capable of discriminating the effects of environmental and anthropogenic factors on soil microbiota, as well as

changes in soil microbial communities related to their interactions with biocontrol agents, the pathogen *Sclerotinia sclerotiorum* and the soybean plant.

1. LITERATURE REVIEW

1.1 Soil biodiversity in a sustainable agriculture perspective

The most important, fundamental, resource for agriculture is the soil. Vast communities of bacteria, fungi, plants, animals, and their detritus inhabit and interact in the soil. The activity of these organisms modifies the nature of the soil, and thus, the growth potential of crops. The soil is not an isolated, closed system, but one that interacts vitally with the above-ground plant and animal communities, biologically and physically (Thomas and Kevan, 1993).

The importance of the biodiversity of soil biota to the integrity, function and long term sustainability of natural and managed terrestrial ecosystems is increasingly recognized (Pankhurst, 1997). There is a need to better understand the biodiversity of soil communities, their functions and their interactions within the agroecosystem.

1.2 Functions of soil communities

Soil organisms live in complex communities. They are largely responsible for key global biogeochemical processes such as storing world's carbon as organic matter, cycling nutrients, creating new biomass, mixing and redistribution of organic matter and organisms, stabilization of soils and sediments, physical shredding of organic matter and its preparation for further decay by other organisms, and biological nitrogen fixation (Paul and Clark, 1996). Below surface biota also provide critical links between the atmospheric, terrestrial and aquatic realms. Some functional groups, for example, the bioturbators (gophers, ants, termites, and earthworms in soils), function across surface interfaces and act to aerate and redistribute organic matter, speeding decay and nutrient cycling. Soil and sediment biota also clean water, bioremediate pollutants, enhance soil fertility, serve as or undergird the food supply, and provide biological control of pests and parasites (Giller et al., 1997; Groffman and Bohlen, 1999; Wall, 1999).

Biological control is a natural function of soil biota. Many pests spend part of their life in the soil and many, if not all, biological agents used to control pathogens responsible for crop diseases were found in soils. In this chapter, I review the literature related to the control of *Sclerotinia sclerotiorum*, the causal agent of Sclerotinia stem rot disease in soybean crop. I describe two biological control agents, mycorrhizal fungi and *Trichoderma virens*, that have been used to control various plant diseases, and explain the possible mode-s of action of each. I also explain how signature lipid biomarkers can be used to evaluate how inoculation with biological control agents affects soil microbiota, and how the soil microbial communities respond to the presence of the pathogen *S. sclerotiorum* as well as the presence of healthy or infected soybean plants.

1.3 Soil biota as a source of pathogens – case of *Sclerotinia sclerotiorum*, causal agent of Sclerotinia stem rot, and its control in soil-plant systems

Soybean (*Glycine max* (L.) Merr.) is one the major crops in the world food agricultural production. For example, in Canada, the field production of soybeans, with more than 2000 to 3000 thousand tonnes from 2002 to 2006, is among the major field crops such as, in order of importance, tame hay, wheat, barley, grain corn, canola (rapeseed), oats, soybeans, flaxseed and rye (Statistics Canada, 2006).

Soybean is qualified the 'miracle' crop because it could be used in many different products for human and animal nutrition, some known and others unexpected, as well in the industry (The American Soybean Association, 2005a,b). Soybean is omnipresent in our lives and fields. However, soybean is susceptible to many pests (weeds, pathogens and animal pests). The total potential loss around the world due to pests was estimated, for the period of 2001 to 2003, to be around 26-29% for soybean (Oerke, 2006). Many diseases, of various biological origins (virus, bacteria, fungi, nematodes), were reported to substantially reduce the harvest yield in soybean in the top 10 soybean-producing countries (these countries produced more than 97.6% of the world's total soybean crop in 1998). The diseases causing the major losses were soybean cyst [*Heterodera glycines* Ichinohe], brown spot [*Septoria glycines* (Hemmi)], charcoal rot [*Macrophomina phaseolina* (Tassi) Goidanich], and sclerotinia stem rot [*Sclerotinia sclerotiorum* (Lib.) de Bary] (Wrather et al.,

2001). In 2000, in the province of Quebec, sclerotinia stem rot was reported to be the major disease attacking soybean (Rioux, 2001).

Since Sclerotinia stem rot could be also devastating for a numerous number of field crops such as canola, sunflower, pea, bean, etc., and vegetables such as carrot, lettuce, cabbage, etc. (Purdy, 1979; Boland and Hall, 1994), there is an urgent need to find efficient ways to control this disease.

The life cycle of *Sclerotinia sclerotiorum*, the causal agent of Sclerotinia stem rot, begins in the soil as vegetative bodies, the sclerotia, that germinate to produce apothecia which release ascospores. Germinating ascospores colonize senescing or dead flower petals in the phyllosphere and, after colonizing such nutrient sources, the pathogen invades adjoining living tissue and initiates disease (Abawi and Grogan, 1979; Adams and Ayers, 1979; Willetts and Wong, 1980; Zhou and Boland, 1998).

Up to now, the main ways to reduce the impact (or to control) of SSR disease on soybean yields is to select resistant cultivars (Grau et al., 1982; Grau and Radke, 1984; Boland and Hall, 1986, 1987; Buzzell et al., 1993; Wegulo et al., 1998; Kim et al., 1999). Also, management practices that can lower the incidence of SSR disease in soybeans include the use of crop rotations with non-host plants like corn or wheat, increasing row width, reducing plant populations and conservation tillage (e.g., no-till) (Grau and Radke, 1984; Buzzell et al., 1993; Kurle et al., 2001; Gracia-Garza et al., 2002). Soybean could be protected from SSR disease by the application of fungicides (Tu, 1997; Bardin and Huang, 2001) as well as of chemicals such as 2,6-dichloroisonicotinic acid, benzothiadiazole, and lactofen that induce resistance in plants (Dann et al., 1998, 1999). The various means used to control SSR disease were reviewed by Tu (1997), Zhou and Boland (1998), as well as Bardin and Huang (2001).

A promising and complementary way of controlling SSR disease in the field is the application of biological control agents (BCAs). The development of biological agents to control Sclerotinia stem rot is an important task considering the widespread distribution of the disease, its importance on agriculture economy (in soybean, for example, see Wrather et al., 1997, 2001) and its wide range of plant hosts (Boland and Hall, 1994).

The keen interest in biological control in the last decade is based on the increased concern over the environmental impact of chemical pesticides and the desire to develop integrated pest- and disease-management practices for more sustainable agriculture. The development of realistic, economical and sustainable biological control strategies requires a clear understanding of the mechanisms of biocontrol, the impact of environmental factors (abiotic and biotic) and the complexity of the interactions that occur among the plant, the pathogen, the biocontrol agent and its surroundings. The best approach is to customize biocontrol strategies by studying naturally occurring biocontrol systems, to understand the ecology of pathogen and its potential biocontrol agents under local growing conditions, to devise biocontrol systems with multiple mechanisms of antagonism, and to integrate biological control with other methods of disease suppression such as reduced chemical treatments (fertilizers and pesticides), appropriate tillage methods, organic amendments, crop rotation, and moderate cultivar resistance (Traquair, 1995; Pankhurst, 1997).

Various biocontrol strategies have been examined for *Sclerotinia sclerotiorum*. The general approach to the biological control of this pathogen is (i) to reduce the inoculum potential of the pathogen by the destruction of spores and vegetative propagules, by the prevention of inoculum production, and by displacement of the pathogen or reduction of its virulence; (ii) to protect plant surfaces from invasion; and (iii) to restrict the progress of the pathogen inside the plant by induced resistance or cross protection (Zhou and Boland, 1998).

There are several BCAs that are effective against *Sclerotinia sclerotiorum* and related species have been reviewed by Tu (1997), Zhou and Boland (1998) as well as Bardin and Huang (2001). Among the various microorganisms evaluated as potential BCAs to control *Sclerotinia sclerotiorum* in the soil, *Bacillus* spp., *Pseudomonas* spp., *Coniothyrium minitans*, *Dictyosporium elegans*, *Penicillium citrinum*, *Sporidesmium sclerotivorum*, *Talaromyces flavus*, *Teratosperma oligocladum*, *Trichoderma* spp. and *Trichoderma virens* proved to be successful.

Although several biocontrol agents for Sclerotinia stem rot have been identified and tested in controlled environments (in vitro culture, growth chamber, greenhouse), there is a major gap in our knowledge of how effective these biocontrol agents are in the field with uncontrolled climatic conditions and soil biotic composition, for example. The selection of two following groups of fungi

are based on evidences of their capacity to control many different pathogens, such as *Sclerotinia sclerotioum*, at least in *in vitro* studies, in the case of *Trichoderma virens*, and their well-recognized beneficial and biocontrol capacities, such as mycorrhizal fungi. Also, in both cases, commercial products were available, thus facilitating field application and study.

1.4 *Trichoderma virens* and mycorrhizal fungi as candidates biological control of Sclerotinia stem rot disease

1.4.1 Interactions of *Trichoderma virens* with soil-borne plant pathogens

One promising BCA for *Sclerotinia sclerotiorum* control is *Trichoderma virens* (formerly classified as *Gliocladium virens* (Rehner and Samuels, 1994), which is sold as SoilGard™ by Certis USA (Columbia, MD, USA). This fungus occurs naturally in agricultural soils (Davet, 1985; Vardavakis, 1990; Park et al., 1992). Several studies have shown that *Trichoderma virens* provided good biological control against pathogenic microorganisms including *Botrytis cinerea* (Di Pietro et al., 1993; Lorito et al., 1994), *Pythium ultimum* (Roberts and Lumsden, 1990; Paulitz and Linderman, 1991; Lumsden et al., 1992a, 1992b; Howell et al., 1993; Wilhite et al., 1994; Koch, 1999), *Rhizoctonia solani* (Lewis and Papavizas, 1985; Lumsden et al., 1992a, 1992b; Howell et al., 1993; Lartey et al., 1994; Mukherjee et al., 1995; Koch, 1999), *Sclerotinia rolfsii* (Papavizas and Collins, 1990; Ristaino et al., 1991, 1994; Lumsden et al., 1992a; Mukherjee et al., 1995; Lewis and Fravel, 1996) and *Sclerotinia sclerotiorum* (Tu, 1980; McCredie and Sivasithamparam, 1985; Mueller et al., 1985; Phillips,1986a, 1986b, 1990; Whipps and Budge, 1990).

Synergistic antifungal activity of cell wall lytic enzymes and antibiotics such as gliotoxin and glioviridin may play a role in biological control by *T. virens* (Roberts and Lumsden, 1990; Lumsden et al., 1992a, 1992b; Di Pietro et al., 1993; Howell et al., 1993; Lorito et al., 1994; Wilhite et al., 1994; Mukherjee et al., 1995). The production of antifungal molecules and the overall biological control can be affected by various factors such as time, moisture, temperature, pH, nutrient status (sources of carbon and nitrogen, their C/N ratio) (Phillips, 1986a; Park et al., 1991; Lumsden et al., 1992a, 1992b; Howell et al., 1993). *In vitro, T. virens* was active as mycoparasite of sclerotia over a broad range of soil moisture levels and over the entire range of agricultural pH. The temperature

seemed to be the main limiting factor of its activity (Phillips, 1986a). Electron microscopy showed that the mycoparasite formed appressoria-like structures on the mycelia of *S. sclerotiorum* and presumably achieved its infection by active penetration (Tu, 1980) and demonstrated internal parasitism (profuse internal sporulation of the mycoparasite) of sclerotial cells of *S. rolfsii, R. solani* (Mukherjee et al., 1995) and *S. sclerotiorum* (Tu, 1980). The parasitized sclerotia were incapable of either myceliogenic or carpogenic germination (Tu, 1980; Mukherjee et al., 1995). In contrast, *T. virens* did not penetrate plant pathogen *Botryodiplodia theobromae*, but, after initial hyphal contact, it produced wall lytic enzymes and-or antifungal substances that caused wrinkling, bursting and collapsing of its mycelium (Gupta et al., 1999).

Thus, *Tichoderma virens* was proven to be able to provide specific biological control against *S. sclerotiorum* in controlled environment (Tu, 1980; McCredie and Sivasithamparam, 1985; Mueller et al., 1985; Phillips, 1986a, 1986b, 1990; Whipps and Budge, 1990). In laboratory and greenhouse studies, the biological control of SSR disease may be improved by synergistic interactions between *T. virens* endochitinases and antibiotics such as gliotoxin and glioviridin (Tu, 1980; Roberts and Lumsden, 1990; Lumsden et al., 1992a, 1992b; Di Pietro et al., 1993; Howell et al., 1993; Lorito et al., 1994; Wilhite et al., 1994; Mukherjee et al., 1995).

In a field experiment, the application of *T. virens* as a wheat bran alginate pellet formulation with each seed at planting enhanced the emergence of snap beans (*Phaseolus vulgaris*), increased nodulation of treated roots and reduced damping-off incidence (Smith, 1996). Paulitz and Linderman (1991) tested the effect of *T. virens* on the colonization of cucumber by vesicular arbuscular mycorrhizal fungi *Glomus etunicatum* and *G. mosseae*. Their results showed that *T. virens* does not have a detrimental impact on these mycorrhizal fungi, and would be compatible if applied as a dual-inoculum. However, it is not known how these fungi (*T. virens* and mycorrhizal fungi) will interact in field conditions and how these interactions could benefit the biological control of a disease such as SSR.

1.4.2 Interactions of mycorrhizal fungi with soil-borne plant pathogens

Mycorrhizas are usually divided into three morphologically distinct groups depending on whether or not there is fungal penetration of root cells: endomycorrhizae, ectomycorrhizae and ectendomycorrhizae (Barea, 1991; Budi et al., 1998). Arbuscular mycorrhizal fungi represent the most widespread, and most ancient, type of plant-fungus association in which the large majority of terrestrial plants must have evolved with compatibility systems towards the fungal symbionts (Gianinazzi-Pearson et al., 1995; Lambais and Mehdy, 1995; Blee and Anderson, 1996). The infection of root tissues by mycorrhizal fungi modifies the morphology and the functioning of roots in promoting the growth of plants by enhancing water and nutrient adsorption (Barea, 1991; Munyanziza et al., 1997; Budi et al., 1998) and by improving their resistance against abiotic/biotic stresses and soil aggregation (see reviews by Barea, 1991; Munyanziza et al., 1997).

Just as pathogenic microorganisms on roots are influenced by saprophytic bacteria and fungi of the rhizoplane and rhizosphere, mycorrhizal fungi are affected by the activities of microbial epiphytes and microfauna in the mycorrhizoplane and mycorrhizosphere (Linderman, 1988, 1994). When mycorrhizal fungi are present, changes in the quality and quantity of root exudates lead to a concomitant change in the microflora on and around the mycorrhizal root. This change is called mycorrhizosphere effect (Linderman, 1988) and can have a pronounced influence on the activity of root pathogens. Similarly to roots, the extramatrical hyphae exude materials that exert a mycosphere effect on microorganisms (Linderman, 1994). Bacteria, including the nitrogen-fixing *Bradyrhizobium* spp, growth promoting bacteria, antagonistic actinomycetes, and saprophytic and antagonistic fungi such as *Penicillium*, *Gliocladium*, and *Trichoderma* spp. in the mycorrhizosphere can modify the biocontrol activity of mycorrhizal fungi (Linderman and Paulitz, 1990).

Cultural practices frequently used in low external-input agricultural systems affect strongly rhizosphere microorganisms, including mycorrhizal fungi. Practices such as no- or reduced tillage, intercropping, and crop rotation have been shown to favor the development of mycorrhiza. Other conventional agricultural practices such as pesticides or fertilizer application can stimulate or injure VAM populations according to the dose and chemical nature of the substances (see reviews by Barea, 1991; Bethlenfalvay, 1992; Munyanziza et al., 1997).

Mycorrhizal fungi are promising candidates for biological control. They are environmentally safe, ubiquitous, mutualistic symbionts of roots which establish stable, long-term associations with the roots of most plants and they were shown repeatedly to protect plants against diseases (Traquair, 1995). Many review articles summarize results on the use of arbuscular mycorrhizal fungi (AMF) as biocontrol agents (among them, see Linderman, 1988, 1994; Smith, 1988; Linderman and Paulitz, 1990; St-Arnaud et al., 1995a; Traquair, 1995; Azcón-Aguilar and Barea, 1996; Harrier and Watson, 2004; Whipps, 2004). Consistent reduction of disease symptoms have been obtained, under controlled conditions, when AMF were used against fungal pathogens such as *Phytophthora*, *Gaeumannomyces*, *Fusarium*, *Chalara* (*Thielaviopsis*), *Pythium*, *Rhizotonia*, *Sclerotium*, *Verticillium*, *Aphanomyces*, and for nematodes such as *Rotylenchus*, *Pratylenchus* and *Meloidogyne* (Azcón-Aguilar and Barea, 1996; Harrier and Watson, 2004; Whipps, 2004). For example, Cylindrocarpon root rot was reduced by endomycorrhizal colonization of potted peach rootstocks with *Glomus aggregatum* under controlled environment conditions using Turface or natural, untreated orchard soils (Traquair, 1995). The disease incidence of Fusarium crown and root rot of tomato was significantly decreased by *Trichoderma harzianum* and *G. intraradices* used alone or in combination (Datnoff et al., 1995). The inoculation of *Glomus mosseae* decreased the conidia number of *Fusarium solani* in the rhizosphere and decreased root rot caused on the common bean *Phaseolus vulgaris* (Hassan Dar et al., 1997). Enrichment of the soil with *G. intraradices* reduced Fusarium root rot of tomato and the population of *F. oxysporum* in soil (Caron et al., 1986). In another study, *G. intraradices* was grown on *Daucus carota* transformed roots in a two-compartiment *in vitro* system in absence of roots and under monoxenic conditions, *G. intraradices* stimulated *F. oxysporum* f. sp. *chrysanthemi* conidial germination and stimulated hyphal growth. This mechanism might reduce soil inoculum through exhaustion or lysis of the germination hyphae (St-Arnaud et al., 1995b). St-Arnaud et al. (1997) showed the inhibition of *F. oxysporum* f. sp. *dianthi* in the non-VAM species *Dianthus caryophyllus* by co-culturing this plant with *Tagetes patula* plants colonized by *G. intraradices*. Inoculation with *G. intraradices* reduced *Pythum ultimum* developement in roots and *P. ultimum* propagule numbers in the *T. patula* mycorrhizosphere (St-Arnaud et al., 1994).

Numerous reviews indicate that, in most cases, mycorrhizal colonization of roots prior to challenge by pathogenic fungi reduces the severity of root diseases (Smith, 1988; Caron, 1989; Linderman, 1994; St-Arnaud et al., 1995a). Various mechanisms have been proposed to explain how AMF reduces the activity of fungal root pathogens (Azcón-Aguilar and Barea, 1996; Harrier and Watson, 2004; Whipps, 2004). They include (1) improved nutrient status of the host plant; (2) damage compensation; (3) competition for host photosynthates; (4) rhizosphere deposition; (5) competition for infection/colonization sites; (6) anatomical and morphological changes in the root system; (7) microbial changes in the mycorrhizosphere; and (8) activation of plant defense mechanisms.

1.5 Measuring soil microbial diversity

The development of a disease in a plant/crop needs the synergism of many macro- and micro-climatic, soil, plant and human factors as well as the required presence of the pathogens in the soil environment. Biological control agents introduced in a soil environment are expected to interact with some of the soil biota, but even when no biocontrol agents are added, the pathogenic organism and the infected plant may also interact with the soil biota and cause changes in the microbial communities. For example, in the case of SSR disease and its biological control, little is known about the interactions, in field conditions, between the proposed BCAs, mycorrhizal fungi and *T. virens*, the pathogenic organism, *S. sclerotiorum*, other components of the soil biota, and the soybean plant. According to our knowledge, these aspects were never investigated using SSR disease as model. So, profiling these changes may lead to an improved understanding of the interactions between the organisms involved in biological control, permitting us to take advantage of naturally-occurring ecological interactions to control plant diseases.

However, the study of genetic and functional diversity in the soil foodweb still presents a significant challenge and very little is known about the vast majority of soil organisms and their ecological function in soil. Some of the reasons for the paucity of knowledge are presented here (Pankhurst, 1997; Wall, 1999; Kirk et al., 2004):

1. The opaqueness of the soil which makes the *in situ* identification of most soil organisms impractical; 2. The large range in size of soil organisms (microbes to earthworms) makes their

interactions and ecological role difficult to assess; 3. The morphology of many organisms changes during their life cycle making them difficult to identify; 4. Methods for extracting and quantifying many microorganisms, fungi and mesofauna from soils have not been determined and techniques for culturing them have have not been developed; 5. The temporal and spatial scale of the niches occupied by different organisms in the soil varies greatly and thus, making difficult to collect representative samples of soil microorganisms; 6. The activity and abundance of different organisms changes in response to changes in the physico-chemical properties of the soil, the quality of organic matter, climate and geography, resulting in few comparisons of the ecological roles of soil taxa in different ecosystems.

Generally, the methods overcoming the limitation of culturability methods could be classified in molecular (e.g., DNA-based profiling methods such as DGGE, T-RFLP), biochemical (e.g., phospholipid fatty acids, total fatty acids, sterols, etc.) and physiological approaches (e.g., community-level physiological profiling using Biolog microplates). The strengths and limitations of each approach have been reviewed by Kirk et al. (2004), Leckie (2005), Spiegelman et al. (2005), and Liu et al. (2006). Singh et al. (2006) compared the effects of plants on the microbial community, using several methods, in three different types of soils. Pots containing soil from three contrasting sites were planted with *Lolium perenne* (rye grass). The change in soil microbial communities caused by the growth of rye grass was studied using physiological (Biolog), biochemical (Phospholipid fatty acid, PLFA) and molecular (DGGE and T-RFLP) fingerprinting methods. Singh et al. (2006) concluded that molecular methods were the most discriminatory, but all methods of microbial fingerprinting gave qualitatively similar results when samples were processed consistently and comparable statistical methods used.

1.6 Signature lipid biomarkers: methods and applications in agroecosystems

1.6.1 Soil lipids

All animal, plant and microbial cells contain carbohydrates, lipids and proteins. Lipids are a rich and heterogenous group of organic molecules that could be operationally defined as being sparingly soluble or insoluble in water, but soluble in selected organic non-polar solvents such as

chloroform/dichloromethane, hydrocarbons (diethyl ether, hexane, etc.) or alcohols (methanol, ethanol, isopropanol, butanol, etc.) (Dinel et al., 1990; Stevenson, 1994; Shahidi and Wanasundara, 2002). This group contains many different types of compounds, such as fatty acids, acylglycerols, sterols and sterol esters, waxes, phosphoglycerides (phospholipids), ether(phospho)glycerides (plasmalogens), glyceroglycolipids (glycosylglycolipids), sphingolipids, fat-soluble vitamins, hydrocarbons (Gunstone et al., 1994; O'Keefe, 2002). The common and unique features of compounds classified as lipids relate to their solubility rather than their structural characteristics (Shahidi and Wanasundara, 2002).

Lipids in soils originate from animal, plant and microbial sources (Dinel et al., 1990; Lichtfouse, 1998; Lichtfouse et al., 1998; Olsson, 1999; Zelles, 1999; Puglisi et al., 2003). The characterization of lipid biomarkers such as total lipid fatty acids (Schutter and Dick, 2000; Drenovsky et al., 2004; Hinojosa et al., 2005), phospholipid fatty acids (PLFAs) (Vestal and White, 1989; Zelles, 1999; Piotrowska-Seget and Mrozik, 2003), neutral lipid fatty acids (Olsson, 1999; Baath, 2003), respiratory quinones (Hiraishi, 1998; Katayama et al., 1998), sterols (Ruzicka et al., 2000; Puglisi et al., 2003; Mille-Lindblom et al., 2004) as well as their composition in stable isotopes (Boschker and Middelburg, 2002) in soil ecosystems is a powerful tool helping us to understand the dynamics/changes in the microbial community structure following environmental and anthropogenic factors (Kennedy and Gewin, 1997). Lipid analysis is also used to monitor transformations in soil organic matter (Dinel et al., 1998; Bull et al., 2000; Wiesenberg et al., 2004), as tracers of early life on earth and elsewhere (Simoneit et al., 1998; Brocks and Summons, 2003), and in forensic science (Forbes et al., 2003) and archeology (Simpson et al., 1999a, 1999b).

Lipids are a diverse class of chemical compounds and their importance for the life of any organism on earth is obvious. However, this richness remains to be fully exploited. The development of methods that use lipid compounds (such as fatty acids) as biomarkers (or diagnostic molecules) could lead to a better understanding of soil processes.

1.6.2 Methods for extracting lipids

The extraction of soil lipids is usually accomplished using a mixture of chloroform, methanol and a citrate or phosphate buffer (modified Bligh and Dyer method (1959) detailed in White and Ringelberg, 1998). Previously, many methods for extracting soil lipids, especially phospholipids, were elaborated and were using generally long extraction times as well as large quantities of solvents: (1) Hance and Anderson (1963) developed an effective method for extracting phospholipids using acid pretreatment and successive extractions with acetone, light petroleum, ethanol and benzene, methanol and chloroform; (2) Kowalenko and McKercher (1970, 1971) used an alternative to the Hance and Anderson (1963) method that consisted in two successive and vigorous extractions of the soil using a mixture of hexane-acetone; (3) Baker (1975), by successively refluxing the two last solvents (ethanol and benzene, methanol and chloroform) of the Hance and Anderson (1963) method using a Soxhlet extractor, was able to achieve comparable results of the Hance and Anderson (1963) in extracting soil phospholipids. Using a modified Bligh and Dyer method, White et al. (1979) developed a method for quantifying lipid phosphate in sediments. This method of extraction remained the one most frequently used for extracting soil lipids and characterizing their phospholipids in microbial ecology studies (Frostegård et al., 1991; Tunlid and White, 1992; White and Ringelberg, 1998; Zelles, 1999). The extraction of lipids by chloroform:methanol mixture is indeed very efficient for many biological materials (Shahidi and Wanasundara, 2002; Christie, 2003). However, the extraction and study of soil lipids is complicated because of their chemical structures and biological origins are very diverse and also because soil particles, such as clays, and organic matter that can interfere with their extraction (Hance and Anderson, 1963; Frostegård et al., 1991; Nielsen and Petersen, 2000).

The efficiency ('robustness') of chloroform:methanol mixtures in extracting lipids from soils with a range of properties was never fully investigated (Tunlid and White, 1992), according to our knowledge, and remains to be assessed. The capacity of chloroform:methanol mixtures to extract fatty acid biomarkers needs to be compared to other efficient solvent mixtures. However, using an extraction mixture that contains chloroform and methanol should also be a concern because these solvents are toxic to humans when inhaled or adsorbed through the skin, and chloroform is carcinogenic (Ikeda, 1992; Golden et al., 1997). The research for less toxic solvents to extract lipids is a long-lasting quest in the field of lipidology (Christie, 1993, 2003).

Many solvents have been tested as alternatives to chloroform-methanol solvent mixtures for lipid extraction (see reviews by Shahidi and Wanasundara, 2002 and Christie, 2003). The performance of these alternative solvent mixtures varies with the material tested. For example, Schäfer (1998) showed that more of the fatty acids contained in cereal and yolk lipids were extracted using a hexane:2-propanol (3:2, v/v) mixture than chloroform:methanol mixture, although the extraction of muscle lipids was more efficient with chloroform:methanol (2:1, v/v) than with other solvent mixtures. Solvents, such as acetone and a hexane:2-propanol mixture, are effective at extracting lipids from wood and other biological materials (Hara and Radin, 1978; Schäfer, 1998; Gonzalez-Vila et al., 2000; Shahidi and Wanasundara, 2002; Christie, 2003; Dodds et al., 2004; Zarnowski and Suzuki, 2004; Tanamati et al., 2005). Acetone is a common laboratory solvent and the hexane:2-propanol mixture is frequently compared with chloroform-methanol solvent mixtures for extracting lipids (Hara and Radin, 1978; Shahidi and Wanasundara, 2002; Christie, 2003).

Moreover, an automated pressurized solvent extraction (PSE) system has been previously used to extract lipids from soils (Macnaughton et al., 1997; Dinel and Nolin, 2000; Spedding et al., 2004; Wiesenberg et al., 2004; Hamel et al., 2005; Jansen et al., 2006). This technique accelerates the extraction process, reduces human contact with solvents, and reduces the volume of solvents used. The capacity of PSE system to extract soil fatty acid biomarkers in a range of soils was not fully optimized using various solvent mixtures.

1.6.3 Analysis of soil lipids

Signature lipid biomarkers analysis is an approach composed of many methods from the high throughput analysis of fatty acids using fast and simple procedures to detailed analysis of specific biomarkers such as sterols, phospholipid fatty acids, neutral lipids, quinones, etc. (White and Ringelberg, 1998; Zelles, 1999; etc.). Among these procedures/methods, we could find: direct profiling of fatty acids from solid soil samples or lipid extracts using pyrolysis-mass spectrometry (Py-MS) and pyrolysis gas chromatography-mass spectrometry (Py-GC-MS) with/without a methylation reagent such as trimethylammonium hydroxide (Dinel et al., 1998; Jandl et al., 2002, 2004); direct profiling of fatty acids by saponification-esterification (Schutter and Dick, 2000; Drenovsky et al., 2004) and mild alkaline transesterification (Drijber et al., 2000; Schutter and Dick,

2000; Hinojosa et al., 2005) followed by analysis with GC-flame ionization detector (FID) or GC-MS; finally, by extraction of soil lipids followed or not by fractionation into lipid classes, derivatization and analysis by GC-FID or GC-MS (Zelles, 1999; Jandl et al., 2005; Kaur et al., 2005; Otto et al., 2005).

It is clear that generating lipid profiles demands intensive laboratory work and data analysis, which may be too time consuming and costly when a large number of samples are being screened to distinguish soil microbial communities among experimental treatments or sites. Simple cataloguing or classification of these samples can be accomplished with techniques that generate a 'fingerprint' of the soil lipids extracted from living organisms and other sources. Fingerprinting techniques such as Fourier transform infra-red and Raman spectroscropic techniques as well as direct infusion MS and Py-MS do not require chromatographic step, and the frequently needed derivatization of the targeted compounds (Goodacre and Kell, 1996; Sumner et al., 2003; Dunn et al., 2005; Villas-Bôas et al., 2005). Generally, fingerprinting involves much larger numbers of measurements (high throughput) than profiling and requires chemometric interpretation of the complexity resulting from simultaneous acquisition of analytical data on hundreds of metabolites (Goodacre et al., 2003; Sumner et al., 2003).

Of the techniques listed above, Py-MS has several advantages, such as speed of analysis, sensitivity, high sample throughput, minimal sample preparation, and low cost. In most Py-MS systems, the chemical compounds in a sample are desorbed and volatilized during a rapid heating phase, followed by ionization with electron impact, and detection by mass spectrometry (Goodacre and Kell, 1996). This technique was successfully used, sometimes with a thermally assisted hydrolysis and methylation step, to discriminate and classify bacteria (Basile et al., 1998a,b; Brandão et al., 2002), fungi (Lilley et al., 2001), yeasts (Timmins et al., 1998), higher plants (Kim et al., 2004) and organic matter (Peuravuori et al., 1999; Magrini et al., 2002; Marche et al., 2003; Jandl et al., 2004). The modified Py-MS system used in this study is pyrolysis metastable atom bombardment time-of-flight mass spectrometry (Py-MAB-TOF-MS). The metastable atom bombardment capacity allows better control of the ionization energy and reduces chemical fragmentation during ionization, compared to electron impact ionization (Faubert et al., 1993). The Py-MAB-TOF-MS system has recently been used for fingerprinting vegetable oils (Sanchez et al., 2002), animal fats (Beaudet et al., 2003), microbes (Wilkes et al., 2005), and steroids (Dumas et

al., 2002). The use of pyrolysis-mass spectrometry, and especially the Py-MAB-TOF-MS system, to specifically fingerprint soil lipids in order to discriminate soils from various agroecosystems was never done. The main advantage of fingerprinting soil lipids is that this approach could allows to obtain a first level of information on the soil samples that could be deepen, if need, by a more detailed analysis of specific signature lipid biomarkers such total ester-linked fatty acids, phospholipid fatty acids or sterols, for example.

Soils are very complex matrix characterized by a wide diversity of physical-chemical properties and living organisms. Many factors could have important impacts on the quantity and the quality of soil lipids extracted: inherent properties of soils, preparation/pre-treatment of the material (representative sampling, sieving and grinding, wetness and dryness (using fresh soil, fresh-frozen soil, air-dried soil, freeze-dried soil), storage time and conditions (exposure to light, heat and oxygen)) and the extraction process (size of subsample, extractant(s), extracting device and conditions, storage conditions of the extract, etc). Ideally, at any moment during the lipid analysis process, the raw material and the resulting lipid extract should not be exposed to light, heat and oxygen in order to keep the chemical integrity of lipid species composing the extract (Ackman, 2000; Shahidi and Wanasundara, 2002). Some of these elements were already assessed to some extent (Hance and Anderson, 1963; Frostegård et al., 1991; Petersen and Klug, 1994; Macnaughton et al., 1997; Nielsen and Petersen, 2000; Schutter and Dick, 2000; Drenovsky et al., 2004; Allison and Miller, 2005). For example, Allison and Miller (2005) demonstrated that the grinding of soil samples reduces the variability in the microbial community composition, but could increase the relative abundance of eukaryote phospholipid fatty acids biomarkers in soils with high rooting densities. Moreover, Schutter and Dick (2000) showed that the storage duration influences the resulting soil fatty acids composition, but this effect was variable depending on the soil type. However, extensive studies remain to be done to fully assess the importance of the various soil properties and pre-treatment factors on the quantity and the quality of soil lipids extracted.

Finally, new developments in instrumentation and methods will permit to expand the use of lipid analysis, as well as the array of lipid biomarkers detectable, in soil science by allowing direct analysis of lipids in biological samples and-or in lipid extracts by Raman (Beattie et al., 2004; Krafft et al., 2005) and infrared (Kujawa et al., 2003; Tapp et al., 2003) spectroscopies, molecular beam

static secondary ion mass spectrometry (Ingram et al., 2003; Touboul et al., 2005), electrospray ionization-mass spectrometry (Rütters et al., 2002; Sturt et al., 2004; Welti and Wang, 2004; Han and Gross, 2005) and matrix-assisted laser desorption/ionization mass spectrometry (Ishida et al., 2003; Schiller et al., 2004).

