Journal of Nematology 31(2):172–183. 1999.

In-vitro Assays of *Meloidogyne incognita* and *Heterodera glycines* for Detection of Nematode-antagonistic Fungal Compounds

James K. Nitao, Susan L. F. Meyer, and David J. Chitwood[1]

Abstract: In-vitro methods were developed to test fungi for production of metabolites affecting nematode egg hatch and mobility of second-stage juveniles. Separate assays were developed for two nematodes: root-knot nematode (*Meloidogyne incognita*) and soybean cyst nematode (*Heterodera glycines*). For egg hatch to be successfully assayed, eggs must first be surface-disinfested to avoid the confounding effects of incidental microbial growth facilitated by the fungal culture medium. Sodium hypochlorite was more effective than chlorhexidine diacetate or formaldehyde solutions at surface-disinfesting soybean cyst nematode eggs from greenhouse cultures. Subsequent rinsing with sodium thiosulfate to remove residual chlorine from disinfested eggs did not improve either soybean cyst nematode hatch or juvenile mobility. Soybean cyst nematode hatch in all culture media was lower than in water. Sodium hypochlorite was also used to surface-disinfest root-knot nematode eggs. In contrast to soybean cyst nematode hatch, root-knot nematode hatch was higher in potato dextrose broth medium than in water. Broth of the fungus *Fusarium equiseti* inhibited root-knot nematode egg hatch and was investigated in more detail. Broth extract and its chemical fractions not only inhibited egg hatch but also immobilized second-stage juveniles that did hatch, confirming that the fungus secretes nematode-antagonistic metabolites.

Key words: bioassay technique, biological control, culture broth, egg hatch, fungus, *Fusarium equiseti, Heterodera glycines,* in-vitro assay, *Meloidogyne incognita,* microbial secondary metabolites, nematicide, nematode.

The search for new microbial strains to use as sources of biological nematicides is an important goal for those seeking to reduce the significant economic damage caused by plant-parasitic nematodes. Fungi exhibit a range of specificities and modes of action in their antagonistic activity toward nematodes, offering an extensive pool of potential candidates to test (Siddiqui and Mahmood, 1996). Like other microbes, fungi can directly parasitize nematodes or secrete nematicidal metabolites and enzymes that affect nematode viability. Evaluating large numbers of fungal strains for nematode-antagonistic properties in greenhouse studies on plants can be restrictively labor intensive and time consuming. A rapid in-vitro method to screen fungal isolates for their potential to secrete nematicidal compounds would assist in targeting fungi to investigate in greater detail. Such an approach has been used successfully with other microbial organisms. For example, Becker et al. (1988) increased the efficiency of their search for rhizobacterial strains to evaluate as control agents of *Meloidogyne incognita* (Kofoid & White) Chitwood by first selecting those strains that produced nematicidal compounds in vitro.

We report procedures developed to test fungi in vitro for production of metabolites affecting root-knot nematode (*M. incognita*) and soybean cyst nematode (*Heterodera glycines* Ichinohe). The assays are used to detect effects on egg hatch and mobility of second-stage juveniles (J2). Our objective was to develop techniques that would permit efficient screening of large numbers of fungal strains. Because microbial growth can interfere with assay results, eggs free of microbial contamination were needed. Although uncontaminated eggs can be produced from monoxenic culture on root explants, obtain-

Received for publication 8 October 1998.

[1] USDA ARS, Nematology Laboratory, Bldg. 011A, Rm. 165B, BARC-West, 10300 Baltimore Avenue, Beltsville, MD 20705-2350.

Email: jnitao@asrr.arsusda.gov

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

The authors thank Gregory Tylka, Patricia Donald, and Linda Rehberger for advice during early stages of the project; Jean Juba (Fusarium Research Center, Pennsylvania State University) and Richard Humber (USDA-ARS, Plant Protection Unit, NY) for fungal identification; Sandra Sardanelli for root-knot nematode eggs during a brief but critical period; Paula Crowley, Christina Bennett, Robert Lee, and Steve Rogers for technical support; and Lynn Carta and Zafar Handoo for comments on the manuscript.

This paper was edited by A. F. Robinson.

ing large numbers of eggs from root explant cultures can be difficult. Alternatively, nematode cultures maintained on greenhouse plants can provide an abundant supply of eggs if the eggs can be surface-disinfested. Therefore, an important goal in developing assay procedures was to find efficacious methods to disinfest the surfaces of eggs collected from the roots of potted plants without damaging the eggs. Additionally, various media for culturing fungi were tested to determine if they affected egg hatch of either nematode, and whether dilution of media would improve egg hatch.

The application of the techniques developed and the preparation of fungus culture broths for assay are illustrated with *M. incognita* and a *Fusarium equiseti* strain. Preliminary tests had indicated that culture broths of this strain possessed nematode-antagonistic activity. To confirm that the activity of culture filtrate was due to metabolites secreted by the fungus, the *F. equiseti* culture broth was chemically extracted, fractionated, and assayed further.

Materials and Methods

Media: Cornmeal, V8 Juice, and yeast/lactose broth media were made by omitting agar from standard solid media protocols. Cornmeal broth was a modification of cornmeal agar medium (Dhingra and Sinclair, 1995). V8-Juice broth was made by leaving out agar and $CaCO_3$ from V8-Juice agar medium (Stevens, 1974); pH was adjusted to 7.2 with 1 N KOH. Yeast extract/lactose broth was a modification of yeast extract/lactose agar medium (Stevens, 1974); this was formulated to contain six times the proportion of yeast extract and lactose specified in the original medium to support higher fungal biomass production. Soybean broth was made by autoclaving 1.5 g dry soybeans and 50 ml water at 121 °C for 30 minutes in an open beaker, filtering the broth through cheesecloth, adding water to return the total volume to 50 ml, and sterilizing in an autoclave for 15 minutes. Potato dextrose broth (PDB) and potato dextrose agar (PDA) were reconstituted from commercially prepared media (Difco Laboratories, Detroit, MI). Gamborg's B5 culture broth (B5) was made by omitting agar from a commercial preparation (Gibco BRL, Life Technologies, Grand Island, NY); pH was adjusted to 6.2 with 1 M KOH.

