The Sterol, Fatty Acid, and Hydrocarobon Composition of Globodera solanacearum[1]

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Abstract: Globodera solanacearum females were found to have less than 0.01% of dry wt as sterols. Seven sterols were detected in the nematode, with stanols (campestanol and stigmastanol) making up more than 50% of the total sterols present. Lipid amounted to 29.4% of the dry weight of the nematode. Triglyceride, free fatty acid, and phospholipid classes were composed predominantly of 20:4, 20:1, and 18:1 fatty acids. Of the total weight of fatty acids found in *G. solanacearum* females, the greatest portion occurred in the triglyceride fraction, followed by the free fatty acid fraction then the phospholipid fraction. Several unidentified hydrocarbons were detected in the nematode. Paraffinic hydrocarbons detected ranged in carbon length from C15 to C29. Total concentration of hydrocarbon composed 0.20% of the dry wt. *Key Words:* Osborne cyst nematode, lipids, stanols, triglycerides, phospholipids.

Several definitive studies have been made of the lipid composition of free-living, animal-parasitic, and plant-parasitic nematodes (2, 6, 8, 9, 15-18). Such studies have led to the realization that free-living and plant-parasitic nematodes are high in lipid content (11-37% of dry weight) compared with animal parasites (4-10% of dry weight). Most nematodes examined thus far have similar lipid compositions, particularly in relation to the kinds of fatty acids and sterols present. It has been suggested (8), however, that plant-parasitic nematodes may be typically high in eighteen carbon unsaturated fatty acids whereas free-living nematodes are high in twenty carbon unsaturated fatty acids.

Sterols are known to occur in nematodes and have been shown to be essential for normal growth and development (1, 3, 4, 7, 11, 21, 26). Several instances have been documented in which nematodes were unable to synthesize sterol precursors and seemingly must depend on exogenous sources to provide the necessary steroid nucleus (4, 24, 27). The major sterols identified in most species have been cholesterol, lathosterol and 7-dehydrocholesterol (16). The only exceptions have been two reports of stanols in *Ascaris lumbricoides* (4) and *Rotylenchulus reniformis* (25).

We know of no information on the hydrocarbon composition of nematodes. That seems unusual considering their high lipid content.

The present study analyzed the plant-parasitic nematode *Globodera solanacearum* for triglyceride fatty acids (TRIG), free fatty acids (FFA), phospholipid fatty acids (PL), free sterols (FS), and paraffinic hydrocarbons (PH).

MATERIALS AND METHODS

Growth and harvesting procedures: Twenty-day-old 'N.C. 95' tobacco seedlings were transplanted into 10-cm pots containing a potting mixture of soil-Weblite 2:1 v/v (Weblite from Weblite Corporation, Roanoke, Virginia) which had been infested with eggs of *G. solanacearum* (20,000 eggs/pot) and grown 6 weeks in the greenhouse until females developed in the roots. The females were washed from the soil, and the flotsam was removed by a combination of sieving, sugar flotation, and elutriation, followed by examination and removal of debris under a dissecting microscope.

Extraction and Analytical Procedures: One gram of freeze-dried female nematodes was ground in a mortar and pestle with chloroform-methanol 2:1 v/v (both solvents were redistilled). The ground nematode material was then transferred to a Soxhlet apparatus containing chloroform-methanol 2:1 v/v and extracted for 18 h. The extract was evaporated to dryness, dissolved in chloroform, and filtered through Whatman no. 1 filter paper. The filtrate was dried and weighed to give a total lipid weight.

Lipid classes were separated on silica gel G (Applied Sciences Laboratories Inc.,

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State College, Pennsylvania) according to a thin layer chromatographic (TLC) technique described by Ginger and Fairbairn (9). Lipid bands corresponding to polar lipids, triglycerides, free fatty acids, sterols, and hydrocarbons were removed from TLC plates and further processed. Triglycerides and polar lipids were hydrolyzed with 20% KOH in 80% ethanol for 45 min at 50 C. The samples were acidified with 6 N HCl and partitioned three times with diethyl ether. The three ether phases were combined and evaporated, and the fatty acids were methylated with 5 ml of BCl_3-methanol (Supelco Inc., Bellefonte, Pennsylvania) at 50 C for 5 min. The methylated samples were partitioned three times with n-hexane and the hexane extracts were combined and evaporated. The samples were rechromatographed on TLC and the fatty acids were removed by eluting the silica gel with diethyl ether. The fatty acids were further purified and separated according to degree of unsaturation using $AgNO_3$-silica gel G TLC (13). The fatty acid bands were eluted with diethyl ether and analyzed by gas-liquid chromatography (GLC) using a Bendix 2600 gas chromatograph (Bendix Process Instrument Division, Ronceverte, West Virginia) equipped with a flame ionization detector. GLC analysis of fatty acids was conducted isothermally at 170 C on a 0.92-meter glass column packed with 10% EGS (Supelco, Bellefonte, Pennsylvania). Identification of the fatty acids was by comparison of GLC retention times with those of known standards. Quantification was based on known concentrations of 18:0.