1.6.4 Use of lipid analysis in soil microbial ecology

Environmental factors such as light, temperature, nutrient availability and so on are known to influence the lipid composition of living biota. Thus, the composition in lipids of a living organism could reflect the environment conditions in which this organism lives as well as being specific to the growth stage of the organism (Lechevalier and Lechevalier, 1989; Rose, 1989). Lipids are recognized to be very valuable biomarkers in chemotaxonomy and could be used to discriminate microorganisms up to the species level (Lechevalier and Lechevalier, 1989). Among the various types of lipids, the fatty acids of a wide range of microorganisms (e.g., bacteria, fungi, algae, protozoa) were extensively characterized. Since fatty acids do not occur in the cells in their free form, but linked to other compounds, their specificity as taxonomic biomarkers is increased when the lipids are fractionated into their different classes (eg., phospholipids, neutral lipids, glycolipids). Phospholipids, as the major class of lipids that are found in the membranes of all living cells, make up a relatively constant proportion of cell biomass and are rapidly turned over on cell death (Lechevalier and Lechevalier, 1989). Thus, in a complex milieu like soil, the characterization of the fatty acids linked to the phospholipids (phospholipid fatty acids, PLFAs) will give us information about microbial communities living in the soil. A change in the composition of soil PLFAs indicates a shift in the soil microbial composition and the knowledge accumulated on the fatty acid composition of the various taxonomic groups of microorganisms will allow us to interpret this shift (Vestal and White, 1989; Tunlid and White, 1992).

The characterization of microbial communities in the environment by the analysis of PLFAs was developed by the work of Dr White at the University of Tennessee (Vestal and White, 1989; Tunlid and White, 1992) and was extensively reviewed (White and Ringelberg, 1998; Zelles and Alef, 1996; Zelles, 1999; Piotrowska-Seget and Mrozik, 2003; Kaur et al., 2005; Leckie, 2005). Briefly, in this method, the crude extract of soil lipids is subsequently fractionated by solid-phase extraction on

silica gel column into the three main lipid classes according to their polarity: neutral lipids, glycolipids and phospholipids. The fatty acids esterified to the phospholipids are released using a mild alkaline methanolysis and analyzed by gas chromatography coupled to flame ionization detector (GC-FID) or mass spectrometry detector (GC-MS). Or, prior to GC analysis, the PLFAs could be fractionated into various chemically relevant groups (hydroxy fatty acids; saturated, monosaturated and polyunsaturated fatty acids; unsaponifiable lipids) using aminopropyl-bonded and benzenesulphonic acid-bonded solid-phase extraction columns. This latest strategy allows an accurate and complete description of the PLFA fraction (Zelles and Alef, 1996; Zelles, 1999).

Also, two methods were developed to rapidly characterize soil microbial community by whole-cell fatty acid analysis. One of the methods used the Microbial Identification System (MIS) protocol developed for releasing whole-cell fatty acids from microorganisms but adapted to soil samples. Briefly, this method is based on a saponification and methylation steps of the fatty acids directly from the soil samples, without lipid extraction (Haack et al., 1994; Cavigelli et al., 1995). In the second method, the ester-linked fatty acids are directly released from the soil sample by a mild alkaline methanolysis, either without lipid extraction. The basis of this method is that ester-bounds in soil are labile and most likely, ester-bound fatty acids should be derived from living organisms (Drijber et al., 2000; Schutter and Dick, 2000). These methods were developed to facilitate the processing of large number of samples and to acquire data that could be valuable complement to the more extended PLFA methodology (Drijber et al., 2000; Drenovsky et al., 2004; Hinojosa et al., 2005). However, these procedures may include fatty acids derived from both cells and soil organic matter (Zelles, 1999; Schutter and Dick, 2000; Petersen et al., 2002; Drenovsky et al., 2004; Hinojosa et al., 2005).

Fatty acids (FAs) could be grouped according to their chemical structure (straight saturated chain FA, branched saturated chain FA, monounsaturated FA, polyunsaturated FA, hydroxy substitued FA, FA with cyclopropyl ring). They could be grouped also according to their biological origin: actinomycetes (e.g., 10Me-16:0, 10Me-17:0, 10Me-18:0, 19Me-19:0), mycorrhizal fungi (16:1ω5c), saprophytic fungi (18:1ω9c, 18:2ω6,9c, 18:3ω6,9,12c), bacteria (e.g., i-14:0, i-15:1, i-15:0, a-15:0, 15:0, i-16:0, 16:1ω7c, i-17:0, a-17:0, 17:0 cyclo, 17:0, 18:1ω7c, 18:1ω5c, 19:0 cyclo ω8c), Gram+ bacteria (e.g., i-14:0, i-15:1, i-15:0, a-15:0, i-16:0, i-17:0, a-17:0) and Gram- bacteria (e.g.,

16:1ω7c, 17:0 cyclo, 18:1ω7c, 18:1ω5c, 19:0 cyclo ω8c) (Lechevalier, 1977; Kroppenstedt, 1985, 1992; Federle, 1986; Lechevalier and Lechevalier, 1988; O'Leary and Wilkinson, 1988; Ratledge and Wilkinson, 1988; Wilkinson, 1988; Vestal and White, 1989; Frostegård et al., 1993; Graham et al., 1995; Olsson et al., 1995; Frostegård and Bååth, 1996; Zelles, 1997, 1999; Olsson, 1999; Hill et al., 2001). The class of lipid from which the fatty acid is derived. For example, the PLFA 16:1ω5 is a specific biomarker of the active biomass of mycorrhizal fungi in soil. The neutral lipid fatty acid 16:1ω5 in mycorrhizal fungi is associated with storage lipids present in vesicles and spores (van Aarle and Olsson, 2003). Multivariate analysis of the fatty acid profiles could be used to detect changes in the microbial community composition (Hedrick et al., 2005).

Fatty acid biomarkers analysis is a suitable method for studying the changes in the soil microbial communities caused by plant diversity and physiology (eg., Söderberg et al., 2002; Dunfield and Germida, 2004; Carney and Matson, 2005), tillage (e.g., Drijber et al., 2000; Feng et al., 2003; Spedding et al., 2004), fertilization (eg., Gryndler et al., 2006; Sullivan et al., 2006), pollution (eg., Kelly et al., 2003), soil properties (e.g., Schutter and Dick, 2000). Fatty acid profiling has been also used in plant pathology in order to better understand the impact of disease on soil microbial communities and also how inoculated biological control agents interact with these soil communities. Using phospholipid fatty acid (PLFA) approach, Hamel et al. (2005) showed that Fusarium crown and root rot of asparagus was associated with a profound cultivar-specific, reorganization of the soil microbial community. Kozdrój et al. (2004) showed that, using total fatty acid profiling, microbial communities in the rhizosphere of maize changed in response to inoculation with *Pseudomonas* spp. and the growth stage of the plant. Larkin and Honeycutt (2006) studied the effects of different cropping systems on soil microbial communities and Rhizoctonia diseases in potato. Many studies used the PLFA approach to study the interactions of arbuscular mycorrhizal fungi with other soil microbiota and plant (e.g., Olsson et al., 1996; Olsson et al., 1998; Green et al., 1999; Söderberg et al., 2002; Gormsen et al., 2004; Johansson et al., 2004; Albertsen et al., 2006). Mazzola (2004) proposed to use phospholipid fatty acid (PLFA) or fatty acid methyl ester (FAME) analysis in order to assess the biological nature of soil suppressiveness. Although, fatty acid profiling (and phospholipid fatty acid profiling) are sensitive enough for monitoring the global changes in the soil microbial community following inoculation of microorganisms and effect of plant disease, this approach was not report frequently used for such studies in field conditions. As far as we are

aware, there have been no published studies that report how soil microbial communities are affected by BCA treatments in a field trial, or whether soil microbial communities are affected when they inhabit the root-associated soil of plants with SSR disease.

1.7 GENERAL OBJECTIVES AND HYPOTHESES

Sclerotinia stem rot (SSR) disease caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is considered one of the most important fungal diseases of soybean. The application of biological control agents is a promising way of controlling SSR disease in the field. Although several biocontrol agents for SSR disease have been identified, there is a major gap in our knowledge of how effective these biocontrol agents are in the field. Also, biological control agents introduced in a soil environment will interact with other soil organisms, the pathogenic organism and the infected plants, which may change the genetic and functional diversity in soil microbial communities. Profiling these changes may lead to an improved understanding of the interactions between these players (biological control agent, pathogen, soil biota and plant) in the biological control phenomenom, permiting us to exploit naturally-occurring ecological relationships and develop more sustainable approaches to control soybean disease. Signature lipid biomarkers approach was used to profile microbial communities in soils. The improvement of laboratory techniques and procedures in the extraction and characterization of soil lipids would benefit researchers and could provide new insight into soil organic matter dynamics and soil microbial ecology.

The **general objective** of this thesis was to study the impacts of introducing biological control agents such as *Trichoderma virens* and arbuscular mycorrhizal fungi in protecting soybean against *Sclerotinia sclerotiorum* pathogen, the causal agent of SSR, and their effects on soil microbial communities in order to develop develop more sustainable approaches to control soybean disease.

The **general hypotheses** underlying this research work were (1) free-living fungi (*Trichoderma virens*) and arbuscular mycorrhizal fungi (*Glomus intraradices* and *Glomus mosseae*) will control SSR in soybean fields, by acting specifically against SSR, by inducing plant resistance mechanisms, through interactions with other microorganisms or by promoting the health of soybean plants, (2) soil inoculation with free-living fungi (*Trichoderma virens*) and arbuscular mycorrhizal

fungi (*Glomus intraradices* and *Glomus mosseae*) will impact the genetic and functional diversity of the soil microbial communties, and (3) changes in soil microbial communities will be detected with signature lipid biomarkers.

The **specific objectives** of this thesis were (1) to find the best solvent mixture for recovering fatty acid biomarkers from soil using a PSE system by comparing the soil lipids extracted with four organic solvent mixtures, namely chloroform:methanol:phosphate buffer (1:2:0.8, v/v/v), chloroform:methanol (1:2, v/v), hexane:2-propanol (3:2, v/v) and acetone, (2) to obtain, using a Py-MAB-TOF-MS system, characteristic and specifc fingerprints of the whole lipid composition of six soils from various agroecosystems, (3) to compare the discrimination among the soils obtained by Py-MAB-TOF-MS lipid fingerprinting to the one obtained by the GC-FID profiling of the fatty acid biomarkers in the same soils; (4) to determine whether SoilGard™ *(T.virens)* and AMF (a mixture of *Glomus intraradices and G. mosseae*), used alone or in combination, could reduce SSR disease incidence in soybeans, (5) to assess the effect of these BCA treatments on soybean growth and yield, (6) to investigate the effect of the inoculation of BCAs on soil microbial communities, and (7) to explore the relationships between the SSR disease and the soil microbial communities in order to better understand how the "health" status of the plant, monitored by the SSR disease incidence, will influence the microbial communities in the vicinity of the plants. The specific objectives 1 to 3 were needed to validate that signature lipid analysis techniques had sufficient efficiency for high-throughput analysis of many soil samples from field sites, as well as sufficient resolution to detect changes in microbial communities from diverse field sites.

The **specific hypotheses** underlying these studies were (1) that the PSE system using chloroform:methanol mixtures (chloroform:methanol:buffer and chloroform:methanol) should be also the most efficient solvents for extracting fatty acid biomarkers in soils with a range of clay and organic matter contents, (2) the Py-MAB-TOF-MS system will be able to rapidly generate a specific and discriminatory fingerprint of a soil based on its composition in lipids, similar to the discrimination that is achieved with fatty acid biomarker profiling by GC-FID. Thus, Py-MAB-TOF-MS could be used as a tool for cataloguing the soils based on their lipid composition and eventually their microbial communities, (3) BCAs have a protective effect on soybean against SSR disease, (4) the combination of the BCAs allows a better protective effect, (5) the introduction of biological

control agents have effects on soil microbial communities, (6) plants infested with SSR disease have effects on soil microbial communities and (7) these effects (BCA treatments, plants infested by SSR disease) can be assessed by fatty acid analysis.

2. COMPARISON OF SOLVENT MIXTURES FOR PRESSURIZED SOLVENT EXTRACTION OF SOIL FATTY ACID BIOMARKERS

2.1 Abstract

The extraction and transesterification of soil lipids into fatty acid methyl esters (FAMEs) is a useful technique for studying soil microbial communities. Traditionally, soil fatty acids were prepared by manually extracting soil lipids with a solvent mixture that contains a citrate or phosphate buffer, chloroform and methanol. The efficiency of the chloroform:methanol mixture in extracting lipids from soil samples with a PSE system, which uses less solvent and has a shorter extraction time than the traditional procedure, remains to be investigated. The objective of this study was to find the best method for extracting fatty acid biomarkers from soils with a range of clay and organic matter contents by comparing the soil lipids extracted with four organic solvent mixtures, namely chloroform:methanol:phosphate buffer (1:2:0.8, v/v/v), chloroform:methanol (1:2, v/v), hexane:2-propanol (3:2, v/v) and acetone. Total lipid fatty acid methyl esters (TL-FAMEs) were identified using gas chromatography and flame ionization detection. The agricultural soils studied had TL-FAME concentrations ranging from 57.3 to 542.2 nmoles g^{-1} soil (dry weight basis). The TL-FAME concentration was greater when soil was extracted with chloroform:methanol:buffer and chloroform:methanol than with the hexane:2-propanol and acetone solvents. The concentration of TL-FAMEs in various chemical (saturated chain, branched saturated chain, monounsaturated chain, polyunsaturated chain, hydroxy substituted fatty acids) and biological groups (bacteria, mycorrhizal fungi, saprophytic fungi, higher plants/faunal biota) was affected by the type of solvent used. The extraction efficiency for the chemical and biological groups followed the general trend of: chloroform:methanol:buffer ≥ chloroform:methanol > hexane:2-propanol = acetone. Discriminant analysis revealed differences in TL-FAME profiles based on soil characteristics and the type of solvent used to extract soil lipids, while principal components analysis indicated that TL-FAME profiles were strongly affected by the presence of saturated chain fatty acids and fatty acids originating from higher plants/faunal biota. Solvent mixtures containing chloroform and methanol were the most efficient for extracting lipids from the agricultural soils in this study. However, for soils with organic content of more than 69.1 g kg^{-1} (soils 3 & 4), we recommend more than 3 static cycles of extraction with the PSE system. Researchers should consider soil properties and the lipid groups to be studied when selecting a solvent mixture.

2.2 Introduction

Soil lipids are chemically and biologically diverse, since they come from plant, animal and microbial cells, and lipid extracts have been found to contain neutral lipids (e.g., sterols, monoacylglycerols, diacylglycerols, triacylglycerols, hydrocarbons, isoprenoids, free fatty acids), glycolipids, phospholipids and many others (e.g., Dinel et al., 1990; Gunstone et al., 1994; Bull et al., 2000; Almendros et al., 2001; O'Keefe, 2002; Otto et al., 2005; Quénéa et al., 2006).

The characterization of fatty acid biomarkers from the total lipids (Schutter and Dick, 2000; Drenovsky et al., 2004; Sullivan et al., 2006), phospholipids (Vestal and White, 1989; Zelles, 1999; Piotrowska-Seget and Mrozik, 2003) and neutral lipids (Olsson, 1999; Bååth, 2003), as well as their stable isotope composition (Boschker and Middelburg, 2002), can reveal changes in the structure, nutritional status and living biomass of soil microbial communities (Vestal and White, 1989; White and Macnaughton, 1997; Zelles, 1999). Fatty acids were extensively characterized in a wide range of microorganisms (e.g., bacteria, fungi, algae, protozoa) and are recognized to be very valuable biomarkers in chemotaxonomy and could be used to discriminate microorganisms up to the species level (Lechevalier and Lechevalier, 1989).

Traditionally, soil fatty acids were prepared by first extracting soil lipids with a solvent mixture that contains a citrate or phosphate buffer, chloroform and methanol (Frostegård et al., 1991; White and Ringelberg, 1998, adapted from Bligh and Dyer, 1959). Indeed, the extraction of lipids by chloroform:methanol mixture is very efficient for many biological materials (Shahidi and Wanasundara, 2002; Christie, 2003). However, the task of extracting and studying soil lipids is complicated because of 1) their diverse chemical structures and biological origins, and 2) soil particles, such as clays, and organic matter that can interfere with their extraction (Hance and Anderson, 1963; Frostegård et al., 1991; Nielsen and Petersen, 2000). Representative and quantitative extraction of soil lipids is desirable, so that comprehensive profiling of these compounds can be performed. Even if a specific group of lipids is targeted, it is essential that the extraction process is optimized to permit comparisons within replicates and among treatments from single and multiple experimental sites.

Tunlid and White (1992) mentioned also that the efficiency of chloroform:methanol mixture in extracting lipids from soil samples was never fully investigated and according to our knowledge, it still remains to be performed. Considering its physical-chemical characteristics, its composition in biotic communities as well as their organization in a three-dimensional environment, a soil could be considered "unique" in its properties and the use of a 'universal' solvent to extract the lipids, if not optimized, could end up in underestimating its composition in microorganisms. Thus, it is imperative to evaluate the performance ('robustness') of chloroform:methanol extractant in a range of soils as well as in comparison with other efficient solvent mixtures in their capacity to extract fatty acid biomarkers. However, using an extraction mixture that contains chloroform and methanol should also be a concern because these solvents are toxic to humans when inhaled or adsorbed through the skin, and chloroform is carcinogenic (Ikeda, 1992; Golden et al., 1997). The research for less toxic solvents to extract lipids is a long-lasting quest in the field of lipidology (Christie, 1993, 2003). This brings us to consider less toxic alternatives in extracting lipids from soils.

Many solvents have been tested as alternatives to chloroform:methanol solvent mixture for lipid extraction (see reviews by Shahidi and Wanasundara, 2002 and Christie, 2003). In this study, we proposed to assess the efficiency of a mixture of chloroform:methanol:buffer (1:2:0.8, v/v/v) in its capacity to extract soil fatty acid biomarkers in soils presenting a range of clay as well as organic matter contents in comparison with chloroform:methanol (1:2, v/v), hexane:2-propanol (3:2, v/v) and acetone. Chloroform:methanol is a similar extractant than chloroform:methanol:buffer (1:2:0.8, v/v/v), but its use doesn't include a the time-consuming post-extraction step of phase separation that is usefull to remove non lipid molecules (amino acids, carbohydrates, salts, etc) that could interfere in the identification and quantification of fatty acids. Hexane:2-propanol is effective at extracting lipids from various biological materials and is commonly used as an equivalent alternative extractant to chloroform:methanol mixtures (Hara and Radin, 1978; Shahidi and Wanasundara, 2002; Christie, 2003; Dodds et al., 2004; Tanamati et al., 2005). This mixture was developed as a low toxicity alternative to chloroform:methanol for extracting lipids from tissues (Hara and Radin, 1978). However, its performance varies with the material tested. For example, Schäfer (1998) showed that more of the fatty acids contained in cereal and yolk lipids were extracted using a hexane:2-propanol (3:2, v/v) mixture than chloroform:methanol mixture, although the extraction of muscle lipids was more efficient with chloroform:methanol (2:1, v/v) than with other

solvent mixtures. Due to its popularity and widespread use, this mixture merits to be investigated in its capacity to extract fatty acid biomarkers from soil samples. Finally, acetone was selected because of its lower toxicity compared to all the other mixtures as well as its capacity to extract lipids form *Eucalyptus globulus* wood wood (González-Vila et al., 2000) and wheat grains (Zarnowski and Suzuki, 2004). This solvent was also never used to extract lipids from soil samples. Even if the polarity index of the solvents and lipid solubilities in these solvents are known, it is difficult to predict the capacity of a given solvent to solubilize lipids in a given biological material and experimentation is often needed to assess solvent efficiency (Christie, 1993).

Moreover, many techniques were designed to facilitate the extraction process from solid matrices (Camel, 2001). Among them, the PSE system has been previously used to extract lipids from soils (Macnaughton et al., 1997; Dinel and Nolin, 2000; Spedding et al., 2004; Wiesenberg et al., 2004; Hamel et al., 2005; Jansen et al., 2006). This technique accelerates the extraction process, reduces human contact with solvents, and reduces the volume of solvents used. The capacity of the PSE system to extract soil fatty acid biomarkers in a range of soils was not fully optimized using various solvent mixtures.

The hypothesis underlying this study are that pressurized solvent extraction using chloroform:methanol mixtures (chloroform:methanol:buffer and chloroform:methanol) should be also the most efficient solvents for extracting fatty acid biomarkers in soils with a range of clay and organic matter contents. The objective of this research was to find the best solvent mixture (or extractant) for recovering fatty acid biomarkers from soil using a PSE system by comparing the soil lipids extracted with four organic solvent mixtures, namely chloroform:methanol:phosphate buffer (1:2:0.8, v/v/v), chloroform:methanol (1:2, v/v), hexane:2-propanol (3:2, v/v) and acetone. In the choice of the best extractant, its toxicity should also be considered. Soil lipids extracted with these solvents were transesterified and characterized chemically and biologically using TL-FAME profiles. We were thus able to quantify and compare the fatty acids extracted with each solvent based on their chemical structure (e.g., saturated, monounsaturated and polyunsaturated fatty acids) as well as biological origin (e.g., fatty acid biomarkers of bacteria, mycorrhizal fungi, saprophytic fungi, higher plants/faunal biota).

2.3 Materials and methods

2.3.1 Soil collection and handling

The soils used in this study (mixed, frigid Typic Endoaquents) were collected from the top 15 cm of agricultural fields in southwestern Québec, Canada in August (soil 4) and September (soils 1, 2 and 3) of 1999 prior to crop harvest. These soils were sampled during a survey of 50 fields in which 4 random composite samples were taken. Each of the four composite soil samples used in this study come from a different field. After collection, half of each soil sample was air-dried, sieved (< 2 mm mesh), stored at room temperature and used for soil physical and chemical analysis, while the other half was frozen immediately and stored at -20°C until microbial biomass carbon and lipid analysis was conducted. Agricultural practices at each collection site and selected soil characteristics are reported in Table 1.

2.3.2 Reagents and glassware

All organic solvents used in this study were HPLC (high pressure liquid chromatography) grade, and all glassware was either rinsed with methanol and chloroform or placed in a furnace at 360°C for at least 2 h. Laboratory equipment that did not tolerate heating at 360°C was rinsed with methanol and then chloroform, and allowed to dry at room temperature (20°C) before use.

2.3.3 Pressurized solvent extraction (PSE) system

Soil lipids were extracted with an ASE 200 accelerated solvent extractor (Dionex Corporation, Sunnyvale, CA, USA) using the operating conditions described by Macnaughton et al. (1997), which consisted of one heating cycle at 80°C and 8280 kPa during 5 min, three static cycles of 15 min each at the same temperature and pressure, rinsing of the transfer lines and sample cell with the solvent and purging with N_2 for 180 s between each sample. Triplicate samples of each soil included in this study were extracted using the PSE system. Between 6 and 8 g of freeze-dried soil was packed into a 11-mL stainless steel ASE vessel that had been rinsed with chloroform:methanol (1:2) solution. Vessels were sealed at both ends with circular cellulose filters to prevent soil

particles from entering the extractor. The following solvents were used: (a) chloroform:methanol:phosphate buffer (1:2:0.8, v/v/v), (b) chloroform:methanol (1:2, v/v), (c) hexane:2-propanol (3:2, v/v) and (d) acetone. Additional chloroform and buffer were added to the extracts from solvent mixture (a) to allow the aqueous and organic phases to separate, so the final ratio of the chloroform:methanol:phosphate buffer was 2:2:1.8 (v/v/v). This procedure produced 20-25 mL of an extract containing soil lipids and solvents. The organic phase was then removed by volatilization under N_2 gas.

2.3.4 Quantification and identification of TL-FAMEs

TL-FAMEs were prepared by mild alkaline methanolysis of total soil lipid extracts according to White and Ringelberg (1998). Each TL-FAME extract was dried completely under N_2 and redissolved with 1-mL of iso-octane containing 25 ng μL^{-1} of methyl-nonadecanoate (C19:0) internal standard. Then, TL-FAMEs (5 µL injected) were analyzed in split mode (50:1) with a gas chromatograph (Hewlett Packard 6890) equipped with a Simplicity Wax capillary column (cross-linked polyethylene glycol; length, 30 m; film thickness, 0.33 µm; Supelco 2-4326), helium as carrier gas (constant at 9.5 psi) and a flame ionization detector. The oven temperature was initially set at 60°C, then raised to 150°C (10°C min^{-1}) and held for 5 min, after which it was raised by 3°C min^{-1} to a final temperature of 230°C and held for 20 min. Inlet and detector temperatures were 200°C and 250°C, respectively. The linear flow velocity was at 32 cm/s.

Identification of peaks was based on comparison of retention times to known standards (Supelco 37 Component FAME Mix cat.#47885-U; Supelco Bacterial Acid Methyl Esters cat.#47080-U; Matreya PUFA-2 cat.#1081; Matreya Bacterial Acid Methyl Esters CP Mix cat.#1114; Matreya cis-11-Hexadecenoic Acid cat.#1208 and Matreya 10-Methyloctadecanoate cat.#1763), used directly or derivatized if needed, containing FAMEs with chain length ranging from 8 to 24 carbon atoms. These standards allowed us to identify more than 50 different FAMEs (see Appendix A).

The concentration of each TL-FAME (nmoles per gram dry soil (DS)) identified was calculated as using C19:0 as internal standard at a concentration of 25 ng μL^{-1} (0.080 nmole C19:0 μL^{-1}) in each sample. The contribution of each identified TL-FAME to the total TL-FAME concentration (all TL-

FAMEs identified) in a sample, expressed as mole fraction (relative richness, % mole), was calculated and used in the multivariate analysis.

2.3.5 TL-FAME nomenclature, chemical and biological groups

We used the standard ω-nomenclature (A:BωC) for designating the fatty acids (IUPAC-IUB, 1977). The TL-FAMEs identified in our samples were grouped according to their chemical class (straight saturated chain, branched saturated chain, monounsaturated chain, polyunsaturated chain, hydroxy substituted fatty acids) and biological origin (biomarkers of bacteria, mycorrhizal fungi, saprophytic fungi, higher plant/faunal biota, general biota). The different biological groups were computed using the following TL-FAMEs: bacteria (i-15:0, a-15:0, 15:0, i-16:0, 16:1ω7, i-17:0, 3-OH-12:0, 17:0, 17:1ω7, 17:0cy, 18:1ω7, 10Me18:0), mycorrhizal fungi (16:1ω5c), saprophytic fungi (18:1ω9c/t, 18:2ω6c/t, 18:3ω6, 18:3ω3) and a general biotic marker (16:0) (Federle, 1986; Vestal and White, 1989; Frostegård et al., 1993; Graham et al., 1995; Olsson et al., 1995; Frostegård and Bååth, 1996; Zelles, 1997, 1999; Olsson, 1999; Hill et al., 2001). Also, all TL-FAMEs with more than 20 carbons (20:0, 21:0, 22:0, 23:0, 24:0, 20:1ω9, 20:2ω6, 20:3ω3, 20:3ω6, 20:4ω6, 20:5ω3, 22:1ω9, 22:2ω6, 22:4ω6, 22:5ω3, 22:6ω3, 24:1ω9) were categorized as TL-FAMEs > 20 C because of the diverse origins (bacterial and fungal cells, plants, protozoa and other animals) of soil lipids in this group (Vestal and White, 1989; Schulten and Schnitzer, 1991; Zelles, 1999; Rezanka and Votruba, 2002; Jandl et al., 2005; Otto et al., 2005).

2.3.6 Statistical analysis

In this study, from each of the four original soil samples, twelve subsamples were removed and extracted with the four extractants in triplicate, thus generating forty-eight samples. This experiment wanted to isolate the effect on the variability that could be attributed to the extraction itself by minimizing sample-to-sample variability, thus using "pseudo-replicates" or "analytical replicates". A follow-up experiment will be needed in order to assess the soil variability on the extraction using true replicates.

A one-way analysis of variance (ANOVA) was performed to determine how solvents (chloroform:methanol:phosphate buffer, chloroform:methanol, hexane:2-propanol and acetone) affected the quantity of TL-FAMEs (nmole/g DS, %mole) in the chemical classes and biological groups defined above. The ANOVA was performed using CoStat, version 6.003 (CoHort Software, Monterey, CA, USA) using LSD (Least significant difference), at α=0.05, as a post-hoc test for mean comparison. The values presented in graphs and tables are untransformed means (± standard errors of the mean) of triplicate soil samples.

Principal components analysis (PCA) and discriminant analysis (DA) were performed on the dataset of individual TL-FAMEs identified in the samples (TL-FAME profiles) to explain sources of variation and to test the discrimination of the samples according to the soil and the solvent mixture. The data, expressed in % mole, were transformed with a $\log_{10}x+1$ transformation, where x is the % mole of each TL-FAME in a sample and analysed using SYSTAT software, version 10 (Systat Software Inc., Richmond, CA, USA).

2.4 Results

The soils chosen for this study had diverse characteristics, with clay contents ranging from 174 to 795 mg kg^{-1}, organic matter levels from 23.5 to 263.3 g kg^{-1} and soil microbial carbon from 153.7 to 452.3 mg C kg^{-1} (Table 1), and are representative of agricultural soils in southwestern Québec. The TL-FAME concentration in these soils ranged from 57.3 to 542.2 nmoles g^{-1} DS (Table 2). In soils 1 and 2, the TL-FAME concentration was greater when soil lipids were extracted with chloroform:methanol:buffer than other solvents (Table 2). Chloroform:methanol:buffer and chloroform:methanol mixtures gave greater TL-FAME concentrations than hexane:2-propanol or acetone in soil 3, but the results for soil 4 were not consistent among extraction procedures (Table 2). Independent of the solvent used, the TL-FAME concentration followed the order soil 4 > soil 3 > soil 2 > soil 1, which coincided with the organic matter and soil microbial carbon content in these soils.

2.4.1 Chemical classes of TL-FAMEs in soil lipid extracts

The TL-FAMEs in soil lipid extracts were grouped according to their chemical structure and analysed to determine whether any chemical groups were selectively extracted by the solvent mixtures tested (Table 2). The TL-FAME profiles contained between 26.8 and 342.0 nmoles/g DS of total saturated chain fatty acids (SAFAs), which is the sum of straight SAFAs and branched SAFAs. Straight SAFAs were the most common, accounting for 29.6 to 62.2 % of the total soil TL-FAMEs. The concentration of total unsaturated chain fatty acids (UFAs), which are composed of monoUFAs (Mono unsaturated chain fatty acids) and polyUFAs (Poly unsaturated chain fatty acids), ranged from 28.8 to 223.1 nmoles/g DS. MonoUFAs accounted for 4.9 to 30.6 % of the soil TL-FAMEs, but the polyUFAs were more common (27.3 to 52.7 % of the soil TL-FAMEs). The hydroxy fatty acids (HYFAs) were less than 2 % of the soil TL-FAMEs and concentrations ranged from 0.0 to 4.8 nmoles/g DS (Table 2).

The concentrations (in nmoles g^{-1} DS) of straight SAFAs in lipid extracts from soils 1, 2 and 3 were significantly ($P<0.05$, LSD test) greater with chloroform:methanol:buffer and chloroform:methanol than with acetone or hexane:2-propanol (Table 2). In soil 4, the concentration of straight SAFAs in lipid extracts was greatest with chloroform:methanol and lowest with hexane:2-propanol (Table 2). In all soils, the branched SAFAs concentrations were significantly ($P<0.05$, LSD test) greater with chloroform:methanol:buffer than other solvents.

The concentrations of monoUFAs, polyUFAs and HYFAs in lipid extracts from soils 1 and 2 were greatest in chloroform:methanol:buffer, followed by chloroform:methanol; there was no difference in the concentrations of monoUFAs, polyUFAs and HYFAs when lipids were extracted with hexane:2-propanol and acetone (Table 2). For soils 3 and 4, the monoUFA and HYFA concentrations were greater ($P<0.05$, LSD test) when lipids were extracted with chloroform:methanol or chloroform:methanol:buffer than with acetone and hexane:2-propanol. PolyUFA concentrations were greater ($P<0.05$, LSD test) in soils 3 and 4 when the lipids were extracted with chloroform:methanol mixture and also as with acetone in soil 4 (Table 2).

2.4.2 Biological groups of TL-FAMEs in soil lipid extracts

Biological groups of TL-FAMEs in soil lipid extracts were analysed to determine whether any were selectively extracted by the solvent mixtures tested (Table 3). The TL-FAME biomarkers were grouped as follows: bacteria were 3.2 to 26.8 % of TL-FAMEs, mycorrhizal fungi were 0.5 to 6.3 % of TL-FAMEs and saprophytic fungi were 7.8 to 20.8 % of TL-FAMEs. We also found that 36.5 to 66.9 % of the TL-FAMEs had a chain length ≥ 20C, which may indicate that these lipids were derived from higher plants and animals. The general microbial biomass marker, 16:0, represented 5.3 to 10.9 % of the TL-FAMEs.

The concentrations of bacteria and mycorrhizal fungi biomarkers in all soils were greatest when chloroform:methanol:buffer was used, and there were significantly (P<0.05, LSD test) greater concentrations of bacteria and mycorrhizal fungi biomarkers when soils were extracted with chloroform:methanol than acetone and hexane:2-propanol mixtures (Table 3). In soils 1 and 2, fungal biomarker concentrations were greater when lipids were extracted with chloroform:methanol:buffer but there was no difference amongst solvents for soil 3. In soil 4, chloroform:methanol:buffer, chloroform:methanol and acetone mixtures were equivalently efficient (P<0.05, LSD test) in extracting fungal biomarkers compared to the hexane:2-propanol mixture. In soil 1, the concentration of TL-FAMEs ≥ 20C was significantly (P<0.05, LSD test) greater when extracted with chloroform:methanol:buffer than with the other solvent. In soil 2, chloroform:methanol:buffer and chloroform:methanol gave an equal concentration of TL-FAMEs ≥ 20C, while in soil 3, the chloroform:methanol extract had a greater TL-FAMEs ≥ 20C concentration than the other solvents. In soil 4, the lipids extracted with acetone had a significantly greater (P<0.05, LSD test) concentration of TL-FAMEs ≥ 20C than lipids extracted with the other solvents (Table 3). The general biomass marker (16:0) concentration tended to be greater when soil lipids were extracted with chloroform:methanol:buffer and chloroform:methanol than acetone and hexane:2 propanol (Table 3).

2.4.3 Classifying soil chemistry and biology with TL-FAME profiles

The PCA of TL-FAMEs led to the identification of eleven principal components (PCs) that explained more than 89 % of the variance in the dataset, however, the two first components, PC1 and PC2, explained more than 51% of the variance. The TL-FAME profiles from soils 3 and 4 gave scores that generally appeared in the negative PC1 quadrant, while the TL-FAME profiles of soils 1 and 2 gave more disperse scores along the PC1 axis (Figure 1). We noticed that the scores coming from soil lipids extracted with chloroform:methanol:buffer and chloroform:methanol were distributed along the PC1 axis; in contrast, the scores from soil lipids extracted with hexane:2-propanol and acetone varied more along the PC2 axis (Figure 1). The composition in TL-FAMEs of hexane:2-propanol and acetone extracts from soils 1 and 2 were similar (Figure 1). The TL-FAME profiles for soil 4 appeared in the upper left-hand corner of Figure 1 (negative PC1 values, positive PC2 values), and were distinct from the scores reported for soils 1, 2 and 3.