Antibacterial and antibacterial-antimycotic solutions were examined for the ability to inhibit microbial growth when added to the hatching medium. Stock solutions were made by reconstituting preformulated lyophilized powders with sterile water (P-3664 and A-7292, Sigma Chemical, St. Louis, MO) and were used within 2 weeks. Antibacterial stock solution consisted of 5,000 units penicillin, 5 mg streptomycin, and 10 mg neomycin/ml in 0.9% sodium chloride. Antibacterial-antimycotic stock solution consisted of 10,000 units penicillin, 10 mg streptomycin, and 25 µg amphotericin B per milliliter in 0.9% sodium chloride. Solutions were mixed into the water control or the culture media, resulting in a final concentration of 1% of the antibacterial or antibacterial-antimycotic stock solutions after combining with the nematode egg suspensions. Significant microbial contamination was monitored by visual examination of assay media under a dissecting scope.

Deionized water was used for all preparations, including media, water controls, and egg suspensions.

Culturing and processing fungus: Fusarium equiseti (Corda) Sacc. (Nematology Lab #L128) was originally isolated from *H. glycines* cysts from the People's Republic of China (Meyer, unpubl.). One-week-old *F. equiseti* cultures on PDA plates (100 × 15 mm) were homogenized into PDB medium (1 PDA plate/1 liter flask containing 250 ml PDB) and incubated at 25 °C on a shaker (240 rpm) for 3 and 7 days. After incubation, the culture broth was centrifuged at 13,700 *g* for 20 minutes, and the supernatant was sequentially passed through syringe filters designed for viscous samples containing particulates (GD/X series 1.0 µm GF/B and 0.45 µm GMF filters, Whatman, Clifton, NJ) and then sterile-filtered (GD/X sterile 0.2 µm PES filter, Whatman).

Disinfesting and assay of root-knot nematode

eggs: Sodium hypochlorite is often used to disinfest plant-parasitic nematode eggs or to separate them from their surrounding gelatinous matrix (Hussey and Barker, 1973; Loewenberg et al., 1960; McClure et al., 1973; Sankaralingam and McGawley, 1994). We modified these techniques to surface-disinfest *M. incognita* eggs for use in assays of culture broths by reducing agitation time and intensity to minimize damage to eggs. *Meloidogyne incognita* cultures were maintained in the greenhouse on tomato plants (*Lycopersicon esculentum* cv. Orange Pixie). Disinfestation and assay preparation were conducted with sterile techniques in a laminar flow hood. For each assay, approximately 45 egg masses were handpicked from the roots, pooled, and agitated in 0.5% sodium hypochlorite for 2 minutes by vigorously drawing the egg suspension in and out of a Pasteur pipet. Debris was allowed to settle from the egg suspension for 30 seconds. Eggs still in suspension were pipetted onto an autoclaved 500-mesh sieve (pore size 25 µm) and washed with 300 ml of sterile water over a 5-minute period. Eggs were washed off the sieve with 10 ml of sterile water into an autoclaved beaker and used directly for assay after an aliquot was removed to estimate the number of eggs per milliliter. Assays were conducted in sterile 24-well tissue culture plates (polystyrene, flat bottom wells; Corning, New York, NY). To test hatch rate under these conditions, 0.1 ml of egg mixture was combined with 0.9 ml of either water or PDB in a well (140 eggs/well). Treatments were each replicated in five wells arranged randomly in the plates.

Tissue culture lids were sealed shut with parafilm, and the plates were placed in 25 °C incubators. After 2 weeks, hatched J2 were counted with the use of an inverted microscope (Nikon TMS, ×2 objective, total magnification ×20). To facilitate counting J2 in the wells, grid lines 2 mm apart were scratched onto the undersides of the tissue-culture plates.

Bioassay of Fusarium equiseti *culture broth and fractionated broth against root-knot nematode:* The assay procedure was tested by measuring *M. incognita* egg hatch in *F. equiseti* culture broth filtrates. Eggs were combined in wells (140 eggs/well) with broth filtrates of 3- and 7-day-old *F. equiseti* cultures, and with water and PDB controls as described in ''Assays of root-knot nematode.'' Treatments were replicated in five wells except for the PDB control, which had four replicates. The bioassay was repeated with five replicates per treatment and 215 eggs/well.

To further test the utility of the assay for detecting nematode-antagonistic compounds and to verify the existence of such metabolites, the fungal broth was chemically extracted and examined further (Fig. 1). *Fusarium equiseti* was grown in PDB medium for 3 days as described above. A total of 6.6 liters of culture broth was processed in four batches by centrifuging at 13,700 *g* for 20 minutes and filtering the supernatant through a coarse-pore glass fritted funnel. Amberlite XAD-16 gel previously washed with methanol and equilibrated in water was added to the broth supernatant in a ratio of 1 volume XAD gel to 6.5 volumes supernatant, and the mixture was placed on a rotary shaker for 1 hour. The broth and gel were poured into a coarse-pore fritted glass funnel where the broth was filtered off with vacuum and discarded. The XAD gel was washed with water (2.5 volumes water × gel bed volume), which was discarded, and then with methanol (2.5 volumes methanol × gel bed volume). The methanol eluate was dried under vacuum (2.7 g), and a portion of the residue (2.5 g) was dissolved in 7% methanol in water and extracted three times

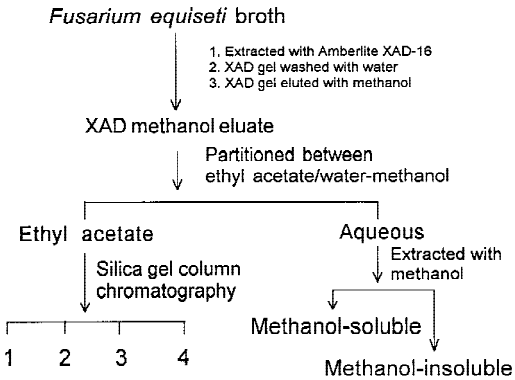

FIG. 1. Outline of chemical extraction and fractionation of *Fusarium equiseti* culture broth.

with ethyl acetate. A portion of the aqueous methanol layer (''aqueous fraction'') was dried under vacuum (0.87 g) and extracted three times (10 ml methanol each), resulting in a methanol-soluble fraction (0.09 g) and a methanol-insoluble residue (0.66 g). The XAD methanol eluate, the aqueous fraction, the ethyl acetate fraction, and the methanol-soluble and methanol-insoluble portions of the aqueous fraction were bioassayed. The ethyl acetate layer was dried under vacuum (0.11 g) and chromatographed on 9.4 g of silica gel (Merck 40–63 μm silica gel, 20:1 chloroform-methanol, increasing methanol to 100% methanol), resulting in 8 fractions: 20:1 chloroform-methanol (15 ml), 10:1 chloroform-methanol (3 fractions, 10 ml each), 4:1 chloroform-methanol (3 fractions, 10 ml each), and 100% methanol (90 ml). After examination by thin-layer chromatography (Merck, silica gel, 250 μm, 60F-254; 8:1 chloroform-methanol; visualized with 20% sulfuric acid and charring), fractions containing shared components were combined into four fractions (fractions 1–4, 0.022–0.035 g each), which were then bioassayed.

