FINGERPRINTING NEMATODE FATTY ACID COMPOSITIONS AS A MEANS FOR IDENTIFICATION

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Abstract

Samples containing dilutions of each of the three nematodes *Rotylenchulus reniformis*, *Meloidogyne incognita*, and *Heterodera glycines* were analyzed using fatty acid methyl-ester (FAME) analysis to determine if these genera could be differentiated from one another. Forty-five different fatty acids were observed from among the experimental samples. Of these 45, 11 were found to be significant to distinguishing among *R. reniformis*, *M. incognita*, and *H. glycines*. Five of these fatty acids − 20:4 ω6,9,12,15c, 18:1 ω5c, 18:2 ω6,9c, 15:1 anteiso A, and 12:0 2OH – are significant (P<0.0001) to separate *H. glycines* from *R. reniformis* (D^2 = 26.07) and *M. incognita* (D^2 = 31.56). These fatty acids are present in greater concentrations in *H. glycines* than either *R. reniformis* or *M. incognita*. The fatty acid 15:1 anteiso A is only expressed in the observed *H. glycines* samples. The remaining six fatty acids − 18:0 3OH, 15:0 iso, 16:1 ω5c, 18:1 ω9c, 14:0, and 17:0 iso – significantly differentiate (P<0.0001) *R. reniformis* from *M. incognita* (D^2 = 3.78). All six of these fatty acids are observed in a much greater concentration in *R. reniformis* than *M. incognita*, such as 16:1 ω5c, whose relative mean concentration is 147 times greater in *R. reniformis*. These results indicate that *R. reniformis*, *M. incognita*, and *H. glycines* can be demarcated by FAME analysis.

Introduction

The nematodes *Rotylenchulus reniformis* and *Meloidogyne incognita* are two of the most economically important cotton nematodes in the southeastern United States. *Heterodera glycines*, the soybean cyst nematode, causes sizable crop damage to soybean crops. Detection techniques for these organisms is labor intensive and usually require field analysis of infected plant root systems and taking soil samples to determine the quantitative impact on the field. Fatty acid methyl-ester (FAME) analysis may provide an easier solution to identifying these organisms in soil samples. FAME analysis was developed to quickly identify bacteria (Sasser, 1990). This form of analysis has been used in conjunction with the Sherlock Analysis System (MIDI Systems, Inc.) to group and characterize bacteria and fungi from many different systems, including plant root systems (McInroy, 1995), decaying artwork (Heyrman, 1999) and even the filamentous fungi (Stahl, 1996). An average profile of the fatty acid expression for each organism is used to quickly characterize or even identify the bacteria within a sample. It is the purpose of this experiment to determine if this process can be used to differentiate between *R. reniformis*, *M. incognita*, and *H. glycines*.

Materials and Methods

Nematode samples were isolated from populations grown at the Auburn University Plant Science Research Center, Auburn, AL. Populations were grown in 500 cm^3 polystyrene pots containing cotton cultivar DPL 555 BG/RR for *R. reniformis* and *M. incognita*, and Croplan Genetics RC 4955 soybean for *H. glycines*. To isolate *R. reniformis* and *M. incognita* vermiform life stages, nematodes were extracted from the soil by combined gravity screening and sucrose centrifugal floatation. For egg extraction, roots were shaken in 0.6% NaOCl for four minutes, and then rinsed through 75 and 25 µm sieves consecutively. All samples were then centrifuged via 1.0 M sucrose-flotation. Each sample was counted to determine the total number of nematode vermiform life stages present and the ratio of vermiform life stages to eggs. The cyst stages of *H. glycines* are extracted by gravity screening and collected on nested sieves with pore sizes of 850 and 250 µm.

A step-wise dilution scheme was devised to determine the minimum number of nematodes needed for a proper analysis. Dilutions for *R. reniformis* and *M. incognita* ranged from 10,000 vermiform life stages and eggs per sample to a single vermiform nematode. Samples for *H. glycines* ranged from 25 cysts to a single cyst (Table 1).

The smaller dilution range was based on the assumption that fewer cysts of *H. glycines* would be needed to perform a complete analysis due to the larger size of the organism compared to that of *R. reniformis* and *M. incognita*. Samples were placed into individual microcentrifuge tubes with 0.5 mL of water. To prepare the smaller samples (1 to 100 nematodes), vermiform life stages were hand-picked to contain the specified number of nematodes. Samples with 250 or more were set up by volume dilutions from the original extractions.

