The Effects of Selected Antimetabolites and Antibiotics on Reproduction, Embryonic Development and Hatch of Meloidogyne hapla

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Abstract: Meloidogyne hapla egg-laying was unaffected by a 3-day immersion in 40 ppm concentrations of 6-azauracil, 5-bromodeoxyuridine, or streptomycin sulfate in physiological saline. Comparable exposure to 1-20 ppm cycloheximide irreversibly inhibited egg-laying, but with exposures of 1, 3, or 9 hr, the effect was partly reversible. Of the few eggs laid after the nematodes were transferred to physiological saline, many were abnormally developed. Most of the unlaid eggs extracted from the uteri of cycloheximide-treated nematodes were nonviable. Oogenesis was irreversibly inhibited by the treatment. Cycloheximide stimulated embryonic development and some early hatching, but later hatching was inhibited. *Key Words:* Cycloheximide, *Meloidogyne hapla*, oogenesis, embryogenesis, egg-laying.

Chemical reproductive sterilization of plant parasitic nematodes is relatively new, although entomologists have given it considerable attention. Kilgore (9) defined insect chemosterilants as "chemicals which deprive insect species of their ability to reproduce". This reproductive inhibition may involve egg-laying or hatching, pupation, or final maturation. Additional extensive information on insect chemosterilization is given by LaBrecque and Smith (10).

The nematicidal effects of selected antibiotics (4) and antimetabolites (3) against root-knot nematodes have been tested. However, specific data on reproduction, embryonic development or hatch was not presented.

Feldmesser *et al.* (6) reported gonad morphology changes and reduced reproduction in saprophagous nematodes caused by 5-fluorouracil. Endo and Schaeffer (5) showed that 6-azauracil inhibited the development of *Heterodera trifolii* in red clover roots beyond the early third larval stage.

Little information is available on the effects of chemicals on the embryonic development of root-knot nematode eggs. Wallace (14) reported that the embryonic development of *Meloidogyne javanica* was stopped in solutions of 0.7-1 M glycerol (about 15 to 20 atmospheres).

The purpose of the experiments reported here was to evaluate the effect of selected antimetabolites and antibiotics on the reproduction, embryonic development, and hatch of *M. hapla*.

MATERIALS AND METHODS

The nematodes used in these experiments were derived from one egg mass on a *M. hapla*-infected tomato plant growing in a field at Macdonald College of McGill University, Ste. Anne de Bellevue, Quebec, Canada. The population was built up and maintained on tomatoes of the variety John Baer grown in 5-inch clay pots, under greenhouse conditions, in soil that had been pasteurized.

The effect of test compounds on egg-laying by M. hapla.—Egg-laying females were dissected from the tomato roots and immersed in physiological saline suitable for egg-laying (8) in which 40 ppm of one of the following antimetabolites or antibiotics were incorporated: 6-azauracil, 5-bromodeoxyuridine, streptomycin sulfate, and cycloheximide.

The treatments were replicated 25 times, as was the control of physiological saline without additional chemicals. Petri dishes (50 X 15 mm), each containing one female, were incubated at 23-26 C, and egg-laying was recorded at various intervals until all nematodes ceased to lay eggs. The solutions containing test chemicals were replaced by physiological saline alone after the 3rd day.

Cycloheximide, the only compound inhibitory at 40 ppm, was tested at 1, 5, 10, and 20 ppm in an experiment similar to the above.

In another experiment, the effect of 1-, 3-, and 9-hr exposure to 20 ppm cycloheximide on reproduction of *M. hapla* was studied. Females that burst during these experiments were discarded. The final egg-count data were analyzed using Duncan's new multiple range

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test for comparison of means with uneven replication.

Viability of unlaid eggs contained in the female body was estimated by incubating them in the physiological saline for about a month. Then they were dissected from the nematodes, and their viability was estimated. Hatched larvae or eggs containing larvae were recorded as viable. Oocytes or eggs not containing larvae were recorded as nonviable.

The effect of cycloheximide on the embryonic development and hatch of M. hapla eggs.—About 200 egg-laying females were dissected from roots of tomato plants and immersed in a saline solution for egg-laying (8). About 24 hr later, the newly laid eggs (one- or two-celled stage) were removed from the saline and washed by gentle agitation in five changes of distilled water. From 150 to 200 eggs were then pipetted into 7-ml solutions of 1, 5, 10, and 20 ppm cycloheximide. The treatments were replicated three times and the eggs incubated at 23–26 C.

For simplicity, only two phases of the embryonic developmental pattern of *M. hapla* were considered: from egg-laying to the appearance of a vermiform larva; and from the formation of a vermiform larva to hatching. The water of the control treatment, and the cycloheximide solutions, were changed four times during the test and replaced by water on the 14th day.