Fractions were assayed for activity against *M. incognita* as described under ''Assays of root-knot nematode,'' with the following modifications. Prior to testing, fungal extracts were dried under high vacuum with a rotary vacuum pump and refrigerated condensor trap (Savant, Holbrook, NY) to remove all traces of organic solvent. Test solutions were made by dissolving fungal extracts in water; if necessary, extract residues were first dissolved in dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO) to solubilize less polar fractions, resulting in a final concentration of 0.5% DMSO. Test solutions were sterilized through 0.2-μm filters, and 0.9 ml of solution was combined with 0.1 ml egg suspension in wells (250 eggs/well). The XAD methanol eluate was tested at 4 μg/ml, 20 μg/ml, 100 μg/ml, and 500 μg/ml; control treatments consisted of a methanol XAD-16 extract of uninoculated PDB prepared identically to the fungal extract and assayed at the same concentrations. All other fungal chemical fractions were assayed at 100 μg/ml and were tested with control treatments of water and 0.5% DMSO in water (250 eggs/well). Preliminary studies indicated that 90% of final *M. incognita* egg hatch under these conditions was completed after 1 week and that viability of hatched J2 in control treatments remained high during this period. Consequently, mobile and immobile J2 and percent hatch were recorded after 1 week, and number of mobile juveniles as a percentage of total hatched juveniles was calculated.

Soybean cyst nematode: Chlorhexidine instead of sodium hypochlorite is commonly used to reduce microbial contamination during *H. glycines* hatching studies in water (Acedo and Dropkin, 1982; Thompson and Tylka, 1997). Our initial observations suggested that chlorhexidine treatment resulted in inconsistent hatching and insufficient efficacy when eggs were placed in fungal broths and culture media. Therefore, we explored other methods to disinfest *H. glycines* eggs. Due to the sensitivity of soybean cyst nematode egg hatch to environmental factors (Dropkin et al., 1958; Tefft et al., 1982), we also tested sodium thiosulfate as a means to neutralize any residual chlorine when sodium hypochlorite was used as a disinfestant.

Heterodera glycines was cultured in the greenhouse on soybean plants (*Glycine max* cv. Essex), and cysts were collected from the roots using a procedure modified from Jenkins (1964). Cysts were washed from the roots and collected on a 100-mesh sieve (pore size 150 μm) nested below a 60-mesh sieve (pore size 250 μm). The cysts were centrifuged for 3 minutes in water and then 3 minutes in sucrose solution (454 g/liter water) to remove debris and soil, and washed thoroughly with tap water on a 400-mesh sieve (pore size 38 μm). Small root fragments were decanted by flushing cysts with deionized water in a small beaker. For each assay, 200–250 tan-colored cysts containing eggs were handpicked and pooled in a 7-ml dounce tissue grinder. Disinfestation and assay preparation were conducted using sterile techniques in a laminar flow hood. Eggs were surface-disinfested as described in de-

tail below, either by crushing the cysts in water and washing with disinfestant on a sieve or by crushing cysts directly in disinfestant solution. The number of eggs per milliliter in the suspension was estimated by sampling an aliquot. Test media were added to egg suspensions in sterile 24-well tissue culture plates, and treatments were replicated in five wells in a randomized arrangement unless noted otherwise. Assays were incubated at 25 °C for 2 weeks and evaluated as with root-knot nematodes.

Soybean cyst nematode eggs disinfested on sieve: Disinfestants tested were 5% formaldehyde, 0.5% sodium hypochlorite (household bleach), and 0.5% chlorhexidine diacetate (Sigma) solutions in water. Formaldehyde was investigated because of its usage as a surface disinfestant of eggs and immature stages of insects (Shapiro, 1984).

Formaldehyde, sodium hypochlorite, and chlorhexidine diacetate solutions were tested on eggs obtained from cysts crushed in sterile water. Eggs were: (i) washed with 0.5% sodium hypochlorite for 3 minutes on a 500-mesh sieve (pore size 25 µm), (ii) similarly washed with 5% formaldehyde solution, (or iii) washed with sterile water on a 500-mesh sieve followed by 0.5% chlorhexidine diacetate for 3 minutes. Eggs were rinsed with sterile water for 5 minutes and resuspended. Water, PDB, or Gamborg's B5 medium (0.9 ml) was combined with egg suspension (0.1 ml) in wells, resulting in a final concentration of 90% media for PDB and B5 treatments for treatments 1 and 2. Both treatments were prepared with and without antibacterial solution. For treatment 3, PDB was diluted with water before addition to the wells to improve hatch, resulting in a final concentration of 20%. Initial numbers of eggs per well were estimated to be 140 in the formaldehyde treatments, 205 in the hypochlorite treatments, and 505 in the chlorhexidine treatments. Corresponding controls received similar numbers of eggs.

Soybean cyst nematode cysts crushed in disinfestant: Disinfestants tested were antibacterial-antimycotic mixture, chlorhexidine diacetate, and sodium hypochlorite solutions in water. As much water as possible was pipetted out of the tissue grinder before adding disinfestant to the cysts.

Antibacterial-antimycotic solution (1% of the stock solution described under *Media* diluted in water), chlorhexidine diacetate (0.5%), and sodium hypochlorite (0.5%) were each tested individually by adding 10 ml of each solution to cysts held in a tissue grinder where cysts were crushed. For antibacterial-antimycotic and chlorhexidine treatments, the eggs were freed with agitation by vigorous bubbling with a Pasteur pipet and allowed to sit for 1 hour in antibacterial-antimycotic solution and 15 minutes in chlorhexidine diacetate. For sodium hypochlorite, eggs were agitated for 2 minutes by bubbling with air emerging from a Pasteur pipet, followed by a 30-second period to permit cyst debris to settle. Eggs were then transferred to a 500-mesh sieve (pore size 25 µm) where they were washed with sterile deionized water for 5 minutes and resuspended in 10 ml of sterile water. For antibacterial-antimycotic and chlorhexidine disinfested eggs, water, PDB, or Gamborg's B5 (0.9 ml) was combined with egg suspension (0.1 ml) in wells. Media were diluted before use to yield final concentrations of 50% and 90% in the wells after adding to the eggs, and antibacterial-antimycotic solution was added to all treatments. For sodium hypochlorite disinfested eggs, water or PDB (0.375 ml) was combined with egg suspension (0.125 ml) in wells. PDB was diluted before use to produce final concentrations of 75% and 90% in the assay, and treatments were tested with and without antibacterial-antimycotic solution. Initial numbers of eggs per well were estimated to be 330, 180, and 280 for the antibacterial-antimycotic, chlorhexidine diacetate assay, and sodium hypochlorite assays, respectively.