Table 1. Dilution scheme of nematode samples for fatty acid analysis. Numbers indicate the total number of nematodes per sample.

R. reniformis vermiform life stages and eggs	*M. incognita* vermiform life stages and eggs	*H. glycines* cysts
10,000	10,000	
5,000	5,000	
1,000	1,000	
500	500	
250	250	
100	100	
50	50	
25	25	25
10	10	10
1	1	1

Fatty acid extraction was performed following the four-step extraction procedure for FAME analysis (Sasser, 1990). The first step, saponification, is designed to lyse the cells, release the fatty acids of the cell, and prepare them for esterification. Methylation transforms the freed fatty acids into esters at the carboxyl terminus. The extraction step removes the hydrophobic fatty acid esters from the aqueous solution by means of an organic solvent. This solvent is washed with a base to remove hydrophilic impurities before analysis. As an additional step, the washed samples were evaporated and reconstituted in 150 µL of the organic solvent. This extra step concentrates the fatty acids within the sample and allows for a more complete analysis. Each sample was then analyzed by the Hewlett Packard 5890 Series II Gas chromatography system (MIDI Systems, Inc.). The analysis utilized an Ultra 2 Crosslinked 5% Phenyl Methyl Siloxane column to determine the fatty acid compositions of the samples.

Statistical analysis of the samples was based on the relative percentage of each fatty acid in a sample. SAS version 9.1.3 (SAS Institute, Inc.) was used to analyze the data. The STEPDISC procedure was used to determine the fatty acids that were statistically significant to differentiating between the nematode genera. The significant fatty acids were then analyzed with the CANDISC procedure and the resulting "between canonical structure" values (phenotypic correlation) were used to define the genera.

Results

A total of forty-five fatty acids were observed from all analyzed samples of *R. reniformis*, *M. incognita,* and *H. glycines* (Table 2).

Table 2. Total fatty acids observed from FAME analysis of *R. reniformis*, *M. incognita*, and *H. glycines*.

Fatty Acids Observed			
9:0	**16:1 ω7c/15 iso 2OH**	18:1 ISO H	**20:4 ω6,9,12,15c**
10:0	**16:1 ω5c**	18:0 ISO	**18:0 3OH**
10:0 2OH	16:0	18:3 ω6c (6,9,12)	19:0 CYCLO ω8c
11:0 2OH	17:1 ISO I/ANTEI B	**18:2 ω6,9c/18:0 ANTE**	20:0 ISO
12:0	15:0 3OH	**18:1 ω9c**	**20:2 ω6,9c**
12:0 2OH	17:1 ANTEISO A	**18:1 ω7c**	20:1 ω9c
14:0	**17:0 ISO**	**18:1 ω5c**	20:1 ω7c
14:0 2OH	17:0 ANTEISO	**18:0**	20:0
15:0 ISO	17:1 ω9c	17:0 ISO 3OH	18:1 ISO H
15:1 ANTEISO A	17:1 ω8c	**19:1 ISO I**	
15:1 ω6c	**16:0 ISO 3OH**	19:0 10 methyl	
16:0 ISO	16:0 2OH	18:0 2OH	

Significant for Identification

The frequency of these fatty acids varied among the three experimental genera. In both *R. reniformis* and *H. glycines*, 45% of the fatty acids were not observed in either of these genera while 15% did not appear in *M. incognita*. Also, the percentage of fatty acids appearing only once out of all analyzed samples was 22%, 9%, and 14% for *M. incognita*, *R. reniformis*, and *H. glycines*, respectively. The remaining fatty acids were spread out at varying percentages among the three genera (Figure 1).

