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LIPIDS: THEIR VALUE AS MOLECULAR MARKERS AND THEIR ROLE IN THE CARBON CYCLE OF ARBUSCULAR FUNGI

By

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ABSTRACT

LIPIDS: THEIR VALUE AS MOLECULAR MARKERS AND THEIR ROLE IN THE CARBON CYCLE OF ARBUSCULAR FUNGI

By

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Arbuscular mycorrhizae are associations between fungi and the roots of vascular plants. Part of this dissertation is devoted to analyzing the fatty acid and sterols of mycorrhizal and non-mycorrhizal *Sorghum* with the aim of identifying universal molecular markers of mycorrhizal infection. The mycorrhizal fungi contain high amounts of unusual lipids that may be used to mark their presence in infected roots. My results show that phospholipid fatty acid 16:1, as well as campesterol are molecules that can be used to consistently identify mycorrhizal infection. In addition, lipid profiles may provide insight as to which fungal species is present in the roots.

In a second experiment, the fatty acids and sterols of several isolates of root pathogenic fungi were surveyed to assess the taxonomic value of lipid profiles. My results show that the genera *Rhizoctonia* and

Pythium can be reliably identified because of their characteristic lipid composition.

Another question that I address in this work is: What is the C turnover time of mycorrhizal lipids? For this purpose, I carried out a pulse-chase experiment in which I followed the incorporation and subsequent turnover of C in mycorrhizal lipids. Mycorrhizal and non-mycorrhizal plants were subjected to a pulse exposure to $^{14}CO_2$, followed by sequential harvesting. Infected plants assimilated more ^{14}C than non-mycorrhizal plants, and had a higher absolute and percentage allocation of ^{14}C to root tissue, below ground respiration, and soil. Despite the increased fixation of C by mycorrhizal plants, mycorrhizal shoots had reduced biomass. This indicates that the C drain imposed by the fungus results in a reduced shoot growth, suggesting that the mycorrhizal fungus was acting as a parasite. The pulse-chase experiment demonstrated that the lipids of mycorrhizal roots are a dynamic pool of C with measurable turnover of ^{14}C. The C turnover time of the mycorrhizal fatty acid 16:1 $\omega 5$ was calculated at 210 h^{-1}. The lipids of non-mycorrhizal roots incorporated less radiolabel, underscoring the difference in the lipid C cycle between the arbuscular mycorrhizae and non-mycorrhizal roots. To my knowledge, this is the first measurement of the C turnover of a biomass component of the mycorrhizal fungus.

To my wife and family.

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INTRODUCTION

Lipids as Molecular Markers

Scientists are confronted with the problem of quantitatively and qualitatively isolating and describing the components of microbial communities. This process is often limited by the fact that microbes exist as part of a consortium of mutually dependent metabolic types, or as with arbuscular mycorrhizal fungi, rely on a host for their nutrition. In addition, bacteria and fungi may exist attached to surfaces, embedded in biofilms, or live inside the host's tissues. The analysis of nucleic acids has shown that soil communities contain a high diversity of microorganisms that are not yet culturable in the laboratory (Torsvik, 1990). This is an important limitation since studies of microorganisms in pure culture are likely to miss important members of the microbiota. In addition, laboratory conditions may not be able to replicate the physical and biological conditions that the microbes find in their natural habitat. It is for this reasons that microbial ecologists rely on the direct analysis of unusual molecules or nucleic acid sequences to detect different groups of microbes *in situ*.

The extraction of specific signature molecules such as DNA and lipids has helped to identify a microbial diversity that could not be observed by subculturing methods (Holben *et al.*, 1988; White, 1994, Cavigelli *et al.*, 1995). Lipids are a set of molecules that has been used to describe the microbial communities of soils (White, 1994). Lipid analysis involves the extraction, derivatization of the lipids present in the sample, followed by identification and quantification of the molecules using chromatography,. Fatty acids are a group of lipids that may be particularly valuable for differentiating microbial taxa. Fatty acids vary in the number of carbons and number of unsaturations. Moreover, fatty acids with the same number of double bonds and carbon atoms may exist in a variety of positional and geometric isomers. Hundreds of different fatty acids exist in microorganisms (Harwood and Russell, 1984). Table 1 gives some examples of fatty acids found in fungi.

Fatty acids may exist free in the cytoplasm or bound to molecules such as phospholipids, triacylglycerols, and diacylglycerols. Many different kinds of fatty acids may be present in the cells of a given species. The number of different lipids and their relative amounts is called the fatty acid profile of a sample. In some instances, fungal isolates may produce a similar diversity of fatty acid molecules but differ significantly in the relative amounts of the fatty acids. However, in other occasions fungi can be differentiated by the type of fatty acids

that they produce as well as the relative content of each molecule (Stahl and Klug, 1996). Because of these reasons, a commercially available method for analyzing fatty acid profiles has proven useful for characterizing large and diverse groups of fungal isolates (Stahl and Klug, 1996).

There are several microbial taxa that contain unusual molecules in their lipid profiles (Table 2). These peculiar lipids may serve as molecular markers for detecting specific groups of microorganisms in the environment. For instance, fatty acids such as 18:2 ω6 are relatively abundant in fungal lipids (Vestal and White, 1989; Stahl and Klug, 1996). The polyenoic fatty acids are those that have more than one carbon to carbon double bond. The concentration of polyenoic fatty acids in natural samples has been used to detect shifts in fungal abundance. It has been observed that polyenoic fatty acids increase in habitats manipulated to make fungi better competitors than other kinds of microorganisms (White *et al.*, 1980).

The analysis of lipid profiles provides quantitative and physiological information that may not be obtained by nucleic acid analysis alone (White, 1994). Phospholipids are ionic molecules composed of 1,2 diacylglycerol with a phosphodiester link that connects the glycerol to a polar head group that is usually a nitrogenous base (Figure 1). The phospholipids are cell and membrane components and

are rapidly degraded after death (White *et al.* 1979). The fatty acids that are ester-linked to phospholipids are referred to as the phospholipid fatty acids (PLFA). Since the PLFA can be extracted, characterized, and quantified, the presence of PLFA markers in environmental samples may serve as an index of the bacterial and fungal biomass in soil (Tunlid and White, 1979; White *et al.*, 1980; Petersen *et al.* 1991). An advantage of analyzing PLFA rather than the total fatty acid content of the sample is that the fatty acids bound to storage molecules are excluded. Storage fatty acids such as those bound to triacylglycerides (Figure 1) may vary in abundance depending on the nutritional status of the microflora (White, 1988). Possible problems for the study of microbial communities using PLFA analysis is that changes in physical and nutritional conditions may alter the fatty acid composition of microbes (White, 1988), and a minimal amount of 10^7 cells is required for adequate sensitivity (White, 1994).

Other lipids besides fatty acids have value as molecular markers. Sterols are characterized by a common four-ringed structure of 1,2-cyclopentano-perhydrophenanthrene (Fig. 2). Different substituents to the rings, methyl groups, and position and amount of unsaturations characterize individual sterols (Figure 2). A wide variety of sterols have been identified in fungi, while sterols are notably absent from most bacteria, insects, and nematodes (Weete, 1980). Unusual sterols have the

potential to serve as molecular markers of eukaryotes in the same way as fatty acids are used for other microbial groups. For example, ergosterol is abundant in Ascomycetes and Deuteromycetes and has been used as an indicator of fungal biomass as well as an indicator of fungal growth rate in field samples (White *et al.*, 1980; Newell *et al.*, 1988; Newell and Fallon, 1991).

Structure and Function of Lipids

Why do fungal species have a variety of fatty acids and sterols? The diversity in the structure of lipids suggests a corresponding functional diversity. Membrane components such as sterols and phospholipids are thought to modulate the transport into and out of the cell by their effect on the viscosity of the lipid bilayer (Weete, 1980). It is generally known that increasing the amount of unsaturation of the fatty acids causes changes in the melting point of the lipids to which the fatty acids are bound. Unsaturated fatty acids remain fluid at lower temperatures than saturated fatty acids. In fungi, this pattern has implications for the adaptation to cold environments. For example, the relative content of unsaturated fatty acids in some fungi increase when they are cultured under lower temperatures (Weete, 1980). With some exceptions, psychrophylic microorganisms have a higher proportion of shorter and/or monounsaturated fatty acids than mesophylic

microorganisms (Harwood and Russell, 1984). Sterols are another component of fungal membranes. It has been hypothesized that sterols have a functional role on membrane permeability because of their impact on the viscosity of lipids (Weete, 1980). Sterols have also been associated with other functions such as acting as precursors of steroid hormones that modulate fungal reproduction (Weete, 1980), and as regulatory agents of fungal enzymes (Weete, 1989).

A different group of lipids with important functional implications on fungi are the triacylglycerides and their bound fatty acids (Figure 1). Triacylglycerides are the main carbon reserves in fungi and may be oxidized for energy or used as a carbon source for the synthesis of sugars (Weete, 1980). As a general rule, bacteria are different from fungi in the sense that bacteria do not accumulate C in the form of triacylglyceride, and use poly-hydroxy butyrate instead (Weete, 1980).

The Lipid Composition of Arbuscular Fungi

Arbuscular mycorrhizal fungi (AMF) are obligate root symbionts with low host specificity and a wide geographic distribution. Six genera from three families have been recognized based on diagnostic spore morphology (Morton and Benny, 1990) and nucleic acid sequences (Simon et al., 1993). The Acaulosporaceae includes the genera *Acaulospora* and *Entrophospora*, the Glomaceae contains the genera

Glomus and *Sclerocystis*, and the Gigasporaceae includes the genera *Gigaspora* and *Scutellospora*. Traditionally, estimating mycorrhizal formation involves root clearing and staining followed by microscopical examination. This process is prone to observer differences, cannot distinguish infection from the various AMF genera, and often lacks correlation to the cost/benefit balance of the AMF-root symbiosis. Other approaches for quantifying AMF have shown limitations. The measurement of chitin and AMF associated pigments may not show a close correspondence with microscopical evaluation of infection (Schmitz et al., 1991). Fluorescein diacetate staining is not reliable for measuring infection or arbuscularization because of the natural autofluorescence of the AMF and interference of root pigmentation (Ames et al., 1982).

Specific fatty acids can be used as molecular markers of AMF at the genus level (Graham et al., 1995). Roots infected with *Glomus* contain several lipids in higher concentrations relative to non-mycorrhizal roots, and this is true across different plant species (Graham et al., 1995; Nagy et al., 1980; Nordby et al., 1981; Pacovsky, 1987; Pacovsky and Fuller, 1988; Pacovsky, 1989). Most of the previous work done on AMF fatty acids has dealt with whole-cell fatty acid (FAME) extracts. These studies show that a significant fraction of whole-cell fatty acids of several AMF genera may be comprised of the unusual fatty acid 16:1 ω5 (Graham et al., 1995). However, there is limited information about specific lipid

fractions such as the phospholipid fatty acids and sterols of mycorrhizal roots. One of my objectives is to analyze the PLFA and sterols of AMF genera that have not been studied to date.

The Carbon Cycle and Arbuscular Fungi

Plants allocate a large fraction of their fixed C to roots and the associated mycorrhizal fungi. Mycorrhizal roots may contribute amounts of C to soil that are comparable to the C inputs from the leaf litter, and a significant fraction of the C turnover in a forest could be attributed to fine roots and their associated fungi (Paul and Clark, 1996). Arbuscular fungi and root tissue are equivalent in their ecological function because both are dependent on soil resources such as P and N, while relying on direct transfers of photoassimilated C from aboveground. However, little is known about how the productivity and mineralization of C in root tissue differs from that in the fungus. Several studies using different plant-fungus combinations show that the AMF affects the allocation pattern of C to the host's tissues and soil. Table 3 shows that mycorrhizal infection has a particularly strong effect on the distribution of C below-ground by increasing the allocation to root respiration and root exudation.

The dominant cytoplasmic constituent of the arbuscular fungus is lipid (Cox and Sanders, 1974). The amount of C stored in the root lipids

of different plant species increases when they are infected with *Glomus* (Cox and Sanders, 1974; Cooper and Lösel, 1978; Nagy et al., 1980; Peng et al., 1993). Because of this, the analysis of fatty acids in infected roots has shown value for estimating fungal carbon cost and the development of colonization (Graham et al., 1995). However, there has been no attempt of measuring the residence time of C in the mycorrhizal lipids. This is relevant since elucidating the C cycle of the mycorrhizal fungus will help to understand an important part of the nutrient cycling in root-microbe symbioses.

About the Dissertation

The unifying theme of this work is the lipids of arbuscular fungi. The overall goal of this thesis is to evaluate particular lipids as molecular markers of arbuscular fungi in roots, and also provide insight into the role of lipid molecular markers in the carbon cycle of the arbuscular fungus-host association. *Sorghum* has been chosen as a common host species for all the experiments. *Sorghum* is an important crop that is known to form arbuscular mycorrhizae, and it's lipid composition is affected by mycorrhizal infection (Pacovsky, 1989).

The dissertation is divided into three experiments. In the first chapter, I studied the lipids of known fungal root pathogens of *Sorghum*. The purpose of this work is to determine if the molecules proposed as

markers of arbuscular fungi are present in other rhizosphere microbes. This is a necessary step for validating a molecular marker, since the presence of the marker in other microbes limits the value of the marker analysis. In addition to the survey, I produced a chemical taxonomy of the fungal root colonizers of *Sorghum* by subjecting the lipid profile data to multivariate statistical analysis.

The second chapter of this dissertation is a study of the fatty acids and sterols of the arbuscular fungus *in situ*. I analyzed the sterols and PLFA of fungal genera for which this information is lacking with the object of identifying possible universal markers of arbuscular fungi. In addition to the marker aspect, I analyzed the complete set of molecules using multivariate analysis. The multivariate analysis illustrates the value of the lipid profiles to distinguish the mycorrhizae of different fungal taxa from non-mycorrhizal roots.

The aim of the final chapter was to measure the turnover rate of C in the lipids of the mycorrhizal fungus. The cost of the mycorrhizal association to the plant has been investigated in only a few cases, and nobody has been able to estimate turnover rates of the fungal partner. In order to achieve this, I carried out a pulse-chase experiment in which I exposed mycorrhizal plants to $^{14}CO_2$, and then followed ^{14}C incorporation and subsequent disappearance in the fungal fatty acids. Overall, fatty acids were used to monitor the populations of arbuscular fungi in the

roots of *Sorghum*, as well as to measure the turnover time of carbon in this important soil organism.

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Table 1. Examples of several fatty acids found in fungi.

Molecule	Nomenclature *
$CH_3(CH_2)_{10}COOH$	12:0
$CH_3(CH_2)_{12}COOH$	14:0
$CH_3-CH_2CH(CH_2)_9COOH$ $\quad\quad |$ $\quad\quad CH_3$	a14:0
$CH_3-CH(CH_2)_{10}COOH$ $\quad |$ $\quad CH_3$	i14:0
$CH_3(CH_2)_3CH=CH(CH_2)_9COOH$	16:1 ω5
$CH_3(CH_2)_7CH=CH(CH_2)_5COOH$	16:1 ω9
$CH_3(CH_2)_4CH=CHCH_2CH=CH\ (CH_2)_7COOH$	18:2 ω6
$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$	18:3 ω3
$CH_3(CH_2)_7CH=CH(CH_2)_{10}COOH$	20:1 ω9
$CH_3(CH_2)_{19}COOH$	21:0

*- Nomenclature is in the form "A:B ω C" where "A" indicates the total number of carbon atoms, "B" the number of unsaturations, and "ω" antecedes the number of carbon atoms between the nearest unsaturation and the methyl end of the molecule. The suffixes "c" and "t" indicate cis and trans geometric isomers. The prefixes "i" and "a" refer to iso and anteiso methyl branching.

Table 2. Marker fatty acids and sterols in the phospholipid fraction of several groups of organisms.

Molecule:	Organism:
12:0	Eukaryotic.
3OH 14:0	Eubacteria.
15:0	Eubacteria in general, cyanobacteria, actinomycetes.
i15:0	Eubacteria in general, gram-positive bacteria[a], cyanobacteria, actinomycetes.
a15:0	Eubacteria in general, cyanobacteria, actinomycetes.
cy15:1	Clostridia.
16:0	Fungi.
i16:0	Gram-positive bacteria[a].
10Me 16:0	Actinomycetes, gram-positive bacteria[a].
16:1 ω3t	Diatoms.
16:1 ω5	Cyanobacteria, Glomus (AMF fungus)[b].
16:1 ω7	Eubacterial aerobes.
16:1 ω7t	Eubacterial aerobes.
16:1 ω9	Eubacteria in general, cyanobacteria, actinomycetes.
16:1 ω13t	Green algae.
16:3 ω6	Micro algae.
17:0	Eubacteria in general, cyanobacteria, actinomycetes.
i17:0	Eubacteria in general, cyanobacteria, actinomycetes.
a17:0	Eubacteria in general, cyanobacteria, actinomycetes.
cy17:0	Eubacterial anaerobes, gram-negative bacteria[c].
17:1 ω6	Sulfate-reducing eubacteria,, actinomycetes.
i17:1 ω7	Sulfate-reducing eubacteria, actinomycetes.
10Me 18:0	Actinomycetes[d].
18:1 ω5	Eubacteria in general, cyanobacteria, actinomycetes.
18:1ω7	Eubacterial aerobes, gram-negative bacteria[c].
18:1ω7t	Eubacteria in general, cyanobacteria, actinomycetes.
18:1 ω9	Fungi, green algae, higher plants, gram-positive bacteria[a].
18:1 ω11	Higher plants.
18:2 ω6	Eukaryotes, cyanobacteria, fungi[e]
18:3 ω3	Fungi, green algae, higher plants.

Table 2 (cont'd).

Molecule:	Organism:
18:3 ω6	Fungi.
cy19:0	Eubacterial anaerobes, gram-negative bacteria.
i19:0	Eubacteria in general, cyanobacteria, actinomycetes.
a19:0	Eubacteria in general, cyanobacteria, actinomycetes.
20:3 ω6	Protozoa.
20:4 ω6	Protozoa.
20:5	Barophyllic, psychrophyllic eubacteria.
20:5 ω3	Diatoms, higher plants.
20:5 ω5	Diatoms.
22:6	Barophyllic, psychrophyllic eubacteria.
26:0	Higher plants.
Polyenoic fatty acids.	Eukaryotes.
Branched chain fatty acids.	Gram-positive bacteria[e].
Monoenoic and cyclopropane.	Gram-negative bacteria[e].
3OH fatty acids.	
Ergosterol	Fungi[f].

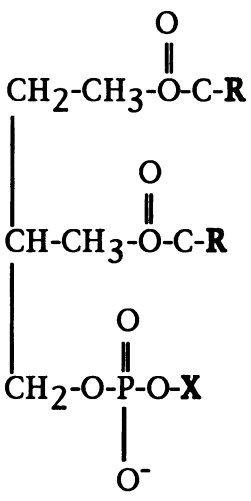
From Vestal and White (1989). Other references are indicated by a superscript number and are listed below.

a- O'Leary, W., M., and Wilkinson, S. G. (1988).
b- Jabaji-Hare, S. (1988).
c- Wilkinson, S. G. (1988).
d- Kroppenstedt, R. M. (1985).
e- Fedrele, T. W. (1986).
f- Newell *et al.*, (1988)

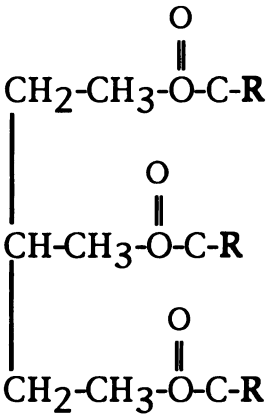
Table 3. Examples of studies showing the effect of mycorrhizae in the C allocation pattern of the host.

Fungal species	Plant species	^{14}C fixation rate. (mg C/g shoot/h).		Belowground C partitioning (% allocation)									
		% allocated belowground.		C lost from roots.[1]		Root respiration.		Root exudates.		VAM fungal respiration		VAM fungus biomass	
		M	NM	M	NM	M	NM	M	NM	M	NM		
Glomus fasciculatus	*Cucumis sativus*	1.3	nd	43.0	nd	33.2	nd	30.1	nd	2.2	2.5	16.0	4.0
Glomus mosseae	*Vicia faba*	8.2	7.9	58.0	52.0	34.5	35.5	22	28.0	4.5	0.5	3.0	1.0
Glomus fasciculatus	6 week old *Glycine max*	3.2	2.6	42.6	27.4	30.1	16.5	5.2	6.6	1.8	1.8	13.7	2.7
Glomus fasciculatus	9 week old *Glycine max*	2.2	2.1	34.8	33.3	22.3	19.6	6.5	10.4	1.3	1.6	4.6	2.8

All values are expressed as the percent of total fixed C except the fixation rate. M= mycorrhizal, NM= non-mycorrhizal, nd= not determined.
1- As exudates + root respiration + extraradical allocation to mycelium. References: *Cucumis sativus-Glomus fasciculatus:* Jakobsen and Rosendahl (1990). *Glomus mosseae-Vicia faba:* Paul and Kucey (1981). *Glomus fasciculatus-Vicia faba:* Harris et al. (1985).



Phospholipid

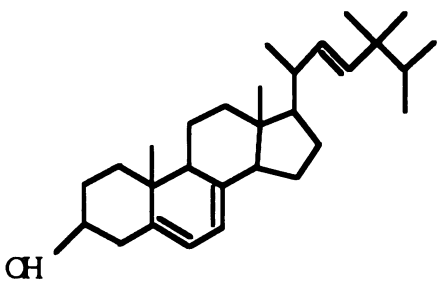


Triacylglyceride

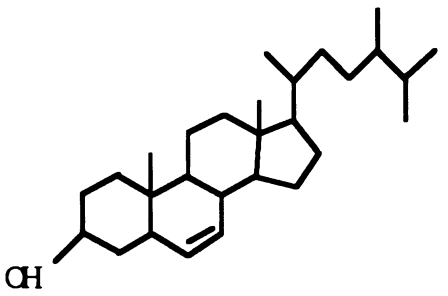
R= aliphatic chains of the fatty acids

X= polar head group.

Figure 1. General structure of a phospholipid and a triacylglycerol.

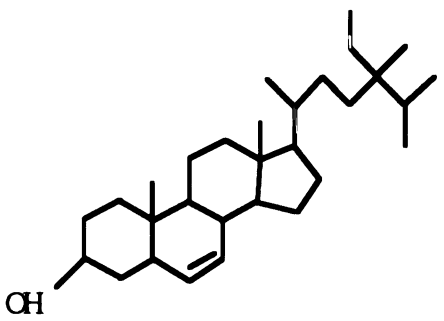


Ergosterol

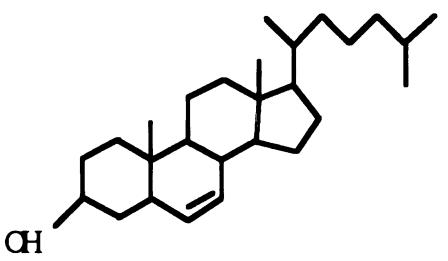


24-methylcholesterol

(Campesterol)



Sitosterol



Cholesterol

Figure 2. Examples of different sterols.

Chapter 1

THE CHEMICAL TAXONOMY OF THREE LIPID FRACTIONS FROM ROOT-ASSOCIATED FUNGI OF *SORGHUM*

Abstract

The whole-cell fatty acid (FAME), phospholipid fatty acid (PLFA), and sterol profiles of 16 species of root inhabiting fungi were analyzed by gas chromatography. The object of this study was to evaluate the usefulness of lipid profiles for distinguishing among the fungal taxa. Hierarchical cluster analysis was used to illustrate relationships between the lipid profiles; principal component analysis was used to determine which lipids defined the grouping patterns.

Twenty different sterol molecules were recovered from the set of fungi. Ergosterol, lanosterol, and a c28:2 sterol were the most abundant sterols. The relatively high amounts of ergost-7-enol and low percentage of ergosterol distinguish the genus *Rhizoctonia* from the rest of the taxa. The lack of sterols is characteristic of the genus *Pythium*.

