Large Scale Hatching, Disinfestation, and Storage of Heterodera schachtii Larvae

E. D. Whitney and D. L. Doney

Research Plant Pathologist and Geneticist, respectively, Crops Research Division, ARS, USDA, Salinas, California 93901.

Use of trade names is for identification, and does not constitute an endorsement or guarantee of the product by the USDA to the exclusion of others of suitable composition.

Accepted for publication 11 March 1970.

ABSTRACT

Methods of hatching, disinfesting, and storing large numbers of second-stage *Heterodera schachtii* larvae without loss of infectivity are described. For greenhouse studies, larvae are hatched by incubating screened cysts for 3-5 days in 4 mM zinc chloride, 10 ppm ethoxyethyl mercury chloride, 0.01% dioctyl sodium sulphosuccinate, 1 mg/ml streptomycin sulfate, and 1,000 units/ml penicillin G potassium. The larvae are further treated in the same solution for 72 hr to reduce contamination. For pure culture studies, the larvae are hatched by incubating recently matured hand-picked cysts as described for greenhouse studies. The larvae are further disinfested, however, by placing them in the above solution plus neomycin sulfate (1 mg/ml) for 7 days. Surface disinfestation of the larvae was complete after storage at 24 C for 30 days as indicated on a wide range of media. Infectivity of the larvae was determined by staining and counting the number of larvae infecting 3-week-old sugarbeet seedlings grown in sand culture. Phytopathology 60:1191-1194.

Attempts to select sugarbeets with single gene resistance to the sugarbeet nematode, *Heterodera schachtii* Schm., have been unsuccessful. There is some evidence that progress could be made in selecting for multigenic resistance (3, 6, 10, 11, 13). Interest in studying this resistance and the nematode root rot disease complex of sugarbeet stimulated studies to provide large quantities of uniform inoculum not readily attainable by using cysts. In addition, minimizing the effects of root rotting pathogens and contaminants infesting cysts that may interact with the nematode is desirable.

To obtain inoculum for experimental studies, several workers (2, 8, 9, 11, 12) developed hatching techniques using root diffusate. The use of synthetic hatching agents has not been reported, but Clark & Shepherd (1) showed that zinc chloride and other metallic ions stimulated the hatching of sugarbeet nematode eggs, which suggested its use for this purpose. Moriarty (9) reported that small quantities of sugarbeet nematode larvae could be surface-disinfested following hatching. The literature pertaining to the freeing of nematodes of microorganisms was reviewed in 1967 by Goodman & Chen (5).

MATERIALS AND METHODS.—*Hatching of larvae.*—Sugarbeet nematode cysts were screened from approximately 25 kg of soil (200-300 cysts/100 g of soil). Approximately 2 kg of soil were roiled in 8 liters of water and allowed to settle for 10 sec. The water was then poured over nested 20- and 60-mesh sieves to remove the cysts. The cysts and organic material retained on the 60-mesh sieves were randomly aliquoted into hatching pans. Two stainless steel, nested 23-cm pie pans, with the bottom of the top pan removed and replaced with 10-mesh stainless steel screen and covered with a moist milk filter disc (Rapid Flow), were used for this purpose. Three spots of solder on the upper lip of the lower pan separated the two pans. Enough water was placed in the bottom pie pan to cover the screen and filter disc of the top pan. The hatch pan, an adaptation of the Baermann funnel method, was used for 24 hr to remove free living nematodes retained with the cysts.

Infectivity of larvae.—Infectivity of larvae was determined by inoculating 3-week-old sugarbeet transplants (F_1 hybrids F58-554H1) grown in individual sand filled containers (200 g) with larvae in 10 ml of liquid. Plants were watered with Hoagland's solution at half-strength. Seven to 10 days after inoculation, plants were removed from the sand and stained in hot lactophenol acid fuchsin for 1 min (7). Larvae infecting the roots of each plant were then counted with the aid of a dissecting microscope.