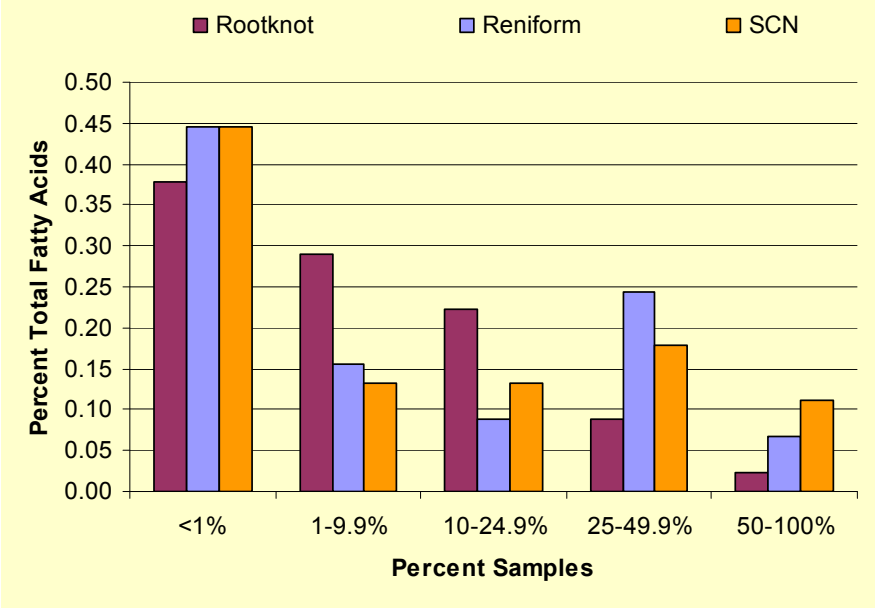

Figure 1. Frequency of observed fatty acids per genus.

Profiles for each genus could be made using the mean relative percentage of each fatty acid (Figures 2, 3, and 4).

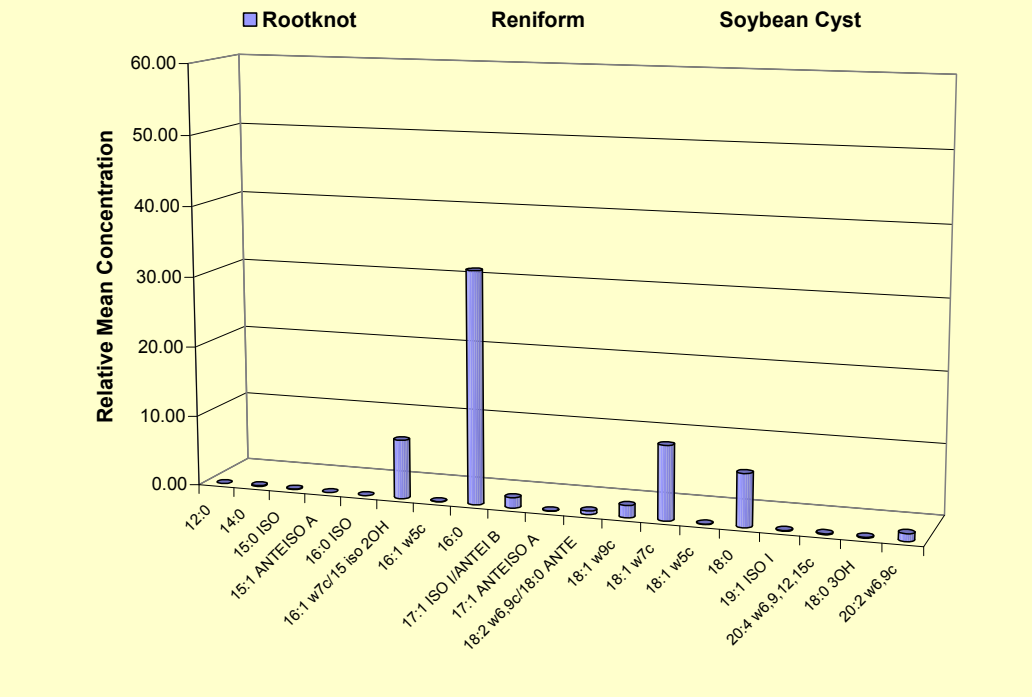

Figure 2. Fatty acid profile of *M. incognita*. Values are mean sample percentage