A total of 25 different phospholipid fatty acids were recovered from the set of fungal isolates. 18:1 ω9, 18:2 ω6, and 16:0 were the most common PLFA and were present in all of the fungi. The PLFA profiles of

the genera *Rhizoctonia* and *Pythium* are unique and serve to reliably identify these taxa. The genus *Pythium* had the highest diversity of PLFA and can be distinguished from the rest of the taxa because of the relatively high amounts of 18:1 ω9 and 16:0, combined with the relatively low content of 18:2 ω6. In addition, the Basidiomycete anamorphs *Rhizoctonia* and *Sclerotium* are recognizable because of the high percentage of 18:2 ω6, and a relatively low amount of 18:1 ω9.

Thirty four different FAME were recovered from all the fungi. In a similar fashion to the PLFA, 18:1 ω9, 18:2 ω6, and 16:0 were the most abundant fatty acids in the FAME. In general, the taxonomic information in the PLFA parallels that of the whole cell fatty acids (FAME). As with the PLFA, the FAME profiles of *Rhizoctonia* and *Pythium* are useful for the identification these genera. The Basidiomycete genera *Rhizoctonia* and *Sclerotium* can similarly be identified because of their high content of FAME 18:2 ω6, and low amount of FAME 18:1 ω9. *Pythium* is different from the rest of the fungal pathogens in the relatively high amounts of FAME 14:0 and 16:0.

The Ascomycete anamorphs included in this study showed a relatively high diversity in their lipid composition and for this reason, lipid profiling showed little value in identifying the ascomycetous genera. For example, the genera *Fusarium*, *Bipolaris*, and *Phoma* are not recognizable by their lipid profiles.

This is the first report of the lipid profiles of *Bipolaris*, *Nigrospora*, and *Periconia*. This research shows the value of lipids for classifying fungal isolates that cause disease in the roots of *Sorghum*.

Introduction

Fungal identification has traditionally been based on morphological and reproductive characters. Some fungal taxa do not sporulate in laboratory conditions and as a result important morphological features for their classification are not available. Chemical profiling of cellular constituents using chromatography is a widely used tool for determining the chemotaxonomy of microbes (Lechevalier and Lechevalier, 1988). Fungal mycelium averages 17% by weight of lipid material, ranging from less than 1% to 55% depending on the species, development stage, and growth conditions (Murray, 1953; Weete, 1980). Specific lipids such as sterols, whole-cell fatty acids (FAME) and ester-linked phospholipid fatty acids (PLFA) have been used to characterize and identify eukaryotic and prokaryotic microorganisms. More than 500 fatty acids have been found in plants and microbes (Harwood and Russell, 1984) and some are unique to specific taxa (Kroppenstedt, 1985; Fedrele, 1986; O'Leary and Wilkinson, 1988; Wilkinson, 1988).

For some fungal groups such as the Ascomycetes and Basidiomycetes, specific lipids lack value as molecular markers because

most taxa share the same diversity of lipid molecules (Lechevalier and Lechevalier, 1988). However, multivariate statistical treatment of fungal lipid profiles may be used to estimate diagnostic differences between isolates by small variations in the relative abundance of the molecules (Blömquist et al. 1992). Such FAME profiles are obtained readily using a commercially available protocol (Microbial Identification System (MIS), Microbial ID, Newark, DE, U.S.A.). The MIS system may be used to identify isolates by comparison of their fatty acid content to a database. The FAMEs are produced by the saponification, derivatization, and extraction of the whole cellular material of the isolates. Because of this, the fatty acids present in phospholipids, triacylglycerides and diacylglycerides, and free fatty acids are part of the FAME profile. This method has proven useful for characterizing fungal isolates at the species and more inclusive taxonomic levels (Stahl and Klug, 1996).

Recent lipid methods for the chemical taxonomy of fungi focus on the fatty acids and sterols obtained from chromatographic analysis of single extracts (Müller and Hallaksela, 1994; Müller *et al.*, 1994; Müller *et al.*, 1995). The results of these experiments suggest that sterols, in addition to fatty acids, may offer taxonomic information that can be used to characterize fungal taxa. The fatty acid content of the phospholipids of fungi may differ from the fatty acid composition of the rest of the cell's lipids. For example, Jabaji-Hare *et al.*, (1984) showed that in mycorrhizal fungi, the relative amount of 18:2 fatty acid is

higher in the phospholipids when compared to other lipid fractions such as the glycolipids and neutral lipids. It is for this reason that studies are needed to investigate if the PLFA and the FAME vary in their value as taxonomic characters

The objectives of this work were: i) To survey the sterol, FAME, and PLFA content of fungi that infect the roots of *Sorghum*. ii) Compare the diagnostic value provided by each lipid fraction at the genus level. iii) Test if the lipid profiles are stable across different developmental stages such as mycelia, conidia, and sclerotia.

Materials and Methods

The fungal species included in the experiment are potential parasites associated with the roots of *Sorghum* (Farr *et al.*, 1989). Fourteen species belonging to nine genera were analyzed (Table 1). *Cephalosporium gramineum* was supplied by D. Fulbright at the Department of Botany and Plant Pathology, Michigan State University. *Periconia macrospinosa* was isolated from soil at the Kellogg Biological Station, Michigan, by G. Thorn, Department of Crop and Soil Sciences, Michigan State University. The rest of the cultures were supplied by G. Adams, Department of Botany and Plant Pathology, Michigan State University. All the cultures were maintained on Potato Dextrose Agar (DIFCO).

Broth Cultures

Environmental conditions such as the C:N ratio, pH, temperature, oxygen concentration, and time of incubation may affect the lipid profiles of fungi (Weete, 1980; Müller et al. 1994). For this reason, the growth conditions must be carefully controlled to achieve reproducibility. I used Malt Extract Broth (DIFCO) as a uniform growth medium. For each culture, 100 ml were placed in 250 ml glass flasks, capped with aluminum foil, and autoclaved at 117 ^{o}C for 15 min. To inoculate the media, mycelium (and spores or conidia when available) were obtained from actively growing PDA cultures and mixed with the broth. The growth period was 7 days. Slower growing fungi such as *C. gramineum, N. sphaerica*, and *P. macrospinosa* were grown for 30, 10, and 10 days respectively. The fungi were grown at 25^{o}C under dark, stationary conditions.

Harvests

Fungal mycelium was separated from the broth by filtration through a Buchner funnel with filter paper (25 µm pore size, Whatman # 114). The mycelium was rinsed with distilled water and blot-dried in gel-blotting paper (Schleicher & Schuell, Keene, NH, U.S.A.). The biomass was then placed on a glass slide, finely cut, mixed, and freeze-dried. Cultures from three different flasks were sampled for FAME and PLFA analysis. Two different flasks were analyzed for sterols. Whenever

possible, specialized structures were analyzed separately from the mycelium. The sclerotia of *S. rolfsii* were removed from 15 day old cultures using a spatula. Conidia of *F. moniliforme* and *Phoma sp.* were obtained from 7 and 10 day old cultures respectively. These formed a sediment layer at the bottom of the flask. The mycelium and growth medium were decanted from the flasks, then the conidial sediments were resuspended with 25 ml of distilled water and centrifuged at 2400 rpm for 2 min. The supernatants were removed, and the conidial pellets saved.

Appendix A is a flowchart that represents the sampling and analysis scheme for the fungal lipids.

Analysis of Whole-Cell Fatty Acids (FAME)

The FAME content of the samples (0.1 g blot-dry weight) was determined using the protocol developed for bacterial fatty acid analysis using the MIDI MIS (MIDI. Newark, Delaware, U.S.A.). The samples were put in 13x100 mm test tubes with Teflon-lined screw caps. One ml of 150 g NaOH/1L 50% MeOH in H_2O was added to each tube, vortexed briefly and saponified at 100 ^{o}C for 30 min. After the 30 minutes the tubes were cooled to room temperature in a water bath. Two milliliters of 54% 6N HCL in MeOH were added at this point. The material was then vortexed briefly, methylated at 80 ^{o}C for 10 min. in a water bath and immediately cooled to room temperature. The fatty acid methyl

esters were extracted by adding 1.25 ml of 1:1 Hexane:Methyl-tert butyl ether and rotating the tubes for 10 min. The tubes were left to stand for a clear separation of the phases and the aqueous phase (bottom) was removed with a Pasteur pipette and discarded. The organic extract remaining in the tube was washed by adding 3.0 ml of 1.2% NaOH in H_2O to each tube, and turning end over end for five minutes. The organic phase was removed, transferred to a crimp-top GC vial and stored at -20°C until analysis. The derivatized extracts were analyzed by gas-liquid chromatography using a non-polar fused silica capillary column (30 m x 0.25 mm) and a flame ionization detector. The time of the run was 38 minutes, which allowed detecting fatty acids up to 28 carbons long (170 °C to 300 °C oven temperature; 250 °C injector temperature ; 300 °C detector temperature). The flow rate of the carrier gasses N, H, and air were 30, 30, and 400 ml $min.^{-1}$ respectively. The GC setup was developed by Microbial I. D. (Newark, DE). The system was coupled to a computer programmed to identify fatty acids based on retention times relative to a known standard mix (that was run every ten samples) and to quantify them relative to other fatty acids in each sample according to the peak width and area data.

Analysis of Phospholipid Fatty Acids (PLFA) and Sterols

Total lipids were extracted from 0.02 g of dry mycelium by use of a Bligh & Dyer (1959) chloroform-methanol extraction modified to

incorporate a phosphate buffer. Fungal material was extracted at room temperature in chloroform/methanol/potassium phosphate buffer (1:2:0.8 by volume; 50 μm , pH 7.4) for at least 2 h. Then, 0.29 volumes of distilled water and chloroform were added resulting in a separation of the aqueous and organic phases. The organic phase was dried by rotary evaporation at 37°C and subsequently fractionated into neutral-, glyco-, and polar lipid using silicic acid (100 mesh) column chromatography as detailed by Guckert *et al.* (1985). This process involves the loading of the lipid extract to a capillary column, followed by the sequential elution of the lipids with solvents of increasing polarity (chloroform, acetone, methanol). The neutral lipids are found in the chloroform, while the polar lipids are obtained by the elution with methanol.

The phospholipids were obtained from the polar lipid fraction. The polar lipids were subjected to a mild alkaline methanolic transesterification (Guckert *et al.*, 1985), producing the phospholipid fatty acid methyl esters (PLFA). The PLFA were then separated, quantified, and identified by capillary column gas chromatography (FID detector, Hewlett-Packard HP-1 50m by 0.2 mm inner diameter non-polar methyl silicone column) using the conditions described by Ringelberg *et al.* (1989). Quantification was based on comparison of peak areas to an internal injection standard (19:0) assuming equi-molar responses within the range of chain lengths between 12-24 C. The structures were verified using a mass selective detector (Hewlett Packard 5791). The extraction,

fractionation, derivatization, and analysis of PLFA and sterols was carried out at the Center for Environmental Biotechnology of the University of Tennessee, Knoxville.

The sterols were recovered from the neutral lipid fraction after saponification in 5% KOH in methanol:water (80:20, v:v). The sterols were derivatized using N, O *bis*(Trimethylsilyl)trifluoroacetamide (BTSFA) that formed trimethyl silyl ethers. The trimethyl silyl ethers of the sterols were then separated, quantified and identified using gas chromatography (as for the PLFA). Sterol and PLFA identification was based on co-injection with standards and relative retention time measurements (Nes and Parish, 1989). All structures were verified using GC-mass spectrometry at an electron energy of 70 eV with the same columns and conditions described above.

Three ml of the growth medium were freeze-dried and analyzed using the same procedure as for the fungal biomass. The amount of FAME, PLFA, and Sterols in the growth medium was lower than the detection limit of the instruments. For this reason, the results from this experiment are assumed to represent the biosynthesis by the isolates.

Fatty acid terminology utilizes "A:B ωC" where "A" indicates the total number of carbon atoms, "B" the number of unsaturations, and "ω" antecedes the number of carbon atoms between the closest unsaturation and the methyl end of the molecule. The suffixes "c" and "t" indicate

cis and trans geometric isomers. The prefixes "i" and "a" refer to iso and anteiso methyl branching.

Statistical Analysis

The lipid profiles were evaluated using Systat version 5.2.1 (SPSS Inc., Chicago, IL, U.S.A.). Principal components analysis (PCA) with the covariance matrix was used to compare the profiles of the isolates. All variables are represented on each principal component. The loading values give insight into which molecules are responsible for the distribution of the taxa in the PCA diagrams. I subjected the data to hierarchical cluster analysis (single linkage method) and constructed dendrograms for the same sets of data used for the PCA. The cluster analysis was used to indicate which isolates have the most similar lipid profiles. Molecules that made up less than 0.25% of the total profile were near the detection limit and were excluded from the analyses.

Results and Discussion

Sterols

A total of 20 sterols were detected and used for the multivariate analysis. Table 2 shows the number and relative amounts of the sterol molecules recovered from each isolate, while Table 3 lists the results of the mass spectral analysis used to identify each molecule. The separate extraction and chromatography allowed the recovery of a higher

diversity of molecules than did previous studies of fungal sterols (Müller and Hallaksela, 1994; Müller *et al.*, 1994; Müller *et al.*, 1995). The most common sterols in the fungal root pathogens of *Sorghum* were ergosterol, lanosterol and a C28:2 sterol (Table 2). However, these three molecules were not universally distributed since some fungal taxa lacked one or more of them.

The absence of sterols may be used to distinguish *Pythium* from other filamentous fungi. Other studies have also shown the absence of sterols in Pythiales (Mc Corkindale, 1969; Weete, 1980). Furthermore, it has been shown that members of the family Pythiaceae may require sterol amendments in order for sporulation to occur in pure cultures (Weete, 1980). The sterol profiles were also useful to differentiate the genus *Rhizoctonia* from the rest of the genera included in the experiment. Component 1, which separates the *Rhizoctonia* species from the rest of the extracts (Figure 1), is mainly accounted for by ergost-7-enol and ergosterol (Table 4). Table 2 shows that ergost-7-enol is enriched in the extracts of the *Rhizoctonia* species, and may partially explain the diagnostic value of the sterols (Figs. 1 and 2). In addition, I found low relative amounts of ergosterol in the extracts of *Rhizoctonia* (Table 2). This is relevant because ergosterol is usually a major sterol of the Basidiomycetes, and has been used to mark the presence of fungi in field samples (Weete, 1980; Harwood and Russell, 1984; Newell and Arsuffi, 1988).

Phospholipid Fatty Acids (PLFA)

A total of 25 PLFA were used for the multivariate analysis (Table 5). The major PLFAs were 18:1 ω9, 18:2 ω6, and 16:0. Combined, these three molecules made up more than 90 % of the PLFA of most of the isolates analyzed (Table 5). In this study, 18:1 ω9, 18:2 ω6, and 16:0 were found in each one of the isolates (Table 5), and others have shown that these three molecules are common fatty acids of fungi and other eukaryotes (Weete, 1980; Müller *et al.* 1994; Stahl and Klug, 1996).

The *Pythium* isolates have the highest diversity of PLFA of the material analyzed. A total of 20 fatty acids in proportions higher than 0.25% were produced by *P. ultimum* alone (Table 5). Other studies have found that dikaryotic fungi such as Ascomycetes and Basidiomycetes produce a reduced variety of fatty acids relative to Oomycetes such as *Pythium* (Stahl and Klug, 1996). Among the root pathogens of *Sorghum*, *Pythium* is exceptional because of the production of several 20 C polyunsaturates and 18:3. This set of molecules have also been found in other members of the Pythiales (Weete, 1974; Stahl and Klug, 1996). The two species of *Pythium* formed a distinct cluster, which suggests that this genus has a diagnostic PLFA profile (Figure 1; Figure 3). Components one and three provide the resolution to separate the *Pythium* isolates from the rest of the extracts. The values for component one are primarily determined by the relative amounts of 18:2 ω6, while the distribution of component three is largely determined by the values of 18:1 ω9, and

16:0 (Table 4). This suggests that it is the relatively high percent of 18:1 ω9 and 16:0 and the low relative concentration of 18:2 ω6 that distinguishes *Pythium* from the rest of the isolates. Sterols as well as the amount of unsaturated fatty acids in the phospholipids are two features that have been associated with the permeability of cellular membranes.

It is remarkable that *Pythium* lacks sterols and has the highest diversity of polyunsaturated PLFA. I hypothesize that this distinctive composition of cellular membrane components has implications for the functioning of this taxon. Besides *Pythium*, *Rhizoctonia* has a characteristic PLFA profile at the genus level (Figure 3). The mycelial and sclerotial extracts of *S. rolfsii* formed a distinct cluster, suggesting that there is a species-specific PLFA signature for this isolate (Figure 3). The principal components diagram and the loading values of the principal components analysis show that it is the relatively high amounts of 18:2 ω6 and the relatively low amounts of 18:1 ω9 that helps distinguish *S. rolfsii* and the *Rhizoctonia* isolates from the rest of the taxa (Table 4; Figure 1).

Whole-Cell Fatty Acids (FAME)

A total of 34 different FAME were identified and included in the analyses (Table 6). As with the phospholipid fatty acids, the major FAME were 18:1 ω9, 18:2 ω6, and 16:0. These three molecules have been found to be common FAMEs in fungi (Stahl and Klug, 1996), and our

data shows that they make up more than 90% of the FAME content of each of the root pathogens of *Sorghum*. Our results suggest that the fatty acid composition of membrane-associated lipids (PLFA) is similar to that of the total lipid content of the fungal cells (FAME). This is also evident from the remarkably similar distribution of the fungal taxa in the principal component diagrams of the PLFA profiles and the FAME profiles (Figure 1).

The cluster diagram shows that FAMEs are useful for the identification of the genera *Pythium* and *Rhizoctonia* (Fig 4). The FAME profile of the genus *Pythium* is recognizable from the rest of the fungi (Figure 1; Figure 4). The *Pythium* species have the highest molar percentage of 16:0 and 14:0 (Table 6). These two molecules are important in determining the distribution of component three (Table 4), and distinguish the *Pythium* isolates from the rest of the genera (Figure 1). Arachidonic acid (20:4) and 18:3 FAMEs are exclusively found in the Pythium isolates (Table 6), and this supports the results found by others (Stahl and Klug, 1996). The genus *Rhizoctonia* has a diagnostic FAME signature (Figure 4). This is a similar pattern as that obtained with the sterol and PLFA profiles (Figs. 1 and 2). The mycelial and sclerotial extracts of *S. rolfsii* formed a distinct cluster (Figure 4), which suggests that the FAME fingerprint for this species is recognizable regardless of the development stage of the organism. As with the PLFA profiles, the FAMEs of the Basidiomycete anamorphs (*Rhizoctonia* and *Sclerotium*) are

distinguishable from the rest of the isolates because of the relatively low amounts of 18:1 ω9, combined with the relatively high amounts of 18:2 ω6 (Figure 1; Tables 4 and 6).

Specialized Structures

Lanosterol is the precursor of ergosterol in fungi (Weete, 1973). In the mycelia and sclerotia of *Fusarium moniliforme* and *Phoma* sp. the production of ergosterol predominates, while the conidia of these two genera are enriched in lanosterol (Table 2). This results suggest less conversion of precursor (lanosterol) to product (ergosterol) in the conidia, relative to the mycelium of each species. Other studies have shown that sterol accumulation may be interrupted once sporulation begins (Stevens and Jones, 1992; Weete, 1981). Because of the relatively high amounts of lanosterol, the sterol profiles of the conidial extracts of *Fusarium* and *Phoma* are more similar to each other than to the mycelia of their respective species (Fig 2). As with the sterols, the relative proportions of fatty acids vary among mycelia of different ages (Weete, 1980; Stevens and Jones, 1992). In this study, 18:1 fatty acids decreased while 18:2 ω6 from PLFA and FAME were enriched in the conidia and sclerotia relative to the mycelia (Table 5; Table 6). This resulted in the lack of similarity of the conidia to the mycelia of the same species (Figure 3; Figure 4).

Lipids and Environmental Samples

Polyenoic fatty acids such as 18:2 ω6 have been shown to increase in microbial communities that favor the proliferation of fungi (White *et al.*, 1980). Our results validate the use of this molecule as a general molecular marker of fungi. Fatty acid 18:2 ω6 was a dominant molecule in the fungal root colonizers of *Sorghum,* accounting for up to 75.9 % of the PLFA, and up to 75.2 % of the FAME (Tables 5 and 6). One potential problem with the use of this molecule as a marker of fungi in soils, is the possible background from plant roots, which may contain significant amounts of this molecule (see chapter 2).

Ergosterol has been used as an indicator of fungi in soil (Grant *et al.*, 1986) and in plant material (Newell *et al.*, 1988). The concentration of this molecule varies between species and is absent from *Pythium* and *Bipolaris* (Table 2). Because of this, any conclusions drawn from the concentration of ergosterol in environmental samples should consider that it is not a universal marker of fungi.

Campesterol and 16:1 ω5 have been proposed as molecular markers of arbuscular mycorrhizae (Pacovsky, 1989; Schmitz *et al.*, 1991). Our data shows that these molecules are absent from non-mycorrhizal fungi that colonize roots and the rhizosphere. This underscores the unique nature of these lipids and supports their use as markers of arbuscular mycorrhizae (see chapter 2).

Conclusions

Stahl and Klug (1996) observed that in general, the FAME profiles of filamentous fungi agree with their taxonomic relationships. They found that in general, Ascomycetes and Basidiomycetes can be distinguished by their FAME composition. The results of our study partially agree with those of Stahl and Klug (1996). For instance, the sterol and FAME profiles of the Basidiomycete anamorphs *Rhizoctonia* and *S. rolfsii* show a close chemical taxonomy when compared to the rest of the taxa. However, the Ascomycete anamorphs included in this experiment showed a relatively high diversity in their lipid profiles and lacked cohesiveness for this reason. For example, the isolates of *Fusarium, Bipolaris,* and *Phoma* have relatively wide differences in their FAME, PLFA, and sterol profiles and lack distinctive lipid signatures at the genus level and higher taxonomic level (Figs. 3 and 4).

I conclude that lipid profiles provide sensible taxonomic information for the classification of fungi at the level of species and genus. To my knowledge, this study provides the first account of the separate use of sterol profiles for the production of the chemical taxonomy of microbes. The taxonomies of the sterol, FAME, and PLFA profiles show some similar trends. For example, the sterol, FAME and PLFA profiles all have specific fingerprints for the genera *Pythium* and *Rhizoctonia* (Figs. 3 and 4). Future work should be aimed at enlarging the available data base of fungal lipids so that broader comparisons can

be carried out. In addition, future studies should test if the presence of fungal parasites in roots can be detected by changes in the lipid composition of infected plant material.

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Table 1. Root-pathogenic fungi analyzed for FAME, PLFA, and sterols.

Bipolaris sorokiniana (Sacc.) Shoem.
Bipolaris victoriae (Meehan & Murphy) Shoem.
Cephalosporium gramineum Nisikado & Ikata
Fusarium equiseti (Corda) Sacc.
Fusarium graminearum Schwabe
Fusarium moniliforme Sheld.
Nigrospora sphaerica Lefebvre & Johnson
Periconia macrospinosa Lefebvre & Johnson
Phoma exocarpina Peck
Phoma sp.
Pythium graminicola Subramanian
Pythium ultimum Trow
Rhizoctonia oryzae Ryker & Gooch
Rhizoctonia solani Kühn
Rhizoctonia zeae Voorhees
Sclerotium rolfsi Sacc.

Table 2. Mean percent of total sterols recovered from each of the root pathogenic fungi.

Sterol:	C27:6	C28:4a	brassica sterol	C28:3a	C27:4	C28:4	ergo-sterol	C28:2a	stella-sterol	C28:3b/ C28:4c
Bipolaris sorokiniana	0	1.6	0	2.4	1.0	0	12.2	0	1.8	0
Bipolaris victoriae	0	0	0	0	0	0	0	0	0	0
Cephalosporium gramineum	11.3	2.2	0	22.4	8.2	0	47.0	0	0	0
Fusarium equiseti	2.5	2.2	2.3	2.3	0.7	0	52.2	0	0	0
Fusarium graminearum	0	0	0	0	0	0	57.0	0	0	0
Fusarium moniliforme	0	6.3	12.0	3.9	1.1	0	27.2	0	0	0
Fusarium moniliforme conidia.	0	0	0	0	0	0	0	0	0	0
Nigrospora sphaerica	5.3	5.4	0	0	0	0	49.3	0	0	0

Table 2 (cont'd).

