Factors Influencing the Hatching of Meloidogyne naasi, and a Comparison with M. hapla

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Supported by a grant from the U.S. Department of the Interior Bureau of Reclamation.
The authors are grateful for the advice and assistance of M. W. Allen and T. A. Shalla.
Accepted for publication 8 October 1969.

ABSTRACT

The general failure of eggs of *Meloidogyne naasi* to hatch in water was not changed by incubation at constant temperatures of −28, 3, 6, 9, 12, 21, 24, 30, or 39 C, by rapid or slow drying, by exposure to pH 3.8, 6.8, or 10.5, or by treatment with potassium permanganate, nicotinic acid, or hydrochloric acid.

Hatching was stimulated by (i) cold-warm treatment consisting of incubation at 6 to 9 C for 7 weeks, followed by incubation at 21 to 24 C; (ii) treatment with 0.4% (by wt) sodium hypochlorite solution.

No morphological differences between cold-warm treated eggs and untreated eggs were apparent by examination with the electron microscope. Hatching stimulation by sodium hypochlorite may indicate the presence of an inhibitory substance in the egg matrix or egg shell.

Hatching of *M. hapla* is inhibited by the cold-warm temperature treatment which stimulates *M. naasi*. Phytopathology 60:457-460.

In 1965, patches of stunted, yellow barley (*Hordeum vulgare* L. 'Hannchen') were seen in the U. S. Dept. of Interior Fish and Game Wildlife Refuge near Tulelake, Calif. (2). Examination of roots revealed galls containing a root knot nematode, *Meloidogyne naasi* Franklin, 1965. The severity of the disease varied erratically from year to year.

Pathogenicity studies were begun in the greenhouse, but attempts to propagate large numbers of the nematode were not successful. Although some of the nematodes completed their life-cycles and laid eggs, very few larvae were recovered from the infected plants. When egg masses were placed in water to obtain larvae in the usual manner (3), few larvae hatched.

This study represents work which evolved from the unsuccessful attempts to recover large numbers of larvae from infected barley roots.

MATERIALS AND METHODS.—*Nematode propagation.*—*Meloidogyne naasi* was obtained from barley at Tulelake, Calif., and *Meloidogyne hapla* Chitwood from tomato (*Lycopersicon esculentum* Mill.) at Davis, Calif. These species were reared separately on Hannchen barley inoculated with single egg masses of each species. The barley was grown in a two-thirds sand and one-third potting soil medium in clay pots or wooden boxes.

To obtain large numbers of egg masses, barley roots were washed free from soil in a gentle stream of tap water. Then egg masses were cut or picked off the roots and placed in double, glass-distilled water at pH 5.9. Nematodes could be stored in this manner for more than 13 weeks with no apparent change in their response to treatments.

Experimental design.—Each replicate consisted of five egg masses in a 15-ml beaker containing 5 ml of glass-distilled water or 5 ml of the treatment solution. The beakers were placed in 10-cm petri dishes, five beakers/dish, and stored in lettuce crisper boxes at various test temperatures or conditions.

The mean number of eggs per replicate, calculated from counts of eggs in 150 replicates, was 2042 ± 76. The coefficient of variability for the number of eggs per replicate was calculated using the means and standard errors of 16 groups of 10 replicates. This coefficient was 9.24 ± 0.75%, with a range of 2.87 to 14.09%.

Counting of eggs and larvae.—After the various physical or chemical treatments, hatched larvae were counted with the aid of a Peters' counting slide and a stereoscopic microscope. Total larval content of the eggs was usually determined also, after grinding the egg masses in a ground-glass tissue homogenizer and counting the freed eggs.

The mean numbers of hatched larvae for the various treatments were compared, using analyses of variance and Duncan's multiple range test.

Temperature effects.—Constant temperatures were maintained in converted refrigerator-incubators which could be controlled within ± 1 C. Temperature changes were accomplished by moving the nematodes from incubator to incubator. A −28 C food freezer was used for freezing treatments. Beakers containing 5 to 15 egg masses were frozen for periods ranging from overnight to 2 weeks, after which the egg masses were thawed and incubated in distilled water at laboratory temperature (approximately 25 C) for either 10 or 15 days.