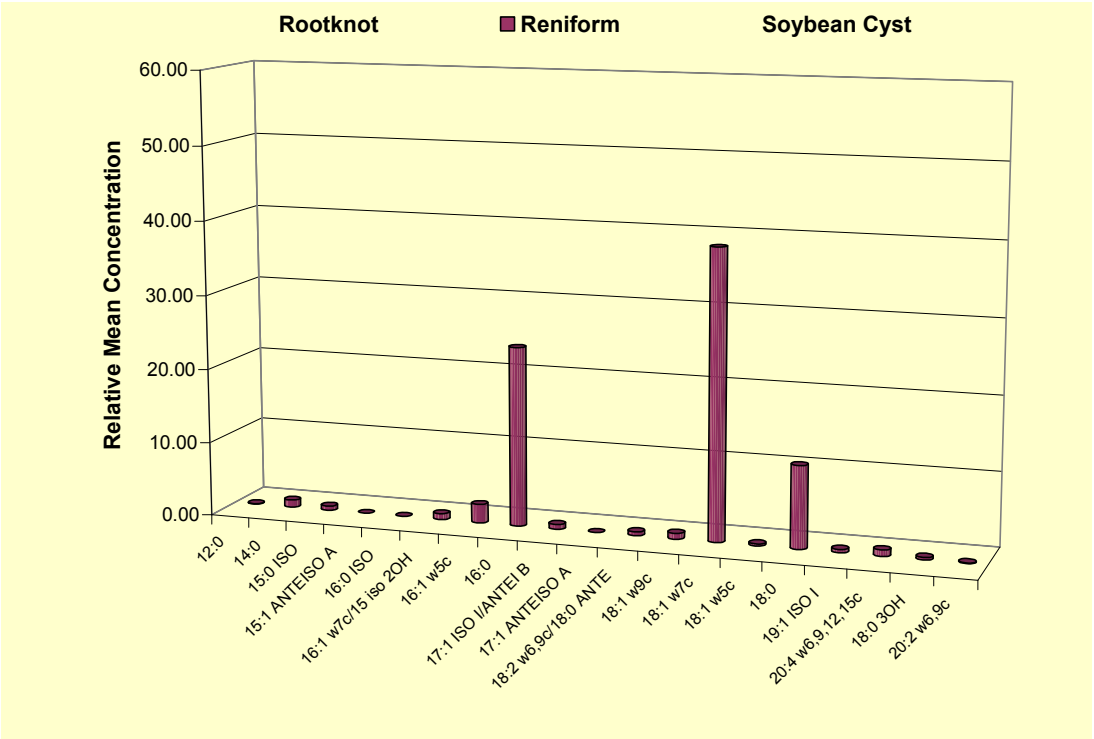

Figure 3. Fatty acid profile of *R. reniformis*. Values are mean sample percentage

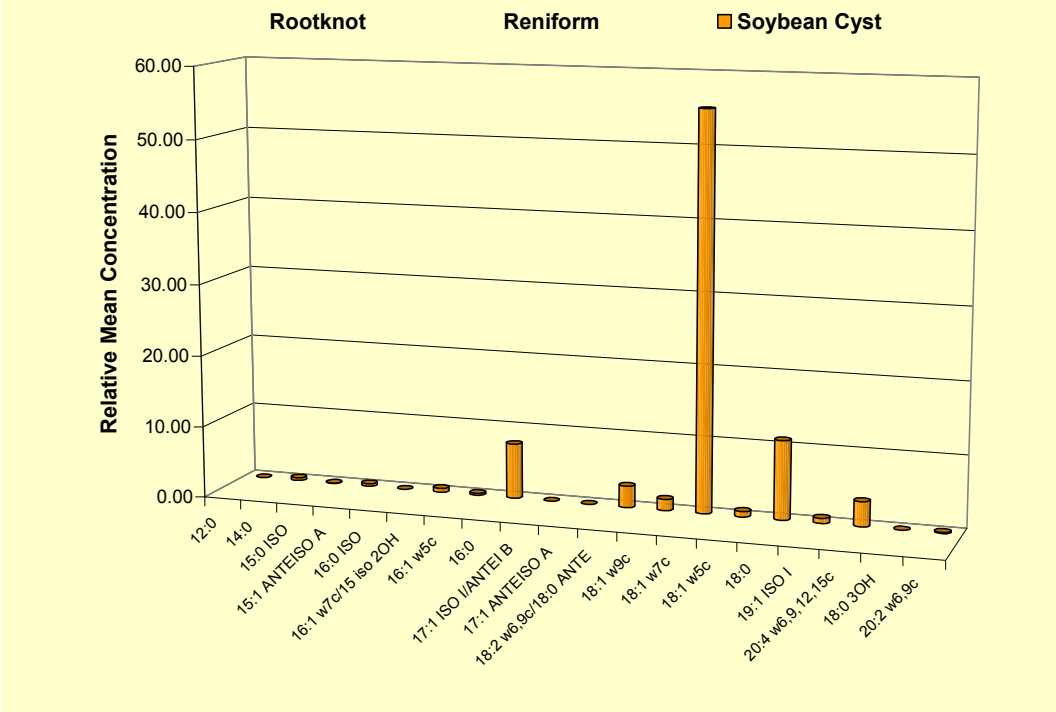

Figure 4. Fatty acid profile of *H. glycines*. Values are mean sample percentage

Fatty acids with percentages less than 1.0 can be excluded because they contribute little to the overall profile. By combining these profiles, visual clues to the variance of fatty acid expression between the three nematode genera can be observed (Figure 5).