Sterol:	C27:6	C28:4a	brassica sterol	C28:3a	C27:4	C28:4	ergo-sterol	C28:2a	stella-sterol	C28:3b/ C28:4c
Periconia macrospinosa	0	4.6	0	3.8	4.1	0	68.0	0	0	0
Phoma exocarpina	0	0	0	0	0	0	24.0	0	0	0
Phoma sp	0.5	0.4	0	14.4	1.1	1.0	27. 5	0	0	2. 4
Phoma sp conidia	0	0	0	2.8	0	0	5.8	0	0	0
Rhizoctonia oryzae	0	0	0	0	0	0	0.3	0	8.6	0
Rhizoctonia solani	0	0	0	0	0	0	0.2	0.1	9.5	0
Rhizoctonia zeae	0	0	0	0	0	0	0.2	0	18.6	0
Sclerotium rolfsii	0	1.7	0	0	1.0	0	11.4	1.5	22.9	0
Sclerotium rolfsii sclerotia	0	3.2	2.7	0	1.4	0	45.8	0	15.0	0

Table 2 (cont'd).

Sterol:	C28:2b	28:1	C28:3c	28:2 c	C28:2d	ergost-7-enol	C29:3	lanosterol	C29:2	C31:2	C30:2
Bipolaris sorokiniana	22.7	0	0	0	49.0	0	0	0.8	5.1	1.9	1.4
Bipolaris victoriae	0	0	0	0	100	0	0	0	0	0	0
Cephalosporium gramineum	2.7	0	0	0	4.9	0	0	0	1.4	0	0
Fusarium equiseti	1.7	0	0	0	11.4	0.8	0	4.0	6.6	11.7	1.8
Fusarium graminearum	0	0	0	0	3.3	0	0	17.9	0	21.9	0
Fusarium moniliforme	1.5	0	0	0	10.3	0	0	8.8	3.2	25.7	0
Fusarium moniliforme conidia.	0	0	3.4	0	0	0	0	88.6	0	8.0	0
Nigrospora sphaerica	0	6.6	0	0	20.3	0	1. 6	5. 4	0	6.2	0

Table 2 (cont'd).

Sterol:	C28:2b	28:1	C28:3c	28:2 c	C28:2d	ergost-7-enol	C29:3	lanosterol	C29:2	C31:2	C30:2
Periconia macrospinosa	3.1	0	0	0	13.0	0	0	0	0	3.7	0
Phoma exocarpina	0	0	0	0	18.2	0	0	19.5	0	38.4	0
Phoma sp	4.5	0	1.5	0	42.8	0	0	0.7	1.3	1.4	0.8
Phoma sp conidia	1.5	0	0	0	10.7	0	0	54.0	0	23.9	1.3
Rhizoctonia oryzae	0.2	0.2	0	0.3	7.4	79.7	0	0.6	0.2	2.6	0
Rhizoctonia solani	0	0	0	0	9.7	77.3	0	0.5	0.1	2.6	0
Rhizoctonia zeae	0.1	0.1	0	0.3	3.5	76.2	0	0.3	0.3	0.4	0
Sclerotium rolfsii	0	0	0	0	3.4	53.4	0	4.2	0	0.5	0
Sclerotium rolfsii sclerotia	0.8	0.3	0	0.6	0	28.9	0	1. 5	0	0	0

n=2. The *Pythium* isolates lacked sterols and are not included.

Table 3. Mass spectral characteristics of sterols recovered from root pathogenic fungi.

Name*	RRt†	Systematic name	M+†	b.p.†	other ions
C27:6	0.47		448	251	433, 376, 361, 350, 333
C28:4a	1.12		466	251	376, 361, 325, 69
brassicasterol	1.14	(22E)-Ergosta-5,7-dienol	470	69	380, 365, 341, 255, 129
C28:3a	1.16		468	363	453, 378, 337, 253
C27:4	1.20		452	362	437, 325, 237, 195, 69
ergosterol	1.26	(22E)-Ergosta-5,7,22-trienol	468	69	453, 378, 363, 337, 253
C28:2a	1.30		470	73	455, 441, 365, 343, 227
stellasterol	1.32		470	69	455, 343, 255, 229
C28:3b/C28:4c	1.33		468 /466	73	363, 337
C28:2b	1.37		470	69	455, 365, 343, 227, 213
C28:1	1.38		472	472	457, 367, 341, 255, 229
C28:3c	1.43		468	363	337, 253, 73
C28:2c	1.46		470	73	455, 380, 365, 355, 343
C28:2d	1.50		470	75	455, 386, 372, 365, 343
fungisterol	1.53	ergost-7-enol	472	75	457, 367, 345, 255, 229
C29:3	1.53		472	69	446, 392, 377, 351, 253
lanosterol	1.61	Lanosta-8,24-dienol	498	69	483, 393
C29:2	1.70		484	69	469, 394, 379, 227
C31:2	1.80		512	69	497, 407, 241
C30:2	1.92		498	73	483, 408, 393, 365, 355

*- The sterols are represented in the form Cx:y, where x= number of carbons in the molecule and y= the number of double bonds.
†- RRt= relative retention time; M+= molecular weight of the protonated compound; b.p.= most abundant fragment on the mass spectrum.

Table 4. Molecules giving the highest loadings for the components.

FAME					
Component 1*		Component 2*		Component 3*	
molecule	loading	molecule	loading	molecule	loading
18:2ω6	-20.4	18:1ω9	-7.0	16:0	3.5
18:1ω9	8.9	14:0	3.3	14:0	2.5
16:0	4.1	16:0	2.3	18:0	-1.6
PLFA					
Component 1*		Component 2*		Component 3*	
molecule	loading	molecule	loading	molecule	loading
18:2ω6	-16.5	18:3ω3	-3.3	18:1ω9	-3.0
18:1ω9	6.4	18:0	-3.2	16:0	2.7
16:0	2.8	18:1ω9	2.0	18:0	-1.2
Sterol					
Component 1*		Component 2*		Component 3*	
molecule	loading	molecule	loading	molecule	loading
ergost-7-enol	-29.6	C28:2d	20.7	ergosterol	18.3
ergosterol	12.7	lanosterol	-15.3	lanosterol	-14.5
C28:2d	7.0	C31:2	-4.4	C28:2d	-8.9

*- The component axes from figure 1 can be used to determine which component (1, 2 or 3) is valuable for identifying each fungal genera. In this table, the higher absolute values for the loadings indicate which molecules have a greater influence on the distribution of the respective components in figure 1. The percent of the total variance accounted by each component is: FAME- component 1= 83.6%; component 2= 11.4%; component 3= 3.7%. PLFA- component 1= 84.7%; component 2= 7.2%; component 3= 4.8%. sterol- component 1= 40.9%; component 2= 24.2%; component 3= 22.4%.

Table 5. Most abundant PLFA in the fungal root pathogens of *Sorghum*.

Fatty acid:	14:0	15:0	16:0	16:1 ω7	16:1 ω9	17:0	18:0	18:1 ω7	18:1 ω9	18:2 ω6	18:3 ω6
Bipolaris sorokiniana	0	0.5 (0.4)	17.2 (2.0)	0.4 (0.1)	0	t	2.0 (0.3)	0.6 (0.1)	10.1 (1.5)	65.1 (1.6)	0
Bipolaris victoriae	0	0.3 (0.3)	22.7 (1.8)	0.7 (0.2)	0	t	3.3 (0.8)	1.2 (0.2)	22.3 (2.2)	54.0 (2.5)	0
Cephalosporium gramineum	t	0.6 (0.1)	24.7 (2.5)	t	0	0.4 (0.1)	1.4 (0.1)	0.7 (0.2)	14.9 (0.2)	53.2 (3.1)	0
Fusarium equiseti	t	t	17.3 (0.9)	1.0 (0.0)	0	t	2.6 (0.1)	0.3 (0.0)	18.5 (0.6)	51.1 (0.6)	0
Fusarium graminearum	0	0	15.6 (0.6)	0.8 (0.2)	0	t	14.1 (2.3)	0	20.8 (1.9)	41.5 (2.6)	0
Fusarium moniliforme	0	0	22.2 (1.9)	0.6 (0.1)	0	t	4.0 (0.6)	t	15.1 (1.1)	53.9 (1.5)	0
Fusarium moniliforme conidia	0.41 (0.0)	t	24.2 (0.4)	1.05 (0.0)	0	t	8.6 (0.3)	2.0 (0.3)	13.6 (0.3)	37.1 (0.7)	0
Nigrospora sphaerica	0	0	19.9 (0.3)	2.6 (0.4)	0	0	1.9 (0.2)	2.6 (0.3)	30.5 (4.5)	39.0 (4.7)	0
Periconia macrospinosa	0	0	21.4 (0.7)	t	0	t	2.4 (0.0)	0.7 (0.1)	15.6 (0.5)	52.0 (1.4)	0

Table 5 (cont'd).

Fatty acid:	14:0	15:0	16:0	16:1 ω7	16:1 ω9	17:0	18:0	18:1 ω7	18:1 ω9	18:2 ω6	18:3 ω6
Phoma sp.	0	0	12.2 (0.6)	1.3 (0.4)	0	t	4.0 (0.2)	1.0 (0.2)	10.9 (2.6)	70.2 (2.4)	t
Phoma sp. conidia	t	t	15.9 (0.2)	1.7 (0.2)	0	0.3 (0.0)	5.6 (0.1)	2.4 (0.1)	17.6 (1.6)	51.7 (1.8)	0
Phoma exocarpina	t	t	22.6 (0.7)	0.7 (0.1)	0	t	13.2 (1.0)	1.2 (0.1)	12.8 (0.9)	40.4 (1.5)	0
Pythium graminicola	10.4 (1.0)	0	27.2 (0.2)	1.9 (0.5)	0	t	0.5 (0.1)	2.1 (0.2)	27.8 (1.4)	20.5 (1.1)	0.7 (0.1)
Pythium ultimum	4.1 (0.5)	t	27.5 (1.5)	4.8 (0.4)	0.3 (0.0)	t	1.8 (0.5)	1.3 (0.3)	22.7 (4.0)	22.2 (5.1)	0.5 (0.2)
Rhizoctonia oryzae	t	t	19.8 (0.6)	0.3 (0.1)	0	0	0.7 (0.2)	0	7.8 (0.7)	69.9 (0.8)	0
Rhizoctonia solani	0	0	17.1 (2.8)	t	0	0	1.0 (0.4)	0	10.5 (2.0)	71.3 (3.3)	0
Rhizoctonia zeae	0	0	20.2 (3.9)	0	0	0	2.8 (0.7)	0	11.3 (1.3)	65.8 (5.8)	0
Sclerotium rolfsii	0	0.7 (0.2)	17.0 (0.6)	t	0	0.3 (0.1)	1.9 (0.0)	t	3.9 (0.3)	75.9 (0.5)	0
Sclerotium rolfsii sclerotia	0	1.4 (0.2)	17.3 (0.9)	t	0	0.9 (0.1)	2.5 (0.1)	1.5 (0.3)	3.2 (0.4)	73.0 (1.6)	0

Table 5 (cont'd).

Fatty acid:	18:3 ω3	20:0	20:1 ω9	20:1 ω11	20:2	20:2 /20:3	20:3 ω6	20:4 ω6	20:5 ω3	20C*	22:0
Bipolaris sorokiniana	7.2 (0.6)	0	0	0	0	0	0	0	0	0	0
Bipolaris victoriae	7.5 (1.6)	t	t	0	t	t	0	0	0	0	0
Cephalosporium gramineum	2.6 (0.2)	t	t	0	0	t	t	0	0	0	t
Fusarium equiseti	0	t	0	t	0	t	t	0	0	0	t
Fusarium graminearum	4.2 (0.2)	t	0	t	0	0	0	0	0	0	t
Fusarium moniliforme	0	0	0	0	0	0	0	0	0	0	0
Fusarium moniliforme conidia	12.9 (0.2)		0	0	0	0	0	0	0	0	0
Nigrospora sphaerica	2.7 (0.3)	0	0	0	0	0	0	0	0	0	0
Periconia macrospinosa	7.3 (0.4)	t	0	t	t	t	0	0	0	0	t

Table 5 (cont'd).

Fatty acid:	18:3 ω3	20:0	20:1 ω9	20:1 ω11	20:2	20:2 /20:3	20:3 ω6	20:4 ω6	20:5 ω3	20C*	22:0
Phoma sp.	0	0	0	t	t	t	0	0	0	0	0
Phoma sp. conidia	3.7 (0.1)	t	t	t	0	t	0	0	0	0	0
Phoma exocarpina	7.7 (1.4)	0.6 (0.2)	0	t	t	t	0	0	0	0	0.3 (0.1)
Pythium graminicola	0	0	t	t	t	t	1.3 (0.2)	5.5 (0.4)	1.7 (0.2)	0	0
Pythium ultimum	0.4 (0.0)	0.7 (0.2)	1.9 (0.3)	0.3 (0.2)	1.0 (0.2)	0.6 (0.2)	0.5 (0.2)	3.1 (1.2)	3.7 (1.8)	0.4 (0.2)	0.5 (0.5)
Rhizoctonia oryzae	0	0.4 (0.2)	0.4 (0.3)	t	t	t	t	0.3 (0.1)	0	0	0
Rhizoctonia solani	0	0	0	0	0	0	0	0	0	0	0
Rhizoctonia zeae	0	0	0	0	0	0	0	0	0	0	0
Sclerotium rolfsii	0	0	0	0	0	0	0	0	0	0	0
S. rolfsii sclerotia	0	0	0	0	0	0	0	0	0	0	0

*- polyenoic molecule. The position of the double bonds is unknown. Values are in percent of total (standard deviation). t= trace (<0.25 %). n=3. Only the molecules accounting for more than 0.25 % are shown.

Table 6. Most abundant FAME in the fungal root pathogens of *Sorghum*.

Fatty acid:	12:0	i12:0	14:0	ai14:0	14:1 ω5	15:0	i15:1 ω10	16:0	i16:0	16:1 ω5	16:1 ω7	16:1 ω9	17:0	17:1 ω8
Bipolaris sorokiniana	0	0.4 (0.1)	0.4 (0.1)	0	0	t	0.6 (0.2)	25.6 (1.4)	3.1 (0.6)	0	0.9 (0.1)	t	0.4 (0.1)	t
Bipolaris victoriae	0	0.4 (0.5)	0.6 (0.2)	0.7 (0.6)	t	t	t	23.8 (2.3)	3.6 (2.4)	0.6 (1.2)	0.9 (0.3)	0	t	t
Cephalosporium gramineum	0	0	0.4 (0.1)	0	0	0.8 (0.1)	0	22.4 (0.4)	0	0	0.7 (0.1)	0	0.6 (0.1)	0.7 (0.4)
Fusarium equiseti	0	0	0.4 (0.1)	0	0	0.3 (0.0)	0	13.1 (0.3)	0	0	1.4 (0.1)	0	t	0
Fusarium graminearum	0	0	0.4 (0.1)	0	0	0	0	13.3 (0.8)	0	0	1.4 (0.2)	0	0	0
Fusarium moniliforme	0	0	0.6 (0.1)	0	0	t	0	19.5 (0.5)	0	0	0.8 (0.1)	0	0	0
Fusarium moniliforme conidia	0	0	2.8 (1.5)	0	0	0	0	21.3 (4.2)	0	0	0.7 (0.6)	0	0	0
Nigrospora sphaerica	0	t	1.7 (0.1)	0	0	t	0	32.0 (0.6)	0	0	2.9 (0.6)	0	0	t
Periconia macrospinosa	0	0	t	0	0	0	0	19.2 (0.8)	0	0	0.4 (0.2)	0	0	0
Phoma exocarpina	0	0.3 (0.9)	1.4 (0.2)	0	0	t	0	27.1 (1.7)	0	0	0.7 (0.6)	0	0	0

Table 6 (cont'd).

Fatty acid:	18:0	18:0 3OH	18:1*	18:1 ω9	18:1ω9 DMA	18:2 ω6	18:3 ω3	20:0	20:1 cω9	20:1 t ω9	20:4 ω6	20:5 cω3	22:1 ω7
Bipolaris sorokiniana	4.7 (0.6)	0	0	23.2 (0.3)	0	40.0 (1.0)	0	0	0	0	0	0	0
Bipolaris victoriae	6.6 (0.9)	0	0	22.3 (2.2)	0	32.1 (1.5)	0	t	0	0	0	0	0
Cephalosporium gramineum	3.3 (0.7)	0	0	23.4 (1.8)	0	44.0 (2.6)	0	0	1.9 (1.9)	0	0	0	0
Fusarium equiseti	5.1 (0.6)	0	0	31.6 (0.3)	1.3 (0.2)	45.5 (1.1)	0	t	0	0	0	0	0
Fusarium graminearum	4.5 (1.7)	0	0	50.0 (4.6)	0	29.6 (3.9)	0	0.4 (0.2)	0	0	0	0	0
Fusarium moniliforme	7.3 (0.9)	0	0	39.9 (0.7)	0	31.3 (1.5)	0	0	0	0	0	0	0
Fusarium moniliforme conidia	5.8 (1.3)	0	0	30.4 (3.2)	0	37.8 (4.0)	0	0	1.1 (1.1)	0	0	0	0
Nigrospora sphaerica	6.4 (0.5)	0	0.76 (1.1)	28.1 (1.1)	0	27.1 (0.9)	0	0.4 (0.2)	0	0	0	0	0
Periconia macrospinosa	3.9 (0.5)	0	0	30.3 (0.9)	0	46.0 (1.4)	0	0	0	0	0	0	0
Phoma exocarpina	11.4 (2.1)	0	0	31.3 (2.0)	0	27.6 (2.8)	0	0	0	0	0	0	0

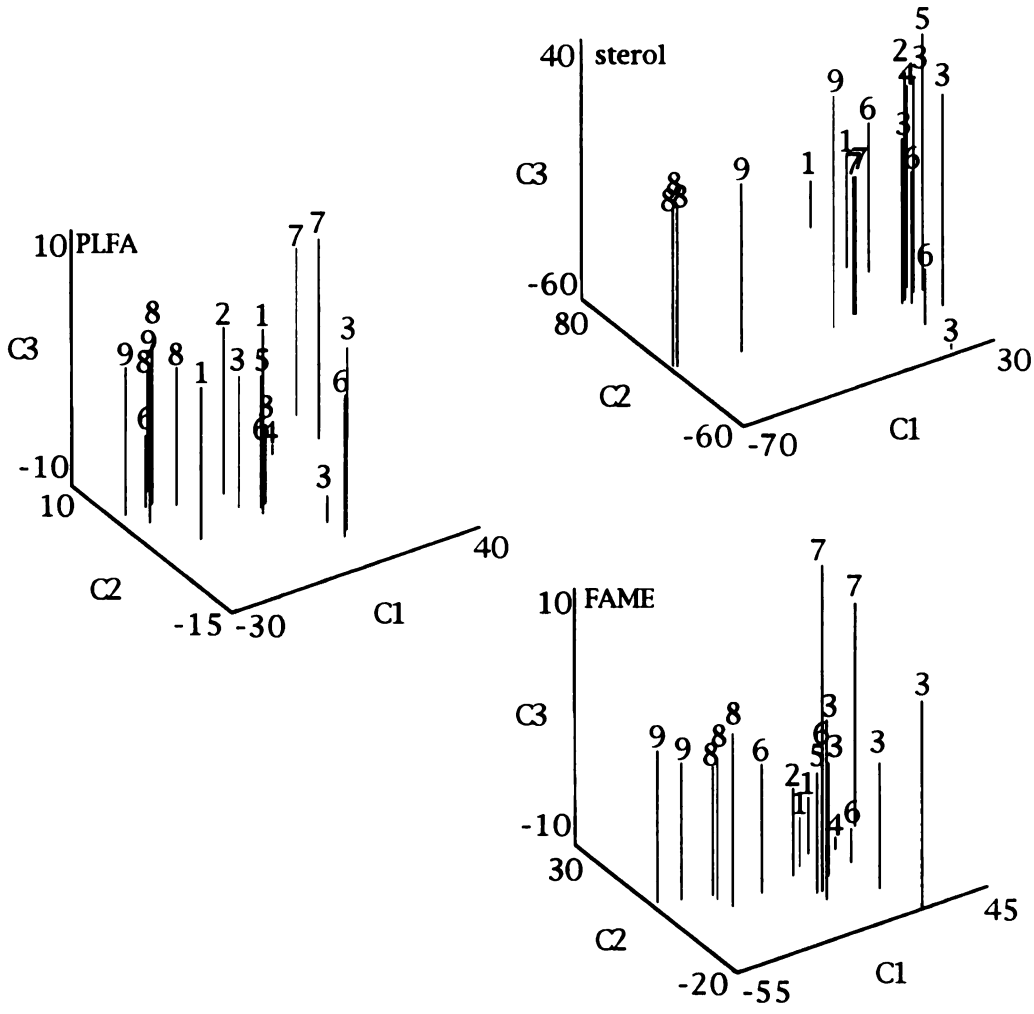
Table 6 (cont'd).

Fatty acid:	12:0	i12:0	14:0	ai14:0	14:1 ω5	15:0	i15:1 ω10	16:0	i16:0	16:1 ω5	16:1 ω7	16:1 ω9	17:0	17:1 ω8
Phoma sp.	0	t	t	0	0	t	0	18.2 (0.5)	0	0	2.7 (0.5)	0	t	t
Phoma sp. conidia	0	0	t	0	0	t	0	16.1 (1.1)	0	0	1.1 (0.3)	0	0.3 (0.0)	t
Pythium graminicola	0.3 (0.0)	0	20.3 (0.4)	0	0.3 (0.1)	0	0	25.6 (0.8)	0	0.6 (0.1)	4.0 (0.5)	0.3 (0.0)	0	t
Pythium ultimum	0.5 (0.1)	0	10.9 (0.9)	0.6 (0.1)	0.9 (0.2)	t	0	21.3 (0.7)	0	0	8.7 (0.9)	0.6 (0.1)	0	t
Rhizoctonia oryzae	t	t	0.3 (0.1)	t	0	0.3 (0.1)	0	15.9 (0.8)	0	0	0. 5 (0.2)	t	0	0
Rhizoctonia solani	t	0	t	t	0	t	0	11.6 (1.3)	0	0	t	t	0	0
Rhizoctonia zeae	t	0	t	0.3 (0.1)	0	t	0	13.8 (1.0)	0	0	t	0.3 (0.1)	0	t
Sclerotium rolfsii	0	0	0	0	0	0.8 (0.3)	0	12.5 (0.5)	0	0	0	0	t	t
Sclerotium rolfsii sclerotia	0	0	0	0	0	1.3 (0.1)	0	11.6 (0.4)	0	0	0	0	0	0

Table 6 (cont'd).

Fatty acid:	18:0	18:0 3OH	18:1*	18:1 ω9	18:1ω9 DMA	18:2 ω6	18:3 ω3	20:0	20:1 cω9	20:1 t ω9	20:4 ω6	20:5 cω3	22:1 ω7
Phoma sp.	3.9 (0.4)	0	0	30.3 (0.8)	0	44.1 (1.4)	0	0	0	0	0	0	0
Phoma sp. conidia	4.7 (0.6)	0	0	20.0 (0.9)	0	57.3 (2.1)	0	0	0	0	0	0	0
Pythium graminicola	2.0 (0.2)	0.3 (0.4)	3.6 (0.2)	22.0 (0.6	0	9.7 (0.7)	t	t	t	1.0 (0.5)	6.2 (0.3)	2.2 (0.1)	t
Pythium ultimum	2.0 (0.3)	0.3 (0.5)	0	30.8 (1.8)	0	13.2 (0.9)	0.6 (0.2)	0.4 (0.3)	1.4 (0.5)	0	2.9 (0.5)	5.1 (1.7)	0
Rhizoctonia oryzae	1.6 (0.4)	0	0	12.4 (1.1)	0	68.4 (1.2)	0	t	t	t	0	0	t
Rhizoctonia solani	1.9 (0.4)	0	0	16.9 (2.6)	0	67.8 (4.2)	0	t	t	0	0	0	t
Rhizoctonia zeae	2.7 (0.4)	0	0	13.1 (1.1)	t	68.7 (1.4)	0	0	t	0	0	0	0
Sclerotium rolfsii	3.6 (0.8)	0	0	6.3 (0.6)	0	75.2 (2.1)	0	0	0	0	0	0	0.8 (0.4)
Sclerotium rolfsii sclerotia	1.4 (0.1)	1.4 (0.2)	0	3.1 (0.3)	0	81.3 (0.7)	0	0	0	0	0	0	0

The values are for mycelial extracts unless stated otherwise. Values are in percent of total fatty acid (standard deviation). t= trace (less than 0.25 %). n=3. *- the position of the unsaturation of this molecule is not resolved.