To remove liquids from larvae, a fritted glass disc (10-mm course) within a glass tube (Corning No. 39570 with one end of the glass tube cut off flush with the disc) attached by means of rubber tubing to a vacuum with trap was used. Larvae were allowed to settle to facilitate the removal of liquids. The fritted disc apparatus was autoclaved for the aseptic removal of liquids.

RESULTS.—*Hatching of larvae.*—To compare rate of hatching and reduction in contaminants during hatching, the water in the hatch pan was replaced with one of the following hatching solutions: (i) 4 mM zinc chloride; (ii) 4 mM zinc chloride, 10 ppm ethoxyethyl mercury chloride (Aretan), 0.01% dioctyl sodium sulphosuccinate (Vatsol), 1 mg/ml streptomycin sulfate, and 1,000 units/ml penicillin G potassium (hatching solution); (iii) root diffusate; and (iv) root diffusate plus the "additives" used with zinc chloride in treatment ii above. A precipitate that formed upon adding Vatsol to the hatch solution was removed by filtering through a Kimwipes (coarse thin paper) in a Büchner funnel. The hatching pans were placed in a constant-temp cabinet, 24 ± 1 C, for 5 days. Relative humidity in the cabinet was maintained at 100% by a 1-min injection of water every 10 min through two spray nozzles delivering 8 liters/hr. Nozzles were directed away from the hatching pans to avoid the addition of water. The newly hatched larvae moved through the

filter disc and were collected in the bottom pan. Three 0.5-ml aliquots from each of three replications of each treatment were counted.

Total larvae hatching in root diffusate, root diffusate plus additives, zinc chloride, and zinc chloride plus additives were 820,000; 1,171,500; 352,500; and 541,000, respectively. Root diffusate was significantly better than zinc chloride as a hatching agent. A significant increase in hatch resulted when the additives were included (Table 1). There was no interaction between the hatch factors and the additives. This indicated that the additives reacted similarly with either zinc chloride or root diffusate. Although none of the hatching solutions reduced contamination of larvae during hatching to a desirable level for use as inoculum, retardation in multiplication of microorganisms by zinc chloride and zinc chloride plus additives was observed (Fig. 1). Retardation in growth was measured by flooding plates of potato-dextrose agar (PDA) with 1 ml of a 10^{-1} dilution of nematode suspension from each hatching treatment.

Disinfestation of larvae.—Larvae from screened cysts washed once in sterile water following hatching were disinfested for 72 hr in 150 ml of hatching solution in a 20- × 150-mm petri dish. Surface sterility of the nematodes was tested by inoculating National Institutes of Health thioglycolate broth (9 ml), PDA, and acidified PDA pH 4.5 with 1 ml of a 10^{-1} dilution of nematode suspension. Four culture tubes of broth and two petri dishes of each type of PDA were used. The test was repeated 4 times. Contamination was reduced by 72% when compared with larvae stored in water.

TABLE 1. Means of treatments and factorial comparisons for *Heterodera schachtii* larval hatch

Treatments	Mean
1 Root diffusate	273,300
2 Root diffusate + additives[a]	390,500
3 Zinc chloride	117,500
4 Zinc chloride + additives	180,500
LSD = .05	56,914
LSD = .01	82,805
Root diffusate vs. zinc chloride	
Root diffusate (1 + 2)	331,933
Zinc chloride (3 + 4)	149,000
LSD = .01	58,548
Additives vs. no additives	
Additives (2 + 4)	285,500
No additives (1 + 3)	195,400
LSD = .01	58,548

[a] Ethoxyethyl mercury chloride, 10 ppm; dioctyl sodium sulphosuccinate, 0.01%; streptomycin sulfate, 1 mg/ml; penicillin G potassium, 1,000 units/ml.