Analysis of the loading values (Table 4) generated by the principal components analysis of TL-FAMEs dataset could give us insights about the chemical structure and biological origin of TL-FAMEs appearing in the scores plot (Figure 1). Bacterial and mycorrhizal biomarkers contribute with high positive loadings to PC1 while TL-FAMEs ≥ 20C contribute with high negative loadings to PC1 (Table 4). The largest positive values along the PC1 axis were from soil 1 and 2 extracted with chloroform:methanol:buffer, which had 31 to 33% bacterial and mycorrhizal biomarkers in the total TL-FAME pool (Table 3). In contrast, TL-FAMEs ≥ 20C represented 49 to 66% of the TL-FAMEs extracted from soils 3 and 4, and was the dominant biomarker group found in hexane:2-propanol and acetone extracts from soils 1 and 2 (Table 3). Eight of the ten TL-FAMEs having positive PC2 loadings were straight chain saturated fatty acids, and most of the negative PC2 loadings were from unsaturated fatty acids (Table 4). It appears that the distribution of TL-FAME profiles along the PC2 axis was related to the concentration of straight SAFAs (Tables 2 and 4, Figure 1). Soil 4 had a greater straight SAFA concentration than the other soils, which explains why it appears in the quadrant with positive PC2 values. The concentration of straight SAFAs in soils 1, 2 and 3 was affected by the solvent used, with hexane:2-propanol and acetone extracting less straight SAFAs than chloroform:methanol:buffer and chloroform:methanol (Table 2). This is consistent with the distribution of TL-FAME profiles for these soils along the PC2 axis (Figure 1).

Discriminant analysis, based on the individual TL-FAMEs found in the soil lipid extracts, provided significant discrimination between soil types and solvent mixtures (Wilks' lambda=0.000 at $p<0.00005$ for all discriminant analysis tests) (Figures 2a, 2b). The best discriminating variables selected by the automatic backward stepwise procedure permitted us to correctly classify 98% of the solvent mixtures and 100% of soils (Figures 2a, 2b).

2.5 Discussion

Lipids can be selectively solubilized by organic solvents, depending on structural features such as the proportion of nonpolar hydrocarbon chains in the fatty acids or other aliphatic moieties and the presence of polar functional groups, such as phosphate and sugar moieties. Neutral lipids are highly soluble in hydrocarbon solvents (e.g. hexane, toluene, benzene or cyclohexane) and some polar solvents (e.g. chloroform and diethyl ether), although they are insoluble in methanol, a polar solvent. The solubility of such lipids in alcoholic solvents increases with the chain length of the hydrocarbon moiety of the alcohol: for example, nonpolar lipids are more soluble in ethanol and completely soluble in n-butanol. In the same way, the solubility of short fatty acids residues in the lipids increases when polar solvents are used. Polar lipids are only sparingly soluble in hydrocarbon solvents unless solubilized in association with other lipids; however, they dissolve readily in more polar solvents, such as methanol, ethanol, or chloroform (Christie, 1982; Christie, 1993; Shahidi and Wanasundara, 2002). The chloroform:methanol:buffer mixture contains an aqueous buffer as well as chloroform and methanol, and it has been proposed that this type of monophasic solution should have a greater ability to break polar bonds and extract lipids from biological materials than the chloroform:methanol mixture alone (Bligh and Dyer, 1959).

Despite methodological differences, the results for total concentration of identified TL-FAMEs as well as the concentrations of TL-FAMEs in chemical and biological groups are in the range of published data (e.g., Bull et al., 2000; Drenovsky et al., 2004; Allison et al. 2005; Jandl et al., 2005; Joergensen and Potthoff, 2005) for agricultural soils. For example, the total concentration of identified TL-FAMEs in the soils used in this study ranged from 57.3 to 542.2 nmoles g^{-1} DS (Table 2). These values are in a similar range to the 160.8 to 341.2 nmoles g^{-1} DS of TL-FAMEs reported

by Drenovsky et al. (2004) for agricultural soils analysed with the Microbial Identification System (MIS, Microbial ID Inc., Newark, DE, USA), another method for directly characterized whole soil fatty acids. The TL-FAME concentration in all soils was greater when solvents containing chloroform and methanol were used, which is consistent with other studies showing chloroform-methanol mixtures to be the most efficient for extracting lipids from biological materials (Fishwick and Wright, 1977; Gunnlaugsdottir and Ackman, 1993; Undeland et al., 1998; Ackman, 2000; Shahidi and Wanasundara, 2002; Gallina Toschi et al., 2003).

The TL-FAMEs ≥ 20C detected and quantified in this study contributed to a large part of the total concentration of TL-FAMEs. Data on these FAMEs are not often presented in papers oriented on the understanding microbial community dynamics because they are not typically biomarkers of bacteria and fungi. However, when they are monitored, it could be observed that TL-FAMEs ≥ 20C contributed to a larger part of the fatty acids pool in agricultural soils (Jandl et al., 2002, 2005). Jandl et al. (2005) showed that these FAMEs and longer ones could came from different biological origins such as above- and below-ground crop residues, organic manure and soil organisms.

However, not all chemical compounds are extracted more efficiently with chloroform:methanol mixtures than other solvents. Several researchers found that the chloroform:methanol:buffer mixture extracted greater quantities of phospholipids than the hexane:2-propanol mixture, but there was no difference in the level of neutral lipids (triacylglycerols, cholesterol esters, free fatty acids) extracted with these mixtures (Erickson, 1993; Gunnlaugsdottir and Ackman, 1993; Undeland et al., 1998). The 2-propanol in the hexane:2-propanol mixture is a less polar solvent than the methanol in the chloroform:methanol:buffer and chloroform:methanol mixtures. Thus, the poor solubility of polar components in hydrocarbon solvents (e.g. hexane), and the lower polarity of 2-propanol compared to methanol, might, in part, explain the trend of lower recovery of polar lipid classes and total TL-FAMEs with the hexane:2-propanol mixture than with the chloroform:methanol:buffer and chloroform:methanol mixtures. Acetone is a solvent of medium polarity often used to extract simple lipids and glycolipids or precipitate phospholipids (Christie, 1993). Even if acetone is the standard solvent for wood extractives (Gonzalez-Vila et al., 2000) and the best for extracting the highest amounts of total resorcinolic lipids from wheat grains (Zarnowski and Suzuki, 2004), our results

show that this solvent was not generally efficient at extracting the various TL-FAME chemical classes and biomarkers from soils selected for this study.

The principal components analysis showed that the TL-FAME profiles of soil 4 were grouped in one quadrant, distinct from the TL-FAME profiles of the other three agricultural soils (Figure 1). This is likely related to the distinct soil properties of soil 4, which contained a greater TL-FAME concentration, organic matter and microbial biomass than the other soils. We believe the TL-FAME profiles for soil 4 to be representative and qualitative, but this remains to be confirmed. Spiking a soil with a known concentration of a particular lipid might seem like one way to evaluate the extraction efficiency of solvent mixtures, except that soils are biologically active and there is a distinct possibility that newly-added lipids would be rapidly metabolized by soil microorganisms. We suspect that solvent mixtures can become saturated in soils with high soil organic matter content, due to the presence of readily available organic molecules (not necessarily lipids). If this is the case, then longer extraction times, a higher solvent mixture:sample ratio, or a solvent mixture containing more chloroform would be required to generate a representative sample of lipids. For example, Iverson et al. (2001) showed that in marine samples containing > 2% lipids, a ratio 1:2 of chloroform:methanol (or the extraction by the Bligh and Dyer method) underestimated the lipid contents of these tissues compared to the Folch method, which uses a ratio of 2:1 chloroform:methanol. These considerations deserve further investigation in soils.

2.6 Conclusions

Fatty acids are major building blocks for many classes of lipids, including acylglycerols and phospholipids, and are widely used as biomarkers in microbial ecology and to characterize soil microbial communities. We found that chloroform:methanol:buffer and chloroform:methanol extracted a greater concentration of TL-FAMEs from soils than the hexane:2-propanol and acetone solvents. The chemical structure and biological groups of TL-FAMEs was affected by the type of solvent used, and the general trend for extraction efficiency was chloroform:methanol:buffer ≥ chloroform:methanol > hexane:2-propanol = acetone. Solvent mixtures containing chloroform and methanol were the most efficient for extracting lipids from the agricultural soils in this study. To generate representative lipid samples from soils with a high organic matter content, we recommend

that researchers consider longer extraction times, a higher solvent mixture:sample ratio, or increase the chloroform content in the solvent mixture. Improved laboratory techniques and procedures that permit researchers to efficiently extract and characterise soil lipids will provide new insight into soil organic matter dynamics and soil microbial ecology.

Table 1. Selected properties of the soils (Typic Endoquents, 0-15 cm depth) used in the experiment.

Soil	Tillage system†	Crop	pH‡	OM§	Sand¶	Clay¶	Silt¶	Textural class	SMB-C#
				g kg^{-1}	g kg^{-1}	g kg^{-1}	g kg^{-1}		mg C kg^{-1}
1	CT	soybean	8.0	23.5	211	450	339	Clay	153.7 (±10.3)
2	NT	soybean	6.8	45.1	543	174	283	Sandy loam	215.3 (±18.4)
3	NT	soybean	7.0	69.1	0	795	205	Clay	384.8 (±11.1)
4	CT	bean	6.1	263.3	149	433	418	Silty clay	452.3 (±23.2)

† CT: conventional tillage, NT: no-tillage.

‡ Soil:water extracts (1:2 soil:solution ratio) (Hendershot et al., 1993).

§ Organic Matter (OM) determined by loss on ignition (360°C for 4 h) (Schulte et al., 1991).

¶ Particle-size analysis (Sheldrick and Wang, 1993).

SMB-C is soil microbial biomass C, mean (± standard error of mean) of 3 replicate measures. SMB-C = Chloroform labile C/K_{EC}, using a K_{EC} of 0.45 (Wu et al., 1990; Voroney et al., 1993; Joergensen, 1996).

Table 2. Chemical classes of TL-FAMEs in soil lipids extracted with various solvents using a PSE system. For each soil, means in the same column followed by the same or no letters are not significantly different (LSD Test, $p<0.05$).

Solvent mixture	Total TL-FAMEs	StraightSAFAs†	BranchedSAFAs	MonoUFAs	PolyUFAs	HYFAs
			mean (nmoles g^{-1} DS)			
			Soil 1			
Acetone	59.7c	25.0c	1.8c	11.0c	21.6c	0.3c
Chloroform:methanol	101.0b	38.2b	5.6b	21.5b	34.7b	0.9b
Chloroform:methanol:buffer	167.1a	49.4a	16.7a	51.1a	47.0a	2.8a
Hexane:2-propanol	57.3c	26.4c	1.8c	9.7c	19.1c	0.4c
			Soil 2			
Acetone	136.1c	66.4b	4.4c	21.5c	42.9c	0.9c
Chloroform:methanol	216.2b	91.7a	13.2b	41.9b	66.7b	2.6b
Chloroform:methanol:buffer	282.1a	93.4a	31.5a	73.0a	79.4a	4.8a
Hexane:2-propanol	137.2c	63.4b	4.1c	20.2c	48.9c	0.7c
			Soil 3			
Acetone	242.9b	85.7b	4.0c	28.9b	124.3bc	0.0c
Chloroform:methanol	360.6a	122.2a	13.5b	69.8a	153.3a	1.9b
Chloroform:methanol:buffer	361.7a	121.8a	30.6a	70.1a	135.6b	3.6a
Hexane:2-propanol	216.8b	77.6b	4.8c	20.2b	114.3c	0.0c
			Soil 4			
Acetone	493.5ab	297.8a	6.4c	24.1b	164.8a	0.7b
Chloroform:methanol	542.2a	321.7a	20.3b	43.7a	153.2a	3.2a
Chloroform:methanol:buffer	438.8b	233.9b	27.7a	54.7a	119.8b	2.6a
Hexane:2-propanol	346.2c	215.3b	6.0c	18.0b	107.0b	0.0c

† SAFAs are saturated chain fatty acids, UFAs are unsaturated chain fatty acids and HYFAs are hydroxy fatty acids.

Table 3. Biologically relevant groups of TL-FAMEs in soil lipids extracted with various solvents using a PSE system.

For each soil, means in the same column followed by the same or no letters are not significantly different (LSD Test, $p<0.05$).

Solvent mixture	Total TL-FAMEs	Bacteria†	Mycorrhizae	Fungi	FAMEs ≥ 20C	General biomass marker 16:0
			mean (nmoles g^{-1} DS)			
			Soil 1			
Acetone	59.7c	5.8c	1.4c	12.4c	30.6c	4.5c
Chloroform:methanol	101.0b	15.3b	3.7b	16.5b	49.8b	8.1b
Chloroform:methanol:buffer	167.1a	44.7a	10.6a	25.2a	61.0a	13.7a
Hexane:2-propanol	57.3c	6.2c	1.3c	9.9d	31.0c	4.2c
			Soil 2			
Acetone	136.1c	13.0c	3.7c	19.1c	82.1b	8.4c
Chloroform:methanol	216.2b	31.7b	7.8b	28.5b	117.1a	16.1b
Chloroform:methanol:buffer	282.1a	72.9a	15.4a	37.1a	118.2a	22.5a
Hexane:2-propanol	137.2c	11.6c	3.5c	14.4d	91.8b	7.3d
			Soil 3			
Acetone	242.9b	9.6c	3.6c	42.7	160.1c	13.8c
Chloroform:methanol	360.6a	26.1b	8.5b	46.9	236.1a	22.2b
Chloroform:methanol:buffer	361.7a	68.3a	17.1a	47.7	178.8b	28.1a
Hexane:2-propanol	216.8b	9.6c	3.7c	36.2	143.8d	12.8c
			Soil 4			
Acetone	493.5ab	15.8c	2.4c	39.5a	321.3a	37.1bc
Chloroform:methanol	542.2a	45.5b	5.7b	49.4a	283.9b	59.1a
Chloroform:methanol:buffer	438.8b	61.0a	9.6a	39.8a	216.3c	46.5b
Hexane:2-propanol	346.2c	14.1c	2.0c	27.1b	204.8c	30.8c

†The TL-FAMEs used to define (or used in) each grouping are detailed in the Materials and Methods section.

Table 4. Selected loading values from principal components analysis of TL-FAME profiles from the dataset of the individual TL-FAMEs identified in soils extracted with various solvents. Values are the ten highest and ten lowest for each principal components (PC) axis. The chemical class and biological group of individual TL-FAMEs are provided.

TL-FAME	Chemical class	Biological group	PC1 loading	TL-FAME	Chemical class	Biological group	PC2 loading
18:2ω6t	PolyUFAs	Fungi	-0.675	18:2ω6c	PolyUFAs	Fungi	-0.617
20:2ω6	PolyUFAs	FAMEs ≥ 20C	-0.581	22:2ω6	PolyUFAs	FAMEs ≥ 20C	-0.611
20:0/19cy†	StraightSAFAs	FAMEs ≥ 20C	-0.568	24:0	StraightSAFAs	FAMEs ≥ 20C	-0.582
21:0	StraightSAFAs	FAMEs ≥ 20C	-0.568	20:5ω3	PolyUFAs	FAMEs ≥ 20C	-0.561
22:2ω6	PolyUFAs	FAMEs ≥ 20C	-0.521	18:1ω9c/t	MonoUFAs	Fungi	-0.542
18:0	StraightSAFAs	-	-0.473	22:6ω3/24:1ω9†	PolyUFAs	FAMEs ≥ 20C	-0.491
22:5ω3	PolyUFAs	FAMEs ≥ 20C	-0.462	20:3ω3	PolyUFAs	FAMEs ≥ 20C	-0.462
22:0	StraightSAFAs	FAMEs ≥ 20C	-0.456	22:1ω9	MonoUFAs	FAMEs ≥ 20C	-0.405
22:1ω9	MonoUFAs	FAMEs ≥ 20C	-0.411	16:1ω5	MonoUFAs	Mycorrhizae	-0.403
14:0	StraightSAFAs	-	-0.385	20:2ω6	PolyUFAs	FAMEs ≥ 20C	-0.400
i-15:0	BranchedSAFAs	Bacteria	0.867	22:5ω3	PolyUFAs	FAMEs ≥ 20C	0.542
17:0cy	BranchedSAFAs	Bacteria	0.873	20:0/19cy†	StraightSAFAs	FAMEs ≥ 20C	0.610
20:1ω9	MonoUFAs	FAMEs ≥ 20C	0.876	10:0	StraightSAFAs	-	0.650
18:3ω3	PolyUFAs	Fungi	0.881	20:2ω6	PolyUFAs	FAMEs20C	0.651
16:1ω5	MonoUFAs	Mycorrhizae	0.885	18:0	StraightSAFAs	-	0.676
18:1ω7/10Me18:0†	MonoUFAs	Bacteria	0.924	16:0	StraightSAFAs	-	0.759
3-OH-C12:0	HYFAs	Bacteria	0.954	15:0	StraightSAFAs	Bacteria	0.811
i17:0	BranchedSAFAs	Bacteria	0.956	13:0	StraightSAFAs	-	0.853
i16:0	BranchedSAFAs	Bacteria	0.973	14:0	StraightSAFAs	-	0.876
16:1ω7	MonoUFAs	Bacteria	0.976	12:0	StraightSAFAs	-	0.888

†Co-eluting TL-FAMEs

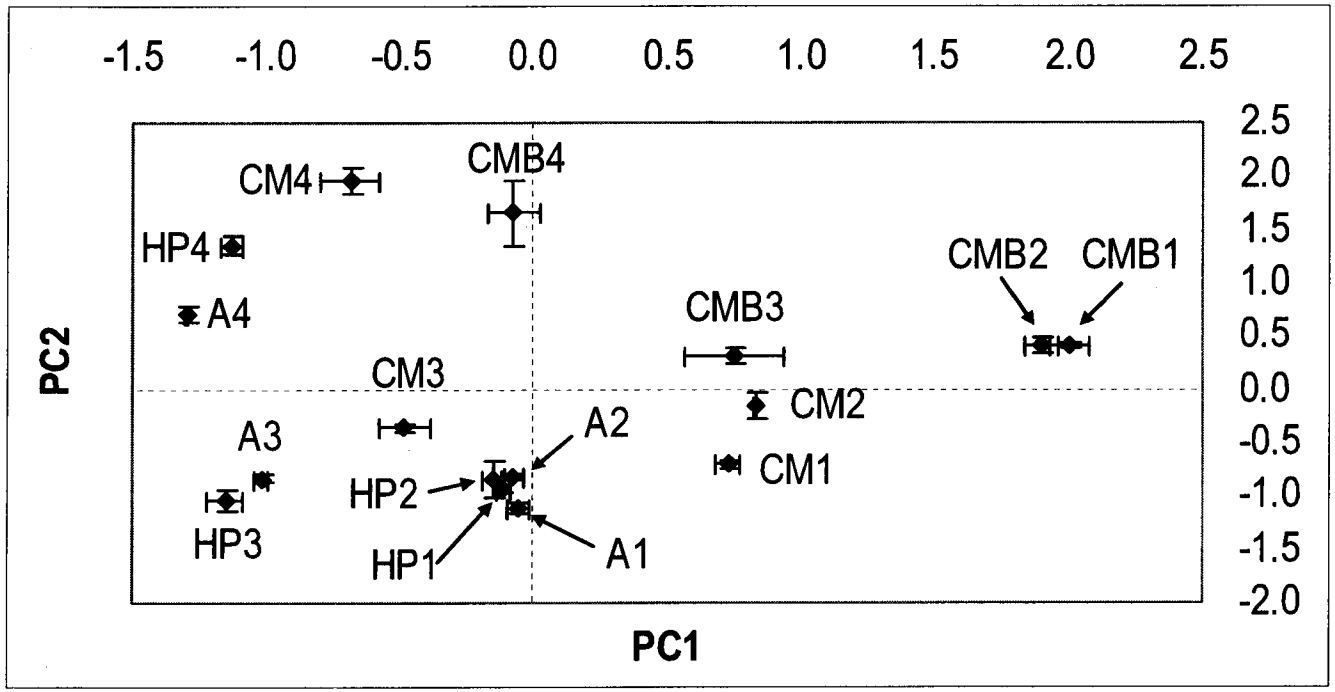


Figure 1. Principal components analysis of the TL-FAME profiles (dataset of the identified individual TL-FAMEs in samples) of the soil lipids extracted with various solvents using a PSE system. The data presented in these figures are means of the replicates' scores and their standard errors of the mean. In the abbreviations used in the figure, the letters designate the extractants (A, acetone; CM, chloroform-methanol; CMB, chloroform-methanol-buffer, HP, hexane-2-propanol) and the numbers (1 to 4), the soils (Table 1).

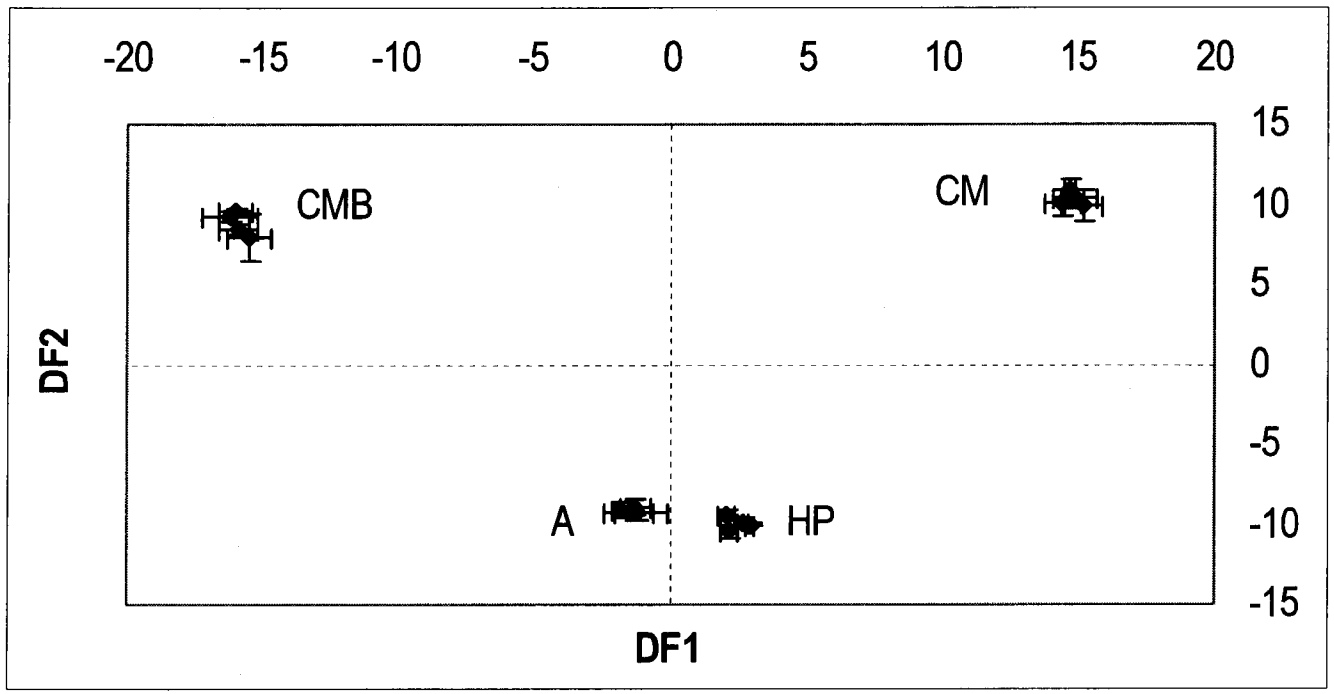


Figure 2a.

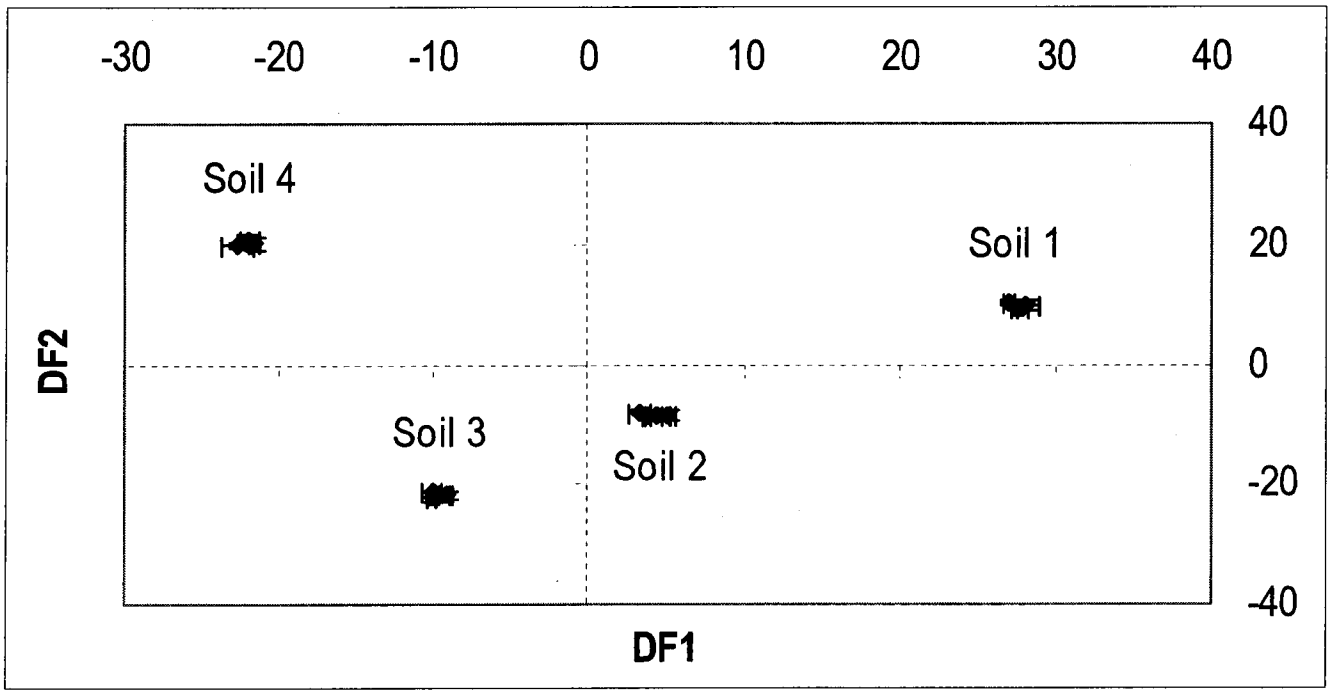


Figure 2b.

Figure 2. Discriminant analysis of the TL-FAME profiles (dataset of the identified individual TL-FAMEs in samples) of the soil lipids extracted with various solvents using a PSE system. Scores plots of discriminant analysis using two different grouping variables: soils (Figure 2a) and extractants (Figure 2b). For each of these DA, discriminant factors (DF) 1 and 2 were plotted. The data presented in these figures are means of the replicates' scores and their standard errors of the mean. In the abbreviations used in the Figure 2a, the letters designate the extractants (A, acetone; CM, chloroform-methanol; CMB, chloroform-methanol-buffer, HP, hexane-2-propanol).

2.7 Connecting paragraph

In Chapter 2, I used four solvent mixtures and an automated pressurized solvent extraction system, to efficiently extract soil lipids. Soil lipids were subject to alkaline methanolysis to release their fatty acid methyl esters (FAMEs) that were subsequently analyzed with a gas chromatograph coupled to a flame ionization detector (GC-FID). Yet, this procedure is relatively time-consuming and would limit the number of samples that could be processed in the laboratory. Chapter 3 presents an exploratory study using pyrolysis-mass spectrometry that could directly and rapidly, without chromatography and derivatization steps, fingerprint microbial communities of soils, based on their composition in lipids, from various agroecosystems.

3. PYROLYSIS-MASS SPECTROMETRY AND GAS CHROMATOGRAPHY-FLAME IONIZATION DETECTION AS COMPLEMENTARY TOOLS IN THE CHARACTERIZATION OF SOIL LIPIDS

3.1 Abstract

Profiling of lipid biomarkers is a very powerful tool for assessing changes in microbial community structure and diversity in soils. However, generating lipid profiles using biomarkers such as phospholipid fatty acids demands intensive laboratory work and data analysis. This approach may not be appropriate when a large number of samples are being screened to distinguish differences among experimental treatments or sites. Simple cataloguing or classification of soil lipids can be accomplished with techniques such as pyrolysis mass spectrometry (Py-MS), which provides a rapid and sensitive 'fingerprint' of these compounds. The objective of this work was to discriminate the soil lipids extracted from different agroecosystems with a modified Py-MS system, the pyrolysis metastable atom bombardment time-of-flight mass spectrometry (Py-MAB-TOF-MS). Soil lipids were extracted with a 1:2 chloroform:methanol solvent from six soils collected from fields under soybean, corn and asparagus production. Then, they were analyzed with Py-MAB-TOF-MS or futher derivatized into fatty acid methyl esters, analyzed by gas chromatography and used to identify microbial communities with the Sherlock Microbial Identification System (MIDI Inc.). Discriminant analysis of the Py-MAB-TOF-MS fingerprints and the ester-linked fatty acid profiles demonstrated significant differences among soils and among crop production systems (soybean, corn, asparagus) (Wilks' lambda=0.000 at $p<0.00005$ for all discriminant analysis tests). Lipid fingerprints generated from analysis of Py-MAB-TOF-MS spectra reflect the overall soil lipid composition (including lipids from microbes, animals, plants and non-living matter). In contrast, the ester-linked fatty acid profiles provide quantitative information on specific groups of soil bacteria and fungi. Although these two methods do not provide exactly the same information, both were able to distinguish differences among soils and crop production systems. Further work is needed to identify diagnostic fragments from Py-MAB-TOF-MS that could be used as biomarkers for more detailed characterization of these microbial communities.

3.2 Introduction

The characterization of lipid biomarkers in soils is proving to be a powerful tool for studying the diversity and structure of living biotic communities as well as the source, turnover and stabilization of non-living organic matter in many environments (e.g. Vestal and White, 1989; Dinel et al., 1990; Zelles, 1999; Bull et al., 2000; Piotrowska-Seget and Mrozik, 2003; Wiesenberg et al., 2004; Otto et al., 2005). A wide array of lipid molecules could be used as biomarkers such as fatty acids, sterols, respiratory quinones, alkanes, etc. In living biota, fatty acids are the major building blocks of the most abundant lipids such as phospholipids, glycolipids and neutral lipids (e.g., sterol esters, monoacylglycerols, diacylglycerols, triacylglycerols) (e.g., Gunstone et al., 1994; Christie, 2003). Total lipid fatty acids (Schutter and Dick, 2000; Drenovsky et al., 2004; Hinojosa et al., 2005), fatty acids linked to phospholipids (Vestal and White, 1989; White and Macnaughton, 1997; Zelles, 1999; Piotrowska-Seget and Mrozik, 2003; Kaur et al., 2005) as well as to neutral lipids (Olsson, 1999; Bååth, 2003) were used to evaluate the impact of land management practices and pollutants on soil microbial communities. Detailed analysis of lipid biomarkers (fatty acids, hydrocarbons, sterols, terpenes, etc.), and more precisely of fatty acid biomarkers, give qualitative (what type of lipid species?) as well as quantitative (how much of each lipid species?) informations on the composition in organic matter and microbial communities of a soil that are specific for each soil due to the jointed action of environmental and anthropogenic factors.

However, it is clear that generating profiles of lipid biomarkers (or fatty acid biomarkers) demands intensive laboratory work (lipid extraction, fractionation, derivatization, chromatography, etc.) and data analysis, which may be too time consuming and costly when a large number of samples are being screened to distinguish soil lipid biomarkers among experimental treatments or sites. Simple cataloguing or classification of these samples can be accomplished with techniques that generate a 'fingerprint' of the soil lipids extracted from living organisms and other sources. Fingerprinting techniques such as Fourier transform infra-red and Raman spectroscropic techniques as well as direct infusion mass spectrometry and pyrolysis mass spectrometry do not require a chromatographic step, and the frequently needed derivatization of the targeted compounds (Goodacre and Kell, 1996; Sumner et al., 2003; Dunn et al., 2005; Villas-Bôas et al., 2005). Generally, fingerprinting involves much larger numbers of measurements (high throughput) than

profiling and requires chemometric interpretation of the complexity resulting from simultaneous acquisition of analytical data on hundreds of metabolites (Goodacre et al., 2003; Sumner et al., 2003).

Of the techniques listed above, pyrolysis mass spectrometry (Py-MS) has several advantages, such as speed of analysis, sensitivity, high sample throughput, minimal sample preparation, and low cost. In most Py-MS systems, the chemical compounds in a sample are desorbed and volatilized during a rapid heating phase, followed by ionization with electron impact, and detection by mass spectrometry (Goodacre and Kell, 1996). This technique was successfully used, sometimes with a thermally assisted hydrolysis and methylation step, to discriminate and classify bacteria (Basile et al., 1998a,b; Brandão et al., 2002), fungi (Lilley et al., 2001), yeasts (Timmins et al., 1998), higher plants (Kim et al., 2004) and organic matter (Peuravuori et al., 1999; Magrini et al., 2002; Marche et al., 2003; Jandl et al., 2004).

The modified Py-MS system used in this study is pyrolysis metastable atom bombardment time-of-flight mass spectrometry (Py-MAB-TOF-MS). The metastable atom bombardment capacity allows better control of the ionization energy and reduces chemical fragmentation during ionization, compared to electron impact ionization (Faubert et al., 1993). The Py-MAB-TOF-MS system has recently been used for fingerprinting vegetable oils (Sanchez et al., 2002), animal fats (Beaudet et al., 2003), microbes (Wilkes et al., 2005), and steroids (Dumas et al., 2002).

Thus, Py-MS systems were proven to be powerful enough to discriminate biological materials of high degree of biochemical similarity (like genotypes of bacteria, virus, plants, etc.) by generating characteristic and rich mass spectrometry data for each of them. The Py-MAB-TOF-MS system uses a softer ionization energy resulting in less fragmentation of the characteristic molecular ions and thus could allow us to tentatively identity lipid species found in a given extract. However, this identification is putative and limited by the degradation of biomolecules in the pyrolysis process, the difference in ionization from one group of molecules to another, as well as the occurrence of isobaric fragments, especially in very rich lipid extract for a highly complex milieu such as a soil. The precise identification of the lipids in a given soil would need to be confirmed by other analytical means. In the other hand, the identification and quantification of fatty acids, by GC-FID and-or GC-

MS, is a well established approach to characterize microbial communities and organic matter in soil. However, it could be consider time-consuming especially with large number of samples to process. Thus, we hypothesize that Py-MAB-TOF-MS system will be able to rapidly generate a specific and discriminatory fingerprint of a soil based on its composition in lipids, similar to the discrimination that is achieved with fatty acid biomarker profiling by GC-FID. So, Py-MAB-TOF-MS could be used as a tool for cataloguing the soils based on their lipid composition.

