Mode of Parasitism of *Meloidogyne* and Other Nematode Eggs by *Dactylella oviparasitica*

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Abstract: Hyphae of *Dactylella oviparasitica* proliferated rapidly through *Meloidogyne* egg masses, and appressoria formed when they contacted eggs. The fungus probably penetrated egg shells mechanically, although chitinase production detected in culture suggested that enzymatic penetration was also possible. In soil, *D. oviparasitica* invaded egg masses soon after they were deposited on the root surface and eventually parasitized most of the first eggs laid. Occasionally the fungus grew into *Meloidogyne* females, halting egg production prematurely. The fungus parasitized eggs in the gelatinous matrix or eggs freed from the matrix and placed on agar or in soil. Specificity in nematode egg parasitism was not displayed, for *D. oviparasitica* parasitized eggs of four *Meloidogyne* spp., *Acrobeloides* sp., *Heterodera schachtii*, and *Tylenchulus semipenetrans*. In tests in a growth chamber, parasitism by *D. oviparasitica* suppressed galling on *M. incognita*-infected tomato plants. *Key Words:* biological control, appressorium, chitinase, *Heterodera schachtii*, *Tylenchulus semipenetrans.*

Dactylella oviparasitica Stirling and Mankau was first isolated from *Meloidogyne* egg masses collected from peach orchards in the San Joaquin Valley, California (12). Laboratory studies indicated (13) that the fungus was parasitic on *Meloidogyne* eggs, and suggested that it contributed to the biological control of root-knot nematodes in the field. It was not known how *D. oviparasitica* invaded egg masses and penetrated eggs, or whether parasitism was limited to eggs of *Meloidogyne*. It seemed possible that the aggregation of eggs in a gelatinous matrix allowed more active parasitism of *Meloidogyne* eggs than of eggs of other nematodes. The objectives of this study were: 1) to follow the invasion and proliferation of *D. oviparasitica* in egg masses, and its penetration into eggs; and 2) to determine whether the parasitism was restricted to a single species or group of nematodes.

MATERIALS AND METHODS

The invasion of *Meloidogyne* egg masses by *D. oviparasitica* was studied on agar plates and in soil. Egg masses of *M. incognita* (Kofoid and White) Chitwood were added to macerated mycelium of *D. oviparasitica* (isolates C, K and S) on water-agar plates by techniques described earlier (13). Plates were incubated at 24 C, and egg masses were examined periodically by dissolving the gelatinous matrix in 1% NaOCl and observing them under a microscope. Egg masses partially dissolved with 1% NaOCl were also fixed in 5% formalin, placed in 2% glycerin, and allowed to dehydrate for 5 weeks. They were then coated with a 10-nm-thick gold coating and ex-

Received for publication 7 July 1978.

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amined in a JEOL JSM-U3 scanning electron microscope at 7KV and a 20-nm aperture.

To determine whether *D. oviparasitica* produced chitinase, seven isolates of the fungus were grown on an agar medium containing colloidal chitin (8).

Moist mycelium of *D. oviparasitica* (isolate S) was also incorporated into autoclaved Hanford sandy loam soil at 1.31 mg dry mycelium / g dry soil (13).

Tomato seedlings inoculated 2 days previously with 50 *M. incognita* larvae were transplanted into infested soil in tubes 10 cm long and 3 cm in diameter. Controls were plants inoculated with *M. incognita* and transferred to autoclaved soil without the fungus. All tubes were maintained in a plant growth chamber at 27 C with 14 h light. Twenty-five, 30, 35, 40, and 50 days after inoculation with *M. incognita,* four replicate plants were harvested from each treatment (five on day 50). Some egg masses were used to study the development of parasitism, while about 10 egg masses from each plant were treated with 1% NaOCl and macerated as described previously (13). Parasitized and unparasitized eggs were counted. On day 50, five additional egg masses from each plant were placed in hatching chambers (3) and hatch was determined after 15 days at 27 C. Eggs were then freed from egg masses as above, and parasitized and unparasitized eggs were counted. The effect of *D. oviparasitica* on infection of plants by *M. incognita* larvae was assessed by counting galls on plants harvested on day 50.