Drying experiments.—Rapid drying was accomplished by removing all of the water from dishes containing five egg masses, and then exposing the dishes to the atmosphere for 5 days. Distilled water was then added, and hatching was observed for 1 week. Slow drying was accomplished in the following manner. Five egg masses were placed in a 15-ml beaker containing a two-thirds sand, one-third soil mixture. Distilled water was added to the beaker until a layer of water was visible. The trays were placed in plastic bags with slits to permit the escape of moisture. Wooden frames held the plastic away from the surface of each tray. The plastic bags were placed in a 22 ± 1-C growth chamber lighted for 12 hr each day. Control trays were moistened daily. Hatched larvae were counted after 1, 2, 3, and 4 weeks.

Leaching and aeration treatments.—To test for the presence of soluble hatching inhibitors, egg masses were placed into 3-cm diam dialysis bags having a 24-Å pore size. The bags were placed in glass-distilled water in a 250-ml beaker. The water in the beakers was removed, and fresh, glass-distilled water was added daily during the storage periods of 3, 4, and 5 weeks. This procedure was followed at each of two temperatures, 9 and 21 C. After the leaching treatment, egg masses were incubated in distilled water at 21 C for 5 days. Control egg masses were kept in distilled water, but were not leached.

Aeration was accomplished by admitting compressed air through a glass manifold and nozzle system to the water in the polypropylene beakers. Evaporated water was replaced twice daily. Egg masses which had been stored for 3 weeks at 9 C were aerated for 5 days at laboratory temperature. The hatch of these eggs was compared with the hatch of controls that had been stored at 9 C but not aerated during the 5-day incubation period, and with other controls that were aerated without the previous cold storage period.

pH experiments.—Phosphate buffers of pH 3.8, 6.8, and 10.5 were used. Egg masses were placed in 5 ml of the appropriate buffer and stored in a 9-C incubator for 8 weeks. Solutions were changed twice each week, and fresh buffer was added to prevent possible change in pH due to the growth of microorganisms. Then, after an incubation period of 5 days at 24 C, hatched larvae were counted.

Root diffusate experiments.—Egg masses that had been incubated for 9 weeks at either 9 or 24 C were placed in glass-distilled water in 15-ml beakers. A 1-day-old Hannchen barley seedling was placed with the egg masses in half the beakers incubated at each temperature. After 5 days' incubation at 24 C, the hatched larvae were counted.

Chemical treatments.—Lots of five egg masses were incubated for 25 min in 0.4% NaOCl, 1, 0.1, 0.01, and 0.0001 M $KMnO_4$, or 6, 3, 1.5, and 0.6 N HCl. Other lots were exposed for 5 days at laboratory temperature, with and without aeration, to 0.01 M nicotinic acid. This compound stimulates hatching of *Heterodera schachtii* (1).

Effects of the potassium permanganate, hydrochloric acid, and nicotinic acid treatments were assayed by direct count of hatch in the chemical at the end of the incubation period, and those of sodium hypochlorite by placing the lots of treated egg masses under an intermittent mist (4) at approximately 21 C for 4 days, then counting the larvae recovered.

In one experiment, a suspension of eggs of *M. naasi* was obtained by treating egg masses for 50 min in a 0.4% solution of sodium hypochlorite. Foreign debris was removed from the suspension with a 200-mesh sieve. Eggs passed through the sieve and were rinsed and suspended in distilled water. Five-ml lots of this suspension, containing 1746 ± 36 eggs and 78 ± 6 free larvae/ml, were incubated at 6, 9, or 21 C for 1 or 2 weeks. They were then stored at 21 C for 5 days before hatched larvae were counted.

Examination of eggs by electron microscopy.—Eggs were freed from gelatinous matrix in a ground-glass tissue homogenizer, passed through a 200-mesh screen to remove debris, killed, fixed in 5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.1, and postfixed in 1% osmium tetroxide with the same buffer. The eggs were embedded in 1.5% water-agar and frozen with dry ice; and the agar was then cut into small blocks. These blocks were dehydrated with acetone and embedded in Epon 812-Araldite 6005 mixture. Sections were cut with a glass knife, stained with 2% aqueous uranyl acetate, poststained with lead citrate (5), and examined with an RCA EMU 3-G electron microscope at 50 kv.

RESULTS.—*Influence of temperature.*—There was no hatch from egg masses which had been frozen. It is believed that the freezing treatment was lethal to the larvae.

At the constant temperatures 6, 12, 21, 30, and 39 C, hatching was very low, ranging from zero to 0.04%. Since *M. naasi* is subjected to changing temperatures in the field, an alternating regime was compared to constant temperatures. Egg masses were given alternating 24-hr exposures to 6 and 12 C for 3, 5, 7, 9, or 11 weeks, or were kept at constant temperatures of 6, 12, or 21 C for these periods. At the end of these times, egg masses were kept at 21 C for 5 days. Then hatched larvae were counted. The most favorable of these treatments was the 7 weeks of daily alternation (Table 1). When the alternating regime was continued too long (11 weeks), the stimulating effect was lost.