Effect of sodium thiosulfate rinse on soybean cyst nematode hatch and larval viability: Sodium thiosulfate neutralizes chlorine in solution and has been used to rinse insect eggs after surface disinfestion with sodium hypochlorite (Shapiro, 1984). We investigated whether soybean cyst nematode egg hatch and J2 viability were improved by rinsing

eggs with sodium thiosulfate solution after disinfestation with sodium hypochlorite. To test for effects on egg hatch, cysts were crushed in 0.5% sodium hypochlorite and agitated as described above. Half of the eggs were washed on a 500-mesh sieve (pore size 25 µm) with sterile filtered 10% sodium thiosulfate (Sigma) in water for 3 minutes, followed by a 5-minute water rinse. The remaining eggs were washed only with water for 5 minutes. Eggs suspended in 0.05 ml of water were combined with 0.45 ml water or PDB (diluted to 25%, 50%, 75%, or 90% final concentration) in a well. No antibacterial agents were added.

To test for effects of sodium thiosulfate rinse on subsequent J2 viability, J2 hatched from eggs treated with sodium thiosulfate and from control eggs washed only with water were examined for mobility. Eggs were surface disinfested with sodium hypochlorite as described above, and half were washed with sterile filtered 1% sodium thiosulfate for 3 minutes followed by sterile water for 5 minutes. The remaining eggs were washed only with sterile water for 5 minutes. The eggs were then transferred for hatching onto nylon screens (30-µm-pore size) in sterile water. The following day, 7–24 (mean 13) J2 were transferred in 0.2 ml of water to wells. Each treatment was replicated in 10 wells. Viability was measured by counting mobile and immobile J2 after 48 hours.

Soybean cyst nematode hatch in various liquid media: H. glycines eggs were placed in various culture media to investigate if hatch could be improved in media other than PDB. Eggs were surface-disinfested by crushing cysts in 0.5% sodium hypochlorite and rinsing with water as described above. Egg suspension (0.125 ml) and either water or medium (0.375 ml) were combined in wells, resulting in a final concentration of 75% medium. The media tested, cornmeal, V8 Juice, yeast extract/lactose, soybean, and potato dextrose broths, were chosen to provide a variety of osmotic concentrations and ingredient components. Antibacterial agents were not added.

Bioassay of Fusarium equiseti *culture broth against soybean cyst nematode:* Following selection of an optimal procedure for egg hatch, the usefulness of the procedure as a method for indicating presence of nematode-antagonistic compounds was tested by measuring *H. glycines* egg hatch in *F. equiseti* culture broth filtrates. Eggs were disinfested by crushing cysts in 0.5% sodium hypochlorite, and 0.125 ml of egg suspension was combined in wells (197 eggs/well) with 0.375 ml of broth filtrates of 3- and 7-day-old *F. equiseti* cultures, and water and PDB controls. Treatments were replicated in five wells.

Statistical analysis: Overall differences among means were tested using one- and two-way analyses of variance (ANOVA) and *t*-tests. Tukey's test was used to test significant differences among individual means if significant overall treatment effects were found at $P < 0.05$. When necessary, data were transformed before analysis to equalize variances among treatments or normalize data. When standard transformations failed to correct significant departures from assumptions of normality or equal variances, Mann-Whitney rank sum test and ANOVA and SNK tests on ranks were used. SigmaStat software (SPSS, Chicago, IL) was used for statistical analyses. Results are reported as mean ± standard error.

Results

Disinfesting and assay of root-knot nematode eggs: Agitation of *M. incognita* egg masses in 0.5% sodium hypochlorite for 2 minutes followed by a 30-second settling period was sufficient time for the eggs to be freed from the gelatinous matrix and to be surface-disinfested. Antibacterial agents or antimycotics were not needed to prevent microbial contamination. Of the total eggs, 42.1% ± 4.0 hatched in water and 81.5% ± 2.4 in 90% PDB. Hatched J2 remained mobile for at least a week.

Bioassay of Fusarium equiseti *culture broth and fractionated broth against root-knot nematode:* The pH of *F. equiseti* broth filtrates at the start of the assay was 7.0 for 3- and 7.6 for 7-day-old cultures. The pH of water and PDB broth controls ranged between 6.0–7.8 and 5.1–5.3, respectively. Average hatch of *M. in-*

cognita in water and PDB controls after 2 weeks was 45.5% ± 1.8 and 62.0% ± 1.1, respectively. In contrast, hatch was greatly reduced in *F. equiseti* culture broth filtrates and was only 5.6% ± 0.5 in broth from the 3-day-old culture and 17.4% ± 1.0 in broth from the 7-day-old culture. All means were significantly different from each other (Tukey's test, $P < 0.05$). The general pattern of egg hatch reduction was similar when the assay was repeated, although hatch inhibition was not as dramatic. Hatch in water and PDB controls was 40.2% ± 2.8 and 85.6% ± 2.9, respectively, compared to 22.1% ± 1.4 and 35.6% ± 4.0 in 3- and 7-day-old culture broth filtrates, respectively. Means were significantly different from each other except when hatch in the water control and 7-day-old culture broth was compared (Tukey's test). When the two trials were combined, overall hatch reduction was 29% and 16% compared to water controls, and 61% and 48% compared to PDB controls for 3- and 7-day-old cultures, respectively.

XAD methanol eluate from *F. equiseti* broth decreased *M. incognita* hatch and J2 mobility relative to those of controls, especially at high concentrations. Although average hatch in fungal extracts was slightly

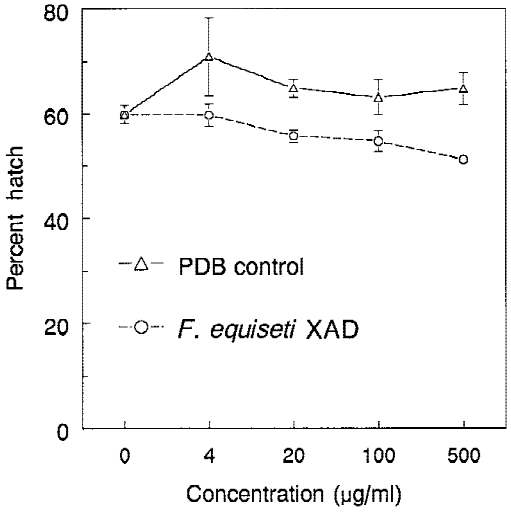

FIG. 2. Percentage of *Meloidogyne incognita* eggs hatched after 1 week in various concentrations of XAD-16 extracts of *Fusarium equiseti* broth (*F. equiseti* XAD) and potato dextrose broth (PDB) control. Data are means ± SE of five replications.