Larvae from hand-picked cysts washed twice in sterile water and disinfested as described above were tested daily for 5 days for surface sterility. The media described above plus four culture tubes of Sabouraud broth were used. The number of bacterial colonies appearing on PDA were counted. The test was repeated once. Larvae thus treated were found to be free of fungi when tested on PDA; however, the reduction

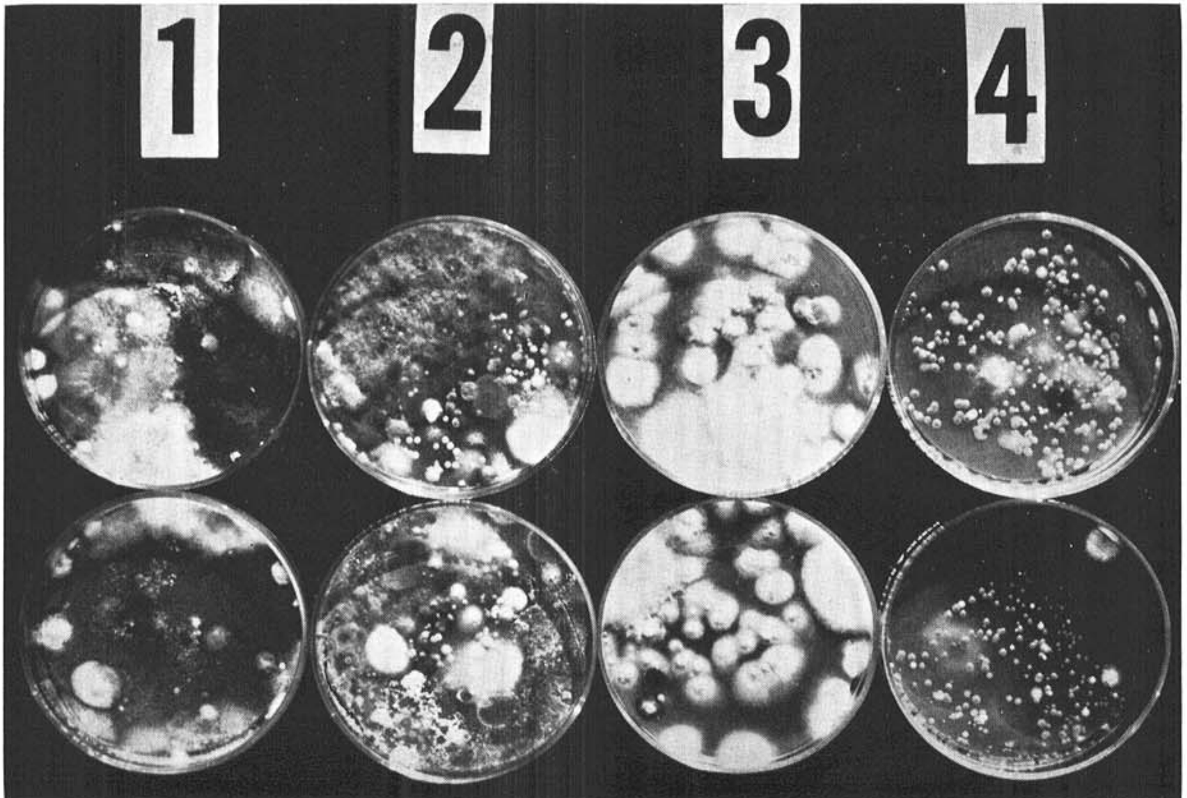

Fig. 1. The effect of zinc chloride 3 and zinc chloride plus additives 4 in retarding microorganism growth as compared with root diffusate 1 and root diffusate plus additives 2 during hatching of *Heterodera schachtii* larvae.

in bacterial contamination was proportional to length of treatment, with a reduction in total population from 1,200 colonies to less than 50 in 5 days. Similar subsequent tests of up to 10 days' duration did not eliminate the bacteria.