The objectives of this work was (1) to obtain, using a Py-MAB-TOF-MS system, characteristic and specifc fingerprints of the whole lipid composition of six soils from various agroecosystems, (2) to compare the discrimination among the soils obtained by Py-MAB-TOF-MS lipid fingerprinting to the one obtained by the GC-FID profiling of the fatty acid biomarkers in the same soils.

3.3 Materials and methods

3.3.1 Soil collection and handling

The soils used in this study were mixed, frigid Typic Endoaquents collected from the top 15 cm of agricultural fields under soybean (Soils A to D), corn (Soil E) and asparagus (Soil F) production located in southwestern Quebec, Canada. After collection, half of each soil sample was air-dried, sieved (< 2 mm mesh), stored at room temperature and used for soil physical and chemical analysis, while the other half was frozen immediately and stored at -20°C until lipid analysis was conducted. The six soil samples used in this study are composite samples from 18 (soil E), 24 (soils A to D) and 25 (soil F) subsamples/plots. Each soil composite was analyzed five times (analytical replicates) by both methods described below. Agricultural practices at each collection site and selected soil characteristics are reported in Table 1.

3.3.2 Preparation of total lipid extracts using a pressurized solvent extraction procedure

All organic solvents used in this study were HPLC grade. Glassware and laboratory equipment were prepared as recommended according to White and Ringelberg (1998). Soil lipids were extracted with an ASE 200 Accelerated Solvent Extractor (Dionex Corporation, Sunnyvale, CA,

USA) using the operating conditions described by Macnaughton et al. (1997) as well as in chapter 2 (section 2.3.3), which consisted of one heating cycle at 80°C and 8280 kPa during 5 min, three static cycles of 15 min each at the same temperature and pressure, rinsing of the transfer lines and sample cell with the solvent and purging with N_2 for 180 s between each sample. Between 6 and 8 g of freeze-dried soil was packed into a 11-mL stainless steel ASE vessel that had been rinsed with chloroform:methanol (1:2) solution. The soil lipids were extracted with a chloroform:methanol (1:2) solution. The lipids were dried under N_2 gas and were quantitatively transferred to a GC vial using chloroform:methanol (1:2) solution, dried under N_2 gas and re-dissolved in 1 mL chloroform-methanol (1:2) prior to analysis with the Py-MAB-TOF-MS.

3.3.3 Analysis of total lipid extracts using the Py-MAB-TOF-MS system

The Py-MAB-TOF-MS (Dephy Technologies, Montreal, Canada) was used to discriminate between six total lipid extracts. The details related to the apparatus design and specifications are detailed elsewhere (Dumas et al., 2002; Wilkes et al., 2005). The ionization gas used was N_2. This gas has an ionization energy of 8.67 eV (85 %) and 11.88 eV (15 %). A sample of 1-µl was applied to the pyroprobe (Pyroprobe 2000 pyrolyzer; CDS Analytical, Oxford, PA, USA). Pyrolysis was achieved by ramping the probe temperature by 20 °C/ms from ambient to 1100-1200 °C, with a final hold time of at least 50 s. The probe was specially modified for enabling helium flow (1-2 mL/min) through the quartz capillary and then enhancing transfer of pyrolysis products into the MAB source. The mass range was scanned between 40 and 1000 m/z. Five analytical replicates of each lipid extract were analyzed.

3.3.4 Preparation of the ester-linked fatty acid methyl esters (EL-FAMEs)

All organic solvents used in this study were HPLC grade. Glassware and laboratory equipment were prepared as recommended in White and Ringelberg (1998). The *in situ* transesterification procedure used in this study is detailed in Schutter and Dick (2000), but with some modifications. Prior to lipid analysis, the soils were freeze-dried and finely ground. Then, 3 to 5 g of soil was incubated with 15 mL of 0.2 M KOH (potassium hydroxide) in methanol for 1-hour at 37°C. The reaction mixture was vortexed every 10-15 minutes. At the end of the reaction, the mixture was

cooled to room temperature and neutralized with 3 mL of 1 N glacial acetic acid. The EL-FAMEs were extracted from the mixture with phase partitioning using 3 x 5 mL hexane-chloroform (4:1). The organic phases were pooled and dried under a gentle steam of N_2, then dissolved in iso-octane prior to analysis by GC-FID.

3.3.5 Analysis of EL-FAMEs by GC-FID

Each EL-FAME extract was dried completely under N_2 and redissolved with 1-mL of iso-octane. The EL-FAME extracts (five analytical replicates, 5 μL injected each) were analyzed in split mode (50:1) with a gas chromatograph (Hewlett Packard 6890) equipped with an Ultra-2 capillary column (cross-linked 5% diphenyl-95% di-methylpolysiloxane; length, 25 m; internal diameter, 0.22 mm; film thickness, 0.33 μm; Agilent J& W 19091B-102), hydrogen is the carrier gas (68.9 kPa), nitrogen is the "makeup" gas (30 mL/min), and air is used to support the flame of the FID. The temperature program ramps from 170°C to 270°C at 5°C per minute with a 2 min at 270°C. Inlet and detector temperatures were 250°C and 300°C, respectively. The settings were the same as those used in the MIS protocol (MIDI, Inc., Newark, Delaware, USA, www.midi-inc.com) (Sasser, 1990; Schutter and Dick, 2000; Buyer, 2002). The retention times of the peaks were converted to equivalent chain length (ECL) values (Sasser, 1990). Identification of peaks was based on comparison of retention times (ECLs) to commercial FAMEs standards (see chapter 2, section 2.3.4, for details about the standards), and led to the identification of 70 and more FAMEs. Peak identifications were cross-validated by sending a subset of our samples to a certificated external laboratory (Laboratoire de Santé Publique du Québec, Sainte-Anne-de-Bellevue, QC) for analysis using the Sherlock Microbial Identification System® (MIDI, Inc., Newark, Delaware, USA). The fatty acids analyzed in our samples by this method are listed in Appendix B. FAMEs are described by standard ω-nomenclature (IUPAC-IUB, 1977).

3.3.6 Statistical analysis

In this study, from each of the six soil samples, one lipid extract as well as one EL-FAME extract were prepared. The lipid extract of each soil was analyzed five times by Py-MAB-TOF-MS and the EL-FAME extract five times by GC-FID. This experiment was qualitative and exploratory and the

use of a limited number of samples was judged sufficient for meeting our objective. However, the differences observed between the soils, as environmental (or biological) 'entities' (or 'bodies'), could not necessarily be only attributed to their distinct lipid composition, because the effect of soil variability was not considered here. A follow-up experiment will be needed for studying the performance of the Py-MAB-TOF-MS system in its capacity to discriminate soils based on their lipid fingerprints using true replicates expressing the natural soil variability.

All the statistical analysis were performed using SYSTAT software, version 10 (Systat Software Inc., Richmond, CA, USA). The Py-MAB-TOF-MS analysis of the total lipid extracts gave us ion masses ranging from 40 to 1000 m/z (m/z, mass-to-charge ratio). The ion intensity for each mass was normalized to percent total ion counts and an analysis of variance (at $p<0.05$) allowed to retain the molecular fragments that discriminated the most the six different soils. Seventeen components from the principal components analysis explained more than 99 % of the total variance

With the GC-FID data, we calculated the percentage area of each EL-FAME as the (peak area of each identified FAME/total peak area of all identified FAMEs) x 100%. The dataset of EL-FAMEs was analyzed using principal components analysis. Six components were extracted explaining more than 90% of the total variance.

Discriminant analyses were conducted on the two datasets themselves and their respective resulting PCA scores as inputs (Radovic et al., 2001; Goodacre et al., 2003) to evaluate the discrimination between soils and the crops as well as to reduce the variance among replicates of a given soil.

3.4 Results and discussion

Soils for this study came from agricultural fields in Southwestern Quebec (Canada) with a range of characteristics (Table 1) such as different levels of soil organic matter, which is expected to support distinct soil lipid biomarkers, more specifically distinct fatty acid biomarkers, and thus distinct soil microbial communities (Palmborg et al., 1998; Cahyani et al., 2002; Biasi et al., 2005; Otto et al., 2005).

3.4.1 Lipid fingerprinting by Py-MAB-TOF-MS

A visual examination of the mass ionization spectra, generated by the Py-MAB-TOF-MS system, of the pyrolyzed soil lipid extracts from soybean, corn and asparagus fields (Figure 1) confirms that each soil has a distinctive lipid fingerprint.

Since the Py-MAB-TOF-MS system did not generate an identity for the lipid biomarkers detected, it is beyond the scope of this thesis to include such detail. Moreover, identification of lipid biomarkers in soil lipid extract will require prealable work with standards of lipid compounds in order to better understand their volatility, fragmentation pattern, and ionization when analyzed by the Py-MAB-TOF-MS system. Thus, more work will be needed before being able to generate a list of compounds identified by this system.

Semi-quantitative evidence of distinctive lipid fingerprints comes also from the principal components and discriminant analysis of spectra generated by the Py--MAB-TOF-MS system. The 523 molecular fragments that were significantly different (at $p<0.05$, LSD test, data not shown) were retained and used as inputs into a PCA to reduce the dimensionality of the dataset, which produced 17 components that explained more than 99 % of the total variance. Figures 2a (PC1 vs PC2) and 2b (PC1 vs PC3) present the plots of the scores of the soils for the three first PCs (PC1, 43.3%; PC2, 19.7%; PC3, 12.2% of variance). PC1 was able to separate the soils in three groups: one of the soybean soil (S1) and the asparagus soil (ASP) were separated from the group formed by the other soils (Figure 2a). The soils from soybean fields (S2, S3 and S3) as well as from the corn field were more efficiently resolved by PC3 (Figure 2b). A discriminant analysis (stepwise backward automatic) was conducted using the PCA scores, extracted from the dataset generated by the Py-MAB-TOF-MS system, as inputs in order to test the discrimination between the soil type and crop type. This analysis provided significant discrimination between soils (soybean S1 to S4, corn, asparagus) and crops (soybean, corn, asparagus) (Wilks' lambda=0.000 at $p<0.00005$ for all discriminant analysis tests) (data not shown).

An examination of the PCA loadings of the molecular fragments (Table 2) reveals that fragments with masses between 250-292 m/z are negatively correlated with PC1 (with loadings > -0.900) and

fragments from 605 to 667 m/z are positively correlated with PC1 (with loadings > 0.900). Fragments from 354 to 482 m/z are negatively correlated with PC2 (with loadings > -0.725) and low m/z fragments (from 113 to 201 m/z) present high positive correlations toward PC2 (with loadings > 0.800). Fragments that are positively (226, 546 to 614, 800 to 803 m/z) and negatively (40 to 63, 281, 440-441 m/z) correlated with PC3 (with loadings > $|0.650|$) are more diverse in their molecular weights. It is interesting to observe that specific ranges of masses are highly correlated with the PC1 and PC2, although is not the case for PC3. The chemical identify of these fragments remains to be determined, and will provide insight into ubiquitous lipids as well as those that are site-specific, perhaps due to the land use history, type of crop grown, soil microbial communities, or other factors.

3.4.2 Fingerprinting fatty acid biomarkers by Py-MAB-TOF-MS

Among the lipid biomarkers found in soils, the fatty acids are extensively used in the characterization of microbial communities (Vestal and White, 1989; Zelles, 1999; Schutter and Dick, 2000; Bååth, 2003; Piotrowska-Seget and Mrozik, 2003; Drenovsky et al., 2004). The fatty acid biomarkers, as diagnostic molecules of the living microbiota, have to be found linked by ester bonds to larger lipid molecules such as phospholipids, neutral lipids and glycolipids (Christie, 2003). The current way to release these fatty acids is by performing a transmethylation either directly from the soil or on the whole lipid extract or on specific lipid fraction (White and Ringelberg, 1998; Christie, 2003). According to our best knowledge, the volatility, the ionization and the fragmentation of the large lipid molecules were never studied. However, the common mechanism of fragmentation of the large lipid molecules found using other ionization sources such as electron impact, chemical ionization, electrospray, etc. is that the fatty acyls are released by the breakage of their ester bonds (Murphy et al., 2001). Thus, if we supposed that the different categories of large lipid molecules could enter intact the gas phase by the pyrolysis process, the fragmentation by the MAB source could result in fatty acyls detected under the following forms: $[RCO + 128]^+$, $[RCO + 74]^+$, $[RCO]^+$ and $[RCO - 1]^+$ where RCO is an acylium ion (acyl cation) in which R is the carbon chain of the fatty acid (CH3-(CH2)n- where n=8 to 18) (Odham and Stenhagen, 1972; Murphy et al., 2001). Table 3 presents the fatty acids that we could currently detected by GC-FID, their molecular formulas, masses as well as the possible fragments of the fatty acids, after release from the larger lipid

molecules by the proposed fragmentation mechanism encountered here during ionization, that could be found in a mass spectrum. In order to explore this aspect, from the original dataset of molecular fragments, we prepared a subdataset composed of one ($[RCO]^+$) of the putative molecular fragments of fatty acid biomakers. The resulting dataset was analyzed by PCA and DA. Figure 3 present the separation of the samples obtained. Discriminant analysis using the original subdataset as well as the scores as inputs provided significant discrimination between soils (soybean S1 to S4, corn, asparagus) and crops (soybean, corn, asparagus) (Wilks' lambda=0.000 at $p<0.00005$ for all discriminant analysis tests) (data not shown). The discrimination of the soils was similar to the one obtain using the whole dataset of molecular fragments (Figure 2). These findings support our hypothesis that Py-MAB-TOF-MS can be used to discriminate amongst soil samples when analyzing soil lipid extracts, similar to the discrimination that is achieved when fatty acid biomarkers are profiled by GC-FID.

An ion monitoring of these specific fatty acid fragments could be target using the Py-MAB-TOF-MS in order to increase the selectivity and sensitivity in the detection of the fatty acid biomakers. However, if we limited our analysis to only selective ions, we maybe reduce the discriminating power of the Py-MAB-TOF-MS. Also, the identification of the biomarkers will remain tentative since many important fatty acids have overlapping masses. The use of TOF-MS detector in its capacity to obtain accurate mass of a molecule could at least help in resolving fatty acids with similar masses but different formulas (see Table 3) (Esch et al., 2007). For more precise identification, chromatographic separation by GC is needed.

3.4.3 Fatty acid profiling by GC-FID

We used an approach to directly extract ester-linked fatty acids (EL-FAMEs) from soil samples using a mild alkaline reagent (Schutter and Dick, 2000). Previously, analysis of EL-FAMEs was used to characterize microbial communities that evolved during 25 years of wheat-fallow cropping after land conversion from a native mixed prairie (Drijber et al., 2000), to study the impact of the season, the soil type and agricultural management practices on microbial communities (Schutter et al., 2001) and to follow microbial changes during composting (Steger et al., 2003). 78 EL-FAMEs were identified in the samples and 62 of them were significantly different among the soils (ANOVA,

at p<0.05) (data not shown). PCA of this dataset extracted 6 principal components explaining 93.3 % of the variance. Figure 4 shows the scores of the samples for the two first PCs (84.3 % variance). The major part of the variance (80.6%) is summarized in PC1. A group formed by the corn and asparagus soils are opposite to the group formed by the soybean soils. However, this analysis was unable to separate the soils sufficiently (Figure 4). When these principal components were used as input variables in the discriminant analysis, there was a significant discrimination among soils and crops (Wilks' lambda=0.000 at p<0.00005 for all discriminant analysis tests) (data not shown). Table 2 show the fatty acids with high loading value for PC1. These fatty acids were all tested significantly different at p<0.001 (LSD test). The fatty acids with the highest positive loadings for PC1 were the ones more significantly (at p<0.001 for all of them, LSD test) abundant in the soils under soybean crop. Also, the highest negative loadings significantly (at p<0.001 for all of them, LSD test) were from the fatty acids more contrastely abundant in soils under corn and asparagus compared to soils under soybean (Table 4). The biological meaning of these differences remains to be determined.

3.4.4 Complementarity of Py-MAB-TOF-MS and GC-FID in studying soil lipids

The pyrolysis of soil lipids with the Py-MAB-TOF-MS system generates many molecular fragments (from 40 to 1000 m/z), but we did not have enough information to categorize these fragments into those from living cells and non-living organic matter. Therefore, we consider that the lipid fingerprints generated from analysis of Py-MAB-TOF-MS spectra reflect the overall soil lipid composition. In contrast, the analysis of EL-FAMEs with GC-FID focuses on fatty acids biomarkers associated with broad groups of organisms. Although, the FAMEs may also come from the non-living fraction of soil organic matter, FAME profiles are believed to reflect the diversity of microorganisms present in a particular soil (Cavigelli et al., 1995; Drijber et al., 2000; Schutter and Dick, 2000; Drenovski et al., 2004; Pankhurst et al., 2005; Hinojosa et al., 2005; Sullivan et al., 2006). While lipid fingerprinting and lipid profiles do not provide exactly the same information, the discriminant analysis of Py-MAB-TOF-MS and FAME data showed that the two methods were able to distinguish differences between soils and cropping systems. Also, the use of soft ionization, such as the MAB source in the system used in this study, that limits extensive fragmentation and provide more parent ions, could be a major help in this task. Soft ionization coupled to mass detector with

larger mass range could allow us to detect not only fatty acid fragments (9:0 to 20:0, for example, in the 140-300 m/z region of a mass spectrum) but also fragments from lipid biomarkers with higher masses such as hydrocarbons, waxes, sterols and steryl esters, glycerolipids, for example. Our results show that Py-MAB-TOF-MS could rapidly distinguish soils by fingerprinting their overall composition in lipid biomarkers. We propose that this approach could be used to distinguish soil microbial communities from various experimental treatments and sites, but further investigation is required. The data presented in this paper are from sites with distinctive site histories and soil properties. Although EL-FAMEs are sufficiently sensitive that they can be used to detect differences between experimental treatments within agronomic research trials (small scale, relatively homogenous soil characteristics), it is not known whether Py--MS is as sensitive. Further work on soil lipid analysis with Py-MS is merited.

There are a number of methods that can be used to generate lipid profiles, but we selected to use an in situ transesterification method (direct mild alkaline transesterification) (EL-FAMEs), which takes less time than the complete phospholipid fatty acid procedure and is easier than the Microbial Identification System (MIS) procedure which requires harsh saponification and methylation steps (Sasser, 1990; Petersen et al., 2002). A trained individual could complete the preparation of between 25 and 50 fatty acids extracts in two days. When fatty acid extracts are injected into a GC-FID system equipped with an autosampler, the injections are mainly automatic, requiring little human supervision. Between 25 and 40 fatty acid extracts can be analyzed per day, depending on how many standards and washings are included in the run. Lipid fingerprinting with Py-MAB-TOF-MS is relatively fast if the apparatus is equipped with an autosampler. Each lipid extract is analyzed in less than 5 minutes, so several hundred samples could be processed per day. If the Py-MAB-TOF-MS does not have an autosampler, a dedicated person could manually inject 30 to 50 samples per day. Clearly, the Py-MAB-TOF-MS system is a more efficient system for screening many samples, such as to differentiate between experimental treatments or sites. The short analysis time permits the user to inject multiple replicates of each sample, thus increasing the analytical precision.

There is a growing body of work that demonstrates the utility of pyrolysis mass spectrometry for profiling targeted compounds such as fatty acids (Basile et al., 1998a,b; Barshick et al., 1999; Jandl

et al., 2004). The works of Schulten and collaborators provides many examples of pyrolysis mass spectrometry applications in characterizing soil organic compounds. They extensively characterized compounds in solid soils and soil extracts generated from pyrolysis field ionization mass spectrometry (for example, see Schulten and Gleixner, 1999; Marche et al., 2003; Smidt et al., 2005). To do such extensive work requires analysis of appropriate standards so that the molecular fragments generated by the Py-MS can be identified as belonging to target compounds and their masses quantified. To our best knowledge, this has not yet been done extensively and specifically for soil lipid biomarkers. With further developments in this area, pyrolysis mass spectrometry could then be used to rapidly characterize soil microbial communities, i.e. to determine changes in the microbial diversity, by identifying specific compounds such as fatty acids or sterols that are functionally important in the metabolism of certain groups of soil microorganisms.

3.5 Conclusions

In conclusion, our results proposed for the first time the use of the Py-MAB-TOF-MS system as a rapid and globay way to fingerprint many types of lipids from soils including the fatty acids. We showed that selective monitoring of microbial fatty acid biomakers could be eventually performed using this tool and could then be used a rapid way to fingerprint them in soils. This application is novel and could offer soil scientists as well as ecologists a efficient tool for the characterization the organic matter composition, and especially the fatty acid biomarkers, in a large sets of samples. However, the behavior of large lipid molecules during the pyrolysis process as well as their fragmentation mechanisms need to be studied in depth using such system. Future studies should investigate the optimization of selective ion monitoring of fatty acid biomarker specific fragments. However, the use of gas chromatography coupled to flame ionization detector or mass spectrometer is required for confirming the identification of the compounds.

Table 1. Selected properties of the soils (Typic Endoquents) used in the experiment

Soil	S1	S2	S3	S4	CORN	ASP
Location	Saint-Césaire 45°25' N 73°00' W	Saint-Césaire 45°25' N 73°00' W	Sainte-Brigide-d'Iberville 45°19' N 73°04' W	La Présentation 45°40' N 73°03' W	Ste-Anne-de-Bellevue 45°30' N 73°35' W	Ste-Anne-de-Bellevue 45°30' N 73°35' W
Crop	Soybean	Soybean	Soybean	Soybean	Corn	Asparagus
Sampling Year	2000	2000	2000	2000	2001	2001
Sampling depth	0-15	0-15	0-15	0-15	0-20	0-15
pH [a]	6.7	6.3	6.2	6.3	6.1	6.0
OM (g kg^{-1}) [b]	42	36	51	40	45	48
Sand (g kg^{-1}) [c]	118	130	140	384	815	530
Clay (g kg^{-1}) [c]	321	353	367	246	96	170
Silt (g kg^{-1}) [c]	561	517	493	370	89	300
Textural Class	Silty Clay Loam	Silty Clay Loam	Silty Clay Loam	Loam	Sandy loam	Loamy sand

[a] Soil:water extracts (1:2 soil:solution ratio) (Hendershot et al., 1993);

[b] Organic Matter (OM) determined by loss on ignition (360°C for 4 h) (Schulte et al. 1991);

[c] Particle-size analysis (Sheldrick and Wang 1993).

Table 2. Selected loading values from PCA of molecular fragment masses detected in lipid extracts from 6 different soils by Py-MAB-TOF-MS system. Values are the ten highest and ten lowest for each principal component (PC) axis.

PC1 loading	Fragment mass (m/z)	PC2 loading	Fragment mass (m/z)	PC3 loading	Fragment mass (m/z)
-0.967	266	-0.865	354	-0.755	40
-0.958	268	-0.830	364	-0.746	47
-0.954	290	-0.826	366	-0.718	58
-0.954	282	-0.817	394	-0.717	42
-0.948	292	-0.803	424	-0.709	63
-0.943	250	-0.781	468	-0.699	281
-0.934	270	-0.777	370	-0.688	50
-0.932	278	-0.769	395	-0.666	56
-0.930	284	-0.766	434	-0.666	440
-0.927	288	-0.753	482	-0.649	441
0.925	666	0.807	137	0.651	580
0.931	663	0.807	113	0.662	547
0.932	639	0.812	123	0.663	614
0.932	633	0.851	149	0.665	596
0.936	605	0.876	153	0.676	226
0.937	635	0.883	173	0.744	546
0.940	621	0.888	181	0.748	801
0.944	637	0.902	201	0.753	803
0.951	667	0.913	159	0.786	800
0.956	665	0.930	160	0.812	802

Table 3. Table of fatty acids detected by GC-FID, their masses, formulas as well as masses of their common fragments.

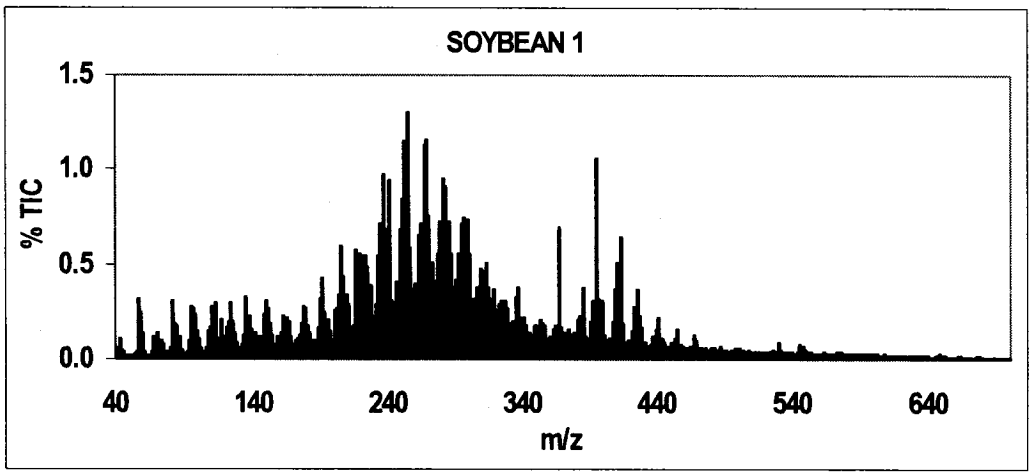
Fatty acid	Formula	Fatty Acid [RCOOH] (m/z)	Fragment $[RCO]^+$ (m/z)
9:0	C9H18O2	158	141
8:0-3OH	C8H16O3	160	143
i-10:0; 10:0	C10H20O2	172	155
9:0-3OH	C9H18O3	174	157
i-11:0; a-11:0; 11:0	C11H22O2	186	169
10:0-2OH; 10:0-3OH	C10H20O3	188	171
12:1	C12H22O2	198	181
12:0	C12H24O2	200	183
i-11:0-3OH; 11:0-2OH; 11:0-3OH	C11H22O3	202	185
13:1	C13H24O2	212	195
12:1-3OH	C12H22O3	214	197
i-13:0; a-13:0; 13:0	C13H26O2	214	197
12:0-2OH; 12:0-3OH	C12H24O3	216	199
i-14:1; 14:1ω5c	C14H26O2	226	209
i-14:0; a-14:0; 14:0	C14H28O2	228	211
i-13:0-3OH; 13:0-2OH	C13H26O3	230	213
i-15:1 isomers 1; i-15:1 isomer 2; a-15:1; 15:1ω6c; 15:1ω5c	C15H28O2	240	223
i-15:0 isomer 3; a-15:0; 15:0	C15H30O2	242	225
16:0 N alcohol	C16H34O	242	225
i-14:0-3OH; 14:0-2OH; 14:0-3OH	C14H28O3	244	227
i-16:1 isomer 1; i-16:1 isomer 2; i-16:1 isomer 3; 16:1ω11c; 16:1ω9c; 16:1ω7c; 16:1ω5c	C16H30O2	254	237

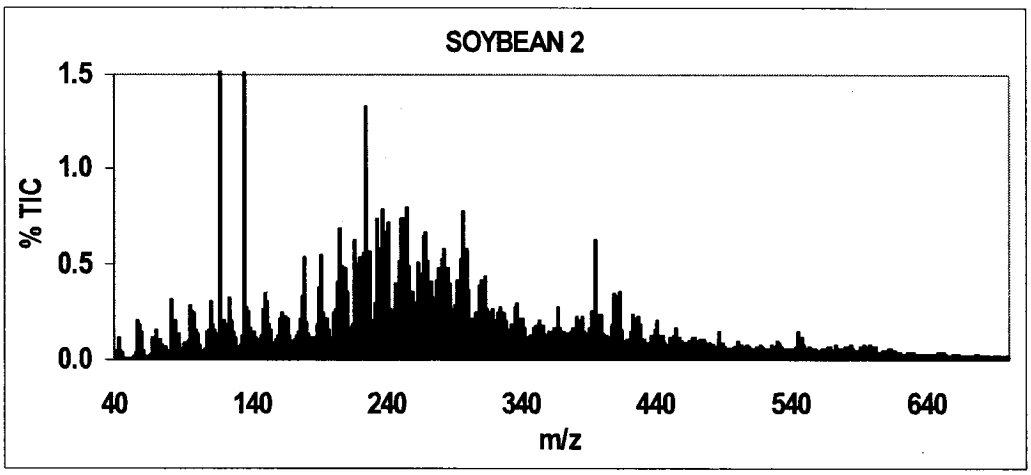
Table 3 (continued). Table of fatty acids detected by GC-FID, their masses (in m/z), formulas as well as masses of their common fragments.

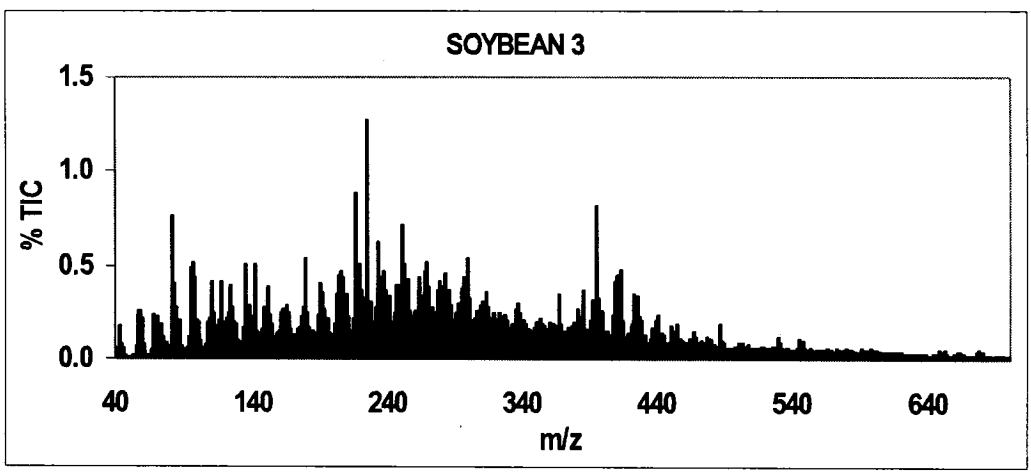
Fatty acid	Formula	Fatty Acid [RCOOH] (m/z)	Fragment $[RCO]^+$ (m/z)
i-16:0; a-16:0; 16:0	C16H32O2	256	239
i-15:0-2OH; i-15:0-3OH; 15:0-2OH; 15:0-3OH	C15H30O3	258	241
i-17:1ω9c; i/a-17:1; a-17:1ω9c; 17:1ω9c; 17:1ω8c; 17:1ω7c; 17:0 cyclo	C17H32O2	268	251
16:1-2OH	C16H30O3	270	253
10Me-16:0; i-17:0; a-17:0; 17:0	C17H34O2	270	253
i-16:0-3OH; 16:0-2OH; 16:0-3OH	C16H32O3	272	255
18:3ω6c(6,9,12)	C18H30O2	278	261
18:2ω6,9c	C18H32O2	280	263
i-18:1; 18:1ω9c; 18:1ω7c; 18:1ω5c	C18H34O2	282	265
a-18:0; 10Me-17:0; i-18:0; 18:0	C18H36O2	284	267
i-17:0-3OH; 17:0-2OH	C17H34O3	286	269
i-19:1; 19:1ω11c; 19:1ω9c; 19:1ω6c; 19:0 cyclo ω10c; 19:0 cyclo ω8c; 11Me-18:1ω7c	C19H36O2	296	279
18:1-2OH	C18H34O3	298	281
10Me-18:0; i-19:0	C19H38O2	298	281
18:0-2OH; 18:0-3OH	C18H36O3	300	283
20:4ω6,9,12,15c	C20H32O2	304	287
20:2ω6,9c	C20H36O2	308	291
20:1ω9c; 20:1ω7c	C20H38O2	310	293
10Me-19:0; i-20:0; 20:0	C20H40O2	312	295

Table 4. Fatty acids with highest loadings from PCA of EL-FAME datasets from 6 different soils (From PCA of the EL-FAMEs, only loadings from PC1 are presented here). Relative abundances of these fatty acids across the 6 soils.

