IDENTIFICATION OF PLANT-PARASITIC NEMATODES

USING FAME ANALYSIS

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IDENTIFICATION OF PLANT-PARASITIC NEMATODES

USING FAME ANALYSIS

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IDENTIFICATION OF PLANT-PARASITIC NEMATODES

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Nicholas Scott Sekora

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THESIS ABSTRACT

IDENTIFICATION OF PLANT-PARASITIC NEMATODES

USING FAME ANALYSIS

Nicholas Scott Sekora

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For several years, chromatographic analysis of fatty acid methyl esters (FAME) has been used to identify bacteria more quickly than traditional methods. The objective of this study was to determine the applicability of FAME analysis for the identification of plant-parasitic nematodes for use in diagnostic laboratories. *Meloidogyne incognita*, *Rotylenchulus reniformis*, and *Heterodera glycines* were statistically distinct ($P < 0.0001$) and could be identified in samples containing at least 100 total individuals for *M. incognita* and *R. reniformis* and 5 cysts for *H. glycines*. Two fatty acids (16:1 ω5c and 18:1 ω5c) indicate the presence of *R. reniformis* when comparing samples containing 100 or greater individuals of either *R. reniformis* or *M. incognita*. Significant

variation ($P < 0.0001$) in the fatty acid profiles of *M. incognita* and *R. reniformis* was observed when either species was increased on tomato, cotton, or soybean plants, but variations of the FAME profile for each nematode allowed for identification of each species regardless of host. Mixed-species samples of *M. incognita* and *R. reniformis* could be differentiated ($P \leq 0.0075$) from one another and single-species samples of each species in 100% of comparisons when samples contained 5000 total individuals and in 95% of comparisons when samples contained 500 total individuals. In samples containing 500 total individuals, it was not possible to differentiate between a (50:50) ratio of *M. incognita* and *R. reniformis* or a sample with a (25:75) ratio of *M. incognita* and *R. reniformis*. However, all other comparisons, including these two ratios separately, were identifiable. Three *Meloidogyne* species (*M. arenaria*, *M. hapla*, and *M. javanica*) and three *M. incognita* races (races 1, 2, and 3) all produced distinct ($P < 0.0001$) fatty acid profiles and could be identified with 85.6% overall accuracy to the race level. Soil containing *R. reniformis* produced a fatty acid profile significantly different ($P < 0.0001$) from soil without *R. reniformis* and contained higher percentages of fatty acids found in nematodes. Certain fatty acids found in *R. reniformis* were found in higher quantities in soil infested with the nematode and may indicate its presence. The compiled library identified nematode samples with 90.4% total accuracy. Genus level identification was accurate at 100% for the samples studied and species level identification was 94.4% accurate. FAME analysis appears to be a promising alternative for identification of plant-parasitic nematodes.

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I. INTRODUCTION

Fatty acid analysis has been performed on numerous types of organisms, most notably bacteria. In 1988, Myron Sasser developed a method that labeled whole-cell fatty acids of bacterial cells that could then be analyzed by an automated gas chromatograph (GC) (Kunitzky *et al.*, 2005). This process hydrolyzes fatty acids from phospholipids, triacylglycerols, sterols, and various other lipid structures and then adds a methyl group to the carboxyl group of fatty acids. This forms a methyl ester that acts as a label that the GC then uses to read and identify the fatty acid. Identification of fatty acids is accomplished with measurements of retention time, the time it takes a specific fatty acid to pass through the GC column (Sherlock Analysis Software, MIDI Systems, Inc.). The analysis software of the GC contains a library of retention times that it matches to the retention time of a fatty acid from an unknown sample. The percentage of each fatty acid is also recorded by the amount of response (measured in the electrical response mV) produced when it passes though the detector at the end of the column. Percentages are based on the response of the fatty acid compared to the response of the entire sample. This system was termed FAME analysis since it utilizes fatty acid methyl esters.

The basic structure of a fatty acid is a carbon skeleton usually containing 12 to 20 carbon atoms bound to a carboxyl group (-COOH) at one end and ending in a methyl group ($-CH_3$). The carbon atoms may be completely saturated with hydrogen atoms to form a linear saturated fatty acid, or double-bonded to one another forming an unsaturated bent fatty acid (McMurray, 2004). To identify the various fatty acids, a numbering scheme is used that starts at the carboxyl carbon – the number one or α (alpha) carbon – and ending at the terminal, or ω (omega), carbon; the carboxyl carbon is labeled C-1. The number of carbon atoms are given, followed by a colon (:) and the number of double bonds in that molecule. The double bond and any other modifications to the carbon backbone - methyl groups ($-CH_3$), hydroxyl groups (-OH) – are indicated by the number of carbon atoms from the carboxyl end. For example, a fatty acid named 18:1 ω5c would contain 18 carbon atoms and a single double bond, the double bond located between the 13C-14C bond. Since the double bond begins at 13C in the 18:1 ω5c example, there are five carbon atoms from 13C to the ω carbon. Therefore, the ω5c notation indicates that the double bond is located five carbon atoms from the ω carbon.

Libraries of fatty acid profiles for thousands of bacteria have been developed to aid in identification (Kloepper et al., 1992, Slabbinck at al., 2008). Identification is based on a profile developed from numerous samples of an individual organism with variations in growth medium, growth time, and other criteria. This gives a "picture" of the expected fatty acid profile of individual bacterial colonies that encompasses some variation in environment. The

developed profiles are compiled in the Sherlock Analysis software (MIDI Systems, Inc.) and are used to identify the bacteria based on the retention times and percentages of fatty acids in the sample. These profiles are used by microbiologists in clinical, field, and other applications to identify isolated bacteria (Cavigelli *et al.*, 1995, Heyrman *et al.*, 1999).

Libraries of profiles have also been developed for selected fungi. Principally, this system is used to characterize fungal groups. Stahl and Klug (1996) used this process to characterize several filamentous fungi. Analyses were used to cluster and group species within this group and to get a better indication of the diversity demonstrated chemically by these organisms. Several species of the soil fungi order Glomales have also been differentiated with this system, including the identification of spores present in soil samples (Graham *et al.*, 1995; Bentivenga and Morton, 1996; Madan *et al.*, 2002). Since FAME analysis has worked well for many bacteria and some eukaryotes such as fungi, it might be used to help differentiate, and possibly even identify, organisms not previously used with this system.

The use the FAME analysis system for the identification, classification, and differentiation of plant-parasitic nematode species might be possible. Plant-parasitic nematodes are an ever-increasing problem with commercial crops. Crop damage and yield loss are two of the greatest problems associated with these organisms. Crops such as corn (*Zea mays* L.), peanuts (*Arachis hypogaea* L.), many fruits and vegetables, and cotton can be drastically affected by plant-parasitic nematode populations. Cotton losses to nematodes in the United States

are estimated at $31.5 million annually (National Cotton Council). Identification and enumeration of the disease-causing nematodes is a time-consuming and involved process that requires trained individuals who can quickly and accurately identify them. The FAME system, thus, might be useful to develop profiles for each individual species, including those difficult to differentiate with traditional methods. Several factors should be taken into account when developing these profiles. Factors such as life stage of the nematode, the number of nematodes present in the sample, and the host impact on nematode species may be critical to the development of the profile for a specific nematode.

Fatty acids are an essential component in the composition and physiology of nematodes. They are structurally important in membranes and organelles of body cells, serve as precursors to hormones, regulate the passage of nutrients and metabolites into and out of a cell, and are also a source of carbohydrates during times of starvation (Barrett and Wright, 1998; Chitwood, 1998). The phospholipids that make up the plasma membranes of cells and their constituents contain the greatest proportion of a nematode's fatty acids (Becker, *et al.* 2003). These phospholipids are arranged in a double membrane that surrounds the entire cell with their fatty acid tails forming a barrier to the outer environment. Energy storage molecules, triacylglycerols, are composed of three fatty acids bound to a glycerol group. These fatty acids can be hydrolyzed and used as building blocks to form carbohydrate molecules with the biochemical pathways of the cell (Horton, *et al.*, 2002).

The first account of fatty acids in plant-parasitic nematodes was by Tracey (1958). He described lipids as “coherent fatty material” in samples that were to be extracted for cellulase and chitinase. Further studies indicated the actual dry weight percentage of these compounds ranges from 11 to 67% in plant-parasitic and free-living nematodes (Chitwood, 1998). Several species of animal-parasitic, plant-parasitic, and free-living nematodes have been analyzed for fatty acid compositions and percentages.

Beams (1965) used gas chromatography to separate fatty acids from *Ascaris lumbricoides* tissues and developed profiles for the cuticle, muscle, and reproductive systems. These profiles contained many saturated and unsaturated fatty acids, though the unsaturated fatty acids were present in much higher concentrations than the saturated fatty acids. The most common fatty acids in these analyses were the 16:0, 18:0, 18:1, and 18:2 fatty acids. These fatty acids showed slight variation among the three tissues. The fatty acids of *Panagrellus redivivus*, a free-living nematode, were analyzed by Sivapalan and Jenkins (1966) using thin-layer chromatography and gas-liquid chromatography. Both analyses also resulted in profiles with the 16:0, 18:1, and 18:2 fatty acids in the greatest concentrations. The concentrations of 16:0 and 18:1 were lower than those described for *A. lumbricoides* (Beams, 1965), while 18:2 was found in *P. redivivus* and not *A. lumbricoides* (Table 1).

Beginning in 1967, Krusberg performed several studies on the fatty acids of other nematode species, including several plant-parasitic species. The first of these studies compared five plant-parasitic nematode species to one another and

the culturing medium on which they were grown (Krusberg, 1967). The five species studied were *Ditylenchus triformis*, *D. dipsaci*, *Pratylenchus penetrans*, *Aphelenchoides ritzemabosi*, and *Tylenchorynchus claytoni*. The fungal pathogen *Pyrenochaeta terrestris* was used to culture *D. triformis*, with the remaining four nematodes grown on alfalfa (*Medicago sativa* L.) callus tissue. The most predominant fatty acids in nematodes studied by Krusberg (1973) and Orcutt (1978) were 18:1 and 20:1, respectively; the other fatty acids varied in their relative concentrations (Table 2). In comparison, the host tissues that the nematodes were grown on demonstrated greater concentrations of the 18:2 fatty acid than any other expressed. These analyses indicate that the fatty acid profiles for each species were distinct from the others in this study.

This type of study was also carried out using *Turbatrix aceti*, which was compared to the two culture media; soy peptone extract and beef liver extract (Krusberg, 1972). The results of this study and a later study by Fletcher and Krusberg (1973), produced fatty acid profiles for *T. aceti* similar to that found by Sivapalan and Jenkins (1966) in the free-living nematode *P. redivivus,* with the 18:1 and 18:2 fatty acids being the most abundant, though there were greater concentrations of 16:0 and 18:0 fatty acids in *T. aceti* (Table 1). The study also indicated that several fatty acids were taken up from the growth medium for utilization by the nematodes. Four fatty acids - iso-15:0, 20:1 ω9c, 20:4 ω3c, and 20:5 ω3c - were actually found to be produced by the nematodes.

A third study by Krusberg (1973) compared the fatty acid compositions of *Meloidogyne incognita* and *M. arenaria* in the egg and female life stages as well

as the larval stage of *M. arenaria*. Though the females of both of these species had the same concentration of 18:1 ω7c, other fatty acids varied in their expression, most notably the 16:0 and 18:0 fatty acids. There was a greater differentiation between the two species in the fatty acid profiles for eggs. A slight difference could be observed between the profile of *M. arenaria* females and larvae (Table 3). These data indicate that it may be possible to differentiate species within genera based on fatty acid profiles. A study by Hutzell and Krusberg (1972) of two free-living nematode species, *Caenorhabditis elegans* and *C. briggsae*, supports this argument. Both of these species displayed the same fatty acids in their analysis, but the actual concentrations of those fatty acids varied. For example, the predominant fatty acid in *C. elegans* was 18:1 ω7c, compared to 18:0 for *C. briggsae*. Nearly every fatty acid observed varied in its concentration between the two nematodes, and the combined profile of each species differed from profiles of nematodes studied beforehand (Table 1).

Another plant-parasitic nematode, *Globodera solanacearum*, was analyzed for its fatty acid composition by Orcutt *et al.* (1978). As with the previous studies, many of the same fatty acids were found in this study, but the expression of each of these fatty acids was also different than those of the previously studied organisms. The 18:0, 18:1, 20:1, and 20:4 fatty acids were found to be the most abundant in *G. solanacearum*. The concentrations of these four fatty acids in *G. solanacearum* were more evenly distributed than observed in other nematodes, which have typically had one or two highly concentrated fatty acids (Table 2).

In 1981, Chitwood and Krusberg (1981, 1981b) analyzed the fatty acids of *T. aceti* and *M. javanica* using thin-layer chromatography. Both of these studies indicated that the same fatty acids were present as in the studies by Beams (1964) and Sivapalan and Jenkins (1966), though the percentages of these fatty acids varied between the two organisms and from those of the nematodes previously mentioned.

Based on the previous studies of plant-parasitic nematode fatty acid compositions, it is believed that FAME analysis will be useful to identify economically important nematode species. If it was indeed possible to differentiate nematode species in this way, a library of several common nematode species needs to be developed for the FAME system. Also, the actual number of nematodes needed to produce accurate fatty acid profiles should be determined. Once profiles for several nematodes species have been developed, a movement toward mixed-species samples should be made to determine the applicability of this research for field use. Mixtures resembling those of typical field samples - for example, 25% *M. incognita* and 75% *R. reniformis* - could help to determine if actual field samples could be analyzed with this system. In conjunction with this, the response data could be used to help demarcate these various ratios. Finally, the possibility of using the response data from these analyses should be evaluated to aid in quantification of nematodes in samples.

Table 1. Fatty acid percentages of nonplant-parasitic nematodes.

Fatty acid	*Ascaris lumbricoides*	*Panagrellus redivivus*	*Turbatrix aceti*	*Caenorhabditis elegans*	*C. briggsae*	Fatty acid	*Ascaris lumbricoides*	*Panagrellus redivivus*	*Turbatrix aceti*	*Caenorhabditis elegans*	*C. briggsae*
10:0	--†	--	Tr	--	--	18:2 ω6c	--	--	10.00	--	--
iso 11:0	--	--	Tr	--	--	18:2 ω9,6c	--	--	--	8.53	4.78
12:0	Tr*	0.10	0.30	--	--	18:3	--	1.50	--	--	--
12:0 branched	Tr	--	--	--	--	18:3 ω6c	--	--	1.80	--	--
12:1	--	0.20	--	--	--	18:3 ω3c	--	--	0.30	--	--
iso 13:0	--	--	0.16	--	--	18:3 ω12,9,6c	--	--	--	0.40	0.17
13:0 branched	Tr	--	--	--	--	18:3 ω3,6,9c	--	--	--	0.15	0.58
iso 14:0	--	--	Tr	--	--	18:2, 18:3	19.77	--	--	--	--
14:0	0.79	0.50	3.00	1.04	0.75	19:0	--	--	--	0.44	0.58
14:1	--	4.00	--	--	--	20:0	2.14	--	0.40	0.71	0.94
iso 15:0	--	--	4.60	2.38	3.99	20:1	--	--	--	--	--
15:0	1.55	--	Tr	0.12	0.15	20:1 ω11c	--	--	0.02	--	--
15:0 branched	Tr	--	--	--	--	20:1 ω9c	--	--	0.70	0.13	0.40
iso 16:0	--	--	Tr	0.11	0.34	20:1 ω7c	--	--	2.20	0.54	0.15
16:0	6.64	3.90	10.20	2.44	6.01	20:2	--	0.60	--	--	--
16:1	0.66	3.70	--	--	--	20:2 ω6c	--	--	0.70	--	--
16:1 ω7c	--	--	0.36	3.04	2.31	20:2 ω9,7c	--	--	--	2.51	1.54
16:1ω5c	--	--	0.02	1.59	0.95	20:3	0.21	4.70	--	--	--
16:2	Tr	--	--	--	--	20:3 ω6c	--	--	5.30	--	--
iso 17:0	--	--	2.90	5.60	6.72	20:3 ω12,9,6c	--	--	--	8.46	5.41
17:0	0.92	--	Tr	0.62	1.34	20:4	--	5.20	--	--	--
17:1	0.36	--	--	--	--	20:4 ω6c	--	--	2.00	--	--
iso 18:0	--	--	1.10	0.42	0.70	20:4 ω3c	--	--	2.60	--	--
18:0	15.45	0.60	14.00	8.13	22.17	20:4 ω15,12,9,6c	--	--	--	7.71	5.01
18:1	16.89	16.30	--	--	--	20:4 ω12,9,6,3c	--	--	--	3.81	1.56
18:1 ω9c	--	--	16.50	5.31	14.60	20:5	--	4.50	--	--	--
18:1 ω7c	--	--	16.50	24.01	13.28	20:5 ω3c	--	--	3.90	--	--
18:1 ω5c	--	--	Tr	3.10	2.05	20:5 ω15,12,9,6,3c	--	--	--	8.72	3.53
18:2	--	20.70	--	--	--	22:0	--	--	--	--	--

* = Trace amounts

† = Not Detected

Data extrapolated from Beames, 1964, Sivapalan and Jenkins, 1966, Krusberg, 1972, and Hutzell and Krusberg, 1972

Table 2. Fatty acid percentages of plant-parasitic nematodes.

Fatty acid	*Ditylenchus triformis*	*D. dipsaci*	*Pratylenchus penetrans*	*Aphelenchoides ritzemabosi*	*Tylenchorynchus claytoni*	*Globodera solanacearum*
12:0	Tr*	Tr	Tr	Tr	Tr	--†
14:0	0.94	2.26	2.71	1.82	2.82	0.52
16:0	2.66	5.77	4.09	6.25	6.88	3.31
16:1	4.03	4.62	1.86	1.04	12.45	1.37
18:0	2.58	2.23	0.89	11.46	1.83	11.53
18:1	64.72	66.59	76.80	29.95	66.54	17.08
18:2	5.75	7.86	0.81	21.61	2.54	2.95
18:3	Tr	0.14	Tr	3.65	0.53	2.22
20:0	1.37	0.10	1.17	Tr	1.59	4.74
20:1	6.35	2.89	6.36	14.32	2.22	20.72
20:2	0.69	0.45	0.61	1.56	0.99	2.00
20:3	4.89	1.29	1.34	4.69	0.35	7.42
20:4	6.01	3.13	2.63	3.65	0.63	23.87
20:5	--	2.68	0.73	Tr	0.63	Tr
22:0	--	--	--	--	--	1.92
22:1	Tr	--	--	--	--	--

* Trace amounts

† = Not Detected

Data extrapolated from Krusberg, 1967, and Orcutt, 1978

Table 3. Fatty acid percentages of three *Meloidogyne* species

	Meloidogyne incognita		*M. arenaria*			
Fatty acid	Females	Eggs	Females	Eggs	Larvae	*M. javanica*
10:0	Tr*	Tr	Tr	Tr	Tr	--†
12:0	0.03	0.02	0.06	0.01	Tr	0.03
iso 13:0	Tr	Tr	Tr	Tr	Tr	--
iso 14:0	--	--	--	--	--	0.02
14:0	1.25	0.87	1.89	1.58	1.75	0.61
iso 15:0	0.78	0.83	0.83	0.68	0.87	0.55
15:0	--	--	--	--	--	0.02
iso 16:0	--	--	--	--	--	0.02
16:0	5.73	5.90	7.42	8.53	9.27	3.33
16:1	--	--	--	--	--	1.84
iso 17:0	0.68	0.15	2.41	2.34	2.57	0.57
17:0	--	--	--	--	--	0.01
iso 18:0	0.25	0.25	0.38	0.54	1.85	0.18
18:0	7.75	10.32	5.23	7.85	5.86	8.78
18:1	--	--	--	--	70.76	64.63
18:1 ω9c	1.50	1.30	Tr	2.90	--	--
18:1 ω7c	75.10	71.80	75.10	68.80	--	--
18:2	--	--	--	--	--	3.22
18:2 ω6c	0.54	1.37	0.60	0.55	0.66	--
18:3	--	--	--	--	0.08	0.19
18:3 ω6c	0.04	0.11	0.02	0.08	--	--
18:3 ω3c	0.01	0.01	0.01	Tr	--	--
20:0	0.38	0.54	0.32	0.46	0.49	2.34
20:1	--	--	--	--	1.67	3.30
20:1 ω9c	--	0.08	--	0.05	--	--
20:1 ω7c	2.44	2.41	2.03	2.35	--	--
20:2	--	--	--	--	--	0.11
20:2 ω6c	0.04	0.20	0.05	0.24	0.08	--
20:3	--	--	--	--	--	1.07
20:3 ω6c	0.34	0.29	0.26	0.15	0.31	--
20:4 ω6c	0.33	0.24	0.29	0.20	0.38	1.00
20:4 ω3c	0.56	0.48	0.62	0.32	0.49	0.98
20:5	--	--	--	--	--	6.52
20:5 ω3c	1.21	1.26	1.24	2.31	1.58	--
22:0	--	--	--	--	--	0.25
22:1	--	--	--	--	--	0.42
24:0	--	--	--	--	--	0.12

* Trace amounts
† = Not Detected
Data extrapolated from Krusberg *et al*., 1973, and Chitwood and Krusberg, 1981

II. IDENTIFICATION AND DIFFERENTIATION OF PLANT-PARASITIC NEMATODE SPECIES USING FAME ANALYSIS

Abstract

Fatty acid methyl ester (FAME) analysis has been utilized to identify bacteria and some fungi, but little progress has been made to identify nematodes using this system. A series of samples containing varying numbers of individuals of *Meloidogyne incognita*, *Rotylenchulus reniformis*, and *Heterodera glycines* was used to determine the applicability of FAME analysis for identification of these three nematode species, quantify the minimum number of individuals required for identification, identify samples containing mixed-species populations, and evaluate the impact of plant host species on nematode fatty acid profiles. All three nematode genera were correctly identified with unique FAME profiles. A minimum of 100 vermiform stage nematodes was required for accurate identification of *M. incognita*, and *R. reniformis* while 25 cysts were required to identify *H. glycines*. Different ratios of mixed-species samples of *M. incognita* and *R. reniformis* could be identified with greater than 83% accuracy. Fatty acid profiles of *M. incognita* and *R. reniformis* varied significantly ($P \leq 0.0001$) when grown on each of three different host plants, though the increased variation within the two species did not inhibit identification of either species. By

using FAME analysis, it is possible to identify multiple plant-parasitic nematode species with greater than 95% accuracy.

Introduction

Several studies have previously been conducted to determine the fatty acids present in animal-parasitic, plant-parasitic, and free-living nematodes (Abu Hatab and Gaugler, 1997; Abu Hatab and Gaugler, 1999; Beams, 1964; Chitwood and Krusberg, 1981; Fletcher and Krusberg, 1973; Gibson *et al.*, 1995; Hutzell and Krusberg, 1982; Krusberg, 1967, 1972; Krusberg et al. 1973; Orcutt et al. 1978; Sivapalan and Jenkins, 1966). From these studies, the percentages of fatty acid classes or individual fatty acids were determined for multiple species of nematodes by gas-liquid chromatography. Many of these studies focused on individual nematode species, but a few studies (Hutzell and Krusberg, 1982; Krusberg, 1967; Krusberg et al. 1973) compared multiple genera or species within genera. These studies indicated that fatty acids present (the fatty acid profile) in each genus or species varied among genera and species, though significance was not determined.

In 1985, the fatty acid methyl ester (FAME) analysis was developed for identification and classification of bacteria using their fatty acid profiles. This system has been adapted for studying fungi (Grahm *et al.*, 1995; Stahl and Klug, 1996) and a few studies have analyzed nematode fatty acid profiles (Ruess et al., 2002), but no studies have focused on use of the FAME system for direct differentiation and identification of plant-parasitic nematodes. The purpose of

this study was to adapt the FAME analysis method to identify plant-parasitic nematodes with the goal of reducing the time and cost required to identify diagnostic samples.

Specific objectives for this study were to 1) evaluate the effect of serial dilutions on the FAME profiles of *Meloidogyne incognita* race 3 (Chit.), *Rotylenchulus reniformis* (Linford and Oliveira), and *Heterodera glycines* (Ichinohe) race 3 (Golden) raised on a single host, 2) evaluate the effect of species mixtures on FAME profiles, and 3) quantify the effect of host plants (*Glycine max* [L.] Merr., *Gossypium hirsutum* L., and *Lycopersicon esculentum* Mill.) on FAME profiles of *M. incognita* and *R. reniformis*.

Materials and Methods

General Culturing

A population of *R. reniformis* was created using multiple populations collected from field sites in Alabama. A stock population of *M. incognita* was collected from the E. V. Smith Research Center in Shorter, AL and increased in the greenhouse. An established population of *H. glycines* was increased from cysts contributed from soybean fields in the Mississippi delta region.

These stock populations of nematodes were maintained at the Auburn University Plant Science Research Center greenhouses. Populations of *R. reniformis* and *M. incognita* were grown on two cultivars of cotton (*G. hirsutum*), 'Stoneville 5599 BGRR' and 'Delta and Pine Land (DPL) 555 BGRR,' respectively, and the population of *H. glycines* was increased on soybean (*G. max*) cv. 'Croplan Genetics RC 4955'. These populations were increased in white

500cm^3 polystyrene pots and physically separated by Plexiglas dividers 61 cm high by 91 cm deep to prevent the formation of mixed populations.

Nematode Extraction

Vermiform adults and juvenile life stages of *R. reniformis* and second stage juveniles (J2s) of *M. incognita* were extracted from the soil of the stock pots and collected using gravity screening over nested 250 μm and 45 μm sieves; the substrate on the 45 μm sieve was rinsed into 150 cm^3 beakers and allowed to settle before initial sucrose centrifugation. Gravid females and eggs of both species were extracted from root tissue by agitation in 6.0% sodium hypochlorite (NaOCl) solution for four minutes, rinsed with water, and collected on nested 75 μm and 25 μm sieves. The vermiform and egg stages for each species collected on the 45 μm sieves were centrifuged in sucrose (specific gravity = 1.13) and rinsed over a 25 μm sieve six times to remove debris. Extractions for each species were then combined and again centrifuged to remove any remaining plant and soil debris. Cysts of *H. glycines* were extracted by gravity screening and rinsed over nested 850 μm and 250 μm sieves. Cysts collected on the 250 μm sieve were hand-picked under a dissecting microscope. Extractions for all species were enumerated to determine the number of eggs, juveniles, females, males, and cysts in each sample.

Objective 1: Differentiation and Dilution Detection for *M. incognita*, *R. reniformis*, and *H. glycines*

The number of individual nematodes per sample was set as a series of dilutions to determine the quantity of each species needed to generate a FAME

profile, and to differentiate among species. Separate samples were prepared for *M. incognita*, *R. reniformis,* and *H. glycines* containing concentrations of 10,000, 5000, 1000, 500, 250, 100, 50, 25, 10, and 1 individual of *M. incognita* and *R. reniformis*, and 25, 10, and 1 cyst of *H. glycines*. These samples were replicated six to 36 times (Table 1). Samples contained a mixture of eggs, J2s, and gravid females for *M. incognita*. Eggs, juvenile life stages, males, infective females, and gravid females were present for *R. reniformis*. Tan cysts were selected for samples of *H. glycines*; these cysts contained eggs as well as first and second stage juveniles. Sample sizes were drastically lower for *H. glycines* due to the size of the cyst stage compared to the life stages present in *M. incognita* and *R. reniformis* samples. Each sample of individuals was extracted and analyzed for FAMEs. The classes analyzed with the SAS procedures STEPDISC, CANDISC, and NLMIXED procedures were species, dilution, and presence of a fatty acid. Species were also compared using the Sherlock Analysis Software (MIDI, Inc., Newark, DE)

Objective 2: Mixed Ratios of *M. incognita* and *R. reniformis*

Samples were prepared containing fixed percentage ratios of *M. incognita* and *R. reniformis* at two dilutions of total individuals with different ratios of *M. incognita* to *R. reniformis* (Mi:Rr). Ratios were set up from extractions containing only vermiform life stages of *M. incognita* and *R. reniformis*. Five mixtures that transitioned from pure samples of *M. incognita* to samples of only *R. reniformis* in 25% increments were prepared at both 5000 and 500 total individuals per sample (Mi:Rr 100:0, 75:25, 50:50, 25:75, and 0:100; Table 2).

Each ratio sample was replicated ten times for both population levels. FAME profiles were analyzed using the ratios of *M. incognita* to *R. reniformis* as the class, and library entries were developed for each ratio.

Objective 3: Host Impact on FAME Profiles for *M. incognita* and *R. reniformis*

Meloidogyne incognita and *R. reniformis* were increased on cotton cv. ‘Stoneville 5599 BGRR’, tomato (*L. esculentum* cv. ‘Roma’), and soybean cv. ‘Hutcheson’. Twenty 500 cm^3 pots of each host species were grown in the greenhouse for 60 days. Plants were grouped by species and spaced 46 cm apart and separated by Plexiglas dividers 61 cm high by 91 cm deep to prevent splashing and mixing among nematode species. Nematodes were extracted from the soil and roots of each pot as described previously. Populations from each plant were kept isolated and three samples of 1000 total individuals were extracted from each population, for a total of 360 nematode samples.

To set up plant tissue controls, twelve seedlings each of cotton, soybean, and tomato host plants were grown in flats for one week. Four samples from each of the seedlings were taken from the root tissue and extracted for fatty acids. A total of 48 samples for each host species were extracted, and the resulting fatty acid profiles were averaged for the four samples from each seedling to give a mean fatty acid profile per seedling.

Fatty Acid Extraction

Fatty acids were extracted from samples using the method described by Sasser (1990). To begin the saponification step, each sample was placed into a

9.0 mL glass, Teflon-lined screwcap test tube and mixed with 1.0 mL of 3.00M NaOH. The samples were vortexed at 3200 rpm with a Vortex Genie II (Fisher Scientific) for 10 seconds and heated in a 100°C waterbath for five minutes. The vortexing was repeated followed by an additional 25 minutes at 100°C. Samples were allowed to cool to room temperature before the methylation step. To methylate the freed fatty acids, 2.0 mL of the methylation reagent (2.93 M HCl in 41.25% aqueous methanol) was added, vortexed and heated to 80°C for 10 minutes, then rapidly cooled under flowing water. To extract the methylated fatty acids from the aqueous solution, 1.25 mL of the organic extraction solvent (50/50 by volume hexanes and methyl tert-butyl ether) was added. Samples were gently mixed by tumbling with a Fisher Scientific Hematology/Chemistry Mixer 346 for 10 minutes and allowed to separate for 15 minutes. After the basal aqueous layer was removed and replaced with 3.0 mL of 0.3 M NaOH, the tumbling procedure was repeated for an additional five minutes to remove remaining aqueous components from the solvent. The organic solvent, containing the extracted fatty acids, was transferred to sample vials and allowed to completely evaporate under a fume hood, after which the samples were reconstituted in 75 μL of the organic extraction solvent and transferred to spring-vial inserts for each sample vial. Vials were crimp-sealed with Teflon-lined aluminum caps and stored at -20°C until analysis.

Samples were analyzed for fatty acid composition by a HP 5890 automated gas chromatography system (Agilent Technologies). Each analysis was accomplished by injecting 2.0 µL of sample into an Ultra 2 Cross-linked 5%

Phenyl Methyl Siloxane column for 25 minutes. Sample data from the Sherlock Sequencer Software (MIDI, Inc.) included total response of each sample (mV) and the response for each detected fatty acid. Fatty acid percentages were calculated from the proportion of each fatty acid within the sample; these percentages were used to create a fatty acid profile for each nematode sample.

Statistical Analysis

The STEPDISC (SAS version 9.1.3; SAS Institute, Inc) procedure was used to analyze the expression of each fatty acid across all samples to determine which fatty acids contributed significantly to the differentiation among classes. Classes for each experiment were dependent upon the character being analyzed (species, host, ratio). The STEPDISC procedure determined fatty acids significant for discrimination among classes based on the ANOVA test F value of a selected fatty acid. The fatty acid with the highest F value, the most significant, was selected first. We selected the default P-values of 0.15 for a variable to enter and remain in this initial analysis. The STEPDISC procedure continued until no more fatty acids were selected based on significance. Significant fatty acids were selected and all selected fatty acids were retested for significance with every selection step. If a selected fatty acid was no longer significant for demarcation when tested against the other selected fatty acids, it was removed. Once all significant fatty acids were selected based on these criteria, the compiled list was used for class differentiation with the CANDISC procedure.

The CANDISC procedure provided canonical discriminant analysis (CDA) of the fatty acid profiles for each nematode sample within a categorical class. The

profiles contained only those fatty acids selected as significant by the STEPDISC procedure. Analyzing each sample with CDA develops canonical class means from sample variances that represent the relative position of each class in a dimensional space. Class means are distributed in space based on variance among classes. The first canonical function (CAN 1) describes the greatest variance among classes and is represented by the x-axis for graphs of canonical class means. Remaining variances among classes are described by subsequent canonical functions (CAN 2 = y-axis, CAN 3 = z-axis) until all variation among classes has been explained. As with all multivariate procedures, the objective was to reduce the number of dimensions to a number smaller than the original number of variables. The number was chosen such that at least 75% of the original multivariance was described. In these experiments, no more than three canonical variates were necessary to achieve that goal.

The fatty acids that increase the variance between classes are indicated by their canonical correlation. A high "between canonical structure" correlation ($r \geq |0.75|$) indicates that the percentage (or presence) of the given fatty acid is responsible for separating the two classes spatially on the given axis. The distance between two classes in space is the Mahalanobis distance (D); this value is the actual numerical displacement between the two means in dimensional space. A sample fatty acid profile is classified to the class that has the minimum squared Mahalanobis distance (D^2) between the sample and a canonical class mean (Johnson, 1998).

Logistic regression of fatty acid expression among classes was determined by the NLMIXED procedure. If a given fatty acid was present in the mean fatty acid profile of a species it was given a value of "1"; if the fatty acid was absent from that sample it was given a value of "0". Logistic regression for detection probability was performed on fatty acids that only occurred in a single species; fatty acids that occurred in more than one species were submitted to stepwise and canonical discriminant analyses. Probability of detection ($\pm$95% confidence) for each fatty acid was achieved by calculating the percentage of positive detections of that fatty acid within all samples of a given dilution. These percentages were plotted against the log of the count of nematodes within a sample dilution to determine the minimum number of nematodes needed for discrimination between species.

The fatty acid profiles resulting from the host experiment were analyzed using the STEPDISC and CANDISC procedures to produce plots representing canonical means for 1) host plants, 2) *M. incognita* on cotton, soybean, and tomato, 3) *R. reniformis* on cotton, soybean, and tomato, 4) *M. incognita* on cotton, soybean, and tomato versus *R. reniformis* on cotton, soybean, and tomato, and 5) pooled host variation within *M. incognita* and *R. reniformis* versus host plants. Fatty acid profiles were also developed using the Sherlock Analysis Software from 1) *M. incognita* on cotton and tomato, 2) *R. reniformis* on cotton, soybean, and tomato, and 3) the cotton, soybean, and tomato host plants themselves.

Sherlock® Analysis Software

A library was developed using the Sherlock Analysis Software by creating entries from nematode fatty acid profiles developed in each study. To determine the usefulness and validity of the newly created library entries with this software, individual samples were compared against their respective composite profiles. This procedure results in comparison and similarity matrices for each study. Comparison matrices list the percentage of time that samples are correctly identified to each newly created library entry. Similarity matrices utilize the fatty acid profiles from each sample and determine their similarity across all selected library entries. Lower numbers indicate samples will match correctly more often, while higher numbers indicate that samples can be mismatched to a neighboring profile.

Sample identification is dependent on the "choice" method. When a sample is analyzed with this software, the fatty acid profile is compared to library entries based on fatty acids present and percentages of each. Library matches are ranked by similarity; the highest similarity is ranked as the most likely match, followed by the next similar entry. "First choice" identification accuracy is based on the proportion of samples that are matched correctly to the highest ranked library entry. "First Second choice" accuracy is dependent on the correct library entry being the first or second most similar entry matched. These matching methods are linked to the comparison matrices for the library; "first-choice" identifications are more accurate when the comparison percentages are closer to 100%. Identification reports list the accuracy using the "First choice" method;

any samples that missed the correct identification are further analyzed using the "First Second choice" method.