FFA from the initial TLC separation were methylated and analyzed as outlined above.

FS were analyzed by GLC as the N,O-bis(trimethylsily) acetamide derivative (Supelco Inc., Bellefonte, Pennsylvania) on 3% SE-30, 1% QF-1, and 3% HiEFF 8-BP (Applied Science Laboratories Inc., State College, Pennsylvania). Isothermal GLC analysis of sterols was conducted on 0.92-m glass columns at 244, 231, and 238 C, respectively, for the above column packings.

Sterol derivatives were prepared by heating the initial TLC sterol fraction in a 1-ml reaction vial with 30 μl of the silylating reagent for 45 min at 50 C. Sterols were tentatively identified by comparing GLC relative retention times (RRT) with known standards and published data (23). Although RRT data of Patterson's (23) were obtained with free sterols and acetate derivatives, we have been able to reproduce almost identical RRT values with the N,O-bis (trimethylsily) acetamide derivatives of many of the same sterols as long as the same column packings, flow rates, and column temperatures are duplicated. Stanols were prepared by dissolving 1 mg each of cholesterol, campesterol, and β-sitosterol in 1 ml of n-hexane. A few milligrams of a 10% palladium powdered charcoal catalyst was added to the solution, and hydrogen gas was bubbled through the mixture until the catalyst precipitated. Identification of nematode sterols was based also on gas-chromatographic mass-spectrophotometer (GC-MS) analysis of trimethylsilyl ether derivatives. Mass spectrometry was conducted on a Varian Mat 112 instrument (Varian Instrument Division, Palo Alto, California) equipped with a 0.92-m glass column packed with 3% QF-1. The column temperature was maintained at 245 C, and spectra were obtained by continuous scanning at an ionization energy of 70 ev.

PH were analyzed by temperature programming on 3% SE-30 from 100 to 250 C at 2/min and were identified by comparison with known standards.

RESULTS

The lipid concentration of *G. solanacearum* females was found to be 29.40% of the dry weight. That is comparable to the range found in other plant-parasitic nematodes (16). Table 1 compares the fatty acids of the TRIG, FFA, PL, and PH as a percentage of total lipid and as a percentage of dry weight. The greatest total weight of fatty acids was found to be in TRIG, followed by FFA and PL. PH were present in very low amounts, making up less than 0.20% of the dry weight.

Table 2 compares the individual fatty acid compositions of the TRIG, FFA, and PL classes as a percent of total fatty acid recovered in each class. The same fatty acids were detected in all lipid classes, and a high degree of unsaturation was characteristic of all classes (TRIG 77.95%, FFA 77.75%,

TABLE 1. Comparison of lipid class fatty acids and total paraffinic hydrocarbons as a percent dry wt and total lipid of *Globodera solanacearum* females.

	Triglycerides	Free fatty acids	Phospholipids	Paraffinic hydrocarbons
% of total lipid	47.27	6.46	4.86	0.48
% of dry weight	13.28	1.81	1.36	0.13

TABLE 2. Fatty acid composition (triglycerides, free fatty acids, and phospholipids) of *Globodera solanacearum* females as a percent of total in each class.[x]

Fatty acid	Triglyceride	Free fatty acid	Phospholipid
14:0	0.40	1.20	0.80
X_1[y]	0.70	trace	1.40
16:0	3.09	5.26	2.90
16:1	1.24	2.26	1.40
18:0	12.00	9.77	9.29
18:1	16.82	16.84	19.98
18:2	2.79	3.61	3.60
18:3	2.24	3.76	trace
20:0	4.48	3.76	8.60
20:1	20.91	15.04	26.37
20:2	1.94	1.80	2.80
20:3	7.62	7.37	5.59
20:4	24.39	27.07	14.59
22:0	1.79	2.26	2.70
20:5	trace	trace	trace

[y]Unknown

[x]Total concentrations of TRIG, FFA, and PL were 132.60, 18.15, and 13.65 mg/gram dry wt of female nematodes.