EL-FAME	PC1 loading	ASP	CORN	S1	S2	S3	S4
		% area					
20:0	-0.997	8.57a	7.83b	2.50cd	2.56c	2.46cd	2.39d
i-14:0-3OH	-0.997	0.15a	0.14b	0.00c	0.00c	0.00c	0.00c
13:0	-0.997	0.14a	0.13b	0.00c	0.00c	0.00c	0.00c
16:0 N alcohol	-0.996	2.46a	2.46a	0.32b	0.29b	0.28b	0.26b
14:0	-0.996	3.53a	3.33b	1.43e	1.61c	1.52d	1.46de
10Me-17:0	-0.993	7.93b	8.71a	0.83c	0.66cd	0.68cd	0.61d
18:3ω6c(6,9,12)	-0.992	6.36b	7.16a	0.94c	0.91cd	0.96c	0.85d
15:1ω6c	-0.989	0.32a	0.33a	0.14b	0.15b	0.14b	0.14b
i-16:1/14:0-3OH	-0.987	0.76a	0.71b	0.33c	0.33c	0.31c	0.30c
10Me-19:0	-0.986	0.82b	0.93a	0.00c	0.00c	0.00c	0.00c
10Me-18:0	0.989	0.30b	0.33b	1.45a	1.40a	1.42a	1.36a
a-17:0	0.989	0.55c	0.52c	1.54a	1.40b	1.51ab	1.47ab
i-15:0	0.991	1.43c	1.35c	4.05b	4.34a	4.26a	4.24a
i-17:0	0.991	0.37b	0.34b	1.59a	1.49a	1.55a	1.54a
19:0 cyclo ω8c	0.992	0.89c	1.01b	2.49a	2.53a	2.54a	2.57a
i-16:0	0.993	1.06c	0.98d	2.13a	2.10ab	2.06b	2.14a
a-15:0	0.994	0.65c	0.64c	2.65b	2.77a	2.69ab	2.71ab
16:1ω7c/i-15:0-2OH	0.994	1.02c	0.98c	3.87b	3.88b	3.82b	4.11a
cy-17:0	0.995	0.23c	0.22c	1.63a	1.51b	1.59ab	1.57ab
16:1ω5c	0.998	0.28c	0.25c	4.80a	4.66b	4.72ab	4.64b


SOYBEAN 1
% TIC
1.5
1.0
0.5
0.0
40
140
240
340
440
540
640
m/z


SOYBEAN 2
% TIC
1.5
1.0
0.5
0.0
40
140
240
340
440
540
640
m/z


SOYBEAN 3
% TIC
1.5
1.0
0.5
0.0
40
140
240
340
440
540
640
m/z

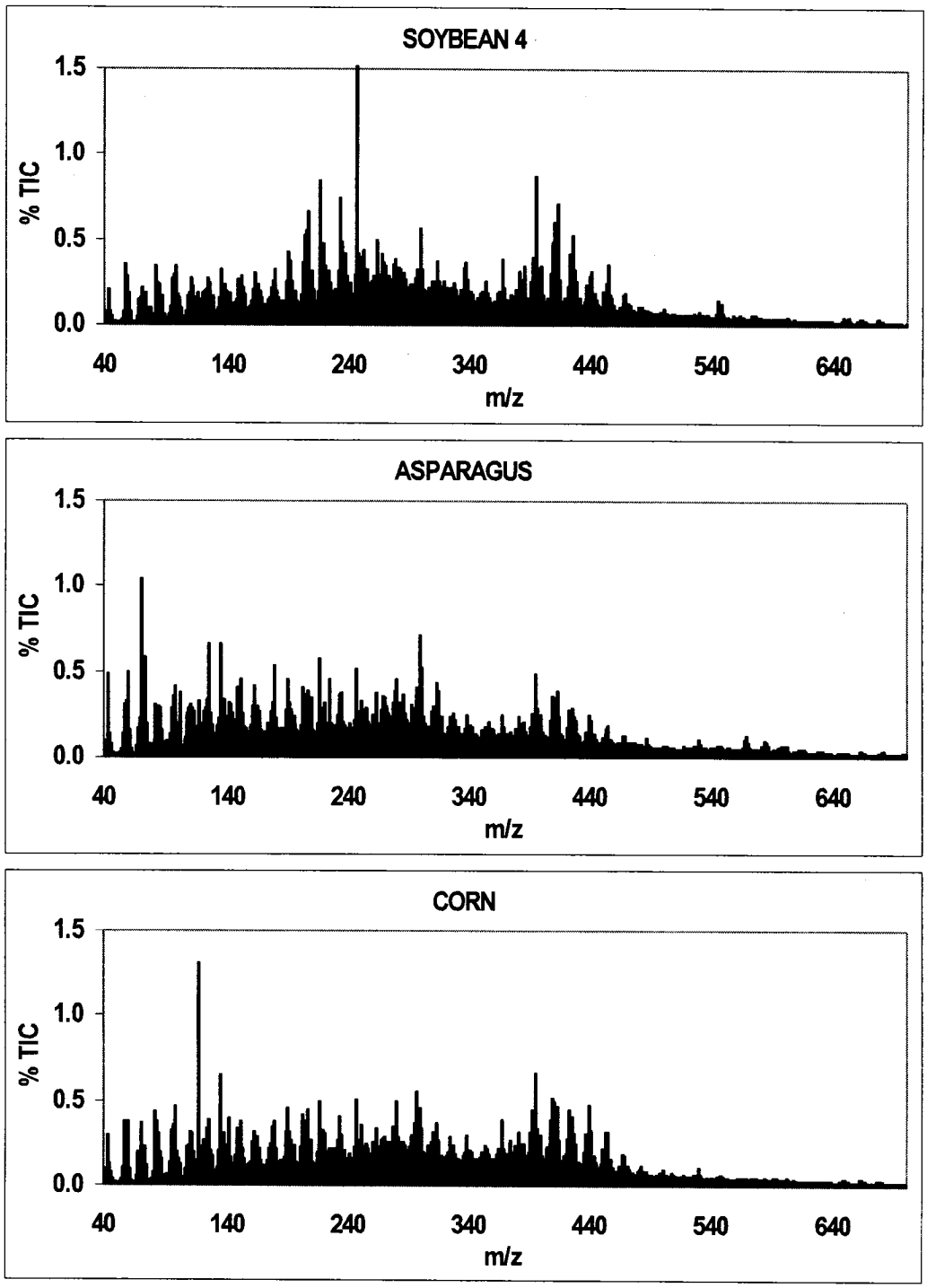
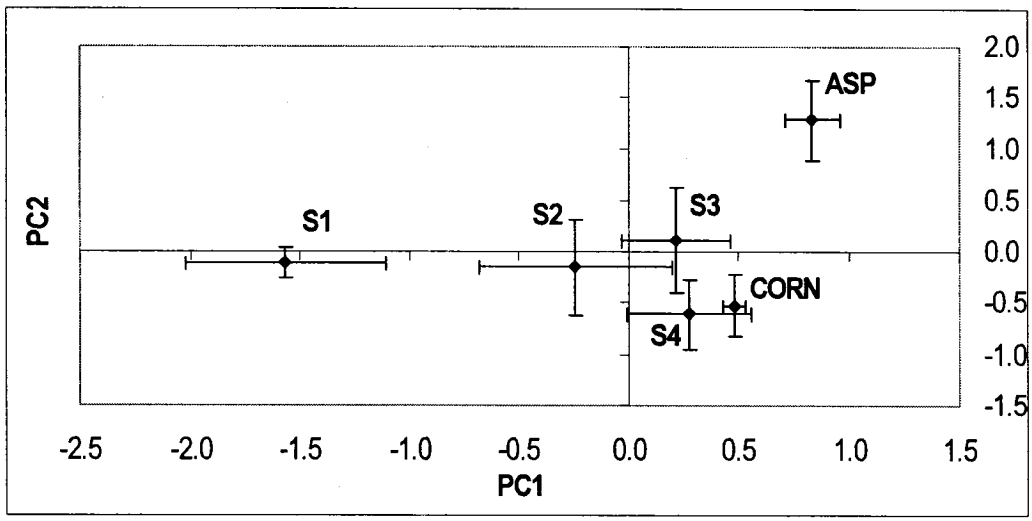
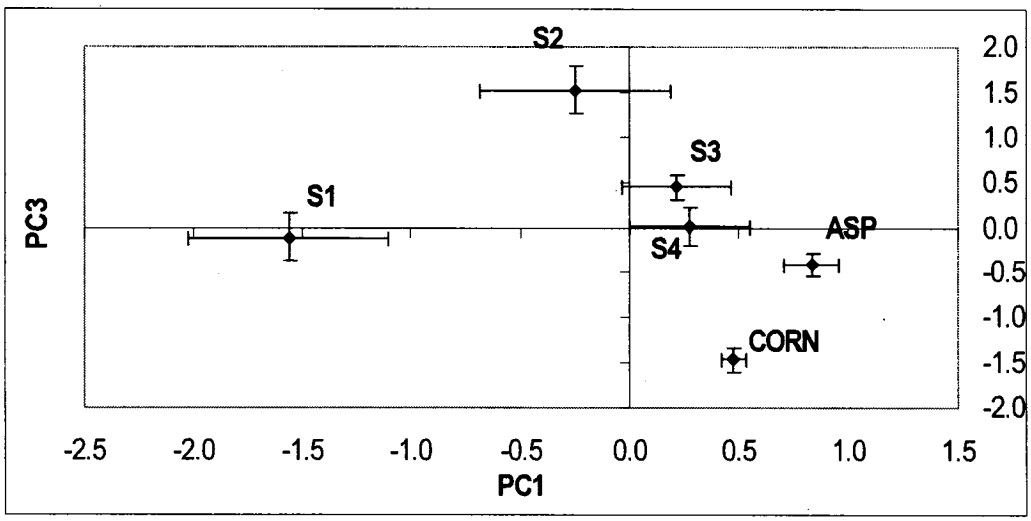


Figure 1. Examples of mass spectra (% Total Ion Count per mass-to-charge ratio, m/z) of pyrolyzed total lipid extracts of soils cropped with soybean (soils 1 to 4), corn and asparagus obtained by Py-MAB-TOF-MS. Each mass spectra is a mean of 5 replicates.

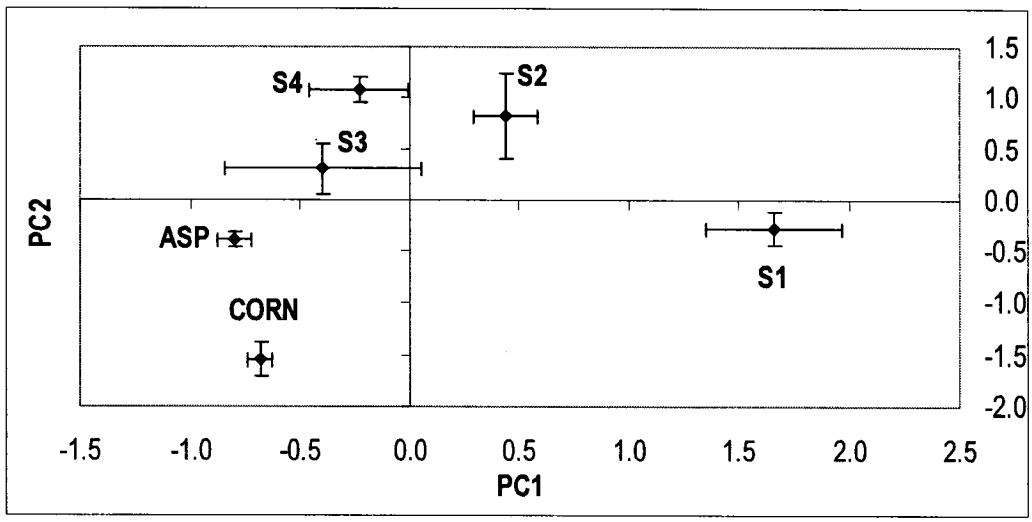


(A)

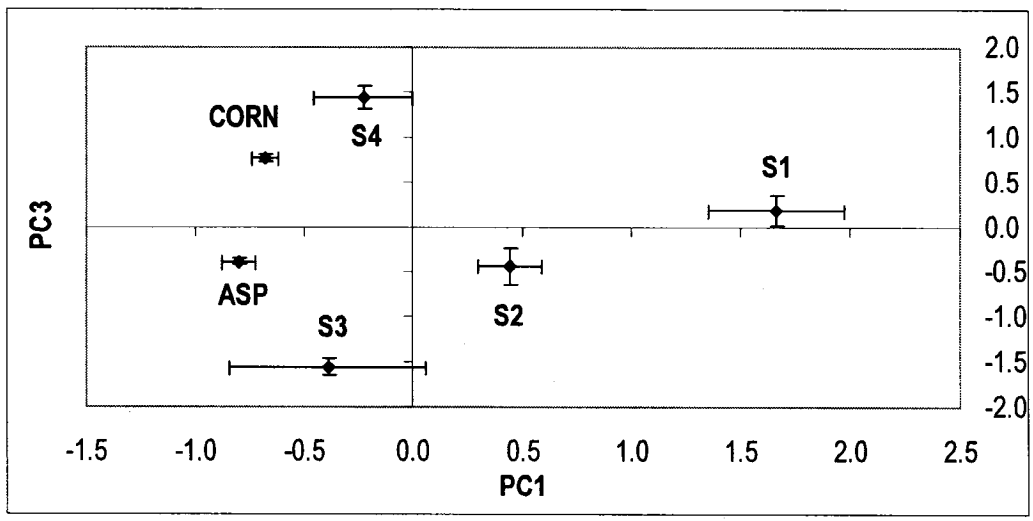


(B)

Figure 2. Plots of the scores from the PCA of molecular fragment masses detected in lipid extracts from 6 different soils by Py-MAB-TOF-MS system. In parenthesis, the percentage of variance explained by each principal component: (A) PC1 (43.3%) vs PC2 (19.7%) plot, (B) PC1 vs PC3 (12.2%) plot. See Table 1 for the description of the abbreviations for the soils.



(A)



(B)

Figure 3. Plots of the scores from the PCA of fatty acid biomarker fragment masses detected in lipid extracts from 6 different soils by Py-MAB-TOF-MS system. In parenthesis, the percentage of variance explained by each principal component: (A) PC1 (52.6%) vs PC2 (19.4%) plot, (B) PC1 vs PC3 (8.6%) plot. See Table 1 for the description of the abbreviations for the soils.

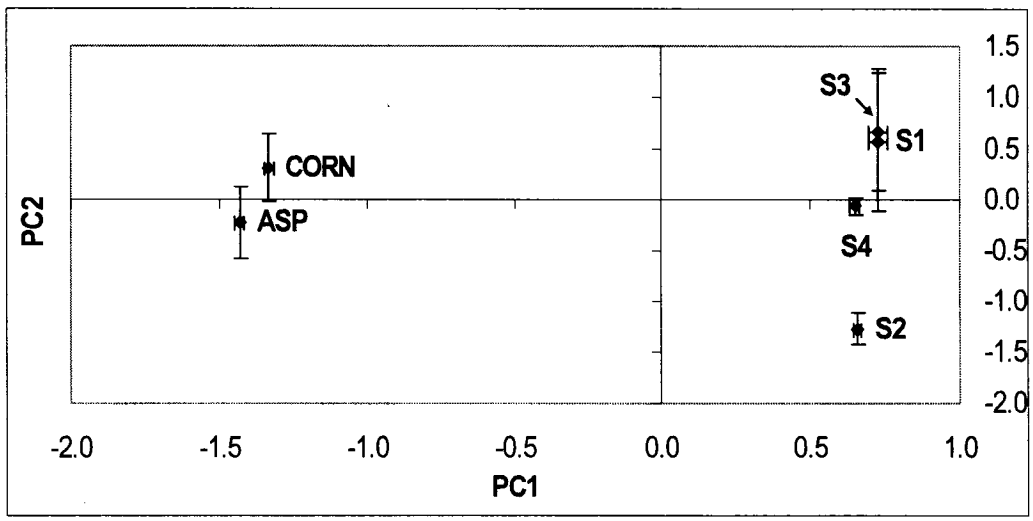


Figure 4. Plot of the scores (PC1 (80.6%) vs PC2 (3.7%)) from the PCA of the dataset of EL-FAMEs from 6 different soils. In parenthesis, the percentage of variance explained by each principal component. See Table 1 for the description of the abbreviations for the soils.

3.6 Connecting paragraph

Chapters 2 and 3 provide information on methods and technologies that can be used to extract soil lipids and characterize soil microbial communities. These studies lead me to select soil fatty acid profiling, based on the analysis by GC-FID of ester-linked fatty acids generated from the direct esterification of soils, as the most suitable method for assessing soil microbial communities in my subsequent field studies. I hypothesized that soil microbial communities could be affected by the presence of plant diseases, like Sclerotinia stem rot of soybean, as well as by the biocontrol agents applied to control this disease. Chapter 4 reports on two biocontrol agents (*Trichoderma virens* (SoilGard™) and arbuscular mycorrhizal fungi) that were applied in the field, singly and in combination, to control Sclerotinia stem rot. Detection of biocontrol agents by DNA-based methods, as well as their effects on disease incidence and soybean yield, are reported here.

4. FIELD ASSESSMENT OF *TRICHODERMA VIRENS* (SOILGARD™) AND MYCORRHIZAL FUNGI AS POTENTIAL BIOCONTROL AGENTS AGAINST SCLEROTINIA STEM ROT IN SOYBEAN

4.1 Abstract

Sclerotinia stem rot (SSR) disease caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is considered one of the most important fungal diseases of soybean. A two-year study was conducted in soybean fields under conventional or no tillage to determine whether *Trichoderma virens* (SoilGard™) and arbuscular mycorrhizal fungi (AMF, a mixture of *Glomus intraradices* and *G. mosseae*), used alone or in combination, could reduce sclerotinia stem rot (SSR) disease incidence in soybeans. Generally, SSR disease indicators were not affected significantly by the biological control treatments. The distribution of viable *S. sclerotiorum* inoculum was probably non-uniform at the study sites, making it difficult to observe significant treatment effects, and weather conditions were not favourable for the development of SSR disease during one of the two study years. Soybean yields were consistent with provincial averages during this study, and were not affected by the levels of SSR disease (up to 25% SSR) found at the field sites. *T. virens*, *G. intraradices* and *G. mosseae* were detected by nested polymerase chain reaction (PCR) analysis in both control and inoculated plots, revealing indigenous presence of these fungi in the soils. Based on our results, it is not known whether the inoculum dose of *T. virens* and the AMF mixture of *G. intaradices:G. mosseae* applied were sufficient to provide protection against SSR disease under field conditions. Further studies are needed to verify the efficiacy and optimize the application method, dose and timing of biological control agents active against SSR disease in soybean fields.

4.2 Introduction

Sclerotinia stem rot (SSR) disease caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is considered one of the most important fungal diseases of soybean, causing significant yield losses wherever soybean is cultivated (Wrather et al. 2001). The life cycle of *Sclerotinia sclerotiorum*, the causal agent of SSR disease in soybean, begins in the soil as asexual structures, the sclerotia, that germinate carpogenically to produce the teleomorphic stage, the apothecia, which then release ascospores. Germinating ascospores colonize senescing or dead flower petals in the phyllosphere, and the pathogen then invades adjoining living tissue and initiates disease (Willetts and Wong 1980).

Several control methods have been reported in literature to reduce the impact of SSR disease on soybean yields. These include the use of resistant cultivars (Buzzell et al. 1993; Wegulo et al. 1998; Kim et al. 1999), management practices such as the use of crop rotations with non-host plants like corn or wheat, increasing row width, reducing plant populations, conservation tillage (e.g., no-till) (Kurle et al. 2001; Gracia-Garza et al. 2002) and the application of chemical inducers of resistance such as 2,6-dichloroisonicotinic acid, benzothiadiazole, and lactofen (Dann et al. 1998, 1999).

A promising and complementary method of controlling SSR disease in the field is the application of biological control agents (BCAs) (Tu 1997; Zhou and Boland 1998; Bardin and Huang 2001). Among the reported BCAs, the mycoparasite *Coniothyrium minitans* is able to control *Sclerotinia sclerotiorum* in a range of crops in glasshouse and field trials (Huang 1977; Trutmann et al. 1980; Budge and Whipps 1991; McLaren et al. 1994; McQuilken et al. 1997; Gerlagh et al. 1999; Huang et al. 2000; Budge and Whipps 2001; Li et al. 2003). Another promising BCA is *Gliocladium virens* (reclassified as *Trichoderma virens* by Rehner and Samuels 1994), and sold as SoilGard™ by Certis USA (Columbia, MD, USA). This fungus occurs naturally in agricultural soils (Davet 1985; Vardavakis 1990; Park et al. 1992) and has provided specific biological control against *S. sclerotiorum* by attacking the sclerotia through the combined actions of endochitinases and the antibiotics gliotoxin and glioviridin, thus preventing their germination into apothecia (Tu 1980; Roberts and Lumsden 1990; Lumsden et al. 1992a, 1992b; Di Pietro et al. 1993; Howell et al. 1993; Lorito et al. 1994; Wilhite et al. 1994; Mukherjee et al. 1995). *T. virens* has been successfully tested in greenhouse/glasshouse trials for controlling a range of plant diseases and more specifically Sclerotinia stem rot in sunflower caused by *Sclerotinia minor* (Burgess and Hepworth 1996), damping-off in cucumber caused by *Sclerotinia sclerotiorum* (Ethur et al. 2005), onion white rot caused *Sclerotium cepivorum* (McLean and Stewart 2000) as well as collar rot of mint (Singh and Singh 2004), damping-off and blight of snap bean (Papavizas and Lewis 1989; Papavizas and Collins 1990) all caused by *Sclerotium rolfsii*. Fewer trials were reported on the use of *T. virens* for controlling plant diseases in fields. Among them, it was shown that it could reduce southern blight (*Sclerotium rolfsii*) in bell pepper (Ristaino et al. 1996) and in carrot (Ristaino et al. 1994), damping-off in snap bean (Smith 1996), Sclerotinia stem rot (*Sclerotinia minor*) of sunflower (Burgess and Hepworth 1996) and Sclerotinia stem rot (*Sclerotinia rolfsii*) of groundnut and betelvine (Maiti et al.

1991). To our knowledge, there are no reports of field studies where *T. virens* was used to control Sclerotinia stem rot (*S. sclerotiorum*) in soybean.

Another group of organisms that may act as BCAs against soil-borne pathogens are arbuscular mycorrhizal fungi (AMF) (Linderman 1988, 1994; St-Arnaud et al. 1995a; Azcón-Aguilar and Barea 1996; Harrier and Watson 2004; Whipps 2004). Consistent reduction of disease symptoms caused by various pathogens have been documented, under controlled conditions, when AMF were used against fungal pathogens belonging to the genera *Phytophthora Gaeumannomyces, Fusarium, Chalara* (*Thielaviopsis*), *Pythium, Rhizotonia, Sclerotium, Verticillium, Aphanomyces*, and against nematodes such as *Rotylenchus, Pratylenchus* and *Meloidogyne species* (Azcón-Aguilar and Barea 1996; Harrier and Watson 2004; Whipps 2004). Various mechanisms have been proposed to explain how AMF reduce the activity of fungal root pathogens (Linderman 1988, 1994; St-Arnaud et al. 1995a; Azcón-Aguilar and Barea 1996; Harrier and Watson 2004; Whipps 2004). They include (1) improved nutrient status of the host plant; (2) damage compensation; (3) competition for host photosynthates; (4) modified rhizosphere deposition; (5) competition for infection/colonization sites; (6) anatomical and morphological changes in the root system; (7) microbial changes in the mycorrhizosphere; and (8) activation of plant defense mechanisms. Soybean is mycorrhizae dependant (Plenchette and Morel 1996), suggesting that the AMF associated with this crop could protect against *S. sclerotiorum* in the field. Also, plant growth and overall health are expected to be better when soybean roots are colonized by AMF, which may reduce the incidence of SSR disease in this crop. Whether AMF colonization of soybean roots acts as a BCA against *S. sclerotiorum* in the field remains to be confirmed.

It is essential to evaluate BCAs against *S. sclerotiorum* under field conditions because the incidence of SSR disease and response of soybean cultivars to the disease are environmentally sensitive (Pennypacker et al., 1999; Bolton et al., 2006). The difficulties in reproducing SSR disease in greenhouse are not trivial: suitable environmental condtions to promote the germination of significant number of sclerotia as well as the release of ascospores, the timing by which the ascospores will be released should coincide with most susceptible stage of growth of soybean (senescing flower petals), etc. (Bolton et al., 2006). The difficulties in reproducing SSR disease in greenhouse are not trivial. It is challenging to select conditions that permit the germination of a

significant number of sclerotia and the release of ascospores at a susceptible stage of soybean growth of soybean in the laboratory., We did not find any report where researchers were able to recreate the complete cycle of SSR disease on soybeans in a greenhouse or growth chamber. In fact, researchers generally bypass the sclerotia germination, ascospores' release and plant infestation by applying mycelia or ascospores at the susceptible growth stage (Rousseau et al., 2004; Chen and Wang, 2005). However, our working hypotheses were: 1) *T. virens* will provide specific biological control over *S. sclerotiorum* by attacking the sclerotia and preventing their germination into apothecia, and 2) AMF will function as a biological control agent by supplying nutrients and water for soybeans, and by blocking infection of roots by ascospores of *S. sclerotiorum*. Thus, only field studies were appropriate for studying SSR disease and testing these hypotheses.

The objectives of this research were (1) to determine whether SoilGard™ *(T.virens)* and AMF (a mixture of *Glomus intraradices and G. mosseae*), used alone or in combination, could reduce SSR disease incidence in soybeans, (2) to assess the effect of these BCA treatments on soybean growth and yield, and (3) to monitor the introduction and establishment of BCAs in fields using PCR-SSCP (Polymerase chain reaction single-strand conformational polymorphism) analysis.

4.3 Materials and Methods

4.3.1 Site description

Based on a 1999 survey of SSR disease incidence of 39 soybean fields in the Montérégie region of southwestern Québec, Canada, four soybean (*Glycine max* (L.) Merrill) fields with a history of SSR disease were selected for this study. The disease incidence in the four fields selected ranged from 22.9 to 31.9%, which was higher than the overall average of 12.5% (R. Jeannotte, unpublished data). Fields A and B were conventionally tilled, while fields C and D were under no-tillage. Fields A and B were ploughed to approximately 18 cm with a mouldboard plough in the fall, and harrowed in the spring before planting, while the no-till fields (fields C and D) were directly seeded with a slot planter in the spring. Studies in these fields were conducted in 2000 and 2001, but it was not possible to use the experimental plots established in 2000 for both study years, since BCAs could

be transferred from one experimental plot to another during routine field operations such as harvest and fall plowing, or natural movement during the winter. Therefore, the 2001 measurements were taken from experimental plots established at an adjacent location within the same field (with similar soil properties, tillage practices and SSR disease incidence). Producers in this region of Quebec generally grow soybeans every second year, in rotation with corn (*Zea mays* L.). The experimental plots established in 2000 were previously under soybean production, while the plots established in 2001 were previously under corn production. The location, tillage system and general soil characteristics of each site are reported in Table 1 and climatic conditions for 2000 and 2001 are given in Table 2.

4.3.2 Experimental design

The experimental design was the same at each site, a randomized complete block design with 6 blocks and four BCA treatments within each block, for a total of 24 plots per site. The BCA treatments were *Trichoderma virens* (TRI), arbuscular mycorrhizal fungi (AMF), *Trichoderma virens* plus arbuscular mycorrhizal fungi (TRI+AMF), and a control (CONTROL) that received no biocontrol agents (see Appendix C). Plots in each field were 3.5 m x 8 m, seeded each spring with soybean (*Glycine max* L. Merr. cv Bayfield) at a rate of 12-16 seeds m^{-1}. The row spacing was 75 cm, giving 5 rows of soybeans per plot.

The TRI treatment received an application of *T. virens* as the commercial product SoilGard™ 12G (Certis USA L.L.C., Columbia, MD, USA; http://www.certisusa.com/). Since sclerotia could be distributed throughout the field, the TRI treatment was applied in three shallow trenches (5 cm deep) between planted rows in each plot about 6-8 weeks after seeding to ensure uniform coverage. In total, 1.25 g m^{-1} of SoilGard™ 12G (corresponding to $12x10^4$ cfu/g of inoculum) was applied between planted rows with 2.50 g m^{-1} of cracked wheat, a bulking agent. This application rate (42.9 kg ha^{-1} of SoilGard™) is equivalent to the dose recommended by Certis USA. The AMF inoculant was a mixture of *Glomus intraradices:Glomus mosseae* (1:1) formulated in a perlite–peat carrier provided by Premier Tech Ltee (Rivière-du-Loup, Quebec, Canada). To promote root mycorrhizal colonization, the AMF inoculum was applied within 5 cm of the planted row within one week of seeding. The AMF application rate was 3.75 g m^{-1} row (minimal inoculum density of 1

propagule g^{-1}), which is equivalent to 53.6 kg ha^{-1} of *G. intraradices:G. mosseae* mixture, as recommended by the manufacturer. The TRI+AMF treatment received an application of *G. intaradices:G. mosseae*, within one week of seeding and an application of SoilGard™ about 6 to 8 weeks after seeding, as described previously. The CONTROL treatment was amended with 2.5 g m^{-1} of cracked wheat and 3.75 g m^{-1} of perlite-peat, both are bulking agents used in SoilGard™ and AMF inocula, respectively.

4.3.3 *S. sclerotiorum* apothecia and SSR disease severity index (SSR-DSI)

The number of *S. sclerotiorum* apothecia was counted in four quadrats of 0.24 m^2 within each plot in mid-August of 2000 and 2001, and the average number of apothecia per plot was calculated. At the end of August, when the plants had reached the pod-filling stage, the incidence of SSR disease was assessed visually by rating 20 consecutives plants in two pre-determined zones in each plot (one zone covering two rows), for a total of 40 soybean plants. Disease severity was classified based on the symptomatic scale described by Grau et al. (1982) where 0 = no symptoms, 1 = only lateral branches showing lesions, 2 = lesions on the main stem, but little or no effect on pod-fill; and 3 = lesions on the main stem that cause poor pod-fill or plant death. The SSR disease severity index (SSR-DSI) was calculated using equation 1, and ranged from 0 (no disease) to 100% (poor pod fill or death of all plants).

$$\text{SSR-DSI} = [\Sigma (DC_i \times \text{plants}_i)/ (\text{total plants}*3)] \times 100\% \quad (1)$$

where DC_i is the ith disease severity class (class = 0, 1, 2, 3), plants_i is the number of plants in the ith disease severity class, and total plants are the 40 soybean plants sampled in each plot.

4.3.4 Plant biomass and root mycorrhizal colonization

Between 5 and 10 soybean plants (shoots and roots) were collected from each plot at the end of August, when they were at the R4 to R6 stages of development (2000) or the pod forming stage (2001) (Fehr et al. 1971). They were placed in plastic bags, transported in coolers with ice packs and stored at 4°C until analysis. In the lab, the above-ground plant parts and the roots were

separated, and roots were washed thoroughly under running tap water, air-dried and cut into 1-2 cm segments. Root samples were cleared with 2.5% KOH by autoclaving at 121°C for 10 minutes, rinsed with water, then rinsed with 1% HCl (hydrochloric acid) and stained with 0.2 % acid fuchsin dissolved in a lactoglycerol solution (1:1:1 ratio of lactic acid (80%), glycerol and distilled water) (Brundrett et al. 1984). The percentage of root length colonized by AMF was estimated using the gridline intersect method (Giovanetti and Mosse 1980) under a dissecting microscope.

4.3.5 DNA extraction

The presence of the BCAs in the field was verified by extracting DNA and conducting nested polymerase chain reaction (PCR) reactions to detect *T. virens* (SoilGard™) and the AMF mixture of *G. intraradices* and *G. mosseae*. Two composite soil samples, from the rows and between the rows, were taken per plot at 0-10 cm depth with a stainless steel piston sampler, well mixed and stored at –20°C prior DNA extraction. Roots were collected as above, washed, dried, cut into 1 cm segments, mixed well and stored at –20°C prior to DNA extraction. Each composite soil sample and two root subsamples from each plot were extracted and then combined to provide one soil or root total DNA extract per plot. The DNA extraction was as follows: five steel beads BB 4.5 mm Copperhead (Crosman, NY,USA) were added to a tube containing 1.5 g of soil sample or 0.7 g of roots sample mixed with 3.5 ml of CTAB (hexadecyltrimethylammonium bromide) lysis buffer [100mM Tris-HCl pH 8.0, 20mM EDTA (Ethylenediaminetetraacetic acid), 1.4M NaCl (sodium chloride), 2.0% (w/v) CTAB, 1.0% (v/v) PVP(polyvinyl pyrrolidone)-40 and 0.2% (v/v) mercapto-2-ethanol]. The tube was shaken at high speed during 2 cycles of 30 sec before being incubated at 60°C for 60 min. A volume of 3.5 ml chloroform was added to the homogenate, then the tube was well shaken and centrifuged to extract 2.0 ml of the aqueous upper phase. A volume of cold isopropanol (-20°C) was mixed with the aqueous phase and DNA were pelleted after an incubation at –20°C for 60 min. DNA pellets were resuspended and purified with QIAquick spin column (QIAGEN, Mississauga, Canada) as described by the supplier. Integrity of the DNA was checked by gel electrophoresis. Gels were photographed using a CCD camera coupled to an AlphaImager software system (AlphaInnotech Corp, San Leandro, USA).

4.3.6 Detection of *G. intraradices* and *G. mosseae* in soil and root samples

The DNA primers set LR1 / NDL22 was used to run a first PCR amplification with soil or root total DNA extracts as described by van Tuinen et al. (1998). Reaction mixtures contained 5 µL of a 100 fold dilution of total DNA extract in a 20 µL of PCR buffer (1 x PCR buffer (QIAGEN) (1.5 mg $MgCl_2$, 0.2 mM dNTPs, 0.5 mg ml^{-1} BSA, 0.5 µM of each primer and 1.25 U of Hot start Taq Polymerase (QIAGEN)). Reactions were performed in an Amplitron II thermocycler (Barnstead/Thermolyne, Dubuque, USA). A nested PCR protocol described by van Tuinen et al. (1998) was used with the primers set LR1 / 8.22 to amplify a DNA fragment of 455 bp specific to *G. intraradices* while the primers set 5.21 / NDL22 was used to amplify a DNA fragment of 367 bp specific to *G. mosseae*. Amplified products were analysed by electrophoresis on 1.2% (w/v) agarose gels, stained and photographed as described above. Further details of the primers and PCR programs used for detection are given in Appendix D.

4.3.7 Detection of *Trichoderma virens* in soil total DNA extracts by nested PCR

The DNA primers set ITS-1F / ITS-4 was used to run a first PCR amplification for the detection of ribosomal ITS regions of fungal DNA in each soil total DNA extract (White et al. 1990; Gardes and Bruns 1993). Reaction mixtures contained 5 µL of a 100 fold dilution of total DNA extract in a 20 µL of PCR buffer (1 x PCR buffer (QIAGEN)) and were performed as described in Appendix E. A nested PCR protocol was used with the primers set Virens-ITS.F1 / Virens-ITS.R1 to amplify a DNA fragment of about 450 bp from *Trichoderma virens* and *T. harzianum* using the PCR conditions described in Appendix E. The amplified products were characterized by a Single-Strand Conformational Polymorphism (SSCP) analysis to assess the bands profiles and to distinguish between the *Trichoderma* spp. detected in each soil total DNA extract. SSCP analysis were run using a 0.7X MDE (Cambrex Bio Science Rockland, Rockland, USA) acrylamide gel under 240 volts at 12°C for an electrophoresis of 5 hours in a Triple Wide Mini-Vertical tank (C.B.S. Scientific Co., Del Mar, USA). Gels were stained with SYBR Gold 1X (Molecular Probes, Eugene, USA) for 10 min as described by supplier, and photographed using the system described above. Further details of the primers and PCR programs used for detection as well as representative examples of gels are given in Appendices E and F.

4.3.8 Plant populations and soybean yield

Soybean grain yield was assessed by harvesting the plants in the center of each plot (2 rows of 4 m each) in September of both years. The development stage at harvest was R8, harvest maturity stage (Fehr et al. 1971). The number of plants in this swath were counted and then thrashed to collect the grain. Grain yield results were expressed on a 13 % moisture basis.

4.3.9 Statistical analysis

Data was transformed to achieve homogeneity of variance, and then evaluated by one-factor analysis of variance using Systat software (Version. 9.1, Systat Software Inc., Richmond, CA, USA). Significant ($P<0.05$) effects of BCA treatments on disease indicators (numbers of apothecia, SSR-DSI) and crop variables (root mycorrhizal colonization and yield) were assessed for each field site and field season. Mean comparisons of significant BCA effects were then made with a LSD test at the 95% confidence level. Correlations between disease indicators during the 2000 and 2001 field seasons were calculated with CoStat software (Version 6.003, CoHort Software, Monterey, CA, USA). Data presented in tables and figures are untransformed means and standard errors of the mean (using 'sem' as abbreviation for 'standard error of the mean').