Results

Objective 1: Differentiation and Dilution Detection for *M. incognita*, *R. reniformis*, and *H. glycines*

Differentiation

A total of 54 fatty acids were observed among the *M. incognita*, *R. reniformis*, and *H. glycines* nematode genera analyzed. Of these 54, three (16:0, 18:0, and 18:1 ω7c) were the most predominant fatty acids among all three nematode genera (Table 3). The remaining 51 fatty acids varied in their expression among species either by the presence or absence of the fatty acid or the level of expression in a particular species (Table 3). A total of 43 (80%) of the observed fatty acids were expressed among nematode species with less than 1.0% mean concentration, but five (9%) were found to be significant ($P \leq 0.0001$) for the discrimination among nematode species tested (Table 4). Of the remaining 11 (20%) fatty acids with mean percentages greater than 1.0%, six (11%) were significant for separating species, for a total of 11 fatty acids significant for differentiation among *M. incognita*, *R. reniformis*, and *H. glycines* (Table 4).

Using CDA, the fatty acid profiles generated for *M. incognita*, *R. reniformis*, and *H. glycines* were separate and distinct ($P < 0.0001$) from each other. Of the 17 fatty acids selected by the STEPDISC procedure, five explained 72% of the total multi variance in the first canonical dimension comparing *H. glycines* to *M. incognita* ($D^2=32$, $P \leq 0.0001$) and *R. reniformis* ($D^2=26$, $P \leq$

0.0001; Figure 1). Five fatty acids were responsible for separating *H. glycines* from *M. incognita* and *R. reniformis*. The fatty acid 20:4 ω6,9,12,15c, which was only expressed in *H. glycines* and not in *M. incognita* or *R. reniformis*, was highly correlated with CAN 1 (r=|0.997|) along the first canonical dimension. The other four fatty acids (18:1 ω5c, 12:0 2OH, 18:2 ω6,9c/18:0 ANTE, and 15:1 ANTEISO A) all further separated *H. glycines* with high correlations with CAN 1 ($r \geq |0.934|$).

The remaining 28% of the multi variance was illustrated by the differences between the fatty acid profiles of *M. incognita* and *R. reniformis*; six fatty acids dominated the differences observed between *M. incognita* and *R. reniformis*. Though the D^2 value (3.78) was lower between *M. incognita* and *R. reniformis* than when comparing either to *H. glycines*, it was still distinct ($P \leq 0.0001$; Figure 1). Two of the six fatty acids responsible for these differences (15:0 ISO and 18:0 3OH) were highly correlated with CAN 2 ($r > |0.994|$); these fatty acids were found in higher percentages in *R. reniformis* samples than in samples containing *M. incognita*. The four other fatty acids (16:1 ω5c, 18:1 ω9c, 14:0, and 17:0 ISO) further separate *M. incognita* and *R. reniformis*. The fatty acid 16:1 ω5c was not present in *M. incognita*, but had a mean percentage of 2.51% in *R. reniformis*. Both 14:0 and 17:0 ISO were found in higher percentages in *R. reniformis* than *M. incognita*, while 18:1 ω9c was higher in *M. incognita*.

By incorporating all of the analyzed samples (257 total samples) into the Sherlock Analysis Software, it was possible to develop library entries for each species. The comparison matrices generated by the software indicated that all

samples would be correctly identified to the correct species. The similarity matrix also indicated that the fatty acid profiles for *M. incognita* and *R. reniformis* had a 0.6% similarity, but both had 0% similarity with *H. glycines*.

Detection

Using a combination of stepwise analysis, CDA, and logistic regression, it was possible to determine that at least 250 total individuals are required to identify *M. incognita* and *R. reniformis*. Using the greater concentrations of individuals (250 -10,000; Table 1), canonical means were generated and plotted using four methods: 1) CDA without a prior STEPDISC procedure in which only fatty acids found in both *M. incognita* and *R. reniformis* are used (Table 5), 2) CDA without a prior STEPDISC procedure of all fatty acids found in both *M. incognita* and *R. reniformis* are used (Table 6), 3) CDA after a STEPDISC of fatty acids found only in *M. incognita* and *R. reniformis* (Table 7), and 4) CDA after a STEPDISC of all fatty acids found in *M. incognita* and *R. reniformis* (Table 8; Figure 2).

Using canonical analysis without a prior STEPDISC procedure, all dilutions of *M. incognita* could be separated from all *R. reniformis* dilutions ($P \leq 0.021$) either using fatty acids found in both species, or when fatty acids unique to *R. reniformis* were included in the CDA. Only 80% of variation among dilutions was explained by the first three canonical dimensions using fatty acids from both *M. incognita* and *R. reniformis*; the percentage decreased to 79% when fatty acids unique to *R. reniformis* were included. In both of these analyses, there was a large degree of separation ($D^2 \geq 3.61$) among dilutions

within each species. When only using the fatty acids found in both *M. incognita* and *R. reniformis*, four of ten comparisons among dilutions within *M. incognita* and six of ten comparisons within *R. reniformis* were considered significantly different ($P \leq 0.037$) from other dilutions of each species (Table 5; Figure 2A). This also happened when the fatty acids unique to *R. reniformis* are included; three of ten dilution comparisons within *M. incognita* and six of ten comparisons among dilutions within *R. reniformis* are significantly different ($P \leq 0.012$) from the other dilutions (Table 6; Figure 2B).

When the STEPDISC procedure was used to determine fatty acids that were significant for differentiation among dilutions before running a CDA, it was also possible to distinguish between both species when either fatty acids present in both species were used, or if the fatty acids unique to *R. reniformis* were included. The first three canonical dimensions explained 94% of variation among dilutions when only fatty acids from both *M. incognita* and *R. reniformis* were included and when fatty acids unique to *R. reniformis* were included (Table 9). There was also no difference in the number of significant comparisons among dilutions within each species if fatty acids unique to *R. reniformis* were included in the analysis or excluded; there were zero significant comparisons ($P \geq 0.112$) among *M. incognita* dilutions and seven of fifteen significant ($P \leq 0.041$) among *R. reniformis* dilutions. In both instances, using the STEPDISC prior to the canonical analysis allows for a tighter grouping of the *M. incognita* dilutions (average D^2 reduced from 0.65 to 0.58) and *R. reniformis* dilutions (average D^2 reduced to 2.49 from 2.76; Figure 2C and 2D), but when the fatty acids unique to

R. reniformis are included, the average separation between *M. incognita* dilutions and *R. reniformis* dilutions is decreased from 8.14 to 7.50 (Tables 7 and 8; Figure 2C and 2D).

The two fatty acids unique to *R. reniformis*, 16:1 ω5c and 18:1 ω5c, were used to develop a detection threshold curve that could be used for differentiating *M. incognita* and *R. reniformis* at varying dilutions (Figure 3). Thus, detection of either fatty acid would indicate the presence of *R. reniformis* unequivocally as they were not found in *M. incognita* at any sample size. These two fatty acids were used to establish the minimum required dilution of 100 total individuals because neither of these fatty acids will be found in *M. incognita* at 100 total individuals, but have at least a 15% predicted chance of detection in *R. reniformis*. These two fatty acids are found in low concentrations in samples containing large numbers of individuals of *R. reniformis*, 1.46% and 1.67%, respectively, and would be difficult to detect in samples containing fewer than 100 individuals per sample (Figure 3).

It was possible to detect a single nematode in this experiment, though accurate identification was not consistent from the decreased numbers of fatty acids detected in the analyses.

Objective 2: Mixed Ratios of *M. incognita* and *R. reniformis*

5000 Individuals

The profiles developed from the means of each ratio containing 5000 total individuals demonstrated a gradual transition in their expression of fatty acids as the sample percentages decreased for *M. incognita* and increased for *R.*

reniformis (Table 10). For instance, the mean percentage of the 16:0 and 18:0 fatty acids was 14.22% and 13.69%, respectively, in samples containing only *M. incognita*; these values decreased gradually until the percentages for each fatty were 8.72% and 9.83%, respectively, for samples of only *R. reniformis*. Other fatty acids, such as 18:1 ω7c, increased in their percentage as the proportion of *R. reniformis* increased within a sample. This was also true for the two fatty acids found in *R. reniformis* and not *M. incognita*, 16:1 ω5c and 18:1 ω5c. Neither of these fatty acids was found in samples containing only *M. incognita*. As the proportion of *M. incognita* decreased in the samples, the concentrations of 16:1 ω5c and 18:1 ω5c increased gradually until their mean concentrations were 1.46% and 1.67%, respectively, in samples that contained no *M. incognita* (Table 10).

The CDA of the ratios explained 98% of the total variation in the first three canonical dimensions and indicated that all five ratios of *M. incognita* to *R. reniformis* were distinct ($P \leq 0.0028$; Figure 4). The greatest difference in fatty acid profiles was between the pure-species samples of *M. incognita* and *R. reniformis*; the fatty acid 18:1 ω9c was primarily responsible for the differences along the first canonical dimension ($r > |0.999|$; Table 11). Five other fatty acids (18:1 ω7c, 16:0, 20:4 ω6,9,12,15c, 20:0, and 15:0 Iso) reinforced the differences with a slightly lower, but still significant correlation value ($r > |0.968|$; Table 11).

The remaining differences among ratios were described by the second canonical dimension among the three mixed-species samples (75:25, 50:50, 25:75; Table 11). Of these three ratios, the comparison between the 25:75 ratio and the 50:50 ratio was the most similar (D^2=14.05), but still significantly

different from each other (*P*=0.0028; Figure 4). The fatty acid 20:1 ω7c was responsible for the differences along the second canonical dimension, which was not found in samples containing only *R. reniformis*.

<u>500 Individuals</u>

A pattern similar to the expression of fatty acids among ratios containing a total of 5000 individuals was observed in ratios containing 500 total individuals (Table 10). The primary difference was found in samples containing 500 total *R. reniformis* individuals, within which the mean expression of the 18:0 ANTE/18:2 ω6,9c peak was 13.90%; this peak was not observed in samples containing 5000 individuals of *R. reniformis*. As a result, the mean percentage of 18:1 ω7c was reduced to 49.85%. The remaining fatty acids were expressed as they had in samples of 5000 individuals. The fatty acids 16:1 ω5c and 18:1 ω5c were also detected as they had been in samples containing 5000 individuals, with a gradual increase in percentage as the proportion of *R. reniformis* within a sample increased.

The canonical analysis of samples containing 500 individuals explained 99% of the total variation in the first two canonical dimensions (Table 11). Canonical means looked very similar to those of samples with 5000 individuals, though the graph was rotated 180° (Figure 5). As with samples containing 5000 total individuals, the majority of the difference among ratios (97%) was between the pure-species samples of *M. incognita* and *R. reniformis*. The 16:0 fatty acids was responsible for the most difference along the first axis, supported by the six other fatty acids 18:1 ω9c, 18:0, 12:0 2OH, 16:1 ω7c/15:0 iso 2OH, 14:0, and 15:0

Iso. All of these fatty acids had relatively high correlation along the first canonical dimension ($r > |0.917|$; Table 11).

Among the mixed-species samples of 500 total individuals, some differences were observed compared to samples containing 5000 individuals (Table 11). The fatty acid 19:1 Iso I had the highest correlation value along the second canonical dimension, followed by 20:4 ω6,9,12,15c. These two fatty acids separated the Mi:Rr ratio 25:75 from 75:25 as well as 50:50 from 75:25, but could not separate the comparison between the 50:50 ratio and the 25:75 ratio, which was not significant (D^2=4.32, P=0.3956; Figure 5) in samples containing 500 individuals. This comparison was also the most similar in samples containing 5000 individuals.

Sherlock Analysis of Ratios

Library entries were created from these samples in the Sherlock Analysis Software from the combined profiles of each ratio at both concentrations of individuals. It was possible to correctly identify a single-species sample of either *M. incognita* or *R. reniformis* from a mixed-species sample of the two species with 100% accuracy. It was also possible to correctly identify 98% of the samples using the "First Choice" method in the Sherlock Analysis Software when 5000 individuals were present; after adding the "First Second Choice" method, all samples were correctly identified. Matching among ratios of mixed-species was possible with 85% accuracy using the "First Choice" method in samples that contained 500 total individuals; of the incorrectly identified samples, 80% of these were samples containing the 50:50 ratio of *M. incognita* to *R. reniformis*.

A correct identification of a sample to the Mi:Rr ratio of 50:50 occurred with 61.5% accuracy; 30.8% of these samples were identified as 25:75. Samples containing an Mi:Rr ratio of 75:25 could be correctly identified at 100% accuracy at both concentrations of individuals. The Mi:Rr 25:75 ratio could be correctly identified in 93.3% of samples at both concentrations, but were misidentified as 50:50 in 6.7% of samples; the misidentification of samples was in samples containing 500 total individuals. When a "First Second Choice" match was attempted, all samples were correctly identified.

All mixed-species samples of *M. incognita* and *R. reniformis* could be identified from single-species samples, regardless of the number of individuals within a sample. Using the Sherlock Analysis Software, it was possible to correctly identify all samples with 94% accuracy using only the "First Choice" method; when the "First Second Choice" method was used, all samples were correctly identified using this software.

Objective 3: Host Impact on FAME Profiles for *M. incognita* and *R. reniformis*

<u>Host Plants</u>

As observed with nematode profiles, the profile for each plant host varied significantly ($P \leq 0.0001$) from the other host plants (Figure 6). Of the 14 fatty acids observed, 16:0 was found in the highest concentration for all three host plants (34% for cotton, 66% for soybean, and 38% for tomato; Table 12). Combined percentages of 16:0 and 18:0 comprised 85.8% of total fatty acids found in soybean tissue, much higher than the percentages found in cotton

(40.3%) and tomato (53.4%). Only three of the fatty acids observed contained an odd numbered chain length (17:0 Iso, 19:0, and 19:0 Cyclo ω10c); 17:0 Iso was found only in tomato plant samples, where as 19:0 and 19:0 Cyclo ω10c were present only in cotton samples. The remaining fatty acids were observed at varying concentrations among host plants with cotton having the greatest difference in fatty acid expression from either soybean or tomato. Cotton tissue had the most fatty acids observed, followed by tomato and soybean.

Canonical analysis of the three host plants' fatty acid profiles explained 100% of variation in the first two canonical dimensions (Table 13). The analysis indicated that cotton had the most different fatty acid profile, with distances of 57.8 ($P \leq 0.0001$) and 50.4 ($P \leq 0.0001$) from the soybean and tomato profiles, respectively (Figure 6). Tomato and soybean were also significantly different ($P \leq 0.0001$) from one another, but the distance value between the two profiles was much less than that of cotton from either plant (D^2=13.0). Based on these results, it is possible that the host plants may have an impact on the fatty acid profiles of *M. incognita* and *R. reniformis*.

Meloidogyne incognita

Host plants parasitized by *M. incognita* have an effect on the fatty acid profile of the nematode. Profiles for populations grown on each of tomato, cotton, and soybean had visual profile differences (Table 14) which were significantly different ($P \leq 0.0001$) from one another using canonical analysis (Figure 7). *Meloidogyne incognita* populations grown on tomato plants also have higher percentages of 16:0 and 18:0 than populations grown on cotton

plants; cotton populations of *M. incognita* have higher percentages of 18:1 ω9c than populations from tomato. Populations of *M. incognita* grown on soybean had fatty acid profiles less similar to profiles of populations grown on cotton (D^2=186) and tomato (D^2=150) than profiles from populations grown on cotton and tomato were from one another (D^2=17.3; Figure 7).

Using CDA of *M. incognita* populations from tomato, cotton, and soybean, 100% of the total variation was explained by the first two canonical dimensions (Table 15). Twelve fatty acids separated soybean populations of *M. incognita* from cotton and tomato populations, with 20:1 ω7c contributing the most to the differences; this fatty acid was expressed at 3.45% and 3.00% in cotton and tomato populations, respectively, but was only detected at 0.12% in soybean population samples (Table 14). Populations grown on soybeans also had a very high mean concentration of 16:1 ω5c (26.4%), a fatty acid that was not found in *M. incognita* populations grown on cotton or tomato. Other fatty acids that separated soybean populations from cotton and tomato populations (10:0 2OH, 15:0 ANTEISO, 16:0 3OH, and 20:4 ω6,9,12,15c) were also only observed in *M. incognita* that had been grown on soybean and not on populations grown on cotton or tomato.

Six fatty acids helped to separate populations of *M. incognita* grown on cotton from populations grown on tomato using CAN 2 (Table 15). The fatty acid peak identified as 18:2 ω6,9c/18:0 ANTE contributed most to the difference between cotton (1.39%) and tomato (0.11%) populations (Table 14). Other fatty acids separating these populations included 14:0 2OH, 19:1 ISO I, which were

expressed at higher percentages in tomato populations (0.06% and 0.91%, respectively) than populations grown on cotton (0.0% and 0.21%, respectively), and 12:0 2OH, that was expressed at a higher percentage in cotton populations (11.0%) versus tomato populations (7.86%).

Rotylenchulus reniformis

Host plants also had a significant impact on the fatty acid profiles of *R. reniformis*. Many of the same fatty acids were observed in populations grown on each of the three hosts, and most of the differences among populations observed were due to differing levels of expression of the fatty acids detected (Table 16). Populations grown on tomato, cotton, and soybean all varied significantly ($P \leq 0.0001$) from one another, though the D^2 values were not as high between soybean and cotton ($D^2=12.0$) or tomato ($D^2=10.5$) as observed with *M. incognita* populations; the distance between cotton and tomato was also less ($D^2=7.5$; Figure 8).

As observed in *M. incognita* populations from tomato, cotton, and soybean, canonical analysis explained 100% of total variation in the first two canonical dimensions for *R. reniformis* populations (Table 17). Many of the fatty acids (22 of 30) were significant in separating soybean populations of *R. reniformis* from populations grown on cotton or tomato. Three fatty acids (16:0 2OH, 16:1 ω5c, and 17:0) were only observed in soybean populations. The fatty acid 17:0 ISO 3OH was not observed in soybean populations, but was expressed in both cotton and tomato populations (0.2% for both). Eleven fatty acids had higher expression in soybean populations than either cotton or tomato

populations, but six were observed at lower concentrations in soybean than either tomato or cotton.

Six fatty acids were significant for separating tomato and cotton populations of *R. reniformis* (Table 17). Of these six fatty acids, four (12:0, 18:1 ω7c, 18:1 ω9c, and 18:2 cis 9,12) were expressed in higher concentrations within tomato populations (Table 16). The two fatty acids 16:1 C and 18:1 cis 9 had higher mean concentrations in populations of *R. reniformis* grown on cotton (0.5% and 0.9%) than tomato populations (0% and 0.7%).

Total Comparison among *M. incognita*, *R. reniformis*, and Hosts

By comparing the three host populations for each of *M. incognita* and *R. reniformis* with canonical analysis, 92% of total variation was explained by the first three canonical dimensions (Table 18). Each population was distinct ($P < 0.0001$) from the others, with no overlap among host or nematode populations. Populations of *M. incognita* from soybean are the most different from the other nematode populations ($D^2 \geq 201.6$) and were responsible for 60% of the differences among populations (Figure 9). Seven fatty acids were primarily responsible ($r > |0.937|$) for the separation of *M. incognita* soybean populations from the other five populations, with 16:1 ω5c the most highly correlated due to its highly increased expression (26%). Separation of the three *R. reniformis* populations from *M. incognita* cotton and tomato populations accounted for 25% of population differences and was explained by five fatty acids ($r > |0.922|$; Table 18). Four of these fatty acids (unknown peak 15.549, 18:1 trans 9, 18:1 cis 9, and the 18:0 ANTEISO/18:2 ω6,9c peak) were only found in *R. reniformis*

populations; 20:1 ω7c was only found in *M. incognita* populations. The differences within each species caused by the different hosts was defined by two fatty acids, 19:1 ISO I and 16:0 2OH ($r > |0.784|$, 7% of total variation). Percent expression of 19:1 ISO I varied among host populations for both species while 16:0 2OH was only found in tomato populations of *M. incognita* and soybean populations of *R. reniformis*.

CDA defined 85% of the total variation among pooled *M. incognita* and *R. reniformis* populations and host plants in the first three canonical dimensions (Table 19). Canonical means indicated that even though the fatty acid profiles of *M. incognita* and *R. reniformis* vary depending on the host they parasitize, this variation does not inhibit differentiation or identification of either species. Both nematode species were distinctly defined ($P < 0.0001$, $D^2 > 100.2$) from the host plants as well as each other ($P < 0.0001$, $D^2=25.4$; Figure 10). These distances allow for a grouping of host plant profiles and a similar grouping of the nematode profiles. Fifteen fatty acids were significantly correlated ($r > |0.750|$; Table 19) with the difference between the plant and nematode groups (73% of variation), but only four of these were highly correlated ($r > |0.900|$); 15:0 ANTEISO was found only in *M. incognita* and *R. reniformis*, 18:0 3OH was expressed in cotton and soybean populations of *M. incognita* as well as soybean plants, 18:1 ω9c was expressed in higher percentages in host plants, and the 18:0 ANTE/18:2 ω6,9c peak was expressed in percentages varying from 2.2% to 21.6% in host plants, 0.28% in *R. reniformis* (Table 16), and not observed in *M. incognita* populations (Table 14).

Separation within the nematode and host plant groups began in CAN2 (14% of total variation; Table 19) in which 20:0 was responsible for separating cotton (4.7%) from soybean (0.6%) and tomato (0.5%; Table 12) and also helping to separate *M. incognita* (0.9%; Table 14) from *R. reniformis* (3.4%; Table 16). The 14:0 fatty acid also separated *M. incognita* from *R. reniformis* because it only occurred in *M. incognita*. Another 9% of the total variation was described by CAN3; this variation further separated *M. incognita* from *R. reniformis*. Six fatty acids were significantly correlated ($r > |0.750|$) with defining *M. incognita* and *R. reniformis* from each other; 16:0 3OH, 16:1 ω7c, and 20:1 ω7c were only found in *M. incognita* and 19:1 ISO I, 16:1 ω5c, and 18:1 ω7c were found in higher percentages in *M. incognita* than *R. reniformis*.

Library entries were created for each of cotton, tomato, and soybean host plants and *M. incognita* and *R. reniformis* populations from each host. By analyzing these data with the Sherlock Analysis software, it was also possible to correctly identify total *M. incognita*, total *R. reniformis*, and host plant with 100% accuracy with "First choice" matching. Individual host influences of cotton, tomato, and soybeans could be identified at near 100% accuracy as "First choice" in *M. incognita* samples. Tomato populations of *R. reniformis* matched 95% correct on "First choice" and 100% on "First Second choice," with the misidentified samples matching to *R. reniformis* cotton populations on the first match attempt. Populations of *R. reniformis* grown on soybean matched correctly on "First choice" with 86% accuracy; misidentified samples matched to tomato populations of *R. reniformis*. Using "First Second choice" matching for

R. reniformis populations on soybeans, matching accuracy reached 100%. "First choice" matching of cotton *R. reniformis* populations was 87%, mismatching to tomato and soybean populations; "First Second choice" matching only reached 94% accuracy since some samples matched as tomato populations first and soybean populations second. Overall, correct matching across all populations of *M. incognita*, *R. reniformis*, and host plants was 96% accurate with "First choice" matching and 99% accurate using "First Second choice" with the Sherlock Analysis software.

Discussion

Objective 1: Differentiation and Dilution Detection for *M. incognita*, *R. reniformis*, and *H. glycines*

Differentiation

We developed and compared FAME profiles for each of the nematode species studied. As Krusberg (1967) found in his studies with other plant-parasitic nematodes, we found 18:1 fatty acids were the predominant fatty acids in *R. reniformis* and *H. glycines*, specifically 18:1 ω7c. The primary fatty acid we observed in *M. incognita* was 16:0, which was contrary to the previous studies by Krusberg *et al.* (1973) in which 18:1 ω7c was predominant. In our study, the analyses of the samples containing fewer than 100 nematodes consistently expressed 16:0 as the only fatty acid present. By removing the samples with less than 100 individuals, the 18:1 ω7c fatty acid expressed with a mean percentage of 20.8%.

Detection

Since it was possible to produce fatty acid profiles for *M. incognita*, *R. reniformis,* and *H. glycines* using FAME analysis and to identify each species using the Sherlock Analysis Software, it should be possible for further studies of additional nematode species. Previous studies of nematode fatty acid compositions (Abu Hatab and Gaugler, 1997; Abu Hatab and Gaugler, 1999; Beams, 1964; Chitwood and Krusberg, 1981; Fletcher and Krusberg, 1973; Gibson *et al.*, 1995; Hutzell and Krusberg, 1982; Krusberg, 1967, 1972; Krusberg et al. 1973; Orcutt et al. 1978; Sivapalan and Jenkins, 1966) were based on actual weights of nematodes studied to determine the percentage of lipids present, but none of these studies indicate the actual number of nematodes within the samples. The dry weight of nematodes used in these studies ranged from 14 mg to 500 mg.

Meloidogyne incognita can be differentiated from *R. reniformis* in samples containing 100 or more individuals, although it may be possible to reduce this threshold. As mentioned, we were able to detect a single nematode, and in some cases identify the individual nematode, but identification accuracy decreased by 20% for every dilution decrease below 100 individuals because of the reduced concentration of fatty acids in samples. Reducing the number of steps that a nematode must be transferred before it is placed in the extraction tubes and/or reducing the volume of fluid the nematode is transferred with, may make it possible to remove any capturing error and obtain consistent results with

a single nematode. Determining the number of nematodes needed for an analysis could make studies more applicable for diagnostic laboratories.

Objective 2: Mixed Ratios of *M. incognita* and *R. reniformis*

A gradual progression in the percentages of fatty acids was observed as ratios of 5000 total individuals that contained increasing proportions of either *M. incognita* or *R. reniformis*. It was possible to detect the fatty acids only found in *R. reniformis* (16:1 ω5c and 18:1 ω5c) in mixed-species samples containing only 25% of *R. reniformis*. Conversely, it was possible to detect 20:4 ω6,9,12,15c, which is characteristic to *M. incognita*, in mixed-species samples containing only 25% *M. incognita*. The presence of a combination of 20:4 ω6,9,12,15c with either 16:1 ω5c or 18:1 ω5c would indicate that a sample contains a mixed population of *M. incognita* and *R. reniformis*.

The increased expression of the 18:0 ANTE/18:2 ω6,9c peak in the 100% *R. reniformis* samples containing 500 individuals was found in 20% of samples. This may be due to the genetic variation within the population. The difficulty of separation of the Mi:Rr 25:75 and 50:50 ratios is probably due to the decreased quantity of lipids within the sample containing 500 individuals compared to samples containing 5000 individuals. Repeating the ratio analysis with 500 individuals will help to clarify if it is possible to differentiate a ratio containing 25% *M. incognita* from a sample containing 50%.

Though there have been several studies on multiple species of nematodes (Hutzell and Krusberg, 1982; Krusberg 1967; Krusberg et al. 1973), there are no studies on mixed-species populations. Mixed-species populations are common in

cotton field samples. A study by Gazaway and McLean (2003) indicated that 39% of soil samples from cotton fields contained one species of plant-parasitic nematode; 61% contained more than a single species. In samples that contained greater than a single plant-parasitic nematode species, 80% of those samples contained two or three species. Other, less common plant-parasitic nematodes can be found in fewer numbers than the predominant species, but could still cause enough variation to reduce the confidence of identification by FAME analysis. By further analyzing more nematode species, it should be possible to reduce the variation found when multiple species are present and even identify those contaminant species.

Objective 3: Host Impact on FAME Profiles for *M. incognita* and *R. reniformis*

The fatty acids found in the three host plants were comprised mainly of 16-carbon, 18-carbon, and 20-carbon fatty acids, as found in alfalfa (*Medicago sativa* L.) callus tissue (Krusberg, 1967), though 16:0 was the predominant fatty acid found in cotton, soybean, and tomato plants compared to 18:2 in alfalfa callus tissue. The other fatty acid percentages varied among plant tissues. Populations of *M. incognita* and *R. reniformis* grown on cotton plants expressed more low-percentage fatty acids than populations grown on tomato and soybean plants, most likely caused by the greater number of fatty acids found in cotton tissue.

Fatty acid profiles of *M. incognita* populations from cotton and tomato were influenced as expected based on the fatty acids present in both hosts. The

three fatty acids 16:0, 18:0, and 18:1 ω9c all appeared to have a direct influence on the fatty acid profiles of *M. incognita*. These results coincide with the findings of other plant-parasitic nematodes in which it appeared that these three fatty acids were directly incorporated from the plant into the nematodes without any modification (Krusberg, 1967). The remaining fatty acids are believed to be synthesized by the nematodes through modification or biosynthesis as proposed by Krusberg (1967, 1972, 1973).

Both 16:0 and 18:0 fatty acids from all three host populations of *R. reniformis* and cotton and tomato populations of *M. incognita* directly reflected the host plant percentages, as was proposed for other plant-parasitic nematodes by Krusberg (1967). The fatty acid 18:1 ω9c is found in extremely low percentages in *R. reniformis* (<0.17%), but 18:1 ω7c is found in relatively high concentrations in tomato populations of *R. reniformis* (11.7%), with lower concentrations in cotton (1.1%) and soybean populations (1.0%). Since 18:1 ω7c is not found in any of the host tissues, it is most likely produced with the other predominant fatty acids by biosynthesis and modification (Krusberg, 1967, 1973).

Since it was possible to identify *M. incognita*, *R. reniformis*, and *H. glycines* with the Sherlock Analysis Software in our studies, it is feasible that increasing the number of nematodes analyzed with this system could allow for identification of nematode samples in diagnostic laboratories. FAME analysis could become a powerful tool to reduce the processing and response times in diagnostic labs. Preparing nematode samples for FAME analysis requires approximately four hours of extraction time, after which the GC analysis is

automated and requires 25 minutes to analyze a single sample. Fifty samples could be completed with four hours of labor and 21 hours of analysis. The same number of samples would take nearly three days of continuous labor to complete using the current methods.

Using FAME analysis, we were able to 1) differentiate and identify *M. incognita*, *R. reniformis*, and *H. glycines*, 2) quantify that 100 total individuals of *M. incognita* or *R. reniformis* were required for near 100% accurate identification, 3) and determine that tomato, cotton, and soybean plants influence the fatty acids found in *M. incognita* and *R. reniformis*, but these influences do not inhibit identification of the these two species by the library we developed with the Sherlock Analysis Software. Based on these results, it would be practical to pursue further development of this method for use by diagnostic laboratories to increase the efficiency of sample processing and reduce labor costs.

Literature Cited

Abu Hatab, M. A., R. Gaugler. 1997. Influence of growth temperature on fatty acids and phospholipids on *Steinernema riobravis* infective juveniles. Journal of Thermal Biology. 22:237-244.

Abu Hatab, M. A., R. Gaugler. 1999. Lipids of in vivo and in vitro cultured *Heterorhabditis bacteriophora*. Biological Control. 15:113-118.

Chitwood, D. J., and L. R. Krusberg. 1981. Diacyl, alkylacyl and alkenylacyl phospholipids of the nematode *Tubatrix aceti*. Comparative Biochemistry and Physiology. 69B:115-120

Chitwood, D. J., and L. R. Krusberg. 1981. Diacyl, alkylacyl, and alkenylacyl phospholipids of *Meloidogyne javanica* females. Journal of Nematology. 13:105-111

Fletcher, C. L., and L. R. Krusberg. 1973. Investigation of some lipids from *Tubatrix aceti*. Comparative Biochemistry and Physiology. 45B:159-165

Gazaway, W. S., K. S. McLean. 2003. A survey of plant-parasitic nematodes associated with cotton in Alabama. Journal of Cotton Science. 7(1):1-7

Gibson, D. M., R. A. Moreau, G. P. McNeil, and B. B. Brodie. 1995. Lipid composition of cyst stages of *Globodera rostochiensis*. Journal of Nematology. 27(3):304-311

Graham, J. H., N. C. Hodge, and J. B. Morton. 1995. Fatty acid methyl ester profiles for characterization of glomalean fungi and their endomycorrhizae. Applied and Environmental Microbiology. 61(1):58-64

Hutzell, P. A., and L. R. Krusberg. 1982. Fatty acid compositions of *Caenorhabditis elegans* and *C. briggsae*. Comparative Biochemistry and Physiology. 73B:517-520

Johnson, D. E. 1998. Applied multivariate methods for data analysis. Duxbury Press, Pacific Grove, CA

Krusberg, L. R. 1967. Analyses of total lipids and fatty acids of plant-parasitic nematodes and host tissues. Comparative Biochemistry and Physiology. 21:83-90

Krusberg, L. R. 1972. Fatty acid compositions of *Tubatrix aceti* and its culture medium. Comparative Biochemistry and Physiology. 41B:89-98

Krusberg, L. R., R. S. Hussey, and C. L. Fletcher. 1973. Lipid and fatty acid compositions of females and eggs of *Meloidogyne incognita* and *M. arenaria*. Comparative Biochemistry and Physiology. 45B:335-341

Kunitsky, C., G. Osterhout, and M. Sasser. . Identification of microorganisms using fatty acid methyl ester (FAME) analysis and the MIDI Sherlock Microbial Identification System. *Encyclopedia of Rapid Microbiological Methods*, MIDI, Inc., Newark, DE

Orcutt, D. M., J. A. Fox, and C. A. Jake. 1978. The sterol, fatty acid, and hydrocarbon composition of *Globodera solanacearum*. Journal of Nematology. 10(3):264-269

Rothstein, M., and P. Gotz. 1968. Biosynthesis of fatty acids in the free-living nematode, *Tubatrix aceti*. Archives of Biochemistry and Biophysics. 126:131-140

Sasser M. J. 1990. Identification of bacteria by gas chromatography of cellular fatty acids. *Technical Note 101*. Microbial ID, Inc., Newark, Del.

Sivapalan, P., and W. R. Jenkins. 1966. Phospholipid and long-chain fatty acid composition of the nematode *Panagrellus redivivus*. Proceedings of the Helminthological Society of Washington. 33:149-157

Stahl, P. D. and M. J. Klug. 1996. Characterization and differentiation of filamentous fungi based on fatty acid composition. Applied and Environmental Microbiology. 62(11):4136-4146

Womersley, C., S. N. Thompson, and L. Smith. 1982. Anhydrobiosis in nematodes II: carbohydrate and lipid analysis in undesiccated nematodes. Journal of Nematology. 14:145-153.

Table 1. Number of nematodes per sample (N) for each of three plant-parasitic nematode species and replications for each dilution (r).

M. incognita		*R. reniformis*		*H. glycines*	
N	r	N	r	N	r
10000	9	10000	6		
5000	9	5000	6		
1000	8	1000	6		
500	9	500	6		
250	9	250	6		
100	13	100	6		
50	24	50	6		
25	12	25	8	25	10
10	12	10	8	10	10
1	36	1	28	1	10

Table 2. Ratio percentages of *Meloidogyne incognita* to *Rotylenchulus reniformis* at two counts of individuals per sample.

Total Individuals	Ratio %	
	M. incognita	*R. reniformis*
5000	100	0
	75	25
	50	50
	25	75
	0	100
500	100	0
	75	25
	50	50
	25	75
	0	100

Table 3. Mean fatty acid percentages for *Meloidogyne incognita*, *Rotylenchulus reniformis*, and *Heterodera glycines*. Means are based on 141 samples of *M. incognita*, 86 of *R. reniformis,* and 30 of *H. glycines* listed in order by fatty acid chain length, location of the double bond, and functional group.

Fatty Acid	*M. incognita*	*R. reniformis*	*H. glycines*
10:0	0.13	1.38	--
12:0 2OH	11.01	13.00	0.16
14:0	0.02	0.46	0.90
16:0	11.25	13.21	2.77
16:1 ω5c	--†	--	1.48
16:1 ω7c/15 iso 2OH	0.05	--	1.53
17:0 ANTEISO	--	2.87	--
18:0	23.69	12.64	1.71
18:0 3OH	0.01	0.05	1.12
18:1 CIS 11/t 9/t 6	--	14.47	--
18:1 CIS 9	--	0.90	--
18:1 TRANS 9/t6/c11	--	9.45	--
18:1 ω5c	0.05	--	2.31
18:1 ω7c	46.91	1.06	60.14
18:1 ω9c	0.49	0.06	2.52
18:2 ω6,9c/18:0 ANTE	1.39	0.62	6.01
18:3 ω6c (6,9,12)	0.22	0.17	1.04
19:0 ANTEISO	--	2.00	--
19:1 ISO I	0.21	0.54	0.98
20:0	0.98	1.66	0.52
20:1 TRANS 11	--	2.07	--
20:1 ω7c	3.48	0.07	3.14
20:2 ω6,9c	--	1.38	0.69
20:4 ω6,9,12,15c	--	--	9.63
TBSA 10Me18:0	--	0.73	0.03
unknown 15:549	--	1.26	--
unknown 18:814	--	13.18	--

† = Not detected

Table 4. Phenotypic canonical correlation of fatty acids for the two canonical discriminant functions of FAME profile analysis for *M. incognita*, *R. reniformis*, and *H. glycines*. Eigenvalue, cumulative percent of total variance, and canonical correlation are listed for each canonical function. Fatty acids listed were determined significant by the STEPDISC procedure. Correlation values are determined to be significant if $r \geq |0.75|$ (**bold**).