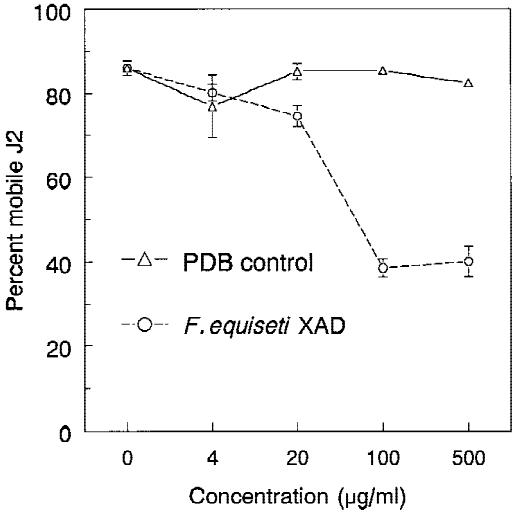

FIG. 3. Percentage of hatched *Meloidogyne incognita* J2 that were mobile 1 week after initiation of assay in various concentrations of XAD-16 extracts of *Fusarium equiseti* broth (*F. equiseti* XAD) and potato dextrose broth (PDB) control. Data are means ± SE of five replications.

lower than controls at concentrations of 20 µg/ml and higher, statistically significant reductions in hatch were detected at 20 µg/ml and 500 µg/ml but not at 100 µg/ml (*t*-test, $P < 0.05$) (Fig. 2). The XAD fungal extract affected J2 mobility more dramatically than it did egg hatch and elicited a concentration-dependent response up to 100 µg/ml (Fig. 3). Fungal XAD extract significantly affected larval mobility at concentrations of 20 µg/ml and higher when compared to controls (*t*-test, $P < 0.05$).

The fungal XAD methanol eluate was separated into aqueous- and ethyl acetate-fractions, and the aqueous fraction was further separated into methanol-soluble and methanol-insoluble fractions. Of these, only the ethyl acetate fraction significantly reduced egg hatch, resulting in a hatch rate half that of all other treatments including the DMSO control (Tukey's test, $P < 0.05$) (Fig. 4). A similar but more pronounced pattern was found with the percentage of mobile J2 after 1 week. The mean percentage of mobile J2 in the ethyl acetate fraction was significantly reduced compared to all other treatments (Tukey's test, $P < 0.05$). No other means were significantly different from each other (Fig. 4). Based on the

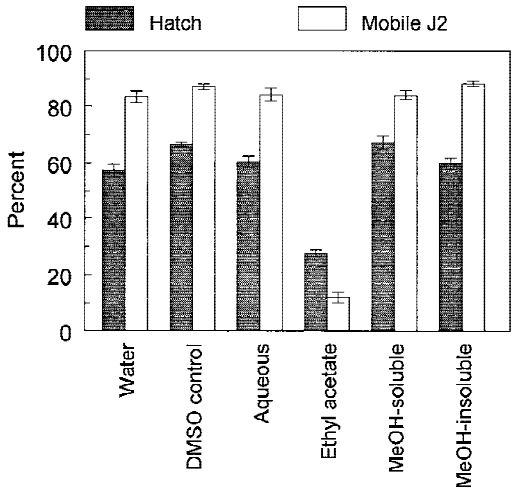

FIG. 4. Percentage of *Meloidogyne incognita* eggs hatched and percent of hatched larvae that were mobile 1 week after initiation of assay. Treatments were chemical fractions from the XAD-16 extract of *Fusarium equiseti* culture broth and tested at 100-µg/ml concentration. DMSO = dimethyl sulfoxide. MeOH = methanol. Data are means ± SE of five replications.

weights of fractions and the volume of culture broth extracted, the original concentrations in the broth are estimated to be 18 µg/ml for the ethyl acetate fraction and 4–6 µg/ml for the chromatographic fractions.

After the ethyl acetate fraction was separated on a silica gel chromatography column, all four resulting fractions reduced egg hatch (Fig. 5) compared to either DMSO or water control (Tukey's test, $P < 0.05$). Percent hatch in fractions 1, 2, and 3 ranged from 5% to 15%, and all were significantly lower relative to fraction 4 (35%) (Tukey's test, $P < 0.05$). All silica gel fractions reduced J2 mobility (ANOVA on ranks, $P < 0.05$) (Fig. 5). Mobility of J2 in fraction 2 was significantly lower than all other treatments (SNK multiple comparison test on ranks, $P < 0.05$), with less than 10% of hatched J2 showing movement compared to more than 95% in control treatments.

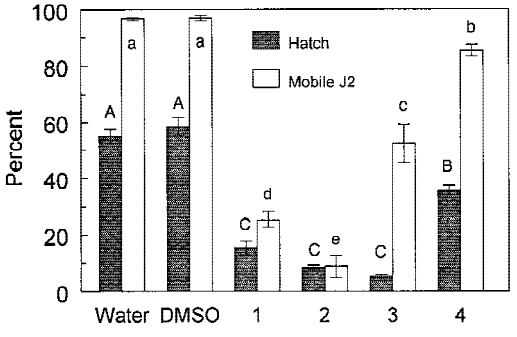

FIG. 5. Percentage of *Meloidogyne incognita* eggs hatched and percent of hatched larvae that were mobile 1 week after initiation of assay. Fractions 1–4 were silica gel chromatography fractions derived from the *Fusarium equiseti* ethyl acetate fraction and tested at 100-µg/ml concentration. DMSO = dimethyl sulfoxide. Data are means ± SE of five replications. Within hatch or mobility measures, means with the same letter are not significantly different (SNK test on ranks).