To determine whether *D. oviparasitica* parasitized nematode eggs which were not embedded in a gelatinous matrix, attempts were made to infect free *M. incognita* eggs which were dispersed, clumped together, or embedded in materials which replaced the matrix. Egg masses were collected from greenhouse-grown tomato, and eggs were freed from the gelatinous matrix by treatment with 1% NaOCl for about 3 min. The eggs were passed through a 45-μm sieve, collected on a 25-μm sieve, and then washed into a centrifuge tube. After centrifugation, most of the supernatant was removed and five drops of the concentrated egg suspension were placed on mycelium of *D. oviparasitica* (isolate K or S) which had been added to the center of water-agar plates. The eggs were left in clumps on the fungus or dispersed over the surface of the mycelium. Other eggs were incorporated into water-agar immediately before it solidified, and then small cubes containing eggs were cut from the agar and placed on water-agar plates containing the fungus. Each treatment was replicated four times. After 7 days at 24 C the eggs were macerated in water in a blender, and parasitized and unparasitized eggs were counted. Hatch was determined by macerating the remaining agar and counting larvae in aliquots of the resulting suspensions.

The parasitism of free *M. incognita* eggs was also studied in soil. A suspension of *M. incognita* eggs obtained from greenhouse-grown tomato plants using NaOCl (9) was diluted to contain about 40,000 eggs/ml. Moist mycelia of *D. oviparasitica* (isolates S and K) were respectively incorporated into Hanford sandy loam soil at 0.88 and 1.06 mg dry mycelium/g dry soil. Thirty 10-g samples of autoclaved soil or of soil containing the fungus were placed in 5.5-cm-diam petri dishes, and 0.5 ml of the egg suspension was added. The soil moisture content was adjusted to 7% with water, and each soil sample was mixed thoroughly to disperse the eggs. Immediately, and again after 2, 4, 6, and 8 days of incubation at 24 C, five replicates of each soil were placed on Baermann funnels for 2 days, and parasitism was assessed indirectly by counting the larvae extracted. On the eighth day, soil in the five remaining dishes was macerated in water in a blender and added to about 300 ml of water in a flask. The soil suspensions were shaken, allowed to settle for 10 seconds, and then decanted through a 25-μm sieve. The material retained on the sieve was centrifuged in sucrose solution (484 g/l) at 1,200 rpm (250 $\times$ *g*). The supernatant was poured through a 25-μm sieve, and the material retained was collected and examined for parasitized eggs.

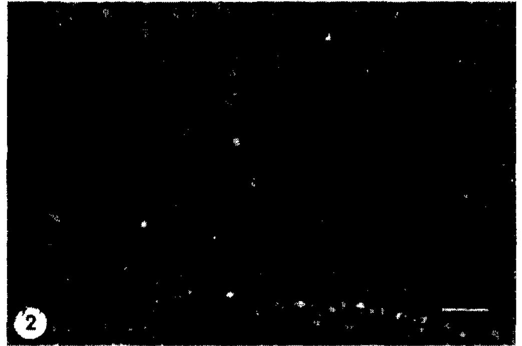
The ability of *D. oviparasitica* to parasitize eggs of different *Meloidogyne* spp. was studied by adding 10 egg masses of *M. arenaria, M. hapla, M. incognita,* and *M. javanica* to water-agar plates containing macerated mycelium of *D. oviparasitica* (isolates S, C, and K), prepared as above. After 15 days at 24 C, eggs remaining in the

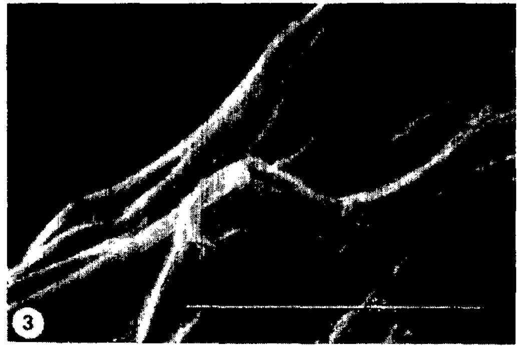
egg masses were rated as follows: 0 = no eggs parasitized; 1 = 1–25% eggs parasitized; 2 = 26–50% eggs parasitized; 3 = 51–75% eggs parasitized; 4 = 76–100% eggs parasitized.