4.4 Results and Discussion

The BCAs examined in this study have been previously tested for their ability to control plant diseases in controlled environments. However to the best of our knowledge, our study is the first of its kind to test the ability of *T. virens* and an AMF mixture containing *G. mosseae* and *G. intraradices* to control SSR disease under field conditions. We did not know *a priori* whether the application rates of *T. virens* and the AMF mixture recommended by the manufacturers would be sufficiently high to control SSR disease. The sites selected for this study had a history of high SSR disease incidence and provided a range of agricultural practices, soil characteristics and climatic conditions (Tables 1 and 2), representative of an important soybean producing area in southwestern Québec, Canada. However, we did not have any information about the spatial distribution of *S. sclerotiorum* within the field sites, nor could we predict whether the growing

seasons would be favorable for the development of SSR disease. Clearly, we could not apply *S. sclerotiorum* to producer's fields, so our approach was to replicate the experiment at 8 sites during a two-year period in the hopes of having some sites with SSR disease and possibly a response to the BCA treatments.

We also tried to reproduce the experiment in the greenhouse, considering the entire disease cycle, from the germination of sclerotia to SSR disease outbreak, but our many attempts were unsuccessful (data not shown). The development of a reliable greenhouse-based test that would recreate the whole cycle of plant disease caused by *S. sclerotiorum* would be very helpful in future studies concerned with the biocontrol of SSR disease in soybean.

4.4.1 Levels of infestation by *Sclerotinia sclerotiorum*

We monitored the presence of *S. sclerotiorum* by counting the number of apothecia and assessing the SSR-DSI. Site A had the highest numbers of apothecia, with an average of 67.0 apothecia m^{-2} in 2000 and 38.9 apothecia m^{-2} in 2001 (Figure 1). Sites B, C and D had fewer apothecia, with average numbers ranging from 2.8 to 15.0 apothecia m^{-2} in 2000 and 1.1 to 11.9 apothecia m^{-2} in 2001 (Figure 1). These values are similar to apothecia numbers reported in other soybean and bean production systems. Schwartz and Steadman (1978) found 6.5 to 13.8 apothecia/m^2 in bean (host plant of *S. sclerotiorum*) and sugar beet (non-host plant) fields. In three separate studies by Boland and Hall (1987, 1988a, 1988b), the number of apothecia in fields under soybean and bean cropping systems ranged from 0.4 to 30.5 per 1.4 m^2 quadrat depending on the crop, the cultivar and the year. Although the levels of infestation were generally high in both study years, the accuracy of apothecial counts would have been improved by surveying more of the plot surface area (our survey covered less than 5% of the total plot area), more than once during the growing season.

In 2000, the SSR-DSI ranged from 16 to 25% at site A, but was negligible (5% or less) at site A in 2001 and for all of the other sites (Table 3). There was a significant ($P<0.05$) linear relationship between the number of apothecia and the SSR-DSI during 2000, probably due to the high numbers of apothecia and high SSR-DSI at site A (Figure 2). Boland and Hall (1988a, 1988b) also found linear relationships between the number of apothecia and SSR-DSI in some fields. There was no

relationship between the number of apothecia and the SSR-DSI in 2001 (Figure 2), probably due to the low level of disease encountered that year.

The lower SSR-DSI in 2001 compared to that in 2000 could be attributed to many factors: fewer sclerotia in soils producing fewer apothecia, unfavourable climatic conditions (for disease) and crop rotation. Although sclerotia were not counted directly, it is well known that a few apothecia can release impressive quantities of ascospores and increase the SSR-DSI when favourable environmental conditions (for disease) are encountered. Schwartz and Steadman (1978) estimated that one apothecium could release an average of 2.32×10^6 ascospores during 9 days of sporulation in the laboratory. The type of relationship that we currently established between the number of apothecia and the SSR-DSI maybe needs to be revisited to find more accurate mathematical function/model of prediction. Yet, the relationship between apothecia numbers and SSR-DSI is greatly influenced by field conditions. For instance, Boland and Hall (1987, 1988a, 1988b) found that SSR disease incidence, measured as the number of plants with or without SSR symptoms, ranged from 0.6 to 92.0 % of the plants surveyed, depending on the crop, the cultivar and the year. The drought during July and August of 2001 (more than 30 days without significant rainfall at most sites, Table 2) likely affected the viability of the apothecia and consequently hindered the spread and infectivity of *S. sclerotiorum* in 2001. Carpogenic germination of *S. sclerotiorum* sclerotia as well as the release and survival of ascospores from apothecia are affected by extremes in temperature, relative humidity and soil moisture (Clarkson et al. 2003; Hao et al. 2003). Rousseau et al. (2006b) showed that an increase in aggregate stability, microbial activity and soil solution concentration in exchangeable ions associated with a crop rotation and compost application correlated negatively with the carpogenic germination, and thus contributes to suppress disease. Finally, the experimental plots established in 2000 were in fields previously under soybean production whereas in 2001, the plots were established after a year of corn production. Apothecia numbers are expected to be lower when soybeans are grown in rotation with corn, a non-host plant for *S. sclerotiorum*, than when they are grown after soybeans. Rousseau et al. (2006a) showed that rotation with corn reduced SSR disease incidence either directly and indirectly by the reduction of weed biomass. The weed biomass is known to favor carpogenic germination and thus disease incidence. Also, in our plots, the weeds were regularly treated or removed.

4.4.2 Effect of BCAs on SSR disease in Soybean

Generally, the BCA treatments had no effect on the number of apothecia occurring at each site although significant ($P<0.05$, LSD test) differences were observed among BCA treatments at site A in 2001 (Figure 1b), which suggest that *T. virens*, alone or in combination with the AMF, may increase the number of apothecia present. There is no clear explanation for this finding, except that field conditions are more variable than controlled environments (laboratory, greenhouse) and may give rise to unexpected results.

There are many challenges to testing BCA treatments against SSR disease in the field. One of the key assumptions in classical statistical design is that the site is uniform and differences are due to experimental treatments, not just field variability. Little is known about the natural spatial distributions of sclerotia in soil and we lack rapid test methods that would provide information on the distribution of viable sclerotia of *S. sclerotiorum* in the field before planting, but we suspect an uneven distribution of sclerotia that could produce apothecia and thus, SSR disease at the study sites. Bae and Knudsen (2007) showed sclerotia arranged in highly aggregated spatial distributions were more significantly colonized by *T. harzianum* compared to sclerotia randomly distributed. They speculated that the sclerotia colonized by *T. harzianum* may serve as a nutrient source for further hyphal growth of the *Trichoderma* and colonization of adjacent sclerotia. Methods to detect and map the spatial distribution of sclerotia, rather than relying on population densities (Jeger et al. 2004), would be helpful to predict the ability of BCAs to control a soilborne disease such as SSR. Instead of the randomized complete block design used in this study, a spatially-explicit analysis could reveal more about the relationships between sclerotia, SSR disease and BCAs. Also, it will be important to be able to predict the growth, in time and space, of fungi used as BCAs in soil systems. This would help researchers and managers to select the best time and rate for BCA application (Knudsen et al., 2006). In the present study, further investigation of how *T. virens*, alone or in combination with the AMF, responds and interacts with *S. sclerotiorum* under field conditions is warranted. In any case, the SSR-DSI was not affected by the BCA treatments at any site during this two year study (Table 3).

4.4.3 Detection of *G. intraradices* and *G. mosseae* by specific nested PCR

For BCA treatments to be effective against SSR disease in soybeans, they must remain viable once they are field released. There is little evidence that *T. virens* or the AMF mixture, alone or in combination, acted against *S. sclerotiorum*, since the number of apothecia present and the SSR-DSI were not generally affected by the BCA treatments (Figure 1, Table 3). The percentage AMF colonization of soybean roots was used as an indirect measurement of AMF viability, and was consistent during both years of this study. Sites A, B and D had from 18 to 24% colonization with AMF, while site C had about 10% AMF root colonization (Figure 3). Inoculation with AMF did not increase mycorrhizal root colonization over the control. Although the TRI treatment did enhance AMF colonization at site B in 2000 relative to the AMF treatment alone or the control, generally AMF root colonization was unaffected by the BCA treatments (Figure 3). Interactions between different *Trichoderma* spp. and mycorrhizal fungi, documented in the literature, may be negative, neutral or positive (Godeas et al. 1999; Vazquez et al. 2000; Martinez et al. 2004; Masadeh et al. 2004). Paulitz and Linderman (1991) found no antagonistic or synergistic interaction between *T. virens* and AMF, which is consistent with most of our results.

Soils from sites A and D were analyzed using a nested Polymerase Chain Reaction (PCR) (van Tuinen et al. 1998) for the specific detection of *G. intraradices* and *G. mosseae*, in June and August of 2000 and 2001. The amplified products specific to each inoculated AMF species were detected in soil DNA extracts from plots of all treatments. The cumulative detection frequency of each AMF species in the soil from sites A and D is presented in Table 4. Both *G. intraradices* and *G. mosseae* were already present, at least at sites A and D, as shown by their detection in all treatments, including non-inoculated controls. *G. intraradices* was detected more frequently in soils (2000 and 2001) and roots (2001) than *G. mosseae*. This leads us to propose that the lack of response to AMF inoculation was due to the fact that the AMF species inoculated were part of the native soil microbial community, at least at sites A and D.

4.4.4 Detection of *T. virens* by a nested PCR-SSCP analysis

The nested PCR used with the primers set Tvh-ITS.F1 / Tvh-ITS.R1, showed that *T. harzianum* and *T. virens* were detected at higher frequency in 2000 field season (Table 4, Appendix F). The average frequency of detection among all treatments during June and August was 29.1% and 12.5 % in field A, while it was 58.3% and 37.5% in field D. The frequency of detection of both *Trichoderma* spp. decreased in 2001, especially in field D. We analyzed the amplified products detected in each plot of both fields by SSCP gel electrophoresis to distinguish the bands pattern of *T. virens* from the one of *T. harzianum*. This SSCP analysis showed that the bands profile of *T. harzianum* was dominant and *T. virens* was detected in very few inoculated plots (Table 4, , Appendix F). The inoculated *T. virens* showed no persistence in these fields. Inoculation with SoilGard™ (*T. virens*) could be less effective as *S. sclerotiorum* antagonists than the *Trichoderma* spp. naturally present in the soil and may have reduced the antagonism of the *Trichoderma* population against the pathogen, resulting in significantly more apothecia in field A in 2000 (Figure 1a). It is not known whether inoculated fungi like *T. virens* and *G. intraradices/G. mosseae* are more antagonistic against *S. sclerotiorum* than naturally occurring populations of *Trichoderma* and *Glomus* spp., and the interactions between naturally-occurring and introduced fungus under field conditions remains to be evaluated.

4.4.5 Effects of BCAs on Soybean Yield

Soybean yields at the study sites were from 2203 to 3667 kg grain ha^{-1} in 2000 and from 2039 to 2492 kg grain ha^{-1} in 2001 (Figure 4). These yields are comparable to the provincial averages in Quebec (Canada), which normally range from 2500 to 3063 kg grain ha^{-1} (Institut de la statistique du Québec, 2005). It should be noted, however, that 2000-01 average yields were low due to unfavourable weather conditions, i.e. greater than normal rainfall from April to June 2000 and drought in August 2001 (Institut de la statistique du Québec, 2005). We also found that soybean yields were not affected by the BCA treatments (Figure 4).

Apart form the environmental factors that will evidently impact disease incidence, plant development and yield, a variety of agricultural practices must be considered to understand the

impact of SSR on seed yield. Factors that affect plant infection and the spreading of disease in the field include: row spacing and plant populations, as well as the cultivars used and their characteristics of disease resistance, maturity, plant architecture (Chun et al. 1987; Buzzell et al. 1993; Park 1993; Saindon et al. 1993; Hoffman et al. 1998; Kim et al. 1999; Arahana et al. 2001; Cober et al. 2003). The experimental design used a wide row spacing (30 cm) that probably inhibited the spread of the disease, however, the soybean cultivar "Bayfield" is known to be susceptible to SSR disease (Sylvie Rioux, personal communication; Auclair et al. 2004; Rousseau et al. 2004).

The relationship between soybean yield and SSR is also influenced by the method used to evaluate disease incidence and severity (Kerr et al. 1978). In our study, yield decreases due to SSR were related more to the number of severely infected plants (plants rated class 3) than to the global index of SSR disease (SSR-DSI). In 2000, we found 122 (of 960) plants with a class 3 disease rating in site A, but only from 1 to 17 (of 960) plants with a class 3 disease rating at the other sites. In 2001, between 0 and 17 (of 960) plants were severely infected in our study sites. When we examined the data from site A in 2000, we found that soybean yields in plots with at least 2.5% of severely infected plants (n=6 plots) were significantly ($P<0.05$, t-test) lower than plots with no severely infected plants (n=18 plots) (data not shown). Although we cannot conclude that the levels of SSR disease observed at site A reduced soybean yield, these findings suggest a critical level of SSR infection that may be linked with a reduction in soybean yield.

4.5 Conclusions

Biocontrol agents may be used as an alternative to, or complement fungicides applied to control plant diseases such as those caused by *S. sclerotiorum*. This study examined *T. virens* and AMF (a mixture of *G. intraradices and G. mosseae*), alone or in combination, for their ability to control Sclerotinia stem rot disease caused by *S. sclerotiorum* in soybean fields during two growing seasons. Generally, SSR disease indicators were not affected significantly by the BCA treatments. The distribution of viable *S. sclerotiorum* inoculum was probably non-uniform at the study sites, making it difficult to observe significant treatment effects, and weather conditions were not favourable for the development of SSR disease during one of the two study years. Soybean yields

were consistent with provincial averages during this study, and were not affected by the levels of SSR disease (up to 25% SSR-DSI) found at the field sites. It is not known whether the inoculum dose of *T. virens* and the AMF mixture of *G. intaradices:G. mosseae* applied were sufficient to provide protection against SSR disease under field conditions. Further study is needed to verify the efficiacy and optimize the application method, dose and timing of BCAs active against SSR disease in soybean fields.

Table 1. Location, agricultural practices and soil characteristics (0-15 cm depth) at the experimental sites under soybean production used in this study. All soils were mixed, frigid Typic Endoaquents.

Field*	Location	SSR-DSI 1999†	Year	Residues‡	pH§	OM‖	Sand¶	Clay¶	Silt¶	Textural class
		%		%		g kg^{-1}				
A	Saint-Césaire	31.9 ± 7.1	2000	2.5	6.7	42	118	321	561	Silty Clay Loam
	45°25' N 73°00' W		2001	0.0	6.8	36	159	257	584	Silty Loam
B	Saint-Césaire	23.1 ± 9.1	2000	0.0	6.3	36	130	353	517	Silty Clay Loam
	45°25' N 73°00' W		2001	8.7	6.8	42	144	327	529	Silty Clay Loam
C	Sainte-Brigide-d'Iberville	28.1 ± 7.6	2000	76.3	6.2	51	140	367	493	Silty Clay Loam
	45°19' N 73°04' W		2001	82.1	6.6	52	151	442	407	Silty Clay
D	La Présentation	22.9 ± 7.1	2000	64.2	6.3	40	384	246	370	Loam
	45°40' N 73°03' W		2001	57.3	6.1	36	450	194	356	Loam

* Fields A and B were under conventional tillage (CT). Fields C and D were under no-tillage (NT).

† SSR-DSI (mean ± sem) in the soybean sites A, B, C and D in 1999. SSR-DSI was estimated in 8 plots (plots of 2 m X 2 m) each on sites A, B, C and in 16 plots on site D.

‡ The percentage of surface residues was determined by the method of Sloneker and Moldenhaner (1977).

§ Soil:water extracts (1:2 soil:solution ratio) (Hendershot et al., 1993).

‖ Organic matter (OM) was determined by loss on ignition (360°C for 4 h) (Schulte et al., 1991).

¶ Particle-size analysis (Sheldrick and Wang, 1993).

Table 2. Monthly averages for precipitation and air temperature at weather stations near the study sites during the 2000 and 2001 field seasons (Environment Canada Meteorological Service, unpublished data).

Month	Monthly Precipitation (mm)						Daily average air temperature (°C)					
	2000			2001			2000			2001		
	I*	II	III	I	II	III	I	II	III	I	II	III
May	147	152	108	67	77	49	13	14	13	15	14	16
June	98	71	89	116	100	81	18	18	18	20	20	20
July	42	85	72	95	154	58	20	21	20	19	19	20
August	103	113	97	82	72	79	19	20	20	21	21	22
September	54	98	94	34	42	33	14	15	14	16	16	17
Total	**444**	**519**	**460**	**394**	**445**	**300**						

*I, Marieville Station (Fields A and B); II, Farnham Station (Field C); III, St-Hyacinthe Station (Field D)

Table 3. Incidence of Sclerotinia stem rot disease in soybean fields inoculated with the biocontrol agents *T. virens* (TRI) and a mixture of *G. intraradices* and *G. mosseae* (AMF), alone or in combination. Values are the mean (± sem) Sclerotinia stem rot disease severity index (SSR-DSI), expressed in percentage (%).

Site	2000				2001			
	TRI+AMF	TRI	AMF	CONTROL	TRI+AMF	TRI	AMF	CONTROL
A	21.3 ± 3.6	16.3 ± 4.1	25.0 ± 4.3	24.2 ± 2.3	3.1 ± 2.0	1.7 ± 0.8	1.7 ± 1.2	0.0 ± 0.0
B	0.6 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.2	0.0 ± 0.0	2.9 ± 1.6	1.3 ± 0.9	5.0 ± 3.6
C	1.5 ± 0.9	2.3 ± 0.9	1.7 ± 1.0	3.3 ± 2.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
D	1.8 ± 0.8	2.2 ± 0.9	1.7 ± 0.8	3.2 ± 1.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.4

Note: The application of biocontrol agents had no significant impact ($P<0.05$) on the SSR-DSI at any sites during the study.

Table 4. Detection of *G. intraradices*, *G. mosseae* and *T. virens* in soybean plots by PCR analysis during 2000 and 2001 field seasons.

Field	Treatment*	2000 soil†						2001 Soil						2001 root	
		June			August			June			August			August	
		G.i.‡	G.m.	T.v.§	G.i.	G.m.	T.v.	G.i.	G.m.	T.v.	G.i.	G.m.	T.v.	G.i.	G.m.
A	TRI + AMF	2	0	2 (1)	2	0	0	1	4	1	2	0	2 (1)	6	0
	TRI	2	0	2	3	2	1	4	0	0	1	0	0	6	0
	AMF	4	0	3	2	0	0	2	4	3	2	0	0	6	0
	CONTROL	2	0	0	4	0	2	2	6	0	1	0	0	6	0
D	TRI + AMF	4	0	0	6	0	2 (1)	4	2	0	4	2	0	6	1
	TRI	2	0	6 (3)	6	0	1	6	6	2 (1)	4	2	0	5	2
	AMF	2	3	2	6	0	0	3	6	0	4	3	0	5	1
	CONTROL	2	0	6	6	0	6	6	6	0	6	6	0	6	1

* 6 plots per treatment.

† The number of plots among 6 plots per treatment sampled at each field in which of *G. intraradices*, *G. mosseae* and *T. virens* have been detected by specific nested PCR analysis of total soil DNA extracts or total soybean's roots DNA extracts.

‡ G.i.: *Glomus intraradices*; G.m.: *Glomus mosseae*;

§ T.v.: *Trichoderma virens/Trichoderma harzianum*. The number in brackets refer to the number of plots among 6 per treatment where *T. virens* was distinguished from *T. harzianum* by SSCP analysis.

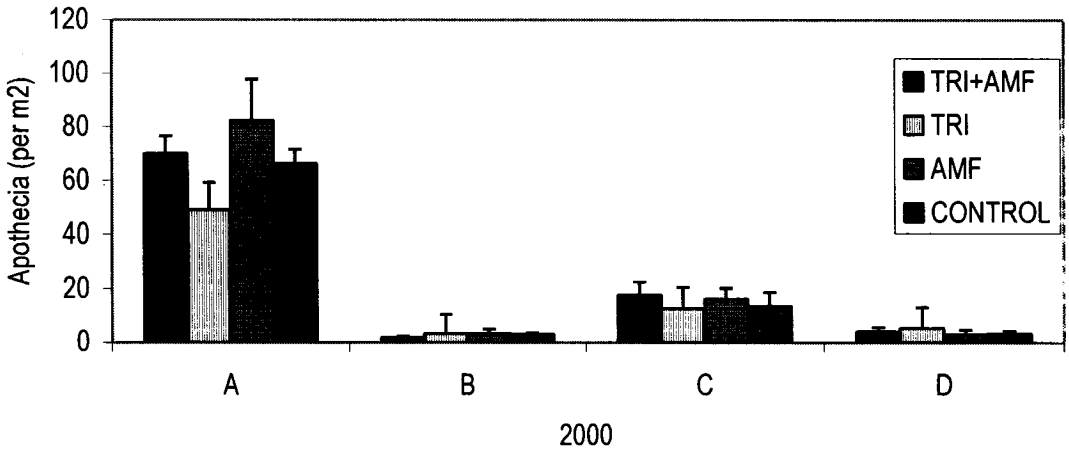


Figure 1a.

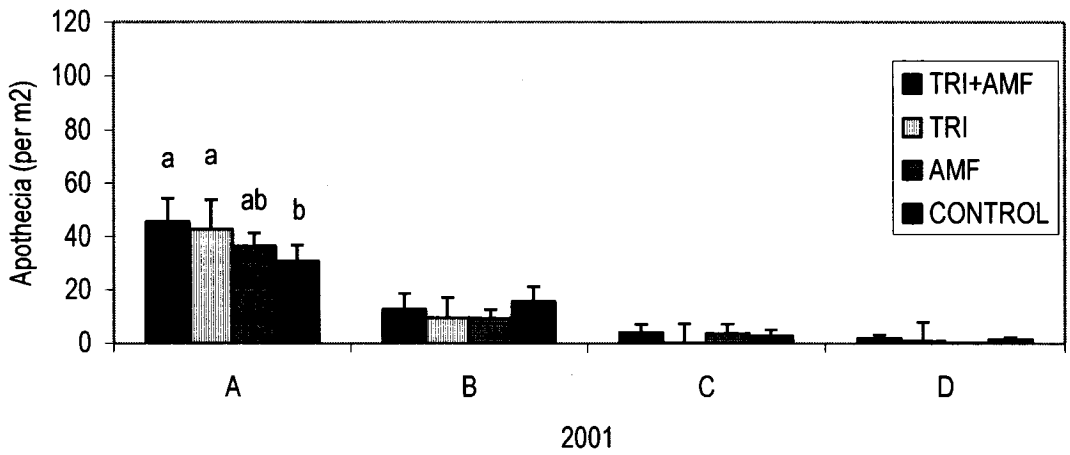


Figure 1b.

Figure 1. Number of apothecia (per m^2) (mean ± sem) in fields inoculated with the biocontrol agents *T. virens* (TRI) and a mixture of *G. intraradices* and *G. mosseae* (AMF), alone or in combination, in 2000 (A) and 2001 (B). Means followed by the same letter within the same site are not significantly different at $P< 0.05$ (LSD test). The absence of letters on bars within a site indicates no difference between biocontrol treatments ($P<0.05$).

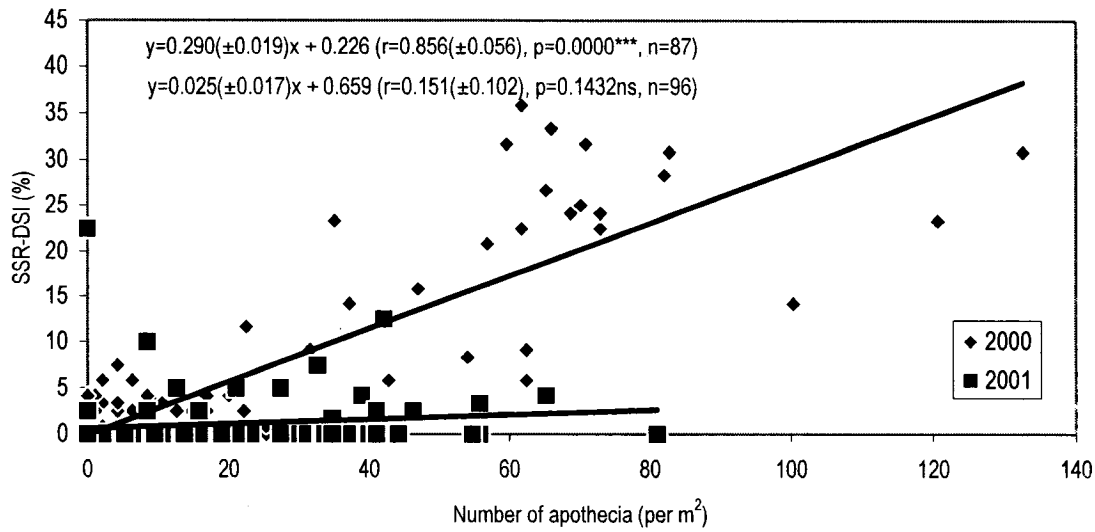


Figure 2. Relationship between the number of apothecia and the incidence of SSR disease, expressed as the Sclerotinia stem rot disease severity index (SSR-DSI), in soybean fields during the 2000 and 2001 seasons.

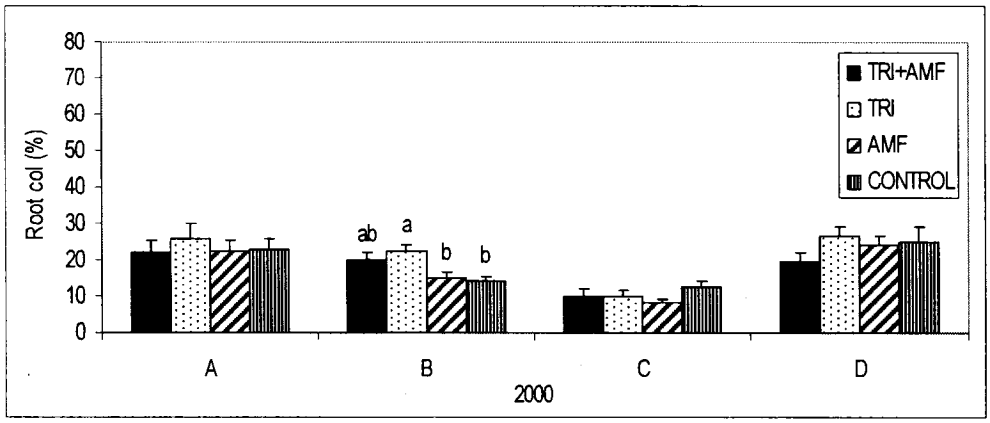


Figure 3a.

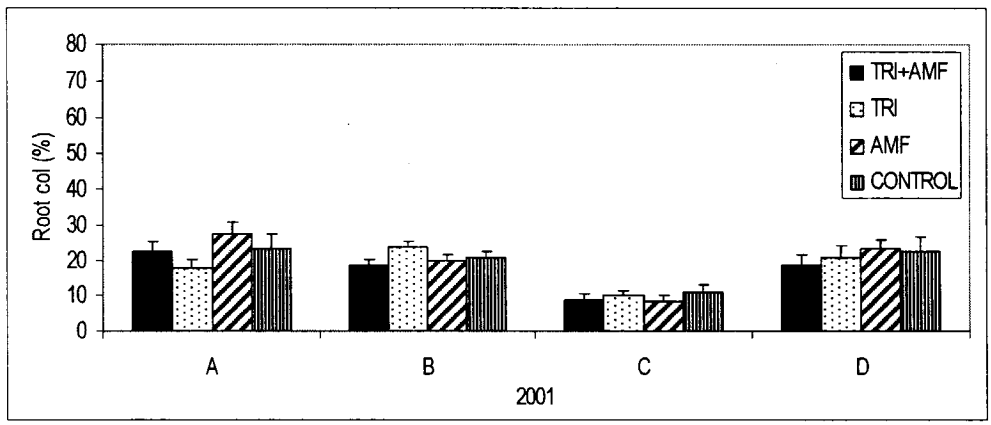


Figure 3b.

Figure 3. AMF root colonization (%) of soybean plants grown in fields inoculated with the biocontrol agents *T. virens* (TRI) and a mixture of *G. intraradices* and *G. mosseae* (AMF), alone or in combination, in 2000 (A) and 2001 (B). Means followed by the same letter within the same site are not significantly different at $P< 0.05$ (LSD test). The absence of letters on bars within a site indicates no difference between biocontrol treatments ($P<0.05$, LSD test).

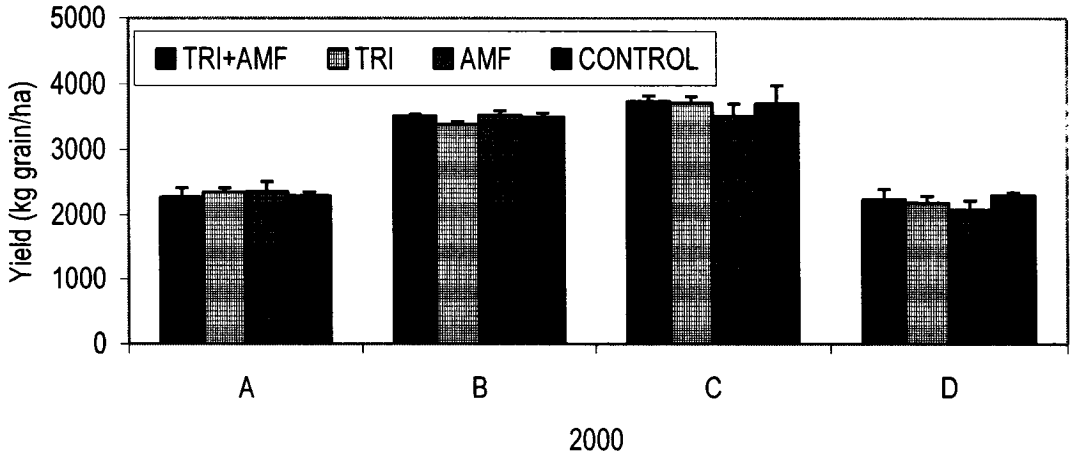


Figure 4a.

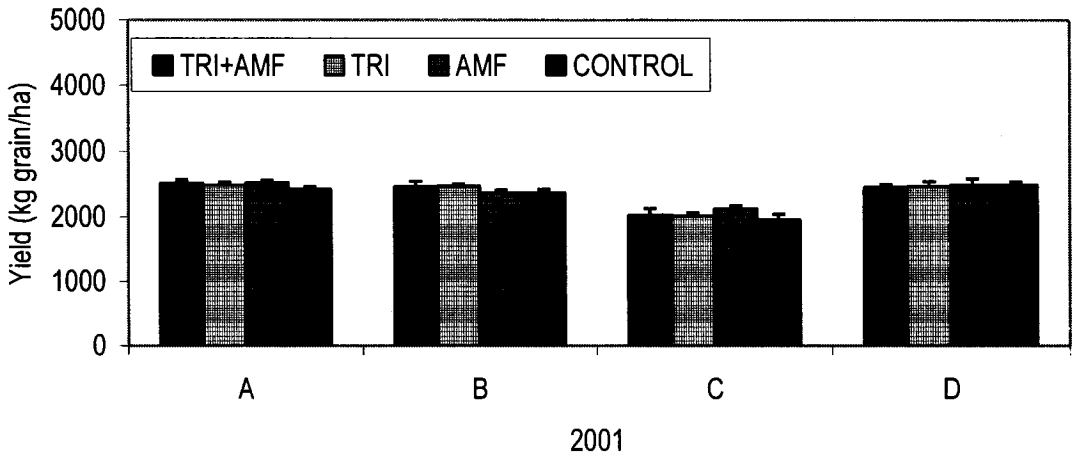


Figure 4b.

Figure 4. Soybean grain yield (kg ha^{-1}, adjusted to 13% humidity) in fields inoculated with the biocontrol agents *T. virens* (TRI) and a mixture of *G. intraradices* and *G. mosseae* (AMF), alone or in combination, in 2000 and 2001. The absence of letters on bars within a site indicates no difference between biocontrol treatments ($P<0.05$).

4.6 Connecting Paragraph

Chapter 4 gives the experimental design and details of a two-year field study that was conducted in soybean fields under conventional or no tillage to determine whether *Trichoderma virens* (SoilGard™) and arbuscular mycorrhizal fungi could reduce Sclerotinia stem rot disease incidence in soybeans. We found that the biocontrol agents had little effect on the incidence of disease. Most fields had very low disease incidence, except for field A during 2000. Chapter 5 investigates the effect of the inoculation of the biological control agents in a soybean field on the soil microbial communities, and explored the relationships between the SSR disease and general soil microbial groups (i.e., Gram+ bacteria, Gram- bacteria, saprophytic fungi, mycorrhizal fungi). These relationships were evaluated by profiling soil fatty acid biomarkers, generated from the direct esterification of soils and analyzed by GC-FID, which I identified as the most reliable method for assessing soil microbial communities.

5. PROFILING OF SOIL MICROBIAL COMMUNITIES USING FATTY ACID ANALYSIS IN A SOYBEAN AGROECOSYSTEM INFESTED WITH SCLEROTINIA STEM ROT DISEASE

5.1 Abstract

Sclerotinia stem rot (SSR) disease caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is considered one of the most important fungal diseases of soybean, causing significant yield losses wherever soybean is cultivated. Biological control is a promising way of controlling SSR disease in the field. A better understanding of the complex interactions between biocontrol agents, the pathogen *Sclerotinia sclerotiorum* and indigenous soil microorganisms as well as the plant; will permit us to optimize control strategies for plant diseases based on an ecological approach. The objectives of this research were (1) to investigate the effect of the inoculation of biological control agents (BCAs) against SSR disease in soybean fields on soil microbial communities, and (2) to explore the relationships between the SSR disease and the soil microbial communities. Following the application of BCA treatments in 2000 and 2001, the concentrations of different bacterial and fungal fatty acid biomarkers were significantly modulated. The concentrations of fatty acid biomarkers specific to bacteria, bacteria Gram+ and actinomycota were selectively and significantly higher in plots with higher number of plants heavily infested with SSR disease. Generally, there was no overall difference in the relative proportions of chemical classes as well as biological groups observed in the field investigated for trial years 2000 and 2001. However, except for the saprophytic and mycorrhizal fungal biomarkers, the concentrations of chemical classes and biological groups of fatty acids were generally higher in 2000. Further study is needed to investigate the nature of the changes induced in the microbial populations by the inoculation of BCAs as well as how they could be related to disease suppression. The selective increase in the concentrations of specific groups of bacteria needs to be investigated in more details in order to understand how these bacteria interact with plants infected by *S. sclerotiorum*.