A test similar to test 2 except for the elimination of the sterile water wash of the larvae was conducted, comparing hatching solution and hatching solution plus neomycin sulfate (1 mg/ml, 750 μg neomycin base per mg) as the disinfestation treatment. Sterility was tested on days 0, 1, 2, 3, 4, and 7 for the first four replications, and daily for 7 days for replication 5. Media used are listed in Table 2. A total of 56 individual tests was made. Only one individual test was found with contaminated larvae (one colony, a species of *Streptomyces*) when treated with hatch solution plus neomycin sulfate for 7 days in contrast to 17 tests showing nematode contamination from bacteria or fungi or both when treated with hatching solution (Table 2). The number of individual tests that remained sterile increased with length of treatment. This increase was most rapid when neomycin sulfate was added (Fig. 2). The number of larvae surface-disinfested in replications 3, 4, and 5 was 61,000; 17,500; and 160,000, respectively. The mean number of larvae was 95/individual sterility test (broth) in the fifth replication. No differences due to treatment were observed in the activity of the nematodes in replications 1 and 2 as measured by movement. Controls were media or broth to which 1 ml of sterile water was added. Controls remained sterile for all tests. Media for all surface-disinfestation tests were incubated at 24 ± 1 C for at least 30 days before readings were made. Disinfestation was carried out under aseptic conditions at room temp in the dark.

Infectivity of larvae.—Infectivity following hatching in each hatching solution was determined by inoculating four replications of five plants each with 2,000 larvae/plant in 10 ml of each hatching solution. The mean number of larvae infecting each plant when the larvae were hatched in root diffusate, root diffusate plus additives, zinc chloride, and zinc chloride plus additives was 349.2, 439.9, 436.1, and 495.3, respectively. These means were not significantly different.

TABLE 2. The effect of hatching solution and hatching solution plus neomycin sulfate on the disinfestation of *Heterodera schachtii* larvae after 7 days of treatment as tested by five media 30 days after inoculation

Treatment	Test media					Total
	PDA[a]	NIHTA	NDA	NIHTB	SB	
Hatch	4/8[b]	2/4	1/4	6/20	4/20	17/56[c]
Hatch + neomycin sulfate	0/8	0/4	0/4	1/20	0/20	1/56

[a] PDA = potato-dextrose agar; NIHTA = National Institute of Health thioglycollate broth plus 1.7% agar; NDA = nutrient dextrose agar; NIHTB = National Institutes of Health thioglycollate broth; SB = sabouraud broth.

[b] Number of culture tubes showing contaminated larvae per total number.

[c] Total of five tests.

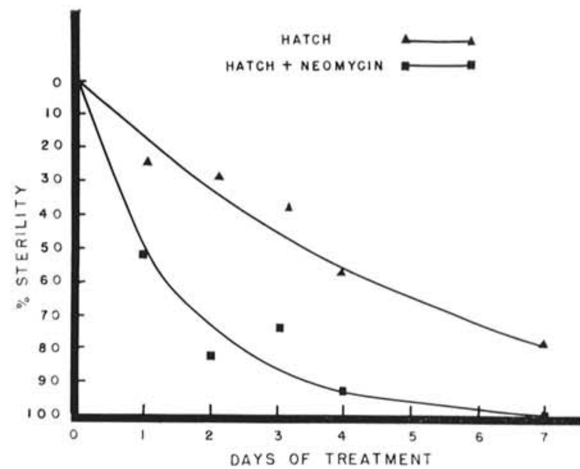

Fig. 2. Comparison of hatching solution and hatching solution plus neomycin sulfate on the disinfestation of *Heterodera schachtii* larvae in relation to time. Each point is the mean per cent of five tests, totaling 56 individual tests.

Infectivity following storage was estimated by inoculating two replications of five plants each with 2,000 larvae. For the test, 10,000 larvae were stored in 50 ml of each hatch solution diluted 10^{-1} in 15- × 100-mm petri dishes.

