Journal of Nematology 17(1):64–68. 1985.

Sterol Composition of the Corn Cyst Nematode, *Heterodera zeae*, and Corn Roots

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Abstract: Sterols from free sterol and steryl ester fractions from *Heterodera zeae* and from total lipids of *Zea mays* roots were analyzed by gas-liquid chromatography (GLC) and by GLC-mass spectrometry. The major free sterols of *H. zeae* were 24-ethylcholesterol (54.4% of total free sterol), 24-ethylcholesta-5,22-dien-3β-ol (13.3%), 24-methylcholesterol (12.5%), and cholesterol (7.2%). The same four sterols comprised 34.6%, 7.2%, 30.3%, and 18.6%, respectively, of the esterified sterols of *H. zeae*. Corn root sterols included 46.6% 24-ethylcholesta-5,22-dien-3β-ol, 16.7% 24-methylcholesterol, 16.4% cycloartenol, 12.7% 24-ethylcholesterol, and 0.5% cholesterol. The sterol composition of *H. zeae* differed greatly from that of the only other cyst nematode previously investigated, *Globodera solanacearum*.

Key words: maize, mass spectrometry, steryl esters, *Zea mays*.

The nutritional requirement for sterol and the lack of de novo sterol biosynthesis in nematodes has been known for several years (2). Nematode sterol metabolism has been the subject of renewed interest because of findings that several compounds with nematicidal activity toward plant-parasitic (10) and animal-parasitic (9) nematodes strongly interfere with the metabolism of plant sterols by the free-living nematode *Caenorhabditis elegans* (5,18).

Because of the difficulty in axenic propagation, investigation of sterol metabolism in plant-parasitic nematodes has necessarily been limited to comparisons of sterol compositions of host and parasite. For example, *Ditylenchus dipsaci* and *Rotylenchulus reniformis* contain much larger proportions of cholesterol than do their hosts (7,25), with the speculation being that *R. reniformis* most likely produces cholesterol by removal of the C-24 methyl or ethyl groups typical of plant sterols (25). Similarly, the surprisingly high percentage of saturated sterols (stanols) in *Globodera solanacearum* has led to the conclusion that this cyst nematode either concentrates trace quantities of naturally occurring plant stanols or else saturates the predominantly unsaturated phytosterols (20). The purpose of the present investigation was to further examine phytosterol dealkylation and saturation in plant-parasitic nematodes by comparison of the sterol compositions of the corn cyst nematode, *Heterodera zeae*, and corn roots.

Materials and Methods

Culture methods: Zea mays 'Pioneer 3184' seeds were planted in quartz sand infested with mature cysts of *H. zeae* (Kent County, Maryland, population). Plants were maintained in a growth chamber at 29 C for 9 weeks, at which time cysts were washed from roots and sand and collected on a 250-μm-pore sieve. The cysts were purified by flotation in 40% sucrose, repeated rinsing with distilled water and centrifugation, and manual removal of the remaining debris with forceps under a dissecting microscope. The cysts were immediately frozen and lyophilized. Root systems from noninfected 'Pioneer 3184' corn were similarly propagated, rinsed with water, frozen, and lyophilized.

Lipid extraction: The lyophilized cysts (170–716 mg dry weight) were homogenized three times in a Ten Broeck tissue homogenizer with 40-ml portions of chloroform/methanol 2:1 (v/v) (11). Lyophilized roots were similarly extracted with three 250-ml portions of chloroform/methanol 2:1 in a Virtis homogenizer. The residue was subsequently extracted with the same solvent in a Soxhlet apparatus for 20 hours, and the residue from the Soxhlet extraction was saponified 4 hours at 65 C in 50 ml of 4% methanolic potassium hydroxide to remove any tightly bound sterols. The crude lipid extracts were purified

Received for publication 19 June 1984.