Response variable		Discriminant variate	
No.	Fatty acid variable	CAN 1	CAN 2
1	12:0 2OH	**0.963**	0.268
2	14:0	0.307	**-0.952**
3	15:0 ISO	0.102	**-0.995**
4	15:1 ANTEISO A	**0.938**	0.346
5	16:1 ω7c/15 iso 2OH	-0.624	**0.782**
6	16:1 ω5c	0.201	**-0.980**
7	17:0 ISO	0.345	**-0.939**
8	16:0 ISO 3OH	-0.611	**0.792**
9	18:2 ω6,9c/18:0 ANTE	**0.951**	0.308
10	18:1 ω9c	-0.230	**0.973**
11	18:1 ω7c	**0.785**	-0.619
12	18:1 ω5c	**0.969**	-0.247
13	18:0	0.604	**-0.797**
14	19:1 ISO I	**0.817**	-0.576
15	20:4 ω6,9,12,15c	**0.997**	-0.079
16	18:0 3OH	-0.108	**-0.994**
17	20:2 ω6,9c	-0.585	0.811
	Eigenvalue	1.854	0.720
	Cumulative %	72.0	100.0
	Canonical Correlation	0.81	0.65

Table 5. Squared Mahalanobis distances (D^2) and class distance probability of similarity (*P*) for canonical discriminant analysis of samples containing 250, 500, 1000, 5000, and 10,000 total individuals of *Meloidogyne incognita* (Mi) and *Rotylenchulus reniformis* (Rr). Canonical analysis contained only fatty acids found in both *M. incognita* and *R. reniformis* with no prior stepwise procedure to select only fatty acids significant for differentiation.

	Mi250		Mi500		Mi1000		Mi5000		Mi10,000	
	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*
Mi250			5.67	0.315	6.14	0.298	13.75	0.002	10.94	0.011
Mi500	5.67	0.315			3.61	0.788	9.19	0.037	13.91	0.002
Mi1000	6.14	0.298	3.61	0.788			8.08	0.103	7.79	0.122
Mi5000	13.75	0.002	9.19	0.037	8.08	0.103			5.03	0.432
Mi10,000	10.94	0.011	13.91	0.002	7.79	0.122	5.03	0.432		
Rr250	16.69	0.000	18.32	0.000	12.63	0.009	19.22	<0.0001	15.51	0.001
Rr500	19.81	0.000	17.09	0.001	15.07	0.004	17.33	0.001	19.69	0.000
Rr1000	18.54	0.000	19.80	<0.0001	16.49	0.001	19.04	0.000	17.25	0.000
Rr5000	23.01	<0.0001	23.65	<0.0001	20.98	<0.0001	24.45	<0.0001	23.01	<0.0001
Rr10,000	41.78	<0.0001	45.71	<0.0001	39.91	<0.0001	46.12	<0.0001	38.73	<0.0001

	Rr250		Rr500		Rr1000		Rr5000		Rr10,000	
	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*
Rr250			14.00	0.007	11.39	0.020	16.64	0.001	32.07	<0.0001
Rr500	14.00	0.007			4.57	0.704	3.80	0.836	17.65	0.001
Rr1000	11.39	0.020	4.57	0.704			4.11	0.734	19.50	0.000
Rr5000	16.64	0.001	3.80	0.836	4.11	0.734			9.39	0.064
Rr10,000	32.07	<0.0001	17.65	0.001	19.50	0.000	9.39	0.064		

Table 6. Squared Mahalanobis distances (D^2) and class distance probability of similarity (*P*) for canonical discriminant analysis of samples containing 250, 500, 1000, 5000, and 10,000 total individuals of *Meloidogyne incognita* (Mi) and *Rotylenchulus reniformis* (Rr). Canonical analysis contained fatty acids found in both *M. incognita* and *R. reniformis* as well as fatty acids unique to *R. reniformis* with no prior stepwise procedure to select only fatty acids significant for differentiation.

	Mi250		Mi500		Mi1000		Mi5000		Mi10,000	
	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*
Mi250			7.33	0.216	6.99	0.313	15.04	0.002	13.28	0.007
Mi500	7.33	0.216			3.81	0.858	9.50	0.066	13.97	0.004
Mi1000	6.99	0.313	3.81	0.858			8.17	0.180	8.23	0.175
Mi5000	15.04	0.002	9.50	0.066	8.17	0.180			5.50	0.496
Mi10,000	13.28	0.007	13.97	0.004	8.23	0.175	5.50	0.496		
Rr250	18.22	0.001	18.76	0.001	12.82	0.021	19.24	0.000	16.07	0.002
Rr500	21.05	0.000	19.23	0.001	16.15	0.006	18.21	0.001	22.31	0.000
Rr1000	20.08	0.000	24.63	<0.0001	19.53	0.001	22.13	<0.0001	23.00	<0.0001
Rr5000	25.05	<0.0001	24.30	<0.0001	21.39	0.000	24.57	<0.0001	23.72	<0.0001
Rr10,000	47.04	<0.0001	46.72	<0.0001	41.91	<0.0001	47.95	<0.0001	39.32	<0.0001

	Rr250		Rr500		Rr1000		Rr5000		Rr10,000	
	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*
Rr250			14.80	0.012	14.47	0.009	16.69	0.003	33.94	<0.0001
Rr500	14.80	0.012			5.43	0.694	4.67	0.812	22.77	0.000
Rr1000	14.47	0.009	5.43	0.694			7.48	0.302	29.16	<0.0001
Rr5000	16.69	0.003	4.67	0.812	7.48	0.302			11.23	0.050
Rr10,000	33.94	<0.0001	22.77	0.000	29.16	<0.0001	11.23	0.050		

Table 7. Squared Mahalanobis distances (D^2) and class distance probability of similarity (*P*) for canonical discriminant analysis of samples containing 250, 500, 1000, 5000, and 10,000 total individuals of *Meloidogyne incognita* (Mi) and *Rotylenchulus reniformis* (Rr). Canonical analysis contained only fatty acids found in both *M. incognita* and *R. reniformis* with prior stepwise procedure to select only fatty acids significant for differentiation.

	Mi250		Mi500		Mi1000		Mi5000		Mi10,000	
	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*
Mi250			2.54	0.263	0.94	0.888	3.37	0.112	0.78	0.918
Mi500	2.54	0.263			1.50	0.676	0.41	0.989	2.03	0.422
Mi1000	0.94	0.888	1.50	0.676			2.03	0.465	0.54	0.977
Mi5000	3.37	0.112	0.41	0.989	2.03	0.465			2.15	0.379
Mi10,000	0.78	0.918	2.03	0.422	0.54	0.977	2.15	0.379		
Rr250	9.52	0.000	11.59	<0.0001	9.29	0.000	13.09	<0.0001	10.49	<0.0001
Rr500	13.97	<0.0001	12.87	<0.0001	11.45	0.000	13.08	<0.0001	13.16	<0.0001
Rr1000	15.55	<0.0001	14.33	<0.0001	11.71	<0.0001	14.57	<0.0001	13.61	<0.0001
Rr5000	19.13	<0.0001	17.64	<0.0001	15.68	<0.0001	18.76	<0.0001	18.12	<0.0001
Rr10,000	32.50	<0.0001	31.59	<0.0001	28.72	<0.0001	32.19	<0.0001	31.36	<0.0001

	Rr250		Rr500		Rr1000		Rr5000		Rr10,000	
	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*
Rr250			5.61	0.026	5.80	0.015	9.53	0.000	22.02	<0.0001
Rr500	5.61	0.026			2.17	0.507	1.72	0.667	7.94	0.003
Rr1000	5.80	0.015	2.18	0.507			3.21	0.193	14.07	<0.0001
Rr5000	9.53	0.000	1.72	0.667	3.21	0.193			5.33	0.024
Rr10,000	22.02	<0.0001	7.94	0.003	14.07	<0.0001	5.33	0.024		

Table 8. Squared Mahalanobis distances (D^2) and class distance probability of similarity (P) for canonical discriminant analysis of samples containing 250, 500, 1000, 5000, and 10,000 total individuals of *Meloidogyne incognita* (Mi) and *Rotylenchulus reniformis* (Rr). Canonical analysis contained fatty acids found in both *M. incognita* and *R. reniformis* as well as fatty acids unique to *R. reniformis* with prior stepwise procedure to select only fatty acids significant for differentiation.

	Mi250		Mi500		Mi1000		Mi5000		Mi10,000	
	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*
Mi250			2.55	0.351	0.95	0.932	3.38	0.165	0.78	0.955
Mi500	2.55	0.351			1.55	0.752	0.46	0.993	2.04	0.523
Mi1000	0.95	0.932	1.55	0.752			2.03	0.572	0.55	0.989
Mi5000	3.38	0.165	0.46	0.993	2.03	0.572			2.16	0.478
Mi10,000	0.78	0.955	2.04	0.523	0.55	0.989	2.16	0.478		
Rr250	9.63	0.000	11.79	<0.0001	9.34	0.001	13.14	<0.0001	10.60	0.000
Rr500	15.27	<0.0001	14.44	<0.0001	12.51	<0.0001	14.12	<0.0001	14.44	<0.0001
Rr1000	16.68	<0.0001	15.71	<0.0001	12.62	<0.0001	15.45	<0.0001	14.72	<0.0001
Rr5000	20.37	<0.0001	19.14	<0.0001	16.70	<0.0001	19.74	<0.0001	19.35	<0.0001
Rr10,000	33.50	<0.0001	32.83	<0.0001	29.52	<0.0001	32.95	<0.0001	32.35	<0.0001
	Rr250		Rr500		Rr1000		Rr5000		Rr10,000	
	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*
Rr250			6.27	0.025	6.34	0.016	10.14	0.000	22.47	<0.0001
Rr500	6.27	0.025			2.18	0.612	1.72	0.762	7.96	0.006
Rr1000	6.34	0.016	2.18	0.612			3.21	0.270	14.08	<0.0001
Rr5000	10.14	0.000	1.72	0.762	3.21	0.270			5.34	0.041
Rr10,000	22.47	<0.0001	7.96	0.006	14.08	<0.0001	5.34	0.041		

Table 9. Phenotypic canonical correlation of fatty acids for the two canonical discriminant functions of FAME profile analysis for *M. incognita* and *R. reniformis* dilutions. Eigenvalue, cumulative percent of total variance, and canonical correlation are listed for each canonical function. Fatty acids listed were determined significant by the STEPDISC procedure. Correlation values are determined to be significant if $r \geq |0.75|$ (**bold**).

Response variable		Discriminant variate	
No.	Fatty acid variable	CAN 1	CAN 2
1	14:0	**0.981**	-0.075
2	16:0	-0.534	-0.198
3	18:2 ω6,9c/18:0 ANTE	-0.473	-0.701
4	18:1 ω7c	**0.865**	0.421
5	18:0	-0.127	0.574
6	20:4 ω6,9,12,15c	**0.870**	-0.455
7	18:0 3OH	0.629	-0.160
8	20:0	0.506	**0.773**
9	16:1 ω5c	**0.981**	-0.079
	Eigenvalue	4.643	0.843
	Cumulative %	74.3	87.8
	Canonical Correlation	0.91	0.68

Table 10. Mean percentages of five ratios of *Meloidogyne incognita* and *Rotylenchulus reniformis* in samples containing 5000 and 500 total individuals listed in order by fatty acid chain length, location of the double bond, and functional group. Ratios are percentages of *M. incognita* to *R. reniformis* (Mi:Rr).

	5000 Total Individuals				
Fatty Acid	Mi:Rr 100:0	Mi:Rr 75:25	Mi:Rr 50:50	Mi:Rr 25:75	Mi:Rr 0:100
12:0 2OH	0.51	1.63	0.56	1.25	1.42
14:0	2.20	3.22	2.60	2.79	2.00
15:0 ISO	0.42	1.53	1.87	1.97	1.72
16:0	14.22	10.50	9.01	9.67	8.72
16:1 ω5c	--†	0.22	0.73	1.68	1.46
16:1 ω7c/15 iso 2OH	2.40	3.63	3.21	3.62	3.02
18:0	13.69	11.31	9.95	9.28	9.83
18:0 3OH	0.32	--	0.31	--	0.07
18:0 ANTE/18:2 ω6,9c	0.87	--	1.67	--	--
18:1 ω5c	--	--	1.12	1.48	1.67
18:1 ω7c	41.56	53.03	56.38	56.91	59.67
18:1 ω9c	8.40	4.03	2.77	2.87	2.07
18:2 ω6,9c/18:0 ANTE	3.29	3.91	1.94	3.20	2.76
18:3 ω6c (6,9,12)	--	--	0.46	0.76	0.40
19:1 ISO I	1.35	1.21	1.64	1.52	1.18
20:0	3.11	0.45	0.22	0.08	0.11
20:1 ω7c	3.08	3.19	3.31	2.41	2.67
20:4 ω6,9,12,15c	4.56	1.55	0.23	0.13	--

	500 Total Individuals				
Fatty Acid	Mi:Rr 100:0	Mi:Rr 75:25	Mi:Rr 50:50	Mi:Rr 25:75	Mi:Rr 0:100
12:0 2OH	3.95	1.72	1.13	1.14	0.41
14:0	--	1.85	1.82	1.51	1.50
15:0 ISO	--	1.45	1.46	1.33	1.05
16:0	19.13	8.93	8.17	8.00	6.54
16:1 ω5c	--	0.52	0.99	0.89	2.23
16:1 ω7c/15 iso 2OH	--	2.67	2.38	2.27	2.25
17:0 ISO	--	--	0.56	0.54	0.26
18:0	18.93	11.42	11.04	11.35	8.78
18:0 3OH	0.37	0.29	0.27	0.32	0.23
18:0 ANTE/18:2 ω6,9c	0.85	--	0.25	--	13.90
18:1 ω5c	--	1.38	1.62	1.58	2.22
18:1 ω7c	43.11	55.42	57.62	59.74	49.85
18:1 ω9c	8.42	3.28	2.48	2.07	1.49
18:2 ω6,9c/18:0 ANTE	1.35	2.88	2.43	2.34	1.89
18:3 ω6c (6,9,12)	--	0.10	0.58	0.55	0.29
19:1 ISO I	0.96	2.30	1.85	1.49	1.08
20:0	--	1.21	1.25	1.13	0.49
20:0 ISO	--	--	0.48	0.49	0.34
20:1 ω7c	1.70	3.16	3.19	3.09	2.57
20:4 ω6,9,12,15c	1.24	1.43	0.37	0.17	--

† = Not detected

Table 11. Phenotypic canonical correlation of fatty acids for the two canonical discriminant functions of FAME profile analysis for *M. incognita* and *R. reniformis* ratios containing 5000 and 500 total individuals. Eigenvalue, cumulative percent of total variance, and canonical correlation are listed for each canonical function. Fatty acids listed were determined significant by the STEPDISC procedure. Correlation values are determined to be significant if $r \geq |0.75|$ (**bold**).

5000 Total Individuals			
Response variable		Discriminant variate	
No.	Fatty acid variable	CAN 1	CAN 2
1	12:0 2OH	0.517	-0.229
2	14:0	0.166	-0.670
3	15:0 ISO	**0.968**	-0.015
4	16:0	**-0.996**	0.025
5	16:1 ω5c	**0.756**	0.629
6	16:1 ω7c/15 iso 2OH	0.710	-0.337
7	18:0	**-0.963**	-0.190
8	18:0 3OH	-0.527	0.061
9	18:0 ANTE/18:2 ω6,9c	-0.215	-0.124
10	18:1 ω5c	**0.778**	0.612
11	18:1 ω7c	**0.996**	0.066
12	18:1 ω9c	**-1.000**	-0.005
13	20:0	**-0.983**	0.111
14	20:1 ω7c	-0.329	**-0.767**
15	20:4 ω6,9,12,15c	**-0.994**	-0.073
	Eigenvalue	113.26	2.94
	Cumulative %	96.0	98.4
	Canonical Correlation	1.00	0.86
500 Total Individuals			
Response variable		Discriminant variate	
No.	Fatty acid variable	CAN 1	CAN 2
1	12:0 2OH	**0.981**	0.142
2	14:0	**-0.936**	0.334
3	15:0 ISO	**-0.917**	0.326
4	16:0	**0.998**	-0.017
5	16:1 ω5c	-0.728	-0.428
6	16:1 ω7c/15 iso 2OH	**-0.951**	0.305
7	17:0 ISO	-0.621	-0.485
8	18:0	**0.981**	-0.000
9	18:1 ω7c	**-0.792**	0.184
10	18:1 ω9c	**0.997**	0.067
11	19:1 ISO I	-0.445	**0.841**
12	20:0	**-0.754**	0.433
13	20:0 ISO	-0.684	-0.578
14	20:4 ω6,9,12,15c	0.643	**0.757**
	Eigenvalue	166.81	3.37
	Cumulative %	97.3	99.2
	Canonical Correlation	1.00	0.88

Table 12. Fatty acid mean percentages for the three host species tomato, cotton and soybean. Fatty acid values are listed in order by fatty acid chain length, location of the double bond, and functional group.

Fatty Acid	Tomato	Cotton	Soybean
10:0 2OH	1.05	0.73	1.50
16:0	38.04	34.34	65.97
17:0 ISO	5.48	--	0.07
18:0	14.30	5.97	19.84
18:0 ANTE/18:2 ω6,9c	6.22	21.65	2.23
18:1 ω9c	2.44	4.84	1.34
18:2 ω6,9c/18:0 ANTE	19.76	7.74	3.35
18:3 ω6c (6,9,12)	4.42	0.18	1.70
19:0	0.05	5.36	--
19:0 CYCLO ω10c/19ω6	0.25	3.30	--
20:0	0.47	4.66	0.56
20:4 ω6,9,12,15c	--†	3.96	0.15
TBSA 10Me18:0	--	1.60	--
unknown 10.928	2.08	0.16	0.60

† = Not detected

Table 13. Phenotypic canonical correlation of fatty acids for the two canonical discriminant functions of FAME profile analysis for tomato, cotton, and soybean plants. Eigenvalue, cumulative percent of total variance, and canonical correlation are listed for each canonical function. Fatty acids listed were determined significant by the STEPDISC procedure. Correlation values are determined to be significant if $r \geq |0.75|$ (**bold**).

Response variable		Discriminant variate	
No.	Fatty acid variable	CAN 1	CAN 2
1	10:0 2OH	**-0.880**	0.476
2	12:0	0.439	**0.898**
3	16:0	-0.679	0.734
4	16:0 2OH	-0.576	**0.818**
5	16:0 ISO 3OH	**0.984**	0.181
6	17:0	**1.000**	0.000
7	17:0 ISO	-0.444	**-0.896**
8	17:1 ω9c	**0.997**	0.077
9	18:0	**-0.956**	0.293
10	18:0 3OH	-0.576	**0.818**
11	18:0 ANTE/18:2 ω6,9c	**0.993**	-0.122
12	18:1 ω6c	**0.997**	0.077
13	18:2 ω6,9c/18:0 ANTE	-0.190	**-0.982**
14	19:0	**0.998**	0.070
15	19:0 ANTEISO	**0.997**	0.077
16	19:0 CYCLO ω10c/19ω6	**1.000**	0.009
17	20:0	**0.996**	0.093
18	20:4 ω6,9,12,15c	**0.994**	0.110
19	TBSA 10Me18:0	**0.997**	0.077
	Eigenvalue	11.83	2.11
	Cumulative %	84.8	100.0
	Canonical Correlation	0.96	0.82

Table 14. Mean fatty acid percentages of three populations of *Meloidogyne incognita* grown on the host plants, tomato, cotton, and soybean. Fatty acid means are listed in order by fatty acid chain length, location of the double bond, and functional group.

Fatty Acid	*Meloidogyne incognita* populations		
	Tomato	Cotton	Soybean
10:0 2OH	--†	--	1.27
12:0 2OH	7.86	11.01	8.01
16:0	18.46	11.25	33.40
17:0 ANTEISO	1.08	--	0.12
18:0	40.25	23.69	7.48
18:1 ω5c	0.06	0.05	0.03
18:1 ω7c	19.81	46.91	8.34
18:1 ω9c	0.14	0.49	5.10
18:2 ω6,9c/18:0	0.11	1.39	0.81
20:0	1.75	0.98	0.06
20:1 ω7c	3.00	3.48	0.12
20:4 ω6,9,12,15c	--	--	1.57
unknown 18.814	8.31	--	--

† = Not detected

Table 15. Phenotypic canonical correlation of fatty acids for the two canonical discriminant functions of FAME profile analysis for *M. incognita* populations increased on tomato, cotton, and soybean plants. Eigenvalue, cumulative percent of total variance, and canonical correlation are listed for each canonical function. Fatty acids listed were determined significant by the STEPDISC procedure. Correlation values are determined to be significant if $r \geq |0.75|$ (**bold**).

Response variable		Discriminant variate	
No.	Fatty acid variable	CAN 1	CAN 2
1	10:0 2OH	**0.989**	0.147
2	12:0 2OH	-0.489	**0.872**
3	14:0 2OH	-0.249	**-0.969**
4	15:0 ANTEISO	**0.989**	0.147
5	15:0 ISO	0.643	-0.766
6	16:0	**0.960**	-0.282
7	16:0 3OH	**0.989**	0.147
8	16:1 ω5c	**0.989**	0.147
9	16:1 ω7c/15 iso	**0.938**	-0.346
10	17:0 ISO	**0.998**	0.055
11	18:0	-0.680	-0.734
12	18:0 3OH	**0.985**	0.175
13	18:1 ω5c	**-0.847**	-0.531
14	18:1 ω7c	-0.723	0.691
15	18:1 ω9c	**0.973**	0.232
16	18:2 ω6,9c/18:0 ANTE	-0.106	**0.994**
17	19:1 ISO I	-0.375	**-0.927**
18	20:0	-0.723	-0.691
19	20:1 ω7c	**-0.999**	0.032
20	20:4 ω6,9,12,15c	**0.989**	0.147
21	unknown 18.814	-0.249	**-0.969**
	Eigenvalue	24.89	3.17
	Cumulative %	88.7	100.0
	Canonical Correlation	0.98	0.87

Table 16. Mean fatty acid percentages of three *Rotylenchulus reniformis* populations grown on three hosts, tomato, cotton, and soybean. Fatty acid means are listed in order by fatty acid chain length, location of the double bond, and functional group.

	Rotylenchulus reniformis populations		
Fatty Acid	Tomato	Cotton	Soybean
10:0	0.17	1.38	3.78
12:0 2OH	13.12	13.00	21.28
14:0 2OH	0.38	0.34	2.59
16:0	15.87	13.21	19.92
17:0 ANTEISO	0.85	2.87	0.23
18:0	14.42	12.64	16.18
18:1 CIS 11/t 9/t 6	16.66	14.47	6.36
18:1 TRANS 9/t6/c11	8.43	9.45	12.35
18:1 ω7c	11.69	1.06	1.00
18:3 ω6c (6,9,12)	0.53	0.17	1.37
19:0 ANTEISO	--†	2.00	--
20:0	3.68	1.66	4.74
20:1 TRANS 11	2.29	2.07	2.31
20:2 ω6,9c	0.06	1.38	--
unknown 15.549	0.93	1.26	1.76
unknown 18.814	4.29	13.18	0.46

† = Not detected

Table 17. Phenotypic canonical correlation of fatty acids for the two canonical discriminant functions of FAME profile analysis for *R. reniformis* populations increased on tomato, cotton, and soybean plants. Eigenvalue, cumulative percent of total variance, and canonical correlation are listed for each canonical function. Fatty acids listed were determined significant by the STEPDISC procedure. Correlation values are determined to be significant if $r \geq |0.75|$ (**bold**).

Response variable		Discriminant variate	
No.	Fatty acid variable	CAN 1	CAN 2
1	10:0	**0.866**	-0.500
2	10:0 2OH	**0.962**	-0.273
3	12:0	-0.330	**0.944**
4	14:0 2OH	**0.987**	-0.161
5	15:0	**0.994**	-0.113
6	15:0 ANTEISO	**-0.817**	0.576
7	15:0 ISO	**-0.935**	0.354
8	16:0	**0.972**	0.233
9	16:0 2OH	**0.984**	-0.179
10	16:0 ANTEISO	**-0.779**	-0.627
11	16:1 C	0.616	**-0.788**
12	16:1 CIS 9	-0.741	-0.672
13	16:1 ω5c	**0.984**	-0.179
14	17:0	**0.984**	-0.179
15	17:0 ISO	**-0.951**	-0.309
16	17:0 ISO 3OH	**-0.999**	-0.053
17	18:0	**0.936**	0.352
18	18:0 ANTEISO/18:2 c	**0.998**	0.065
19	18:1 CIS 11/t 9/t 6	**-0.926**	0.378
20	18:1 CIS 9	0.498	**-0.867**
21	18:1 TRANS 9/t6/c11	**0.906**	-0.424
22	18:1 ω7c	-0.335	**0.942**
23	18:1 ω9c	-0.556	**0.831**
24	18:2 CIS 9,12/18:0a	-0.555	**0.832**
25	18:3 ω6c (6,9,12)	**0.992**	0.126
26	19:1 ISO I	**-0.814**	-0.580
27	20:0	**0.859**	0.512
28	20:2 ω6,9c	-0.662	-0.749
29	unknown 15.549	**0.828**	-0.560
30	unknown 18.814	**-0.834**	-0.552
	Eigenvalue	2.09	1.28
	Cumulative %	62.1	100.0
	Canonical Correlation	0.82	0.75

Table 18. Phenotypic canonical correlation of fatty acids for the three canonical discriminant functions of FAME profile analysis for *M. incognita* and *R. reniformis* populations each increased on tomato, cotton, and soybean plants. Eigenvalue, cumulative percent of total variance, and canonical correlation are listed for each canonical function. Fatty acids listed were determined significant by the STEPDISC procedure. Correlation values are determined to be significant if $r \geq |0.75|$ (**bold**).

Response variable		Discriminant variate		
No.	Fatty acid variable	CAN 1	CAN 2	CAN 3
1	10:0	-0.070	**0.789**	0.037
2	10:0 2OH	**0.993**	0.057	-0.086
3	12:0	-0.156	0.193	-0.203
4	12:0 2OH	-0.299	**0.818**	-0.201
5	14:0	0.202	**0.768**	0.340
6	14:0 2OH	-0.143	0.733	0.015
7	15:0	-0.116	0.686	-0.015
8	15:0 ANTEISO	0.194	0.396	-0.114
9	15:0 ISO	0.350	-0.085	0.495
10	16:0	**0.891**	0.167	0.254
11	16:0 2OH	-0.074	-0.562	**0.784**
12	16:0 3OH	**0.994**	-0.002	-0.082
13	16:0 ANTEISO	-0.216	0.456	0.167
14	16:0 ISO 3OH	-0.088	0.629	-0.001
15	16:1 C	-0.200	**0.835**	0.128
16	16:1 CIS 9	-0.200	0.423	0.181
17	16:1 ω5c	**0.995**	-0.006	-0.088
18	16:1 ω7c	**0.995**	-0.019	-0.088
19	16:1 ω7c/15 iso	**0.781**	-0.568	0.226
20	17:0	-0.088	0.629	-0.001
21	17:0 ISO 3OH	-0.254	0.451	0.051
22	18:0	-0.316	-0.734	0.498
23	18:0 3OH	**0.937**	0.149	-0.165
24	18:0 ANTE/18:2 ω6,9c	-0.181	0.379	0.196
25	18:0 ANTEISO/18:2 c	-0.263	**0.922**	-0.025
26	18:1 CIS 11/t 9/t 6	-0.303	0.673	-0.018
27	18:1 CIS 9	-0.309	**0.939**	0.038
28	18:1 TRANS 9/t6/c11	-0.298	**0.953**	0.021
29	18:1 ω7c	-0.186	**-0.833**	-0.504
30	18:1 ω9c	**0.979**	-0.104	-0.168
31	18:2 CIS 9,12/18:0a	-0.223	0.365	-0.142
32	18:2 ω6,9c/18:0	0.182	-0.621	-0.695
33	18:2 ω6,9c/18:0 ANTE	-0.219	0.427	0.148
34	18:3 ω6c (6,9,12)	-0.083	0.659	-0.046
35	19:0 ANTEISO	-0.162	0.352	0.213
36	19:1 ISO I	-0.253	-0.458	**0.816**
37	20:0	-0.401	0.685	0.064
38	20:1 ω7c	-0.280	**-0.936**	-0.098
39	20:2 ω6,9c	-0.153	0.364	0.206
40	20:4 ω6,9,12,15c	**0.995**	-0.019	-0.088
41	unknown 10.928	-0.174	**0.796**	-0.027
42	unknown 15.549	-0.281	**0.954**	0.043
43	unknown 16.582	-0.298	0.064	0.611
44	unknown 18.814	-0.268	0.075	0.654
	Eigenvalue	15.19	6.24	1.78
	Cumulative %	60.0	84.7	91.7
	Canonical Correlation	0.97	0.93	0.80

Table 19. Phenotypic canonical correlation of fatty acids for the three canonical discriminant functions of FAME profile analysis for *M. incognita* and *R. reniformis* populations each increased on tomato, cotton, and soybean plants and the host plants. Eigenvalue, cumulative percent of total variance, and canonical correlation are listed for each canonical function. Fatty acids listed were determined significant by the STEPDISC procedure. Correlation values are determined to be significant if $r \geq |0.75|$ (**bold**).

Response variable		Discriminant variate		
No.	Fatty acid variable	CAN 1	CAN 2	CAN 3
1	10:0	-0.364	-0.061	0.547
2	10:0 2OH	**0.765**	0.436	-0.277
3	12:0	-0.073	-0.171	0.182
4	12:0 2OH	**-0.821**	-0.058	0.471
5	14:0	0.419	-0.141	**0.793**
6	14:0 2OH	-0.328	-0.141	0.488
7	15:0	-0.282	-0.123	0.459
8	15:0 ANTEISO	-0.377	0.205	0.400
9	15:0 ISO	-0.547	0.333	0.159
10	16:0	0.721	0.217	-0.348
11	16:0 2OH	0.232	0.085	-0.533
12	16:0 3OH	0.126	**0.953**	0.220
13	16:0 ANTEISO	-0.379	-0.161	0.354
14	16:0 ISO 3OH	**0.760**	-0.274	0.435
15	16:1 C	-0.407	-0.172	0.560
16	16:1 CIS 9	-0.354	-0.149	0.332
17	16:1 ω5c	0.060	**0.965**	0.220
18	16:1 ω7c	0.065	**0.966**	0.210
19	16:1 ω7c/15 iso	-0.168	**0.776**	-0.144
20	16:1 ω7c/15 iso 2OH	0.730	-0.275	0.454
21	17:0	**0.823**	-0.287	0.386
22	17:0 ANTEISO	-0.324	-0.216	0.321
23	17:0 ISO	0.371	0.065	-0.394
24	17:0 ISO 3OH	-0.348	-0.224	0.415
25	18:0	-0.434	-0.114	-0.646
26	18:0 3OH	-0.091	**0.898**	0.322
27	18:0 ANTE/18:2 ω6,9c	**0.868**	-0.279	0.297
28	18:0 ANTEISO/18:2 c	-0.490	-0.217	0.601
29	18:1 CIS 11/t 9/t 6	-0.508	-0.228	0.478
30	18:1 CIS 9	-0.540	-0.237	0.612
31	18:1 TRANS 9/t6/c11	-0.530	-0.233	0.618
32	18:1 ω6c	0.730	-0.275	0.454
33	18:1 ω7c	-0.487	-0.013	-0.360
34	18:1 ω9c	**0.843**	0.399	0.220
35	18:2 CIS 9,12/18:0a	-0.381	-0.178	0.294
36	18:2 ω6,9c/18:0	-0.300	0.292	-0.216
37	18:2 ω6,9c/18:0 ANTE	0.698	-0.133	-0.301
38	18:3 CIS 6,12,14	-0.538	-0.235	0.604
39	18:3 ω6c (6,9,12)	0.387	-0.028	-0.446
40	19:0	0.737	-0.252	0.457
41	19:0 ANTEISO	-0.287	-0.120	0.283
42	19:0 CYCLO ω10c/19ω6	**0.763**	-0.279	0.425
43	19:1 ISO I	-0.157	-0.263	0.055
44	20:0	0.039	-0.477	0.735
45	20:1 ω7c	-0.494	-0.102	-0.460
46	20:2 ω6,9c	-0.300	-0.110	0.295
47	20:4 ω6,9,12,15c	**0.750**	0.012	0.493
48	TBSA 10Me18:0	0.531	-0.337	0.593
49	unknown 10.928	0.376	-0.102	-0.297
50	unknown 15.549	-0.512	-0.224	0.620
51	unknown 18.814	-0.486	-0.170	0.105
	Eigenvalue	36.32	10.69	5.93
	Cumulative %	58.3	75.6	85.1
	Canonical Correlation	0.99	0.96	0.93

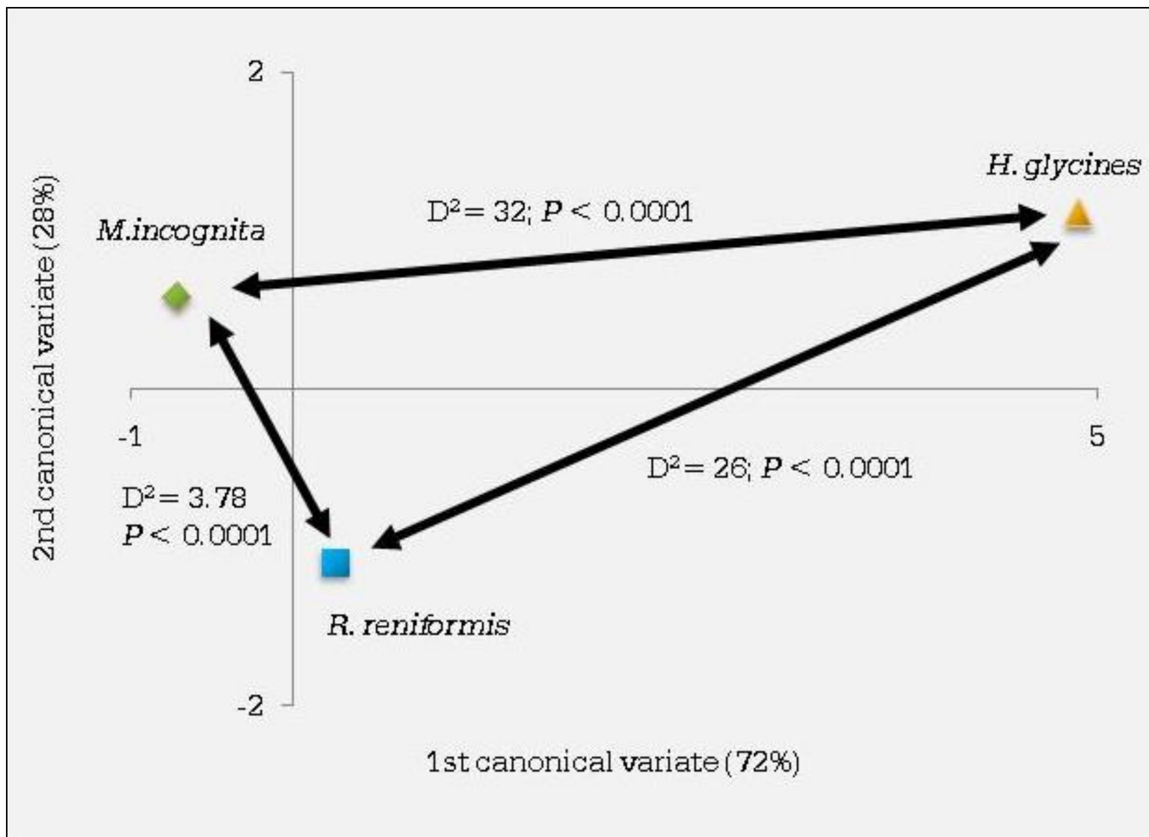

Figure 1. Canonical means of three nematode species. X-axis is the first canonical dimension and y-axis is the second canonical dimension. D^2 values listed are real-distances between points squared.