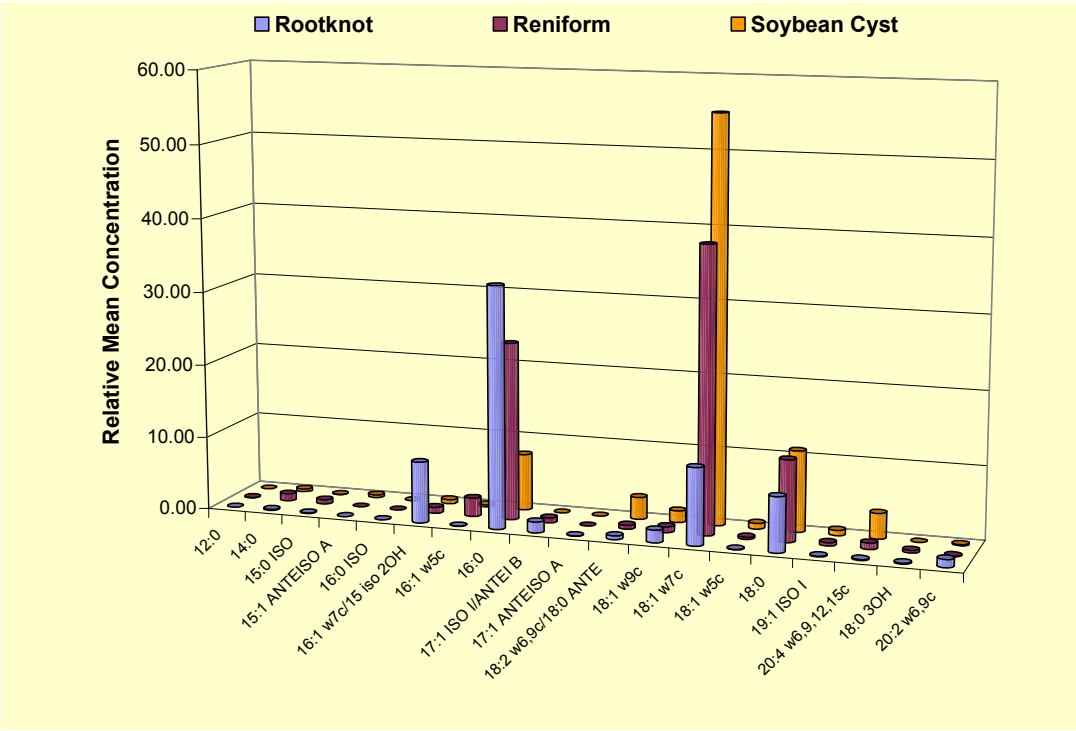

Figure 5. Combined fatty acid profiles for three nematode genera.

To determine which of the forty-five observed fatty acids are significant to differentiating between *R. reniformis*, *M. incognita*, and *H. glycines*, the STEPDISC procedure was used. This procedure uses discriminant analysis to determine which of the fatty acids contribute to variation between genera (SAS Institute, Inc.). This analysis yielded 17 fatty acids that can be used to separate the genera (Table 2, significant fatty acids are listed in bold). Next, the CANDISC procedure was used to compare all 17 of the significant fatty acids and determine which are the most indicative of genus identification.

Of these 17 fatty acids, five were significant in the first canonical variate to differentiate *H. glycines* from *R. reniformis* and *M. incognita* and explained 72% of the variation among the genera. Six fatty acids described the remaining 28% of variation in the second canonical variate and could be used to discriminate between *R. reniformis* and *M. incognita* (Figure 6).