Tests were conducted after storage for 1, 2, 3, and 4 weeks in the dark at room temp or at 5 C. Neither length of storage nor treatment of the larvae reduced the infectivity of the larvae stored at 5 C. This was also true for larvae stored at room temp for 2 weeks in diluted zinc chloride or root diffusate. When the additives were included in the storage solution, storage of the larvae at room temp for more than 1 week resulted in a reduction in infectivity with each additional week of storage. The effects of the additives on larval infectivity were similar when used with root diffusate or zinc chloride at room temp (Table 3).

Infectivity of the larvae after surface disinfestation (7 days) was determined by inoculating 16 seedlings each with 1,000 larvae. The control was larvae stored in water. The test was repeated once. The mean per cent of larvae infecting sugarbeet seedlings in water,

TABLE 3. Mean number of *Heterodera schachtii* larvae from each hatching treatment infecting each sugarbeet plant when inoculum is stored at room temp for 1, 2, 3, and 4 weeks

Weeks of storage	Treatments			
	1[a]	2	3	4
1	549.4a[b]	534.6a	621.7a	527.7a
2	577.3a	301.6b	628.0a	317.7b
3	432.1b	146.3c	430.9b	153.0c
4	297.0c	110.7c	294.0c	91.4c

[a] 1 = Root diffusate. 2 = Root diffusate; ethoxyethyl mercury chloride, 10 ppm; dioctyl sodium sulphosuccinate, 0.01%; streptomycin sulfate, 1 mg/ml, and penicillin G potassium, 1,000 units/ml. 3 = Zinc chloride, 4 mM. 4 = Zinc chloride, 4 mM; plus additives in 2 above.

[b] Mean of two replications of five plants inoculated with 2,000 larvae. Values followed by the same letter do not differ significantly at the 5% level of probability.

hatching solution, and hatching solution plus neomycin sulfate was 30.2, 25.8, and 30.7, respectively. There was no significant reduction in the infectivity of the larvae due to either treatment with antibiotics for surface disinfestation of larvae except for the hatching solution in test 1. The number of nematodes infecting each individual root varied from 114 to 491 in test 1 and 119 to 409 in test 2.

Effect of surfactants on hatching, disinfestation, and infectivity of larvae.—The effects of Triton GR-5 (a 60% solution of a sulfonated alkyl ester) and Triton X-100 (iso-octyl phenoxy polyethoxy ethanol), on hatching, larval infectivity, and disinfestation of nematode larvae were compared with Vatsol (at the same rate of active ingredients, 0.01%) as the surfactant in the hatching solution. Screened cysts were aliquoted randomly into hatching pans, selected at random for each treatment, and incubated for 48 hr. Each treatment was replicated 5 times. Hatched larvae and larvae treated an additional 72 hr in each solution were tested for surface disinfestation. The solution from each treatment diluted 10^{-2} was tested for contaminated larvae on PDA and acidified PDA (4.5) by flooding four petri dishes of each medium with 1 ml of solution. Infectivity of larvae was determined after a 72-hr disinfestation treatment. Three replications of eight plants each were inoculated with 1,000 larvae/plant. Plants were harvested and stained for counting 7 days after inoculation.

Total larval hatches with Vatsol and Triton X-100 as the surfactant in the hatching solution were higher than with Triton GR-5. The estimated hatches were 2,248,000, 2,102,000, and 1,566,400, respectively. No visible differences in surface sterility after hatching or disinfestation were apparent, nor were there any differences in the infectivity of the larvae. The per cent infectivity of the larvae for each of the above surfactants was 19.8, 14.9, and 20.4, respectively. Triton X-100 did not cause a precipitate to form when added as the surfactant to prepare the hatching solution.

DISCUSSION.—The data reported herein show that large numbers of second-stage *H. schachtii* larvae can be hatched and partially surface-disinfested and used for inoculum when complete disinfestation is not required. The following procedure is suggested: (i) use cysts screened from infested soil; (ii) place cysts in hatching pans containing water for 24 hr to remove excessive free living nematodes; (iii) place cysts in hatching solution for 3-5 days; (iv) remove the hatching solution from the larvae and replace with fresh hatching solution for 72 hr; and (v) wash larvae with sterile water.