C1=component1
C2=component2
C3= component 3

1) *Bipolaris*	6) *Phoma*
2) *Cephalosporium*	7) *Pythium*
3) *Fusarium*	8) *Rhizoctonia*
4) *Nigrospora*	9) *Sclerotium*
5) *Periconia*	

Figure 1. Principal components anlyses of the lipid profiles.

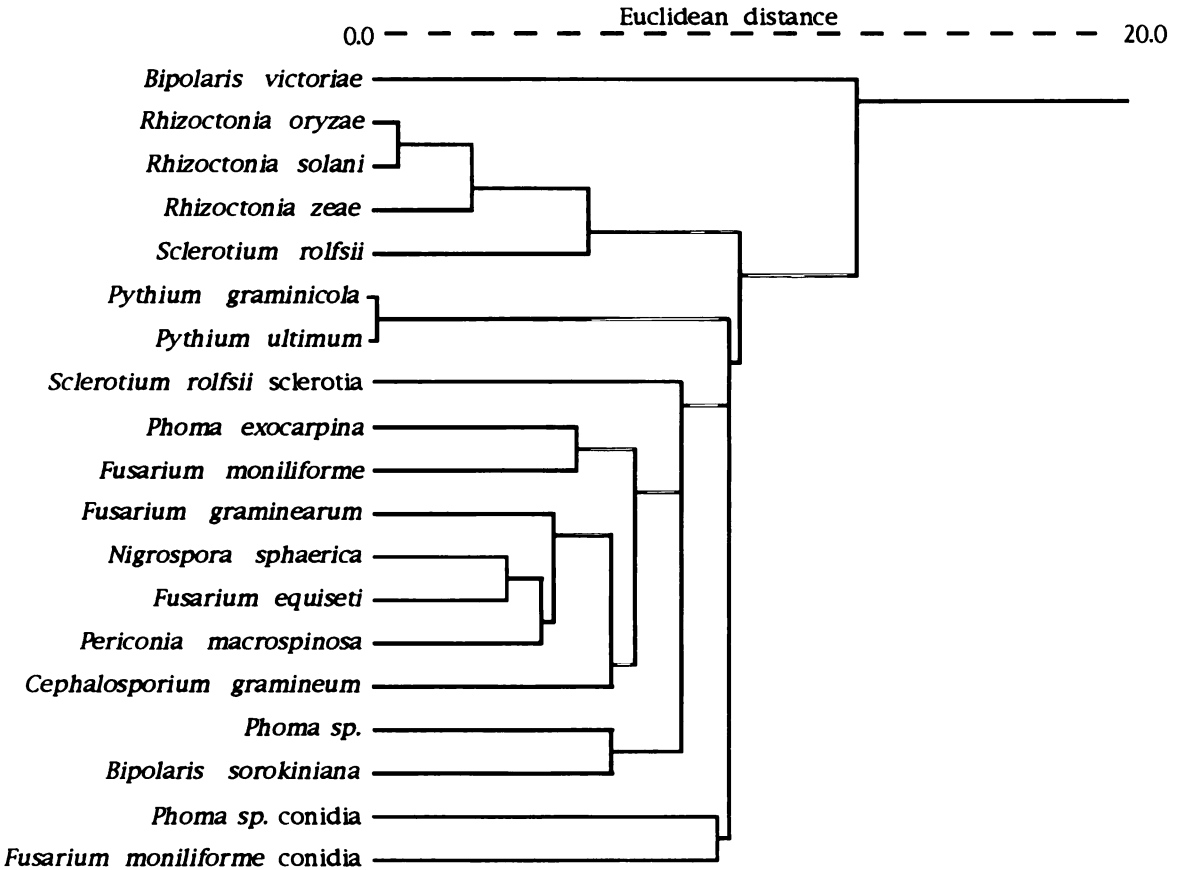


Figure 2. Tree diagram of the sterol profiles from the mycelium, conidia, or sclerotia.

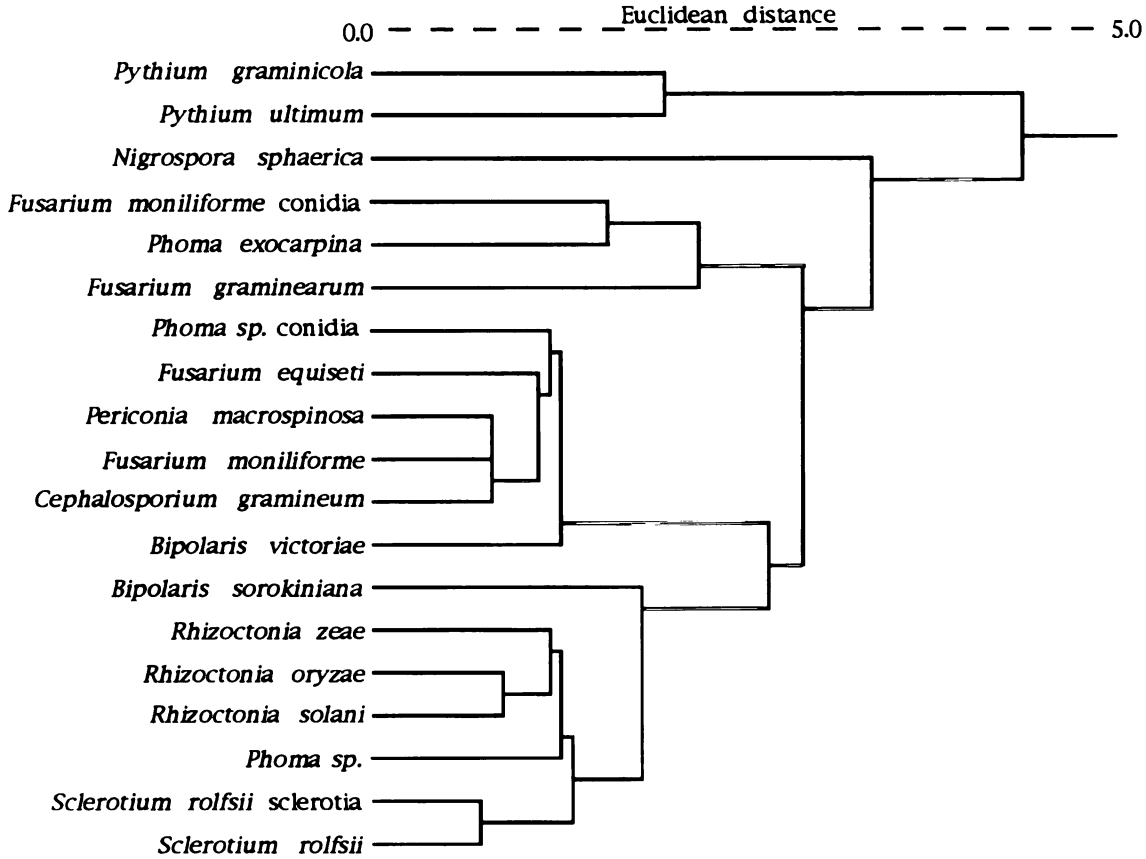


Figure 3. Tree diagram of the phospholipid fatty acid profiles from the mycelium, conidia, or sclerotia

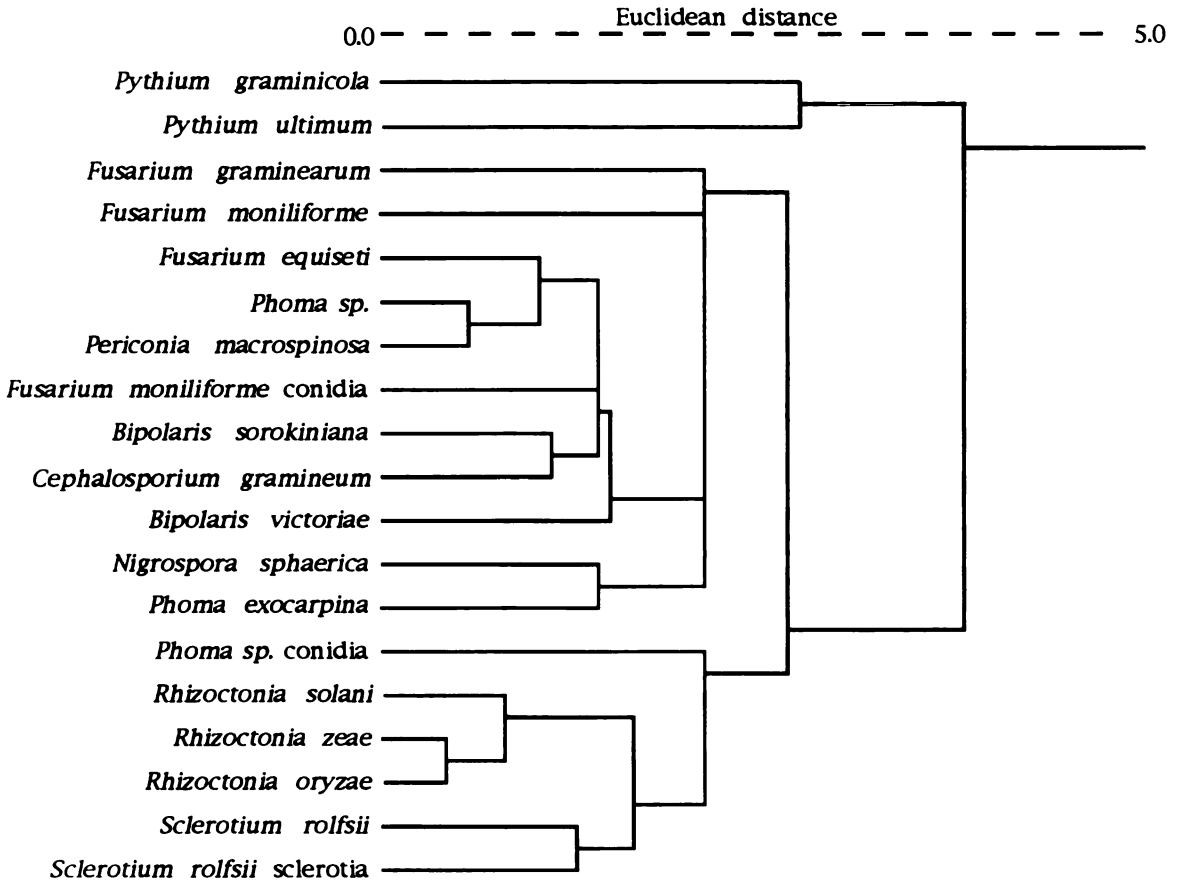


Figure 4. Tree diagram of the whole-cell fatty acid profiles from the mycelium, conidia, or sclerotia

Chapter 2

IDENTIFICATION OF POSSIBLE MOLECULAR MARKERS IN THE LIPIDS OF ARBUSCULAR MYCORRHIZAE

Abstract

We analyzed the total fatty acid methyl esters (FAME), phospholipid fatty acids (PLFA) and sterols of the mycorrhizal and non-mycorrhizal roots of *Sorghum* by gas chromatography. The purpose of this work was to identify lipid markers of mycorrhizal infection. To achieve this, the roots of *Sorghum* infected with several species of glomalean fungi were compared to non-mycorrhizal roots. Fungi belonging to the genera *Glomus*, *Gigaspora*, *Acaulospora*, and *Scutellospora* were analyzed for their lipid content.

Increases in hexadecenoic acid (16:1) in the root phospholipids indicates the presence of arbuscular mycorrhizae. Mycorrhizal infection is associated with increased amounts of either PLFA 16:1 ω5 or PLFA 16:1 ω7 in the roots of *Sorghum*, depending on which fungal symbiont is present. The 16:1 ω7 isomer was highest in the *G. occultum* symbiosis, accounting to more than twice the molar percentage of this fatty acid in the control roots. Phospholipid fatty acid 16:1 ω5 increased in the rest

of the fungal treatments, and it was present in at least 50 % higher molar percentage when compared to non-mycorrhizal roots.

The mycorrhizal roots of all the fungal treatments had at least 21.1% more total FAME concentration than uninfected roots. The mycorrhizae of *Glomus etunicatum, Glomus intraradices, Acaulospora mellea*, and *Scutellospora erytropa* have relative amounts of 16:1 ω5 FAME which range from 4.9% to 13.3 % of the total fatty acids. In contrast, the concentration of 16:1 ω5 in non-mycorrhizal roots, as well as in the mycorrhizae of *Glomus occultum* and *Gigaspora rosea* was beyond the detection limit of the instruments. Hierarchical cluster analysis shows that the FAME profiles are valuable in the diagnosis of non-mycorrhizal roots and provide insight as to which fungus is present in the mycorrhizal association.

Besides the FAME and PLFA, the sterols are valuable for marking the presence of arbuscular fungi in roots. Infected roots can be distinguished from non-mycorrhizal roots because the molar percentage of campesterol is at least 23% higher when the roots are colonized.

This is the first report of the sterol content of the mycorrhizae of *Acaulospora* and *Scutellospora*, and the first account of the phospholipid fatty acids of roots infected by *Acaulospora, Gigaspora,* and *Scutellospora*.

Introduction

The dominant cytoplasmic constituent of the glomalean fungus is lipid (Cox and Sanders, 1974), comprising more than half of the dry weight of the fungus in some cases (Beilby and Kidby, 1980). The difference between mycorrhizal and non-mycorrhizal lipids is not only quantitative, but it is also qualitative. For example, the roots of several plant species infected with *Glomus* contain various unsaturated fatty acids which are not detectable in non-mycorrhizal roots (Graham *et al.*, 1995; Nagy *et al.*, 1980; Nordby *et al.*, 1981; Pacovsky, 1987; Pacovsky and Fuller, 1988; Pacovsky, 1989). The high lipid content combined with presence of unusual molecules provides the opportunity for the use of lipids as molecular markers of arbuscular mycorrhizal fungi. For instance, fatty acid 16:1 ω5 has been used to estimate the presence of *Glomus* vesicles in the roots of monocotyledons and dicotyledons (Graham *et al.*, 1995; Pacovsky, 1987; Pacovsky and Fuller, 1988; Pacovsky, 1989).

Recent studies of the fatty acids of glomalean mycorrhizae have concentrated on the analysis of the whole-cell fatty acids (FAME) (Graham *et al.*, 1995). The fatty acids that are bound to other cell components are separated by methylation and analyzed by gas chromatography to produce the FAME profile. Because of this, the FAME analysis includes the fatty acids bound to neutral lipids (such as triacylglycerides and diacylglycerides), those bound to polar lipids (such

as phospholipids), as well as free fatty acids. The neutral and polar lipid fractions of glomalean fungi may differ in the abundance of 16:1 ω5, indicating the importance of separating the lipids prior to the fatty acid analysis (Jabaji-Hare, 1984; Olsson *et al.*, 1995). However, information on the fatty acid composition of specific lipid fractions such as the phospholipids is lacking for several glomalean genera. This is relevant because phospholipids fatty acids have been shown to be good measures of the extra-radical phase of *Glomus* (Olsson *et al.*, 1995), and may be used as indicators of the biomass of microbes in the environment (Vestal and White, 1989). Sterols are another group of lipids which have proven useful for quantifying arbuscular mycorrhizae (Nagy *et al.*, 1980; Nordby *et al.*, 1981; Schmitz *et al.*, 1991; Dalpé *et al.* 1994). However, information about the sterols of genera such as *Acaulospora* and *Scutellospora* is lacking.

The objectives of this experiment are: 1) To test the value of FAME, PLFA and sterols as markers of mycorrhizae, and 2) To gather information on the PLFA and sterols of glomalean taxa for which this information is not available. To do this, I inoculated *Sorghum* with different species of glomalean fungi which belong to different genera. Following a growth period, the roots were harvested and analyzed for the FAME, PLFA, and sterol composition. This information will be used in chapter 3 as the basis of our studies investigating the C transformation rates in the lipids of mycorrhizal roots.

Materials and Methods

Fatty acid terminology utilizes "A:B ωC" where "A" indicates the total number of carbon atoms, "B" the number of unsaturations, and "ω" precedes "C", the number of carbon atoms between the closest unsaturation and the methyl end of the molecule. The suffixes "c" and "t" indicate cis and trans geometric isomers. The prefixes "i" and "a" refer to iso and anteiso methyl branching.

Soil, Inoculum, and Growth Conditions

A sandy clay loam (60.0% sand, 20.0% silt, and 20% clay) pH 6.9, and cation exchange capacity 10.2 me/100g was used as a growth medium. The soil was supplied by the Plant Biology greenhouses at Michigan State University. It contained 4.5% by weight organic matter, 24 mg kg^{-1} total P, 80 mg kg^{-1} K, 1642 mg kg^{-1} Ca, and 220 mg kg^{-1} Mg. The soil was passed through a 2 mm sieve and 0.8 liters (740 g) of air-dry soil were added to each pot. The pots were closed with a plastic lid and then irradiated with cobalt-60 at the Phoenix Memorial Laboratory, University of Michigan. Irradiation was carried out for 13 hours with a gamma dose rate of 3826.1 Gy/hr. Each pot received 120 ml of nutrient solution which contained 1.5 mM $CaCl_2$, 0.5 mM K_2SO_4, 2.5 mM NH_4NO_3, 0.25 mM $MgSO_4$, 25 μM H_3BO_3, 20 μM FeDDHA, 20 μM $ZnSO_4$, 0.5 μM $CaSO_4$, 0.4 μM H2MoO$_4$, and 0.6 μM $CoCL_2$. The pH was adjusted to 6.8 with a

KOH solution. The nutrient solution was filtered (0.2 μm) before being dispensed to the pots. The pots were watered with 140 ml of distilled water every two days throughout the duration of the experiment.

Surface-sterilized seeds (70% ethanol for 30 sec., then 20% commercial bleach for 20 min., then washed with distilled water) of *Sorghum bicolor* L. Moench were germinated in a petri dish for two days over a sterile moist filter paper before planting. Two seeds were planted per pot and the plants were thinned to one per pot after the first week of the experiment.

The pots were placed randomly in a growth chamber and they were randomized once more four weeks after the start of the experiment to minimize positional effects. The photosynthetically active radiation ranged from 450 to 500 $\mu M\ m^{-2}\ s^{-1}$ and the photoperiods lasted 14 hours. The average temperature was $25^{o}C$, and the average relative humidity was 75%. Plants were grown for 8 weeks, left for four days without watering, and harvested.

The inocula were obtained from the International Culture Collection of Arbuscular and Vesicular-Arbuscular Fungi at West Virginia University. These consisted of *Acaulospora mellea* Spain & Schenck FL266-2, *Gigaspora rosea* Nicolson & Schenck BR151A-3, *Glomus intraradices* Schenck & Smith UT143-2, *Glomus etunicatum* Becker & Gerdemann SC306A, *Glomus occultum* Walker IA702, *Scutellospora erythropa* (Koske & Walker) Walker & Sanders MA453B-2*. The inocula

consisted of a mixture of soil, roots, and spores which contained at least 12 spores g^{-1}. A minimum of 25 g of inoculum were used per pot. Each pot in the mycorrhizal treatments received inoculum from a single species. Six replicates of each species and six uninoculated controls were included in the experiment. A chlamydospore-free filtrate of each kind of inoculum was added to all pots to achieve a homogeneous diversity of saprophytic microbes throughout the treatments.

Root Harvesting and Preservation

The roots were separated from the soil by wet sieving (2 mm mesh). Root material retained in the sieve was finely cut and mixed in distilled water. The roots and supernatant were then washed with water through a 200 µm sieve, and root fragments with diameters of > 1.0 mm were lyophilized before lipid analysis, or stored in 5% formalin until clearing and staining.

Appendix A is a diagram of the methodology used to obtain the lipid profiles.

Whole-Cell Fatty Acids (FAME)

The Microbial Identification System (Microbial ID, inc., Newark DE) was used to determine the total FAME profile of the roots. Samples of 0.02 g dry weight were analyzed using the Microbial ID protocol. The method was modified to include an internal standard (50 µl of 15:0,

0.001 g/ml in hexane) added after the saponification step. This made it possible to obtain quantitative data in addition to the relative molar percentage of the molecules. The fatty acids were quantified by comparison of the peak areas with those of the standard peak.

Phospholipid Fatty Acids (PLFA)

The total lipids were extracted from 0.2 g of freeze-dried roots by a chloroform-methanol extraction (Bligh & Dyer, 1959) modified to incorporate a phosphate buffer. The root material was extracted at room temperature with the chloroform/methanol/potassium phosphate buffer (1:2:0.8 by volume; 50 μm , pH 7.4) for at least 2 h, at which time 0.29 volumes of organic-free water and chloroform were added to separate the aqueous and organic phases. After rotary evaporation at 37^{o}C, the total lipid was fractionated into Neutral-, Glyco-, and Polar lipid fractions using silicic acid (100-200 mesh) column chromatography (Guckert *et al.*, 1985). This way, the sterols and phospholipids were recovered from the same total lipid extract.

The phospholipids were obtained from the polar lipid fraction. The polar lipid was subjected to a mild alkaline methanolic transesterification (Guckert *et al.*, 1985) from which the PLFA-methyl esters were recovered. The PLFA were then separated, quantified, and identified by capillary column gas chromatography (FID detector, Hewlett-Packard HP-1 50 m by 0.2 mm inner diameter non-polar methyl

silicone column) using the conditions described by Ringelberg *et al.* (1989). Quantification was based on comparison of peak areas to an internal injection standard (19:0) assuming equi-molar responses within the range of chain lengths between 12-24 C. The structures were verified using a mass selective detector (Hewlett Packard 5791).

Sterols

The sterols were recovered from the neutral lipid fraction after saponification in 5% KOH in methanol:water (80:20, v:v). The extract was derivatized using N, O *bis*(Trimethylsilyl)trifluoroacetamide (BTSFA). This formed trimethyl silyl ethers, which were then separated and identified using gas chromatography (as for the PLFA). Sterol identification was based on co-injection with the standard cholestane, relative retention time, and by comparison of the mass spectra with previously reported spectra. The structures were verified using GC-mass spectrometry at an electron energy of 70eV with the same columns and conditions described above (Table 1).

The concentration and mol % of each molecule were determined with the following formulas:

mol g^{-1}= ((area of sample peak)(area of standard $peak^{-1}$))(mol μl^{-1} of standard)(dilution factor of sample in μl)(grams of sample)$^{-1}$.

mol %= (mol of individual peak)(total mol in sample)$^{-1}$(100).

Microscopic Determination of the Percent Mycorrhizal Infection.

The roots were cleared and stained using the technique of Kormanik and McGraw (1982). The percentage root length occupied by vesicles, arbuscles, and hyphae was determined by the line intercept method (Giovanetti and Mosse, 1980). The values are shown in Table 2. This was modified to use the lines of an eyepiece graticule to determine the observation points. The specimens were observed at 40 x and the magnification was increased when necessary to diagnose specific structures. Three subsamples of 100 intercepts were observed for each root system. I did not observe any mycorrhizal infection in the control treatment.

Statistical Analysis

The lipid profiles of the six pot cultures in each fungal treatment were evaluated by hierarchical cluster analysis using the average linkage method of Systat 5.2.1 (SPSS Inc., Chicago, IL, U.S.A.). I used a one-way ANOVA followed by the Tukey's mean comparisons to test for differences in the relative amount of each molecule between the treatments. Molecules in concentrations of <1500 ρmol or making up <0.25 % of the total profile were excluded from the statistical and multivariate analyses.