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The authors thank Malcolm J. Thompson of the Insect Physiology Laboratory for supplying authentic reference sterols. Mention of a company name or a proprietary product does not imply endorsement by the U.S. Department of Agriculture.

by partition between chloroform/methanol/0.88% NaCl 8:4:3 (11).

Sterol isolation: Nematode neutral lipids were isolated from lipid extracts by elution with 250 ml $CHCl_3$ through a 9-g column (11-mm i.d.) of 70–230-mesh silica gel 60 (E. Merck, Darmstadt, W. Germany). The neutral lipids were then fractionated on similar columns by elution with 35-ml portions of 0, 2, 5, 10, 22, 30, 50, and 100% diethyl ether in hexane; these fractions contained hydrocarbons, steryl esters, unidentified substances, triglycerides, fatty alcohols and free 4-methylsterols, free 4-desmethylsterols and 1,3-diglycerides, 1,3- and 1,2-diglycerides, and monoglycerides, respectively. Sterols in the steryl ester fraction were isolated by saponification for 4 hours at 65 C in 2 ml of 4% methanolic potassium hydroxide, addition of 2 ml H_2O, partition three times with 4-ml portions of hexane, and subsequent separation of the combined hexane extracts on silicic acid columns as described earlier. The free 4-desmethylsterol fraction was similarly saponified and purified to separate the 4-desmethylsterols from diglyceride contaminants.

Corn root lipid extracts were not fractionated but instead were directly saponified in 10 ml of 4% methanolic KOH. The 4-methylsterols and 4-desmethylsterols were purified as described above.

Sterol identification: Sterols were analyzed by gas-liquid chromatography (GLC) in a Varian model 3700 gas chromatograph fitted with a flame ionization detector and with packed glass columns (2-mm i.d. × 2 m) containing 2% SE-30 or 2% OV-17 liquid phases as well as a 12.8-m × 0.25-mm J & W DB-1 fused silica capillary column (0.25-μm film). Quantification was obtained by use of a Shimadzu model C-R1B recording integrator. Sterols were acetylated at room temperature for 16 hours in 0.8 ml of pyridine/acetic anhydride 3:1; the steryl acetates were purified by elution with 5% diethyl ether in hexane on silicic acid columns and were analyzed by GLC similarly to the free sterols. Tentative identifications were confirmed by gas chromatography-mass spectrometry (GC-MS) of the acetates in a Finnigan model 4510 instrument equipped with a 15-m × 0.32-mm DB-1 capillary column and connected to an Incos data system.

Steryl acetates were subsequently fractionated according to degree and position of unsaturation by chromatography on 11-mm-i.d. glass columns containing 4.5 g silicic acid coated with 20% silver nitrate by elution with 20-ml portions of 0, 1, 2, 3, 4, 5, 6, 10, and 25% diethyl ether in hexane (26). The 2% ether fraction was collected in 1-ml aliquots to completely separate stanols from monoene sterols. Column fractions were analyzed by argentation thin-layer chromatography (5) and GLC.

With the exception of argentation columns, all column chromatographic separations were monitored by thin-layer chromatography (TLC) on precoated Anasil H chromatoplates (Analabs, North Haven, Connecticut) with a solvent system of hexane/diethyl ether/acetic acid 80:20:1. Results are presented as the means of three separate harvests of nematodes and two separate harvests of noninfected roots.

Results

Lipid comprised approximately one-third of the dry weight of *H. zeae* cysts (Table 1). Gravimetric analysis of silicic acid column fractions examined by TLC indicated that neutral lipids comprised 87.5% of the total lipid and that triglycerides comprised 85% of the neutral lipid. Small quantities of diglycerides, monoglycerides, free fatty acids, free sterols, fatty alcohols, and steryl esters were also identified by TLC. Subsequent GLC quantification indicated that sterols comprised 0.049% of the dry weight of *H. zeae* cysts, with most of the sterols being nonesterified (Table 1). The polar lipid fraction from *H. zeae* contained very little sterol, comprising less than 1% of that of the neutral lipid sterol.