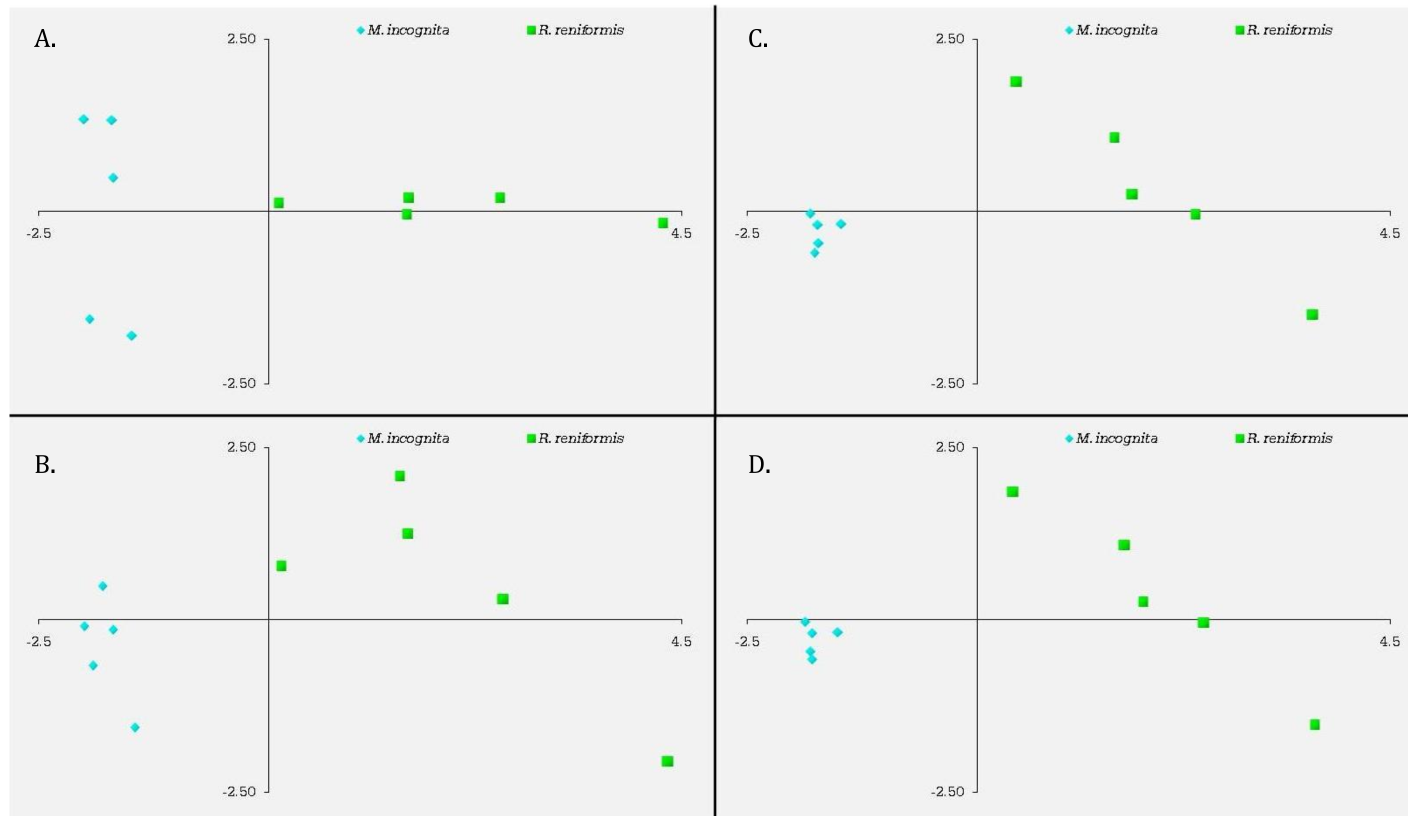

Figure 2. Canonical means for detection of fatty acids that can be used to differentiate *Rotylenchulus reniformis* and *Meloidogyne incognita* in samples containing greater than 100 individuals. A.) Canonical means distribution without using the STEPDISC procedure before analyzing samples with only those fatty acids found in both *R. reniformis* and *M. incognita*. B.) Canonical means distribution of samples containing all fatty acids found in both *R. reniformis* and *M. incognita* without a precursor STEPDISC. C.) Canonical means of fatty acid profiles with fatty acids only found in both *R. reniformis* and *M. incognita* and also analyzed by STEPDISC. D.) STEPDISC-analyzed canonical distribution of all fatty acids found in *R. reniformis* and *M. incognita*.

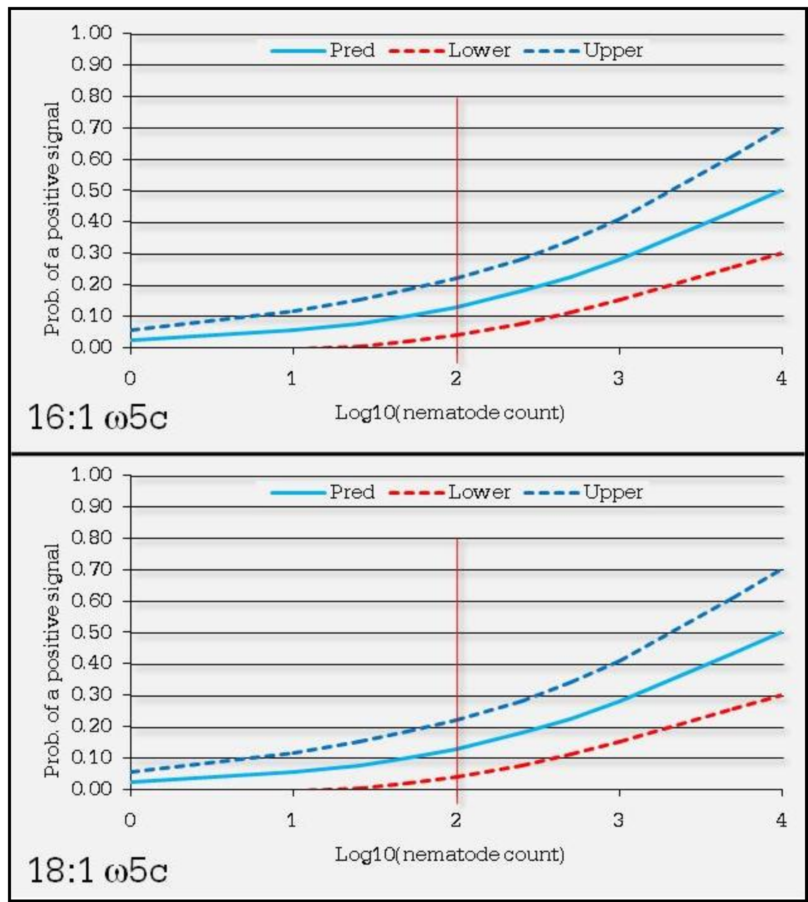

Figure 3. Prediction graphs for the probability of detection for 16:1 ω5c and 18:1 ω5c in *Rotylenchulus reniformis*. X-axis is the log of total individuals within a sample vs. the y-axis of probability of detection within a sample – vertical solid red line indicates 100 total individuals per sample.

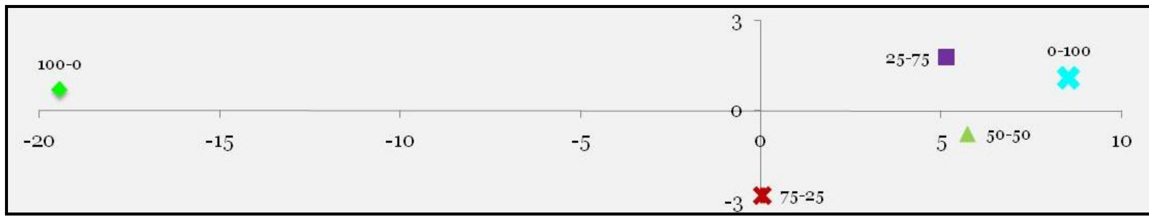

Figure 4. Canonical means for five ratios of *Meloidogyne incognita* to *Rotylenchulus reniformis*, labeled by percentage of *M. incognita* and *R. reniformis* per ratio. The first canonical dimension is the x-axis and the y-axis is the second canonical dimension. D^2 values are greater than 14.05 and significant at $P \leq 0.0028$ for any given comparison.

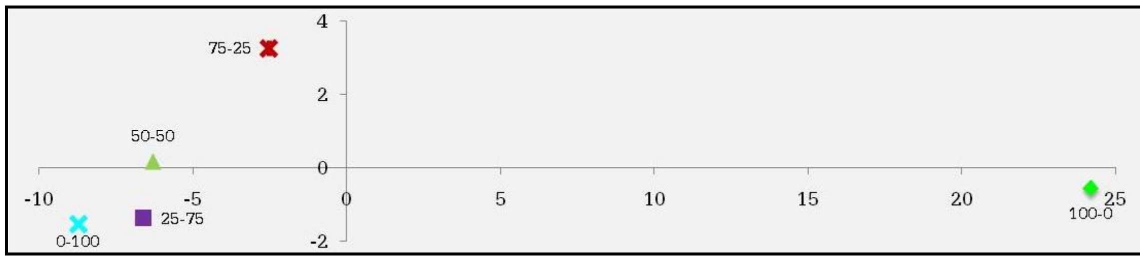

Figure 5. Canonical means of five percentage ratios of *Meloidogyne incognita* to *Rotylenchulus reniformis* for samples containing 500 total individuals. The x-axis represents the first canonical dimension while the y-axis represents the second canonical dimension. D^2 values are greater than 11.11 at $P \leq 0.0075$ for all comparisons except the 50-50 to 25-75 comparison (D^2=4.32, P=0.3956).

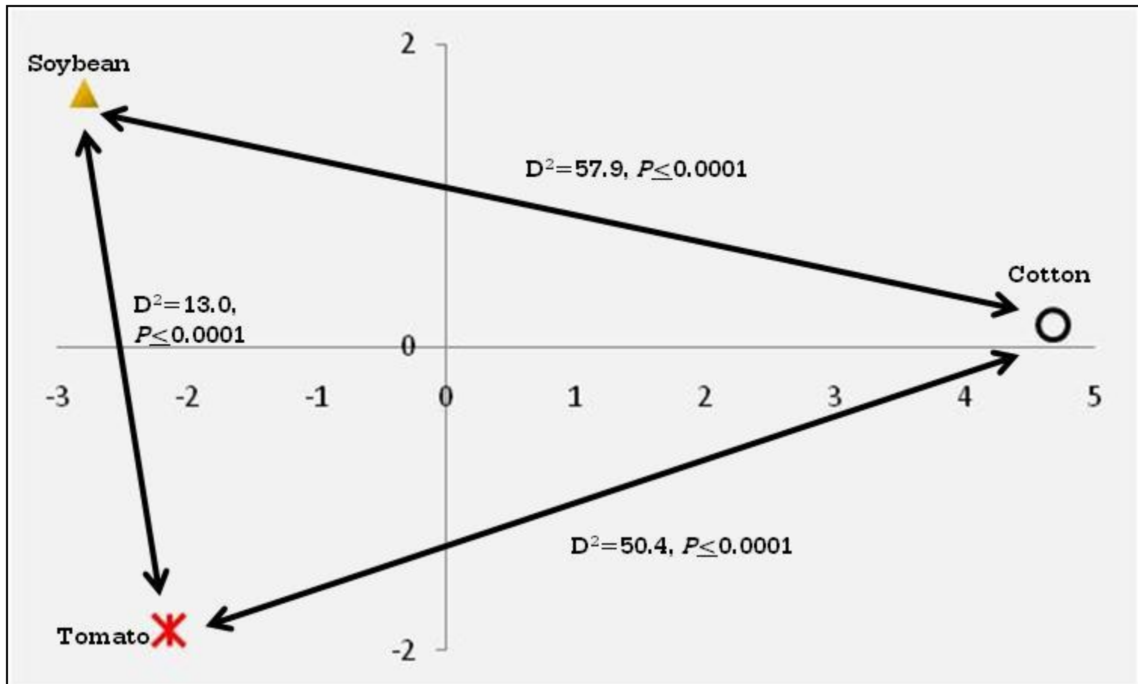

Figure 6. Canonical means for the host plants tomato, cotton, and soybean. The first canonical dimension is represented by the x-axis, and the y-axis represents the second canonical dimension. D^2 values listed are real-distances between points squared.

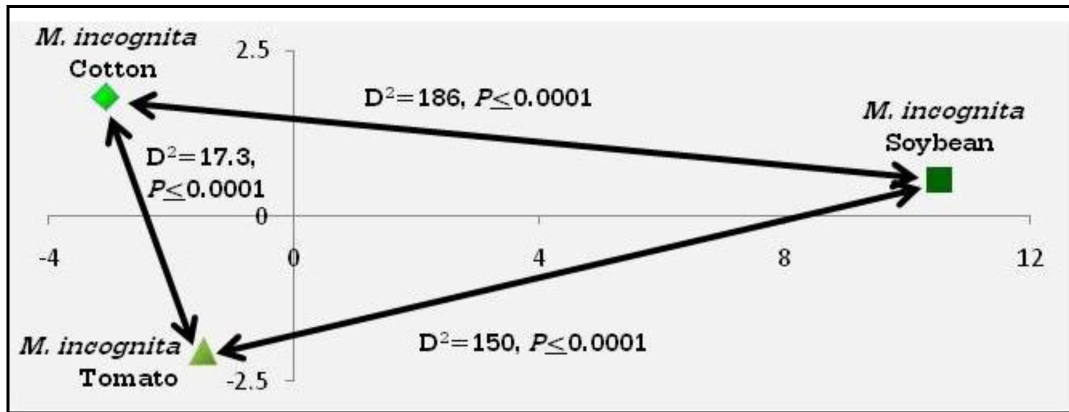

Figure 7. Canonical means of *Meloidogyne incognita* grown for sixty days on three separate hosts, tomato, cotton, and soybean. The x-axis represents the first canonical dimension and the y-axis represents the second canonical dimension. D^2 values listed are real-distances between points squared.

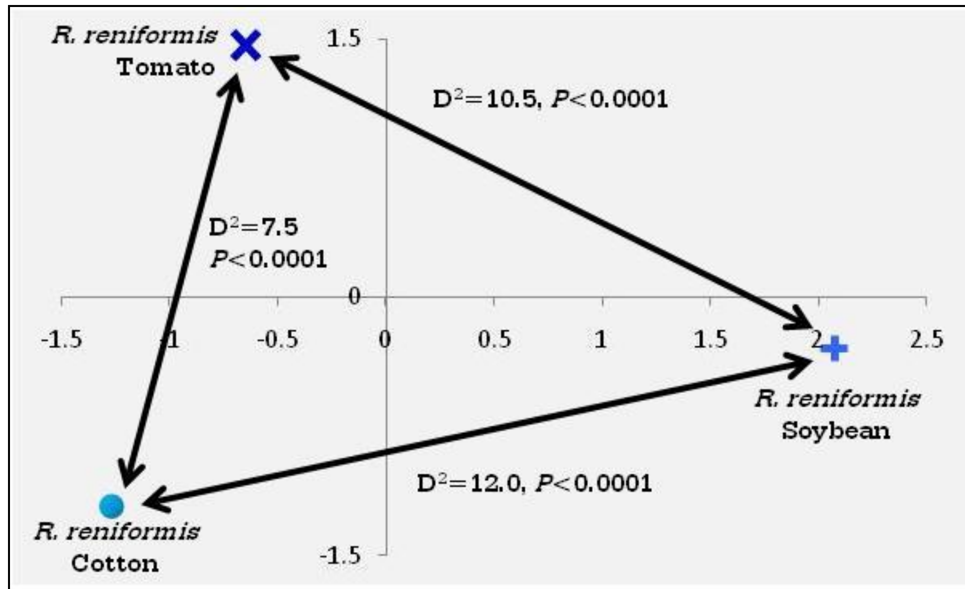

Figure 8. Canonical mean distribution of *Rotylenchulus reniformis* populations grown on three hosts, tomato, cotton, and soybean. The x-axis represents the first canonical dimension and the y-axis represents the second canonical dimension. D^2 values listed are real-distances between points squared.

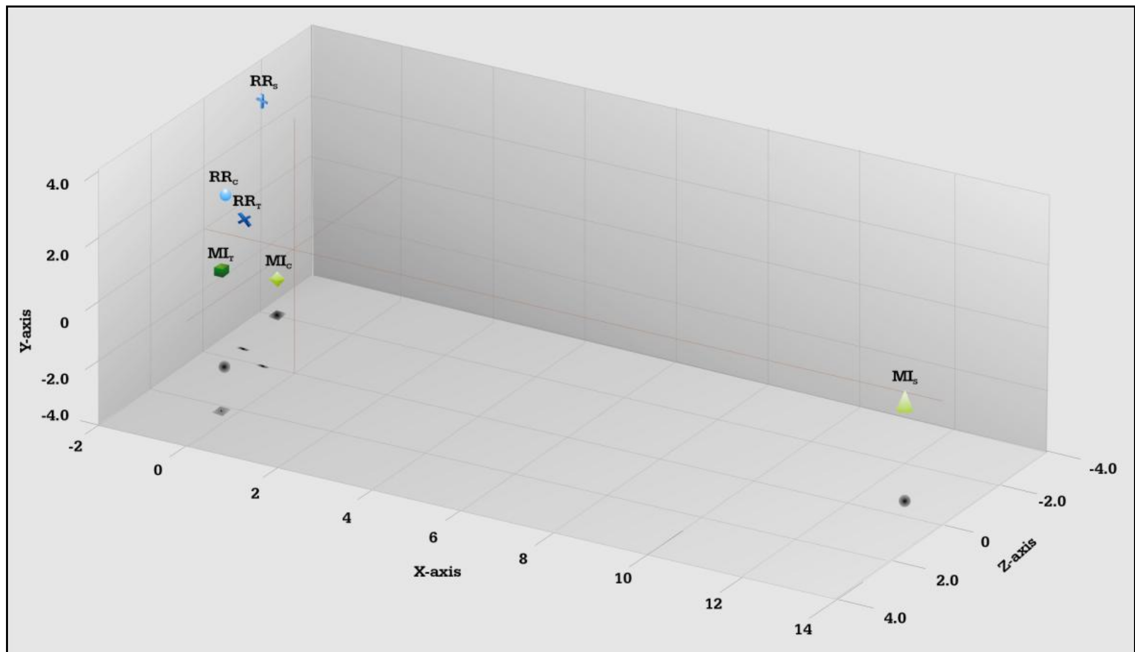

Figure 9. Canonical means of *Meloidogyne incognita* (MI) and *Rotylenchulus reniformis* (RR) on three hosts, tomato (T), cotton (C), and soybean (S). Means are distributed along the first (x-axis), the second (y-axis), and third (z-axis) canonical axes. Point positions are indicated by height (y-axis) and shadow (x-axis and z-axis intercept).

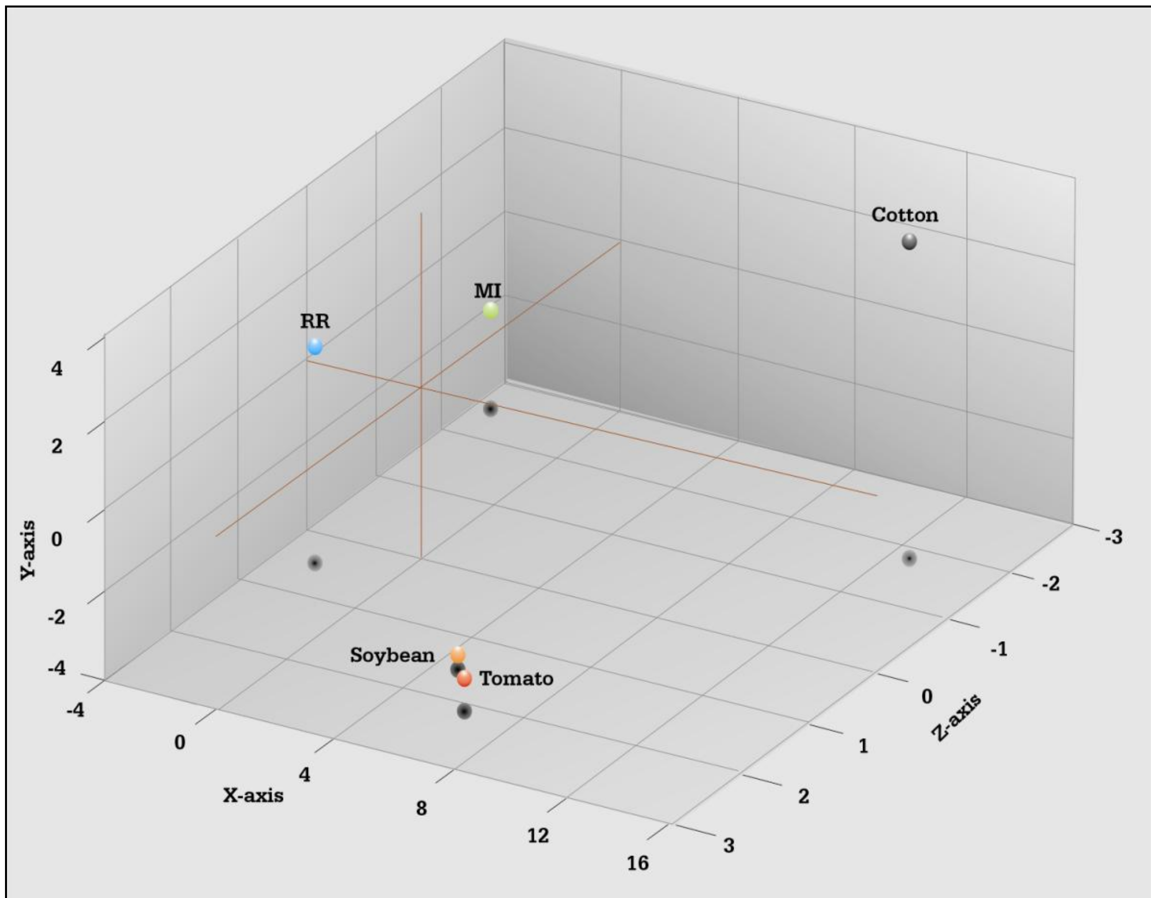

Figure 10. Canonical mean comparison for fatty acid profiles of *Rotylenchulus reniformis* (RR) and *Meloidogyne incognita* (MI), as well as tomato, cotton, and soybean host tissues. Placement is defined for the first (x-axis), second (y-axis), and third (z-axis) canonical axes by height (y-axis) and shadow (x-axis and z-axis intercept).

III. DIFFERENTIATION OF MELOIDOGYNE SPECIES AND RACES WITH FAME ANALYSIS

Abstract

Fatty acid methyl ester (FAME) analysis can be used as a means for differentiating among plant-parasitic nematode genera. Species such as *Rotylenchulus reniformis*, *Heterodera glycines*, and *Meloidogyne incognita* all have significantly different fatty acid profiles (Squared Mahalanobis distances [D^2] $\geq$7.22, $P \leq 0.0005$) at concentrations greater than 100 individuals. The objective of this study was to evaluate the use of this system to identify species and races within a single genus. Fatty acids were extracted and analyzed from samples containing 1000 individuals of each *Meloidogyne* species *M. arenaria* (race 2), *M. hapla*, *M. incognita* (races 1, 2, and 3), and *M. javanica*. The resulting profiles generated by the Sherlock® Analysis Software were then statistically analyzed with the STEPDISC and CANDISC procedures of SAS version 9.1.3. The profiles of each *Meloidogyne* species and race were significantly different. The first canonical axis defines 66.0% of the difference among species and 23.6% is defined by the second axis for a total of 89.6% defined by the first two axes. The four *Meloidogyne* species separated with a minimum D^2 between *M. incognita* and *M. arenaria* (D^2=15.9, P<0.0001). When the species are separated by race, the minimum distance was between *M.*

arenaria race 2 and *M. incognita* race 1 (D^2=15.8, P<0.0001). D^2 values among *M. incognita* races are all significant at $P < 0.0001$ with a minimum distance between *M. incognita* race 1 and *M. incognita* race 3 of 57.8. A total of 82.5% of the differences among *M. arenaria*, *M. hapla*, *M. javanica*, and the three races within *M. incognita* were explained by the first two canonical axes; 57.6% in the first and 24.9% in the second. By incorporating these profiles into a Sherlock® Analysis Software library, the FAME method can be used to distinguish among four *Meloidogyne* species and three races to provide an alternative source of identification.

Introduction

There are four species within the genus *Meloidogyne* (Chit.), the root-knot nematode, that cause the majority of the known economic damage to agricultural crops across the United States (Chitwood, 1949; Bridge and Starr, 2007). Damage to cotton crops in the United States from the root-knot nematode for 2007 was estimated at $24,145,182, accounting for 84% of the total cotton losses from nematode damage and 23% of total cotton disease losses (National Cotton Council). Certain species of *Meloidogyne* are host-specific, but the host potential for *Meloidogyne* spp. covers most cropping plants (Thorne, 1961; Bridge and Starr, 2007). Identification of these nematodes is complicated by races within *Meloidogyne* species. Races are host-specific; for instance, two races within *M. incognita* may both infect tomato, but only one of these two races may infect tobacco. To identify *Meloidogyne* species and races, the methods most often used are the North Carolina Differential Host Test (Taylor and Sasser, 1978),

microscopic study of perineal patterns (Hooper, 1986), esterase phenotypes (Esbenshade and Triantaphyllou, 1985; Venkatachari et al., 1991), and PCR methods (Powers and Harris, 1993). These methods of identification are time-consuming, require trained individuals to perform the identification, and may require life stages not commonly found in soil samples. Other methods using mitochondrial DNA have been proposed (Okimoto et al., 1991), but these methods have not been adapted by diagnostic laboratories for identification of *Meloidogyne* samples. Currently in our lab, the North Carolina Differential Host Test is used to identify species and races within the *Meloidogyne* genus based on species identifications of Chitwood (1949) and race identifications of Taylor and Sasser (1978).

Studies comparing species within *Meloidogyne* and *Caenorhabditis* indicate that the fatty acid profiles of these species are distinct and vary enough for differentiation (Hutzell and Krusberg, 1982; Krusberg *et al*., 1973). It should be possible to further differentiate additional species within *Meloidogyne*, and possibly races within species, based on fatty acid profiles.

The objectives for this study are to 1) determine if *M. arenaria* (Chit.), *M. hapla* (Chit.), *M. incognita* (Chit.), and *M. javanica* (Chit.) can be differentiated using FAME analysis, 2) establish if races within *Meloidogyne* species can be identified with FAME analysis, 3) evaluate the combined statistical separation of four *Meloidogyne* species and three *M. incognita* races within the *Meloidogyne* genus, and 4) assess identification of species and races within the *Meloidogyne* genus using the Sherlock® Analysis Software.

Materials and Methods

General Culturing

A stock population of *Meloidogyne incognita* race 3 was collected from the Plant Breeding Unit of the E. V. Smith Research Center in Shorter, AL and increased at the Auburn University Plant Science Research Center greenhouses on *Lycopersicon esculentum* Mill. var. 'Roma' in 500 cm^3 polystyrene pots. The remaining populations in this study (*M. incognita* [Chit.] races 1 and 2 [Hartmann and Sasser], *M. arenaria* race 2 [Hartmann and Sasser], *M. hapla*, and *M. javanica*) were increased from samples collected around the country and increased on *L. esculentum* var. 'Rutgers' at the Clemson University greenhouses; these populations were maintained in 45 cm clay pots and physically separated by Plexiglas dividers 61 cm high by 91 cm deep to prevent the formation of mixed populations.

Nematode Extraction

Second stage juvenile life stages (J2s) of *Meloidogyne* populations were extracted from the soil of the stock pots using gravity screening and gravid females and eggs of all species and races were extracted from root tissue using NaOCl. Both extractions for each species were then combined and centrifuged utilizing a sucrose gradient to remove any remaining debris. Extractions for all species were enumerated to determine the number of eggs, females, and J2s in samples.

Sample Preparation and Fatty Acid Extraction

Samples to be analyzed contained a total of 1000 individuals from one of the six *Meloidogyne* species or races. Each *Meloidogyne* population was replicated 20 times for a total of 120 samples. Fatty acids were extracted from samples using the method described by Sasser (1990). After the extraction procedure was completed, the organic solvent was transferred to sample vials and allowed to evaporate under a fume hood. Dried samples were reconstituted in 75 μL of organic extraction solvent and transferred to spring-vial inserts for each sample vial. Vials were sealed and stored at -20°C until analysis.

Samples were analyzed for fatty acid composition by a HP 5890 automated gas chromatography system (Agilent Technologies) equipped with an Ultra 2 Cross-linked 5% Phenyl Methyl Siloxane column; 2.0 µL of sample was injected into the column for each analysis. Sample data from the Sherlock® Sequencer Software (MIDI, Inc.) included total response of each sample (mV) and the response for each detected fatty acid. Fatty acid percentages were calculated from the proportion of each fatty acid within the sample; these percentages were used to create a fatty acid profile for each nematode sample.

Statistical Analysis

For this experiment, it was desirable to make three separate comparisons: 1) comparing the four *Meloidogyne* species, 2) comparing the three *M. incognita* races, and 3) comparing all six species and races within *Meloidogyne*. For the first comparison, class values were defined by "species," in which all analyses

were grouped by their respective *Meloidogyne* species; *M. incognita* races were pooled in this analysis to represent variation within the species. To compare the races of *M. incognita*, the three races were compared to one another using the "race" class. A complete analysis of all species and races within species was conducted by using "race" as a class for all species and races; if only a single race within a species was analyzed, its "race" classification was the same as its "species" classification.

The STEPDISC (SAS version 9.1.3; SAS Institute, Inc) procedure was used to analyze the expression of each fatty acid across all samples to determine which fatty acids contributed significantly to the differentiation among classes; classes for each experiment were dependent upon the character being analyzed. In this case, classes were either "species" or "race" depending on the comparison. The STEPDISC procedure determined fatty acids significant for discrimination among classes based on the ANOVA test *F* value of a selected fatty acid (Johnson, 1998). The compiled list of fatty acids was used for class differentiation with the CANDISC procedure. The CANDISC procedure provided canonical discriminant analysis (CDA) of the fatty acid profiles for each nematode sample within its respective categorical class.

Sherlock® Analysis Software

A library was developed using the Sherlock® Analysis Software by creating entries from fatty acid profiles of the *Meloidogyne* species and races developed in this study. To determine the usefulness and validity of the newly created library entries with this software, individual samples were compared against their

respective composite profiles to create comparison and similarity matrices for each *Meloidogyne* species and race. Identification reports were also used to evaluate identification accuracy using the “First choice” and “First Second choice” methods.

Results

Objective 1: *Meloidogyne* Species

The four *Meloidogyne* species have similar fatty acid profiles (Table 1), but the percentage of each fatty acid present varies significantly among all species ($D^2 \geq 15.9$, $P < 0.0001$; Table 2). This variation is best observed between *M. incognita* and *M. javanica*; these two species differ the most in their expression of the fatty acids 18:1 ω7c and 18:0. In *M. javanica*, 18:1 ω7c is present at 54.5%, which is similar to *M. arenaria* (57.5%) and *M. hapla* (58.9%) but greater than *M. incognita* (45.3%). The difference in 18:1 ω7c between *M. javanica* and *M. incognita* is primarily due to the higher percentage of 18:0 found in *M. incognita* (23.2%), which is twice that of *M. javanica* (11.5%). Differences between *M. arenaria* and *M. hapla* are less pronounced than those between *M. incognita* and *M. javanica*. Most fatty acids vary less than a difference of 0.5%. However, 12:0 2OH is found in *M. arenaria* (3.3%) at more than double the percentage found in *M. hapla* (1.4%) and 18:1 ω7c is expressed slightly higher in *M. hapla* (58.9%) than *M. arenaria* (57.5%; Table 1).

The first three canonical dimensions explained 100% of the total multivariance among *Meloidogyne* species. Ten fatty acids were significant ($r \geq |0.757|$) for delineation along the first canonical dimension and defined 66.3% of

the overall multivariance (Table 3). Of these fatty acids, 16:1 ω5c was the highest correlated along CAN 1 (r=|0.965|; Table 3) and separated *M. incognita* (0.18%) from the other species, primarily *M. javanica* (4.58%; Table 1). The three fatty acids 20:2 ω6,9c, 20:0 Iso, and 20:4 ω6,9,12,15c were all found in greater concentrations in *M. javanica* than the other three species (Table 1). *Meloidogyne javanica* and *M. hapla* had the same mean concentration of 18:1 ω9c (2.07%), which was higher than both *M. arenaria* (1.61%) and *M. incognita* (1.52%; Table 1). Both fatty acids 14:0 2OH and 18:1 ω5c were found in the greatest concentration in *M. arenaria* (0.19% and 1.88%, respectively); *M. javanica* had the highest concentrations of 17:0 Iso and 10:0 3OH (0.90% and 0.26%, respectively). *Meloidogyne incognita* had the greatest concentration of 12:0 2OH (4.52%), followed by *M. arenaria* (3.28%), *M. javanica* (2.05%), and *M. hapla* (1.37%; Table 1).

The second canonical dimension described 23.3% of the total multivariance among species. Two fatty acids were significant ($r \geq |0.768|$; Table 3) along the second canonical dimension. Both 14:0 and 15:0 Iso were found at the highest concentration in *M. hapla* (0.90% and 1.27%, respectively) and were lowest in *M. incognita* (0.15% and 0.48%, respectively; Table 1). The concentrations of these two fatty acids were also higher in *M. arenaria* (0.52% and 0.98%, respectively) than *M. javanica* (0.48% and 0.91%, respectively; Table 1).

There was enough variation in the third canonical dimension to separate *M. hapla* from *M. arenaria* and describe the remaining 10.4% of multivariance,

though there were no fatty acids determined to be significant ($r \geq |0.75|$; Table 3) for this separation (Figure 1).

Objective 2: *Meloidogyne incognita* Races

Fatty acid expression varied significantly ($D^2 \geq 18.2$, $P < 0.0001$; Figure 2) among the three races of *M. incognita* studied. Many of the same fatty acids were observed among the three races, but each fatty acid was expressed at different concentrations among the three races (Table 4). The fatty acid concentrations of *M. incognita* race 1 and *M. incognita* race 2 were most similar, while *M. incognita* race 3 concentrations were more distinct from *M. incognita* race 1 and *M. incognita* race 2. Though there are five fatty acids (14:0 2OH, 16:0 2OH, 17:0 Anteiso, and unknown peaks at 16.582 and 18.814) that were found in *M. incognita* race 3 and not *M. incognita* race 1 or *M. incognita* race 2 and three (10:0, 16:1 ω5c, and 18:0 Ante/18:2 ω6,9c) found in *M. incognita* race 1 or *M. incognita* race 2 and not *M. incognita* race 3, most differences among the profiles can be observed in the expression of four fatty acids (12:0 2OH, 16:0, 18:0, and 18:1 ω7c; Table 4). These four fatty acids have similar expression between *M. incognita* race 1 and *M. incognita* race 2 but are very different in *M. incognita* race 3. *Meloidogyne incognita* race 3 has nearly twice the concentration of 12:0 2OH (7.86%), 16:0 (18.46%), and 18:0 (40.25%) than *M. incognita* race 1 (2.14%, 7.37%, and 14.38%) or *M. incognita race 2* (3.55%, 8.74%, and 15.09%). In contrast, 18:1 ω7c concentrations in *M. incognita* race 1 (58.94%) and *M. incognita* race 2 (57.01%) are three times the concentration of *M. incognita* race 3 (19.81%; Table 4).

CDA explained 100% of the total multivariance among *M. incognita* race 1, *M. incognita* race 2, and *M. incognita* race 3 in two canonical dimensions. Nine fatty acids were significant for differentiating *M. incognita* race 3 from *M. incognita* race 1 and *M. incognita* race 2 along the first canonical dimension (87.4% of total multi variance; Table 6). All nine of these fatty acids were highly correlated along CAN 1 ($r \geq |0.871|$), but five of these (18:3 ω6,9,12c, 18:0 Ante/18:2 ω6,9c, 17:0 Anteiso, 18:0 3OH, and 17:0 Iso) were correlated along CAN 1 at greater than |0.985| (Table 6). Expression of these fatty acids is not uniform across all three races; none are found in all three of the races. In *M. incognita* race 3, 17:0 Anteiso was found at 1.08%, but was not found in *M. incognita* race 1 or *M. incognita* race 2 (Table 4). Similarly, 18:0 3OH was found in *M. incognita* race 1 (0.05%) and not *M. incognita* race 2 or *M. incognita* race 3. Both 17:0 Iso and 18:3 ω6,9,12c are found in *M. incognita* race 3 (0.08% and 0.26%), but 17:0 Iso was expressed in *M. incognita* race 1 (0.24%) and not *M. incognita* race 2, while 18:3 ω6,9,12c was found in *M. incognita* race 2 (0.21%) and not *M. incognita* race 1. The only other fatty acid that was not expressed in all three races was 10:0, which was found in *M. incognita* race 1 (0.05%) and *M. incognita* race 2 (0.08%), but not *M. incognita* race 3. The fatty acid 18:1 ω5c, found at concentrations of 1.65% and 0.91% in *M. incognita* race 1 and *M. incognita* race 2, respectively, was found at a concentration of 0.06% in *M. incognita* race 3. Differential expression of the two remaining fatty acids significant for identification along CAN 1 has already been discussed (16:0, and 18:0).