Later, constant low temperature (6 or 9 C) followed by a warm temperature was found to be as effective as alternating low temperatures followed by a warm temperature (Table 2). Six weeks or more of cold incubation provided greatest hatching stimulation. Three weeks was the minimal effective cold period.

Larval hatch was also dependent on sufficient exposure to a warm temperature following the cold treatment. After sufficient exposure to cold, most hatching occurred in 5 days' incubation at 21 C (Fig. 1).

There has been no previous report that root knot nematodes are stimulated to hatch by cold temperatures. If root knot nematodes other than *M. naasi* are so stimulated, *M. hapla* Chitwood might be suspected to respond, since it inhabits north temperate regions. However, cold storage reduced, rather than stimulated, the hatching of eggs of *M. hapla* (Table 3). Hatching of this species was more complete than hatching of *M. naasi* under all conditions tested.

Influence of drying.—After rapid drying and the addition of distilled water, no hatching occurred. Sodium hypochlorite treatment (discussed below) of rapidly dried egg masses produced no hatching. Presumably, eggs receiving this treatment died. Slow drying inhibited, rather than stimulated, hatching (Fig. 1).

Influence of leaching, aeration, and pH.—Leaching may have had a favorable effect on hatching. After 4 and 5 weeks of leaching at 9 C, larval hatch was higher than that from unleached egg masses kept at the same temperature. Differences between leached and unleached did not quite reach the 5% level of statistical significance, however. If leaching has an effect, it is much

TABLE 1. Per cent of *Meloidogyne naasi* eggs hatched in 5 days at 21 C after several periods of time at three constant temperatures, or after alternating exposure to two low temperatures

Temp.	% *M. naasi* eggs hatched[a]				
C	*3 Weeks*	*5 Weeks*	*7 Weeks*	*9 Weeks*	*11 Weeks*
6	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
12	0.02 ± 0.01	0.02 ± 0.01	3.58 ± 1.77	3.58 ± 1.77	3.58 ± 1.77
21	0.09 ± 0.04	0.09 ± 0.04	0.09 ± 0.04	0.09 ± 0.04	0.09 ± 0.04
Alternating 24 hr exposures to 6 and 12	0.99 ± 0.36[b]	1.93 ± 0.43[b]	16.50 ± 2.73[b]	11.73 ± 3.60[b]	3.88 ± 0.86

[a] Mean of 10 replicates ± S. E. of this mean; the initial mean number of eggs for the 200 replicates was 1737 ± 89.
[b] Significantly higher ($P < .01$) than all other treatments at this time period. Duncan's Multiple Range test.

TABLE 2. Per cent of *Meloidogyne naasi* eggs hatched after storage at several constant low temperatures for various periods of time followed by incubation at 21 C for 5 days

Storage temp.	% Eggs hatched[a]				
C	*3 Weeks*	*5 Weeks*	*7 Weeks*	*9 Weeks*	*12 Weeks*
6	1.7 ± 0.3	16.1 ± 2.7	19.2 ± 4.59	37.6 ± 4.7	44.2 ± 4.7
9	9.2 ± 1.4	10.3 ± 3.6	28.6 ± 7.1	33.7 ± 8.2	
12	4.2 ± 2.0	6.0 ± 3.8	1.7 ± 0.6	1.1 ± 0.5	5.0 ± 1.7
21	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
LSD[b] (.05)	3.2	8.6	12.3	11.5	7.8
LSD[b] (.01)	4.3	11.6	16.6	15.5	10.7

[a] Mean of 10 replicates. The initial mean number of eggs for the 150 replicates was 2042 ± 76.
[b] Between mean for any temperature and mean for 21 C after analysis of variance.

smaller than the temperature effect. At 21 C, hatching was never over 2% for leached or unleached egg masses.

The hatching increase resulting from aeration of cold-treated eggs also approached, but did not equal, the increase required for significance at the 5% level.

The hatch in distilled water (pH 5.9) was significantly greater ($P < 0.01$) than that at any pH (3.8, 6.8, or 10.5) obtained with buffered solutions. The phosphoric acid and sodium hydroxide used to obtain these other hydrogen ion concentrations probably inhibited hatching.