Corn roots contained 7.2% lipid and 0.55% sterol on a dry weight basis (Table 1). The Virtis homogenizer, Soxhlet apparatus, and saponified residue extracts contained, respectively, 79.2%, 20.6%, and 0.2% of the total lipid and 98.8%, 1.0%, and 0.2% of the total sterol.

All sterols from *H. zeae* and *Z. mays* were identical to authentic standards by GLC (Table 2), argentation TLC, and GC-MS. However, we lacked authentic material for 24-ethyl-5α-cholest-22-en-3β-ol, cycloartanol, 24Z-ethylidene-5α-cholest-7-en-3β-ol, and 24-methylenecycloartanol. The relative retention times (RRTs) of the free

TABLE 1. Lipid and sterol content of *Heterodera zeae* cysts and *Zea mays* roots.

	H. zeae	*Z. mays*
% of dry weight as lipid	35.6	7.2
% of lipid as sterol	0.14	7.7
% of dry weight as sterol	0.049	0.55
% of total sterol as esterified sterol	15.4	*

* Not determined.

TABLE 2. Gas-liquid chromatographic relative retention times of sterols from *Heterodera zeae* or *Zea mays*, expressed relative to cholesterol. GLC was performed isothermally on a DB-1 fused silica capillary column (12.8 m × 0.32-mm i.d., 0.25-μm film) or on packed glass columns (2 m × 2-mm i.d.) containing 2% SE-30 or 2% OV-17 stationary phases.

	DB-1	SE-30	OV-17
Cholesterol	1.00	1.00	1.00
Cholestanol	1.02	1.02	1.02
24-methylcholesta-5,22-dien-3β-ol	1.11	1.11	1.14
Lathosterol	1.12	1.12	1.19
24-methylenecholesterol	1.27	1.28	1.37
24-methylcholesterol	1.29	1.30	1.33
24-methylcholestanol	1.31	1.32	1.35
24-ethylcholesta-5,22-dien-3β-ol	1.40	1.41	1.45
24-ethyl-5α-cholest-22-en-3β-ol	1.43	1.43	1.47
24-ethylcholesterol	1.60	1.63	1.67
24-ethylcholestanol	1.63	1.65	1.69
Isofucosterol	1.66	1.68	1.85
Cycloartanol	1.70	1.72	1.67
24-ethyllathosterol	1.81	1.83	1.96
Cycloartenol	1.86	1.89	2.05
24Z-ethylidene-5α-cholest-7-en-3β-ol	1.89	1.90	2.20
24-methylenecycloartanol	2.14	2.15	2.28

Trivial and systematic names: cholesterol, cholest-5-en-3β-ol; cholestanol, 5α-cholestan-3β-ol; lathosterol, 5α-cholest-7-en-3β-ol; 24-methylenecholesterol, 24-methylenecholest-5-en-3β-ol; 24-methylcholesterol, 24-methylcholest-5-en-3β-ol; 24-methylcholestanol, 24-methyl-5α-cholestan-3β-ol; 24-ethylcholesterol, 24-ethylcholest-5-en-3β-ol; 24-ethylcholestanol, 24-ethyl-5α-cholestan-3β-ol; isofucosterol, 24Z-ethylidene-cholest-5-en-3β-ol; cycloartanol, 4,4,14α-trimethyl-9β,19-cyclo-5α-cholestan-3β-ol; 24-ethyllathosterol, 24-ethyl-5α-cholest-7-en-3β-ol; cycloartenol, 4,4,14α-trimethyl-9β,19-cyclo-5α-cholest-24-en-3β-ol; 24-methylenecycloartanol, 4,4,14α-trimethyl-24-methylene-9β,19-cyclo-5α-cholestan-3β-ol.

sterols vs. cholesterol were identical to those of the acetate derivatives vs. cholesteryl acetate, except for the three 4,4-dimethylsterols (cycloartanol, cycloartenol, and 24-methylenecycloartanol), which had the following RRTs as their respective acetates: 1.59, 1.75, and 2.00 on DB-1; 1.61, 1.77, and 2.01 on SE-30; and 1.53, 1.85, and 2.06 on OV-17. Such large decreases in RRTs as acetates are characteristic of 4,4-dimethylsterols (21,26).