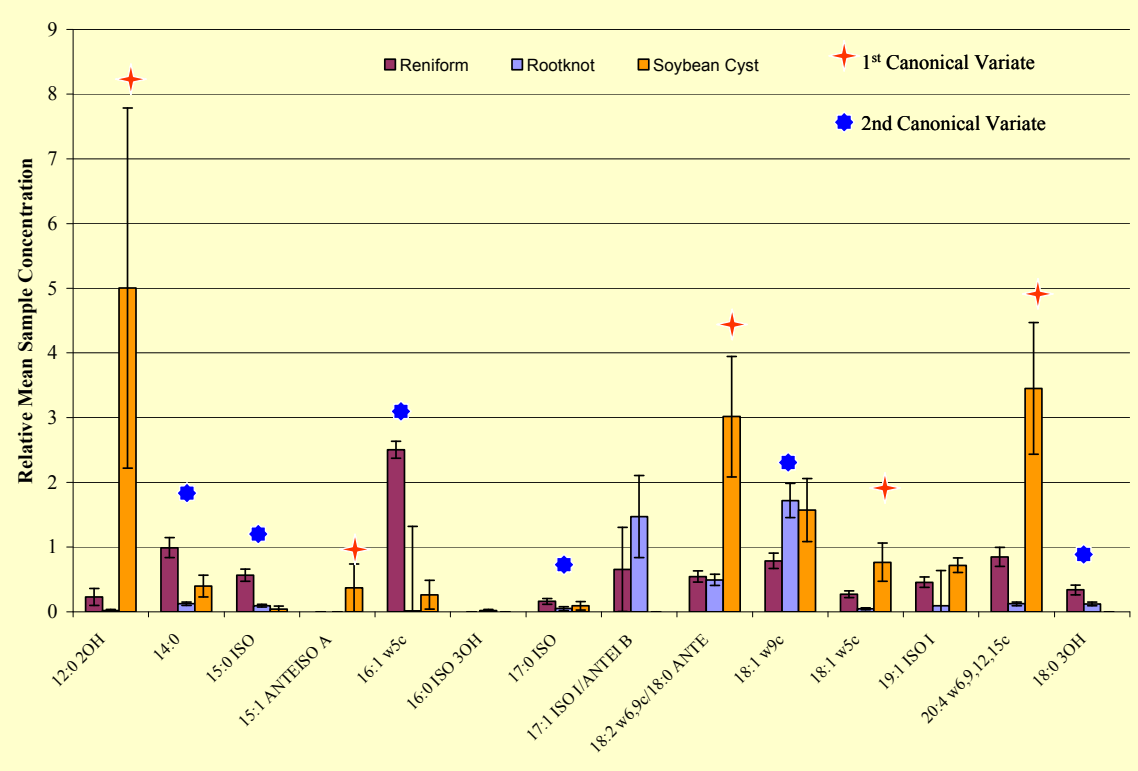

Figure 6. Fatty acids significant for differentiation between the three nematode genera.

The distance of separation squared (D^2) values for the separation of *H. glycines* from *R. reniformis* and *M. incognita* were 26.07 and 31.56, respectively (Figure 7).

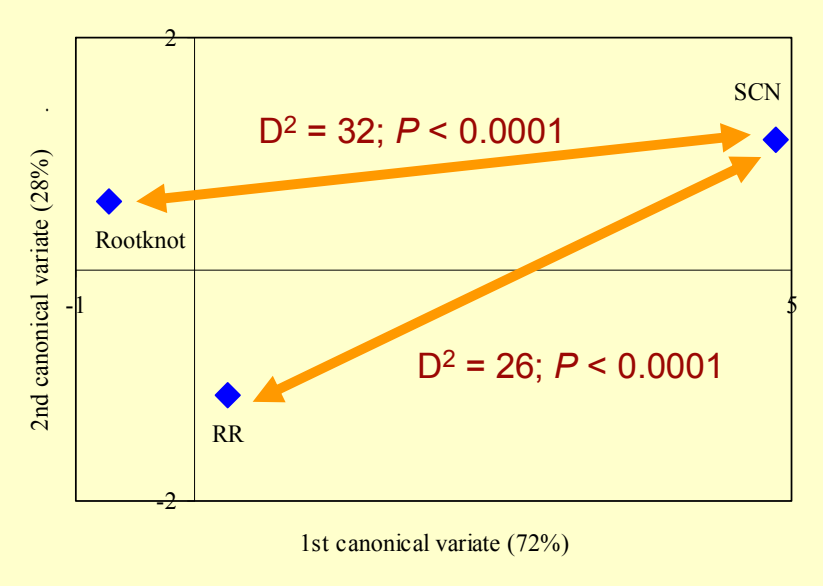

Figure 7. Distribution of the first canonical variate for discriminating *H. glycines* from *R. reniformis* and *M. incognita*.