Eggs of other nematode species were also added to mycelium of *D. oviparasitica* by the above technique. Eggs of *Diplenteron* sp. were obtained from cultures with mixed bacteria on peanut butter agar (peanut butter, 15 g; water, 1 liter; agar, 16 g). Agar containing the nematodes was shaken in water, and the suspension was passed through 149-μm and 38-μm sieves to separate eggs from adults and larvae. The eggs and some larvae retained on the 38-μm sieve were concentrated by centrifugation. Eggs of *Acrobeloides* sp. were obtained in the same way with 74-μm and 25-μm sieves. Citrus roots heavily infected with *Tylenchulus semipenetrans* and females and cysts of *Heterodera schachtii* were also placed on mycelium of the fungus.

RESULTS

D. oviparasitica invaded *Meloidogyne* egg masses rapidly; hyphae were observed proliferating through the gelatinous matrix 18 hours after egg masses were placed on the fungus. The appressoria which formed on the hyphae when they contacted eggs (Figs. 1, 2, 3) varied in shape and size from simple round knoblike swellings about 4 μm in diameter to more complex elongate swellings with several lobes. They often followed the contour of the egg shell and appeared tightly appressed to the egg surface. Scanning electron micrographs (Fig. 3) showed that a substance occurred between the appressorium and the egg shell which may have had adhesive properties. Penetration of eggs from the appressoria was not observed because newly penetrating hyphae were obscured by the embryo. Sixty hours after inoculation, some eggs contained visible hyphae, and the number of parasitized eggs then increased rapidly. Once *D. oviparasitica* invaded egg masses on agar, many of the eggs were eventually parasitized, particularly those in early stages of development. Eggs containing second-stage larvae usually escaped parasitism, although parasitized larvae within eggs have been observed previously (13). During the early





FIGS. 1-3. Photomicrographs (1, 2) and scanning electron micrograph (3) of an appressorium of *Dactylella oviparasitica* on the surface of a *Meloidogyne incognita* egg. In each figure, bars represent 10 μm.

stages of infection, hyphae of *D. oviparasitica* completely occupied the embryo of the egg. After the embryo contents were consumed, the hyphae became vacuolate and their protoplasm eventually disappeared. At this stage it was difficult to recognize previously parasitized eggs, since they looked empty unless observed at a magnification of at least 400×, when fungal cell walls could sometimes be seen. The fungus did not produce resting spores such as chlamydospores in nematode eggs, although conidia have been seen occasionally in egg masses in other studies (12).

Cleared zones occurred around colonies of *D. oviparasitica* on a medium containing colloidal chitin, suggesting that the fungus produced chitinase in culture.

In the experiment with *M. incognita* in soil, egg laying began 18–20 days after plants were inoculated with the nematode. Eggs were either deposited directly in soil, or accumulated in the root, later becoming exposed as pressure from the female and eggs ruptured the root cortex. Observations of egg masses from soil containing *D. oviparasitica* on day 25 showed that few of them contained parasitized eggs but many contained fungal hyphae. Parasitized eggs were observed in almost all egg masses on day 30, and the proportion of parasitized eggs then increased with time (Table 1a). There were more eggs in egg masses invaded by *D. oviparasitica* than in controls (Table 1a) because parasitized eggs remained in the egg mass and did not hatch. Young egg masses containing parasitized eggs could generally be identified under a dissecting microscope since they were tan or golden-brown in color rather than the normal yellowish-white. Observations of egg masses on days 40 and 50 showed that the outer layer of eggs were usually all parasitized, but newly deposited eggs around the vulva of the female were not parasitized. The fungus did not invade many of these newly produced eggs when egg masses were transferred to hatching chambers, for the proportion of parasitized eggs increased only slightly in the next 15 days (Table 1b). Some of the parasitized eggs were almost devoid of mycelium at the end of the experiment, suggesting that at 27 C *D. oviparasitica* is barely visible in parasitized eggs 40–45 days after invasion.