Compounds for which we lacked authentic reference material were identified as steryl acetates by argentation column chromatography, argentation TLC, GC-MS, and GLC RRTs (Table 2) that deviated no more than 0.01 unit from calculated values (21). For example, 24-ethyl-5α-cholest-22-en-3β-yl acetate emerged from the argentation column between the stanyl acetates and Δ^5-steryl acetates. Its mass spectrum [mass/charge m/z (relative intensity): 456 (9), 353 (9), 344 (14), 329 (2), 317 (2), 315 (19), 257 (36), 255 (8), 215 (5) and 55 (100)] was similar to literature spectra (17,27) and included two fragments (m/z 353 and 344) indicative of a C-22 double bond (12,15,17). The acetate of 24Z-ethylidene-5α-cholest-7-en-3β-ol migrated slightly faster than isofucosteryl acetate during argentation chromatography. Its mass spectrum contained a molecular ion at m/z 454 (1); abundant fragments at m/z 356 (25) and 313 (100) indicated a fragmentation pattern typical of a $\Delta^{7,24(28)}$-sterol (24). The mass spectrum of cycloartanyl acetate [m/z: 470 (1), 455 (2), 410 (8), 395 (9), 367 (3), 357 (1), 355 (1), 341 (3), 297 (6), 288 (10), 273 (3), and 55 (100)] largely consisted of fragments two m/z units larger than those in a spectrum of authentic cycloartenyl acetate and was similar to a literature spectrum (13). In the mass spectrum of 24-methylenecycloartanyl acetate [m/z: 482 (1), 467 (1), 422 (9), 407 (6), 379 (4), 353 (1), 300 (4), 297 (2), 285 (1), and 55 (100)], all side-chain containing fragments were 14 m/z units greater than those in the spectrum of authentic cycloartenyl acetate.

The major sterols of *H. zeae* were cholesterol, 24-methylcholesterol, 24-ethylcholesta-5, 22-dien-3β-ol, and 24-ethylcholesterol (Table 3). With the exception of cholesterol, these were also the most abundant sterols in *Z. mays* roots. In addition, the 4-methylsterol fraction of *Z. mays* contained large quantities of cycloartenol and smaller amounts of cycloartanol and 24-methylenecycloartanol, whereas corresponding fractions from *H. zeae* cysts did not contain these or any other 4α-methyl- or 4,4,14α-trimethylsterols. (The limit of

TABLE 3. Relative percentages of sterols in *Zea mays* roots and in free sterol and steryl ester fractions from *Heterodera zeae* cysts.

	H. zeae		
	Free sterols	Steryl esters	*Z. mays*
Cholesterol	7.2	18.6	0.5
Cholestanol	0.4	0.5	0.02
24-methylcholesta-5,22-dien-3β-ol	0.5	1.0	0.1
Lathosterol	0.2	0.6	0.1
24-methylenecholesterol	0.1	0.6	0.1
24-methylcholesterol	12.5	30.3	16.7
24-methylcholestanol	1.8	1.4	1.5
24-ethylcholesta-5,22-dien-3β-ol	13.3	7.2	46.6
24-ethyl-5α-cholest-22-en-3β-ol	3.1	1.1	1.4
24-ethylcholesterol	54.4	34.6	12.7
24-ethylcholestanol	5.1	1.7	1.0
Isofucosterol	0.7	1.4	0.3
Cycloartanol	*	*	1.5
24-ethyllathosterol	0.5	0.6	0.3
Cycloartenol	*	*	16.4
24Z-ethylidene-5α-cholest-7-en-3β-ol	0.3	0.4	0.5
24-methylenecycloartanol	*	*	0.3

* Not detected.

detection was 20 ng, or 0.01 to 0.02% of the total *H. zeae* sterol).