For the disinfestation of larvae for pure culture studies, use the above procedures with the following changes: (i) use recently matured hand-picked cysts from plants inoculated solely with *H. schachtii* and grown in sand; (ii) wash larvae twice in sterile water prior to the disinfestation treatment of hatching solution plus neomycin sulfate for 7 days; (iii) perform disinfestation treatment under aseptic conditions; (iv) test larvae for surface disinfestation using several media.

Larval hatches of from 5^6 to 10^6 have been consistently achieved each week when screened cysts and hatching solution are used. The number hatched declines with each succeeding hatching period (3-5 days); therefore the cysts must be replaced after approximately 2-3 weeks.

Zinc chloride was used initially as a hatching stimulant. However, its beneficial effects in retarding growth of microorganisms suggested its use as a disinfestant. Although root diffusate was superior to zinc chloride with respect to rate of hatch, this may not be true in all cases, as shown by Clark & Shepherd (1). This difference is undoubtedly due to variation in the quality of the root diffusate from one collection to another. The beneficial effect of zinc chloride as a disinfestant, the consistent hatch without loss in larval infectivity, and the ease with which it can be used and obtained warrants its use in preference to root diffusate.

When extremely large numbers of larvae are required, the accumulation of larvae over a period of at least 4 weeks is possible under refrigeration, as suggested by the data of Golden & Shafer (4).

Although washing of larvae prior to the disinfestation treatment in sterile water was not done in disinfestation test 3, we suggest the practice.

The data showed that Triton X-100 could be used to replace Vatsol in the hatching and partial disinfestation of larvae.

LITERATURE CITED

1. CLARK, A. M., & A. SHEPHERD. 1965. Zinc and other metallic ions as hatching agents for the beet cyst nematode, *Heterodera schachtii* Schm. Nature 208: 502.
2. CURTIS, G. J. 1965. An improved method of hatching larvae of cyst-forming nematodes. Nematologica 11: 213-217.
3. FINKNER, R. E., & J. F. SWINK. 1956. Breeding sugar beets for resistance to nematodes. Agron. J. 48:389-392.
4. GOLDEN, A. M., & T. SHAFER. 1960. Survival of emerged larvae of the sugar-beet nematode (*Heterodera schachtii*) in water and soil. Nematologica 5: 32-36.
5. GOODMAN, R. M., & T. A. CHEN. 1967. Two methods for surface disinfestation of *Trichodorus christiei.* Phytopathology 57:1216-1220.
6. JORGENSEN, E. C., & C. H. SMITH. 1966. Evaluation of selected varieties of sugarbeets for response to the sugarbeet nematode, *Heterodera schachtii.* Plant Dis. Reptr. 50:650-654.
7. MCBETH, C. W., A. L. TAYLOR, & A. L. SMITH. 1941. Note on staining nematodes in root tissue. Helminthol. Soc. Wash. Proc. 8:26.
8. MORIARTY, F. 1963. A nylon sieve for hatching *Heterodera* larvae. Nematologica 9:157-158.
9. MORIARTY, F. 1964. The monoxenic culture of beet eelworm (*Heterodera schachtii* Schm.) on excised roots of sugar beet (*Beta vulgaris* L.). Parasitology 54:289-293.
10. PRICE, C. 1965. Breeding sugar beets for resistance to the cyst nematode *Heterodera schachtii.* J. Amer. Soc. Sugar Beet Technol. 13:397-405.
11. SHEPHERD, A. M. 1958. Experimental methods testing resistance to beet eelworm (*Heterodera schachtii* Schmidt.). Nematologica 3:127-135.
12. SHEPHERD, A. M. 1959. Increasing the rate of larval emergence from cysts in hatching tests with beet eelworm, *Heterodera schachtii* Schmidt. Nematologica 4:161-164.
13. SWINK, J. F. 1954. Breeding for resistance to the sugarbeet nematode. Amer. Soc. Sugar Beet Technol. Proc. 8:109-111.