TABLE 3. Paraffinic hydrocarbon composition of *Globodera solanacearum* females.

Carbon no.	% of total
15	0.18
16	0.75
17	3.20
18	2.17
19	3.20
20	0.87
21	8.19
22	1.15
23	7.26
24	1.24
25	1.64
26	1.24
27	1.13
28	9.17
29	1.11
X_1	7.59
X_2	8.12
X_3	3.11
X_4	3.64
X_5	17.95
X_6	12.12
X_7	4.97

X_1-X_7, unknown hydrocarbons.
1.34 mg total hydrocarbon extracted from 1 gm dry wt of female nematodes.

and PL 74.33% unsaturated). The three most abundant fatty acids found in the TRIG, FFA, and PL classes were eicosatetraenoic (20:4), eicosenoic (20:1), and octadecenoic (18:1) acids. These 3 fatty acids made up 62.11, 58.94, and 60.94% of the total fatty acid in the respective classes. Also detected in each class were smaller amounts of tetradecanoic (14:0), hexadecanoic (16:0), hexadecenoic (16:1), octadecanoic (18:0), octadecadienoic (18:2), otcadecatrienoic (18:3), eicosanoic (20:0), eicosadienoic (20:2), eicosatrienoic (20:3), docosanoic (22:0), and eicosapentaenoic (20:5) acids.

Table 3 represents the PH composition of *G. solanacearum* females. PH ranging in carbon length from C15 to C29 composed 42.50% of the total hydrocarbon concentration. The remaining 57.50% consisted of unidentified components, probably branched-chain or unsaturated hydrocarbons.

Seven sterol components were detected in extracts of *G. solanacearum* females. Analysis of the components by continuous scanning GC-MS indicated that the sterols had molecular weights of 458, 474, 484, 486, 486′, and 488 (Table 4). Mass spectra of the 458 and the 486 components were compared with mass spectra of authentic cholest-5-en-3β-ol and found to be identical. Tentative identification of the remaining sterol components was based on GC-MS fragmentation patterns of the sterols, molecular weights, and RRT data derived from GLC. Components having molecular weights of 474, 484, 488 and 486′ were respectively identified tentatively as 24-methyl-5α-cholestan-3β-ol,

TABLE 4. Relative retention times, molecular weights, and relative percentages of *Globodera solanacearum* sterols and standards.

Sterol	Relat. reten. time[a]	Molecular wt	Relat. %
Unknown	1.00	458	11.50
cholest-5-en-3β-ol	1.00	458	
Unknown 2	1.11	—	9.20
24-methylcholest-5-en-3β-ol	1.11	476	
Unknown 3	1.13	474	43.13
24-methyl-5α-cholestan-3β-ol	1.14	474	
Unknown 4	1.15	486′	19.80
5α-stigmast-22-en-3β-ol	—	486′	
Unknown 5[b]	1.19	486	
stigmast-5-en-3β-ol	1.19	486	7.67
Unknown 6[b]	1.19	484	
stigmast-5,24(28)-dien-3β-ol	—	484	
Unknown 7	1.22	488	8.70
5α-stigmastan-3β-ol	1.21	488	

[a]All relative retention times based on cholesterol. Chromatographic conditions: 3% QF-1 on Gas Chrom Q, 100-120-mesh; temperature programmed from 150 to 230 C at 2 C/min on 0.92-m glass column.
[b]Unknown 5 and 6 were not resolved on QF-1, so relative percentage was combined calculation.
Where blanks occur in the table, either standards were not available or molecular weights were not determined.

stigmasta 5,24(28)-dien-3β-ol, 5α-stigmastan-3-β-ol, and 5α-stigmast-22-en-3β-ol. The 484 and 486′ components both had characteristic molecular ions at me/387, which is indicative of $\Delta^{24(28)}$ C-29 sterols (14). Sterols with molecular weights of 474 and 488 have to be saturated C-28 and C-29 stanols, respectively. GLC data (Table 4) using 3% QF-1 also support the GC-MS data. Three percent SE-30 was ineffective for separating stigmast-5,24(28)-dien-3β-ol, stigmast-5-en-3β-ol, and 5α-stigmastan-3β-ol. 3% QF-1 did not resolve stigmast-5,24(28)-dien-3β-ol. HiEFF-8-BP did show the presence of a sterol component having an RRT corresponding to stigmast-5,24(28)-dien-3β-ol (23). This packing would not resolve stigmast-5-en-3β-ol and 5α-stigmastan-3β-ol. Sterols made up 0.01% of the dry weight of the female nematodes. The relative percentages of stanols were surprisingly high, with 24-methyl-5α-cholestan-3β-ol and 5α-stigmastan-3β-ol making up more than 50% of the total sterols. Cholest-5-en-3β-ol and 5α-stigmast-22-en-3β-ol respectively made up 9.20% and 19.80% of the total sterol. 5α-stigmast-22-en-3β-ol and stigmast-5-en-3β-ol combined, made up 7.67% of the total sterol.