Results and Discussion

Whole-Cell Fatty Acids (FAME)

Mycorrhizal infection is associated with an increase in the total fatty acid content of roots. The mycorrhizal treatments had at least 21.1 % more total fatty acid content than the controls (Table 3), and the difference was statistically significant ($p<0.05$) for all treatments except *G. rosea*. Likewise, Pacovsky (1989) and Peng *et al.*, (1993) found increases in fatty acid concentration in the roots of different host-fungus combinations. Arbuscular mycorrhizae may improve the phosphorus nutrition of the host plant. One question that we may ask is whether the increased lipid concentration in infected roots is due to nutritional effects, or to the presence of the fungus. Pacovsky (1989) carried out experiments in which the non-mycorrhizal *Sorghum* plants were amended with P fertilizer to achieve a comparable P nutrition to that of the mycorrhizal plants. He showed that the increase in fatty acid content of mycorrhizal roots is not related to the phosphorus nutrition of the host. Because of this, the increase in the fatty acids is attributed to the presence of the fungal biomass in the infected roots.

A total of 20 different fatty acids with molar percentages higher than 5 % were recovered from mycorrhizal and non-mycorrhizal roots (Table 3). Besides the effect on the concentration of total lipids in roots, arbuscular mycorrhizae are also associated with a qualitative change in the lipid composition. For example, the molar percentage of fatty acid

16:1 ω5 was significantly higher ($p < 0.05$) for all mycorrhizal treatments, except for *G. rosea* and *G. occultum*. *Glomus occultum* is characterized because it produces 16:1 ω7 instead of the 16:1 ω5 isomer (Table 3). This opens the possibility of reliably discerning between the infection by *G. occultum* and that of other glomalean species in field samples and controlled experiments. Molecular markers for detecting *G. occultum* should be particularly useful since this fungus is difficult to stain and quantify microscopically (Table 2). The roots infected by *G. rosea* are distinguishable because of the increased percentage of 18:1 ω9 relative to the non-mycorrhizal roots ($p < 0.01$). Others have shown that this fatty acid is also valuable for differentiating the spores of *Gigaspora* from those of other genera (Bentivenga and Morton, 1994).

Several lipids such as 18 C and 20 C unsaturated fatty acids have been proposed as mycorrhizal markers (Pacovsky, 1987), while 18 C polyenoic fatty acids has been used as molecular markers of fungi in general (Vestal and White, 1989). Our results show that the usefulness of 18 and 20C fatty acids as molecular markers depends on the fungal species present in the roots. For example, I did not find these molecules to be generally higher in the mycorrhizae of the different fungal treatments, except for the increase in fatty acid 18:1 ω9 in *G. rosea* (Table 3).

The results from this study show that the occurrence of FAME 16:1 ω5 can not be predicted using genetic similarities or morphologic

differences. The occurrence of 16:1 ω5 as a dominant FAME is shared by members of the Glomineae (*Acaulospora* and *Glomus*) and Gigasporineae (*Scutellospora*). In spite of the common occurrence of 16:1 ω5, the Glomineae and Gigasporineae have different development patterns and spore morphologies which separate them taxonomically (Morton and Benny, 1990). Despite the fact that these two different glomalean families are similar in their content of 16:1 ω5, members within another glomalean family, the Gigasporaceae, show different patterns of 16:1 ω5 abundance.

Studies using nuclear gene sequences and spore morphology place the genus *Scutellospora* together with *Gigaspora* in the family Gigasporaceae (Morton and Benny, 1990; Simon et al., 1993). Nucleic acid sequences indicate that *Gigaspora* diverged from *Scutellospora* and may be one of the most recent evolutionary lines of the arbuscular fungi (Simon et al., 1993). It is remarkable that *Gigaspora* is distinguished from the rest of the genera by having the 18:1 ω9 as a dominant FAME instead of 16:1 ω5 (Table 3). For this reason, I hypothesize that the loss of 16:1 ω5 FAME as a dominant fatty acid is a derived character within the Gigasporaceae. The presence of 16:1 ω7 in *G. occultum* may also have important evolutionary implications. *Glomus occultum*, together with *G. leptotichum* produce 16:1 ω7 as the most abundant 16 C monoenoic fatty acid (Bentivenga and Morton, 1996) (Table 3). *Glomus leptotichum* is thought to be one of the more ancestral members of the

arbuscular fungi (Bentivenga and Morton, 1996) and it is for this reason that I propose 16:1 ω7 is an ancestral character within the Glomales. The unusual nature of the G. occultum fatty acids is also demonstrated in Figure 1, which shows that the FAME composition of roots infected with this fungus are distinct from uninfected roots and to mycorrhizal roots of other fungal taxa.

Phospholipid Fatty Acids (PLFA)

Sixteen different PLFA in molar percentages of at least 0.25 % were recovered form the set of mycorrhizal and non-mycorrhizal roots of *Sorghum* (Table 4). All mycorrhizal treatments except *G. occultum* have a higher mean concentration of total root PLFA than the controls (Table 4). The difference is significant ($p < 0.01$) for the *A. mellea* and *G. etunicatum* treatments. This agrees with the results of Nagy *et al.* (1980), which found that the total amount of phospholipid in the roots of *Citrus* increased with the formation of *Glomus* mycorrhizae.

All fungal treatments except *G. occultum* have at least 2.6 times the molar percentage of 16:1 ω5 than uninfected roots ($p < 0.05$; Table 4). As with the FAME, roots infected *G. occultum* have 16:1 ω7 as the major unsaturated 16 C PLFA (Table 4). This suggests that PLFA 16:1 may be a general indicator of arbuscular mycorrhizal infection. Other studies have shown that polyunsaturated 20 C PLFA can be used as a measure of *Glomus* mycelium in soil (Olsson *et al*, 1995). In our study of root

extracts, I found that the concentration of root PLFA 20:1 and 20:4 did not always increase with inoculation (Table 4). While 20:1 and 20:4 may be good indicators of the extraradical phase, our results suggest that they are not consistent markers of glomalean fungi in roots (Table 4).

Phospholipid 16:1 ω5 was detected in the control roots (Table 4). The control treatment of this experiment is not designed to produce sterile roots, and the non mycorrhizal roots may have supported a rhizoplane microbial community. I hypothesize that the PLFA 16:1 ω5 present in the control roots may be accounted for by rhizoplane microorganisms. For instance, 16:1 ω5 may be found in low amounts (<0.6 % of total FAME content) in the fungal root pathogens *Pythium* and *Bipolaris* (see chapter 1 of this thesis; Gandhi and Weete, 1991). However, a significant background of 16:1 is unlikely because heavy colonization by root pathogenic microorganisms is likely to produce recognizable symptoms of disease and cause root death.

A Comparison of the FAME and PLFA

The mycorrhizae of *G. rosea* are remarkable because of the distribution of 16:1 ω5 in FAME and PLFA. *Gigaspora rosea* follows the pattern of the rest of the fungal treatments in that it is associated with an increase of 16:1 ω5 in mycorrhizal phospholipids (Table 4). However, the concentration of 16:1 ω5 in whole-cell extracts of roots infected with *G. rosea* is beyond the detection limit of the instruments (Table 3). I

hypothesize that this is explained by a lack of accumulation of 16:1 ω5 in the neutral lipids of *G. rosea*. This difference in the fatty acid composition of phospholipids and total lipid underscores the value of separating the PLFA from the total lipid before the fatty acid analysis.

Sterols

A total of six different sterols were recovered from the mycorrhizal and non-mycorrhizal roots of *Sorghum* (Table 5). The total concentration of sterols is statistically indistinguishable in mycorrhizal and control roots (Table 5). Similarly, Schmitz *et al.*, (1991) found that mycorrhizal infection is not associated to increases in the total concentration of sterols in roots. However, the concentration of campesterol in the roots of various plant species increases with the infection of *Glomus* and *Gigaspora* (Ho, 1977; Nagy *et al*, 1980; Nordby *et al.*, 1981; Schmitz *et al.*, 1991; Dalpé *et al.* 1994). The fungal symbiont may be a contributor to this increase, since *Glomus* spores have campesterol as a major sterol (Beilby and Kidby, 1980; Dalpé *et al.* 1994). In this study, all the fungal treatments had at least 23 % more campesterol than non-mycorrhizal roots (Table 5). The difference was significant for all fungal treatments ($p < 0.05$; Table 5) except for the roots inoculated with *A. mellea*. This suggests that the use of campesterol to measure mycorrhizal formation may be restricted to specific fungal taxa. An advantage of campesterol as a molecular marker

of glomalean fungi is the fact that other root infecting microorganisms should not contribute a significant background signal to sterol measurements of mycorrhizae, since campesterol is absent from non-mycorrhizal fungi (see chapter 1 of this thesis), bacteria, and nematodes. Previous work has shown that ergosterol increases with mycorrhiza formation in the roots of *Trifolium* and *Zea* (Frey *et al.*, 1992). However, in this experiment the concentration of this molecule was beyond the detection limit of our instruments.

Multivariate Analysis

Cluster analysis is a procedure by which multiple variables such as fatty acids or sterols are compared. As a result, a tree diagram is produced which illustrates the similarity between the different fungal treatments. Closely related cases form distinct clusters in the tree diagram. The FAME, PLFA, and sterol profiles extracted from mycorrhizal and control roots were examined using cluster analysis. Our results show that the whole set of FAME molecules provides a signature that identifies control roots (Figure 1). Additionally, the FAME profiles provide insight about which fungus is present. Figure 1 shows that the roots infected with *G. rosea* and *A. mellea* are recognizable by their FAME profiles. The PLFA and sterol profiles were similarly analyzed by hierarchical cluster analysis. However, for both sterols and PLFA, the control roots and the different fungal treatments did not show distinct clustering patterns

(data not shown). This suggests that infection has a more pronounced effect on the FAME than on the PLFA and sterol profiles.

Mycorrhizal Infection as Determined by Microscopy

The different AMF species varied in the amount of infection as well as the formation of storage (vesicles) and exchange structures (arbuscles) (Table 2). The lowest score for the percent infection was observed in *G. occultum* with 8.8 %, while the highest values were observed in *G etunicatum* with 44.3 %. The different AMF species varied in their amount of arbuscle and vesicle formation. For example, no vesicles were observed in *G. rosea* or *S. erytropa*. This agrees with previous studies which have shown that *Gigaspora* and *Scutellospora* form few or no vesicles inside the host's roots (Gerdemann and Trappe, 1974). The percent arbuscularization ranged from 3.9 in *S. erytropa* to 20.2 in *G. etunicatum*. I compared the infection data to the lipid data from the six replicates in each fungal treatment to test if the concentration of the specific lipids increase with the microscopical estimates of infection. No correlation of the amount of specific lipids and the infection estimates was found (data not shown). I hypothesize that the lipid content will follow microscopical measurements when wide differences in the intensity of infection are compared. However, this experiment was not designed to obtain a wide range of percent infection within each fungal treatment. Others have also found that tests for determining the

relationship of total fatty acids to percent infection have shown contrasting evidence. Graham *et al.* (1995) found that 16:1 fatty acid correlates with the percent infection, while others did not find a significant relationship (Pacovsky, 1987). Similarly, measures of intra-radical biomass of glomalean fungi such as chitin analysis fail to correspond to percent infection (Paul and Clark, 1989). I hypothesize that this may be due to several reasons: 1) The inability of the staining procedure to discern between active and non-living mycelium; 2) The line intercept method may not be a good measure of the intensity of infection since heavily colonized root intercepts score the same as intercepts with few hyphae, and 3) Possible fluctuations in the lipid composition of mycorrhizae during the life cycle of the symbionts.

Conclusions

The fatty acid composition of the fungal symbiont is not affected significantly by the species of host (Bentivenga and Morton, 1994). This suggests that the use of fatty acid markers to indicate the presence of arbuscular mycorrhizae may be useful across different host species and field collected samples. This study shows that the lipids of glomalean fungi provide the possibility of marking the presence of these organisms *In Situ* and may provide insight as to which fungal symbiont is present in the host's roots. Future studies will be able to use this methodology to

carry out experiments where the populations of glomalean fungi are monitored *in situ*.

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Table 1. Mass spectral characteristics of sterols recovered from mycorrhizal and non-mycorrhizal roots.

Name	Rt*	Systematic name	M+*	b.p.*	other ions
campesterol	17.73	Ergost-5enol	472	129	457, 382, 367, 343, 255, 129
stigmasterol	18.20	(22E)-Stigmasta-5,24(28)-dienol	484	83	394, 379, 355, 255, 129
stigmastanol	18.28		486	75	374, 353, 345, 257
sitosterol	18.99	(24R)-Stigmast-5-enol	486	129	471, 396, 381, 357, 255
unknown-1	19.14	unknown	488	55	473, 386, 296, 218, 129
unknown-3	20.05	unknown	nd*	75	386, 343

*- Rt= retention time in minutes; M+= molecular weight of the protonated compound; b.p.= most abundant fragment on the mass spectrum; nd- not determined.

Table 2. Microscopical estimates of infection.

Inoculum:	Percent arbuscles	Percent vesicles	Percent infection
A. mellea	11.2(4.8)	0.7(0.5)	36.6(8.6)
G. rosea	12.6(9.4)	0.0(0.0)	20.4(10.2)
G. etunicatum	20.2(8.7)	0.6(0.3)	44.3(5.1)
G. intraradices	12.8(3.7)	3.6(0.9)	44.0(8.7)
*G. occultum**	5.1(4.4)	0.2(0.3)	8.8(4.4)
S. erytropa	3.9(1.5)	0.0 (0.0)	20.4(4.5)

The values are the mean with the standard error in parenthesis. n=6 pots.

*- The mycorrhizae of *G. occultum* stained noticeably weaker than the rest of the treatments, the microscopical estimates may be biased for this reason.

Table 3. FAME of mycorrhizal and non-inoculated *Sorghum* roots.

Fatty acid:	Control	*Acaulospora mellea*	*Glomus etunicatum*	*Glomus intraradices*	*Glomus occultum*	*Gigaspora rosea*	*Scutellospora erytropa*
total (μmol/g)	34.0(1.5)	44.4(1.5)	51.5(2.2)	60.1(3.0)	77.7(4.5)	41.2(2.5)	93.3(8.0)
Mol%:							
14:0	0.0	0.1(0.1)	0.0	0.0	0.1(0.1)	0.0	0.4 (0.1)
16:1ω7	0.0	0.0	0.0	0.0	4.1 (0.5)	0.0	0.7 (0.1)
16:1ω5	0.0	4.9(0.4)	11.4(1.5)	13.3(2.0)	0.0	0.0	5.6(0.6)
16:0	14.0(0.3)	22.3(0.6)	18.6(0.2)	19.2(0.5)	17.1(0.8)	16.0(0.6)	16.1(0.5)
17:1	0.0	0.0	0.0	0.1(0.1)	1.1(0.9)	0.2(0.2)	5.6(1.7)
18:3	0.1(0.1)	0.0	0.1(0.1)	0.5(0.1)	0.5(0.1)	0.0	0.9(0.2)
18:2	22.7(0.7)	22.0(0.7)	17.7(0.8)	18.9(0.8)	19.5(1.1)	21.2(1.0)	18.3(0.9)
18:1 ω9	10.7(0.2)	9.5(0.4)	8.4(0.4)	8.4(0.1)	9.0(0.5)	11.9(0.4)	8.9(0.2)
18:1 *	0.0	0.0	0.0	0.1(0.1)	0.9(0.2)	0.0	1.7(0.3)
18:0	1.8(0.3)	1.7(0.0)	1.5(0.0)	1.1(0.1)	0.9(0.1)	1.4(0.3)	1.2(0.1)
19:1	11.1(0.5)	8.5(0.5)	9.8(0.4)	11.0(0.6)	12.3(1.0)	14.1(0.6)	9.7(1.1)
18:0 2OH	4.8(0.2)	3.5(0.1)	2.7(0.1)	3.1(0.3)	3.6(0.2)	4.1(0.1)	3.1(0.2)

Table 3 (cont'd).

Fatty acid:	Control	*Acaulospora mellea*	*Glomus etunicatum*	*Glomus intraradices*	*Glomus occultum*	*Gigaspora rosea*	*Scutellospora erytropa*
18:0 3OH'	0.0	0.0	0.0	0.2(0.1)	0.0	0.1(0.1)	0.5(0.1)
20:1 ω9	0.0	0.0	0.0	0.0	0.0	0.6(0.4)	0.0
20:0 iso	1.9(0.3)	3.0(0.3)	1.5(0.2)	1.7(0.5)	2.1(0.6)	0.2(0.2)	1.8(0.2)
20:0	8.2(0.2)	6.3(0.3)	6.4(0.4)	4.1(0.3)	4.9(0.5)	5.4(0.3)	4.4(0.3)
21:0 iso	5.1(0.4)	4.9(0.3)	5.9(1.2)	6.9(0.4)	5.4(1.0)	7.7(0.3)	4.4(0.6)
21:0	14.8(0.4)	9.9(0.4)	12.0(0.7)	11.4(0.5)	17.8(0.8)	15.9(0.8)	16.1(1.1)
22:0	0.8(0.3)	1.0(0.2)	1.0(0.2)	0.0	0.1(0.1)	0.4(0.4)	0.2(0.1)
24:0	4.2(0.1)	2.4(0.2)	2.6(0.2)	0.0	0.2(0.1)	0.4(0.4)	0.2(0.0)

Values are average (standard error). Only the fatty acids accounting for at least 0.5% are shown. n= 6 cultures. The values in the top row indicate the concentration of total FAME in the sample, while the rest of the rows indicate the percent of the total FAME accounted by the specific molecule.
*- This is an unresolved mixture of several 18:1 fatty acids.

Table 4. PLFA of mycorrhizal and non-inoculated *Sorghum* roots.

fatty acid:	control	*Acaulospora mellea*	*Glomus etunicatum*	*Glomus intraradices*	*Glomus occultum*	*Gigaspora rosea*	*Scutellospora erytropa*
total µmol/g	0.5(0.0)	0.9(0.1)	0.9(0.1)	0.6(0.1)	0.5(0.1)	0.7(0.1)	0.7(0.1)
mol%:							
I15:0	0.7(0.2)	0.8(0.1)	0.9(0.2)	0.6(0.2)	0.6(0.1)	0.6(0.1)	1.2(0.5)
15:0	0.5(0.1)	0.9(0.1)	0.6(0.1)	0.5(0.1)	0.6(0.1)	0.7(0.1)	0.7(0.1)
16:1ω7	1.2(0.1)	0.9(0.1)	0.9(0.1)	1.3(0.2)	2.5(0.5)	0.9(0.1)	1.9(0.5)
16:1ω5	0.6(0.1)	1.6(0.1)	2.9(0.3)	0.9(0.1)	0.5(0.1)	2.4(0.1)	2.2(0.2)
16:0	28.9(1.7)	33.9(0.4)	29.8(1.0)	29.8(1.5)	27.0(2.4)	31.2(1.4)	29.9(1.4)
cy17:0	1.7(0.1)	0.6(0.1)	0.6(0.1)	1.1(0.1)	1.2(0.2)	0.6(0.1)	1.3(0.1)
18:2ω6	35.0(1.2)	35.5(0.8)	36.4(0.9)	35.2(1.4)	34.7(1.4)	33.6(2.3)	33.4(1.6)
18:3ω3	5.5(0.1)	5.2(0.1)	5.7(0.3)	5.8(0.4)	4.1(0.2)	5.8(0.4)	4.2(0.1)
18:1ω9	11.4(0.9)	8.8(0.3)	9.8(0.1)	11.1(0.5)	9.4(0.4)	10.4(0.7)	8.3(0.2)
18:1ω7	3.9(0.3)	4.1(0.2)	4.3(0.2)	3.8(0.3)	5.4(0.4)	4.8(0.4)	4.1(0.5)
18:0	3.0(0.2)	1.9(0.1)	2.0(0.1)	2.5(0.1)	2.3(0.1)	2.3(0.1)	2.3(0.1)
cy19:0	2.6(0.2)	0.9(0.1)	1.0(0.1)	1.5(0.2)	2.0(0.2)	1.2(0.1)	1.5(0.1)
20:4ω3	0.1(0.0)	1.5(0.1)	0.7(0.0)	0.0	0.0	1.2(0.1)	0.0

Table 4 (cont'd).

fatty acid:	control	*Acaulospora mellea*	*Glomus etunicatum*	*Glomus intraradices*	*Glomus occultum*	*Gigaspora rosea*	*Scutellospora erytropa*
20:1ω9	0.2(0.0)	0.2(0.0)	0.3(0.0)	1.3(0.2)	0.2(0.0)	0.3(0.0)	0.2(0.0)
22:0	1.3(0.2)	0.7(0.2)	1.1(0.1)	1.1(0.2)	2.0(0.3)	1.1(0.1)	1.7(0.1)
24:0	3.5(0.5)	2.4(0.2)	3.0(0.2)	3.5(0.3)	7.6(1.1)	3.0(0.3)	7.1(0.5)

Values are average (standard error). Only the fatty acids accounting for at least 0.25% are shown. n= 6. The values in the top row indicate the concentration of total PLFA in the sample, while the rest of the rows indicate the percent of the total PLFA accounted by the specific molecule.

Table 5. Sterols of mycorrhizal and non-inoculated *Sorghum* roots.

sterol:	control	*A. mellea*	*G. etunicatum*	*G. intraradices*	*G. occultum*	*G. rosea*	*S. erytropa*
total ηmol/g	35.0(15.8)	37.4(7.5)	30.0(11.6)	68.8(20.8)	89.7(40.1)	24.7(3.4)	54.6(29.0)
mol%:							
Campesterol	18.9(0.8)	23.3(1.4)	25.8(0.9)	29.1(4.1)	31.8(1.4)	25.7(1.5)	24.7(1.1)
Stigmasterol	37.2(3.8)	38.9(1.0)	37.4(1.6)	37.4(6.1)	48.3(1.6)	32.6(1.9)	46.5(2.5)
Stigmastanol	6.5(1.5)	5.4(0.9)	5.0(0.8)	4.5(1.2)	0.0	4.8(0.9)	3.1(0.5)
Sitosterol	25.4(2.1)	26.1(1.1)	25.0(1.3)	24.7(2.4)	18.0(0.6)	30.7(3.1)	19.8(0.7)
unknown-1	8.2(1.0)	4.8(0.9)	4.0(0.4)	3.1(0.5)	1.9(0.8)	4.4(1.2)	3.6(0.6)
unknown-3	3.8(1.0)	1.6(0.4)	2.8(0.8)	1.1(0.5)	0.0	1.8(0.3)	2.3(0.5)

Values are average (standard error). n= 6. The values in the top row indicate the concentration of total sterols in the sample, while the rest of the rows indicate the percent of the total sterols accounted for by the specific molecule.

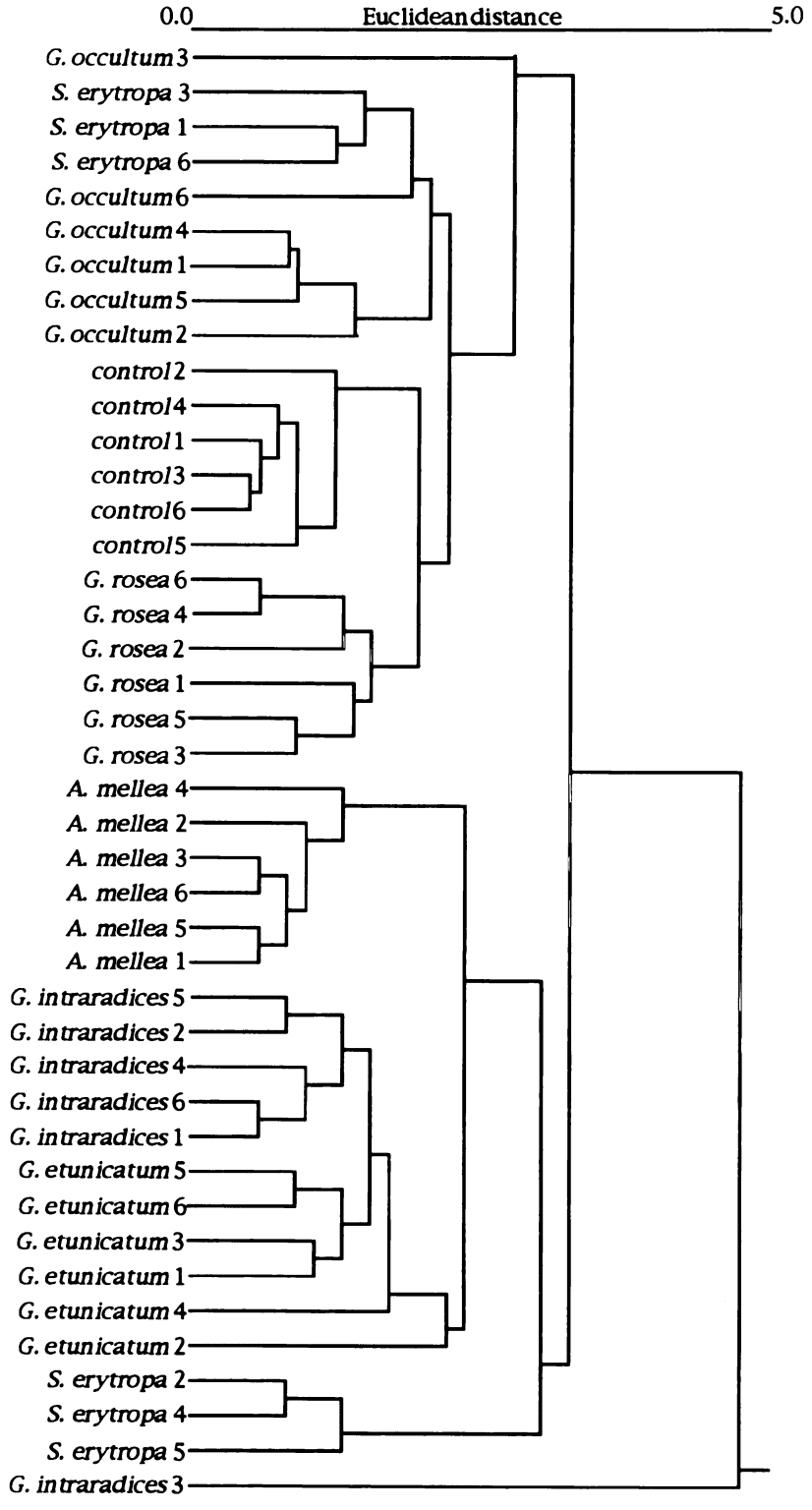

Figure 1. Hierarchical cluster analysis of the FAME profiles.