The remaining 12.6% of multi variance was described by CAN 2, which helped to further differentiate *M. incognita* race 1, *M. incognita* race 2, and *M. incognita* race 3. Four fatty acids (12:0 2OH, 14:0, 16:1 ω7c/15:0 Iso 2OH, and 18:1 ω7c) were significant in this separation ($r \geq |0.874|$), but three of these, 12:0 2OH, 14:0, and 16:1 ω7c/15:0 Iso 2OH, were nearly perfectly correlated along CAN 2 ($r \geq |0.993|$; Table 6). Among the four significant fatty acids, 14:0 was the only fatty acid not found in all three races; concentrations in *M. incognita* race 1 and *M. incognita* race 3 were 0.33% and 0.14%, respectively, but absent in *M. incognita* race 2 (Table 4). The concentration of 12:0 2OH was higher in *M. incognita* race 3 (7.86%) than *M. incognita* race 1 (2.14%) or *M. incognita* race 2 (3.55%); 16:1 ω7c/15:0 Iso 2OH and 18:1 ω7c was expressed lower in *M. incognita* race 3 (0.11% and 19.81%) than in either *M. incognita* race 1 (1.18% and 58.94%) or *M. incognita* race 2 (2.84% and 57.08%; Table 4).

Objective 3: *Meloidogyne* Species and Races

By combining the fatty acid profiles we developed for *M. arenaria*, *M. hapla*, *M. javanica*, *M. incognita* race 1, *M. incognita* race 2, and *M. incognita* race 3, it was possible to differentiate all six populations ($D^2 \geq 15.8$, $P < 0.0001$; Table 5). The fatty acid profiles of five of the six *Meloidogyne* populations appear similar with minor variations in expression, but the profile of *M. incognita* race 3 appears less similar to the other profiles. The percentage of 18:1 ω7c in *M. incognita* race 3 was 19.81% (Table 4); the percentage of this fatty acid in other populations ranged from 54.50% in *M. javanica* to 58.94% in *M. incognita* race 1 (Table 1 and Table 4). Also, concentrations of 16:0 and 18:0 in *M. incognita* race

3 (18.46% and 40.25%; Table 4) were more than twice as high as any other *Meloidogyne* population studied (Table 1, Table 4).

Using CDA confirmed that *M. incognita* race 3 was the most different profile analyzed; the average D^2 for *M. incognita* race 3 was 72.8, ranging from 57.8 to 101.5 between canonical means for *M. incognita* race 1 and *M. javanica*, respectively (Table 6). The first three canonical dimensions described 93.0% of the total multivariance among *Meloidogyne* populations. The first canonical dimension explained 57.6% of the total multivariance and primarily separated *M. incognita* race 3 from *M. javanica*. Six fatty acids (18:1 ω7c, 18:1 ω9c, 12:0 2OH, 18:2 ω6,9c/18:0 Ante, 18:1 ω5c, and 16:1 ω7c/15:0 Iso 2OH) were responsible for the separation of populations along the first canonical dimension (Table 7). All of these fatty acids except 12:0 2OH were found in higher concentrations in the five *Meloidogyne* populations compared to *M. incognita* race 3; 12:0 2OH was expressed at more than twice the concentration of any other population.

A single fatty acid (18:0 Ante/18:2 ω6,9c) was significant for differentiation (r=|0.829|) among populations along CAN 2 (24.9% of total multivariance; Table 7). Expression of 18:0 Ante/18:2 ω6,9c was observed in *M. arenaria*, *M. incognita* race 1, and *M. incognita* race 2 at 0.09%, 0.33%, and 0.55%, respectively; this fatty acid was not observed in *M. hapla*, *M. incognita* race 3, or *M. javanica*. The differential expression of 18:0 Ante/18:2 ω6,9c along CAN 2 helped to separate *M. incognita* race 3 and *M. javanica* from the remaining populations on the y-axis (Figure 3).

Even though no significant fatty acids were observed in CAN 3 ($r \leq |0.672|$; Table 7), separation along this dimension was observed primarily between *M. hapla* and *M. javanica*. *Meloidogyne incognita* race 1 and *M. arenaria* were also separated from *M. incognita* race 2 along CAN 3 (Figure 3).

Objective 4: Identification of *Meloidogyne* Species and Races using the Sherlock® Analysis Software

By analyzing samples of each *Meloidogyne* species with the library entries developed from this study, it was possible to correctly identify 90.6% of the samples. Identification accuracy was greater than 90% for *M. hapla* (94.4%), *M. incognita* (90.3%), and *M. javanica* (100%), but was reduced in *M. arenaria* (77.8%). The reduction in *M. arenaria* was caused by misidentification to *M. hapla* in 16.7% of samples. *Meloidogyne incognita* also mismatched 8.3% of samples to *M. hapla* and 1.4% to *M. arenaria*. The remaining misidentification of samples in *M. arenaria* and *M. hapla* occurred because 5.6% of the samples in each species could not be identified.

The samples of the three races of *M. incognita* were correctly identified with 80.5% accuracy. Samples of *M. incognita* race 3 were identified with 100% accuracy. For *M. incognita* race 1 samples, correct identification occurred at 64.7% accuracy; 5.9% of *M. incognita* race 1 samples were identified as *M. incognita* race 2, while 29.4% were identified as either *M. arenaria* or *M. hapla*. Similarly, *M. incognita* race 2 samples were mismatched to *M. incognita* race 1 (7.7%), *M. incognita* race 3 (7.7%), and *M. arenaria* or *M. hapla* (7.7%), but 76.9% of the samples were correctly identified to the *M. incognita* race 2 library

entry. Though there was some misidentification of samples, the correct *M. incognita* race was still identified greater than 64% of the time.

Discussion

We were able to clearly identify all four *Meloidogyne* species and three *M. incognita* races from each other using FAME analysis. The fatty acid profile generated for *M. javanica* was similar to the profile reported by Chitwood and Krusberg (1981), but there were variations in the percentages of fatty acids observed. The same pattern was also observed comparing our fatty acid profiles for *M. incognita* and *M. arenaria*; percentages of fatty acids present varied from those reported by Krusberg et al. (1973), but the same fatty acids were found in our study. The differences in percentages may be due to the advancement of technology since the previous studies were performed or the methods on which the nematode isolates were increased. As these and other studies indicated (Hutzell and Krusberg, 1982), fatty acid profiles among species within *Meloidogyne* expressed the same fatty acids, but the expression of those fatty acids was not uniform among species. Enough differences were observed among species in our study to separate the four *Meloidogyne* species and three *M. incognita* races studied using the Sherlock® Analysis Software with 85.6% overall accuracy. The species within *Meloidogyne* share an average of 17% similarity among fatty acid profiles; this similarity increases to only 18% within races of *M. incognita*. Because of these low similarities and the high degree of identification accuracy, identifying species and races of *Meloidogyne* with FAME analysis may be a practical means of identification to reinforce other methods of identification.

The Sherlock® Microbial Identification System has been used to identify bacterial samples since 1985. FAME analysis with this software has revolutionized bacterial identification in a way that has increased the efficiency of diagnostic laboratories around the world. Since many plant disease diagnostic laboratories already have a FAME analysis system, it should be easy to incorporate nematode identification with this system. By using the developed library of nematode fatty acid profiles, identification of *Meloidogyne* species and races would be much faster and more economically feasible than traditional methods that can require more time and resources.

Literature Cited

Bridge, J. and J.L. Starr. 2007. Plant Nematodes of Agricultural Importance. Academic Press, San Diego, CA

Chitwood, B. G. 1949. Root-knot nematodes, Part I. Proceedings of the Helminthological society of Washington. 5(2):68-75

Chitwood, D. J., and L. R. Krusberg. 1981. Diacyl, alkylacyl, and alkenylacyl phospholipids of *Meloidogyne javanica* females. Journal of Nematology. 13:105-111

Esbenshade, P. R., and A.C. Trianthphyllou. 1985. Use of enzyme phenotypes for identification of *Meloidogyne* species. Journal of Nematology. 17(1):6-20

Hooper, D. J. 1986. Handling, fixing, staining, and mounting nematodes. Laboratory Methods for Work with Plant and Soil Nematodes, Her Majesty's Stationery Office, London. 58-80

Hutzell, P. A., and L. R. Krusberg. 1982. Fatty acid compositions of *Caenorhabditis elegans* and *C. briggsae*. Comparative Biochemistry and Physiology. 73B:517-520

Johnson, D. E. 1998. Applied multivariate methods for data analysis. Duxbury Press, Pacific Grove, CA

Krusberg, L. R., R. S. Hussey, and C. L. Fletcher. 1973. Lipid and fatty acid compositions of females and eggs of *Meloidogyne incognita* and *M. arenaria*. Comparative Biochemistry and Physiology. 45B:335-341

Okimoto, R, H. M. Chamberlin, J. L. Macfarlane, and D. R. Wolstenholme. 1991. Repeated sequence sets in mitochondrial DNA molecules of root knot nematodes (*Meloidogyne*): nucleotide sequences, genome location and potential for host-race identification. Nucleic Acids Research. 19(7):1619-1626

Powers, T. O. and T. S. Harris. 1993. A polymerase chain reaction method for identification of five major *Meloidogyne* species. Journal of Nematology. 25(1):1-6

Sasser M. J. 1990. Identification of bacteria by gas chromatography of cellular fatty acids. *Technical Note 101*. Microbial ID, Inc., Newark, Del.

Sekora, N. S., K. K. Lawrence, E. van Santen, J. A. McInroy. 2008. Fingerprinting nematode fatty acid compositions as a means for identification. Proceedings of the National Beltwide Cotton Conference, Vol. 1:235-244. National Cotton Council, Memphis TN. Online: **www.cotton.org/beltwide/proceedings.**

Taylor, A. L. and Sasser, J. N. 1978. Biology, identifiaction and control of root-knot nematodes (*Meloidogyne* species). Department of Plant Pathology, North Carolina State University and the United Sates Agency for International Development. Raleigh, NC

Thorne, G. 1961. Principles of Nematology. McGraw-Hill Book Company, Inc., New York, NY

Venkatachari, S., L. A. Payan, D. W. Dickson, and T. E. Hewlett. 1991. Comparisons of isozyme phenotypes in five *Meloidogyne* spp. with isoelectric focusing. Journal of Nematology. 23(4):457-461

Table 1. Fatty acid profiles for four *Meloidogyne* species. Means are listed in order by fatty acid chain length, location of the double bond, and functional group.

Fatty Acid	*M. arenaria*	*M. hapla*	*M. incognita*	*M. javanica*
10:0	0.70	0.16	0.04	0.38
10:0 2OH	0.03	--	--	--
12:0 2OH	3.28	1.37	4.52	2.05
14:0	0.52	0.90	0.15	0.48
14:0 2OH	0.19	0.03	0.02	0.18
15:0 ISO	0.98	1.27	0.48	0.91
15:1 ANTEISO A	0.19	--	--	0.05
16:0	7.39	7.19	11.52	8.69
16:0 2OH	--†	--	0.05	--
16:1 ω5c	0.68	0.55	0.18	4.58
16:1 ω7c/15 iso 2OH	1.85	1.36	1.38	1.32
17:0	--	--	0.01	--
17:0 ANTEISO	--	--	0.36	--
17:0 ISO	0.79	0.82	0.11	0.90
18:0	13.34	12.96	23.24	11.49
18:0 3OH	0.03	0.25	0.02	0.26
18:0 ANTE/18:2 ω6,9c	0.09	--	0.29	--
18:1 ω5c	1.88	1.54	0.87	1.72
18:1 ω7c	57.45	58.89	45.27	54.50
18:1 ω9c	1.61	2.07	1.52	2.07
18:2 ω6,9c/18:0 ANTE	1.85	2.55	1.57	1.97
18:3 ω6c (6,9,12)	0.35	0.63	0.16	0.50
19:1 ISO I	1.63	1.44	1.07	1.54
20:0	1.56	1.67	1.88	1.41
20:0 ISO	0.06	0.34	0.01	0.40
20:1 ω7c	3.45	3.70	3.48	4.15
20:1 ω9c	--	--	--	0.01
20:2 ω6,9c	--	0.13	0.02	0.19
20:4 ω6,9,12,15c	0.01	0.19	--	0.24
unknown 16:582	--	--	0.14	--
unknown 18:814	--	--	2.77	--

† = Not detected

Table 2. Squared Mahalanobis distances (D^2) and class distance probability of similarity (*P*) for canonical discriminant analysis of four *Meloidogyne* species.

	MA		MH		MI		MJ	
	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*
MA			16.7	<.0001	15.9	<.0001	43.9	<.0001
MH	16.7	<.0001			22.0	<.0001	45.4	<.0001
MI	15.9	<.0001	22.0	<.0001			53.5	<.0001
MJ	43.9	<.0001	45.4	<.0001	53.5	<.0001		

MA: *M. arenaria*, MH: *M. hapla*, MI: *M. incognita*, and MJ: *M. javanica*

Table 3. Phenotypic canonical correlation of fatty acids for the two canonical discriminant functions of FAME profile analysis for four *Meloidogyne* species. Eigenvalue, cumulative percent of total variance, and canonical correlation are listed for each canonical function. Fatty acids listed were determined significant by the STEPDISC procedure. Correlation values are determined to be significant if $r \geq |0.75|$ (**bold**).

Response variable		Discriminant variate		
No.	Fatty acid variable	CAN 1	CAN 2	CAN 3
1	10:0	0.594	0.360	-0.719
2	12:0 2OH	**-0.809**	-0.582	-0.080
3	14:0	0.575	**0.806**	0.142
4	14:0 2OH	**0.757**	-0.002	-0.654
5	15:0 ISO	0.640	**0.768**	0.039
6	16:1 ω5c	**0.965**	-0.262	-0.007
7	16:1 ω7c/15 iso 2OH	0.551	0.621	-0.557
8	17:0 ISO	**0.844**	0.516	-0.146
9	18:0 3OH	**0.849**	0.325	0.416
10	18:1 ω5c	**0.790**	0.523	-0.320
11	18:1 ω7c	0.735	0.659	-0.162
12	18:1 ω9c	**0.865**	0.495	0.080
13	20:0 ISO	**0.893**	0.292	0.341
14	20:2 ω6,9c	**0.906**	0.094	0.413
15	20:4 ω6,9,12,15c	**0.886**	0.231	0.401
	Eigenvalue	6.02	2.12	0.95
	Cumulative %	66.3	89.6	100.0
	Canonical Correlation	0.92	0.81	0.67

Table 4. Fatty acid profiles for three *Meloidogyne incognita* races. Means are listed in order by fatty acid chain length, location of the double bond, and functional group.

Fatty Acid	*M. incognita* race 1	*M. incognita* race 2	*M. incognita* race 3
10:0	0.05	0.08	--
12:0 2OH	2.14	3.55	7.86
14:0	0.33	--	0.14
14:0 2OH	--†	--	0.06
15:0 ISO	0.60	0.38	0.46
16:0	7.37	8.74	18.46
16:0 2OH	--	--	0.15
16:1 ω5c	0.44	0.10	--
16:1 ω7c/15 iso 2OH	1.18	2.84	0.11
17:0	0.04	--	--
17:0 ANTEISO	--	--	1.08
17:0 ISO	0.24	--	0.08
18:0	14.38	15.09	40.25
18:0 3OH	0.05	--	--
18:0 ANTE/18:2 ω6,9c	0.33	0.55	--
18:1 ω5c	1.65	0.91	0.06
18:1 ω7c	58.94	57.08	19.81
18:1 ω9c	2.23	2.18	0.14
18:2 ω6,9c/18:0 ANTE	2.60	1.99	0.11
18:3 ω6c (6,9,12)	--	0.21	0.26
19:1 ISO I	1.35	0.95	0.91
20:0	2.02	1.86	1.75
20:0 ISO	0.02	--	--
20:1 ω7c	3.94	3.50	3.00
20:1 ω9c	--	--	--
20:2 ω6,9c	0.06	--	--
unknown 16:582	--	--	0.42
unknown 18:814	--	--	8.31

† = Not detected

Table 5. Phenotypic canonical correlation of fatty acids for the two canonical discriminant functions of FAME profile analysis for three *Meloidogyne incognita* races. Eigenvalue, cumulative percent of total variance, and canonical correlation are listed for each canonical function. Fatty acids listed were determined significant by the STEPDISC procedure. Correlation values are determined to be significant if $r \geq |0.75|$ (**bold**).

Response variable		Discriminant variate	
No.	Fatty acid variable	CAN 1	CAN 2
1	10:0	**-0.951**	-0.308
2	12:0 2OH	-0.005	**1.000**
3	14:0	-0.090	**0.996**
4	15:0 ISO	0.680	0.734
5	16:0	**0.929**	-0.371
6	16:1 ω7c/15 iso 2OH	0.122	**0.993**
7	17:0 ANTEISO	**-0.988**	-0.154
8	17:0 ISO	**0.985**	-0.171
9	18:0	**0.871**	0.491
10	18:0 3OH	**0.986**	0.168
11	18:0 ANTE/18:2 ω6,9c	**0.988**	0.153
12	18:1 ω5c	**0.951**	0.309
13	18:1 ω7c	0.486	**0.874**
14	18:3 ω6c (6,9,12)	**-0.991**	-0.136
	Eigenvalue	11.91	1.72
	Cumulative %	87.4	100.0
	Canonical Correlation	0.96	0.80

Table 6. Squared Mahalanobis distances (D^2) and class distance probability of similarity (*P*) for canonical discriminant analysis of *Meloidogyne arenaria* (MA), *M. hapla* (MH), *M. incognita* race 1 (MIR1), *M. incognita* race 2 (MIR2), *M. incognita* race 3 (MIR3), and *M. javanica* (MJ).

	MA		MH		MIR1		MIR2		MIR3		MJ	
	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*
MA			19.5	<.0001	15.8	<.0001	33.1	<.0001	60.0	<.0001	46.9	<.0001
MH	19.5	<.0001			19.2	<.0001	57.9	<.0001	64.4	<.0001	45.1	<.0001
MIR1	15.8	<.0001	19.2	<.0001			27.6	<.0001	57.8	<.0001	49.0	<.0001
MIR2	33.1	<.0001	57.9	<.0001	27.6	<.0001			80.5	<.0001	88.5	<.0001
MIR3	60.0	<.0001	64.4	<.0001	57.8	<.0001	80.5	<.0001			101.5	<.0001
MJ	46.9	<.0001	45.1	<.0001	49.0	<.0001	88.5	<.0001	101.5	<.0001		

MA: *M. arenaria*, MH: *M. hapla*, , MIR1: *M. incognita* race 1, MIR2: *M. incognita* race 2, MIR3: *M. incognita* race 3, and MJ: *M. javanica*

Table 7. Phenotypic canonical correlation of fatty acids for the two canonical discriminant functions of FAME profile analysis for four *Meloidogyne* species, including three *Meloidogyne incognita* races. Eigenvalue, cumulative percent of total variance, and canonical correlation are listed for each canonical function. Fatty acids listed were determined significant by the STEPDISC procedure. Correlation values are determined to be significant if $r \geq |0.75|$ (**bold**).

Response variable		Discriminant variate		
No.	Fatty acid variable	CAN 1	CAN 2	CAN 3
1	10:0	0.598	0.225	0.044
2	12:0 2OH	**-0.969**	-0.028	-0.210
3	14:0	0.543	0.466	0.672
4	14:0 2OH	0.242	0.604	-0.235
5	15:0 ISO	0.574	0.485	0.581
6	16:1 ω5c	0.568	0.698	-0.436
7	16:1 ω7c/15 iso 2OH	**0.804**	-0.518	-0.144
8	17:0 ISO	0.677	0.592	0.283
9	18:0 3OH	0.622	0.651	0.178
10	18:0 ANTE/18:2 ω6,9c	0.407	**-0.829**	-0.275
11	18:1 ω5c	**0.941**	0.118	0.214
12	18:1 ω7c	**0.973**	-0.169	0.156
13	18:1 ω9c	**0.971**	-0.157	0.054
14	18:2 ω6,9c/18:0 ANTE	**0.943**	-0.127	0.246
15	20:0 ISO	0.603	0.684	0.099
16	20:2 ω6,9c	0.632	0.687	-0.001
17	20:4 ω6,9,12,15c	0.558	0.695	0.044
	Eigenvalue	14.11	6.09	2.58
	Cumulative %	57.6	82.5	93.0
	Canonical Correlation	0.97	0.92	0.83

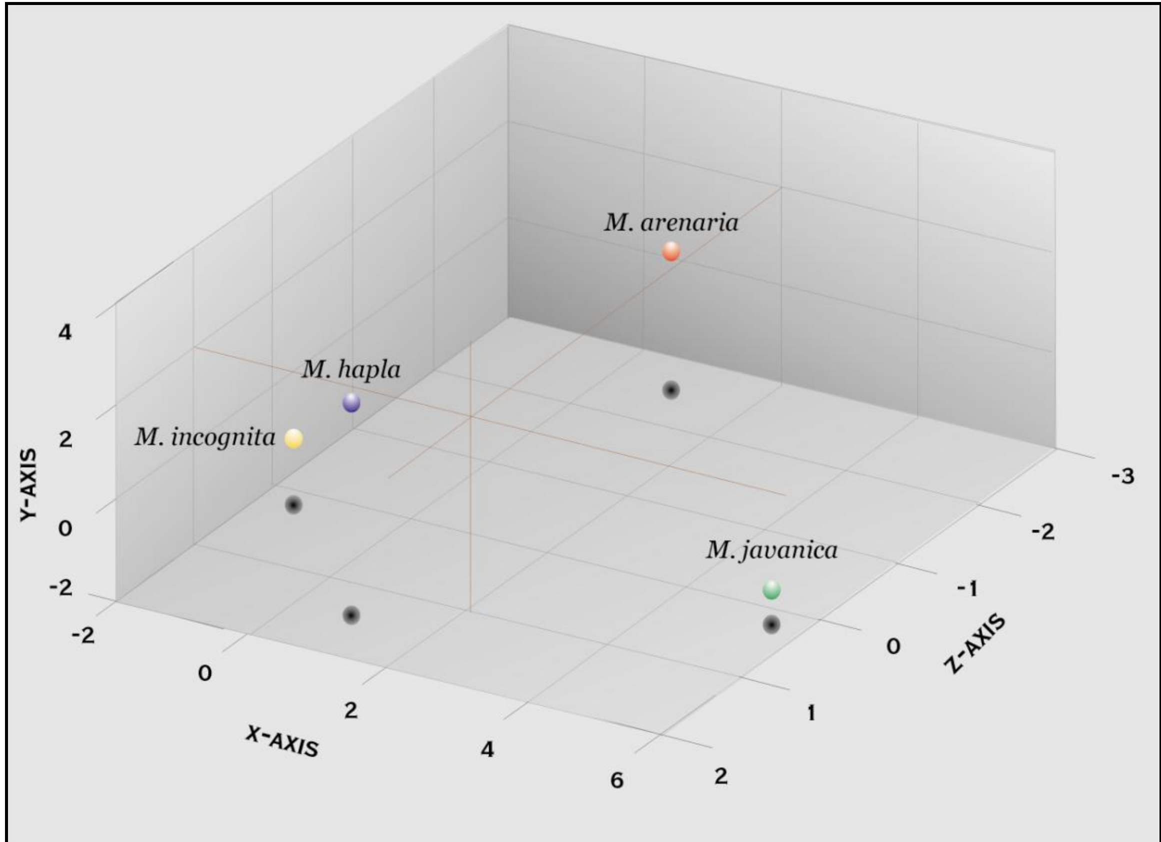

Figure 1. Graph of canonical means for *Meloidogyne* species. Placement is defined for the first (x-axis), second (y-axis), and third (z-axis) canonical axes by height (y-axis) and shadow (x-axis and z-axis intercept).

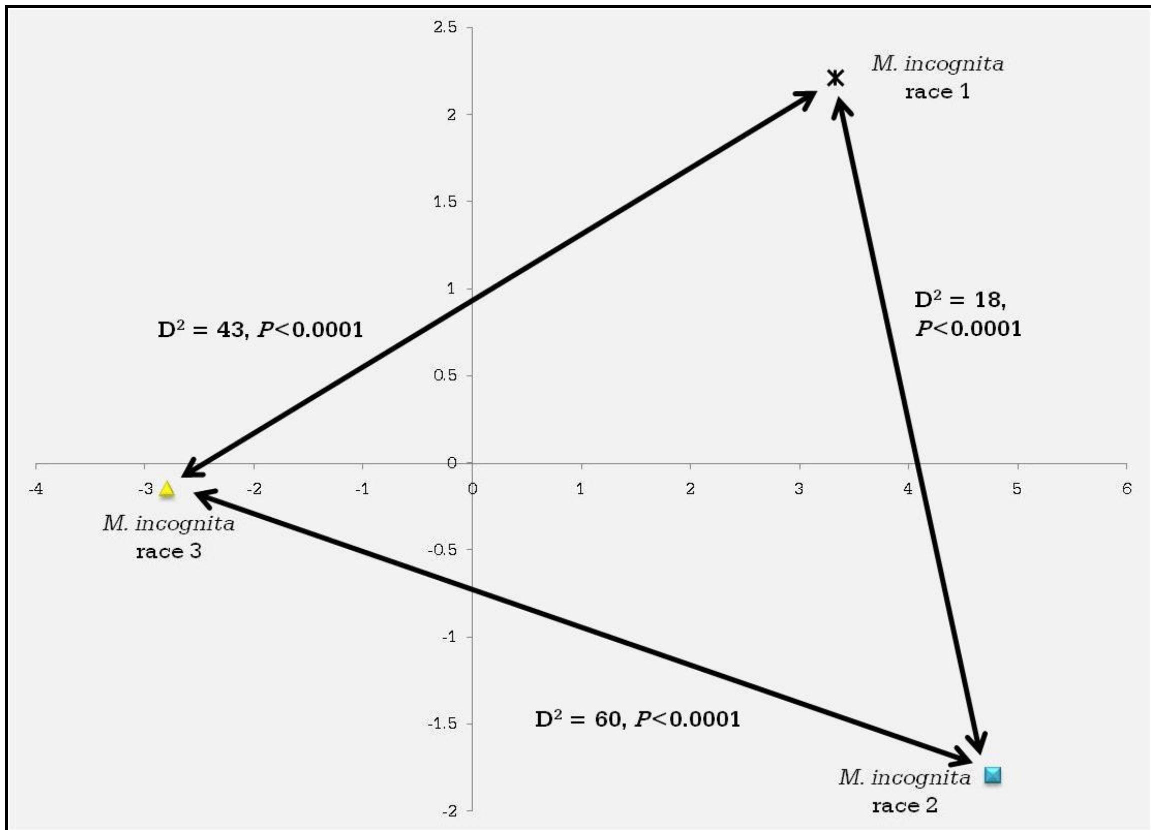

Figure 2. Canonical mean distribution for three *Meloidogyne incognita* races. The x-axis represents the first canonical dimension and the y-axis represents the second canonical dimension. D^2 values listed are real-distances between points squared.

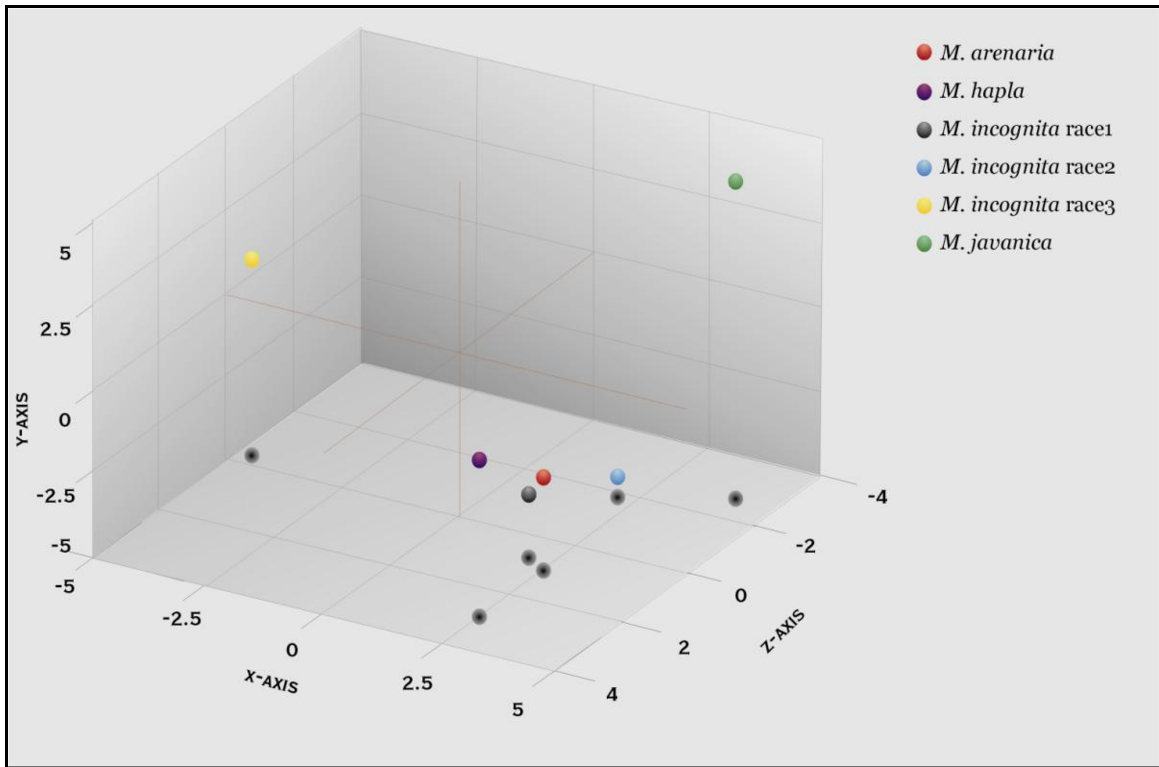

Figure 3. Canonical distribution of three *Meloidogyne* species and three races of *M. incognita*. Placement is defined for the first (x-axis), second (y-axis), and third (z-axis) canonical axes by height (y-axis) and shadow (x-axis and z-axis intercept).

IV. USING FAME ANALYSIS TO COMPARE, DIFFERENTIATE, AND IDENTIFY MULTIPLE NEMATODE SPECIES

Abstract

We have adapted the Sherlock® Microbial Identification system for identification of plant-parasitic nematodes based on their fatty acid profiles. Fatty acid profiles of 12 separate plant-parasitic nematode species have been determined using this system. Additionally, separate profiles have been developed for *Rotylenchulus reniformis* and *Meloidogyne incognita* based on their host plant, four species and three races within the *Meloidogyne* genus, and three life stages of *Heterodera glycines*. Statistically, 85% of these profiles can be delimited from one another; the specific comparisons between the cyst and vermiform stages of *H. glycines, M. hapla* and *M. arenaria*, and *M. arenaria* and *M. javanica* cannot be segregated using canonical analysis. By incorporating each of these fatty acid profiles into the Sherlock® Analysis Software, 20 library entries were created. While there was some similarity among profiles, all entries correctly identified the proper organism to genus, species, race, life stage, and host at greater than 86% accuracy. The remaining 14% were correctly identified to genus, although species and race may not be correct due to the underlying variables of host or life stage. These results are promising and indicate that this library could be used for diagnostics labs to increase response time.

Introduction

By using the FAME extraction method, we have been able to develop fatty acid profiles for several species, races, and life stages of plant parasitic nematodes as well as quantify the impact of various host species on the fatty acid profiles of *Meloidogyne incognita* and *Rotylenchulus reniformis* (Sekora *et. al*, 2008a, 2008b, 2009a, 2009b, and 2009c). The overall goal of our continuing research is to develop a library of plant-parasitic nematode fatty acid profiles for use with the Sherlock® Analysis Software (MIDI, Inc) that can identify nematode samples in plant disease diagnostic laboratories at a more economical cost than current practices. To evaluate the applicability of the developed library for nematode sample identification, the twenty library entries of this library were analyzed to quantify any overlap among profiles that would hinder sample identification.

Three objectives were outlined to analyze fatty acid profiles within the library using both statistical analysis and the Sherlock® Analysis Software, 1) statistical analysis of fatty acid profiles based on species classification, 2) additionally analyzing species fatty acid profiles while keeping any unique profiles (race, host plant, life stage) as separate profiles, and 3) using the Sherlock® Analysis Software to analyze the similarity among all 20 developed profiles.

Materials and Methods

General Culturing

Populations of *R. reniformis* (Linford and Oliveira) and *Heterodera glycines* (Ichinohe) race 3 (Golden) were collected from populations found in

field sites across Alabama. *R. reniformis* populations were increased on *Gossypium hirsutum* (L.) cv. 'Stoneville 5599 BGRR,' *Lycopersicon esculentum* (Mill.) cv. 'Roma,' and *Glycine max* (L.) cv. 'Hutcheson.' The mixed population of *H. glycines* was increased on Croplan Genetics *G. max* cv. 'RC 4955.' A stock population of *M. incognita* race 3 (Chit.) was collected from the Plant Breeding Unit of the E. V. Smith Research Center in Shorter, AL and increased on *L. esculentum* cv. 'Roma,' *G. hirsutum* cv. 'Delta and Pine Land (DPL) 555 BGRR', and *G. max* cv. 'Hutcheson'. These populations of *R. reniformis*, *M. incognita* race 3, and *H. glycines* were increased in 500cm^3 polystyrene pots at the Auburn University Plant Science Research Center greenhouses. Pots were physically separated by Plexiglas dividers (61 cm high by 91 cm deep) to prevent the formation of mixed populations.

The remaining *Meloidogyne* species in this study (*M. incognita* [Chit.] races 1 and 2 [Hartmann and Sasser], *M. arenaria* [Chit.] race 2 [Hartmann and Sasser], *M. hapla* [Chit.], and *M. javanica* [Chit.]) were increased from populations collected around the country and increased on *L. esculentum* var. 'Rutgers' at the Clemson University greenhouses; these populations were maintained in 45 cm clay pots and also physically separated by Plexiglas dividers 61 cm high by 91 cm deep. Populations of *Aphelencoides fragariae*, *Aphelenchus avenae*, *Bursaphelenchus xylophilus*, *Ditylenchus dipsaci*, *Pratylenchus penetrans*, and *Radopholus similis* were contributed from lab cultures maintained at Clemson University.

Nematode Extraction

Second stage juvenile life stages (J2s) of *Meloidogyne* populations, juvenile life stages and vermiform adults of *R. reniformis*, as well as J2s, mature females, and cysts of *H. glycines* were extracted from the soil of stock pots using combined gravity screening and sucrose centrifugation. Gravid females and eggs of all *Meloidogyne* species and races were extracted from root tissue using NaOCl. Both extractions for each species and race were then combined and centrifuged utilizing a sucrose gradient to remove any remaining plant and soil debris. Individuals of *A. fragariae*, *A. avenae*, *B. xylophilus*, *D. dipsaci*, *P. penetrans*, and *R. similis* were collected by rinsing multiple plates of each species over nested sieves to remove culture and plant debris. Extractions for all species and races were enumerated to determine the number of each life stage in samples.

Fatty Acid Extraction

A total of 867 samples were prepared from races, hosts, and life stages of the 12 nematode species. Fatty acids from samples were extracted using the method described by Sasser (1990). After the extraction procedure was completed, the organic solvent was transferred to sample vials and allowed to evaporate under a fume hood. The dried samples were reconstituted in 75 μL of organic extraction solvent and transferred to spring-vial inserts for each sample vial. Vials were sealed and stored at -20°C until analysis.

Samples were analyzed for fatty acid composition by an HP 5890 automated gas chromatography system (Agilent Technologies) equipped with an Ultra 2 Cross-linked 5% Phenyl Methyl Siloxane column; 2.0 µL of sample was injected into the column for each analysis. Sample data from the Sherlock® Sequencer Software (MIDI, Inc.) included total response of each sample (mV) and the response for each detected fatty acid. Fatty acid percentages were calculated from the proportion of each fatty acid within the sample; these percentages were used to create a fatty acid profile for each nematode sample.