DISCUSSION

G. solanacearum females are similar to other plant-parasitic nematodes in the kinds of fatty acids present and amounts of total lipid. The fatty acid composition differs quantitatively from most previous findings in that 18:1 fatty acids were not predominant (8, 15-18). In our analysis of *G. solanacearum*, 20-C unsaturated fatty acids made up the bulk of polar and neutral lipids of the nematode. The predominant fatty acid in the neutral lipid classes (TRIG and FFA) was 20:4, and 20:1 was predominant in the PL class. That is in contrast with previous reports that 18:1 was the predominant fatty acid in five other species of plant-parasitic nematodes (15). Also of interest was the finding that neutral lipids were the major source of polyunsaturated fatty acids and that both polar and neutral classes contained high percentages of polyunsaturates. Again, this contrasts with reports on other plant-parasitic nematodes (15). *Globodera rostochiensis*, a plant parasite, has recently been reported (2) to contain the same major fatty acid components we detected in *G. solanacearum* (20:4, 20:1, and 18:1). Those are the only reports we know of that found highly unsaturated fatty acids other than 18:1 as the major components of plant-parasitic nematodes.

A variety of hydrocarbons occur in *G. solanacearum*, the majority of which are

probably branched-chained or unsaturated. The concentration of hydrocarbons in this nematode contributes little to the overall lipid concentration. Whether such analyses are of value in relation to the physiology or biochemistry of nematodes awaits further evaluation.

The sterol composition is of considerable interest because of the high percentage of stanols that occur in the nematode, and the low concentration of total sterol observed. The only stanols reported in nematodes were in the animal parasite *Ascaris lumbricoides* (4) and the plant parasite *R. reniformis* (25). In *A. lumbricoides*, cholest-5-en-3β-ol, 24-methyl-5α-cholestan-3β-ol, and 5α-stigmastan-3β-ol combined made up of 38% of the total sterols present. In *R. reniformis*, 5α-cholestan-3β-ol, 24-methyl-5α-cholestan-3β-ol, 5α-stigmastan-3β-ol, and an unidentified stanol made up about 5.0% of the total sterol composition.

Because there have been only a few reports of stanols in higher plants (12, 19, 20) we wonder why stanols occur in *A. lumbricoides*, *R. reniformis*, and now in *G. solanacearum*, sometimes in rather large quantities. Either the nematode is concentrating small amounts of stanols which occur naturally in the plant tissues and/or it is saturating the more frequently observed phytosterols (campesterol, stigmasterol, β-sitosterol) to satisfy some of its own sterol requirements.

The occurrence of a sterol with a totally saturated steroid nucleus and a single unsaturation in the side chain is rare in plants (10). The occurrence of a sterol resembling 5α-stigmast-22-en-3β-ol in *G. solanacearum* along with a large percentage of stanols suggests that the nematode has the hydrogenase enzymes necessary to saturate the phytosterols.

Other interesting observations made from this study are the very low sterol concentrations (about 0.01% of dry wt) and the very high unsaturated fatty acid concentrations of *G. solanacearum*. Krusberg (16) has pointed out that animal-parasitic nematodes are generally lower in total lipid than plant-parasitic forms. The reverse seems true for sterols, for they made up 0.21% and 0.38% of the respective dry weights of *A. lumbricoides* (4) females and males. That is considerably higher than for the plant nematodes *G. solanacearum* (0.01%), *Ditylenchus triformis* (0.09%), and *Ditylenchus dipsaci* (0.06%). Madosingh *et al.* (22) observed that sterol synthesis in rats is inhibited by unsaturated fatty acids. Those workers indicated that unsaturated fatty acids inhibit the rate-limiting enzyme hydroxymethylglutaryl CoA reductase, required in the production of mevalonate, which is required for sterol synthesis. Since nematodes cannot incorporate mevalonic acid into sterol biosynthesis (1, 5, 27) it is unlikely the same mechanism is operating in nematodes in controlling sterol levels.

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