Chapter 3

PATTERNS OF CARBON ALLOCATION AND FATTY ACID TURNOVER IN MYCORRHIZAL *SORGHUM*

Abstract

Radioactive tracers were used to study the carbon allocations to plant tissues, to below-ground respiration, and to soil in the *Sorghum bicolor-Glomus clarum* mycorrhizal association. *Sorghum bicolor* L. Moench was grown in sterile soil or inoculated with the arbuscular-mycorrhizal fungus (AMF) *Glomus clarum* Nicolson & Schenck. Mycorrhizal and non-mycorrhizal plants 51 day old, were subjected to a 3 hour $^{14}CO_2$ pulse exposure followed by a 24 day chase period with sequential harvesting. Infected plants assimilated 21 % more ^{14}C than non-mycorrhizal plants, and had a higher percentage and absolute allocation of ^{14}C to root tissue, below-ground respiration, and soil. Mycorrhizal plants had a higher content of total lipids and total fatty acids. The mycorrhizal fatty acid 16:1 ω 5 comprised up to 29.5 % of the total fatty acid content of mycorrhizal roots. Non mycorrhizal roots had only trace levels of this molecule. The pulse-chase experiment demonstrated that the lipids of mycorrhizal roots are a dynamic pool of C with measurable incorporation and turnover of ^{14}C. Thin layer chromatography was used to physically separate the fatty acids

extracted from the roots. The radiolabel content of particular fatty acids was determined by radiography. The ^{14}C turnover time of the mycorrhizal fatty acid 16:1 ω5 was calculated at 210 h^{-1}. The lipids of non-mycorrhizal roots incorporated less radiolabel and there was not a distinct turnover of the assimilated ^{14}C, underscoring the difference in the lipid C cycle between the arbuscular mycorrhizae and non-mycorrhizal roots.

Introduction

A majority of the land plants are associated with mycorrhizal fungi, and the allocation of C from host to fungus is a fundamental part of the economy of the mycorrhizal symbiosis. It has been shown the infection with mycorrhizal fungi increases the flow of fixed C to the root system and soil (Pang and Paul, 1980; Snellgrove *et al.* 1982; Jakobsen and Rosendahl, 1990). For this reason, roots and their associated mycorrhizal fungi are an important supply of reduced C to soil ecosystems and affect the amount of C compounds available to the soil biota (Finlay and Söderstrom, 1992). This increase in the quantity of C available to soil organisms influences root interactions with pathogens, decomposers, the grazing of roots and mycelium by fauna, and soil aggregation (Finlay and Söderstrom, 1992).

The arbuscular-mycorrhizal fungus (AMF) appears to be exclusively dependent on the host plant for it's C nutrition (Ho and Trappe, 1973),

and it has been observed that the fungus will rely on it's own C reserves when separated from the host plant. For example, Olsson *et al.* (1993) observed a reduction in the storage lipids of the fungus after harvesting the host and incubating the mycorrhizal biomass in the soil. The AMF has an extraradical phase that increases the absorptive area of the root system, and an intraradical phase that acts both as the exchange surface between the symbionts and as the carbon storage compartment of the fungus. The amount of C that is allocated to the intraradical phase of the AMF has never been measured directly because the close association of the root and the fungus limits the feasibility of a physical separation (Tinker *et al.* 1994). Because of this, little is known regarding the C cycle of arbuscular-mycorrhizal roots (AM) and the associated fungus.

Plants contribute up to 50% of their photoassimilated C to their roots and ultimately to the below-ground ecosystem (Warembourg and Paul, 1973). The mycorrhizal fungus is an important factor both in the retention and loss of C in terrestrial ecosystems since the AMF can act as a net C immobilzer, while mineralizing C at a high rate. For example, Horwath (1993) hypothesized that slow root turnover values in AM are explained by immobilization of C by the fungal symbiont. However, the molar growth yield of the AMF has been proposed to be low because of the C cost of active transport and the associated high respiratory loss that results in a relatively short permanence of C in the AMF biomass (Harris and Paul, 1987).

Lipids play an important role in the C economy of the arbuscular mycorrhizal fungus. Ericoid mycorrhizal fungi accumulate C in carbohydrates such as glycogen and mannose (Stribley and Read, 1974). Fungal-specific sugars are not abundant in AMF, and lipid synthesis may have an analogous role to the sugars of the ericoid fungi. For example, Cox *et al.* (1975) showed the incorporation of photoassimilated ^{14}C in lipid droplets of AMF cells. Mycorrhizal roots contain high amounts of several fatty acids that are absent in non-mycorrhizal roots (Nagy *et al.*, 1980; Nordby *et al.*, 1981). The higher lipid content of mycorrhizae has an effect on the C dynamics of the roots. Infected roots may have higher construction costs than non-mycorrhizal roots because of the increased lipid content (Eissenstat *et al.*, 1993). Specifically, fatty acid 16:1 ω5 has been proposed as an indicator of the C cost of the mycorrhizal symbiosis (Graham and Hodge, 1993), since this molecule is a predominant component of the cell mass of the AMF (Beilby, 1980; see chapter 2 of this thesis).

Carbon turnover is a result of the exchange of C into and out of a biological compartment (Robertson, 1957). The C turnover time is the amount of time required to replace the C present in a C pool. The C turnover time is an important variable because it may indicate if the C pool of interest is acting as a long-term storage pool. Determining the turnover of AMF in roots is necessary for understanding the role of these microbes in the retention and cycling of C. The AMF lipids offer the

opportunity to determine the turnover of an important biomass component of the AMF by a pulse-incorporation of radiolabeled C, followed by sequential measurements of the radioactive signal in specific lipids.

The objectives of this experiment are: 1) Measure the patterns of C incorporation and retention in the plant tissues, root lipids, soil, and soil atmosphere of mycorrhizal and non-mycorrhizal *Sorghum*, 2) Measure the turnover of C in the fungal-specific lipids of the mycorrhizal roots.

Materials and Methods

Environmental Conditions and Inoculation

Plants were grown in 2 Kg of sieved (5 mm) sandy loam of pH 6.2, cation exchange capacity 4.5 me/100 g, 1.2 % by weight organic matter, 12.5 mg kg^{-1} total P, 88.0 mg kg^{-1} K, 647.5 mg kg^{-1} Ca, 121.5 mg kg^{-1} Mg. The soil was collected from the Ap horizon of a Kalamazoo loam from Kellogg Biological Station, Michigan. The soil was irradiated with cobalt-60 (13 hours with a gamma dose rate of 3826.1 Gy/hr) and stored at -20°C until the start of the experiment. Each pot received 300 ml of nutrient solution (1.5 mM $CaCl_2$, 0.5 mM K_2SO_4, 2.5 mM $NH4NO_3$, 0.25 mM $MgSO_4$, 25 µM H_3BO_3, 20 µM FeDDHA, 20 µM $ZnSO_4$, 0.5 µM $CaSO_4$, 0.4 µM $H2MoO_4$, and 0.6 µM $CoCL_2$, pH to 6,8 with KOH). Each pot was watered to field capacity with distilled water every two days.

Surface-sterilized seeds of *Sorghum bicolor* L. Moench (70% ethanol for 30 sec., then 20% commercial bleach for 20 minutes, then washed with distilled water) were germinated in a petri dish for two days over a sterile moist filter paper before planting. Two seeds were planted per pot and thinned to one after the first week of the experiment.

The pots in the mycorrhizal treatment (M) were inoculated with 50 g of *Glomus clarum* Nicolson & Schenck (INVAM BR147B-4) by adding a mixture of infected roots and soil-borne spores. The non-mycorrhizal treatment (NM) received a chlamydospore-free and root-free filtrate of the mycorrhizal inoculum. All M and NM plants were inoculated with *Azospirillum braziliense* (ATCC # 29729). The bacteria were grown in PSS (Hylemon *et al.*, 1973) at 30 °C for 72 h and a 1.0 ml cell suspension (0.015 g ml^{-1}) was added to the soil 7 days after planting.

A total of 40 plants (20 M and 20 NM) were placed inside a 5.43 m^3 Plexiglas chamber with a sealed wood frame and base. The plants were grown with natural light supplemented with high pressure sodium lamps placed outside the chamber. The photosynthetically active radiation ranged from 250 to 1200 $\mu M\ m^{-2}\ s^{-1}$ during the 16 hour photoperiod. The temperature ranged from 23 to 29 ^{o}C.

$^{14}CO_2$ Pulse Labeling

Plants were subjected to a single exposure of $^{14}CO_2$ 51 days after the start of the experiment. A total amount of 92 μM of labeled C in the

form of $Na_2{}^{14}CO_3$, with a specific activity of 154.2 MBq/mg C was used. The labeled $Na_2{}^{14}CO_3$ was mixed with unlabeled Na_2CO_3 to form a solution of 0.77 M. The concentration of the CO_2 in the chamber was maintained at ambient concentration manually by monitoring the internal CO_2 concentration with an infra-red gas analyzer and producing CO_2 as needed. The $^{14}CO_2$ was produced by reacting the Na_2CO_3 solution with an excess 85% lactic acid. A total of 170.2 MBq were added to the chamber during a 3 hour period. The canopy was opened at the end of the pulse-labeling and the atmosphere within the canopy was purged to the outside of the greenhouse at a rate of 17.7 m^3 hr^{-1}.

Harvests

Five harvests were carried out 1, 3, 6, 12, and 24 days after the $^{14}CO_2$ exposure. At each harvest time, the shoots were separated from the roots by clipping and the whole cylinders and shoots were placed at -20°.

Plant Biomass

The shoots were dried at 65 °C for 24 h and their mass was determined gravimetrically. After the biomass determination, the shoots were ground and stored at 5 °C until the nutrient and biomass analyses were carried out. The roots were separated from the soil by wet sieving. After washing, the material was freeze dried and the biomass of the fine (< 1 mm diameter), and coarse (> 1 mm diameter) roots was recorded.

The freeze-dried roots were stored at 4°C until the lipid and radioactivity analyses.

Shoot Phosphorus and ^{14}C Content

The shoots of the first harvest were analyzed for P content. Samples of 0.5 g were ashed (500 °C for 5 hours). The ashes were digested for 1 hr in 25 ml 3N HNO_3 in 1000 mg x kg^{-1} LiCl. The digests were filtered and mixed with a 0.3 N NaOH solution (1:9 vol:vol). These were then analyzed colorimetrically with a Flow injection Analyzer (Lachat Chemicals Inc., Wisconsin, USA) (Murphy and Riley, 1962). The mean total phosphorus in the shoot of the M and NM plants was statistically indistinguishable as planned. M plants had a mean P content of 12.2 mg, while that of the NM was 10.3 mg.

Ground shoot subsamples of known weight were analyzed for specific activity and C content. The samples were combusted with a biological sample converter (Europa Scientific Roboprep-CN) in series with a mass spectrometer (Europa Scientific 20-20 Stable Isotope Analyzer). The mass spectrometer provided the area under the curve for the CO_2 peak. The C content of the samples was determined by comparison of the sample areas to those generated by sucrose standards of known mass. The CO_2 evolved by combusting the samples was trapped in Carbon 14 Cocktail (R. J. Harvey Instrument Co., Hillsdale, N. J.) and the radioactivity was measured by liquid scintillation. The

effectiveness of the traps was estimated by combusting and trapping the CO_2 from samples of ^{14}C leucine of known mass and specific activity.

Root ^{14}C Content and Lipid Analyses

Coarse and fine root subsamples were cut, mixed, and analyzed for the radiolabel content in the same way as the shoot material. The specific activity of the coarse and fine roots was statistically equal (data not shown). The total radiolabel content of the root system was determined by the adding the values of the coarse roots and fine roots.

Appendix B is a flowchart that represents the sampling and analysis scheme for the root lipids.

The presence of mycorrhizal infection was determined by the measurement of molecular marker fatty acid 16:1 ω5. Samples of 0.02 g dry weight were analyzed using the methodology and software of the Microbial Identification System (Microbial ID, inc., Newark DE). Samples of freeze-dried fine roots (0.02 g) were placed in a glass centrifuge tube with a Teflon-lined screw cap. Two ml of saponification reagent (150 g NaOH in 1L 50% MeOH) were added to each tube. The tubes were vortexed briefly, and heated at 100°C. After the 30 minutes, the tubes were cooled to room temperature in a water bath. At this point, 50 µl of an internal standard (15:0, 0.001 g/ml in hexane) was added to the saponified mixture. The methylation was carried out by adding 4.0 ml of 54% 6N HCL in MeOH to each tube. The tubes were then vortexed

briefly, heated to 80°C for 10 min. in a water bath and immediately cooled to room temperature.

The fatty acid methyl esters were extracted by adding 1.25 ml of 1:1 hexane: methyl-tert butyl ether and rotating the tubes end over end for 10 min. The organic phase (top) was removed with a Pasteur pipette into a 13x100 screw capped glass culture tube. A second extraction of the remaining root debris was carried out and the organic phases were combined. The pooled extracts were washed by adding 3.0 ml of 1.2% NaOH in H_2O to each tube, and turning for five minutes. If necessary, the tubes were centrifuged at low rpm for a clear separation of the phases. The organic phase was removed, transferred to a glass 13x100 culture tube, and dried under a stream of N_2. The dry samples were stored at -20°C. Before analysis, the samples were resuspended in 1.25 ml of 1:1 hexane: methyl-tert butyl ether and placed in crimp-top GC vials. The fatty acids were quantified based on the relative mass response of the detector to the standard peak.

Fatty acid terminology utilizes "A:B ωC" where "A" indicates the total number of carbon atoms, "B" the number of unsaturations, and "ω" antecedes the number of carbon atoms between the nearest unsaturation and the methyl end of the molecule. The suffixes "c" and "t" indicate cis and trans isomers. The prefixes "i" and "a" refer to iso and anteiso methyl branching.

The total lipids of root subsamples (30 mg) were obtained using the method of Bligh and Dyer (1959). The extract was dried under N_2 and re-suspended in 1 ml of chloroform. A subsample of known volume was placed in a glass fiber filter of known mass (type A/E, Gelman Sciences, Ann Arbor, U.S.A.) and dried at 75 °C for one hour. The weight of the lipid subsample was measured gravimetrically and the specific activity of the lipid material in the filter was determined using the same procedure as for the plant biomass. With these data, the lipid mass and radioactivity per unit of root weight were calculated. A second subsample of the total lipids was taken for the measurement of the radiolabel content of the root fatty acids.

The fatty acid methyl esters (FAME) were obtained by methylating the lipid extract using the procedure of Morrison and Smith (1964). The methylation product was dried and re-suspended in hexane. Subsamples of the methylation product were taken for liquid scintillation analysis and argentation TLC. The argentation TLC allows for a physical separation of the FAMEs by the number of double bonds and C chain length (Morris, 1966). The plate preparation, developments, and visualization were carried out following the methods detailed by Cahoon and Ohlrogge (1994). The FAMEs bands were identified by comparison with authentic standards (16:1, 18:1, 20:1 and 22:1 Sigma Chemical, St. Louis U.S.A.). A commercial standard for fatty acid 16:1 ω 5 was not available. For this purpose the FAME extract from a

E. coli clone (pDES 16) that produces 16:1 ω5, 16:1 ω7, and 18:1 ω9 as the dominant monoenoic fatty acids was included (Schultz *et al.*, 1996). The argentation TLC achieved a full resolution of saturated, dienoic, 16:1 ω5, 16:1 ω7, 18:1 ω7, 18:1 ω9, and 20:1 FAME. The plates were analyzed by radiography using a Packard Instant Imager (Packard Instrument Company, Illinois, U.S.A.) at a scan time of 1.5 h to measure the radiation contributed by each fatty acid band. Up to three main bands corresponding to 16:1 ω5, saturated fatty acids, and polyenoic fatty acids were observable in the radiographs (Figure 1). Some bands which were not visualized produced a detectable radioactive band in the radiogram and were quantified. The readings of the liquid scintillation analysis of the same extracts as those used for the TLC plates were used to calculate the radioactivity per unit of root mass contributed by the different FAME bands.

Transport of a substance (such as C) into and out of a compartment (such as 16:1 ω5 fatty acid) results in the incorporation and turnover of C in 16:1 ω5. The turnover time is the interval of time necessary for the amount of C that goes in and out of 16:1 ω5 to be equal to the total amount of C present in 16:1 ω5 (Robertson, 1957). The turnover of the labeled C in the mycorrhizal fatty acid 16:1 ω5 was calculated using four assumptions: 1) A constant amount of 16:1 ω5 in the roots (referred to as the steady state), 2) A single pool of 16:1 ω5 with a decomposition curve of one slope, 3) No recycling of the label

between 16:1 ω5 and other pools, and 4) The maximum specific activity of the lipid occurs 24 hours after labeling and corresponds to the time of the first harvest. Given these assumptions, the turnover of ^{14}C in the 16:1 ω5 fatty acid should conform to the exponential first order function:

$$A_t=A_i(e^{-kt})$$

Where A_i is the net increase in labeled C in 16:1 ω5 after incorporation in terms of mass of ^{14}C per root; A_t is the mass of the tracer in 16:1 ω5 at time t measured in mass of ^{14}C per root; k is the turnover rate constant in units of inverse time, t is the time between the end of the pulse labeling and the measurement. The turnover rate constant k is the slope of the line defined by the line of Ln A_t against time. The turnover time is $1/k$. The turnover rate (ϕ_i) is calculated using the relationship:

$$\phi_i=kM_i$$

Where M_i represents the total mass of 16:1 ω5 in the root system. For example, using the average mass of 16:1 ω5 for the first harvest (8.95 mg), I calculated a C turnover rate of -42.5 μg plant^{-1} h^{-1}.

Below-Ground Respiration

The soil atmosphere was sampled from a port located at the bottom of the cylinder. The below-ground respiration of the 8 plants of the last harvest was measured starting at 8 hours after the pulse label.

The soil atmosphere was flushed for 10 minutes before the first sampling period, then each pot was sampled by continuously extracting air from the below-ground airspace at a rate of 0.02 m^3/hour. The CO_2 was trapped with 350 ml of 3 M NaOH, and the Bq/hour were estimated using a Packcard 1500 Tri-carb Liquid Scintillation Analyzer. Six sampling periods of 8 hours were carried out sequentially, with one hour intervals between samplings. The below-ground atmosphere was not sealed from the above ground atmosphere. This system assumes that the atmosphere sampled from the below-ground sampling has negligible exchange with the atmosphere from the above ground canopy. This was achieved by flushing the atmosphere of the above ground canopy with $^{14}CO_2$-free air at a rate of 17.7 m^3 hr^{-1} concurrently with the sampling of the below-ground respiration.

Soil

Soil subsamples were observed under a dissecting microscope and all fine roots were removed. Root-free soil samples were dried at 65 °C and the specific activity of the soil C was determined in the same manner as the plant biomass. To test for the presence of radiolabel in soil carbonates, randomly selected soil samples were acidified (0.5 ml 1N HCL per gram of soil) and their specific activity was compared to non-acidified samples. No detectable amount of soil ^{14}C was present in the

form of carbonate. The measured radiolabel in the soil is therefore accounted for by the cellular and extracellular organic matter.

All the soils from the M treatment were sampled for the concentration and radioactivity of mycorrhizal spores. The spores were obtained from 15 g soil samples by wet sieving (38 μm mesh), followed by the method of sucrose-gradient centrifugation modified from Daniels and Skipper, (1982). The soil was sieved by washing with tap water through a 420 μm sieve into a 38 μm sieve that collected the spores. The spores were then transferred with tap water from the 38 μm sieve into a 50 ml centrifuge tube, and the spore suspension was centrifuged (5 min. at 2000 rpm) using a swinging bucket type rotor. The water and root debris in the supernatant were decanted, and the spore pellet was re-suspended in 20 ml of 45 % sucrose in H_2O. The spore suspension was then balanced and centrifuged for 1.5 minutes at 2000 rpm. The sucrose solution containing the spores and hyphae was decanted into a clean centrifuge tube and centrifuged for 1.5 min. at 2000 rpm. The supernatant containing the spores was transferred into a 38 μm sieve and the spores were rinsed with distilled water. The spores were then transferred into a petri dish for counting under a dissecting microscope. After this, the number of mycorrhizal spores per gram of soil was calculated. The spores were separated from the solution by filtration (Gelman Sciences type A/E glass fiber filter, Ann Arbor, MI), dried (65°C,

24h). The spores with the glass filter were then analyzed for the specific activity in the same way as the soil and plant material.

Statistical Analysis

The pots were placed in four blocks as a Split-Plot design with the main plots determined by harvest time and the sub-plots determined by the mycorrhizal treatments. The main plots and sub-plots were completely randomized. The harvest times and mycorrhizal addition were analyzed as treatments using the Split-Plot Analysis of Variance of SAS version 6.11 (SAS Institute Inc., Cary, N. C., U. S. A.). n= 4 plants per mycorrhizae and harvest treatment combination.

Results and Discussion

Carbon Allocation

The infection by *G. clarum* of the *Sorghum* plant affects the amount of C assimilation and the distribution of the fixed ^{14}C to the *Sorghum* tissues and soil. On average, M individuals assimilated a total of 21 % more labeled C than the NM (Table 1). The ^{14}C allocation to the below-ground portion of the individuals is the sum of the amount of ^{14}C recovered from the root biomass, the soil, and the below-ground respiration. In this experiment, mycorrhizal infection is associated with a higher C incorporation plus an increased below-ground C allocation. The M plants allocated an average of 2.2 MBq below-ground; the average

for the NM plants was 1.5 MBq (Table 1). Mycorrhizal individuals allocated 52.1 % of their fixed ^{14}C to below-ground, while the NM plants distributed 43.5 % of their label below-ground (Table 1). Other pulse-chase experiments to study the transfer of C from host to fungus have shown that mycorrhizal plants allocate more of their C fixation to the mycorrhizal roots when compared to non-mycorrhizal plants (Pang and Paul, 1980; Snellgrove *et al.* 1982; Jakobsen and Rosendahl, 1990). These experiments involved different growth media, pulse and chase periods, host-fungus combinations, and plant ages. Despite the differences in experimental design, all of these studies observed distinct effects of the mycorrhizal fungus on the C assimilation and allocation (Tinker *et al.*, 1994).