Statistical Analysis

Two comparisons among nematode fatty acid profiles were made in this study, 1) comparing fatty acid profiles at the species level and 2) comparing profiles generated for each species, race, life stage, and host. Species comparisons were made by including all races, life stages, and hosts under a single "species" class for each nematode species. Total profile comparisons of various life stages, hosts, or races were classified to a "variable" class and did not combine any profiles from a species.

The STEPDISC (SAS version 9.1.3; SAS Institute, Inc) procedure was used to analyze the percentage of each fatty acid across all samples within a given class to determine which fatty acids contributed significantly to the differentiation among classes (species or variable) based on the ANOVA test F value of a selected fatty acid (Johnson, 1998). The compiled list of fatty acids was used for class differentiation with the CANDISC procedure. The CANDISC procedure provided

canonical discriminant analysis (CDA) of the fatty acid profiles for each nematode sample within its respective categorical class.

Sherlock® Analysis Software

A library was developed using the Sherlock® Analysis Software by creating entries from fatty acid profiles of the 19 nematode species, races, life stages, and hosts in this study. To determine the usefulness and validity of the newly created library entries, individual samples were compared against their respective composite profiles to create comparison and similarity matrices for each library entry. Identification reports were also used to evaluate identification accuracy using the "First choice" and "First Second choice" methods among samples.

Results

Differentiation by Species

A total of 54 fatty acids were observed among the 12 nematode species studied (Table 1). Of these 54 fatty acids, an average of 11 (19%) were expressed within each nematode species profile. The maximum number of fatty acids observed in a species profile was 49 (91%) fatty acids within *R. reniformis*; a minimum of 7 (13%) fatty acids were observed within the profile of *B. xylophilus*. Most of these fatty acids (80%) were expressed at percentages less than 1.0% among all species profiles; *H. glycines* expressed 19 (35%) fatty acids above 1.0%, the most of any other profile. Two fatty acids (16:0 and 18:1 ω7c) were observed in the highest concentrations among the nematode species. Six species (*A. fragariae*, *A. avenae*, *B. xylophilus*, *D. dipsaci*, *P. penetrans*, and *R. reniformis*)

expressed 16:0 as their primary fatty acid; percentages ranged from 16.21% in *R. reniformis* to 50.26% in *B. xylophilus*. For the remaining six species (*M. arenaria*, *M. hapla*, *M. incognita*, *M. javanica*, *H. glycines*, and *R. similis*), 18:1ω7c was the fatty acid with the highest percentage and ranged from 38.21% in *M. incognita* to 58.89% in *M. hapla*. The fatty acid most commonly expressed at the second highest percentage was 18:0 in seven species (*A. fragariae*, *A. avenae*, *B. xylophilus*, *M. arenaria*, *M. hapla*, *M. incognita*, and *M. javanica*). The expression of 18:0 in these seven species ranged from 11.49% in *M. javanica* to 27.68% in *B. xylophilus*, with a mean of 18.83%.

CDA explained 92.9% of the total multivariance in the first three canonical dimensions (Table 3). Based on this analysis, 98% of the total comparisons among species were significant ($D^2 \geq 3.8$, $P \leq 0.492$; Table 2). The comparison between *M. hapla* and *M. javanica* was not significant ($D^2=2.3$, $P=0.949$) based on species comparisons. Thirty-four of the fatty acids were significant for differentiation among fatty acid profiles at the species level. The first canonical dimension explained 68.0% of the total multivariance (Table 3) and primarily separated *H. glycines* from the remaining fatty acid profiles (Figure 1). Two fatty acids (20:4 ω6,9,12,15c and 16:1 ω7c) were primarily responsible for differentiation along the first canonical axis (Table 3). Both of these fatty acids were found in the highest concentrations in the *H. glycines* profile (9.77% and 3.96%, respectively; Table 1). The four species within the Meloidogyne genus (*M. arenaria*, *M. hapla*, *M. incognita*, and *M. javanica*) expressed both of these fatty acids, but at concentrations less than those of *H. glycines* (mean of 1.36% and

0.50%, respectively). *Pratylenchus penetrans* was the only other nematode species to express 20:4 ω6,9,12,15c, but its mean concentration (0.08%) was also lower than that of *H. glycines*. The fatty acid 16:1 ω7c was not observed in any other nematode species outside the *Meloidogyne* genus or *H. glycines*.

Five fatty acids (12:0 2OH, 10:0, TBSA 10Methyl 18:0 peak, 16:1 cis 9, and 20:1 trans 11) explained 15.8% of the total multivariance along the second canonical dimension (Table 3). These fatty acids helped separate *R. reniformis* from the *Meloidogyne* species group and the remaining nematode species group (*A. fragariae*, *A. avenae*, *B. xylophilus*, *D. dipsaci*, *P. penetrans*, and *R. similis*; Figure 1). All five of these fatty acids are found in *R. reniformis* (Table 1). Three of the five fatty acids (12:0 2OH, 10:0, TBSA 10Methyl 18:0 peak) were found in the highest concentrations in *R. reniformis* (15.67%, 1.76%, and 0.46%, respectively). Both 16:1 cis 9 and 20:1 trans 11 were only found in *R. reniformis* (0.14% and 2.21%, respectively).

The third canonical dimension explained 9.0% of the total multivariance (Table 3) and separated the group containing *A. fragariae*, *A. avenae*, *B. xylophilus*, *D. dipsaci*, *P. penetrans*, and *R. similis* from the *Meloidogyne* species group (Figure 1). A single fatty acid, 18:1 ω7c, separated these groups. Within the *Meloidogyne* species, 18:1 ω7c had a mean expression of 52.26% and ranged from 38.21% in *M. incognita* to 58.89% in *M. hapla* (Table 1). This fatty acid was only found in three species (*D. dipsaci*, *P. penetrans*, and *R. similis*) from the other group. Expression of 18:1 ω7c within these profiles was 46.24% in *R. similis*, 30.55% in *D. dipsaci*, and 19.09% in *P. penetrans*.

Differentiation by Species, Race, Life Stage, and Host

Of the same 55 fatty acids observed in the species analysis, a mean of 22 (40%) fatty acids were observed within the profiles when separated based on life stage, host plant, and race (Tables 1 and 4). The fatty acid profiles of *R. reniformis* from cotton and soybean plants expressed the largest number of fatty acids (41, 76%) among all profiles; the fatty acid profile of *B. xylophilus* contained the least fatty acids (7, 13%). An average of 17 (31%) fatty acids were expressed at percentages greater than 1.0% among all fatty acid profiles; *H. glycines* females expressed 16 (30%) fatty acids at percentages greater than 1.0%. Both 18:1 ω7c and 16:0 were the two fatty acids expressed at the highest concentrations among the 20 fatty acid profiles. The mean percentage of 18:1 ω7c observed was 35.18% across all fatty acid profiles. Nine profiles (*M. arenaria*, *M. hapla*, *M. incognita* race 1, *M. incognita* race 2, *M. incognita* race 3 from cotton, *M. javanica*, *R. similis, H. glycines* cysts, and *H. glycines* J2s) expressed 18:1 ω7c as their primary fatty acid; percentages ranged from 46.24% in *R. similis* to 60.14% in *H. glycine*s cysts. Across all profiles, 16:0 was expressed at a mean concentration of 20.37% (ranging from 2.77% in *H. glycines* cysts to 50.26% in *B. xylophilus*) and was the primary fatty acid observed within seven profiles (*A. fragariae*, *A. avenae*, *B. xylophilus*, *D. dipsaci*, *M. incognita* race 3 from soybean, *P. penetrans*, and *H. glycines* females). The third most common fatty acid among all profiles was 18:0 (15.04%) and was found to be the second most abundant fatty acid in ten profiles (*A. fragariae*, *A. avenae*, *B. xylophilus*, *M. arenaria*, *M.*

hapla, *M. incognita* race 1, *M. incognita* race 2, *M. incognita* race 3 from cotton, *M. javanica*, and *R. similis*).

A total of 74.3% of the total multivariance was explained by the first three canonical dimensions using CDA (Table 6). Among the 20 profiles, 98% of the possible comparisons were significantly different ($D^2 \geq 8.57$, $P \leq 0.005$; Table 5). Three comparisons were not significant when analyzing the twenty fatty acid profiles. These comparisons were between *M. arenaria* and *M. hapla* (D^2=6.6, P=0.1571), *M. hapla* and *M. javanica* (D^2=6.2, P=0.1725), and *H. glycines* cysts and *H. glycines* J2s (D^2=3.1, P=1.000). Forty-six fatty acids were determined to be significant for differentiating among the twenty fatty acid profiles. A single fatty acid (20:4 ω6,9,12,15c) was responsible for separating profiles along the first canonical dimension (45.9% of multivariance; Table 6). The three *H. glycines* profiles (cysts, females, and J2s) were separated from the remaining profiles along the first canonical dimension. Among the three *H. glycines* profiles, 20:4 ω6,9,12,15c was observed at 9.63%, 9.46%, and 10.22%, respectively. In comparison, *M. arenaria*, *M. hapla*, *M. incognita* race 3 from soybean, *M. javanica*, and *P. penetrans* expressed 20:4 ω6,9,12,15c at concentrations less than 1.6% (Tables 1 and 4).

The second canonical dimension explained 15.8% of the total multivariance among fatty acid profiles (Table 6). Four fatty acids (16:1 ω7c, 19:0 cyclo ω8c, 16:0 3OH, and 16:1 ω5c) were responsible for separation along the second canonical axis and separated profiles within the *Meloidogyne* genus and *R. reniformis* from the six nematode species *A. fragariae*, *A. avenae*, *B.*

xylophilus, *D. dipsaci*, *P. penetrans*, and *R. similis* (Figure 2). Only profiles within the *Meloidogyne* genus (*M. arenaria*, *M. hapla*, *M. incognita* race 1, *M. incognita* race 2, *M. incognita* race 3 from cotton, *M. incognita* race 3 from soybean, *M. incognita* race 3 from tomato, and *M. javanica*) and *H. glycines* (cysts, females, and J2s) had 16:1 ω7c in their profiles (Tables 1 and 4). Both 19:0 cyclo ω8c and 16:0 3OH were only found in profiles of *R. reniformis* and *M. incognita* race 3 that varied by host; 19:0 cyclo ω8c was found in *R. reniformis* and *M. incognita* race 3 when both were grown on soybean, and 16:0 3OH was found in *M. incognita* race 3 from soybean and *R. reniformis* from cotton. The fourth fatty acid, 16:1 ω5c, was found in *R. reniformis* from soybeans, the three life stages of *H. glycines*, *M. arenaria*, *M. hapla*, *M. incognita* race 1, *M. incognita* race 2, *M. incognita* race 3 from soybean, and *M. javanica*. The percentage of this fatty acid varied among profiles from 0.10% in *M. incognita* race 2 to 26.43% in *M. incognita* race 3 from soybeans.

Four fatty acids (18:1 TRANS 9/t6/c11, the unknown 15.549 peak, 12:0 2OH, and 18:0 ANTEISO/18:2 c) were responsible for defining 12.6% of the total multivariance along the third canonical dimension (Table 6). Differentiation along the third dimension separated *H. glycines* females from cysts and J2s as well as the *Meloidogyne* genus from the *R. reniformis* profiles (Figure 2). Three of the four fatty acids (18:1 TRANS 9/t6/c11, the unknown 15.549 peak, and 18:0 ANTEISO/18:2 c) were observed in three *R. reniformis* profiles and no other profile (Tables 1 and 4). Fourteen of the twenty profiles contained 12:0 2OH, but the highest percentages were found in *R. reniformis* (mean 15.67%). Profiles

within the *Meloidogyne* genus expressed 12:0 2OH at an average of 4.91% across eight profiles; *H. glycines* cysts, *H. glycines* J2s, and *P. penetrans* expressed 12:0 2OH at less than 1.0% (0.16%, 0.34%, and 0.91%, respectively).

Identification with the Sherlock® Analysis Software

We were able to develop a library of fatty acid profiles containing 13 entries based on 440 samples. While some samples were mismatched depending on host, race, or species, 98.9% of the samples analyzed were correctly identified to the genus level with the Sherlock® Analysis Software (Table 7). *Heterodera* and *Rotylenchulus* correctly matched to their correct genus in 100% of samples. *Meloidogyne* genus samples were correctly identified at 98.4% to the genus level, 1.6% being matched to the *Rotylenchulus* genus.

At the species level, *Meloidogyne* species were identified at 90.7% accuracy (Table 7). Samples were correctly identified to the four species within *Meloidogyne* (*M. arenaria*, *M. hapla*, *M. incognita*, and *M. javanica*) at 77.8%, 94.4%, 90.73%, and 100% accuracy, respectively. *Meloidogyne arenaria* samples matched to *M. hapla* 16.7% of the time, while *M. incognita* samples matched to *M. arenaria* and *M. hapla* in 1.58% and 7.70% of *M. incognita* samples. Races within *M. incognita* matched correctly in 87.3% of samples. Races one, two, and three of *M. incognita* matched correctly at 64.7%, 76.9% and 98.5%, respectively. Race 1 matched to race 2 in 5.9% of samples, but also to *M. arenaria* and *M. hapla* at 5.0% and 24.4%, respectively. Race 2 samples were incorrectly identified to *M. arenaria*, *M. hapla*, *M. incognita* race 1, and *M. incognita* race 3 in 1.3%, 6.4%, 7.7%, and 7.7% of all samples, respectively.

In samples of *M. incognita* race 3 and *R. reniformis* that were increased on different hosts, samples of these nematodes matched to the correct host 94.5% of the time (Table 7). *Meloidogyne incognita* race 3 samples could be correctly identified to host at 98.5% accuracy, with 1.5% of cotton samples matching to *M. incognita* race 1. Samples of *R. reniformis* from each host were correctly identified at 91.8% accuracy. A total of 2.0% and 4.0% of *R. reniformis* samples from cotton were identified as coming from soybean and tomato plants, respectively. Samples of *R. reniformis* from soybean were identified as tomato samples in 5.0% of samples. *Rotylenchulus reniformis* tomato samples were incorrectly identified in 13.6% of samples.

All life stages of *H. glycines* were correctly identified in 100% of samples. By comparing all 13 entries within the developed library, 91.2% of all samples were correctly identified to race, host, or life stage level. Species level identification of samples across these entries was 96.9% accurate.

Discussion

FAME analysis was developed to identify bacterial species more quickly and easily than differential biochemical testing. The Sherlock® Analysis Software is currently able to accurately identify 1700 species of bacteria and yeast, many to the subspecies or strain level (Kunitsky, 2005). Hoping to exploit the usefulness of this system, researchers have begun to adapt this system for use with other groups of organisms, most notably the fungi. FAME analysis has been used to characterize, classify, and identify more than 150 different species of fungi (Bentivenga and Morton, 1996; Graham et al., 1995; Stahl and Klug, 1996).

Fatty acid profiles of thirteen nematode species studied were distinct and characteristic using both CDA statistical analysis and the Sherlock® Analysis Software. In this study, 18:1 fatty acids were the predominant fatty acids in nine nematode fatty acid profiles (*M. arenaria*, *M. hapla*, *M. incognita* race 1, *M. incognita* race 2, *M. incognita* race 3 increased on cotton, *M. javanica*, *R. similis*, *H. glycines* cysts, and *H. glycines* J2), which is similar to what Krusberg (1967) observed in his studies with other plant-parasitic nematodes. Krusberg (1967) and Krusberg et al. (1973) found that the primary fatty acids in *D. dipsaci*, *P. penetrans*, *M. incognita*, and *M. arenaria* was 18:1, but in our studies we observed 16:0 as the most abundant fatty acid in *D. dipsaci*, *M. incognita* race 3 increased on soybean, and *P. penetrans*, as well as *A. avenae*, *A. fragariae*, *B. xylophilus*, and *H. glycines* females. The 16:0 fatty acid was found in the greatest abundance when samples contain less than 100 nematodes (Sekora et. al, 2008). Additional studies have been conducted using FAME analysis to observe variation in fatty acid profiles of *A. avenae* and *A. composticola* caused by starvation (Chen et al., 2001). This indicates that the seven profiles generated within which 16:0 was the predominant fatty acid may be based on diluted samples, but could also be caused by variations in the feeding cycle of those nematode species. Additional samples of these species would help resolve this variation and help explain differences observed among these studies.

Using stepwise comparisons and canonical analysis, we could not differentiate three comparisons (*M. arenaria* to *M. hapla*, *M. hapla* to *M. javanica*, and *H. glycines* cysts to *H. glycines* J2s) among the 190 profile

comparisons. The profile we developed for *M. arenaria* was similar to that published by Krusberg et. al (1973), but we found the percentage of 18:0 to be twice the original reported value. The percentage of 16:0 and 18:1 fatty acids in our profile for *M. javanica* was similar to that published by Chitwood and Krusberg (1981). By comparing profiles for the four species of *Meloidogyne*, we found fatty acids in these species were similar in percentages of fatty acids present as compared to percentages of the same fatty acids from other genera. However, fatty acid profiles of the four *Meloidogyne* species varied significantly ($P < 0.0001$) and differences could be observed when comparing within the genus (Chapter III). Studies by Krusberg et al. (1967) and Hutzell and Krusberg (1982) indicated that fatty acid profiles of species within the same genus could vary just as much as profiles among genera. The possibility that fatty acid profiles vary as much among species as they do among genera is promising and indicates that using fatty acid profiles as a means for identification at greater levels of specificity (race or life stage) may produce the same degree of variation.

The similarities we observed between cysts and J2s of *H. glycines* were not surprising considering that a mature cyst contains eggs with varying stages of embryonic development, including J1s and J2s (Baldwin and Mundo-Ocampo, 1991). A study by Gibson et al. (1995) indicated that mature cysts of *Globodera rostochiensis* could be identified by the presence of J2s within cysts. A mature *H. glycines* cyst should therefore produce a fatty acid profile comparable to that of *H. glycines* juveniles alone. The fatty acid composition of a cyst itself may be similar to that of females and juveniles, but crushed and emptied cysts would

need to be analyzed to determine the actual fatty acid profile of a cyst. Determining fatty acid composition of the cyst may not be necessary since the Sherlock® Analysis Software is able to differentiate samples containing cysts and juveniles from samples containing only juveniles in all samples studied, indicating that differences in the fatty acid profile due to the cyst has already been accounted for by the software.

The Sherlock® Analysis Software requires a minimum amount of lipids to be present in samples to create a library entry that can reliably identify an unknown sample (Sasser, 1990). Lipid concentrations are the limiting factor to creating library entries, but not to identifying samples. A sample may contain less than the amount of lipids to create a library entry; however, the software will attempt to match that sample to an existing library entry. Generating library entries based on samples containing concentrated lipids allows the system to detect minute fatty acids and produces a more robust fatty acid profile than using dilute samples.

Several aspects of an unknown sample can be determined from the fatty acid profile of that sample. Characteristics such as life stage, host of origin, and race could be determined by the amount of lipid present in a sample. For instance, if a sample of *Meloidogyne* was analyzed, the species and/or race could be identified based on the percentage of 16:0, 18:0, and 18:1 ω9c in the samples. It might be possible to use a fatty acid such as 19:0 cyclo ω8c to determine the host of origin when needed to recommend crop rotations for certain species and races of nematodes. This fatty acid can be present in *Rhizobium* species that

induce root nodules in soybean plants (Tighe, 2000). The presence of this fatty acid in a sample may indicate that the nematode contains this bacterial species when feeding on *Rhizobium* infested root systems. Of the three hosts studied, only nematodes increased on soybean plants contained the 19:0 cyclo ω8c fatty acid.

Many of the diagnostic laboratories in our area identify plant-parasitic nematodes to the genus level. Genus identification is accurate for recommendation of nematicide applications. However, the species and races of *Meloidogyne* samples must be determined to make the proper crop rotation recommendations because of the host specificity exhibited by different species and races. The library developed herein can identify these genera of nematodes with 99% accuracy, and only decreases to 97% for identifications to the species level. Diagnostic laboratories utilizing this software could identify species or races within *Meloidogyne* with at least 87% accuracy. Identification of nematode genera, species, races, and life stages with this system can reduce the total work required for sample processing in diagnostic laboratories.

Based on the success of this research, it is possible to use FAME analysis and the Sherlock® Analysis Software as an alternative means for identification of plant-parasitic nematodes in diagnostic laboratories. Using this approach would reduce the time required for identifications to the species and race levels, which can take up to 45 days to complete for *Meloidogyne* species and races (Taylor and Sasser, 1978). It may even be possible to detect and identify plant-parasitic nematodes directly from extracted soil samples based on comparative studies of

soil FAME profiles. Studies by Madan (2002) and Ruess (2002) have indicated that FAME analysis could be used to detect, and possibly quantify, fungal and nematode species in soil extractions. In addition to identification, information found in the development of this library could have potential to be valuable in other areas of agriculture, such as using fatty acids as a means for resistance induction in crop plants. Zinovieva et al. (1995) observed that treating tomato seeds with varying concentrations of the 20:4 ω6,9,12,15c fatty acid reduced *M. incognita* numbers and increased the production of nematoxic compounds by treated plants. Many disciplines have the potential to gain from using FAME analysis as a means for identification of plant-parasitic nematodes.

Literature Cited

Baldwin, J. G. and M. Mundo-Ocampo. 1991. Heteroderinae, cyst- and non-cyst-corming nematodes. *Manual of Agricultural Nematology*. Marcel Dekker, Inc. New York, NY.

Bentivenga, S. P., and J. B. Morton. 1996. Congruence of fatty acid methyl ester profiles and morphological characters of arbuscular mycorrhizal fungi in Gigasporaceae. Proceedings of the National Academy of Sciences USA. 93:5659-5662

Chen, J., H. Ferris, K. M. Scow, and K. J. Graham. 2001. Fatty acid composition and dynamics of selected fungal-feeding nematodes and fungi. Comparative Biochemistry and Physiology Part B. 130:135-144

Chitwood, D. J., and L. R. Krusberg. 1981. Diacyl, alkylacyl, and alkenylacyl phospholipids of *Meloidogyne javanica* females. Journal of Nematology. 13:105-111

Gibson, D. M., R. A. Moreau, G. P. McNeil, and B. B. Brodie. 1995. Lipid composition of cyst stages of *Globodera rostochiensis*. Journal of Nematology. 27(3):304-311

Graham, J. H., N. C. Hodge, and J. B. Morton. 1995. Fatty acid methyl ester profiles for characterization of glomalean fungi and their endomycorrhizae. Applied and Environmental Microbiology. 61(1):58-64

Hutzell, P. A., and L. R. Krusberg. 1982. Fatty acid compositions of *Caenorhabditis elegans* and *C. briggsae*. Comparative Biochemistry and Physiology. 73B:517-520

Krusberg, L. R. 1967. Analyses of total lipids and fatty acids of plant-parasitic nematodes and host tissues. Comparative Biochemistry and Physiology. 21:83-90

Krusberg, L. R., R. S. Hussey, and C. L. Fletcher. 1973. Lipid and fatty acid compositions of females and eggs of *Meloidogyne incognita* and *M. arenaria*. Comparative Biochemistry and Physiology. 45B:335-341

Kunitsky, C., G. Osterhout, and M. Sasser. 2005. Identification of microorganisms using fatty acid methyl ester (FAME) analysis and the MIDI Sherlock Microbial Identification System. Encyclopedia of Rapid Microbiological Methods, MIDI, Inc., Newark, DE

Madan, R., C. Pankhurst, B. Hawke, and S. Smith. 2002. Use of fatty acids for identification of AM fungi and estimation of the biomass of AM spores in soil. Soil Biology and Biochemistry. 34:125-128

Ruess, L., M. M. Häggblom, E. J. García Zapata, J. Dighton. 2002. Fatty acids of fungi and nematodes-possible biomarkers in the soil food chain? Soil Biology and Biochemistry. 34:745-756.

Sasser M. J. 1990. Identification of bacteria by gas chromatography of cellular fatty acids. *Technical Note 101*. Microbial ID, Inc., Newark, Del.

Sekora, N. S., K. S. Lawrence, E. van Santen, J. A. McInroy. 2008. A Step-Wise Dilution Scheme to Determine the Number of Nematodes Required for Accurate FAME Identification. Southeastern Biology 55(3):243.

Sekora, N. S., K. K. Lawrence, E. van Santen, J. A. McInroy. 2008a. Fingerprinting nematode fatty acid compositions as a means for identification. Proceedings of the National Beltwide

Cotton Conference, Vol. 1:235-244. National Cotton Council, Memphis TN. Online: www.cotton.org/beltwide/proceedings.

Sekora N. S., K. K. Lawrence, E. van Santen, J. A. McInroy. 2008b. Host influence on the fatty acid profiles of selected plant-parasitic nematodes. Phytopathology 98:S143

Sekora, N. S., K. S. Lawrence, E. van Santen, J. A. McInroy. 2009a. Delineating mixed populations of *Rotylenchulus reniformis* and *Meloidogyne incognita* with FAME analysis. Proceedings of the National Beltwide Cotton Conference, (In Press). National Cotton Council, Memphis TN. Online: www.cotton.org/beltwide/proceedings.

Sekora N. S., K. S. Lawrence, P. Agudelo, E. van Santen, J. A. McInroy. 2009b. FAME analysis as an alternative means for distinguishing *Meloidogyne* species and races. Phytopathology (In Press).

Sekora, N. S., K. S. Lawrence, E. van Santen, J. A. McInroy. 2009c. Identifying selected nematode species based on fatty acid profiles using FAME analysis. Southeastern Biology (In Press).

Stahl, P. D. and M. J. Klug. 1996. Characterization and differentiation of filamentous fungi based on fatty acid composition. Applied and Environmental Microbiology. 62(11):4136-4146

Taylor, A. L. and Sasser, J. N. 1978. Biology, identifiaction and control of root-knot nematodes (*Meloidogyne* species). Department of Plant Pathology, North Carolina State University and the United Sates Agency for International Development. Raleigh, NC

Tighe, S. W., P. de Lajudie, K. Dipietro, K. Lindström, G. Nick, and B. D. W. Jarvis. 2000. International Journal of Systematic and Evolutionary Microbiology. 50(2):787-801

Zinovieva S. V., O. L. Ozeretskovskaya, L. I. Iliinskaya, N. I. Vasyukova, and Z. V. Udalova. 1995. Biogenic elicitor (arachidonic acid) induced resistance in tomato to *Meloidogyne incognita*. Russian Journal of Nematology. 3(1): 68-69.

Table 1. Fatty acid profiles for twelve nematode species. Each species is based on the average expression of fatty acids among all races, life stages, or hosts analyzed. Means are listed in order by fatty acid chain length, location of the double bond, and functional group.

Fatty Acid	AD	AP	BR	DT	MA	MH	MI	MJ	PT	RD	RR	SCN
10:0	--†	--	--	--	0.70	0.16	0.20	0.38	0.77	--	1.76	--
10:0 2OH	1.71	--	--	--	0.03	--	1.27	--	0.11	--	0.06	--
12:0	--	0.29	--	--	--	--	--	--	1.48	--	0.11	0.01
12:0 2OH	--	--	--	--	3.28	1.37	6.51	2.05	0.91	--	15.67	0.25
13:0	--	0.31	--	--	--	--	--	--	1.02	--	0.02	--
14:0	--	0.60	--	--	0.52	0.90	0.20	0.48	2.77	--	0.32	2.95
14:0 2OH	--	0.20	--	--	0.19	0.03	0.06	0.18	0.14	--	1.09	--
15:0 ANTEISO	--	--	--	--	--	--	0.24	--	--	--	0.19	0.54
15:0 ISO	--	--	--	--	0.98	1.27	0.41	0.91	--	--	0.32	1.58
15:1 ANTEISO A	--	--	--	--	0.19	--	--	0.05	--	--	0.07	--
16:0	34.94	42.15	50.26	40.18	7.39	7.19	15.84	8.69	36.85	--	16.21	7.52
16:0 2OH	--	0.20	--	--	--	--	0.15	--	0.28	29.24	0.01	--
16:0 3OH	--	--	--	--	--	--	0.20	--	--	--	--	--
16:0 ANTEISO	--	--	--	--	--	--	--	--	--	--	0.18	--
16:1 CIS 9	--	--	--	--	--	--	--	--	--	--	0.14	--
16:1 ω5c	--	--	--	--	0.68	0.55	8.99	4.58	--	--	0.41	5.01
16:1 ω7c	--	--	--	--	1.85	1.36	0.93	1.32	--	--	--	3.96
17:0	--	0.41	--	--	--	--	0.04	--	1.02	--	0.03	1.28
17:0 ANTEISO	--	--	--	--	--	--	0.60	--	--	--	1.32	--
17:0 ISO	--	--	--	--	0.79	0.82	0.29	0.90	--	--	0.15	0.44
17:1 ISO I/ANTEI B	--	--	--	--	--	--	--	--	--	--	0.37	2.37
18:0	26.24	19.94	27.68	12.82	13.34	12.96	20.18	11.49	12.19	--	14.32	3.15
18:0 3OH	0.38	0.75	--	1.64	0.03	0.25	0.20	0.26	--	3.72	0.07	1.04
18:0 ANTE/18:2 ω6,9c	0.99	6.99	2.34	--	0.09	--	0.44	--	--	10.91	0.19	1.94
18:0 ANTEISO/18:2c	--	--	--	--	--	--	--	--	--	--	0.28	--
18:1 CIS 11/t 9/t 6	--	--	--	--	--	--	--	--	--	--	12.46	--
18:1 CIS 9	--	--	--	--	--	--	--	--	--	--	0.86	--
18:1 TRANS 9/t6/c11	--	--	--	--	--	--	--	--	--	--	10.00	--
18:1 ω5c	0.73	--	--	--	1.88	1.54	0.54	1.72	--	--	--	1.91
18:1 ω7c	--	--	--	30.55	57.45	58.89	38.21	54.50	19.09	--	4.58	42.16
18:1 ω9c	20.50	13.21	11.94	3.22	1.61	2.07	2.03	2.07	0.01	--	0.09	3.70
18:2 CIS 9,12/18:0a	--	--	--	--	--	--	--	--	--	--	0.19	--
18:2 ω6,9c/18:0 ANTE	6.14	6.87	2.99	--	1.85	2.55	1.38	1.97	--	46.24	0.41	5.63
18:3 CIS 6,12,14	--	--	--	--	--	--	--	--	--	0.14	0.02	--
18:3 ω6c (6,9,12)	0.64	--	4.05	--	0.35	0.63	0.25	0.50	4.52	--	0.68	1.05
19:0	--	0.47	--	--	--	--	0.18	--	2.26	0.80	--	--
19:0 CYCLO ω8c	--	--	--	--	--	--	0.33	--	--	--	0.01	--
19:0 ANTEISO	--	--	--	--	--	--	--	--	0.18	--	2.00	--
19:1 ISO I	1.08	--	0.73	5.44	1.63	1.44	0.70	1.54	0.06	1.66	0.29	1.18
20:0	3.15	2.18	--	1.67	1.56	1.67	1.34	1.41	9.25	--	3.33	0.94
20:0 ISO	0.39	0.36	--	0.33	0.06	0.34	0.02	0.40	0.44	--	0.03	0.67
20:1 TRANS 11	--	--	--	--	--	--	--	--	--	0.13	2.21	--
20:1 ω7c	1.23	0.41	--	0.58	3.45	3.70	2.81	4.15	4.81	0.82	0.36	3.98
20:1 ω9c	0.39	--	--	0.75	--	--	--	0.01	0.33	--	--	0.02
20:2 ω6,9c	0.10	1.27	--	1.00	--	0.13	0.04	0.19	--	--	0.72	2.21
20:4 ω6,9,12,15c	--	--	--	--	0.01	0.19	1.57	0.24	0.08	5.20	--	9.77
TBSA 10Me18:0	--	--	--	--	--	--	--	--	--	--	0.46	0.03
unknown 10:928	--	--	--	--	--	--	--	--	--	--	0.39	--
unknown 15:549	--	--	--	--	--	--	--	--	--	--	1.30	--
unknown 16:582	--	--	--	--	--	--	0.42	--	--	--	0.48	--
unknown 18:814	0.24	--	--	1.82	--	--	8.31	--	--	--	5.97	--

AD: *Aphelenchoides fragariae*, *AP: Aphelenchus avenae*, BR: *Bursaphelenchus xylophilus*, DT: *Ditylenchus dipsaci*, MA: *Meloidogyne arenaria*, MH: *M. hapla*, MI: *M. incognita*, MJ: *M. javanica*, PT: *Pratylenchus penetrans*, RD: *Radopholus similis*, RR: *Rotylenchulus reniformis*, and SCN: *Heterodera glycines*
† = Not Detected

Table 2. Squared Mahalanobis distances (D^2) and class distance probability of similarity (P) for canonical discriminant analysis of twelve nematode species.

	AD		AP		BR		DT		MA		MH	
	D^2	P	D^2	P	D^2	P	D^2	P	D^2	P	D^2	P
AD			26.4	<.0001	14.4	<.0001	36.0	<.0001	74.9	<.0001	70.0	<.0001
AP	26.4	<.0001			12.7	<.0001	33.7	<.0001	84.5	<.0001	77.1	<.0001
BR	14.4	<.0001	12.7	<.0001			19.0	<.0001	66.3	<.0001	61.4	<.0001
DT	36.0	<.0001	33.7	<.0001	19.0	<.0001			45.3	<.0001	40.1	<.0001
MA	74.9	<.0001	84.5	<.0001	66.3	<.0001	45.3	<.0001			3.9	0.492
MH	70.0	<.0001	77.1	<.0001	61.4	<.0001	40.1	<.0001	3.9	0.492		
MI	52.7	<.0001	56.9	<.0001	41.4	<.0001	25.5	<.0001	15.3	<.0001	14.0	<.0001
MJ	75.5	<.0001	82.9	<.0001	66.3	<.0001	44.4	<.0001	3.8	0.4962	2.3	0.9493
PT	46.5	<.0001	42.4	<.0001	31.8	<.0001	26.6	<.0001	52.3	<.0001	47.1	<.0001
RD	50.1	<.0001	45.1	<.0001	30.1	<.0001	15.9	<.0001	31.1	<.0001	26.9	<.0001
RR	77.1	<.0001	78.5	<.0001	67.8	<.0001	52.0	<.0001	41.3	<.0001	40.7	<.0001
SCN	459.4	<.0001	445.7	<.0001	446.2	<.0001	435.1	<.0001	394.9	<.0001	380.8	<.0001

	MI		MJ		PT		RD		RR		SCN	
	D^2	P	D^2	P	D^2	P	D^2	P	D^2	P	D^2	P
AD	52.7	<.0001	75.5	<.0001	46.5	<.0001	50.1	<.0001	77.1	<.0001	459.4	<.0001
AP	56.9	<.0001	82.9	<.0001	42.4	<.0001	45.1	<.0001	78.5	<.0001	445.7	<.0001
BR	41.4	<.0001	66.3	<.0001	31.8	<.0001	30.1	<.0001	67.8	<.0001	446.2	<.0001
DT	25.5	<.0001	44.4	<.0001	26.6	<.0001	15.9	<.0001	52.0	<.0001	435.1	<.0001
MA	15.3	<.0001	3.8	0.4962	52.3	<.0001	31.1	<.0001	41.3	<.0001	394.9	<.0001
MH	14.0	<.0001	2.3	0.9493	47.1	<.0001	26.9	<.0001	40.7	<.0001	380.8	<.0001
MI			15.5	<.0001	31.4	<.0001	12.4	<.0001	22.9	<.0001	404.4	<.0001
MJ	15.5	<.0001			50.1	<.0001	30.0	<.0001	43.1	<.0001	385.4	<.0001
PT	31.4	<.0001	50.1	<.0001			21.7	<.0001	47.6	<.0001	438.2	<.0001
RD	12.4	<.0001	30.0	<.0001	21.7	<.0001			39.4	<.0001	420.9	<.0001
RR	22.9	<.0001	43.1	<.0001	47.6	<.0001	39.4	<.0001			437.3	<.0001
SCN	404.4	<.0001	385.4	<.0001	438.2	<.0001	420.9	<.0001	437.3	<.0001		

AD: *Aphelenchoides fragariae*, *AP: Aphelenchus avenae*, BR: *Bursaphelenchus xylophilus*, DT: *Ditylenchus dipsaci*, MA: *Meloidogyne arenaria*, MH: *M. hapla*, MI: *M. incognita*, MJ: *M. javanica*, PT: *Pratylenchus penetrans*, RD: *Radopholus similis*, RR: *Rotylenchulus reniformis*, and SCN: *Heterodera glycines*

Table 3. Phenotypic canonical correlation of fatty acids for the three canonical discriminant functions of FAME profile analysis for 12 nematode species. Eigenvalue, cumulative percent of total variance, and canonical correlation are listed for each canonical function. Fatty acids listed were determined significant by the STEPDISC procedure. Correlation values are determined to be significant if $r \geq |0.75|$ (**bold**).