Although the same sterols were present in both the steryl ester and free sterol fractions from *H. zeae*, the relative proportions of several of the sterols differed (Table 3). The steryl ester fraction contained greater percentages of cholesterol and 24-methylcholesterol, whereas the free sterol fraction contained substantially larger proportions of 24-ethylcholesta-5,22-dien-3β-ol and 24-ethylcholesterol.

DISCUSSION

The high lipid content, high neutral lipid content, and abundance of triglycerides in *H. zeae* are features shared by most other Heteroderoidea thus far examined (3,6,16,20). The sterol content of *H. zeae* (0.05%) is several times that of the related cyst nematode *G. solanacearum* (0.01%) and is approximately that of *D. dipsaci* (0.06%) (7,20). The predominance of free sterols in *H. zeae* is a characteristic of *C. elegans* (5) but not *D. dipsaci*, in which free and esterified sterols occur in approximately equal proportions (7).

Our results confirm previous findings that 24-ethylcholesta-5,22-dien-3β-ol is the principal sterol and 24-ethylcholesterol and 24-methylcholesterol are other major sterols in *Z. mays* roots (1,22). Although cholesterol is not usually abundant in plant material, small quantities have previously been detected in corn roots (14,22). Similarly, cycloartenol has previously been reported as the major 4,4,14α-trimethylsterol of corn roots (22). To our knowledge, cholestanol, 24-methylcholesta-5,22-dien-3β-ol, 24-ethyl-5α-cholest-22-en-3β-ol, and 24Z-ethylidene-5α-cholest-7-en-3β-ol have not previously been detected in corn roots.

With the exception of the three 4,4,14α-trimethylsterols in *Z. mays*, the sterols from *H. zeae* and corn roots were identical. However, sterol fractions from *H. zeae* did contain substantially larger proportions of cholesterol and 24-ethylcholesterol and smaller percentages of 24-ethylcholesta-5,22-dien-3β-ol than did its host. Because every sterol of *H. zeae* was identified in *Z. mays*, differences in sterol composition between the nematode and its host could result from selective uptake, metabolism of plant 24-alkylsterols, or both. Although unequivocal demonstration of phytosterol metabolism in *H. zeae* would require use of radiolabelled phytosterols in an axenic culture system, *C. elegans* and *Turbatrix aceti* are capable of C-24 ethyl group removal (5,8), and *C. elegans* possesses the ability to reduce the C-22 double bond (Lozano, pers. comm.). However, because a few plant-feeding insects lack the phytosterol dealkylation pathway present in most phytophagous insects (23), one should exercise caution in extrapolation of results obtained from one nematode species to another.

One of the more interesting aspects of nematode sterol metabolism is the recently discovered ability of *C. elegans* to produce 4-methylsterols from 4-desmethylsterol precursors (4), a pathway not reported in any other organism. Although not detected in *H. zeae* cysts, 4-methylsterols could possibly occur in other life stages of this nematode.

Cholesterol, 7-dehydrocholesterol, and lathosterol are the most commonly abundant sterols reported to occur in nematodes. To our knowledge, 24-methylcholesta-5,22-dien-3β-ol and 24Z-ethylidene-5α-cholest-7-en-3β-ol have not previously been detected in nematodes. Perhaps the most striking comparative difference between *H.*

zeae and other nematodes is the relative lack of stanols in *H. zeae,* compared to the only other cyst nematode examined thus far, *G. solanacearum,* in which stanols comprise a majority of the sterols (20). It is possible that this difference is a function of differing ages of the females analyzed or is host related, although stanols have been detected in higher plants only in trace quantities (19). Whether other *Globodera* and *Heterodera* species resemble *G. solanacearum* or *H. zeae* in stanol abundance has not been determined, but the possibility that the two major genera of cyst nematodes could have characteristic sterol compositions warrants further investigation of cyst nematode sterols.

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