The difference in the ^{14}C allocation below-ground between M and NM individuals can be used to quantify the additional diversion of C to the root/soil system caused by the fungal symbiont. This should be a legitimate comparison for this experiment, since the total shoot phosphorus content of the M and NM plants is statistically equal. This suggests that the differences in allocation pattern between the M and NM should be due to the C sink imposed by the fungus and not by differences in the nutritional status of the M and NM. My results demonstrate that infection with *Glomus clarum* is associated with an additional 0.7 MBq of labeled C distribution to below-ground (Table 1). This represents 15 % of the total fixed ^{14}C of the M plants and is the C

demand imposed by the fungal symbiont. The 15 % increased allocation of C to mycorrhizae may be accounted for by several factors: a) Fungal respiration, b) Root respiration, c) Allocation to mycelial biomass, d) Allocation to root biomass, e) Mycelial respiration (internal and external), and f) Exudation from roots or hyphae.

In this experiment, I did not measure the photosynthetic rate of the individuals directly. However, this can be estimated from the radioactivity data. Table 1 shows that the plants in the M treatment assimilated a larger amount of total label. Nevertheless, the M shoots have less biomass than the NM shoots in the first harvest (Table 2). On average, the photoassimilation rate of radiolabel by the M plants was 30 % higher than that of the NM plants (0.26 MBq g shoot^{-1} h^{-1} for the M treatment, vs. 0.20 MBq g shoot^{-1} h^{-1} for the NM treatment). The ability of plants to compensate for the needs of the mycorrhizal partner was first suggested by Paul and Kucey (Paul and Kucey 1981; Kucey and Paul; 1982). My data with a different plant type than that of Paul and Kucey further shows the ability of the plant to adapt it's photosynthetic rate to the C demand of the fungus. In this study, there was not a complete compensation, for the M shoots have less biomass than the NM (Table 2). It has been hypothesized that the effect on the photosynthetic rate is explained by a the improvement of the water balance, increased leaf tissue P, higher specific leaf area, or

phytohormones associated with mycorrhizal infection (Harris *et al.*, 1985).

Allocation to Plant Tissues

The growth of the shoots was higher than root growth during the chase period in both M and NM individuals (Table 2). This difference in shoot and root growth rate is reflected in the radioactivity measurements, which show that the shoots incorporated more radiolabel than the roots in both NM and M (Table 3). The total amount of label in the shoots and roots was relatively stable throughout the harvest periods and this was true for both the M and the NM treatments (Table 3). This is illustrated by the lack of a harvest effect for the total ^{14}C content of both shoots and roots in the analysis of variance (Table 4). This pattern indicates that the majority of the C remaining in the plants 24 h after the ^{14}C pulse was already incorporated into long-term storage or structural components and is not turned over to a significant degree. This should be expected for a rapidly growing plant that is allocating a great deal of it's photosynthate to the production of structural and long-term storage C pools.

Our results imply that the C allocation from *Sorghum* to *Glomus* is a C drain that may be regarded as a potential loss of shoot growth unless full photosynthetic compensation occurs. Non-mycorrhizal shoots had a higher biomass than the M shoots (Tables 2 and 4). However, the total

amount of radiolabel allocated to the M and NM shoot tissues is statistically indistinguishable because the specific activity of the M shoots is higher than that of the NM shoots (Table 5). Other studies show contrasting results in regards to the beneficial, or parasitic nature of mycorrhizal fungi. In some instances, previous work found that the cost associated with mycorrhizae represents a C drain that is not completely compensated for by the host's C fixation. For example, Harris et al. (1985) used $^{14}CO_2$ labeling to study the tripartite symbiosis of *Rhizobium-Glomus-Glycine*. Six week old mycorrhizal-rhizobial plants had a higher fixation rate than the controls. However, the growth rate of the symbiotic plants was less than that that of the non-inoculated controls because of the allocation of photosynthate to the symbionts. Snellgrove *et al.* (1982) found a similar kind of pattern in *Allium* infected with *Glomus*. They carried out a pulse-chase experiment and observed that mycorrhizal plants transported 7% more C to the roots, while having a reduced leaf mass relative to non-mycorrhizal plants. In contrast to the two previous references, other studies have found that mycorrhizal plants had similar or higher growth rates relative to non-mycorrhizal plants, implying that the host compensated for the C demand from the symbionts with an increased photosynthetic rate (Pang and Paul 1980; Paul and Kucey, 1981). The degree of compensation is dependent on the efficiency of the symbiosis relative to the soil-plant nutrient status. Higher fertility may result in parasitism, whereas the

AMF may act as a mutualist when it can help obtain limiting resources such as soil P. It should be possible to modify the rates of fertilizer amendment to achieve a more similar growth patterns of M and NM plants.

Mycorrhizal infection was associated to increased allocation and long-term incorporation of C to the root system. Mycorrhizal roots had significantly higher specific activity and total radiolabel content than the NM roots (Tables 3, 4, and 5). This contrasts with previous studies that did not measure an increase in the incorporation of labeled C to the root biomass of mycorrhizal plants (Paul and Kucey, 1981; Harris *et al.*, 1985).

Allocation to Below-Ground Respiration

The amount of ^{14}C recovered from the below-ground atmosphere was consistently higher in the M pots relative to the NM (Figure 2; Table 4). Each M plant evolved a total of 0.55±0.08 MBq, while NM individuals respired a total of 0.40±0.05 MBq. This agrees with previous studies that show increased CO_2 evolution of mycorrhizal plants relative to non-mycorrhizal controls using pulse-chase experiments with different plant-fungus combinations. (Pang and Paul, 1980; Kucey and Paul, 1982; Snellgrove *et al.* 1982; Harris *et al.*, 1985). For both treatments, 72% of the total below-ground respired ^{14}C was recovered within 34 hours after the pulse label. The root biomass of the M plants was statistically equal

to that of the NM (Tables 2 and 4), which suggests a higher specific respiration rate of the M plants. The observed increase in below-ground $^{14}CO_2$ recovery from the M soil may be accounted for by any one of several factors: a) An increase in the specific respiration rate of the host, b) The respiration of the AMF, c) Increased rhizosphere respiration in the M treatment, and/or d) As shown on chapter 2, mycorrhizal roots have increased amounts of fatty acids relative to uninfected roots. The biosynthesis of fatty acids is an energetically expensive process, requiring the expense of relatively high amounts of ATP and NADPH. Because of this, the production of additional fatty acids in mycorrhizal roots may be partly responsible for the increased below-ground respiration in M individuals. As stated above, soil may be a contributor to the M and NM below-ground respiration because of the observed (although non-significant) reduction of soil ^{14}C content during the 24 day chase period periods. Other studies show that the microbial respiration of root exudates, can be an important source of below-ground CO_2 production. For example, Martin and Kemp (1986) studied the translocation of C to the roots of wheat plants by pulse labeling and measurement the $^{14}CO_2$ content of the shoot, soil + roots, and rhizosphere of fumigated and untreated soil. Their results show that the rhizosphere of non-fumigated soil had increased respiration (twice) compared to the soil + root of the same treatment.

Allocation to Soil

The average specific activity of the M soil was at least twice that of the NM soil, and this difference was measured across all of the harvest periods (Tables 4 and 5). This result, combined with the observed higher accumulation of radiolabel in the M soil atmosphere, demonstrate a higher allocation of fixed ^{14}C to the M soil relative to the NM. Other studies show contrasting evidence regarding increases in soil C allocation associated with infection. Snellgrove (1982) found a marginal difference, while Jakobsen and Rosendahl (1990) found that the allocation of C to the extraradical phase in mycorrhizal cucumber was twice that of non-mycorrhizal plants and represented 3.1% of the total C fixation by the host. Paul and Kucey (1981) observed that extraradical mycorrhizal hyphae accounted for one percent of the fixed C in the *Vicia-Glomus* symbiosis, suggesting that the C allocation to fungal structures may explain in part the increased C allocation to soil. In this study, the specific activity of the M soil varies throughout the harvest period, although there is a net decrease since the soil specific activity at day 76 is lower than that measured at the first harvest (Table 5). However, this decrease is not statistically significant (Table 4), suggesting an overall C mineralization in the M and NM soils for the time frame of the chase period. The M soil shows a net decrease in total label content during the first 12 days of the chase period (from 0.4 to 0.2 MBq), followed by a net increase in labeled C during the last 12 days of the chase period (from

0.2 to 0.3 MBq) (Table 3). I hypothesize that these fluctuations may be explained by the mineralization of root exudates during the first half of the chase period followed by the turnover of mycorrhizal tissues from day 12 to 24 after the pulse.

Concentration of Lipids in Mycorrhizal and Non-Mycorrhizal Roots

The roots of the M plants had significantly higher concentrations of total lipid, total fatty acids, and fatty acid 16: 1 ω5 when compared to the controls (Table 6). The increased accumulation of lipids in mycorrhizae has been documented by previous studies (see chapter 2 of this thesis). For example, the roots of *Allium*, clover, ryegrass, and *Citrus* have a higher trygliceride concentration when infected by *Glomus* than when the roots are non-mycorrhizal (Cox and Sanders, 1974; Cooper and Lösel, 1978; Nagy et al., 1980), and the concentration of fatty acids in mycorrhizal roots may be twice that of uninfected roots (Peng et al., 1993). Several studies show that a significant fraction of the lipids of the AMF genus *Glomus* may be comprised of the unusual fatty acid 16:1 ω5 (Graham et al., 1995), and this molecule has been proposed as the principal AMF storage molecule in intra-radical vesicles (Pacovsky and Fuller, 1988). This effect in the concentration of root lipids indicates that the arbuscular fungus may be favoring the accumulation of C in the root system. As a result, the infection with an arbuscular fungus has an associated increase in the cost of root production (Eissenstat *et al.*,

1993). The effect of arbuscular mycorrhizae in the lipid composition of roots has been demonstrated for several fungus-plant combinations and different growth conditions, suggesting that the increased storage of C in roots is a widespread pattern in mycorrhizal symbioses. In spite of this, the growth efficiency of the AMF has been shown to be relatively low (Harris and Paul, 1987). My results suggests that the net effect of the AMF is to increase the C storage in root lipids, despite the high losses of C associated with the metabolism of the mycorrhizal fungus.

The average concentration of the total lipid, total fatty acids, and the molecular marker 16: 1 ω5 remained in a relatively steady state throughout the 24 day chase period (Table 6). Other studies have shown that the concentration of 16:1 ω5 fatty acid in roots increases with the formation of the storage structures of the fungus. For instance, Graham and Hodge (1993) observed a rapid accumulation of 16:1 ω5 in roots after 65 days in the *Glomus -Citrus* association, when the development of storage vesicles takes place. My results suggest that during the chase period, *G. clarum* was not concentrating stored C in fatty acids to a significant extent. However, the total amount of lipids in the roots increased because of the growth in root biomass (Table 2).

Throughout the harvest period, fatty acid 16:1 ω5 accounts for up to 29.5 % of the total fatty acid content of the M roots (Table 6). Previous studies of the *Sorghum-Glomus* symbiosis show that molar percentages of 13.3 % correspond to microscopical estimates of root

colonization of 44 % (see chapter 2 of this thesis). Assuming an overall correlation of fungal infection with the relative amounts of 16:1 ω5 in roots, I estimate that the percent infection of the M roots in this experiment exceeds 44 % root colonization. This is a reasonably high amount of mycorrhizal infection in the M roots. Arbuscular mycorrhizal fungi cannot be grown in pure culture, therefore, it is impossible to get an accurate ratio of 16:1 ω5 to fungal biomass that could be applied to the lipid results to calculate fungal weight.

Radiolabel and Fine Root Lipids

The radiolabel content of the total lipid, total fatty acids, monoenoic fatty acid, saturated fatty acids, and 16:1 ω5 fatty acid is higher in the M relative to the NM fine roots (Tables 7 and 8). The difference is pronounced during the first 12 days of the harvest period as indicated by the significant interaction between harvests and mycorrhizal treatment (Table 8). In general, the total extractable lipid, total fatty acid, and 16:1 w5 fatty acids of the M roots followed a similar pattern of high radiolabel incorporation before the first harvest, followed by a decrease in the label content throughout the chase period (Table 8). I showed in chapter 2 that mycorrhizae formation in *Sorghum* has an effects the qualitative and quantitative composition of total fatty acids, phospholipid fatty acids, and sterols. This experiment further demonstrates that the amount of C incorporation and loss from the

lipids of the roots of *Sorghum* are affected by mycorrhizal infection. The incorporation pattern of M roots is different from the ^{14}C incorporation and turnover pattern of NM roots. The results for the first harvest show that the total lipids and the different fatty acid classes of NM roots incorporated 30 % or less of the label incorporated by the corresponding fractions in the M treatment (Table 6). Furthermore, the turnover of radiolabel in NM lipids was slight or absent during the chase period, indicating that NM root lipids were acting as relatively long term C storage or structural compounds with limited net oxidation or mineralization.

The saturated fatty acids are characterized by a lack double bonds in the C chain. The most abundant saturated fatty acid in the M and NM roots in palmitic acid 16:0. Table 6 shows that the average concentration of 16:0 in M roots is higher than that of NM throughout all the harvest periods, suggesting that part of the increase in 16:0 is due to the presence of this fatty acid in the fungal biomass. The incorporation and turnover of radiolabel in the saturated fatty acids of M roots is different from that of NM roots and follows a similar pattern to the incorporation and turnover of ^{14}C in the mycorrhizal fatty acid 16:1 $\omega 5$ (Table 7). I hypothesize that this pattern is explained by the fact that a portion of the 16:0 in M roots is of fungal origin, which explains the similarity in the C cycle between 16:1 and saturated fatty acids.

Polyenoic fatty acids are characterized by having 2 or more unsaturations in the carbon chain. In the M and NM roots of *Sorghum*, the predominant polyunsaturated fatty acid is linoleic acid 18:2. The incorporation and turnover of ^{14}C in polyenoic fatty acids follows a relatively similar pattern in M and NM roots (Table 7). This contrasts with the data from the saturated fatty acids, total fatty acids, and 16:1 $\omega 5$ where M lipids incorporated more label before the first day of the harvest period and had a marked turnover of radiolabel that was not observed in the NM treatment. One major difference between polyenoic fatty acids and the rest of the fatty acid classes is that the relatively high incorporation of radio tracer between days 0 and 1 of the chase period is not observed in the M polyenoic fatty acids. Table 6 shows that the mass of 18:2 fatty acid per gram of root is mostly lower or equal in M relative to the NM roots, suggesting that the mycorrhizal fungus is not contributing significantly to the concentration of this molecule. It is for this reason that I believe 18:2 fatty acid to be mostly a component of plant biomass rather than of fungal biomass. If we accept the hypothesis that polyenoic fatty acids present in the roots of *Sorghum* are mostly of plant origin regardless of mycorrhizal infection, this may explain the similar C cycle of 18:2 fatty acid of M and NM roots.

The total lipids of M toots incorporated 97.1 KBq per plant before day 1 of the chase period, whereas total fatty acids incorporated or total of 38.2 KBq (Table 7). With these values I estimated that the total fatty

acids of M roots account for 39% of the radiolabel incorporated by total lipids between days 0 and 1 of the chase period. This implies that other non-fatty acid, lipid-soluble molecules are also responsible for the relatively high incorporation of radiolabel of M root lipids. The results from Chapter 2 support this hypothesis, since we showed that the concentration non-fatty acid lipids in the roots of *Sorghum*, such as sterols may be affected by mycorrhizal infection. In addition, other lipid soluble molecules such as waxes and carotenoids can potentially be involved in the increased ^{14}C incorporation of M roots.

Turnover of the Root Fatty Acid 16:1 ω5

The ten fold drop in the ^{14}C content of 16:1 ω5 shows that the C pool in the fatty acid 16:1 ω5 is dynamic and suggests that the mycorrhizal lipid C is either oxidized or converted to non-lipid compounds within the time frame of the chase period of 24 days. Despite the observed transience of C in the mycorrhizal lipids, the increased mass of root fatty acids in the M treatment indicates that fatty acids serve as compartments for net C immobilization in mycorrhizal roots.

The equation of the solid line in Figure 3 is: y=4.3-0.00475*(x). The slope of the solid line represents the rate constant k. Using these values, the rate constant for the turnover of C in 16:1 ω5 was determined to be -0.00475 h^{-1} (Figure 3), which gives a turnover time of 210 hours.

The turnover time of fatty acid 16:1 ω5 is within the turnover time frame of the fungal arbuscles reported by Cox and Tinker, (1976). They calculated that fungal arbuscles have a life span of 4 to 15 days within the root cells, after which time they are re-absorbed by the plant cell. My results verify the relatively fast turnover of mycorrhizal structures relative to uninfected roots. Such short turnover is probably a prerequisite to an efficient symbiotic system, since a long-term permanence of fungal material in the roots may indicate that the fungus is acting as a parasite.

We observed a relatively fast incorporation of labeled C and a subsequent slower turnover rate during the chase period (Figure 3). Pulse-chase studies of detrital microflora have shown a similar pattern of C incorporation and turnover of microbial lipids. King *et al.*, (1977) observed that labeled C is rapidly incorporated into lipids and attain a maximum specific activity within 20 hours, while the turnover of the label occurs over a much longer span of time that may last several days. This pattern of high incorporation rate followed by a slow turnover rate in the mycorrhizal fatty acid 16:1 ω5 provides insight into the C physiology of the mycorrhizal fatty acids. In systems that are in the steady state, the mass of the substance of interest remains constant, and the turnover rate must equal the incorporation rate (Robertson, 1957). The incorporation and turnover of C tracer in fatty acid 16:1 ω5 does

not follow that of a single C pool in the steady state. This may be explained several ways:

a) Increase in the C pool in root fatty acid 16:1 ω5- Table 6 shows that the concentration of fatty acid 16:1 ω5 in the roots remains constant throughout the chase period. However, the total pool of C in root fatty acid 16:1 ω5 increases throughout the experiment because of the increase in root biomass.

b) Recycling of the tracer- Recycling of tracer may occur between lipid and non-lipid pools. In fungi, carbohydrates may be converted to fat (Weete, 1980). Actetyl-CoA is the main precursor for fatty acid synthesis, and sugars are converted to Acetyl-CoA when subject to glycolysis followed by decarboxylation. The reverse process of conversion of fat to carbohydrate is also true (Weete, 1980). Triacylglycerides are an important source of energy and C skeletons in fungi. The triacylglycerides can be degraded to it's component fatty acids by enzymatic action. The C in fatty acids such as 16:1 ω5 can then be transferred to sugar molecules by the process of β oxidation followed by the glyoxylate pathway and reverse glycolysis. Besides the possibility of C recycling between fungal lipids and non-lipid molecules, there is also a likelihood that some recycling also occurs between the fungus and the host plant. There are contrasting observations regarding the role of lipids in the C economy of the host-fungus relationship. It has been hypothesized that the storage of C in fungal-specific lipids creates a

metabolic sink that favors the net movement of C to the fungal symbiont (Finlay and Söderstrom, 1992). However, others have reported the movement of fat globules from the fungus to the host's cell, suggesting that there may be a limited movement of C from fungus to host (Mosse, 1973).

c) Multiple pools of fatty acid 16:1 ω5- Fatty acid 16:1 ω5 may exist free in the cytoplasm or be bound to different lipid classes such as triacylglycerides, diacylglycerides, or phospholipids. Triacylglyceride serves as long-term storage of C, while phospholipids serve a structural function by being part of cellular membranes. I hypothesize that 16:1 ω5 obtained from whole-cell extracts is in effect obtained from a mixture of different C pools, with the possibility that each pool has a C cycle of different time span.

In this study, I isolated the extraradical spores of the arbuscular fungus to measure their concentration in the M soil, as well as their ^{14}C turnover. The spores of arbuscular fungi are rich in C storage compounds (Beilby, 1980), and should be a good index of the C storage in the fungal biomass outside of the roots. The possibility of physically separating the spore biomass from the soil should allow for the direct measurement of the incorporation and turnover of C in the fungal biomass without having to recur to the analysis of fungal-specific molecules. However, in this experiment the sporulation of the external phase of the fungus was low, with concentrations of less than 1 spore per

gram of soil recorded for all the harvest periods. Because of this, the mycorrhizal spores accounted for less than 0.8 % of all the soil radiolabel content, and I was not able to detect a distinct pattern of ^{14}C incorporation and turnover in the external phase of the mycorrhizal fungus. This information is valuable, however, because it indicates that the increased ^{14}C content of the M soil relative to the NM soil cannot be explained by fungal sporulation. Other factors such as fungal hyphae, root exudation, and C immobilization in soil saprophytes may explain the response in radiolabel content of the M soil.

Conclusions

This study shows that the infection of *Glomus clarum* affects the C assimilation and allocation pattern of *Sorghum bicolor*. The higher root respiration and large movement of ^{14}C underground is a consequence of mycorrhizal interactions. The allocation of 6.3 % of the photosynthate to soil is higher than other results obtained in our laboratory that tended to show values of 1-3%. The value of 6.3 % of soil allocation represents 18 % of the below-ground production. This is a large enough amount of C to have an effect in the activity of rhizosphere microbiota.

To this date, there are no studies that have measured the C cycle of the fungal symbiont separately from the C cycle of the host because the fungus is an integral part of the mycorrhizal root. The separation of the mycorrhizal fatty acids is useful from both a quantitative and

qualitative standpoint, since it allows for the first time the calculation of the turnover of an important cytoplasmic component of the arbuscular fungus. 16:1 ω5 is not a long term structural molecule and does not necessarily give a total turnover of the AMF biomass. However, fatty acids account for a high percentage of the AMF cytoplasm, so the turnover rate of the mycorrhizal fatty acids represents the C turnover an important C pool of the fungus. In this experiment I measured the incorporation followed by a sustained decrease in the content of ^{14}C of the 16:1 ω5 fatty acid during the 24 day chase period, which results in a ^{14}C turnover time in 16:1 ω5 of 210 hours. This pattern is absent in the non-mycorrhizal roots and represents an important difference in the C cycle of the mycorrhizal fungus and the host. This technique opens the possibility of further experiments to study environmental impacts on the turnover of mycorrhizal components.

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Table 1. Average distribution of labeled C per individual for the 24 day harvest period.

Treatment:	M		NM	
	total MBq	% allocation	Total MBq	% allocation
Shoot	2.2 (0.1)	47.9 (1.3)	2.1 (0.1)	56.5 (1.2)
Root	1.3 (0.1)	28.9 (1.9)	1.0 (0.1)	27.3 (1.9)
Soil	0.3 (0.0)	6.3 (0.9)	0.1 (0.0)	2.4 (0.3)
below-ground respiration *	0.6 (*)	11.9 (0.5)	0.4 (*)	10.6 (1.2)
Shoot respiration**	0.2 (**)	5.0 (0.2)	0.2 (**)	6.1 (0.7)
Total per individual	4.6 (0.2)		3.8 (0.2)	

The numbers are the average for the five harvests. The standard error of the mean is in parenthesis.
*- Calculated using the recovery of respired ^{14}C between 8 and 59 hours after the pulse label. Only the plants of the last harvest were sampled for below-ground respiration. Mycorrhizal plants had an average below-ground respiration of 0.55 MBq, while NM pants had an average of 0.40 MBq. The values are assumed equal for the individuals of all the harvest periods.
**- The shoot respiration was not determined separately for each individual. To calculate the shoot respiration, I assumed that the radiolabel which is not accounted for by the shoot, root, soil, and below-ground respiration is the shoot respiration. Another assumption is that the shoot respiration was uniform for all M and NM individuals. This way, I estimate a maximum value of 0.2 MBq of shoot ^{14}C respiration that is uniform for all plants.

Table 2. Plant biomass (g) after the pulse-label.

Day:	52		54		57		64		76	
	M	NM	M	NM	M	NM	M	NM	M	NM
Shoot biomass	5.8 (0.1)	6.4 (0.1)	6.1 (0.4)	6.7 (0.3)	7.1 (0.3)	7.6 (0.4)	9.6 (0.1)	9.7 (0.5)	14.7 (0.5)	16.9 (1.3)
Fine root biomass	5.6 (0.3)	5.6 (0.4)	5.1 (0.5)	5.2 (0.5)	5.4 (0.7)	4.8 (0.4)	5.0 (0.4)	5.3 (0.3)	7.4 (0.1)	6.2 (0.4)
Total root biomass	7.4 (0.5)	8.1 (0.6)	6.7 (0.5)	7.4 (0.5)	7.4 (0.8)	7.2 (0.4)	7.0 (0.4)	8.2 (0.3)	10.9 (0.2)	10.0 (0.4)

The values are the average (standard error of the mean) of each plant. n= 4 plant per treatment combination. M= inoculated; NM= non-inoculated. The plants were 51 day old at the time of the pulse exposure to $^{14}CO_2$.