The percentage data (N) of this test were transformed to arcsin N and analysed using Duncan's new multiple range test.

RESULTS

The results of the tests to assess the effect of the antimetabolites and antibiotics on egg-laying by *M. hapla* are in Tables 1, 2, and 3.

Table 1 shows that egg-laying of *M. hapla* females was affected only by the cycloheximide.

Table 2 shows that even 1 ppm of cycloheximide strongly inhibited egg-laying, although the effect was increased at the higher concentrations.

Egg-laying activity in non-feeding nematodes was maintained for about 7 days, by which time almost all the eggs in the ovary were deposited, and the female body was depleted and relatively transparent.

Most of the unlaid eggs excised from females kept in saline solution for about 1 month after egg-laying ceased, following the cycloheximide treatments, were not viable, whereas the few eggs found in the controls after an equal time in the saline solution, were mostly viable (Tables 2, 3). This strongly suggests that oogenesis in the females treated with cycloheximide was irreversibly inhibited, although the nematodes were not initially killed, as evidenced by phloxine tests (7). The majority of nonviable "eggs" remaining in cycloheximide-treated females were arrested oocytes.

Table 3 shows that egg-laying of females in 20 ppm cycloheximide was strongly and significantly inhibited even by 1 hr of exposure. Some of the few eggs laid after the females were transferred to the saline solution were abnormal. Their color was gray compared to the darker, normal eggs and oocytes excised from the uteri of the females. The protoplasm of the abnormal eggs was netlike and less dense than normal, and it occupied the whole space inside the eggshell without any evidence of the perivitellar spaces often seen in newly laid eggs. Unlike normal eggs, they were readily stained with gentian violet (13), indicating that the

TABLE 1. The effect of selected antimetabolites and antibiotics on *in vitro* egg-laying by *Meloidogyne hapla* females in physiological saline

Treatment (40 ppm active)	Cumulative† egg counts per female at various intervals (days)					
	1	3‡	5	7	9	
Untreated control	54.4	99.5	123.2	128.5	128.5	(a)
6-azauracil	52.1	87.9	108.6	116.7	116.7	(a)
5-bromodeoxyuridine	54.6	98.9	124.1	132.4	132.4	(a)
Streptomycin sulfate	52.2	97.9	111.7	116.2	116.2	(a)
Cycloheximide	9.6	9.6	9.6	9.6	9.6	(b)

† Cumulative average egg counts for 25 nematodes.

‡ Antimetabolites and antibiotics were replaced by physiological saline solution alone on the 3rd day.

Means followed by the same letter are not different at the $P = 0.05$ level as determined by Duncan's new multiple range test.

TABLE 2. The effect of cycloheximide concentration on egg-laying by *Meloidogyne hapla*

Conc. (ppm in physiological saline)	Cumulative† egg counts at various intervals (days)					Unlaid eggs	
	1	3‡	5	7	9	Viable	Nonviable
0	51.1	103.1	133.1	140.8	140.8(a)	2.3	0.5
1	43.1	60.9	66.9	67.6	67.6(b)	1.6	5.7
5	30.1	31.1	31.1	31.1	31.1(c)	1.1	6.7
10	20.4	20.8	20.8	20.8	20.8(c)	1.3	12.2
20	14.4	14.4	14.4	14.4	14.4(c)	0.9	18.6

† Average per female nematode.
‡ The cycloheximide was replaced by physiological saline alone on the 3rd day.
Means followed by the same letter are not different at the $P = 0.05$ level as determined by Duncan's new multiple range test.

TABLE 3. Exposure time effects of 20 ppm cycloheximide on egg production by *Meloidogyne hapla*

Immersion time (hr)		Cumulative† egg counts at various intervals (days)					Unlaid eggs	
		1	3	5	7	9	Viable	Nonviable
(Control)		42.3	86.3	94.8	96.3	96.3c	1.5	0.8
1	1.9‡	19.6	25.4	26.9	27.2	27.2b	1.4	7.0
3	4.6‡	11.7	12.6	13.2	13.2	13.2ab	0.3	9.8
9	3.8‡	6.8	7.1	7.1	7.1	7.1a	1.6	10.1

† Average per female nematode.
‡ Average number of eggs per nematode just before transfer from the cycloheximide to a saline solution at 1, 3, and 9 hr, respectively.
Means followed by the same letter are not different at the $P = 0.05$ level as determined by Duncan's new multiple range test.