The D^2 value between *R. reniformis* and *M. incognita* was 3.78 (Figure 8).

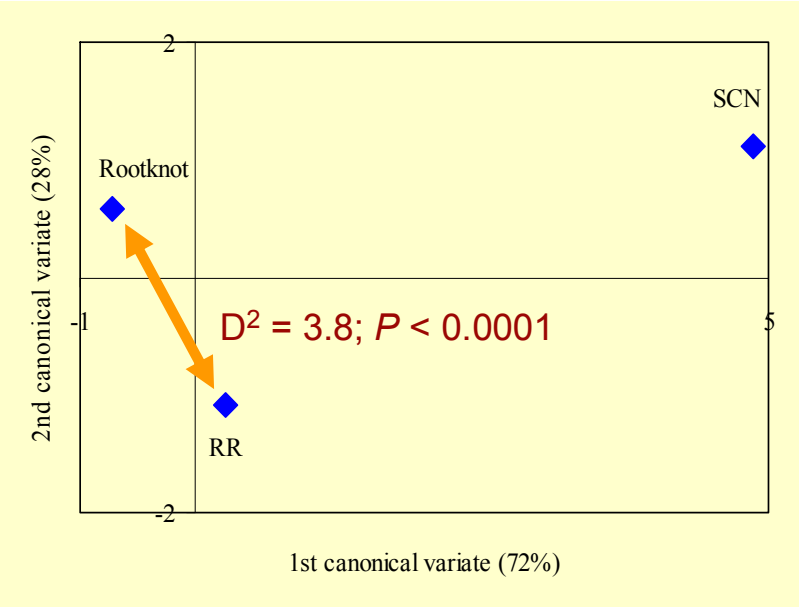

Figure 8. Second canonical variate used to distinguish *R. reniformis* from *M. incognita*.

All three of the D^2 values were significant at $P<0.0001$.

Discussion

At the present, pure cultures of each genus can be used to definitively segregate each of the three genera. *Heterodera glycines* can be clearly identified from *R. reniformis* and *M. incognita* by five fatty acids. The fatty acid 20:4 ω6,9,12,15c is perfectly correlated along the first canonical axis and is an ideal candidate for defining this genus from the other two. In addition, the four fatty acids 18:1 ω5c, 18:2 ω6,9c, 15:1 anteiso A, and 12:0 2OH

support this discrimination strongly along the same axis. For *H. glycines*, 15:1 anteiso A only appears in this genus and not *R. reniformis* or *M. incognita*. The other four fatty acids are present in higher concentrations for *H. glycines* than either of the other two genera.

To distinguish *R. reniformis* from *M. incognita*, the fatty acids 18:0 3OH and 15:0 iso are the best candidates based on their nearly perfect correlation along the second canonical axis. This distinction is reinforced by 16:1 ω5c, 18:1 ω9c, 14:0, and 17:0 iso. Though the fatty acid 16:1 ω5c has a relative mean sample concentration of 2.5% in *R. reniformis* and is virtually nonexistent in *M. incognita*, differentiation is more strongly supported by the ratios of the other five fatty acids. For example, 14:0 and 15:0 iso mean sample concentrations are 7.5 and 6.3 times higher, respectively, in *R. reniformis* than *M. incognita*, but the mean concentration of 18:1 ω9c for *M. incognita* is twice as high as that of *R. reniformis*.

Based on these results of this experiment, fatty acids can be used to differentiate between *R. reniformis*, *M. incognita*, and *H. glycines*. The fatty acid profile of each of these nematodes varies from the other two. Even though all three of these genera have relatively higher concentrations of the fatty acids 16:0, 18:1 ω7c, and 18:0 compared to the other fatty acids, the fatty acids present in lower concentrations are more significant to discrimination between the genera. Because of this, the relative ratios of fatty acids should generally be used to define the genus, not just looking at what fatty acids are present. For instance, the ratios of the fatty acids 16:1 ω5c/15 iso 2OH to 16:1 ω5c are 0.33:1, 417:1, and 2.15:1 for *R. reniformis*, *M. incognita*, and *H. glycines*, respectively. By using the ratios instead of "signal peaks," as are sometimes used to identify bacteria, a more definitive identification may be possible. Mixed-genus samples need to be run to determine if all genera present can be identified. At present, identification can be tentatively made if one of the three profiles is given and the possibilities are *R. reniformis*, *M. incognita*, or *H. glycines*.

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