Response variable		Discriminant variate		
No.	Fatty acid variable	CAN 1	CAN 2	CAN 3
1	10:0	-0.297	**-0.810**	0.429
2	10:0 2OH	-0.119	0.387	0.167
3	12:0	-0.079	0.210	0.225
4	12:0 2OH	-0.381	**-0.866**	0.212
5	14:0	0.556	0.026	0.103
6	15:0 ISO	0.503	-0.315	-0.586
7	15:1 ANTEISO A	-0.093	-0.211	-0.256
8	16:0	-0.368	0.688	0.557
9	16:0 3OH	-0.182	0.061	-0.544
10	16:1 CIS 9	-0.251	**-0.804**	0.515
11	16:1 ω5c	0.274	0.080	-0.705
12	16:1 ω7c	-0.160	0.114	-0.564
13	16:1 ω7c/15 iso 2OH	**0.805**	0.029	-0.510
14	17:0	0.132	0.299	0.270
15	17:0 ISO	0.333	-0.180	-0.637
16	17:1 ISO I/ANTEIB	0.036	0.089	-0.028
17	18:0	-0.570	0.377	-0.149
18	18:0 3OH	0.662	0.290	0.178
19	18:0 ANTE/18:2 ω6,9c	0.006	0.523	0.476
20	18:1 ω5c	0.727	0.098	-0.457
21	18:1 ω7c	0.418	0.180	**-0.868**
22	18:1 ω9c	0.062	0.736	0.415
23	18:2 ω6,9c/18:0 ANTE	0.637	0.532	0.211
24	18:3 ω6c (6,9,12)	0.028	0.283	0.326
25	19:0	-0.069	0.268	0.178
26	19:0 CYCLO ω8c	-0.171	0.088	-0.554
27	19:1 ISO I	0.144	0.308	-0.219
28	20:0	-0.233	-0.230	0.429
29	20:0 ISO	0.320	0.505	0.103
30	20:1 TRANS 11	-0.251	**-0.804**	0.515
31	20:1 ω7c	0.381	0.294	-0.705
32	20:1 ω9c	-0.063	0.450	0.222
33	20:4 ω6,9,12,15c	**0.997**	-0.036	0.041
34	TBSA 10Me18:0	-0.246	**-0.805**	0.516
	Eigenvalue	27.46	6.39	3.63
	Cumulative %	68.0	83.9	92.9
	Canonical Correlation	0.98	0.93	0.89

Table 4. Fatty acid profiles for three *Meloidogyne incognita* races, three host plants for *M. incognita* race 3 and *Rotylenchulus reniformis*, and three life stages of *Heterodera glycines*. Means are listed in order by fatty acid chain length, location of the double bond, and functional group.

Fatty Acid	MIR1	MIR2	MIR3$_C$	MIR3$_S$	MIR3$_T$	RR$_C$	RR$_S$	RR$_T$	SCNC	SCNF	SCNV
10:0	0.05	0.08	0.13	0.52	--	1.38	3.72	0.17	--	--	--
10:0 2OH	--†	--	--	1.27	--	0.01	0.10	--	--	--	--
12:0	--	--	--	--	--	--	--	0.11	0.01	--	--
12:0 2OH	2.14	3.55	11.01	8.01	7.86	13.00	20.90	13.12	0.16	--	0.34
13:0	--	--	--	--	--	--	0.02	--	--	--	--
14:0	0.33	--	0.02	0.32	0.14	0.46	0.29	0.20	0.90	7.15	0.81
14:0 2OH	--	--	--	--	0.06	0.34	2.54	0.38	--	--	--
15:0	--	--	--	--	--	0.03	0.74	0.08	--	--	--
15:0 ANTEISO	--	--	--	0.24	--	0.21	0.01	0.36	--	0.54	--
15:0 ISO	0.60	0.38	0.08	0.54	0.46	0.41	0.04	0.50	0.54	3.70	0.49
15:1 ANTEISO A	--	--	--	--	--	--	0.07	--	--	--	--
16:0	7.37	8.74	11.25	33.40	18.46	13.21	19.56	15.87	2.77	16.15	3.64
16:0 2OH	--	--	--	--	0.15	--	0.01	--	--	--	--
16:0 3OH	--	--	--	0.61	--	0.01	--	--	--	--	--
16:0 ANTEISO	--	--	--	--	--	0.41	0.02	0.11	--	--	--
16:1 C	--	--	--	--	--	0.46	0.65	0.04	--	--	--
16:1 CIS 9	--	--	--	--	--	0.36	0.01	0.07	--	--	--
16:1 ω5c	0.44	0.10	--	26.43	--	--	0.41	--	1.48	11.59	1.95
16:1 ω7c	1.18	2.84	0.05	0.48	0.11	--	--	--	1.53	8.87	1.48
17:0	0.04	--	--	--	--	--	0.03	--	--	1.28	--
17:0 ANTEISO	--	--	--	0.12	1.08	2.87	0.23	0.85	--	--	--
17:0 ISO	0.24	--	0.02	0.84	0.08	0.23	0.07	0.16	0.46	0.42	0.45
17:1 ISO I/ANTEI B	--	--	--	--	--	--	--	0.37	--	2.37	--
18:0	14.38	15.09	23.69	7.48	40.25	12.64	15.89	14.42	1.71	4.86	2.89
18:0 3OH	0.05	--	0.01	0.55	--	0.05	0.04	0.13	1.12	0.74	1.26
18:0 ANTE/18:2 ω6,9c	0.33	0.55	--	--	--	0.34	--	0.04	2.16	1.72	--
18:0 ANTEISO/18:2c	--	--	--	--	--	0.19	0.41	0.25	--	--	--
18:1 B	--	--	--	--	--	0.35	0.03	0.13	--	--	--
18:1 CIS 11/t 9/t 6	--	--	--	--	--	14.47	6.24	16.66	--	--	--
18:1 CIS 9	--	--	--	--	--	0.90	0.93	0.73	--	--	--
18:1 TRANS 9/t6/c11	--	--	--	--	--	9.45	12.13	8.43	--	--	--
18:1 ω5c	1.65	0.91	0.05	0.03	0.06	--	--	--	2.31	1.20	2.22
18:1 ω7c	58.94	57.08	46.91	8.34	19.81	1.06	0.98	11.69	60.14	11.64	54.70
18:1 ω9c	2.23	2.18	0.49	5.10	0.14	0.06	0.03	0.17	2.52	5.19	3.39
18:2 CIS 9,12/18:0a	--	--	--	--	--	0.13	0.03	0.40	--	--	--
18:2 ω6,9c/18:0 ANTE	2.60	1.99	1.39	0.81	0.11	0.62	--	0.21	6.01	4.35	6.53
18:3 CIS 6,12,14	--	--	--	--	--	0.02	0.02	0.02	--	--	--
18:3 ω6c (6,9,12)	--	0.21	0.22	0.29	0.26	0.17	1.34	0.53	1.04	--	1.06
19:0	--	--	--	0.18	--	--	--	--	--	--	--
19:0 CYCLO ω8c	--	--	--	1.00	--	--	0.02	--	--	--	--
19:0 ANTEISO	--	--	--	--	--	2.00	--	--	--	--	--
19:1 ISO I	1.35	0.95	0.21	0.07	0.91	0.54	0.11	0.23	0.98	--	1.37
20:0	2.02	1.86	0.98	0.06	1.75	1.66	4.65	3.68	0.52	--	1.37
20:0 ISO	0.02	--	--	--	--	0.03	--	--	0.10	1.87	0.02
20:1 TRANS 11	--	--	--	--	--	2.07	2.26	2.29	--	--	--
20:1 ω7c	3.94	3.50	3.48	0.12	3.00	0.07	0.09	0.91	3.14	--	4.81
20:1 ω9c	--	--	--	--	--	--	--	--	--	--	0.02
20:2 ω6,9c	0.06	--	--	0.03	--	1.38	--	0.06	0.69	4.95	0.98
20:4 CIS 5,8,11,14	--	--	--	--	--	0.18	0.15	0.21	--	--	--
20:4 ω6,9,12,15c	--	--	--	1.57	--	--	--	--	9.63	9.46	10.22
TBSA 10Me18:0	--	--	--	--	--	0.73	--	0.18	0.03	--	--
unknown 10:928	--	--	--	--	--	0.14	0.80	0.24	--	--	--
unknown 15:549	--	--	--	--	--	1.26	1.73	0.93	--	--	--
unknown 16:582	--	--	--	--	0.42	0.60	--	0.37	--	--	--
unknown 18:814	--	--	--	--	8.31	13.18	0.45	4.29	--	--	--
unknown 19:735	--	--	--	--	--	--	0.06	0.05	--	--	--

MIR1: *M. incognita* race 1, MIR2: *M. incognita* race 2, MIR3$_C$: *M. incognita* race 3 increased on cotton, MIR3$_S$: *M. incognita* race 3 increased on soybean, MIR3$_T$: *M. incognita* race 3 increased on tomato, RR$_C$: *R. reniformis* increased on cotton, RR$_S$: *R. reniformis* increased on soybean, RR$_T$: *R. reniformis* increased on tomato, SCNC: *H. glycines* cyst life stage, SCNF: *H. glycines* female life stage, and SCNV: *H. glycines* juvenile life stage

†= Not Detected

Table 5a. Squared Mahalanobis distances (D^2) and class distance probability of similarity (*P*) for canonical discriminant analysis of twelve nematode species, including three life stages of *Heterodera glycines*, three host plants for *Rotylenchulus reniformis* and *Meloidogyne incognita*, and three races of *M. incognita*.

	AD		AP		BR		DT		MA		MH	
	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*
AD			27.2	<.0001	14.8	<.0001	38.6	<.0001	105.1	<.0001	89.8	<.0001
AP	27.2	<.0001			13.3	<.0001	36.1	<.0001	113.7	<.0001	95.7	<.0001
BR	14.8	<.0001	13.3	<.0001			22.1	<.0001	97.5	<.0001	82.1	<.0001
DT	38.6	<.0001	36.1	<.0001	22.1	<.0001			68.1	<.0001	53.8	<.0001
MA	105.1	<.0001	113.7	<.0001	97.5	<.0001	68.1	<.0001			6.6	0.157
MH	89.8	<.0001	95.7	<.0001	82.1	<.0001	53.8	<.0001	6.6	0.157		
MIR1	77.7	<.0001	84.6	<.0001	71.7	<.0001	43.1	<.0001	9.7	0.001	8.6	0.005
MIR2	140.9	<.0001	143.3	<.0001	130.1	<.0001	100.7	<.0001	31.3	<.0001	50.5	<.0001
MIR3C	68.4	<.0001	69.4	<.0001	55.9	<.0001	30.4	<.0001	39.1	<.0001	27.9	<.0001
MIR3S	250.5	<.0001	249.2	<.0001	235.2	<.0001	233.3	<.0001	286.7	<.0001	275.2	<.0001
MIR3T	51.7	<.0001	58.0	<.0001	40.4	<.0001	30.8	<.0001	47.6	<.0001	34.5	<.0001
MJ	90.7	<.0001	97.1	<.0001	82.4	<.0001	54.7	<.0001	9.8	0.001	6.2	0.173
PT	49.4	<.0001	46.1	<.0001	35.1	<.0001	27.4	<.0001	75.9	<.0001	62.1	<.0001
RD	56.5	<.0001	51.7	<.0001	37.3	<.0001	16.9	<.0001	51.1	<.0001	38.8	<.0001
RRC	104.6	<.0001	103.9	<.0001	93.2	<.0001	76.8	<.0001	85.9	<.0001	75.2	<.0001
RRS	110.6	<.0001	111.6	<.0001	99.6	<.0001	86.1	<.0001	93.7	<.0001	87.4	<.0001
RRT	83.0	<.0001	84.4	<.0001	72.9	<.0001	54.9	<.0001	66.1	<.0001	55.6	<.0001
SCNC	463.8	<.0001	446.0	<.0001	451.2	<.0001	425.3	<.0001	377.9	<.0001	365.8	<.0001
SCNF	1139.0	<.0001	1097.0	<.0001	1112.0	<.0001	1118.0	<.0001	910.5	<.0001	935.3	<.0001
SCNV	489.8	<.0001	480.9	<.0001	481.1	<.0001	457.4	<.0001	413.6	<.0001	400.8	<.0001

AD: *Aphelenchoides fragariae*, AP: *Aphelenchus avenae*, BR: *Bursaphelenchus xylophilus*, DT: *Ditylenchus dipsaci*, MA: *Meloidogyne arenaria*, MH: *M. hapla*, MIR1: *M. incognita* race 1, MIR2: *M. incognita* race 2, MIR3C: *M. incognita* race 3 increased on cotton, MIR3S: *M. incognita* race 3 increased on soybean, MIR3T: *M. incognita* race 3 increased on tomato, MJ: *M. javanica*, PT: *Pratylenchus penetrans*, RD: *Radopholus similis*, RRC: *Rotylenchulus reniformis* increased on cotton, RRS: *Rotylenchulus reniformis* increased on soybean, RRT: *Rotylenchulus reniformis* increased on tomato, SCNC: *Heterodera glycines* cyst life stage, SCNF: *Heterodera glycines* female life stage, and SCNV: *Heterodera glycines* juvenile life stage

Table 5b. Squared Mahalanobis distances (D^2) and class distance probability of similarity (*P*) for canonical discriminant analysis of twelve nematode species, including three life stages of *Heterodera glycines*, three host plants for *Rotylenchulus reniformis* and *Meloidogyne incognita*, and three races of *M. incognita*.

	MIR1		MIR2		MIR3$_C$		MIR3$_S$		MIR3$_T$		MJ	
	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*
AD	77.7	<.0001	140.9	<.0001	68.4	<.0001	250.5	<.0001	51.7	<.0001	90.7	<.0001
AP	84.6	<.0001	143.3	<.0001	69.4	<.0001	249.2	<.0001	58.0	<.0001	97.1	<.0001
BR	71.7	<.0001	130.1	<.0001	55.9	<.0001	235.2	<.0001	40.4	<.0001	82.4	<.0001
DT	43.1	<.0001	100.7	<.0001	30.4	<.0001	233.3	<.0001	30.8	<.0001	54.7	<.0001
MA	9.7	0.001	31.3	<.0001	39.1	<.0001	286.7	<.0001	47.6	<.0001	9.8	0.001
MH	8.6	0.005	50.5	<.0001	27.9	<.0001	275.2	<.0001	34.5	<.0001	6.2	0.173
MIR1			33.4	<.0001	17.3	<.0001	265.0	<.0001	27.7	<.0001	12.5	<.0001
MIR2	33.4	<.0001			73.1	<.0001	333.9	<.0001	85.8	<.0001	56.0	<.0001
MIR3$_C$	17.3	<.0001	73.1	<.0001			244.4	<.0001	9.3	<.0001	31.1	<.0001
MIR3$_S$	265.0	<.0001	333.9	<.0001	244.4	<.0001			238.1	<.0001	218.7	<.0001
MIR3$_T$	27.7	<.0001	85.8	<.0001	9.3	<.0001	238.1	<.0001			37.9	<.0001
MJ	12.5	<.0001	56.0	<.0001	31.1	<.0001	218.7	<.0001	37.9	<.0001		
PT	50.9	<.0001	107.0	<.0001	36.3	<.0001	247.0	<.0001	35.4	<.0001	62.3	<.0001
RD	25.9	<.0001	82.4	<.0001	13.1	<.0001	239.0	<.0001	21.4	<.0001	40.2	<.0001
RR$_C$	68.5	<.0001	126.7	<.0001	38.0	<.0001	265.5	<.0001	39.8	<.0001	77.3	<.0001
RR$_S$	79.0	<.0001	135.3	<.0001	48.3	<.0001	259.6	<.0001	51.9	<.0001	85.2	<.0001
RR$_T$	46.5	<.0001	103.2	<.0001	20.2	<.0001	252.2	<.0001	24.8	<.0001	57.4	<.0001
SCNC	374.5	<.0001	402.8	<.0001	403.2	<.0001	586.3	<.0001	422.3	<.0001	363.9	<.0001
SCNF	974.9	<.0001	819.7	<.0001	1100.0	<.0001	1177.0	<.0001	1084.0	<.0001	947.3	<.0001
SCNV	409.6	<.0001	438.8	<.0001	436.4	<.0001	607.9	<.0001	452.4	<.0001	396.6	<.0001

AD: *Aphelenchoides fragariae*, *AP: Aphelenchus avenae*, BR: *Bursaphelenchus xylophilus*, DT: *Ditylenchus dipsaci*, MA: *Meloidogyne arenaria*, MH: *M. hapla*, MIR1: *M. incognita* race 1, MIR2: *M. incognita* race 2, MIR3$_C$: *M. incognita* race 3 increased on cotton, MIR3$_S$: *M. incognita* race 3 increased on soybean, MIR3$_T$: *M. incognita* race 3 increased on tomato, MJ: *M. javanica*, PT: *Pratylenchus penetrans*, RD: *Radopholus similis*, RR$_C$: *Rotylenchulus reniformis* increased on cotton, RR$_S$: *Rotylenchulus reniformis* increased on soybean, RR$_T$: *Rotylenchulus reniformis* increased on tomato, SCNC: *Heterodera glycines* cyst life stage, SCNF: *Heterodera glycines* female life stage, and SCNV: *Heterodera glycines* juvenile life stage

Table 5c. Squared Mahalanobis distances (D^2) and class distance probability of similarity (*P*) for canonical discriminant analysis of twelve nematode species, including three life stages of *Heterodera glycines*, three host plants for *Rotylenchulus reniformis* and *Meloidogyne incognita*, and three races of *M. incognita*.

	PT		RD		$\mathrm{RR_C}$		$\mathrm{RR_S}$		$\mathrm{RR_T}$	
	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*	D^2	*P*
AD	49.4	<.0001	56.5	<.0001	104.6	<.0001	110.6	<.0001	83.0	<.0001
AP	46.1	<.0001	51.7	<.0001	103.9	<.0001	111.6	<.0001	84.4	<.0001
BR	35.1	<.0001	37.3	<.0001	93.2	<.0001	99.6	<.0001	72.9	<.0001
DT	27.4	<.0001	16.9	<.0001	76.8	<.0001	86.1	<.0001	54.9	<.0001
MA	75.9	<.0001	51.1	<.0001	85.9	<.0001	93.7	<.0001	66.1	<.0001
MH	62.1	<.0001	38.8	<.0001	75.2	<.0001	87.4	<.0001	55.6	<.0001
MIR1	50.9	<.0001	25.9	<.0001	68.5	<.0001	79.0	<.0001	46.5	<.0001
MIR2	107.0	<.0001	82.4	<.0001	126.7	<.0001	135.3	<.0001	103.2	<.0001
$\mathrm{MIR3_C}$	36.3	<.0001	13.1	<.0001	38.0	<.0001	48.3	<.0001	20.2	<.0001
$\mathrm{MIR3_S}$	247.0	<.0001	239.0	<.0001	265.5	<.0001	259.6	<.0001	252.2	<.0001
$\mathrm{MIR3_T}$	35.4	<.0001	21.4	<.0001	39.8	<.0001	51.9	<.0001	24.8	<.0001
MJ	62.3	<.0001	40.2	<.0001	77.3	<.0001	85.2	<.0001	57.4	<.0001
PT			23.1	<.0001	72.1	<.0001	75.9	<.0001	47.9	<.0001
RD	23.1	<.0001			63.3	<.0001	72.1	<.0001	40.5	<.0001
$\mathrm{RR_C}$	72.1	<.0001	63.3	<.0001			21.2	<.0001	14.6	<.0001
$\mathrm{RR_S}$	75.9	<.0001	72.1	<.0001	21.2	<.0001			20.5	<.0001
$\mathrm{RR_T}$	47.9	<.0001	40.5	<.0001	14.6	<.0001	20.5	<.0001		
SCNC	434.3	<.0001	408.7	<.0001	462.5	<.0001	473.6	<.0001	438.7	<.0001
SCNF	1122.0	<.0001	1115.0	<.0001	1119.0	<.0001	1139.0	<.0001	1116.0	<.0001
SCNV	463.3	<.0001	441.1	<.0001	493.9	<.0001	503.8	<.0001	469.8	<.0001

AD: *Aphelenchoides fragariae*, *AP: Aphelenchus avenae*, BR: *Bursaphelenchus xylophilus*, DT: *Ditylenchus dipsaci*, MA: *Meloidogyne arenaria*, MH: *M. hapla*, MIR1: *M. incognita* race 1, MIR2: *M. incognita* race 2, $\mathrm{MIR3_C}$: *M. incognita* race 3 increased on cotton, $\mathrm{MIR3_S}$: *M. incognita* race 3 increased on soybean, $\mathrm{MIR3_T}$: *M. incognita* race 3 increased on tomato, MJ: *M. javanica*, PT: *Pratylenchus penetrans*, RD: *Radopholus similis*, $\mathrm{RR_C}$: *Rotylenchulus reniformis* increased on cotton, $\mathrm{RR_S}$: *Rotylenchulus reniformis* increased on soybean, $\mathrm{RR_T}$: *Rotylenchulus reniformis* increased on tomato, SCNC: *Heterodera glycines* cyst life stage, SCNF: *Heterodera glycines* female life stage, and SCNV: *Heterodera glycines* juvenile life stage

Table 5d. Squared Mahalanobis distances (D^2) and class distance probability of similarity (*P*) for canonical discriminant analysis of twelve nematode species, including three life stages of *Heterodera glycines*, three host plants for *Rotylenchulus reniformis* and *Meloidogyne incognita*, and three races of *M. incognita*.

	SCNC		SCNF		SCNV	
	D^2	*P*	D^2	*P*	D^2	*P*
AD	463.8	<.0001	1139.0	<.0001	489.8	<.0001
AP	446.0	<.0001	1097.0	<.0001	480.9	<.0001
BR	451.2	<.0001	1112.0	<.0001	481.1	<.0001
DT	425.3	<.0001	1118.0	<.0001	457.4	<.0001
MA	377.9	<.0001	910.5	<.0001	413.6	<.0001
MH	365.8	<.0001	935.3	<.0001	400.8	<.0001
MIR1	374.5	<.0001	974.9	<.0001	409.6	<.0001
MIR2	402.8	<.0001	819.7	<.0001	438.8	<.0001
$MIR3_C$	403.2	<.0001	1100.0	<.0001	436.4	<.0001
$MIR3_S$	586.3	<.0001	1177.0	<.0001	607.9	<.0001
$MIR3_T$	422.3	<.0001	1084.0	<.0001	452.4	<.0001
MJ	363.9	<.0001	947.3	<.0001	396.6	<.0001
PT	434.3	<.0001	1122.0	<.0001	463.3	<.0001
RD	408.7	<.0001	1115.0	<.0001	441.1	<.0001
RR_C	462.5	<.0001	1119.0	<.0001	493.9	<.0001
RR_S	473.6	<.0001	1139.0	<.0001	503.8	<.0001
RR_T	438.7	<.0001	1116.0	<.0001	469.8	<.0001
SCNC			620.7	<.0001	3.1	1.000
SCNF	620.7	<.0001			630.9	<.0001
SCNV	3.1	1.000	630.9	<.0001		

AD: *Aphelenchoides fragariae*, *AP: Aphelenchus avenae*, BR: *Bursaphelenchus xylophilus*, DT: *Ditylenchus dipsaci*, MA: *Meloidogyne arenaria*, MH: *M. hapla*, MIR1: *M. incognita* race 1, MIR2: *M. incognita* race 2, $MIR3_C$: *M. incognita* race 3 increased on cotton, $MIR3_S$: *M. incognita* race 3 increased on soybean, $MIR3_T$: *M. incognita* race 3 increased on tomato, MJ: *M. javanica*, PT: *Pratylenchus penetrans*, RD: *Radopholus similis*, RR_C: *Rotylenchulus reniformis* increased on cotton, RR_S: *Rotylenchulus reniformis* increased on soybean, RR_T: *Rotylenchulus reniformis* increased on tomato, SCNC: *Heterodera glycines* cyst life stage, SCNF: *Heterodera glycines* female life stage, and SCNV: *Heterodera glycines* juvenile life stage

Table 6. Phenotypic canonical correlation of fatty acids for the three canonical discriminant functions of FAME profile analysis for 19 nematode variables. Eigenvalue, cumulative percent of total variance, and canonical correlation are listed for each canonical function. Fatty acids listed were determined significant by the STEPDISC procedure. Correlation values are determined to be significant if $r \geq |0.75|$ (**bold**).

Response variable		Discriminant variate		
No.	Fatty acid variable	CAN 1	CAN 2	CAN 3
1	10:0	0.251	-0.151	0.633
2	10:0 2OH	0.071	0.692	-0.013
3	12:0	0.084	0.042	-0.131
4	12:0 2OH	0.421	-0.215	0.778
5	13:0	0.075	0.071	-0.170
6	14:0	-0.451	-0.010	-0.082
7	14:0 2OH	0.229	-0.198	0.577
8	15:0	0.199	-0.173	0.550
9	15:0 ANTEISO	0.061	0.101	0.481
10	15:0 ISO	-0.442	-0.055	-0.121
11	15:1 ANTEISO A	0.062	-0.167	0.040
12	16:0	0.347	0.539	-0.239
13	16:0 3OH	0.016	**0.915**	0.219
14	16:0 ANTEISO	0.219	-0.241	0.540
15	16:1 C	0.264	-0.253	0.717
16	16:1 CIS 9	0.205	-0.227	0.511
17	16:1 ω5c	-0.146	**0.893**	0.167
18	16:1 ω7c	0.008	**0.920**	0.199
19	16:1 ω7c/15 iso 2OH	-0.646	-0.132	-0.341
20	17:0	-0.148	0.056	-0.223
21	17:0 ANTEISO	0.267	-0.237	0.518
22	17:0 ISO	-0.334	0.346	0.050
23	17:1 ISO I/ANTEIB	-0.057	0.012	-0.128
24	18:0	0.439	-0.109	-0.292
25	18:0 3OH	-0.624	0.295	-0.017
26	18:0 ANTE/18:2 ω6,9c	-0.011	0.180	-0.294
27	18:0 ANTEISO/18:2 c	0.305	-0.293	**0.766**
28	18:1 CIS 11/t 9/t 6	0.293	-0.305	0.676
29	18:1 TRANS 9/t6/c11	0.321	-0.316	**0.803**
30	18:1 ω5c	-0.716	-0.147	-0.273
31	18:1 ω7c	-0.434	-0.230	-0.478
32	18:1 ω9c	-0.081	0.400	-0.417
33	18:2 CIS 9,12/18:0a	0.216	-0.229	0.445
34	18:2 ω6,9c/18:0 ANTE	-0.630	0.105	-0.323
35	18:3 ω6c (6,9,12)	0.002	0.063	-0.067
36	19:0	0.068	0.145	-0.150
37	19:0 ANTEISO	0.173	-0.189	0.434
38	19:0 CYCLO ω8c	0.012	**0.918**	0.210
39	19:1 ISO I	-0.164	-0.059	-0.359
40	20:0	0.249	-0.251	0.211
41	20:0 ISO	-0.292	0.055	-0.401
42	20:1 ω7c	-0.376	-0.225	-0.484
43	20:1 ω9c	0.063	0.128	-0.293
44	20:4 ω6,9,12,15c	**-0.971**	0.071	0.190
45	unknown 10.928	0.248	-0.226	0.653
46	unknown 15.549	0.315	-0.308	**0.800**
	Eigenvalue	30.84	10.62	8.49
	Cumulative %	45.9	61.7	74.3
	Canonical Correlation	0.98	0.96	0.95

Table 7. Comparison matrix for 13 nematode fatty acid profiles generated using the Sherlock® Analysis Software. Comparisons are listed by column with numbers indicating the percentage of samples from each column matching to the indicated row.

	SCNV	SCNC	SCNF	MA	MH	MIR1	MIR2	$MIR3_C$	$MIR3_T$	MJ	RR_C	RR_S	RR_T
SCNV	100.0	.	.	.	.	.	.	.	.	.	.	.	.
SCNC	.	100.0	.	.	.	.	.	.	.	.	.	.	.
SCNF	.	.	100.0	.	.	.	.	.	.	.	.	.	.
MA	.	.	.	77.8	.	5.0	1.3	.	.	.	.	.	.
MH	.	.	.	16.7	94.4	24.4	6.4	.	.	.	.	.	.
MIR1	.	.	.	.	.	64.7	7.7	3.0	.	.	.	.	.
MIR2	.	.	.	.	.	5.9	76.9	.	.	.	.	.	.
$MIR3_C$	.	.	.	.	.	.	7.7	97.0	.	.	.	.	.
$MIR3_T$	.	.	.	.	.	.	.	.	100.0	.	.	.	.
MJ	.	.	.	.	.	.	.	.	.	100.0	.	.	.
RR_C	.	.	.	5.6	5.6	.	.	.	.	.	94.0	.	13.6
RR_S	.	.	.	.	.	.	.	.	.	.	2.0	95.0	.
RR_T	.	.	.	.	.	.	.	.	.	.	4.0	5.0	86.4

MA: *Meloidogyne arenaria*, MH: *M. hapla*, MIR1: *M. incognita* race 1, MIR2: *M. incognita* race 2, $MIR3_C$: *M. incognita* race 3 increased on cotton, $MIR3_T$: *M. incognita* race 3 increased on tomato, MJ: *M. javanica*, RR_C: *Rotylenchulus reniformis* increased on cotton, RR_S: *Rotylenchulus reniformis* increased on soybean, RR_T: *Rotylenchulus reniformis* increased on tomato, SCNC: *Heterodera glycines* cyst life stage, SCNF: *Heterodera glycines* female life stage, and SCNV: *Heterodera glycines* juvenile life stage

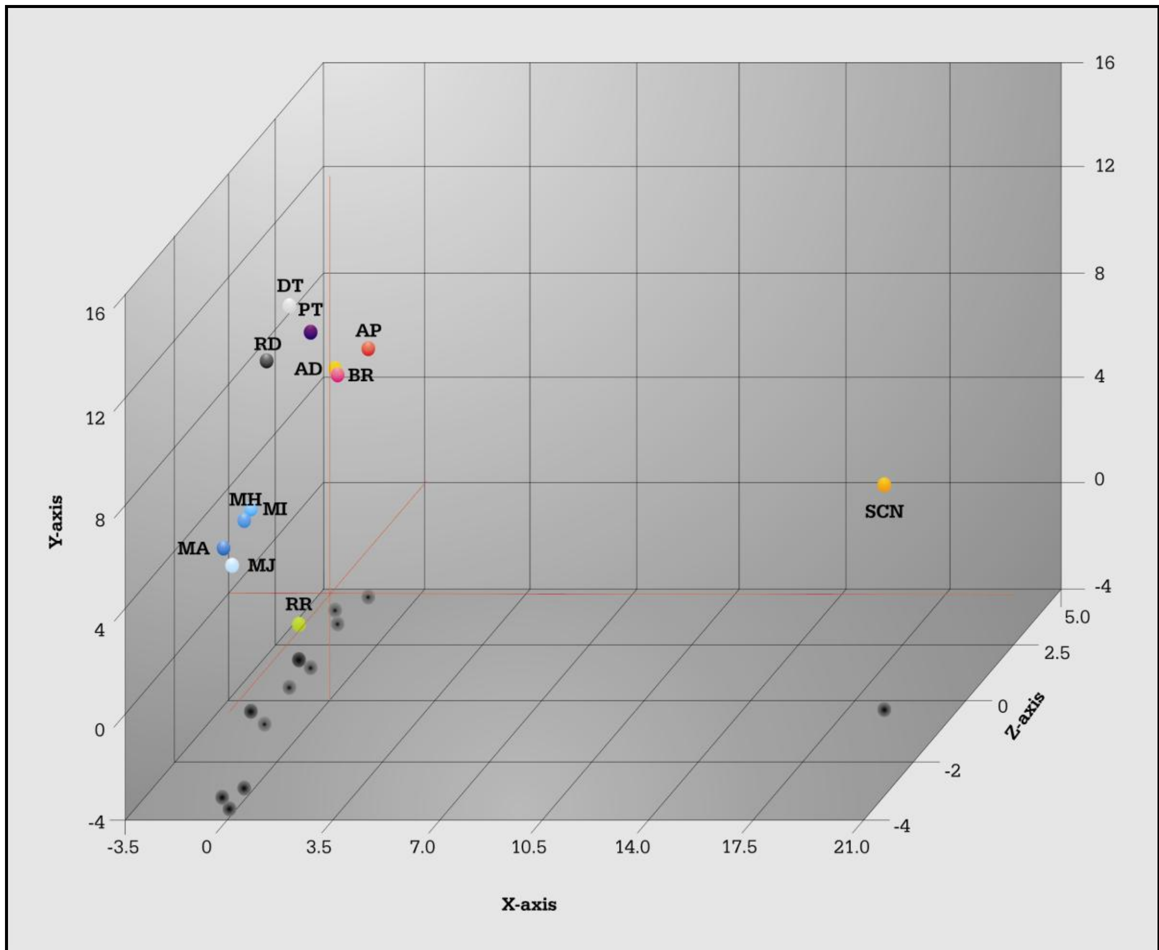

Figure 1. Graph of canonical means for twelve nematode species. Placement is defined for the first (x-axis), second (y-axis), and third (z-axis) canonical axes by height (y-axis) and shadow (x-axis and z-axis intercept).
AD: *Aphelenchoides fragariae*, *AP: Aphelenchus avenae*, BR: *Bursaphelenchus xylophilus*, DT: *Ditylenchus dipsaci*, MA: *Meloidogyne arenaria*, MH: *M. hapla*, MI: *M. incognita*, MJ: *M. javanica*, PT: *Pratylenchus penetrans*, RD: *Radopholus similis*, RR: *Rotylenchulus reniformis*, and SCN: *Heterodera glycines*

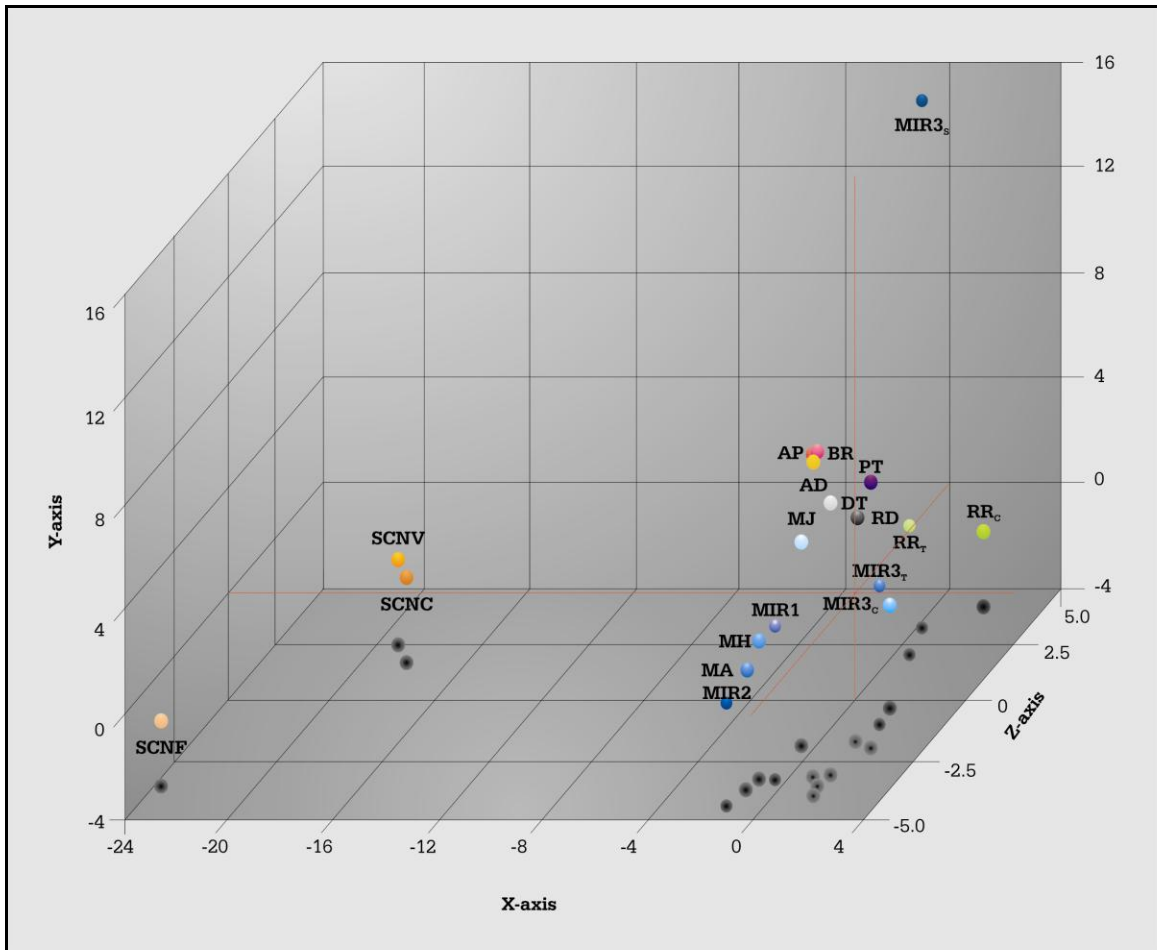

Figure 2. Graph of canonical means for twelve nematode species, including three life stages of *Heterodera glycines*, three host plants for *Rotylenchulus reniformis* and *Meloidogyne incognita*, and three races of *M. incognita*. Placement is defined for the first (x-axis), second (y-axis), and third (z-axis) canonical axes by height (y-axis) and shadow (x-axis and z-axis intercept).