Soybean cyst nematode eggs disinfested on sieve: Washing eggs on top of a sieve with disinfestant was ineffective. Although no contamination occurred with formaldehyde-treated eggs placed in water to hatch, many of the wells in media without antibacterial agents were overgrown with microbial contamination after 2 weeks; four of five wells with B5 and two of five wells with PDB were contaminated. Eggs placed in water hatched at 16.2% ± 0.7 and 20.0% ± 1.4 for wells with and without antibacterial agents, respectively. None of the wells supplemented with antibacterial agents exhibited microbial growth, but egg hatch was very low in media treatments compared to water treatments (1.8% ± 0.7 and 3.5% ± 1.5 for B5 and PDB, respectively). Eggs washed with sodium hypochlorite on a sieve also were inadequately surface-disinfested; microbial contamination was evident irrespective of antibacterial treatment in the media, as two of five PDB wells with antibacterial agents and three of five B5 wells without antibacterial agents were contaminated. Similarly, chlorhexidine diacetate treatment of eggs resulted in contamination of all five wells containing PDB and one well containing water.

Soybean cyst nematode cysts crushed in disinfestant: Treating eggs with antibacterial-antimycotic solution and amending the media with antibacterial-antimycotic mixture during the assay did not prevent microbial growth; all PDB and B5 wells were extensively contaminated. Chlorhexidine diacetate treatment for 15 minutes also did not sufficiently surface-disinfest the eggs under these conditions; one of five wells contain-

ing water, four of five wells with PDB, and one of five wells with B5 were contaminated.

In contrast to all other techniques tested, crushing the cysts and agitating the eggs in 0.5% sodium hypochlorite surface-disinfested the eggs and provided satisfactory egg hatch. Microbial contamination was not detected in any wells. Egg hatch after 2 weeks (Fig. 6) was reduced significantly by the antibacterial-antimycotic mixture and by PDB with and without the mixture (significant PDB and antibacterial-antimycotic main effects in two-way ANOVA, $P < 0.05$). However, the negative impact of the antibacterial-antimycotic solution on egg hatch occurred in the water but not in either the 70% or 90% PDB treatments (significant PDB × antibacterial-antimycotic interaction effect, two-way ANOVA, $P < 0.05$; significant Tukey's test comparing antibacterial effect within water treatment, $P < 0.05$).

Effect of sodium thiosulfate rinse on soybean cyst nematode hatch and J2 viability: Overall, egg hatch decreased with increasing PDB concentration (Fig. 7). Sodium thiosulfate wash appeared to have a detrimental effect on hatch, especially with increasing PDB concentration; however, a significant difference between water and sodium thiosulfate treatments was found only at 90% PDB (*t*-test, $P < 0.05$).

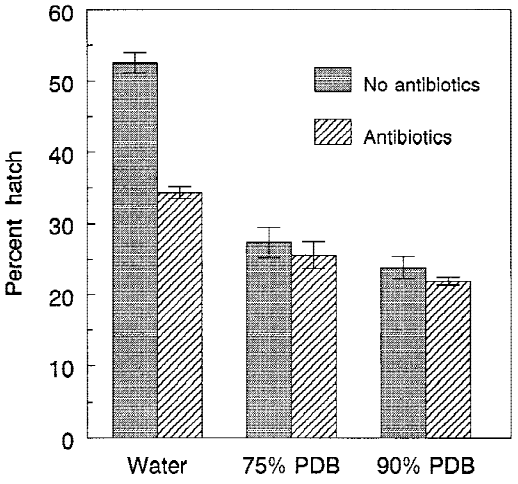

FIG. 6. Percentage of *Heterodera glycines* eggs hatched after 2 weeks in various concentrations of potato dextrose broth (PDB) with and without antibacterial-antimycotic mixture (penicillin, streptomycin, and amphotericin B). Eggs were surface-disinfested with 0.5% sodium hypochlorite in a tissue grinder. Data are means ± SE of five replications.

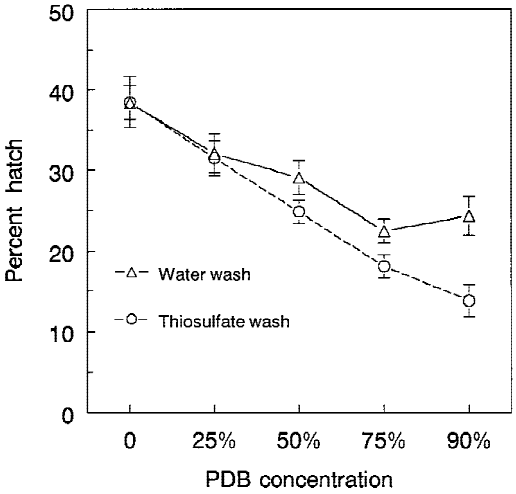

FIG. 7. Percentage of *Heterodera glycines* eggs hatched after 2 weeks in various concentrations of potato dextrose broth (PDB). Eggs were surface-disinfested with 0.5% sodium hypochlorite and washed either with water or with 10% sodium thiosulfate. Data are means ± SE of five replications.

Treating eggs with 1% sodium thiosulfate had no effect on mobility of J2 48 hours after hatching. The proportion of mobile J2 from eggs washed only with water was 87.0% ± 2.1 and from eggs washed with sodium thiosulfate was 91.3% ± 2.0 (not significantly different, $P > 0.05$, Mann-Whitney rank sum test, n = 20; data combined from two experiments).

Soybean cyst nematode hatch in various liquid media: Hatch in every medium was significantly lower than in water (Fig. 8). Although hatch in lactose-yeast and cornmeal media was slightly higher than in PDB, this difference was not significant ($P > 0.05$, Tukey's test on log transformed data). V8 Juice medium resulted in the lowest hatch and was approximately a third of the hatch in water.

Bioassay of Fusarium equiseti *culture broth against soybean cyst nematode:* Egg hatch was 13.3% ± 0.6 in water control and 26.6% ± 0.9 in PDB control treatments. Hatch in the *F. equiseti* broths from 3- and 7-day-old cultures was 10.3% ± 1.0 and 8.3% ± 0.5, respectively. Mean hatch in the PDB control treatment was significantly higher compared to all other treatments, as was hatch in water com-

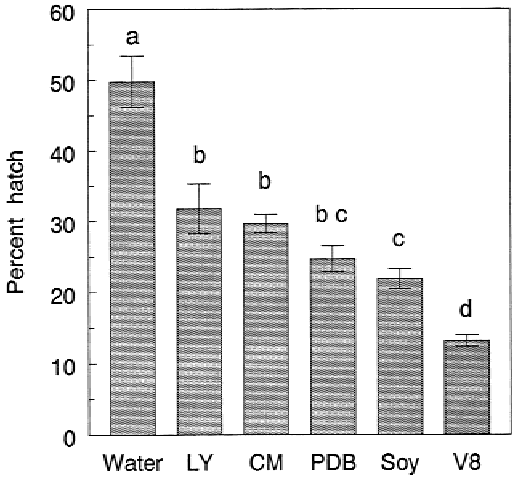

FIG. 8. Percentage of *Heterodera glycines* eggs hatched after 2 weeks in various liquid media. LY = lactose-yeast; CM = cornmeal; PDB = potato dextrose broth; Soy = soybean; V8 = V8 Juice. Data are untransformed means ± SE of 10 replications and are pooled from two trials. Means with the same letters are not significantly different (Tukey's test on log-transformed data).

pared to the 7-day-old culture broth; however, differences in hatch between water control and 3-day-old broth treatments and between 3- and 7-day-old broth treatments were not significant (Tukey's test).