Table 3. Total radiolabel content (MBq per plant) of the plant tissues and soil.

Day:	52	54	57	64	76
			Shoot		
M	2.2 (0.2)	2.6 (0.3)	2.2 (0.2)	2.0 (0.1)	2.1 (0.2)
NM	2.1 (0.4)	2.2 (0.1)	2.1 (0.1)	1.7 (0.2)	2.4 (0.3)
			Whole root:		
M	1.5 (0.6)	1.4 (0.1)	1.5 (0.4)	1.2 (0.3)	1.2 (0.3)
NM	1.0 (0.2)	1.0 (0.1)	1.0 (0.3)	1.1 (0.4)	1.2 (0.2)
			Fine root:		
M	1.1 (0.5)	0.9 (0.1)	1.1 (0.3)	0.7 (0.1)	0.9 (0.3)
NM	0.6 (0.1)	0.7 (0.1)	0.7 (0.2)	0.5 (0.1)	0.8 (0.1)
			Soil:		
M	0.4 (0.1)	0.3 (0.1)	0.2 (0.1)	0.2 (0.1)	0.3 (0.1)
NM	0.2 (0.1)	0.1 (0.0)	0.1 (0.0)	0.1 (0.2)	0.1 (0.0)

The values are the average (standard error of the mean) of each individual. n= 4 plant per treatment combination. M= inoculated; NM= non-inoculated. The plants were 51 day old at the time of the pulse-exposure to $^{14}CO_2$. nd= not determined.

Table 4. Results of the ANOVA for the plant biomass, soil, and respiration variables.

	Harvest	Mycorrhizae	Harvest × Mycorrhizae
Variable:			
Shoot biomass (g)	+	+	-
Shoot specific activity (MBq/gC)	+	+	-
Shoot total radiolabel (MBq)	-	-	-
Whole root biomass (g)	+	-	-
Fine root biomass (g)	+	-	-
Root specific activity (MBq/gC)	-	+	-
Root total radiolabel (MBq)	-	+	-
Soil specific activity (KBq/gC)	-	+	-
Soil total radiolabel (MBq)	-	+	-
Below-ground respiration radiolabel (KBq)	*	+	*

"-" indicates $p > 0.05$; "+" indicates $p \leq 0.05$.
*- The below-ground respiration was only mesured for the individuals of the last harvest.

Table 5. Specific activity (MBq/gC) of the plant tissues and soil.

Day:	52	54	57	64	76
			Shoot:		
M	0.9 (0.1)	0.9 (0.1)	0.7 (0.1)	0.5 (0.0)	0.3 (0.0)
NM	0.8 (0.1)	0.8 (0.1)	0.6 (0.1)	0.4 (0.1)	0.3 (0.1)
			Whole root:		
M	0.5 (0.2)	0.5 (0.1)	0.5 (0.1)	0.4 (0.2)	0.2 (0.1)
NM	0.3 (0.1)	0.4 (0.0)	0.4 (0.1)	0.4 (0.1)	0.3 (0.1)
			Fine root:		
M	0.5 (0.2)	0.5 (0.0)	0.6 (0.1)	0.4 (0.1)	0.3 (0.1)
NM	0.3 (0.0)	0.4 (0.0)	0.4 (0.2)	0.3 (0.1)	0.3 (0.0)
			Soil:		
M	0.018 (0.006)	0.015 (0.004)	0.009 (0.003)	0.011 (0.004)	0.014(0.004)
NM	0.009 (0.004)	0.004 (0.000)	0.004 (0.001)	0.002(0.001)	0.002 (0.001)

The values are the average (standard error of the mean) of each individual. n= 4 plant per treatment combination. M= inoculated; NM= non-inoculated. The plants were 51 day old at the time of the pulse-exposure to $^{14}CO_2$. nd= not determined.

Table 6. Lipid concentrations in fine roots (mg/g).

Day	52		54		57		64		76	
	M	NM	M	NM	M	NM	M	NM	M	NM
Total lipid	26.7(3.0)	19.3(1.9)	n d	n d	n d	n d	29.5(1.9)	21.4(1.7)	24.1(2.4)	20.6(2.5)
Total fatty acid	6.1(1.2)	4.3(0.4)	5.6(0.2)	5.1(0.9)	6.8(1.2)	4.6(0.6)	6.5(1.8)	4.9(0.9)	6.1(1.4)	5.6(0.2)
Fatty acids from whole-cell extracts:										
16:1ω5	1.7(0.6)	t	1.5(0.3)	t	1.5(0.3)	0.2(0.2)	1.7(0.8)	t	1.8(0.7)	t
16:0	1.5(0.3)	0.8(0.1)	1.4(0.1)	0.9(0.1)	1.4(0.2)	0.8(0.3)	1.5(0.4)	1.0(0.1)	1.4(0.3)	0.9(0.1)
17:1	t	0.1(0.0)	0.3(0.1)	t	0.4(0.2)	0.3(0.2)	0.2(0.1)	0.4(0.1)	0.2(0.1)	0.2(0.2)
18:2 ω6	0.3(0.0)	0.5(0.1)	0.4(0.0)	0.5(0.1)	0.7(0.0)	0.6(0.1)	0.5(0.1)	0.6(0.1)	0.3(0.0)	0.6(0.1)
18:1 ω9	0.3(0.0)	0.3(0.0)	0.3(0.0)	0.4(0.1)	0.4(0.1)	0.5(0.1)	0.3(0.1)	0.4(0.1)	0.3(0.1)	0.4(0.0)
18:1 *	0.3(0.1)	t	0.2(0.0)	t	0.3(0.0)	t	0.2(0.1)	t	0.2(0.1)	t
19:1	0.6(0.1)	0.9(0.1)	0.4(0.1)	1.1(0.2)	0.6(0.1)	0.8(0.1)	0.5(0.1)	0.7(0.3)	0.4(0.1)	1.1(0.1)
21:0 iso	0.3(0.1)	0.5(0.1)	0.2(0.0)	0.7(0.2)	0.4(0.2)	0.4(0.0)	0.3(0.1)	0.5(0.2)	0.2(0.1)	0.7(0.1)
21:0	0.7(0.1)	0.7(0.1)	0.5(0.1)	0.9(0.2)	0.7(0.1)	0.7(0.1)	0.7(0.2)	0.8(0.2)	0.7(0.2)	1.1(0.1)

The values are the average (standard error of the mean) of each plant. n= 4 plant per treatment combination. M= inoculated; NM= non-inoculated. Only the lipids which made up at least 0.25 mg/g are shown. The plants were 51 day old at the time of the pulse exposure to $^{14}CO_2$. nd= not determined. t= trace (< 0.05 mg/g).

*- This is an unresolved mixture of several 18:1 isomers.

Table 7. Total radiolabel in the fine root biomass accounted for by each lipid type (KBq/plant).

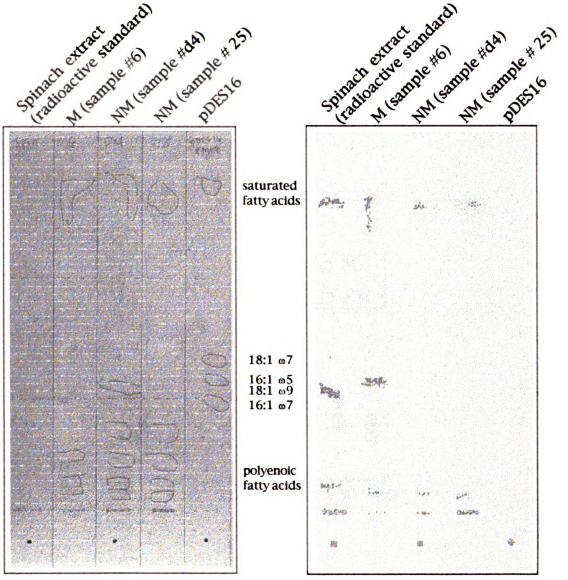
Day:	52	64	76
	Total lipid-extractable material		
M	97.1 (20.0)	60.2 (9.0)	34.4 (2.3)
NM	28.1 (9.5)	32.2 (1.7)	28.4 (5.8)
	Total fatty acids		
M	38.2 (12.2)	14.9 (4.3)	10.9 (4.6)
NM	11.2 (4.3)	12.3 (2.1)	6.8 (4.2)
	Polyenoic fatty acids		
M	1.8 (0.7)	4.1 (0.5)	2.6 (1.2)
NM	2.1 (1.0)	2.7 (0.5)	2.9 (0.6)
	16:1 ω 5 fatty acid		
M	13.9 (4.0)	2.5 (0.5)	1.7 (0.7)
NM	0.9 (0.4)	0.3 (0.2)	0.4 (0.2)
	Saturated fatty acids		
M	18.9 (5.5)	6.3 (1.4)	4.4 (2.3)
NM	4.4 (2.0)	4.6 (1.0)	3.4 (1.4)

The values are the average (standard error of the mean) of each plant. n= 4 plant per treatment combination. M= inoculated; NM= non-inoculated. The plants were 51 day old at the time of the exposure to $^{14}CO_2$.

Table 8. Results of the ANOVA for the lipid concentration and radiolabel content of fine roots.

	Harvest	Mycorrhizae	Harvest × Mycorrhizae
Variable:			
Total lipid (mg/g)	-	+	-
Total fatty acids (mg/g)	-	+	-
16:1 ω5 (mg/g)	-	+	-
Total lipid (KBq/plant)	-	+	+
Total fatty acid (KBq/plant)	+	+	-
16:1 fatty acid (KBq/plant)	+	+	+

"-" indicates $p > 0.05$; "+" indicates $p \leq 0.05$.



Argentation TLC plate after staining with dichlorofluorescein and visualization under UV light, followed by marking of the fatty acid bands with pencil.

Radiograph of the same plate as the image on the left

Figure 1. Argentation chromatography of the fatty acid extracts from M and NM *Sorghum* roots.

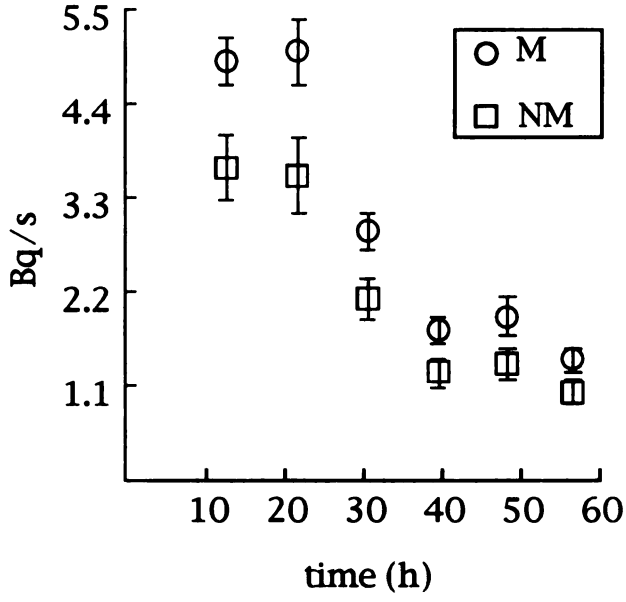


Figure 2. ^{14}C trapped from the below-ground atmosphere.

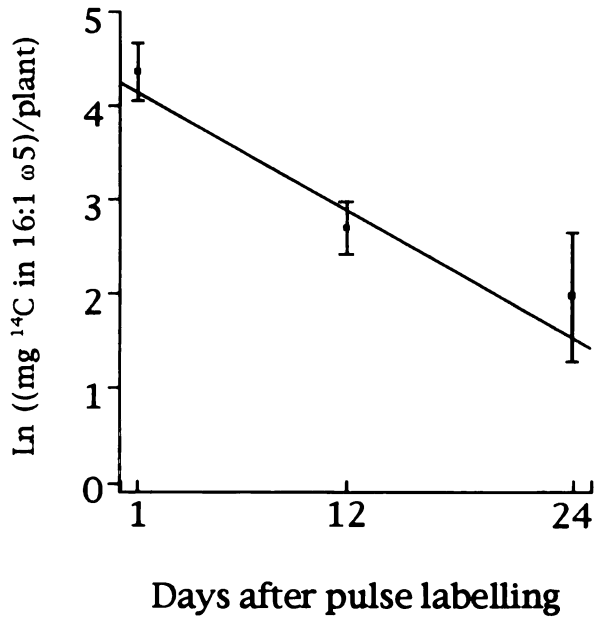


Figure 3. Ln(A_t) versus time for the ^{14}C turnover in the 16:1 ω5 fatty acids from the roots of *Sorghum* infected with *G. clarum*.

OVERALL CONCLUSIONS

About the Lipid Composition of Mycorrhizae

The principal aim of this work was to characterize the species of fungus present in mycorrhizal roots by lipid analysis and to measure the C turnover of mycorrhizal lipids. I have shown that mycorrhizal infection has an impact in the kinds and amounts of lipids in the roots of *Sorghum*. Chapter 2, identifies several lipids such as campesterol and 16:1 fatty acids which are useful for discerning between mycorrhizal and non-mycorrhizal roots. Multivariate analysis of the lipid profiles was shown to be a valuable approach for the identification of the fungal species engaged in the symbiosis. In chapter one, I further validated the lipid markers of mycorrhizal fungi by demonstrating that campesterol and 16:1 fatty acids are present only in small amounts in the biomass of the non-mycorrhizal fungi that can potentially share the habitat with the AMF.

There are limitations to the use of lipid analysis for the identification of mycorrhizal fungi in roots. I have shown that 16:1 ω5 FAME is not detectable in uninfected roots and for this reason its presence reliably indicates mycorrhizal roots. However, other fungal

lipid markers like 16:1 ω5 PLFA, 16:1 ω7 PLFA, and campesterol are present in detectable amounts in non-mycorrhizal roots. For this reason, it is necessary to obtain information about the lipid composition of infected and uninfected roots to make a correct assessment of infection when using this set of lipid markers. This technology should be useful in ecological studies in greenhouse or field conditions where we know the host species, plant age, as well as the dominant species of arbuscular fungi in the soil community. This way, the lipid markers and lipid profiles of samples can be compared to a known data base of infected and uninfected roots, so that a putative identification of the fungal species present in the roots can be carried out. An additional advantage of the use of lipids for the analysis of arbuscular fungi is that we not only have the potential to identify the species of fungus present in the root, but there is the potential to estimate the amount of biomass of the fungus per unit mass of root. This possible because if we make the assumption of a constant ratio of lipid to fungal biomass, the mass of the fungus in the root can be directly calculated from the lipid data. For instance, Jabaji Hare (1988) has measured the ratio of phospholipid to biomass in the vesicles of arbuscular fungi. Using this information, Olsson (1995) was able to calculate the biomass of *Glomus* in the growth medium of mycorrhizal plants by measuring the relative amount of 16:1 ω5 in the phospholipid extracts of the samples. Using the same

approach, we can calculate the biomass of *Glomus clarum* in the roots of *Sorghum*. The assumptions are that 13.1 % of the dry weight of the fungus is FAME fatty acid, and that approximately 50 % of the FAME is 16:1 ω5 (Jabaji Hare, 1988). Using the data for the fatty acid concentration from chapter 3, we can estimate that the mass of fungus per unit mass of root ranges from 23.0 to 27.5 mg/g. Nonetheless, it is important to remark that this type of estimate is speculative, since assumptions have to be adopted regarding a constant lipid to biomass ratio in different fungal cell types, fungal species, fungal age, and environmental conditions.

The results of my work show some interesting patterns with possible implications in the functionality of the lipids AMF which are worthy of further analysis. For example, it is widely known that alterations in the kinds of fatty acids and sterols present in lipids may have an impact on the melting point of lipids and the fluidity of membranes. Because of this, we can hypothesize that organisms enriched in unsaturated fatty acids (such as 16:1 ω5) are able to maintain membrane function at lower temperatures than other organisms richer in saturated fatty acids. Chapter 2 showed that the genus *Gigaspora* forms mycorrhizae which have a reduced amount of 16:1 ω5 fatty acid when compared to the rest of the mycorrhizal species included in the experiment. Relevant questions are: Does *Gigaspora* has

an adaptation to a different temperature regime than the rest of the arbuscular fungi? Does the qualitative differences in the sterol composition between mycorrhizal and non-mycorrhizal roots have implications in the viscosity of cellular lipids, or do they serve as precursors for the production of steroid hormones which affect plant and fungal growth? These are important questions which should be addressed in future work.

About the C Cycle of the Lipids of Arbuscular Fungi

In the third chapter of this thesis, I provide the turnover time of C in the mycorrhizal fatty acid 16:1 ω5. As shown in Chapter 2, lipids can be an important component of the biomass of the arbuscular fungus. Because of this, the C turnover in the mycorrhizal fatty acids represents the length of the C cycle of the most abundant C pool of the arbuscular fungus. This experiment sets the precedent for future work to expand our understanding of the C cycle of AMF. A variety of factors are known to affect the C budget and the C cycling of plants and their associated fungi. For example, physical conditions such as available oxygen, temperature, and water status are known to affect the respiration rate of organisms, while biological factors such as growth habit, fruit set, and plant age are associated with changes in the respiration rate and C cycling in plant tissues. Further, we do not know if the different

arbuscular fungal species vary in their inherent C turnover times, respiration rate and patterns of C storage and utilization. These are important variables, since the C demand, accumulation and loss by the fungus may be determinants of the mutualistic or parasitic nature of the symbioses. Future studies should address how the C turnover of the fungus is affected by various biological and physical conditions mentioned above. This will be an important step to improve our understanding of the complex economy of the mycorrhizal symbiosis.

LIST OF REFERENCES

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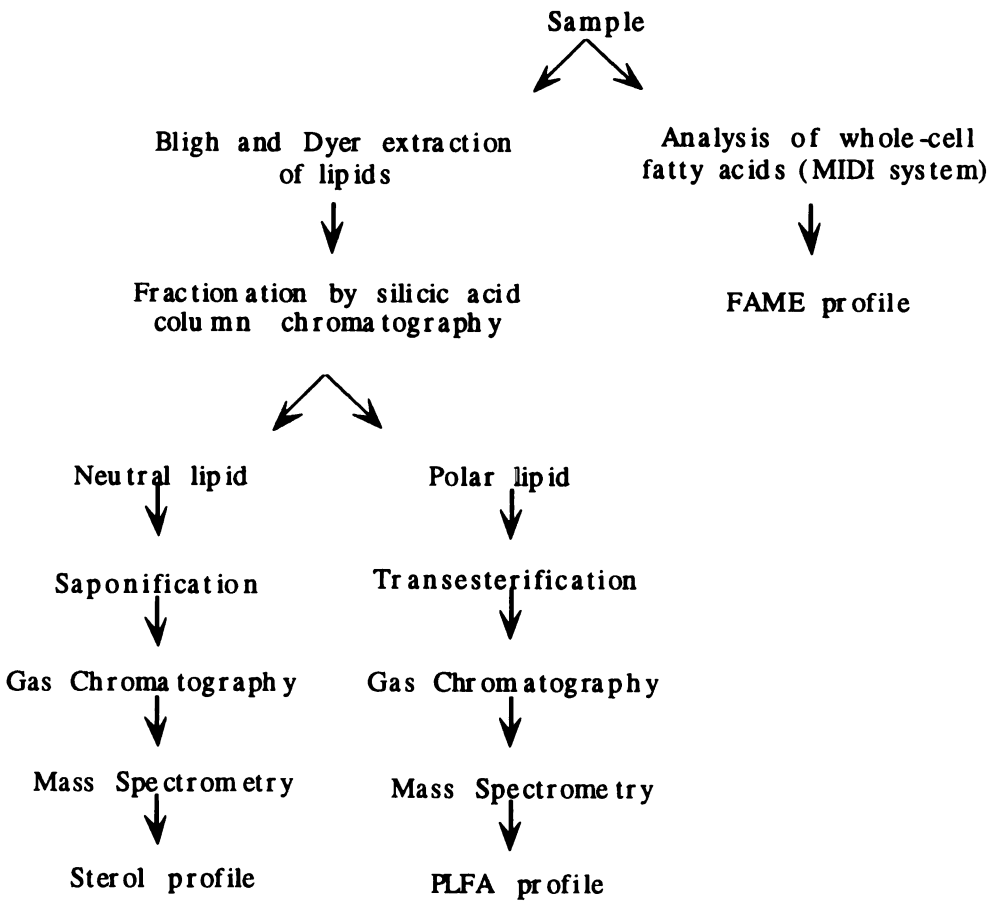
Jabaji-Hare, S., Deschene, A., and Kendrick, B. 1984. Lipid content and composition of vesicles of a vesicular-arbuscular mycorrhizal fungus. Mycologia. 76(6), 1024-1030.

Olsson, P.A., Bååth, E., Jakobsen, I., and Söderström, B. 1995. The use of phospholipid and neutral fatty acids to estimate biomass of arbuscular mycorrhizal fungi in soil. Mycol. Res. 99, 623-629.

APPENDIX A

APPENDIX A

FAME, PLFA, AND STEROL ANALYSIS OF ROOTS AND FUNGAL MATERIAL

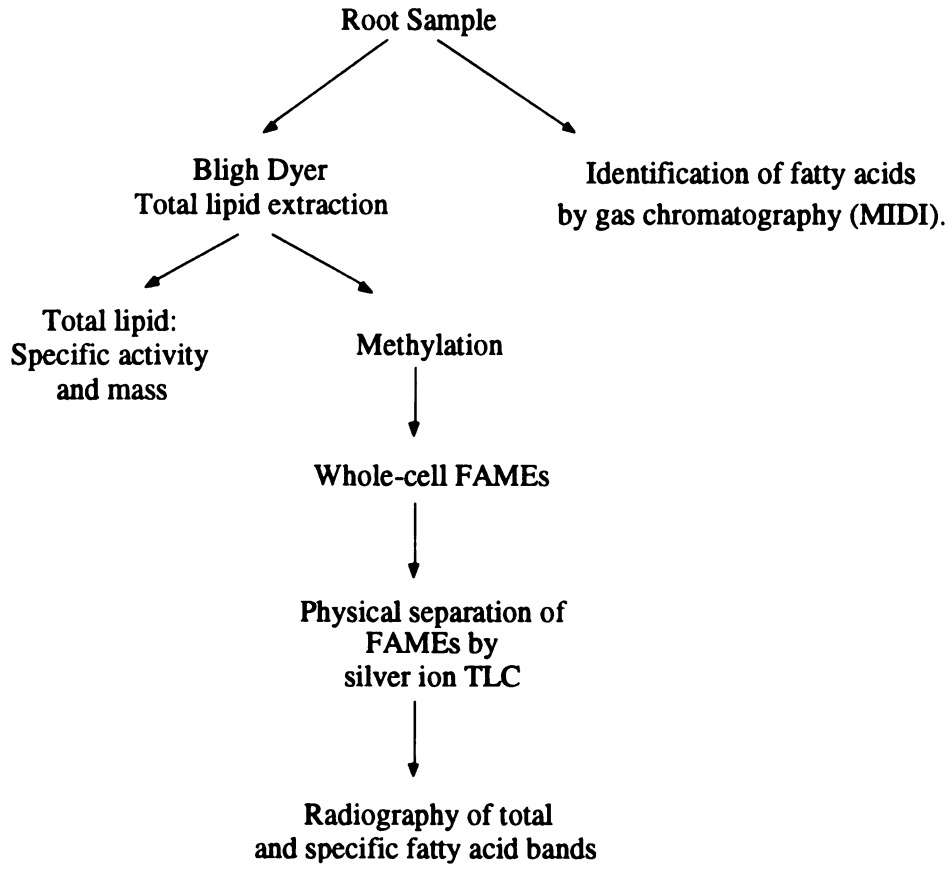


Flowchart representing the procedure used to analyze the FAME, sterol, and PLFA profiles of the root and fungal material in chapters 1 and 2.

APPENDIX B

APPENDIX B

ROOT LIPID EXTRACTION AND RADIATION MEASUREMENTS



Flowchart representing the sampling and analysis scheme for the root lipids in chapter 3.