5.2 Introduction

SSR disease caused by *S. sclerotiorum* (Lib.) de Bary is considered one of the most important fungal diseases of soybean, causing significant yield losses wherever soybean is cultivated (Wrather et al. 2001). Sclerotia of *S. sclerotiorum* germinate carpogenically to produce the teleomorphic stage, the apothecia, which then release ascospores. Germinating ascospores colonize senescing or dead flower petals in the phyllosphere, and the pathogen then invades

adjoining living tissue and initiates disease (Willetts and Wong 1980). Up to now, the main ways to control SSR disease were by chemical control (e.g. fungicides), disease resistance by cultivar breeding, cultural control (e.g. increasing the width between rows, reducing plant populations) and biological control (Tu, 1997; Zhou and Boland, 1998; Bardin and Huang, 2001). In our previous chapter, arbuscular mycorrhizal fungi and *Trichoderma virens* were assessed in a two-year field trial in their capacity to control SSR disease in soybean as well as the effect of these BCA treatments on soybean growth and yield. Mycorrhizal fungi that established in a soil environment will interact with other soil microorganisms (Olsson et al., 1996, 1998; Green et al., 1999; Hodge, 2000; Söderberg et al., 2002; Johansson et al., 2004; Albertsen et al., 2006). The interactions of *Trichoderma virens* with indigenous microbial populations in field conditions is less known. Also, the plant type and its growth stage as well as any modification of its physiology caused by disease, for example, could be an important determinant of the microbial community structure in its surroundings soil (rhizosphere) (Garbeva et al., 2004; Johnson et al., 2005; Romeiro et al., 2005; Mougel et al., 2006; Roesti et al., 2006). However, the effect of plant diseases attacking the aerial parts of plants, like Sclerotinia stem rot, on soil microbial community is unclear.

Fatty acids biomarkers analysis is a suitable method for studying the changes in the soil microbial communities caused by many factors such as plant diversity (Söderberg et al., 2002; Carney and Matson, 2005), tillage (Spedding et al., 2004), fertilization (Sullivan et al., 2006), pollution (Kelly et al., 2003), soil properties (Schutter and Dick, 2000), etc. Fatty acid profiling has been also used in plant pathology in order to better understand the impact of disease on soil microbial communities and also how inoculated biological control agents interact with these soil communities (e.g., Kozdrój et al., 2004; Hamel et al., 2005; Larkin and Honeycutt, 2006). Many studies used the phospholipid fatty acid (PLFA) approach to study the interactions of arbuscular mycorrhizal fungi with other soil microbiota and plant (e.g., Olsson et al., 1996, 1998; Green et al., 1999; Söderberg et al., 2002; Johansson et al., 2004; Albertsen et al., 2006). Mazzola (2004) proposed to use PLFA or fatty acid methyl ester (FAME) analysis in order to assess the biological nature of soil suppressiveness. Although, fatty acid profiling (and phospholipid fatty acid profiling) are sensitive enough for monitoring the global changes in the soil microbial community following inoculation of microorganisms and effect of plant disease, this approach was not report frequently used for such studies in field conditions. As far as we are aware, there have been no published studies that report

how soil microbial communities are affected by BCA treatments in a field trial, or whether soil microbial communities are affected when they inhabit the root-associated soil of plants with SSR disease.

The purpose of this work was (1) to investigate the effect of the inoculation of BCAs against SSR disease in soybean fields on soil microbial communities, and (2) to explore the relationships between the SSR disease and the soil microbial communities in order to better understand how the "health" status of the plant, monitored by the SSR disease incidence, will influence the microbial communities in the vicinity of the plants. The soil microbial communities will be assessed by profiling the fatty acids biomarkers and evaluated the effects of BCA treatments and SSR disease on the quantity of total FAMEs present in the soil, the proportions of different chemical groups of fatty acids in the soil, the abundance of fatty acids representing certain soil microorganisms (bacteria vs fungi, gram+ vs gram-, etc) as well as the microbial diversity assessed by multivariate analysis.

5.3 Materials and Methods

A two-year field study was conducted in soybean fields under conventional or no tillage located in southwestern Quebec. At each site (2 under each tillage regime), the experimental design was the same: a randomized complete block design with 6 blocks and four BCA treatments within each block, for a total of 24 plots per site. The BCA treatments were *Trichoderma virens* (TRI), arbuscular mycorrhizal fungi (AMF), *Trichoderma virens* plus arbuscular mycorrhizal fungi (TRI+AMF), and a control (CONTROL) that received no biocontrol agents. The effect of the BCAs on the Sclerotinia stem rot (SSR) disease were monitored, in each plot, by counting the number of apothecia from *Sclerotinia sclerotiorum*, the causal agent of the disease, and by assessing the incidence of SSR disease in the crop. The effects of the BCAs on the plants were evaluated by measuring the biomass of the plants, colonization of the roots by AMF as well as the soybean grain yield. The establishement of the BCAs was studied using PCR approaches. Detailed description of the sites selected, the experimental design, methodology used for assessing the SSR disease and soybean yield were presented in chapter 4.

5.3.1 Soil collection and handling

Soil samples were collected from each plot, in each field, at the end of August, when the soybean plants were at the R4 to R6 stages of development (2000) or the pod forming stage (2001) (Fehr et al., 1971). In each plot, seven subsamples were taken, using a soil auger of 3 cm diameter, from the top 10 cm of planted rows and pooled to generate one composite sample per plot (total weight of the composite per plot: ca 200-250 g of field moist soil). The samples were frozen immediately and stored at -20°C until analysis. Fatty acid analysis was conducted on 23 (one sample lost) soil samples collected from field A in 2000 (6 blocks x 4 treatments), as well as 12 soil samples collected from field A in 2001 (three blocks x four treatments). General physical and chemical characteristics of these soils are described in Table 1 of chapter 4.

5.3.2 Preparation of the ester-linked fatty acid methyl esters (EL-FAMEs)

All organic solvents used in this study were HPLC grade. Glassware and laboratory equipment were prepared as recommended in White and Ringelberg (1998). The *in situ* transesterification procedure used in this study is detailed in Schutter and Dick (2000), but with some modifications. Prior to lipid analysis, the soils were freeze-dried and finely ground. Then, 3 to 5 g of soil was incubated with 15 mL of 0.2 M KOH in methanol for 1-hour at 37°C. The reaction mixture was vortexed every 10-15 minutes. At the end of the reaction, the mixture was cooled to room temperature and neutralized with 3 mL of 1 N glacial acetic acid. The EL-FAMEs were extracted from the mixture with phase partitioning using 3 x 5 mL hexane-chloroform (4:1). The organic phases were pooled and dried under a gentle steam of N_2, then dissolved in iso-octane prior to analysis by GC-FID.

5.3.3 Analysis of EL-FAMEs by GC-FID

The EL-FAMEs were analyzed in split mode (50:1) with a gas chromatograph (Hewlett Packard 6890) equipped with a 25-m Ultra-2 capillary column and a flame ionization detector (FID), following the settings of the MIS protocol (MIDI, Inc., Newark, Delaware, USA, www.midi-inc.com) (Sasser, 1990; Schutter and Dick, 2000; Buyer, 2002) as also detailed in section 3.3.5. The retention times

of the peaks were converted to equivalent chain length (ECL) values (Sasser, 1990). Identification of peaks was based on comparison of retention times (and ECLs) to commercial FAME standards. Also, the identification of the peaks in our samples was cross-validated by sending a subset of our samples in a certificated external laboratory (Laboratoire de Santé Publique du Québec, Sainte-Anne-de-Bellevue, QC) using the Sherlock Microbial Identification System® (MIDI, Inc., Newark, Delaware, USA). The fatty acids analyzed in our samples by this method are listed in Appendix B. The concentration of each identified FAME (nmoles per gram dry soil (DS)) was calculated as using C19:0 as internal standard at a concentration of 24.5 ng μL^{-1} (0.0784 nmole C19:0 μL^{-1}) in each sample. The contribution of each identified FAME to the total FAME concentration (all FAMEs identified) in a sample was expressed as mole fraction (relative richness, % mole). We used the standard ω-nomenclature (A:BωC) for designating the fatty acids (IUPAC-IUB, 1977). The FAMEs identified in our samples were grouped according to their chemical class: Straight saturated chain FAs or StraightSAFAs (12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0), BranchedSAFAs (i-14:0, i-15:0, a-15:0, i-16:0, i-17:0, a-17:0, 10Me-16:0, 10Me-17:0, 10Me-18:0, 10Me-19:0), fatty alcohol (16:0 N alcohol), CycloFAs (17:0 cyclo, 19:0 cyclo ω8c), HYFAs (14:0-2OH, 15:0-3OH, 18:0-2OH, 18:0-3OH), MonoUnsaturatedFAs or MonoUFAs (16:1ω11c, 16:1ω5c, 17:1ω8c, 18:1ω9c, 18:1ω7c, 18:1ω5c, 19:1ω11c/19:1ω9c (co-eluting fatty acids), 20:1ω9c, 20:1ω7c), PolyUFAs (18:3ω6,9,12c). EL-FAMEs identified in our samples were also grouped according to their biological origin and computed using the following EL-FAMEs: actinomycetes (10Me-16:0, 10Me-17:0, 10Me-18:0, 10Me-19:0), mycorrhizal fungi (16:1ω5c), saprophytic fungi (18:1ω9c, 18:2ω6,9c, 18:3ω6,9,12c), bacteria (i-14:0, i-15:1, i-15:0, a-15:0, 15:0, i-16:0, 16:1ω7c, i-17:0, a-17:0, 17:0 cyclo, 17:0, 18:1ω7c, 18:1ω5c, 19:1ω6c/unknow 18.846/cyclo19:0ω10c/19ω6, 19:0 cyclo ω8c), Gram+ bacteria (i-14:0, i-15:1, i-15:0, a-15:0, i-16:0, i-17:0, a-17:0) and Gram- bacteria (16:1ω7c, 17:0 cyclo, 18:1ω7c, 18:1ω5c, 19:1ω6c/unknow 18.846/cyclo19:0ω10c/19ω6, 19:0 cyclo ω8c) (Lechevalier, 1977; Kroppenstedt, 1985, 1992; Federle, 1986; Lechevalier and Lechevalier, 1989; O'Leary and Wilkinson, 1989; Ratledge and Wilkinson, 1989; Wilkinson, 1989; Vestal and White, 1989; Frostegård et al., 1993; Graham et al., 1995; Olsson et al., 1995; Frostegård and Bååth, 1996; Zelles, 1997, 1999; Olsson, 1999; Hill et al., 2001).

5.3.4 Statistical analysis

Statistical analyses were performed using SYSTAT software (Version 10, Systat Software Inc., Richmond, CA, USA) and CoStat software (Version 6.003, CoHort Software, Monterey, CA, USA). Analysis of variance (using LSD, at α=0.05, as a post-hoc test for mean comparison) was performed to determine the effect of BCA treatments, for each study year (2000 and 2001), on individual FAMEs, total quantities of identified FAMEs in soil samples, chemical classes as well as biological groups of FAMEs. Principal components analysis (PCA) was performed for each study year, on the dataset of soil EL-FAMEs identified in field A to explain sources of variation and to test the discrimination of the samples according to the treatments. The data, expressed in % mole, were transformed with a $\log_{10}x+1$ transformation, where x is the % mole of each EL-FAME in a sample and analyzed using SYSTAT software, version 10 (Systat Software Inc., Richmond, CA, USA).

One-way ANOVA was also used to compare the concentrations of individual FAMEs, total quantities of identified FAMEs in soil samples, chemical classes as well as biological groups of FAMEs in the 'healthy' plots compared to the 'diseased' plots. A 'healthy' plot was one where less than 12.5% of plants had a disease severity index of 3 or greater (lesions on the main stem that cause poor pod-fill or plant death) on the symptomatic scale described in Grau et al. (1982) (see Chapter 4). A 'diseased' plot contained more than 12.5% of plants with a disease severity index of 3 or greater. Data presented in tables and figures are untransformed means and standard errors of the mean (using 'sem' as abbreviation for 'standard error of the mean').

5.4 Results and discussion

5.4.1 Selection of the site

The levels of Sclerotinia stem rot disease incidence (SSR-DSI) in the three of the four fields investigated were very low in both years (Figure 1): SSR-DSI in fields B, C and D ranged from 0.0 to 22.5 with an average of 1.1 ± 0.2 (n = 135) during the study. However, in field A during the first year of the trial (2000), a greater SSR-DSI was detected, ranging from 5.8 to 35.8 with an average of 21.7 ± 1.8 (n = 24) (Figure 1). In 2001, the SSR-DSI in field A was from 0.0 to 12.5 with an

average of 1.6 ± 0.6 (n = 24). Fatty acid analysis was conducted on 23 (one sample lost) soil samples collected from field A in 2000 (6 blocks x 4 treatments), as well as 12 soil samples collected from field A in 2001 (three blocks x four treatments).

The fatty acids were prepared using a direct *in situ* transesterification method that was documented to be complementary to phospholipid fatty acid methodology, easy to perform and adequate for discriminating the dynamics of microbial communities (Drijber et al., 2000; Hinojosa et al., 2005). In our thirty-five samples, we identified forty-six fatty acids. These fatty acids were considered individually or grouped according to their chemical nature and biological origin (see references cited in Materials and Methods section of this chapter).

5.4.2 Effect of the inoculation of *Trichoderma virens* and mycorrhizal fungi as potential biocontrol agents against Sclerotinia stem rot on microbial communities in soils under soybean crop

We performed an analysis of variance to detect any significant change in concentrations of the soil fatty acids after BCA treatments in fields A (Table 1) for both years. Table 1 presented the fatty acids and their concentrations that were significantly affected (at $p<0.05$, LSD) by the BCA treatments in field A either in years 2000 or 2001. The concentrations of i-16:1, 11Me-18:1ω7c, 19:1ω11c/19:1ω9c were significantly higher (at $p<0.05$, LSD) in plots inoculated by AMF than in the others in 2000. The concentrations of 18:3ω6,9,12c, a fungal biomarker, was similar in TRI, AMF and control plots compared to plots inoculated by TRI+AMF in 2000. The concentration of 20:1ω7c was significantly higher (at $p<0.05$, LSD) in CONTROL plots compared to the others.

Thus, the fatty acids (i-16:1, 11Me-18:1ω7c, 19:1ω11c/19:1ω9c) increased in plots inoculated by AMF constitute less than 1% of the average TotalFAs (Total fatty acids) (Table 1, 2000). The literature is scarce about the biological origin of these fatty acid biomarkers. They are more likely to be of bacterial origin (Ratledge and Wilkinson, 1989; Zelles, 1999). Right now, it is too early to link these increases to the direct inoculation of AMF. The interactions of AMF with soil bacteria were demonstrated in several papers (Olsson et al., 1996, 1998; Söderberg et al., 2002; Johansson et al., 2004; Albertsen et al., 2006). Future studies will be required to verify if the fatty acids observed

here come from Rhizobium bacteria that are omnipresent in the rhizosphere of soybean. According to our knowledge, this is one of the first assessments by fatty acid profiling of the effect of AMF inoculated in field on soil bacteria.

No inoculation, or inoculation of AMF or TRI alone probably did have any significant effect on the concentrations of 18:3ω6,9,12c (Table 1, 2000). However, co-inoculation of TRI and AMF significantly (at $p<0.05$, LSD) 'repress' or lower the expression of this fungal fatty acid suggesting an antagonistic effect such as observed, in controlled experiments, by Green et al. (1999), Martinez et al. (2004), Tiunov and Scheu (2005).

In field A (in 2001), the inoculation of AMF and TRI+AMF seems to have an adverse effect on concentrations of the fatty acids (Table 1). Most of them were significantly higher (at $p<0.05$, LSD) in CONTROL and-or in TRI plots. The results are not consistent from one year to the other. These results suggest that effect of BCA treatments on soil microbial communities could be modulate by other factors such as climatic conditions. This factor, also with the different previous crop as well as the presence of SSR disease, constitute the major differences between 2000 and 2001 that we could observed in our experimental plots.

ANOVA testing the effects of the BCAs treatments on the fatty acids classified by their chemical groups reveals that for the two soils at both years, only the PUFAs group is significantly different (at $p<0.05$) among the treatments in 2000, essentially because this group is composed only of 18:3ω6,9,12c (see above). A fungal group composed of the summation of the fatty acid biomarkers of mycorrhizal fungi (16:1ω5c) and saprophytic fungi (18:1ω9c, 18:2ω6,9c, 18:3ω6,9,12c) was significantly different among the treatments in 2000 precisely because of the significant changes of the fatty acid 18:3ω6,9,12c as stated above.

Finally, a principal components analysis (PCA) was performed on the individual fatty acid biomarkers from the 2000 and 2001 datasets separately. PCA with data from 2000 extracted seven principal components explaining more than 86% of all the variance in the dataset (more than 58% for PC1 and PC2). Figure 2a presents the PCA scores plot for 2000. The treatments overlap in Figure 2a and an analysis of variance using the PCA scores for each sample as variables didn't

show any statistical significant difference between the treatments in 2000. With the 2001 data, PCA extracted five principal components explaining more that 93% of the variance (more than 73% for PC1 and PC2). Analysis of variance using the PCA scores reveals that PC2 significantly discriminated the four treatments (at $p<0.05$, LSD). The fatty acids 11Me-18:1ω7c, 12:0, i-15:1, 15:0-3OH, 16:1ω11c, 14:0-2OH have high positive loading values (>0.7) (data not shown) on PC2 axis. The concentrations of these fatty acids (see Table 1) contributed to the difference between the control plots and the treatments' plots observed in Figure 2b.

These results raised the question on the origin of these changes. It was not possible to demonstrate clear establishment of the BCAs using PCR techniques (Chapter 4). Also, we observed no significant difference in root colonisation by AMF as well as in the concentration of 16:1ω5c, the fatty acid biomarker specific to AMF, following the inoculation by AMF in fields (data not showed). Thus, our results suggest that the inoculation at the beginning of the season initiated changes that continued until the end of the season. Also, up to now, the significance of these changes in microbial communities in the context of biological control of SSR disease is not known. These aspects remain to be assessed in further studies.

5.4.3 Relationships between SSR-DSI and fatty acids

One of the goals of this research was to characterize the microbial communities in soil in the vicinity of plants infested to different extents with SSR. Since we observed a good range of SSR-DSI in field A (see Figure 1, and section 5.4.1), in 2000, only the fatty acids from this field were considered in the analysis.

In this research, the SSR-DSI was estimated according to the method described in Grau et al. (1982) in which forty soybean plants per plot were individually rated according to a symptomatic scale from 0 to 3, 0 meaning that the plant presents no symptoms and 3, lesions on the main stem resulting in plant death and poor-pod fill are observed. Different ways of expressing the disease incidence were tested in order to find better relationships between the SSR disease expression and potential effects on the fatty acid biomarkers. Based on our field observations, soybean plants rating from 0 to 2 were not necessarily affected by the disease in term of plant death or yield

reduction. However, plants rating with a 3 on the symptomatic scale were most likely to die from the infection by the pathogen. Thus, we computed a parameter expressing the number of plants, among the forty recorded by plot, rating 3. In order to detect of possible effects of these plants on the soil microbial communities, we separated the plots in two categories according to the following criterium: a plot with 5 plants and more with a rate of 3 on the symptomatic scale, over the 40 plants monitored, is designated to be "diseased" and a plot with less than 5 plants with a rate of 3 on the symptomatic scale, over the 40 plants monitored, is designated to be healthy. The criterium was arbitrary stated. However, the average percentage of plants rated 3 is 12.7%. So, the threshold was set at 12.5%, 5 plants over 40. Then, analysis of variance comparing categories A ("diseased") vs B ('healthy") was performed on the concentrations (expressed in nmol g^{-1} DS) of individual fatty acids, their chemical classes as well as biological groups. Table 2 shows clearly that the concentrations of bacterial fatty acid biomarkers, especially from Gram+ as well as actinomycota, are higher in the soil samples from 'diseased' plots compared to the concentrations from more 'healthy' plots. Surprisingly, the fatty acid biomarkers specific to saprophytic fungi as well as mycorrhizal fungi were not altered (data not shown). It is important to state that the parameters significantly different between the two conditions were also tested for possible underlying treatment and block effects. For the data presented in Table 2, there were no significant treatment and block effects.

Bacterial populations respond clearly to the presence of heavily infested plants in a plot. Higher concentrations of specific bacterial fatty acid biomarkers were observed and more specifically Gram+ as well as the actinomycota fatty acids biomarkers. These results could suggest that the health status of a plant heavily infested in its phyllosphere by a disease such as SSR could even modulate the soil microbial communities in its vicinity in a very specific way. At this moment, it is not possible to say if these shifts are beneficial or detrimental to the plant. However, the absence of significant effect of the plant health status on the fungal biomarkers could promote the hypothesis of the induction of supporting bacterial communities.

Plant-microbial interactions in the rhizosphere are very complex. Heavy infestion of a plant by a necrotrophic fungus such as *S. sclerotiorum* most likely ends in the death of the plant. However, we speculate that the plants could also mobilize specific set of microorganisms to assist in its defense

against pathogens and recovery from pathogenic attack. A satisfactory plant-soil model needs to be developed for in-depth study of the changes in soil microbial communities, observed by fatty acid profiling, as well as their functions, following the infection of the soybean plant by *S. sclerotiorum*.

5.4.4 Concentrations of fatty acid chemical classes and biomarker groups from field A in 2000 and 2001

Table 3 displays the averages (in nmol g^{-1} DS as well as in % mole) found in soils collected from field A in 2000 and 2001. An analysis of variance was used to test the difference between the concentrations and relative proportions of the chemical classes and biological groups between both years. The % mole of the chemical class PolyUFAs was significantly higher in 2000 (at $p<0.01$, LSD). For all the other chemical classes and biological groups, the relative proportions did not change from year to year in this field (Table 3). However, the quantities, in nmol g^{-1} DS, of TotalFAs, BranchedSAFAs, HYFAs, PolyUFAs and StraightSAFAs were significantly higher in 2000 (at least at $p<0.05$, LSD). Also, the quantities of unspecificFAs (fatty acids not specifc to any biological group) as well as the fatty acid biomarkers of bacteria, Gram+, actinomycota were significantly higher (at least at $p<0.05$) in 2000. No significant difference was observed in the quantities of the saprophytic as well as the mycorrhizal fungi specific fatty acid biomarkers (Table 1).

The factors that could account for differences or similarities in the concentrations and proportions of fatty acid biomarkers found in 2000 compared to 2001 are mainly the soil properties, the climatic conditions, previous crop, disease incidence and plant stage at the sampling time. The locations in field A at which the experimental plots in 2000 and 2001 were established present only slight different soil properties such as organic matter levels, sand and clays levels as well as higher percentage of surface residues in 2000 (Table 1, chapter 4). Moreover, the climatic conditions in 2001 were more different than 2000 with a period of drought in July and August 2001 (more than 30 days without significant rainfall at most sites, Table 2, chapter 4). As well, the previous crop for the location of 2000 was soybean and corn for the one in 2001. We already mentioned that SSR-DSI were higher in 2000 compared to 2001. Also, we collected soil samples in August of both years, but

the soybean plants were at different stages in their development: R4 to R6 stages (2000) or the pod forming stage (2001) (Fehr et al., 1971).

Even if the above factors were slightly different between the two years, the proportions (% mole) of fatty acids from chemical classes as well as biological groups were generally similar from one year to the other (Table 3). Our results could suggest that the relative proportions of the fatty acid biomarkers specific to microbial communities in these soils were not affected, to a measurable extent, by the factors mentioned above. Or the soil factor, and-or the plant factor, being the more 'stable' factors in the agroecosystem compared to climatic conditions, disease or previous crop, could have supported a microbial community structure able to face these changes in their environment, for example a drought in 2001, without major disturbances in the community members as revealed by the fatty acid profiling. There is a growing body of evidences showing that the soil type, plant type as well as agricultural management regime are the major determinant of fhe structure of microbial community (Garbeva et al., 2004; Johnson et al., 2005; Singh et al., 2006). These determinants could then be able to re-induce equilibrium in the microbial community after severe events like a drought, for example.

However, the above factors could together explained why the concentrations of fatty acid from the chemical classes TotalFAs, BranchedSAFAs, HYFAs, PolyUFAs and StraightSAFAs and those from the biomarker groups unspecificFAs, bacteria, Gram+, actinomycota were significantly higher in 2000. The concentrations of saprophytic as well as the mycorrhizal fungi fatty acid biomarkers were not different between 2000 and 2001. Our results could suggest that these fungal communities were more 'stable' compared to bacterial communities. The factors listed above that contribute to the difference and similarity of locations in 2000 and 2001 are maybe not sufficient enough to induce measurable changes in the specific fungal and mycorrhizal fatty acid biomarkers contents, but bacterial populations could be sensitive enough to the changes and respond with variations in their specific fatty acid biomarkers concentrations. The profiling of fatty acids could be helpful in identifying the changes associated with soil disease suppression (Janvier et al., 2007), but this remains to be confirmed.

Further study needs to be performed in order to address more specifically the question of the major determinants of microbial community structure in field conditions.

5.4.5 Conclusions

According to our knowledge, this study is the first (1) to report significant effects of the introduction of biological control agents, such as arbuscular mycorrhizal fungi and *Trichoderma virens*, on the soil microbial community, profiled by their fatty acid biomarkers, and in soybean field infested with Sclerotinia stem rot disease; and (2) to show that the soybean plants with severe infection by Sclerotinia stem rot will alter the microbial communities, profiled using their fatty acid biomarkers, in the soil environment surrounding them. The concentrations of different of bacterial and fungal fatty acid biomarkers were significantly modulated following the application of the BCA treatments in 2000 and 2001. The concentrations of fatty acid biomarkers specific to bacteria, bacteria Gram+ and actinomycota were selectively and significantly higher in plots with higher number of plants heavily infested with SSR disease. Generally, there was not overall difference in the relative proportions of chemical classes as well as biological groups observed in the field investigated for trial years 2000 and 2001. However, the concentrations of chemical classes and biological groups of fatty acids were generally higher in 2000, excepted for the saprophytic and mycorrhizal fungal biomarkers. Further study will be needed in order to investigate the nature of the changes induced in the microbial populations by the inoculation of BCAs as well as how they could be related to disease suppression. Moreover, the selective increase in the concentrations of specific groups of bacteria will need to be investigated in more details in order to understand how these bacteria interact with plants presenting heavy infection by SSR disease.

Table 1. Fatty acids significantly altered by BCA treatments in field A in 2000 and 2001. For each fatty acid, means in the same row, from the same year of trial, followed by the same or no letters are not significantly different ($p<0.05$, LSD).

Field	A (2000)				A (2001)			
Treatment	TRI+AMF+	TRI	AMF	CONTROL	TRI+AMF+	TRI	AMF	CONTROL
	nmole g-1 DS				nmole g-1 DS			
12:0	0.35	0.30	0.31	0.33	**0.00b**	**0.00b**	**0.00b**	**0.18a**
i-15:1	0.19	0.20	0.25	0.22	**0.00b**	**0.16a**	**0.00b**	**0.15a**
14:0-2OH	0.20	0.21	0.22	0.22	**0.00b**	**0.00b**	**0.00b**	**0.13a**
i-16:1	**0.00c**	**0.21b**	**0.27a**	**0.00c**	0.00	0.00	0.00	0.00
16:1ω11c	0.36	0.38	0.40	0.35	**0.00b**	**0.19ab**	**0.00b**	**0.29a**
i-15:0-3OH	0.00	0.00	0.00	0.00	**0.00b**	**0.18a**	**0.00b**	**0.15a**
15:0-3OH	0.22	0.24	0.24	0.27	**0.00b**	**0.00b**	**0.00b**	**0.20a**
17:1ω8c	0.36	0.46	0.67	0.46	**0.00b**	**0.35a**	**0.00b**	**0.00b**
18:3ω6,9,12c	**0.00b**	**0.17a**	**0.18a**	**0.18a**	0.00	0.00	0.00	0.00
11Me-18:1ω7c	**0.00b**	**0.00b**	**0.19a**	**0.00b**	**0.00b**	**0.00b**	**0.00b**	**0.28a**
19:1ω11c/19:1ω9c	**0.00b**	**0.00b**	**0.24a**	**0.00b**	0.00	0.00	0.00	0.00
20:1ω7c	**0.77ab**	**0.58b**	**0.48b**	**0.92a**	0.88	0.77	0.90	0.61

Table 2. Fatty acid biomarkers, chemical classes of fatty acids and biological groups significantly different in "healthy" vs "diseased" plots. For each individual fatty acid, chemical class or biological group, means in the same row followed by the same or no letters are not significantly different (p<0.05, LSD).

	A ("Diseased" plots) (n=13)	B ("Healthy" plots) (n=10)
	mean ± sem (nmoles g^{-1} DS)	
i-15:0	2.82 ± 0.06a	2.58 ± 0.08b
a-15:0	2.00 ± 0.05a	1.80 ± 0.07b
10Me-16:0	2.03 ± 0.06a	1.74 ± 0.10b
10Me-18:0	0.98 ± 0.04a	0.85 ± 0.04b
19:0 cyclo ω8c	1.32 ± 0.04a	1.17 ± 0.05b
BranchedSAFAs	11.71 ± 0.29a	10.53 ± 0.40b
PolyUFAs	0.13 ± 0.03a	0.05 ± 0.02b
Bacteria	19.85 ± 0.55a	17.65 ± 0.92b
Gram+	10.59 ± 0.25a	9.54 ± 0.36b
Actinomycota	3.36 ± 0.10a	2.89 ± 0.15b

Table 3. Concentrations (mean ± sem) of fatty acid chemical classes and biomarker groups in field A, trial years 2000 and 2001. For each chemical class or biological group, means in the same row, comparing data from both years expressed in the same unit, followed by the same or no letters are not significantly different (p<0.05, LSD).

		A (2000) n=23		A(2001) n=12	
		nmole g-1 DS	% mole	nmole g-1 DS	% mole
Chemical classes	TotalFAs	43.80 ± 1.55a	-	34.26 ± 3.11b	-
	BranchedSAFAs	11.19 ± 0.26a	25.83 ± 0.41	8.17 ± 0.29b	25.01 ± 1.21
	CycloFAs	2.90 ± 0.16	6.74 ± 0.41	2.27 ± 0.18	6.75 ± 0.25
	HYFAs	2.39 ± 0.13a	5.44 ± 0.17	1.67 ± 0.11b	5.11 ± 0.37
	MixedFAs*	4.19 ± 0.25	9.44 ± 0.39	3.12 ± 0.48	8.68 ± 0.66
	MonoUFAs	7.43 ± 0.50	16.56 ± 0.70	7.77 ± 1.82	20.22 ± 2.56
	PolyUFAs	0.09 ± 0.02a	0.19 ± 0.04a	0.00 ± 0.00b	0.00 ± 0.00b
	StraightSAFAs	15.61 ± 0.50a	35.8 ± 0.46	11.26 ± 0.47b	34.22 ± 1.44
Biological groups	UnspecificFAs	19.99 ± 0.75a	45.68 ± 0.72	14.69 ± 0.6b	44.91 ± 2.25
	Bacteria	18.89 ± 0.54a	43.38 ± 0.41	15.56 ± 1.99b	44.38 ± 1.55
	Gram+	10.13 ± 0.23a	23.38 ± 0.36	7.14 ± 0.29b	21.76 ± 0.99
	Gram-	6.72 ± 0.28	15.29 ± 0.27	6.78 ± 1.76	17.53 ± 2.54
	Actinomycota	3.16 ± 0.10a	7.26 ± 0.13	2.42 ± 0.09b	7.4 ± 0.36
	Saprophytic fungi	3.53 ± 0.27	7.84 ± 0.48	2.98 ± 0.66	7.97 ± 1.29
	AMF	1.39 ± 0.13	3.1 ± 0.25	1.03 ± 0.23	2.74 ± 0.44

*MixedFAs: fatty acids containing more than one chemical feature.

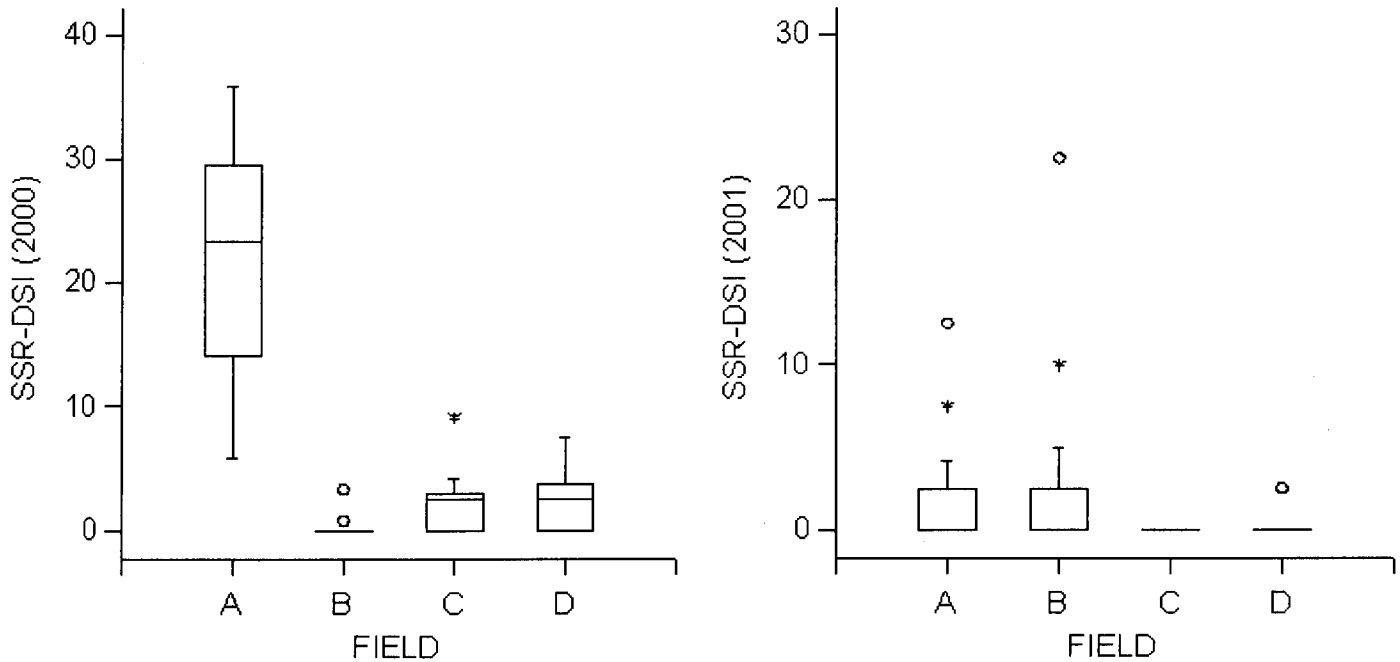


Figure 1. Box-plot SSR-DSI in fields used to study BCAs in 2000 and 2001.