DISCUSSION

Incidental microbial growth during in-vitro assays of culture broths can seriously complicate the interpretation of egg hatch data due to uncontrolled effects on hatch. The direction of such effects is unpredictable since hatch rates can be increased as well as decreased by fungi and bacteria (Cronin et al., 1997; Meyer et al., 1990). Thus, if fungal isolates are to be properly evaluated for their ability to produce nematode-antagonistic metabolites, the fungi being tested must be removed from the broth by sterile filtration before the assay, the eggs must be surface-disinfested, and the assay must be conducted aseptically.

Procedures to extract and surface-disinfest root-knot nematode eggs with sodium hypochlorite (Loewenberg et al., 1960; McClure et al., 1973; Sankaralingam and McGawley, 1994) required only minor modification to assay fungal culture broths with *M. incognita*. These techniques were also readily adapted for the bioassay-directed fractionation of *F. equiseti* fungal broth. Extraction and fractionation of the broth confirmed that this fungus secretes nematode-antagonistic compounds or modifies broth components into antagonistic compounds. Not only was egg hatch inhibited by *F. equiseti* broth extract, but more than 90% of those J2 that did hatch were immobilized within 1 week after the start of the assay. The responsible compounds were nonpolar, as they were partitioned into the ethyl acetate fraction and had low retention on silica gel. At least several different compounds appeared to be nematode-antagonistic because egg hatch and J2 mobility were reduced in multiple chromatographic fractions (Fig. 5).

Culture filtrates of *Fusarium oxysporum* and *Fusarium solani* are also toxic to J2 of *M. incognita* (Hallman and Sikora, 1996; Mani et al., 1986). Nonpolar, long-chain alkanes were tentatively identified as the nematicidal components of the *F. solani* culture filtrate (Mani et al., 1986), but the toxic compounds of *F. oxysporum* have not been characterized. Among the array of metabolites produced by *Fusarium* spp. (Savard et al., 1990), some are known to be mycotoxins that are also nematicidal (Ciancio, 1995). The toxicity of the nematode-antagonistic compounds from *F. equiseti* to nontarget organisms will not be known until further characterization of the active components is accomplished.

Soybean cyst nematode egg hatch was also lower in *F. equiseti* culture broths relative to the PDB controls; but, when compared to the water control, the reduction was significant only in the broth of the 7-day-old culture. Fractions of the culture broth have yet to be tested on soybean cyst nematode to ascertain whether fractions active against *M. incognita* are similarly active against *H. glycines*.

For soybean cyst nematodes, disinfesting the eggs rapidly and efficiently was the most challenging aspect when cultures from greenhouse plants were used. Typically, sterilization of plant-parasitic nematodes in-

volves a sequence of chlorhexidine diacetate, streptomycin, and mercuric chloride washes (Huettel and Rebois, 1985). This technique requires more than 2 hours to complete and uses a highly toxic mercury compound that requires special disposal procedures. Although chlorhexidine diacetate alone sufficiently disinfests eggs being hatched in water (Acedo and Dropkin, 1982; Wong et al., 1993), in our studies it was inadequate for eggs placed in culture broth, and, after 2 weeks, microbial contamination was extensive. Formaldehyde, which is used as a disinfestant of insect eggs, was also ineffective under these conditions.

Sodium hypochlorite was effective in disinfesting *H. glycines* eggs, but the method of treatment was important. Optimum results were obtained when tan-colored cysts were crushed in 0.5% sodium hypochlorite, directly releasing the eggs into the disinfestant, whereas washing the eggs with sodium hypochlorite on a sieve resulted in inconsistent success. The superiority of sodium hypochlorite over formaldehyde and chlorhexidine diacetate was likely due in part to the ability of sodium hypochlorite to dissolve the gelatinous matrix and the outer surface of nematode eggs (Di Vito et al., 1986; Esser, 1972; Hussey and Barker, 1973), resulting in the physical removal of microbiota. To avoid injuring the eggs, exposure time must be carefully controlled. Agitation for 2 minutes followed by 0.5 minute to allow debris to settle apparently did not harm the juveniles within the eggs; hatch rates in water were comparable to those reported for soybean cyst nematode eggs either treated with chlorhexidine or not exposed to any disinfestant (Wong et al., 1993; Yen et al., 1995). Moreover, we have used eggs treated with the sodium hypochlorite procedure to inoculate soybean root tips for in-vitro monoxenic cultures, suggesting that J2 from these eggs were healthy and viable. Sodium thiosulfate was tested as a means to remove trace chlorine, but neither egg hatch nor J2 mobility 2 days after hatch was improved by this treatment. Washing eggs with 10% sodium thiosulfate apparently was detrimental if eggs were subsequently placed in high concentrations of PDB, as hatch was reduced under these conditions. Although sodium hypochlorite did not affect juveniles, it may increase permeability of the egg shells to toxins, enhancing their effect during the bioassay.

In most cases, *H. glycines* hatch was reduced in uninoculated culture media compared to water controls, a result observed with other plant-parasitic nematode species (Khan and Husain, 1989; Mehta et al., 1990). In the study comparing different broth media, cornmeal and lactose-yeast media tended to be better hatching media than PDB. However, the hatching of soybean cyst nematode was highly variable, as demonstrated by the higher *H. glycines* hatch in PDB compared to water in the bioassay of *F. equiseti* broth.

With regard to media effects on soybean cyst nematode hatch, transferring the eggs from test media to water during the assay would likely improve hatch by decreasing exposure to toxins but would be time consuming if aseptic conditions were to be maintained and large numbers of fungi were being screened routinely. Zinc ion is an artificial stimulant of soybean cyst nematode hatch (Shepherd and Clarke, 1971; Tefft et al., 1982) and has been used to improve the sensitivity of assays measuring the inhibitory action of herbicides (Wong et al., 1993). Although helpful in studying specific hatch inhibitors, the use of zinc should be approached cautiously when testing fungal broths. Metabolites secreted by fungi, such as citric acid and oxalic acid, are known to bind to metal ions (Gadd, 1993), and a reduction in hatch due to the interaction of Zn^{++} ions with fungal products could be interpreted incorrectly as a sign of nematode-antagonistic properties.