Effect of barley root diffusates.—Hatching of cold-conditioned eggs was inhibited ($P < 0.01$) by barley seedlings. However, the seedlings did not continue growth after placement with the egg masses. Under these conditions, compounds other than hatching stimulants in the root diffusate may have predominated. Stimulation of *Meloidogyne* eggs of three other species was usually observed only with vigorous seedlings (7).

Effects of chemicals.—Sodium hypochlorite stimulated ($P < 0.01$) hatching of *M. naasi* eggs. This effect has also been noted in independent research at another laboratory (6).

The hatching of eggs freed of gelatinous matrix by sodium hypochlorite treatment was high at 21 C, and was not increased by incubation at 6 or 9 C (Table 4). Larvae obtained from the sodium hypochlorite treatment caused galling of barley roots, indicating that some of them, at least, were infective.

None of the potassium permanganate or hydrochloric acid treatments dissolved the gelatinous matrix or stimulated hatching. Nicotinic acid treatments were also ineffective.

Electron microscopy studies.—Examination of the egg shell, the cuticle of the developing larvae, and organs and organelles in cold-warm treated and laboratory-temperature treated eggs of the same age re-

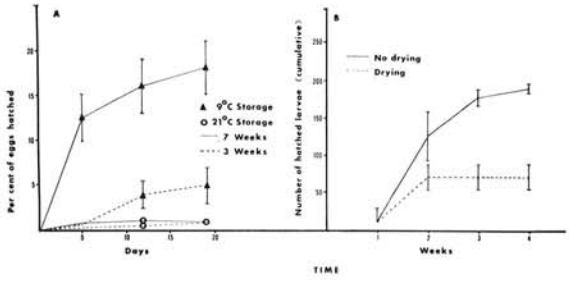

Fig. 1. Hatch of *M. naasi* eggs after **A)** storage at 9 C for 3 and 7 weeks, followed by incubation at room temperature for varying periods of time; **B)** with time, in soil kept moist or dried.

TABLE 3. Per cent of *Meloidogyne hapla* eggs hatched after storage at constant low temperatures for various periods of time followed by incubation at 21 C for 5 days

Storage temp.	% Eggs hatched[a]			
C	*1 Week*	*2 Weeks*	*3 Weeks*	*8 Weeks*
6	44.7 ± 3.7	51.0 ± 4.5	57.0 ± 1.4	52.8 ± 3.1
9	56.4 ± 2.6	45.9 ± 3.8	58.8 ± 3.4	70.7 ± 2.4
21	66.5 ± 4.8	83.0 ± 2.4	85.9 ± 2.2	
LSD[b] (.05)	13.7	9.6	7.6	
LSD[b] (.01)	18.8	13.2	10.4	

[a] Mean of 10 replicates; the initial mean number of eggs for the 90 replicates was 1261 ± 53.
[b] Between means for any temperature and mean for 21 C after analysis of variance.

TABLE 4. No. *Meloidogyne naasi* larvae hatched in several temperature regimes from eggs that had been freed of gelatinous matrix with sodium hypochlorite

Temp. treatments, all followed by 5 days at 21 C	No. hatched larvae[a]
1 week at 6 C	1580 ± 254
9 C	2126 ± 108
21 C	1941 ± 128
2 weeks at 6 C	1614 ± 114
9 C	1799 ± 154
21 C	1934 ± 108

[a] Mean of five replicates and the standard error of this mean.

vealed no cytological or morphological differences that might be correlated with the hatching stimulation caused by the cold treatment.

DISCUSSION.—Of the factors studied, only exposure to low temperature followed by warm temperature, and treatment with sodium hypochlorite, stimulated hatching of *M. naasi* eggs.

The cold-warm incubation requirement for hatching could explain much of the previously observed behavior of *M. naasi* in field and greenhouse. The incubation requirement is probably fulfilled naturally in the field with seasonal progress from winter to spring. Once the minimal low temperature storage time has been met, the gradually rising soil temperature in the growing season would provide suitable conditions for hatching.

Sodium hypochlorite treatment of eggs may be a useful and rapid method for obtaining hatchable eggs for pathogenicity studies. The hatching response to sodium hypochlorite indicates that a hatching inhibitor may be present in the egg mass matrix or associated with the egg shell. Larvae hatching from eggs treated with sodium hypochlorite are infective, and appear to be normal.

Lack of cytological differences between sections of temperature-treated and untreated eggs indicates that the temperature treatment produces a chemical, rather than a structural effect, perhaps inactivating a hatching inhibitor.

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