TABLE 1a. Average number[a] of parasitized and unparasitized eggs in *Meloidogyne incognita* egg masses on tomato plants growing in autoclaved soil, or soil containing *Dactylella oviparasitica*, 25, 30, 35, 40, and 50 days after inoculation with the nematode.

	Autoclaved soil	Soil containing *D. oviparasitica*	
Day	No. eggs	No. eggs	% parasitized eggs
25	299	310	1
30	810	656	19
35	945	994	21
40	1050	1028	45
50	1217	1410	48

TABLE 1b. Hatch and parasitism of eggs collected from the above plants on day 50 and placed in hatching chambers.

Autoclaved soil		Soil containing *D. oviparasitica*		
Hatch	Unparasitized eggs	Hatch	Unparasitized eggs	Parasitized eggs
1018	240	555	41	800 (57%)

[a]Data are means of four replicates (five on day 50) of 8–10 egg masses per replicate.

On day 50, roots of plants in soil containing *D. oviparasitica* had 66±59 galls, compared with 197±73 galls on control plants (mean ± S.E.). Suppression of galling was most apparent on plants with more than half of the eggs parasitized, for they had only 11, 29, and 32 galls.

No parasitized females were observed in this experiment, although they did occur in similar experiments. When *D. oviparasitica* parasitized all the eggs in an egg mass it sometimes invaded and completely filled the body of the female which produced the eggs (Fig. 4). The fungus was isolated from infected females and its identity confirmed. Parasitized females ceased egg production prematurely, became flaccid, and changed from white to grayish-brown. Usually less than 10% of the females were parasitized.

D. oviparasitica invaded *M. incognita* eggs which had been removed from the gelatinous matrix, whether they were dispersed, present in clumps, or incorporated into water-agar (Table 2). The fungus most actively parasitized eggs which were in clumps, and fewer eggs were parasitized when they were dispersed or incorporated in water-agar. Hatch was similar in all treatments (Table 2).

When free *M. incognita* eggs were added to soil, the numbers of larvae hatched during the first 4 days were similar for autoclaved soil and for soil containing *D. oviparasitica* (Fig. 5). The number of larvae extracted from autoclaved soil increased significantly on days 6 and 8 but remained constant in soil containing either isolate of *D. oviparasitica*, suggesting that

FIG. 4. The body of a *Meloidogyne incognita* female recently invaded by *Dactylella oviparasitica*. Bar represents 10 μm.

TABLE 2. Parasitism and hatch of *Meloidogyne incognita* eggs dispersed over mycelium of *Dactylella oviparasitica* (isolates K and C), added in clumps or incorporated into water-agar.

	% eggs parasitized[a]		% hatch[a]	
	Isolate S	Isolate K	Isolate S	Isolate K
Eggs in clumps	85.9 a	78.8 a	14.1 a	20.4 a
Eggs dispersed	77.1 ab	63.7 b	20.4 a	27.8 a
Eggs in water-agar	68.8 b	52.4 b	21.1 a	24.5 a

[a]Percentages of eggs added originally. Means are from four replicates containing an average of 3,200 eggs in clumps, 2,210 dispersed eggs, or 4,010 eggs in water agar, per replicate. In each column, numbers followed by the same letter are not significantly different ($P = 0.05$) by Duncan's multiple-range test.