These assay procedures permit routine screening of large numbers of potential microbial agents and are currently being used in our laboratory to test hundreds of fungal isolates against root-knot and soybean cyst nematodes. With the use of an inverted microscope, just over 1 minute per well is required to count hatched larvae when an optimum number of 250 to 300 eggs are used

per well. These techniques will greatly facilitate the search for safer nematicides having fewer negative effects on the environment as well as assist in identifying the mechanisms by which nematode-antagonistic fungi act.

Literature Cited

Acedo, J. R., and V. H. Dropkin. 1982. Technique for obtaining eggs and juveniles of *Heterodera glycines*. Journal of Nematology 14:418–420.

Becker, J. O., E. Zavaleta-Mejia, S. F. Colbert, M. N. Schroth, A. R. Weinhold, J. G. Hancock, and S. D. Van Gundy. 1988. Effects of rhizobacteria on root-knot nematodes and gall formation. Phytopathology 78: 1466–1469.

Ciancio, A. 1995. Observations on the nematicidal properties of some mycotoxins. Fundamental and Applied Nematology 18:451–454.

Cronin, D., Y. Moënne-Loccoz, A. Fenton, C. Dunne, D. N. Dowling, and F. O'Gara. 1997. Role of 2,4-diacetylphloroglucinol in the interactions of the biocontrol pseudomonad strain F113 with the potato cyst nematode *Globodera rostochiensis*. Applied and Environmental Microbiology 63:1357–1361.

Dhingra, O., and J. B. Sinclair. 1995. Basic plant pathology methods, second edition. Boca Raton, FL: Lewis Publishers.

Di Vito, M., N. Greco, and A. Carella. 1986. Effect of *Meloidogyne incognita* and importance of the inoculum on the yield of eggplant. Journal of Nematology 18: 487–490.

Dropkin, V. H., G. C. Martin, and R. W. Johnson. 1958. Effect of osmotic concentration on hatching of some plant-parasitic nematodes. Nematologica 3:115–126.

Esser, R. P. 1972. Effect of sodium hypochlorite concentrations on selected genera of nematodes. Proceedings of the Helminthological Society of Washington 39: 108–114.

Gadd, G. M. 1993. Interactions of fungi with toxic metals. New Phytologist 124:25–60.

Hallman, J., and R. A. Sikora. 1996. Toxicity of fungal endophyte secondary metabolites to plant-parasitic nematodes and soil-borne plant pathogenic fungi. European Journal of Plant Pathology 102:155–162.

Huettel, R. N., and R. V. Rebois. 1985. Culturing plant-parasitic nematodes using root explants. Pp. 155–158 *in* Zuckerman, B. M., W. F. Mai, and M. B. Harrison, eds. Plant nematology laboratory manual. Amherst, MA: University of Massachusetts Agricultural Experimental Station.

Hussey, R. S., and K. R. Barker. 1973. A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. Plant Disease Reporter 57: 1025–1028.

Jenkins, W. R. 1964. A rapid centrifugal-flotation technique for separating nematodes from soil. Plant Disease Reporter 48:692.

Khan, T. A., and S. I. Husain. 1989. Effect of culture filtrates of soil fungi on the mortality and hatching of reniform nematode (*Rotylenchulus reniformis*). Indian Journal of Nematology 19:35–40.

Loewenberg, J. R., T. Sullivan, and M. L. Schuster. 1960. The effect of pH and minerals on the hatching and survival of *Meloidogyne incognita incognita* larvae. Phytopathology 50:215–217.

Mani, A., C. L. Sethi, and Devkumar. 1986. Isolation and identification of nematoxins produced by *Fusarium solani* (Mart) Sacc. Indian Journal of Nematology 16: 247–251.

McClure, M. A., T. H. Kruk, and I. Misaghi. 1973. A method for obtaining quantities of clean *Meloidogyne* eggs. Journal of Nematology 5:230.

Mehta, N., K. K. Walia, and D. C. Gupta. 1990. Effect of culture filtrates of *Rhizoctonia solani* and *R. bataticola* cultured on different media on hatching of *Meloidogyne javanica* larvae. Plant Disease Research 5:96–99.

Meyer, S. L. F., R. N. Huettel, and R. M. Sayre. 1990. Isolation of fungi from *Heterodera glycines* and in-vitro bioassays for their antagonism to eggs. Journal of Nematology 22:532–537.

Sankaralingam, A., and E. C. McGawley. 1994. Influence of *Rhizoctonia solani* on egg hatching and infectivity of *Rotylenchulus reniformis*. Journal of Nematology 26: 486–491.

Savard, M. E., R. Greenhalgh, and J. W. ApSimon. 1990. Recent advances in the chemistry of secondary metabolites isolated from *Fusarium* species. Pp. 213–259 *in* Atta-ur-Rahman, ed. Studies in natural products chemistry. New York: Elsevier.

Shapiro, M. 1984. Micro-organisms as contaminants and pathogens in insect rearing. Pp. 130–142 *in* E. G. King and N. C. Leppla, eds. Advances and challenges in insect rearing. New Orleans, LA: Agricultural Research Service, United States Department of Agriculture.

Shepherd, A. M., and A. J. Clarke. 1971. Molting and hatching stimuli. Pp. 267–287 *in* B. M. Zuckerman, W. F. Mai, and R. A. Rohde, eds. Plant-parasitic nematodes, vol. 2. New York: Academic Press.

Siddiqui, Z. A., and I. Mahmood. 1996. Biological control of plant-parasitic nematodes by fungi: A review. Bioresource Technology 58:229–239.

Stevens, R. B., ed. 1974. Mycology guidebook. Seattle, WA: University of Washington Press.

Tefft, P. M., J. F. Rende, and L. W. Bone. 1982. Factors influencing egg hatching of the soybean cyst nematode *Heterodera glycines* race 3. Proceedings of the Helminthological Society of Washington 49:258–265.

Thompson, J. M., and G. L. Tylka. 1997. Differences in hatching of *Heterodera glycines* egg-mass and encysted eggs in vitro. Journal of Nematology 29:315–321.

Wong, A. T. S., G. L. Tylka, and R. G. Hartzler. 1993. Effects of eight herbicides on in-vitro hatching of *Heterodera glycines*. Journal of Nematology 25:578–584.

Yen, J. H., T. L. Niblack, and W. J. Wiebold. 1995. Dormancy of *Heterodera glycines* in Missouri. Journal of Nematology 27:153–163.