AD: *Aphelenchoides fragariae*, *AP: Aphelenchus avenae*, BR: *Bursaphelenchus xylophilus*, DT: *Ditylenchus dipsaci*, MA: *Meloidogyne arenaria*, MH: *M. hapla*, MIR1: *M. incognita* race 1, MIR2: *M. incognita* race 2, $MIR3_C$: *M. incognita* race 3 increased on cotton, $MIR3_S$: *M. incognita* race 3 increased on soybean, $MIR3_T$: *M. incognita* race 3 increased on tomato, MJ: *M. javanica*, PT: *Pratylenchus penetrans*, RD: *Radopholus similis*, RR_C: *Rotylenchulus reniformis* increased on cotton, RR_S: *Rotylenchulus reniformis* increased on soybean, RR_T: *Rotylenchulus reniformis* increased on tomato, SCNC: *Heterodera glycines* cyst life stage, SCNF: *Heterodera glycines* female life stage, and SCNV: *Heterodera glycines* juvenile life stage

V. IDENTIFYING PLANT-PARASITIC NEMATODES IN SOIL SAMPLES BY FAME ANALYSIS

Abstract

Our objective was to develop a FAME profile of *Rotylenchulus reniformis* to detect the plant-parasitic nematode in soil samples. Forty pots of cotton were grown for sixty days under greenhouse conditions. Three 1.0 g-samples were taken from each pot of two groups, those that were grown in the absence of *R. reniformis*, and the other inoculated with 2000 *R. reniformis* individuals per 500 cm^3 for the growing period. Each of the 120 samples was extracted and analyzed using FAME gas chromatography. The resulting fatty acid profiles for all samples were analyzed using the STEPDISC and CANDISC procedures of SAS (SAS Institute, Inc). A total of sixty-four fatty acids were detected. Of these, six were found to be significant for differentiating between samples containing or lacking *R. reniformis*. The total Mahalanobis distance (D^2) between the soil samples with *R. reniformis* and without was 13.67 ($P \leq 0.0001$). Of these fatty acids, 12:0 2OH appeared to indicate the presence of *R. reniformis*. This fatty acid was found at a concentration of less than 0.04% in soil samples lacking *R. reniformis*. In samples containing *R. reniformis*, the mean sample percentage was 1.27%. Since this is a fatty acid found in the FAME profile of *R. reniformis*, it may be possible to use the presence of 12:0 2OH as an indicator of *R. reniformis* in soil samples.

Introduction

There has been a trend toward identification of organisms directly from soil samples using FAME analysis. Studies evaluating the identification and detection of bacteria (Kloepper et al., 1992; Kloepper et al., 1992), fungi (Bentivenga and Morton, 1996; Graham et al., 1995; Madan et al., 2002), and nematodes (Ruess et al., 2002) from soil have indicated that there may be some applications for FAME using whole-soil extractions. These studies focused on looking for "signal" fatty acids that were detectable when the organism of study was present, and absent in samples when the target organism was not present. Studies by Bentivenga and Morton (1996), Graham et al. (1995), Madan et al. (2002), and Ruess et al. (2002) examined fatty acids of fungi and nematodes extracted from soil in the same way comparisons of nematode species through FAME analysis were accomplished (Chapters II, III, and IV). Currently, there have been no studies to detect or identify plant-parasitic nematode species by directly analyzing overall fatty acid profile of soil samples using total soil fatty acid methyl ester (TSFAME) analysis.

Our previous research has developed a FAME profile for *Rotylenchulus reniformis* (Chapter IV) and has shown that this nematode can be detected and identified in populations greater than 100 individuals in pure culture (Sekora *et al.* 2008). By using the developed FAME profile, it should be possible to detect *R. reniformis* in soil samples generated under controlled conditions. Our objectives for this study were 1) use TSFAME profiles to analyze differences between soil containing *R. reniformis* and soil lacking *R. reniformis*, and 2)

determine if *R. reniformis* could be detected and identified using the previously generated FAME profile.

Materials and Methods

Rotylenchulus reniformis Population Establishment and Soil Treatment Setup

A population of *R. reniformis* (Linford and Oliveira) was created using populations collected from multiple field sites around Alabama. This stock population of nematodes was maintained at the Auburn University Plant Science Research Center greenhouses on cotton (*Gossypium hirsutum* L.) cv. 'Stoneville 5599 BGRR' in 500cm^3 polystyrene pots.

Forty polystyrene pots were filled with a 75:25 mixture of autoclaved field soil to autoclaved sand and planted with cotton cv. 'Stoneville 5599 BGRR.' Twenty of these pots were inoculated with 2000 total individuals of *R. reniformis* each. Cotton plants in all 40 pots were allowed to grow for 60 days under greenhouse conditions. Each soil treatment was physically separated by Plexiglas dividers 61 cm high by 91 cm deep to prevent cross-contamination of treatments.

Fatty Acid Extraction

A total of 120 1.0 gram samples were prepared for TSFAME extraction, 3 samples from each of the 40 pots. Fatty acids from samples were extracted using the method described by Sasser (1990) and tripling the volume of reagent used in each step. After the extraction procedure was completed, the organic solvent was transferred to sample vials and allowed to evaporate under a fume hood. Dried samples were reconstituted in 75 μL of the organic extraction solvent and

transferred to spring-vial inserts for each sample vial. Vials were sealed and stored at -20°C until analysis.

Samples were analyzed for fatty acid composition by a HP 5890 automated gas chromatography system (Agilent Technologies) equipped with an Ultra 2 Cross-linked 5% Phenyl Methyl Siloxane column; 2.0 µL of sample was injected into the column for each analysis. Sample data from the Sherlock® Sequencer Software (MIDI, Inc.) included total response of each sample (mV) and the response for each detected fatty acid. Fatty acid percentages were calculated from the proportion of each fatty acid within the sample; these percentages were used to create a fatty acid profile for each soil sample.

Statistical Analysis

Two comparisons among soil treatment fatty acid profiles were made in this study, 1) comparing the average fatty acid profile of each soil treatment and 2) comparing soil fatty acid profiles for each pot of a given soil treatment. The soil treatment comparison was made by analyzing all samples based on their "Soil_Type," with or without *R. reniformis*. Comparisons among pots were made by classifying individual samples based on their "Pot" designation and pooled all samples taken from a given pot.

The STEPDISC (SAS version 9.1.3; SAS Institute, Inc) procedure was used to analyze the percentage of each fatty acid across all samples within a given class to determine which fatty acids contributed significantly to the differentiation among classes (species or variable) based on the ANOVA test *F* value of a selected fatty acid (Johnson, 1998). The compiled list of fatty acids was used for class

differentiation with the CANDISC procedure. The CANDISC procedure provided canonical discriminant analysis (CDA) of the fatty acid profiles for each soil sample within its respective categorical class.

In an effort to determine if soil lacking *R. reniformis* could be used as a correction factor to reveal the *R. reniformis* FAME profile in TSFAME samples, the mean fatty acid percentage values for soil devoid of *R. reniformis* were subtracted from the TSFAME profile of each sample from pots inoculated with *R. reniformis*. Fatty acids that possessed a negative value in a sample after the subtraction were given a value of "0" for that sample. A correction value to counteract the dilution of the extracted FAME profile was developed by dividing the percentage of a fatty acid found in the soil samples into the reference percentage for that fatty acid in the developed FAME profile of *R. reniformi*s (Sekora *et al.*, 2009). Each fatty acid in the mean soil profile was then multiplied by the average correction factor (Equation 1). The corrected profiles were also compared to the reference FAME profile of *R. reniformis* using the STEPDISC and CANDISC procedures of SAS.

Equation 1. $$p = \frac{\%_i \times k}{\sum_{i=1}^{j=n}(\%_i \times k)} \times 100$$

Where: p = Adjusted fatty acid percentage, $\%_i$ = percent of fatty acid i in sample, k = correction factor, and n = total number of fatty acids observed.

Sherlock® Analysis Software

A library was developed using the Sherlock® Analysis Software (MIDI, Inc) by creating entries from fatty acid profiles of the 19 nematode species, races, life stages, and hosts in this study. To determine the usefulness and validity of the newly created library entries, individual samples were compared against their

respective composite profiles to create comparison and similarity matrices for each library entry. Identification reports were also used to evaluate identification accuracy using the "First choice" and "First Second choice" methods among samples.

Results

Objective 1: Comparison of Soil FAME Profiles

A total of 64 fatty acids were observed from the 120 samples analyzed. The most predominant fatty acid found in both soil treatments was 16:0. Soil not containing *R. reniformis* had a greater mean concentration of 16:0 (17.55%) than soil containing *R. reniformis* (15.38%). The second most common fatty acid in both soil treatments was 19:1 ω6c, which was also expressed at a greater concentration in soil lacking *R. reniformis* (15.46%) than in soil with *R. reniformis* present (12.78%). Three fatty acids (18:1 ω7c, 18:1 ω9c, and 19:0 cyclo ω10c) were found at greater percentages in soil with *R. reniformis* present (10.12%, 9.95%, and 9.13%) than noninoculated soil (1.29%, 8.19%, and 7.49%). The 18:1 ω7c fatty acid was the principal fatty acid found in *R. reniformis* (59.67%) as well as several other nematode species (Sekora et al., 2009; Sekora et al., 2009a). Other fatty acids present in *R. reniformis* that were found in higher quantities in inoculated soil were 12:0 2OH and 20:4 ω6,9,12,15c; these fatty acids were found at 1.27% and 0.97%, respectively, in inoculated soil and 0.03% and 0.66% in noninoculated soil.

Based on CDA, soil containing *R. reniformis* had a statistically different FAME profile than soil without *R. reniformis* (D^2=13.67, P < 0.0001). Analysis

by pot indicated that 18 of the 20 pots inoculated with R. reniformis were significantly different than noninoculated pots ($D^2 \geq 236.3$, $P \leq 0.0404$); the first three canonical dimensions explained 72.4% of the total multivariance (Table 2). Fifty-six of the sixty-four total fatty acids were determined to be significant for differentiation among pots by the STEPDISC procedure. Of these 56 fatty acids, six fatty acids (15:0 ANTEISO, 16:0 ISO, 17:0 ANTEISO, 17:0, 15:0 ISO, and 18:1 ω9c) were significant for differentiation along the first and second canonical dimensions. The five fatty acids responsible for separating the two soil treatments along the first canonical axis (15:0 ANTEISO, 16:0 ISO, 17:0 ANTEISO, 17:0, and 15:0 ISO) explained 47.1% of the total multivariance among pots (Figure 1). All five of these fatty acids were found at concentrations in noninoculated pots that were nearly twice those found in pots inoculated with *R. reniformis* (Table 1).

The second canonical dimension explained 15.27% of the total multivariance among pots. Within this dimension, 18:1 ω9c was responsible for further separating *R. reniformis* infested pots from the pots not containing *R. reniformis* (Figure 1). Although the third canonical dimension described 10.0% of the multivariance, no fatty acids were significant for differentiating pots along this dimension.

Objective 2: Detection of *Rotylenchulus reniformis* in Soil

By using the mean fatty acid profile from pots not containing *R. reniformis*, it was possible to extract a FAME profile similar to that of *R. reniformis* from pots inoculated with the nematode. Five fatty acids (14:0, 15:0

ISO, 16:0, 18:1 ω9c, and 20:0) were not found at percentages similar to those of the *R. reniformis* FAME profile in any pot sampled. Even though percentages of the remaining 11 fatty acids varied among pots, a few were expressed in the same patterns as in the *R. reniformis* profile. For example, 18:1 ω7c is the predominant fatty acid in 15 of the 20 pots. Of the 11 usable fatty acids in the reference profile, a mean of 8 fatty acids were expressed within one standard deviation of their respective means within each pot. These data indicate that it may be possible to extract the fatty acid profile of *R. reniformis* from soil samples analyzed using TSFAME analysis.

Discussion

Extracting TSFAMEs has been shown to be preferable to other methods such as phospholipid fatty acid (PLFA) analysis in situations where sample sizes are small, time is a limiting factor, or when comparing the overall soil fatty acid profile among treatments or sites (Drenovsky et al., 2004). White et al. (1979) stated that PLFA analysis indicates the presence of living microbes in soil communities, which could then be used to monitor changes in the soil community (Bossio et al., 1998; Calderon et al., 2001; Peacock et al., 2001). However, PLFA analysis required 16 times more soil (8.0 g) for extraction and evaluation than TSFAME analysis (0.5 g) in studies by Drenovsky et al. (2004). While TSFAME does not indicate the differences in soil communities as accurately as PLFA, differences over time can still be observed (Klug and Tiedje, 1993; Cavigelli et al., 1995; Buyer and Drinkwater, 1997; Ibekwe and Kennedy, 1999).

Even though the majority of fatty acids observed from the soil analysis were not significant for differentiation of the two soil treatments, those that were significant may be crucial to indicating differences in soil populations of other nematodes, bacteria, fungi, and other soil organisms based on the presence or absence of *R. reniformis*. Soil properties such as texture, color, and ecology are visibly different in soils lacking *R. reniformis* than those of soil containing the nematode. The differences observed in the fatty acid profiles of soil samples with and without *R. reniformis* may be consistent enough to indicate the presence of this nematode in the soil sample. Previous research has indicated that the relative mean concentrations of 12:0 2OH and 18:1 ω7c in samples containing 5000 individuals of *R. reniformis* are 1.42% and 59.67%, respectively (Sekora *et al.*, 2009). It may be possible to use 12:0 2OH and 18:1 ω7c as indicators of *R. reniformis* in soil samples analyzed by TSFAME analysis and perhaps indicate population numbers based on percentages.

By determining if fatty acids such as 12:0 2OH and 18:1 ω7c are indicative of *R. reniformis* and other plant-parasitic nematodes in soil communities, it should be possible to test or monitor field sites using TSFAME to detect, identify, and possibly even quantify populations of plant-parasitic nematodes in agricultural fields. TSFAME analysis would be helpful for diagnostic laboratories that could extract TSFAME profiles and identify plant-parasitic nematodes from soil samples without directly isolating nematodes from those samples and then extracting fatty acids from the isolated nematodes.

Literature Cited

Bentivenga, S. P., and J. B. Morton. 1996. Congruence of fatty acid methyl ester profiles and morphological characters of arbuscular mycorrhizal fungi in Gigasporaceae. Proceedings of the National Academy of Sciences USA 93:5659-5662.

Bossio, D.A., Scow, K.M., Gunapala, N., Graham, K.J., 1998. Determinants of soil microbial communities: effects of agricultural management, season, and soil type on phospholipid fatty acid profiles. Microbial Ecology 36:1–12.

Buyer, J.S., Drinkwater, L.E., 1997. Comparison of substrate utilization assay and fatty acid analysis of soil microbial communities. Journal of Microbiological Methods 30:3–11.

Calderon, F.J., Jackson, L.E., Scow, K.M., Rolston, D.E., 2001. Short-term dynamics of nitrogen, microbial activity, and phospholipid fatty acids after tillage. Soil Science Society of America Journal 65:118–126.

Drenovsky, R. R., G. N. Elliott, K. J. Graham, K. M. Scow. 2004. Comparison of phospholipid fatty acid (PLFA) and total soil fatty acid methyl esters (TSFAME) for characterizing soil microbial communities. Soil Biology and Biochemistry 36:1793-1800.

Graham, J. H., N. C. Hodge, and J. B. Morton. 1995. Fatty acid methyl ester profiles for characterization of glomalean fungi and their endomycorrhizae. Applied and Environmental Microbiology 61(1):58-64.

Ibekwe, A.M., Kennedy, A.C., 1999. Fatty acid methyl ester (FAME) profiles as a tool to investigate community structure of two agricultural soils. Plant and Soil 206:151–161.

Kloepper , J. W., R. Rodriguez-Kabana, J. A. McInroy, and R. W. Young. 1992. Rhizosphere bacteria antagonistic to soybean cyst (*Heterodera glycines*) and root-knot (*Meloidogyne incognita*) nematodes: Identification by fatty acid analysis and frequency of biological control activity. Plant and Soil 139(1):75-84.

Kloepper , J. W., J. A. McInroy, and K. L. Bowen. 1992. Comparative identification by fatty acid analysis of soil, rhizosphere, and geocarposphere bacteria of peanut (*Arachis hypogaea* L.). Plant and Soil 139(1):85-90.

Klug, M.J., Tiedje, J.M., 1993. Response of microbial communities to changing environmental conditions: chemical and physiological approaches, in: Guerrero, R., Pedros-Alio, C., *Trends in Microbial Ecology*. Spanish Society for Microbiology, Barcelona, pp. 371–374.

Madan, R., C. Pankhurst, B. Hawke, and S. Smith. 2002. Use of fatty acids for identification of AM fungi and estimation of the biomass of AM spores in soil. Soil Biology and Biochemistry 34:125-128.

Peacock, A.D., Mullen, M.D., Ringelberg, D.B., Tyler, D.D., Hedrick, D.B., Gale, P.M., White, D.C., 2001. Soil microbial community responses to dairy manure or ammonium nitrate applications. Soil Biology & Biochemistry 33:1011–1019.

Ruess, L., M. M. Häggblom, E. J. García Zapata, J. Dighton. 2002. Fatty acids of fungi and nematodes-possible biomarkers in the soil food chain? Soil Biology and Biochemistry 34:745-756.

Sasser, M. J. 1990. Identification of bacteria by gas chromatography of cellular fatty acids. *Technical note 101*. Microbial ID Inc., Newark, DE.

Sekora, N. S., K. S. Lawrence, E. van Santen, J. A. McInroy. 2008. A step-wise dilution scheme to determine the number of nematodes required for accurate FAME identification. Southeastern Biology 55:3 p. 243.

Sekora, N. S., K. K. Lawrence, E. van Santen, J. A. McInroy. 2008a. Fingerprinting nematode fatty acid compositions as a means for identification. Proceedings of the National Beltwide Cotton Conference, Vol. 1:235-244. National Cotton Council, Memphis TN. Online: www.cotton.org/beltwide/proceedings.

Sekora, N. S., K. S. Lawrence, E. van Santen, J. A. McInroy. 2009. Delineating mixed populations of *Rotylenchulus reniformis* and *Meloidogyne incognita* with FAME analysis. Proceedings of the National Beltwide Cotton Conference, (In Press). National Cotton Council, Memphis TN. Online: www.cotton.org/beltwide/proceedings

Sekora, N. S., K. S. Lawrence, E. van Santen, J. A. McInroy. 2009a. Identifying selected nematode species based on fatty acid profiles using FAME analysis. Southeastern Biology (In Press).

White, D.C., Davis, W.M., Nickels, J.S., King, J.D., Bobbie, R.J., 1979. Determination of the sedimentary microbial biomass by extractable lipid phosphate. Oecologia 40:51–62.

Table 1. Fatty acid profiles of two soil treatments, soil samples not containing *Rotylenchulus reniformis* (SNRR) and soil samples containing *R. reniformis* (SRR). Means are listed in order by fatty acid chain length, location of the double bond, and functional group.

Fatty Acid	SNRR	SRR	Fatty Acid	SNRR	SRR
10:0	0.01	0.15	16:1 ω7c/15 iso 2OH	2.58	1.97
10:0 2OH	0.34	1.57	16:1 ω9c	0.16	0.08
10:0 3OH	0.18	--	17:0	0.76	0.37
11 methyl 18:1 ω7c	0.29	0.15	17:0 10 methyl	0.38	0.23
11:0 ANTEISO	--†	0.44	17:0 2OH	0.12	0.07
12:0	1.10	0.08	17:0 ANTEISO	1.27	0.68
12:0 2OH	0.03	1.27	17:0 CYCLO	0.92	0.82
12:0 3OH	0.07	--	17:0 ISO	0.61	0.65
13:0 ANTEISO	0.54	0.05	17:0 ISO 3OH	0.72	0.51
14:0	2.21	1.01	17:1 ω7c	4.77	4.27
14:0 2OH	0.22	0.32	18:0	2.69	4.54
14:0 3OH/16:1 ISO I	0.71	0.44	18:0 2OH	0.57	--
14:0 ISO	0.32	0.01	18:0 3OH	0.02	0.22
15:0 3OH	0.03	--	18:0 ANTE/18:2 ω6,9c	0.74	1.96
15:0 ANTEISO	2.64	0.92	18:0 ISO	0.07	--
15:0 ISO	2.03	1.17	18:1 2OH	0.11	0.11
15:0 ISO 2OH/16:1ω7c	0.14	0.04	18:1 ω7c	1.29	10.12
15:0 ISO 3OH	0.06	0.18	18:1 ω9c	8.19	9.95
15:1 ANTEISO A	0.04	0.01	18:2 ω6,9c/18:0 ANTE	8.32	5.68
15:1 ISO G	0.08	--	18:3 ω6c (6,9,12)	1.19	1.07
15:1 ω6c	0.16	0.02	19:0	0.02	0.17
15:1 ω8c	3.13	3.05	19:0 CYCLO ω10c/19ω6	7.49	9.13
16:0	17.55	15.38	19:0 ISO	0.06	0.03
16:0 10 methyl	0.06	0.48	19:1 ω6c/.846/19cy	15.46	12.78
16:0 2OH	0.46	0.28	20:0	1.65	1.99
16:0 3OH	0.46	0.25	20:1 ω7c	--	0.39
16:0 ANTEISO	0.15	0.06	20:4 ω6,9,12,15c	0.66	0.97
16:0 ISO	1.68	0.90	ANTEISO 17:1 ω9c	0.05	--
16:0 ISO 3OH	0.07	0.06	ISO 17:1 ω10c	0.36	0.21
16:0 N alcohol	1.08	0.99	ISO 17:1 ω9c	0.60	0.86
16:1 ISO I/14:0 3OH	0.11	0.01	TBSA 10Me18:0	0.14	0.01
16:1 ω5c	0.88	0.44	unknown 14.263	0.01	0.02

† = Not detected

Table 2. Phenotypic canonical correlation of fatty acids for the three canonical discriminant functions of FAME profile analysis of 40 pots, 20 not containing *Rotylenchulus reniformis* (SNRR) and 20 containing *R. reniformis* (SRR). Eigenvalue, cumulative percent of total variance, and canonical correlation are listed for each canonical function. Squared Mahalanobis distance (D^2) between the two soil treatments is also listed. Fatty acids listed were determined significant by the STEPDISC procedure. Correlation values are determined to be significant if $r \geq |0.75|$ (**bold**).

Response variable	Discriminant variate			Response variable	Discriminant variate		
Fatty acid variable	CAN 1	CAN 2	CAN 3	Fatty acid variable	CAN 1	CAN 2	CAN 3
10:0	-0.251	0.454	0.135	17:0	**0.756**	-0.136	-0.102
10:0 2OH	-0.407	0.430	0.117	17:0 ANTEISO	**0.807**	-0.088	0.294
10:0 3OH	0.501	-0.131	0.501	17:0 CYCLO	0.154	0.225	0.451
11 methyl 18:1 ω7c	0.474	-0.255	0.273	17:0 ISO	-0.078	0.274	0.340
11:0 ANTEISO	-0.417	0.388	0.057	17:0 ISO 3OH	0.066	0.192	0.298
12:0	0.589	-0.246	0.465	17:1 ω7c	0.199	0.669	0.027
12:0 2OH	-0.420	0.557	0.072	18:0	-0.446	-0.614	-0.418
12:0 3OH	0.384	-0.044	-0.074	18:0 2OH	0.368	0.412	-0.632
13:0 ANTEISO	0.619	-0.059	0.050	18:0 3OH	-0.182	-0.362	-0.151
14:0	0.696	-0.246	0.121	18:0 ANTE/18:2 ω6,9c	-0.119	-0.257	-0.172
14:0 2OH	-0.154	0.620	-0.040	18:0 ISO	0.367	0.417	-0.567
14:0 3OH/16:1 ISO I	0.332	-0.240	0.543	18:1 ω7c	-0.616	0.018	-0.144
14:0 ISO	0.702	-0.011	0.309	18:1 ω9c	-0.208	**-0.791**	-0.242
15:0 ANTEISO	**0.830**	0.040	0.081	18:2 ω6,9c/18:0 ANTE	0.195	-0.706	0.203
15:0 ISO	**0.755**	0.161	-0.136	18:3 ω6c (6,9,12)	-0.237	0.160	0.518
15:0 ISO 3OH	-0.315	0.273	0.079	19:0	-0.190	0.235	-0.038
15:1 ANTEISO A	0.310	-0.087	0.228	19:0 CYCLO ω10c/19ω6	0.008	0.263	0.210
15:1 ISO G	0.550	0.162	-0.034	19:0 ISO	0.153	-0.057	0.169
15:1 ω6c	0.554	-0.295	0.346	19:1 ω6c/.846/19cy	-0.102	0.009	0.318
15:1 ω8c	-0.150	0.311	0.647	20:0	-0.203	0.297	0.260
16:0	0.482	-0.223	-0.444	20:1 ω7c	-0.445	-0.334	-0.254
16:0 10 methyl	-0.323	0.246	0.080	20:4 ω6,9,12,15c	-0.312	0.286	0.168
16:0 2OH	0.202	-0.028	0.443	ANTEISO 17:1 ω9c	0.354	-0.011	0.227
16:0 ANTEISO	-0.201	0.080	0.246	ISO 17:1 ω10c	-0.042	0.031	0.236
16:0 ISO	**0.817**	0.165	-0.047	ISO 17:1 ω9c	-0.136	0.127	-0.190
16:0 ISO 3OH	0.041	0.085	0.237	unknown 14.263	-0.018	0.073	0.100
16:0 N alcohol	-0.088	0.147	0.502				
16:1 ω5c	0.305	-0.396	0.267	Eigenvalue	384.58	124.64	81.86
16:1 ω7c/15 iso 2OH	-0.132	-0.048	0.491	Cumulative %	47.1	62.4	72.4
16:1 ω9c	-0.332	0.192	0.243	Canonical Correlation	1.00	1.00	0.99

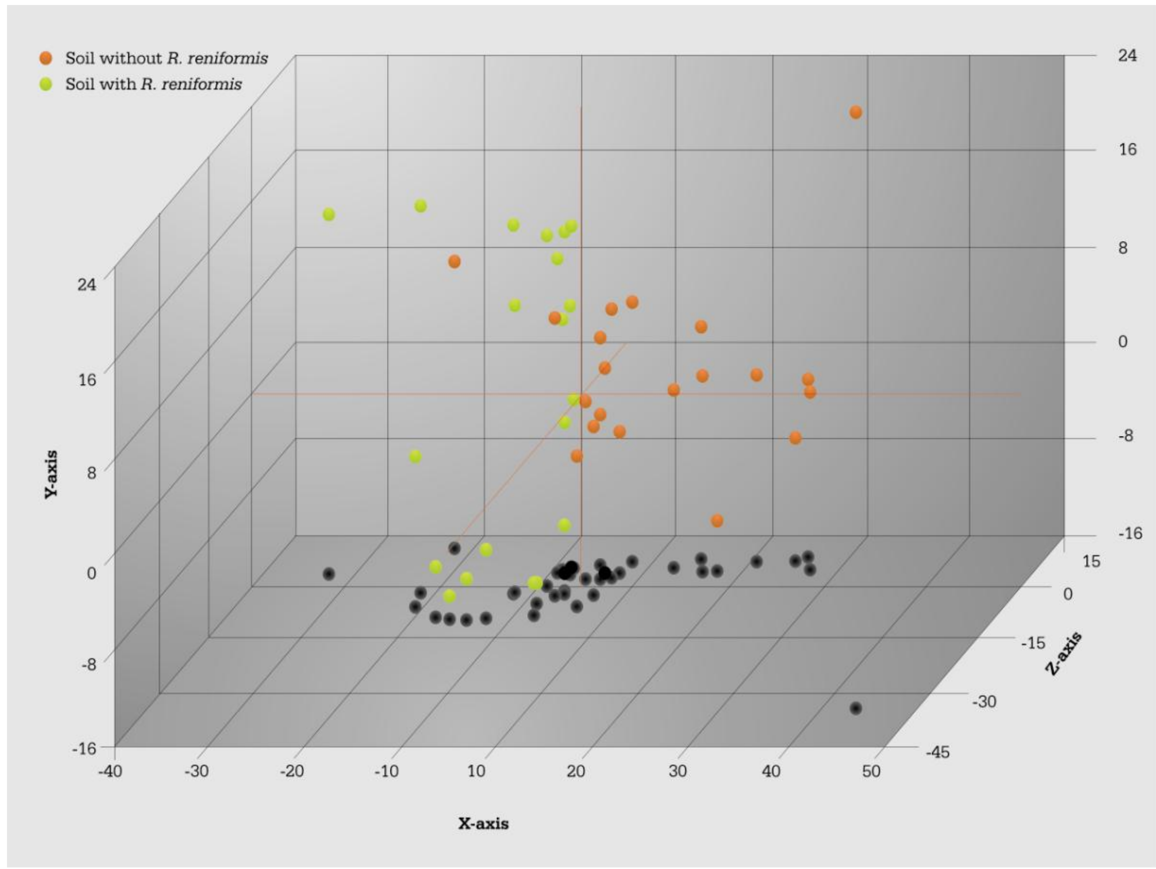

Figure 1. Graph of canonical means for twenty pots each of two soil treatments, soil samples not containing *Rotylenchulus reniformis* (SNRR) and soil samples containing *R. reniformis* (SRR). Points represent the average of three samples per pot. Placement is defined for the first (x-axis), second (y-axis), and third (z-axis) canonical axes by height (y-axis) and shadow (x-axis and z-axis intercept).

VI. SUMMARY

Traditional practices for identifying plant-parasitic nematodes include enumerating soil extractions, examination of plant roots, and host differential tests. These tests can be very time consuming and require trained personnel to accurately identify nematodes. Also, the cost of processing these samples is focused around work-hours of personnel; processing large numbers of samples can take up to a week to finish. Developing a method that accurately identifies plant-parasitic nematodes and reduces the work-hours required could be very valuable to the agricultural world, especially to disease diagnostics labs.

For several years, chromatographic analysis of fatty acid methyl esters (FAME) has been used to identify bacteria faster than the typical media tests, gram-staining, and other methods that are necessary. This system labels the fatty acids found in the bacterial cells and allows them to be detected with an automated gas chromatography system. Sample preparation requires about four hours to extract the fatty acids and 25 minutes to analyze each sample; up to 72 samples can be extracted at once in the 4-hour extraction. The total time required to extract and analyze 72 samples is 4 hours of labor and 24 hours of time on the automated gas chromatography system, for a total of 28 hours. Adapting this system for use with nematode samples would greatly reduce the time and labor required for processing samples.

To determine the applicability of the FAME system for use with plant-parasitic nematodes, samples containing dilutions of each of the three nematodes *Rotylenchulus reniformis*, *Meloidogyne incognita*, and *Heterodera glycines* were analyzed using FAME. Forty-five different fatty acids were observed from among the experimental samples, and eleven of these fatty acids are significant for distinguishing among *R. reniformis*, *M. incognita*, and *H. glycines*. Five of these fatty acids are significant to separate *H. glycines* from *R. reniformis* and *M. incognita*. The remaining six fatty acids significantly differentiate *R. reniformis* from *M. incognita*. These results indicate that *R. reniformis*, *M. incognita*, and *H. glycines* can be demarcated by FAME analysis and that other species could also be identified with this system.

To quantify the minimum number of nematodes needed to discriminate between *R. reniformis* and *M. incognita* by FAME analysis, a series of dilutions ranging from 10,000 total individuals to a single individual was used. While a single nematode can be detected, 100 total individuals allows for more consistent differentiation of the two nematode genera. At this concentration, five of the six fatty acids significant for discrimination between *R. reniformis* and *M. incognita* were present. Both 16:1 ω5c and 18:1 ω5c, fatty acids found in *R. reniformis* and not *M. incognita*, are detectable in samples containing at least 100 individuals of *R. reniformis*.

Because the three host plants of tomato, cotton, and soybean can each be parasitized by both *R. reniformis* and *M. incognita* while having unique FAME profiles, it is believed that each of these hosts will affect the FAME profiles of *R.*

reniformis and *M. incognita*. At the end of a sixty day growing period, nematodes were extracted from soil and roots of each host pot. Both *R. reniformis* and *M. incognita* produce statistically different FAME profiles depending on host species. However, these profiles are still distinctly different between the two nematode species. Host plants did not inhibit differentiation of *R. reniformis* and *M. incognita* using FAME.

Samples containing five separate ratios of *M. incognita* to *R. reniformis* (100-0, 75-25, 50-50, 25-75, 0-100) were prepared at both 500 and 5000 total individuals per sample to determine if mixed-species samples could be separated using FAME. Gradual shifts in fatty acid profiles as the percentages of *M. incognita* or *R. reniformis* increased were observed throughout the ratios. Statistical analysis indicated that all ratios are significantly different in samples containing 5000 total individuals. In samples containing 500 individuals, all ratios are significantly different except when comparing the 50-50 ratio to the 25-75 ratio. Samples containing mixed species of *M. incognita* and *R. reniformis* could be identified from one another and single species samples with 85% accuracy using FAME analysis.

Building on the success of FAME analysis in the differentiation of *M. incognita*, *R. reniformis*, and *H. glycines*, it should be possible to further demarcate among other species and races of plant-parasitic nematodes. Fatty acids were extracted from samples containing individuals of each *Meloidogyne* species *M. arenaria* (race 2), *M. hapla*, *M. incognita* (races 1, 2, and 3), and *M. javanica*. The resulting profiles were analyzed using statistical means and the

Sherlock® Analysis Software. All profiles were significantly different among species and races. The four *Meloidogyne* species are distinguished easily with a minimum D^2 between *M. incognita* and *M. arenaria*. When the species are separated by race, the minimum distance lies between *M. arenaria* race 2 and *M. incognita* race 1. D^2 values among *M. incognita* races are all significant at $P < 0.0001$. The Sherlock Analysis Software identified these six species and races at 85% accuracy to the race level. By incorporating these profiles with eight other developed profiles into a Sherlock® Analysis Software library, it was possible to differentiate the plant-parasitic nematodes studied with greater than 94% accuracy to the race level. Any discrepancies are generally restricted to differences in life stage, host, or mixtures containing that species.

Additional research to identify *R. reniformis* from total soil FAME (TSFAME) extractions has indicated that it may be possible to detect this nematode in infested soil. A study comparing two soil treatments, soil from cotton pots 60 days after inoculation with 2000 total individuals or *R. reniformis* and soil from cotton pots not inoculated with *R. reniformis* 60 days beforehand, demonstrated that there were detectable differences in the FAME profile of the two soil treatments. Two fatty acids that are found consistently in the FAME profile of *R. reniformis*, 12:0 2OH and 18:1 ω7c, were observed in greater quantities in soil containing *R. reniformis*. By using further manipulation of the soil profiles from inoculated pots, a FAME profile resembling that of *R. reniformis* was extrapolated. Though this profile was significantly different from the reference profile of *R. reniformis* ($P \leq 0.0001$), 8 of the 11 fatty acids found in

these profiles were within a single standard deviation of the reference profile and within the limits of identification by the Sherlock® Analysis Software.

Based on the success of this research, it is possible to use FAME analysis and the Sherlock® Analysis Software as an alternative means for the identification of plant-parasitic nematodes in diagnostic laboratories. Using this approach would reduce the time required for identifications to the species and race levels, which can take up to 45 days to complete for *Meloidogyne* species and races. Information gained from developing and studying these FAME profiles has the potential to lead to other discoveries in agriculture, bioengineering, and breeding. Many disciplines have the potential to gain from using FAME analysis as a means for identification of plant-parasitic nematodes.