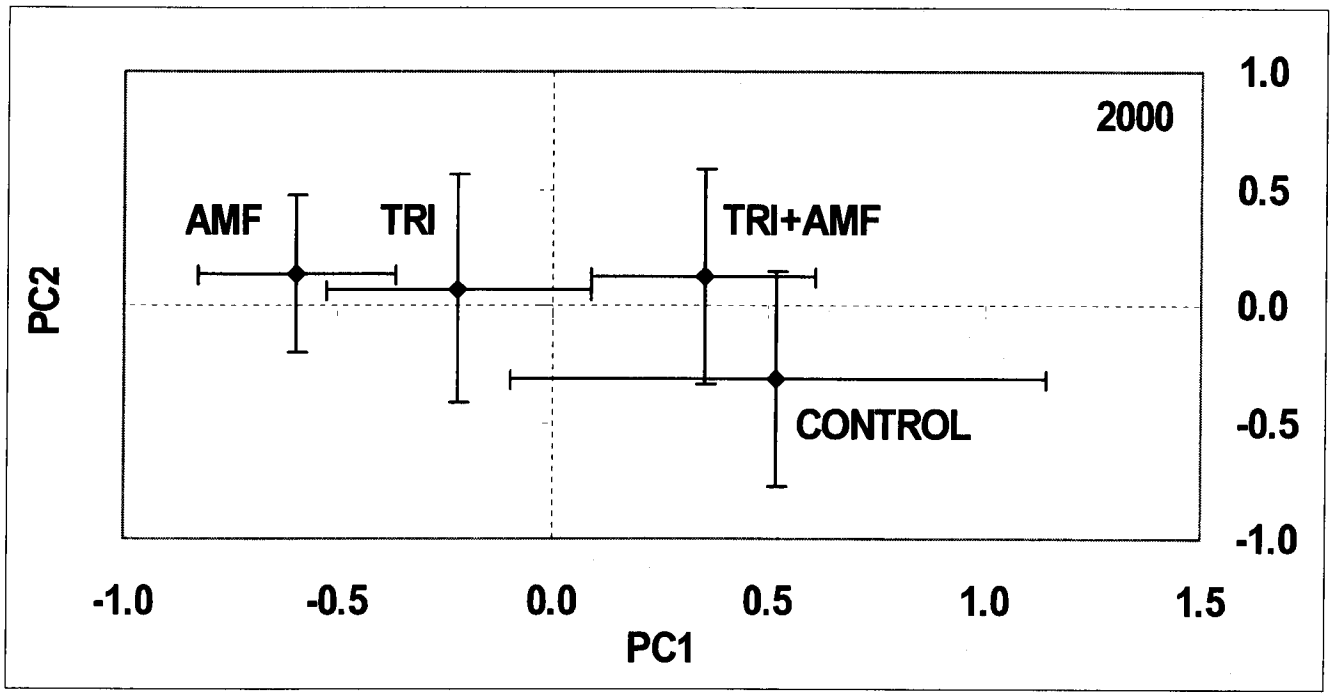

2000
AMF
TRI
TRI+AMF
CONTROL
PC2
PC1
1.0
0.5
0.0
-0.5
-1.0
-1.0
-0.5
0.0
0.5
1.0
1.5

Figure 2a.

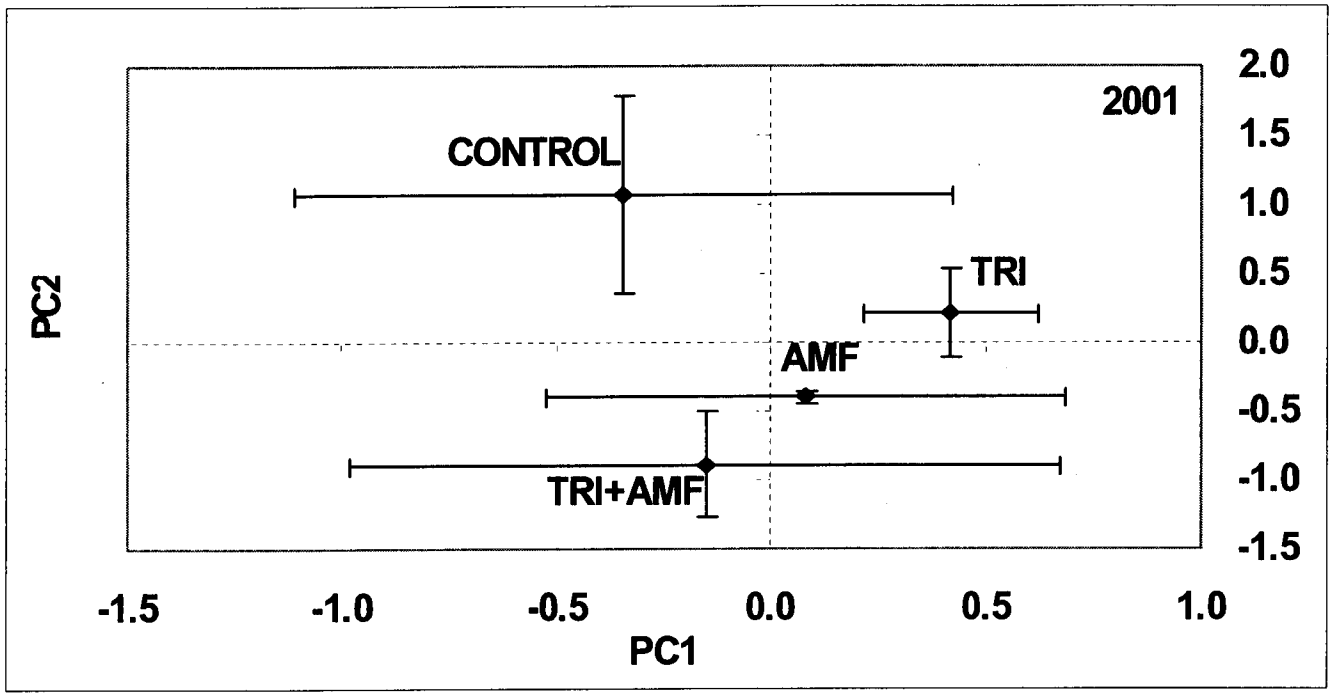


Figure 2b.

Figure 2. Principal components analysis of the individual EL-FAMEs detected in soil samples from field A in 2000 (Figure 2a) and in 2001 (Figure 2b). The data presented in these figures are means of the replicates' scores, by treatment (TRI+AMF, AMF, TRI, CONTROL) and their standard errors of the mean.

GENERAL CONCLUSIONS

1. Total lipid fatty acid methyl ester (TL-FAME) was greater when soil was extracted with chloroform:methanol:buffer and chloroform:methanol than with the hexane:2-propanol and acetone solvents. The concentration of TL-FAMEs in various chemical (saturated chain, branched saturated chain, monounsaturated chain, polyunsaturated chain, hydroxy substituted fatty acids) and biological groups (bacteria, mycorrhizal fungi, saprophytic fungi, higher plants/faunal biota) was affected by the type of solvent used. The extraction efficiency for these chemical and biological groups followed the general trend of: chloroform:methanol:buffer ≥ chloroform:methanol > hexane:2-propanol = acetone. When selecting a solvent for soil lipid extraction, researchers should consider the soil properties and the lipid groups under study.

2. Discriminant analysis of the pyrolysis metastable atom bombardment time-of-flight mass spectrometry (Py-MAB-TOF-MS) fingerprints and the ester-linked fatty acid profiles demonstrated significant differences among soils from three crop production systems (soybean, corn, asparagus). Lipid fingerprints generated from analysis of Py-MAB-TOF-MS spectra reflect the overall soil lipid composition (including lipids from microbes, animals, plants and non-living matter). In contrast, the ester-linked fatty acid profiles provide quantitative information on specific groups of soil bacteria and fungi. Further work is needed to identify diagnostic fragments from Py-MAB-TOF-MS that could be used as biomarkers for more detailed characterization of these microbial communities. With further developments in this area, pyrolysis mass spectrometry could be used to rapidly characterize soil microbial communities, i.e., to determine changes in the microbial diversity and to identify specific compounds such as fatty acids or sterols that are functionally important in the metabolism of certain groups of soil microorganisms.

3. Sclerotinia stem rot (SSR) disease indicators were not affected by the biological control treatments applied during a 2-year field study. The distribution of viable *Sclerotinia sclerotiorum* inoculum was probably non-uniform at the study sites, making it difficult to observe significant treatment effects, and weather conditions were not favourable for the development of SSR disease during one of the two study years. Soybean yields were consistent with provincial averages during this study, and were not affected by the levels of SSR disease (up to 25% SSR) found at the field

sites. *Trichoderma virens, Glomus intraradices* and *Glomus mosseae* were detected by nested polymerase chain reaction analysis in both control and inoculated plots, revealing indigenous presence of these fungi in the soils. Based on our results, it is not known whether the inoculum dose of *T. virens* and the AMF mixture of *G. intaradices:G. mosseae* applied were sufficient to provide protection against SSR disease under field conditions. Further studies are needed to verify the efficiency and optimize the application method, dose and timing of biological control agents active against SSR disease in soybean fields.

4. Generally, there was not overall difference in the relative proportions of chemical classes as well as biological groups observed in the field investigated for trial year 2000 and 2001. Excepted for the saprophytic and mycorrhizal fungal biomarkers, the levels of chemical classes and biological groups of fatty acids were generally higher in 2000. The application of BCA treatments in 2000 and 2001 modulated the levels of different of bacterial and fungal fatty acid biomarkers. The levels of fatty acid biomarkers specific to bacteria, bacteria Gram+ and actinomycota were selectively and significantly higher in plots with higher number of plants heavily infested with SSR disease. However, biomarkers specific to saprophytic and mycorrhizal fungi were not affected by the presence of SSR disease. Further studies will be needed in order to investigate the nature of the changes induced in the microbial populations by the inoculation of BCAs as well as how they could be related to disease suppression. Moreover, the selective increase in the levels of specific groups of bacteria will need to be investigated in more details in order to understand how these bacteria interact with plants presenting heavy infection by SSR disease.

5. Improvement in methodologies of extraction and characterization of soil lipid biomarkers, as an approach for characterizing soil biota, may permit researchers to conduct more extensive field studies investigating soil and plant health issues.

CONTRIBUTIONS TO SCIENCE

The work presented in this thesis contributed to the expansion of scientific knowledge in several ways:

1. In Chapter 2, for the first time, we showed that the chloroform-methanol mixtures are the best extractants for recovering fatty acid biomarkers in conjunction with the use of a PSE system. To the best of our knowledge, the efficiency of chloroform-methanol mixtures in extracting soil lipids was not formally demonstrated, at least using a PSE system. Also, I examined how lipids originating from certain microbial groups (bacteria, fungi) and chemical classes (saturated and unsaturated fatty acids) were affected by the solvent mixture. I also found that lipid recovery was probably affected by soil properties such as organic matter content and clay content. This work contributes to the promotion of new technologies, namely the PSE system, which permits researchers to extract lipids in a safe and efficient way.

2. In Chapter 3, we presented the first report on the use of a Py-MAB-TOF-MS system to obtain characteristic and specific fingerprints of the whole lipid composition of six soils from various agroecosystems. This experiment is a proof-of-concept in which the capacity to fingerprint of the Py-MAB-TOF-MS was demonstrated to complement the more specific analysis of the fatty acid biomarkers by GC-FID in the same soils. I was able to distinguish soil samples based on the molecular composition of soil lipids from soybean, corn and asparagus fields. Coupled with multivariate statistical analysis, the Py-MAB-TOF-MS system could be easily used in large-scale ecological studies to rapidly acquire information on lipid biomarkers from a large set of samples. The initial results were promising, but further work is necessary to confirm the capacity and precision of this system for lipid biomarker identification.

3. In Chapter 4, we presented the first study of BCAs (*Trichoderma virens* and *G. intaradices:G. mosseae*) inoculated in fields under soybean crop for controlling SSR disease. Although the work was not entirely successful, since the inoculation of BCAs in fields under the conditions tested was not efficient in significantly reducing SSR-DSI, number of apothecia and soybean yield, it provides some impetus for moving such research beyond controlled laboratory and greenhouse studies.

4. In Chapter 4, we reported for the first time a PCR-SSCP method, developed by the team of our collaborator Richard Hogue, for the detection of *Trichoderma* spp. in our field studies. Also, using this method, we reported the first results on the natural populations of *Trichoderma* spp. in soybean fields under different tillage systems in Quebec. There is evidence that *Trichoderma* spp. has a role in disease suppression; being able to detect this organism may help us to understand the underlying factors governing disease outbreaks and suppression in Quebec agroecosystems, and elsewhere.

5. In Chapter 5, we presented the first study showing evidence, by fatty acid biomarker analysis, of the effect of the introduction of BCAs on the soil microbial communities of a soybean agroecosystem. This study points out the importance of using sensitive methods (DNA-based profiling and signature lipid biomarker analysis, for example) in order to reveal the impacts, that could be subtle, of the introduction of BCAs on the soil microbial communities.

6. In Chapter 5, we reported for the first time changes in specific groups of microorganisms, profiled by fatty acid biomarker analysis, in plots with higher number of plants heavily infested with SSR disease. These results are important because they imply the need to develop a plant-soil model that could study the relationships between the levels of infection of a plant by a disease, such as SSR, and its impacts on microbial communities. The nature of these relationships are intriguing and I can not rule out the possibility that they could be either beneficial or detrimental for the infested plant. If there are beneficial relationships between soil microbes and a diseased plant that helps the plant to resist or recover from diseases, this would be an excellent discovery. Further work is needed to unravel such plant root-soil microbial interactions, which can be revealed through soil lipid analysis.

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APPENDICES

APPENDIX A

Fatty acids detected by GC-FID using a Simplicity Wax capillary column[1]

FATTY ACID	FORMULA	MW[2]	FATTY ACID	FORMULA	MW
10:0	C10H20O2	172	16:1ω5	C16H30O2	254
10:0-2OH	C10H20O3	188	16:0	C16H32O2	256
11:0	C11H22O2	186	i-16:0	C16H32O2	256
12:0	C12H24O2	200	16:0-2OH	C16H32O3	272
12:0-2OH	C12H24O3	216	17:0 cyclo	C17H32O2	268
12:0-3OH	C12H24O3	216	17:1ω7	C17H32O2	268
13:0	C13H26O2	214	17:0	C17H34O2	270
14:1ω5	C14H26O2	226	i-17:0	C17H34O2	270
14:0	C14H28O2	228	18:3ω6	C18H30O2	278
14:0-2OH	C14H28O3	244	18:3ω3	C18H30O2	278
14:0-3OH	C14H28O3	244	18:2ω6c/t	C18H32O2	280
15:1ω5	C15H28O2	240	18:1ω9c/t	C18H34O2	282
15:0	C15H30O2	242	18:1ω7	C18H34O2	282
i-15:0	C15H30O2	242	18:0	C18H36O2	284
a-15:0	C15H30O2	242	19:0 cyclo	C19H36O2	296
16:1ω7	C16H30O2	254	19:0	C19H38O2	298

[1]The details of the method used for the dectection of the fatty acids and the sources of the standards of fatty acids are given in Chapter 2, section 2.3.4.

[2]MW: Molecular Weight.

APPENDIX A

(continued)

FATTY ACID	FORMULA	MW
10Me-18:0	C19H38O2	298
20:5ω3	C20H30O2	302
20:4ω6	C20H32O2	304
20:3ω6	C20H34O2	306
20:3ω3	C20H34O2	306
20:2ω6	C20H36O2	308
20:1ω9	C20H38O2	310
20:0	C20H40O2	312
21:0	C21H42O2	326
22:6ω3	C22H32O2	328
22:5ω3	C22H34O2	330
22:4ω6	C22H36O2	332
22:2ω6	C22H40O2	336
22:1ω9	C22H42O2	338
22:0	C22H44O2	340
23:0	C23H46O2	354
24:1ω9	C24H46O2	366
24:0	C24H48O2	368

APPENDIX B

Fatty acids detected by GC-FID using an Ultra-2 column[1]

FATTY ACID	FORMULA	MW[2]	ECL[3]	FATTY ACID	FORMULA	MW	ECL
9:0	C9H18O2	158	9.000	12:0-2OH	C12H24O3	216	13.177
8:0-3OH	C8H16O3	160	9.392	12:1-3OH	C12H22O3	214	13.288
i-10:0	C10H20O2	172	9.599	i-14:1	C14H26O2	226	13.381
10:0	C10H20O2	172	10.000	12:0-3OH	C12H24O3	216	13.453
9:0-3OH	C9H18O3	174	10.408	i-14:0	C14H28O2	228	13.618
i-11:0	C11H22O2	186	10.608	a-14:0	C14H28O2	228	13.708
a-11:0	C11H22O2	186	10.701	14:1ω5c	C14H26O2	226	13.901
12:0 ALDE	C12H24O	184	10.912	14:0	C14H28O2	228	14.000
11:0	C11H22O2	186	11.000	i-13:0-3OH	C13H26O3	230	14.109
10:0-2OH	C10H20O3	188	11.153	13:0-2OH	C13H26O3	230	14.192
10:0-3OH	C10H20O3	188	11.422	i-15:1 isomer 1	C15H28O2	240	14.391
12:1	C12H22O2	198	11.918	i-15:1 isomer 2	C15H28O2	240	14.440
12:0	C12H24O2	200	12.000	a-15:1	C15H28O2	240	14.530
i-11:0-3OH	C11H22O3	202	12.099	i-15:0	C15H30O2	242	14.623
11:0-2OH	C11H22O3	202	12.16	a-15:0	C15H30O2	242	14.713
11:0-3OH	C11H22O3	202	12.438	15:1ω6c	C15H28O2	240	14.850
i-13:0	C13H26O2	214	12.613	15:1ω5c	C15H28O2	240	14.903
a-13:0	C13H26O2	214	12.701	15:0	C15H30O2	242	15.001
13:1	C13H24O2	212	12.934	i-14:0-3OH	C14H28O3	244	15.119
13:0	C13H26O2	214	13.000	14:0-2OH	C14H28O3	244	15.203

[1]The details of the method used for the dectection of the fatty acids are given in Chapter 3, section 3.3.5.

[2]MW: Molecular Weight.

[3]Fatty acids with same ECL value co-eluted.

APPENDIX B

(continued)

FATTY ACID	FORMULA	MW	ECL	FATTY ACID	FORMULA	MW	ECL
i-16:1 isomer 1	C16H30O2	254	15.443	i-17:0	C17H34O2	270	16.63
i-16:1 isomer 2	C16H30O2	254	15.457	a-17:0	C17H34O2	270	16.723
i-16:1 isomer 3	C16H30O2	254	15.487	17:1ω9c	C17H32O2	268	16.763
14:0-3OH	C14H28O3	244	15.487	17:1ω8c	C17H32O2	268	16.794
16:0 N alcohol	C16H34O	242	15.55	17:1ω7c	C17H32O2	268	16.818
i-16:0	C16H32O2	256	15.627	17:0 cyclo	C17H32O2	268	16.889
a-16:0	C16H32O2	256	15.719	17:0	C17H34O2	270	17
16:1ω11c	C16H30O2	254	15.763	16:1-2OH	C16H30O3	270	17.041
16:1ω9c	C16H30O2	254	15.772	i-16:0-3OH	C16H32O3	272	17.156
16:1ω7c	C16H30O2	254	15.817	16:0-2OH	C16H32O3	272	17.233
i-15:0-2OH	C15H30O3	258	15.817	10Me-17:0	C18H36O2	284	17.409
16:1ω5c	C16H30O2	254	15.909	i-18:1	C18H34O2	282	17.459
16:0	C16H32O2	256	16	16:0-3OH	C16H32O3	272	17.517
i-15:0-3OH	C15H30O3	258	16.134	18:3ω6c(6,9,12)	C18H30O2	278	17.576
15:0-2OH	C15H30O3	258	16.223	i-18:0	C18H36O2	284	17.633
i-17:1ω9c	C17H32O2	268	16.419	18:2ω6,9c	C18H32O2	280	17.721
10Me-16:0	C17H34O2	270	16.43	a-18:0	C18H36O2	284	17.721
i/a-17:1	C17H32O2	268	16.486	18:1ω9c	C18H34O2	282	17.77
15:0-3OH	C15H30O3	258	16.507	18:1ω7c	C18H34O2	282	17.824
a-17:1ω9c	C17H32O2	268	16.521	18:1ω5c	C18H34O2	282	17.917

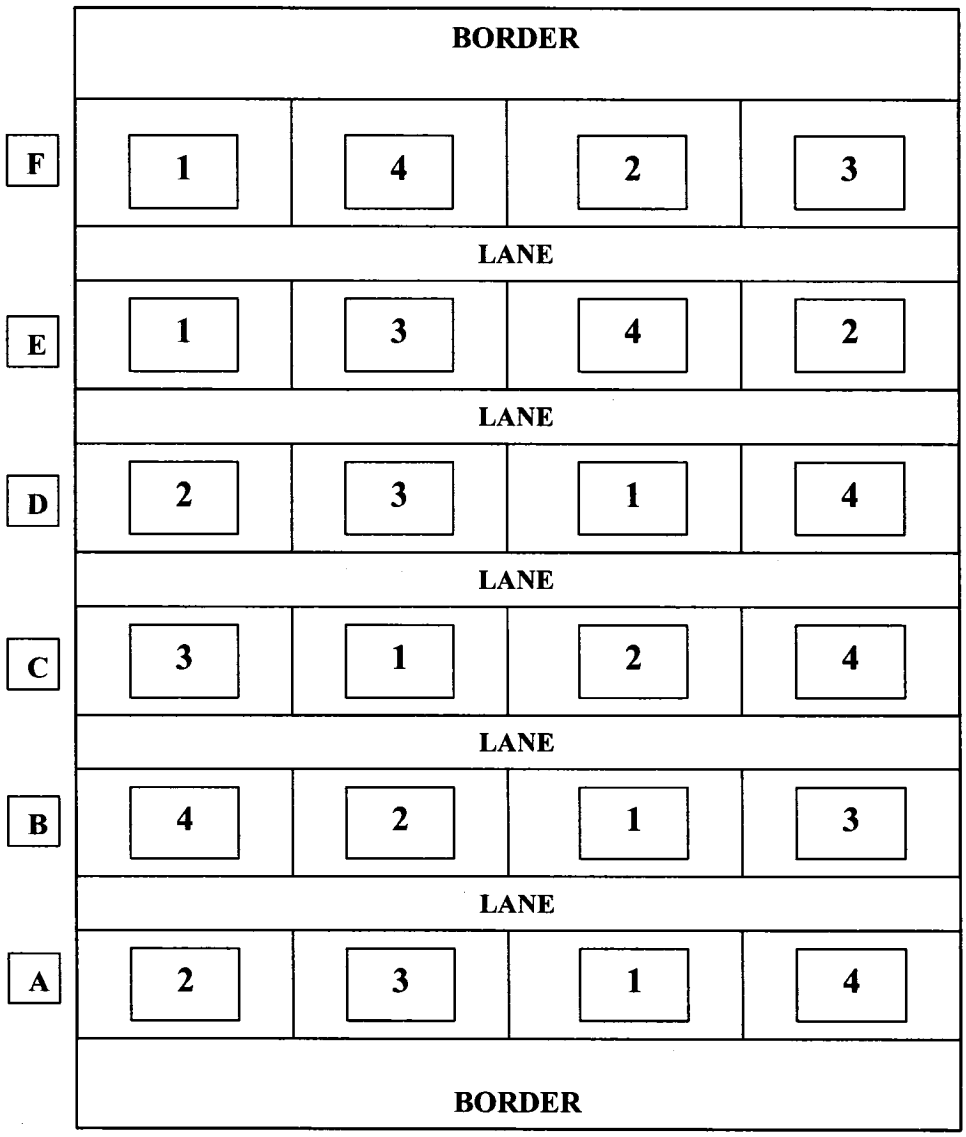
APPENDIX B

(continued)

FATTY ACID	FORMULA	MW	ECL
18:0	C18H36O2	284	18
11Me-18:1ω7c	C19H36O2	296	18.08
i-17:0-3OH	C17H34O3	286	18.162
17:0-2OH	C17H34O3	286	18.261
10Me-18:0	C19H38O2	298	18.39
i-19:1	C19H36O2	296	18.473
i-19:0	C19H38O2	298	18.632
19:1ω11c	C19H36O2	296	18.752
19:1ω9c	C19H36O2	296	18.752
19:1ω6c	C19H36O2	296	18.856
19:0 cyclo ω10c	C19H36O2	296	18.856
19:0 cyclo ω8c	C19H36O2	296	18.903
19:0	C19H38O2	298	19.001
18:1-2OH	C18H34O3	298	19.089
18:0-2OH	C18H36O3	300	19.269
10Me-19:0	C20H40O2	312	19.366
20:4ω6,9,12,15c	C20H32O2	304	19.398
18:0-3OH	C18H36O3	300	19.551
i-20:0	C20H40O2	312	19.641
20:2ω6,9c	C20H36O2	308	19.731
20:1ω9c	C20H38O2	310	19.77
20:1ω7c	C20H38O2	310	19.825
20:0	C20H40O2	312	19.999

APPENDIX C

Experimental design in the soybean fields



APPENDIX C

(continued)

Legend: Randomized complete block design with 6 blocks (A to F) and four BCA treatments within each block, for a total of 24 plots per site. The BCA treatments were *Trichoderma virens* plus arbuscular mycorrhizal fungi (1), *Trichoderma virens* (2), arbuscular mycorrhizal fungi (3), , and a control (4) that received no biocontrol agents.

APPENDIX D

List of primers and PCR programs used in the detection of arbuscular mycorrhizal fungi (*Glomus* spp.)

Primers	Sequence 5' – 3'	PCR program and reference
LR1	GCA TAT CAA TAA GCG GAG GA	15 min at 95^{0}C, followed by 35 cycles: 30s at 95^{0}C, 30s at 58^{0}C, 30s at 72^{0}C, a final polymerization of 10 min at 72^{0}C and a cooling step at 4°C until the analysis by electrophoresis on 1.2 % (w/v) agarose gel was performed (van Tuinen et al., 1998).
NDL22	TGG TCC GTG TTT CAA GAC G	
8.22	AAC TCC TCA CGC TCC ACA GA	15 min at 95^{0}C, followed by 30 cycles: 30s at 95^{0}C, 30s at 60^{0}C, 30s at 72^{0}C, a final polymerization of 10 min at 72^{0}C and a cooling step at 4°C until the analysis by electrophoresis on 1.2 % (w/v) agarose gel was performed (van Tuinen et al., 1998).
5.21	CCT TTT GAG CTC GGT CTC GTG	

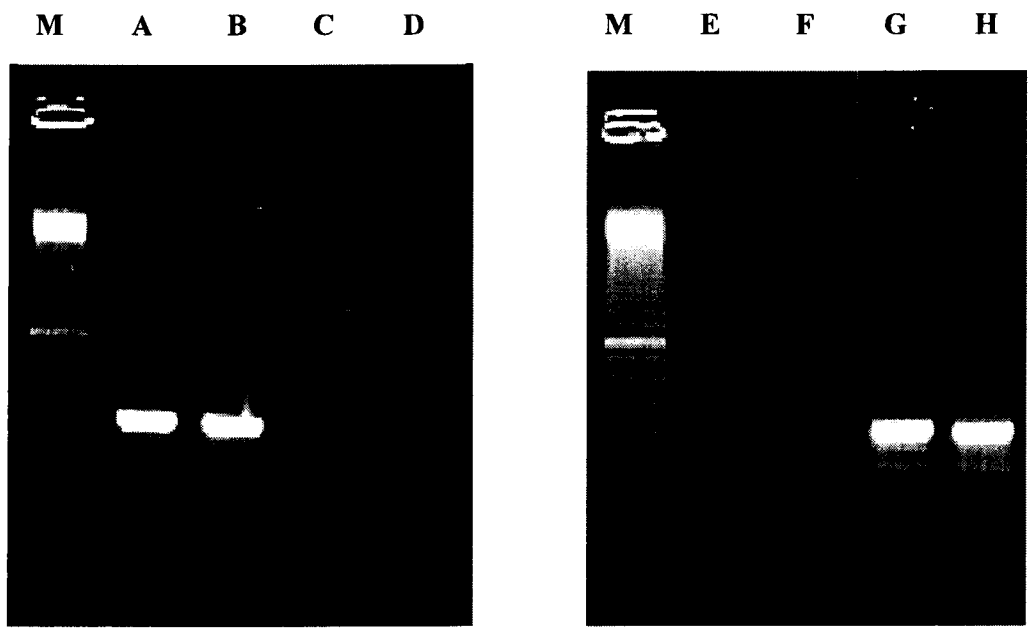
APPENDIX E

List of primers and PCR programs used in the detection of *Trichoderma* spp.

Primers	Sequence 5' – 3'	PCR program and reference
ITS1-F	CTT GGT CAT TTA GAG GAA GTA A	15 min at 95^{0}C, followed by 35 cycles: 30s at 95^{0}C, 30s at 55^{0}C, 30s at 72^{0}C; a final polymerization of 10 min at 72^{0}C and a cooling step at 4°C until the analysis by electrophoresis on 1.2 % (w/v) agarose gel was performed (Wirsel et al. 2001).
ITS4	TCC TCC GCT TAT TGA TAT GC	
A11-ITS-F1	GTT GCC TCG GCG GGG TCA CG	15 min at 95^{0}C, followed by 35 cycles: 30s at 95^{0}C, 30s at 68^{0}C, 30s at 72^{0}C, a final polymerization of 10 min at 72^{0}C and a cooling step at 4°C until the analysis by electrophoresis on 1.2 % (w/v) agarose gel was performed (White et al., 1990).
A11-ITS-R1	GCC GCG CTC CCG GTG CGA GTT	
Virens-ITS-F1	GTT GCC TCG GCG GGA TCT CT	15 min at 95^{0}C, followed by 35 cycles: 30s at 95^{0}C, 30s at 68^{0}C, 30s at 72^{0}C, a final polymerization of 10 min at 72^{0}C and a cooling step at 4°C until the analysis by electrophoresis on 1.2 % (w/v) agarose gel was performed (Hogue and Daigle, 2005).
Virens-ITS-R1	GCC GCG CTC CCG ATG CGA GTG	

APPENDIX F

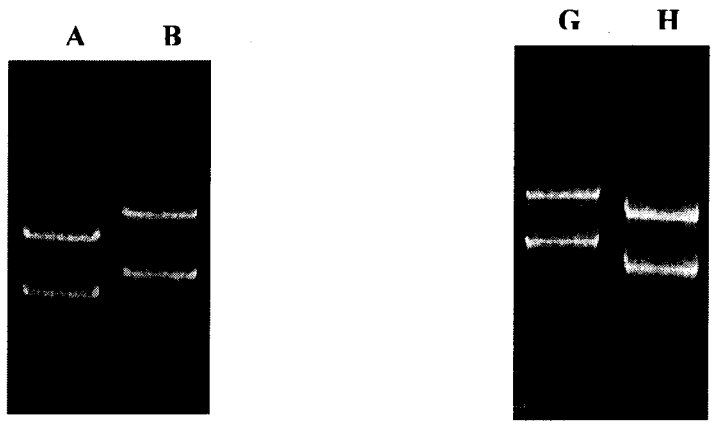
Detection of *Trichoderma virens* in soil total DNA extracts by nested PCR



APPENDIX F

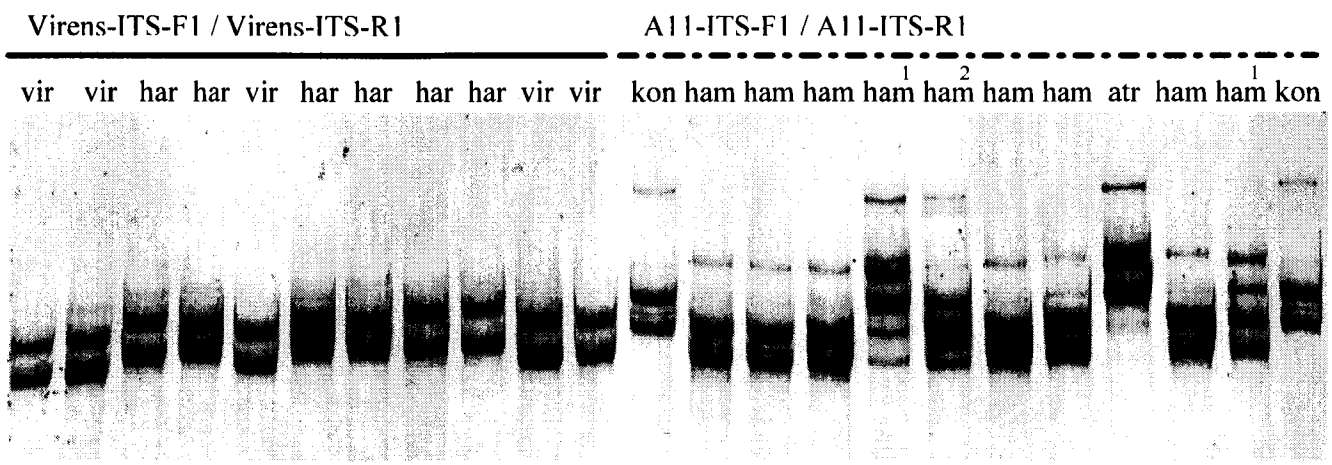
(continued)

II



APPENDIX F

(continued)



III

APPENDIX F

(continued)

Legends:

I. PCR amplication of *Trichoderma* spp. DNA with specific primers (see Appendix 5). Genomic DNAs of *Trichoderma virens* (A, C), *T. harzianum* (B, D), *T. koningii* (E, G) and *T. hamatum* (F, H) were amplified by PCR with the pair of primers Virens-ITS-F1/ Virens-ITS-R1 (A, B, E, F) or the pair of primers A11-ITS-F1 / A11-ITS-R1 (C, D, G, H). DNA ladder markers of 100 bp increment were used to identify the specific amplication product of 450 pb.

II. Single-Strand Conformational Polymorphism (SSCP) analysis of the PCR amplification products using specific primers. The amplicons of the Virens-ITS-F1/ Virens-ITS-R1 PCR (A and B) and those of the A11-ITS-F1 / A11-ITS-R1 PCR (G et H) primers (Appendix 5) were analyzed by electrophoresis using a 0.7X MDE acrylamide gel under 240 volts at 12°C during 4.5 hours in a TBE 1.5X buffer. Gels were stained with SYBR Gold 1X (Molecular Probes, Eugene, USA) for 10 min as described by supplier.

III. Detection of *Trichoderma* spp. by PCR-SSCP analysis of soil total DNA extracts from soybean fields. The DNA extracts were amplified by PCR using the primers (Appendix 5) Virens-ITS-F1 / Virens-ITS-R1 or A11-ITS-F1 / A11-ITS-R1 for the specific detection of *T. virens* (vir), T. harzianum (har), *T. koningii* (kon) and *T. hamatum* (ham). Analysis of the SSCP profiles of the amplification products leads to species identification and the detection of two species of *Trichoderma* in some samples. *T. hamatum* and *T. atroviride* (ham^1) as well as *T. hamatum* and *T. koningii* (ham^2) were detected in the same soil samples. SSCP analysis were performed electrophoresis using a 0.7X MDE acrylamide gel under 240 volts at 12°C during 4.5 hours in a TBE 1.5X buffer. Gels were stained with SYBR Gold 1X (Molecular Probes, Eugene, USA) for 10 min as described by supplier.