TABLE 4. The effect of cycloheximide on embryonic development and hatch of *Meloidogyne hapla* eggs

Cycloheximide (ppm)	% Embryos attaining vermiform stage in 6 – 11 days†			% Larvae hatched in 12 – 18 days					
	6	8	11	12	13	14‡	15	16	18
(Control)	16.8	78.2	85.6	2.5	25.9	69.6	77.2	82.0	82.0
1	26.0	80.0	81.0	10.7	51.5	71.0	72.1	72.6	72.6
5	30.5	79.1	81.1	23.2	62.1	71.4	72.1	72.1	72.1
10	39.1	82.3	82.3	30.7	55.5	61.5	62.1	62.1	62.1
20	25.5	77.5	81.0	9.4	27.9	37.7	38.7	40.1	40.1

† Average of three replicates, each composed of 150–200 eggs.
‡ Cycloheximide solutions were replaced by water on the 14th day.

vitelline membrane was either defective or absent. No cleavage or further development of the abnormal eggs could be seen during the several days in which they were observed. The percentages of these abnormal nonviable eggs, laid after the females were transferred to the physiological saline solution, were 3.4, 9.6, and 79.1 in females treated 1, 3, and 9 hr, respectively, in 20 ppm cycloheximide. The few eggs that were laid by individual females treated for 9 hr and then transferred to the saline solution were either all normal in appearance or all abnormal, indicating that the induced sterility was not reversible.

Two or three times, in preliminary experiments, as many as 40–60 eggs, all abnormal, were laid by individuals in physiological saline after immersion in solutions

of 10 or 20 ppm cycloheximide for 2 to 3 days.

The stimulating effect of cycloheximide on the embryonic development and hatch of *M. hapla* eggs is shown in Table 4.

On the 6th day, more than twice as many embryos had reached the vermiform larval stage in eggs treated with 10 ppm cycloheximide than in the controls. After the 8th and 11th day there were no differences among the treatments.

That cycloheximide stimulated the embryonic development of *M. hapla* eggs is also demonstrated by the percentage of larvae hatched on the 12th day when many more larvae had hatched in all treatments than the control (Table 4). From the 14th day onward, stimulation of hatch, resulting from the stimulatory effect on embryonic development, was less evident in the cycloheximide-treated eggs.

The cycloheximide shortened by about 1 day the normal time needed for the newly laid eggs to develop and hatch. This resulted in a relatively high percentage of early hatching. However, by the end of the test the cumulative percentage hatch in all cycloheximide treatments was less than in the non-treated controls.

DISCUSSION

Most of the unlaid eggs excised from cycloheximide-treated females (about 1 month after egg-laying had ceased) were not viable (Tables 2, 3), whereas the majority of those found in untreated females unable to deposit their eggs were viable. This suggested that maturation of oocytes to eggs was irreversibly inhibited in treated females.

When egg-laying females were treated with 20 ppm cycloheximide for 1, 3, or 9 hr, egg-laying was inhibited, but in these trials the inhibition was partly reversible, although a number of those eggs laid after the females were transferred to a saline solution were nonviable. Feldmesser *et al.* (6) reported that 5-fluorouracil reduced the rates of reproduction of saprophytic nematodes, and Sitaramaiah *et al.* (12) showed that fatty acids suppressed egg-laying in *Meloidogyne javanica*, and the few eggs which were laid failed to hatch.

In the present study, cycloheximide caused a reduction both in egg production and in percentage hatch, the latter being more pronounced, especially when gravid adult females of *M. hapla* were treated 9 hr with 20 ppm of cycloheximide.

Bennett *et al.* (1) reported that cycloheximide inhibited DNA and protein synthesis in mammalian cells, and Mahler and Cordes (11) have stated that cycloheximide interferes with cell-free protein synthesis in yeasts, fungi, and higher organisms in general. Thus, it is possible that protein synthesis was inhibited in adult female *M. hapla* nematodes treated with cycloheximide. The abnormal eggs laid by females treated with cycloheximide had no visible vitelline membrances and were readily stained with gentian violet. Abnormal eggs in this respect were similar to immature oocytes excised from the uteri of gravid females, but their protoplasm was netlike and less dense, and it appeared gray instead of the normally darker color. This suggests that the formation of the vitelline membrane and possibly protein synthesis was inhibited by the cycloheximide. However, no biochemical studies were made to determine its mode of action on nematode reproduction.

Although cycloheximide inhibited reproduction of adult *M. hapla* females, it stimulated embryonic development and promoted earlier hatching; nevertheless, total hatch was inhibited by all cycloheximide treatments. Perhaps only highly diluted cycloheximide reached the embryo to cause stimulation of embryonic development because of the presence of the vitelline membrane. The vitelline membrane of *Meloidogyne javanica* is dissolved by enzymatic action, just before hatching (2). When the membrane was dissolved, in the *M. hapla* of these tests, a sufficiently high concentration of cycloheximide may have entered the eggs to inhibit hatching.

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