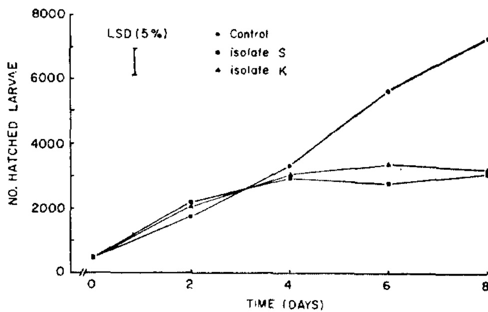


FIG. 5. Effect of *Dactylella oviparasitica* (isolates S and K) on hatch of *Meloidogyne incognita* eggs free in soil. Each point is the mean of five replicates. Bar represents the least significant difference (5%) between means.

eggs were being parasitized by the fungus. On day 8, parasitized eggs were extracted from soil containing *D. oviparasitica* but not from autoclaved soil.

D. oviparasitica parasitized eggs of all the *Meloidogyne* spp. tested, although isolate S proved less virulent than the other isolates in this experiment (Table 3). Eggs of *Diplenteron* sp., *Acrobeloides* sp., and *T. semipenetrans* were also parasitized by the fungus. *D. oviparasitica* parasitized *H. schachtii* eggs in egg masses outside the female, and also invaded white females and cysts. More eggs were parasitized in white females than in cysts because most of the eggs in cysts contained second-stage larvae, and these eggs were generally not parasitized by *D. oviparasitica*.

DISCUSSION

Observations on the proliferation of *D. oviparasitica* in *Meloidogyne* egg masses showed that appressoria formed on eggs soon after the egg mass was invaded. Appressoria are important in attachment and penetration of the host by plant-parasitic fungi (2) and since the appressoria of *D. oviparasitica* are morphologically similar to those fungi, they probably have a similar function. Plant-parasitic fungi most commonly penetrate their hosts mechanically, but enzymatic penetration or a combination of both also occur in some species (2). Many species of entomophagous fungi produce chitinase and other enzymes which enable them to penetrate the insect cuticle (4, 6, 7). However, even in species known to enzymatically digest the cuticle, some mechanical penetration also occurs (5, 10). *D. oviparasitica* probably penetrates egg shells mechanically, but since chitin is the

TABLE 3. Parasitism of eggs of four *Meloidogyne* spp. by *Dactylella oviparasitica* (isolates K, C, and S) on agar.

Species	Rating of parasitism[a]		
	Isolate K	Isolate C	Isolate S
M. arenaria	4.0 a	3.7 a	1.0 b
M. hapla	3.7 a	4.0 a	1.1 b
M. incognita	3.6 a	3.5 a	1.1 b
M. javanica	4.0 a	4.0 a	1.2 b

[a]Eggs remaining at the end of the experiment were rated as follows: 0, no eggs parasitized; 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, 76–100% of eggs parasitized. Each number is the mean rating on 10 egg masses. Means followed by the same letter are not significantly different ($P = 0.05$) by Duncan's multiple-range test.

main constituent of *Meloidogyne* egg shells (1), the production of chitinase in culture suggests that enzymatic penetration is also possible.

Studies using plants infected with *Meloidogyne* supported earlier suggestions (13) that under natural conditions *D. oviparasitica* may enter egg masses soon after they are exposed to soil. Most of the eggs produced by females during the first 15–20 days of egg production eventually were parasitized. The ability of the parasite to move into more eggs declined as the number of parasitized eggs in an egg mass increased. At this stage, hyphae outside the egg became thickened and were morphologically similar to those inside the egg, suggesting that when nutrition was adequate fungal hyphae became assimilative and extension growth slowed. A similar effect was observed in culture, for the radial growth of *D. oviparasitica* was less on nutritionally rich media such as YPSS and glucose-peptone than on media such as CMA (12).

The invasion of females by *D. oviparasitica* appeared to depend on the fungus invading egg masses as soon as the first eggs were deposited, and being active enough to grow through the egg mass before many eggs were laid. The fungus appeared to enter females through the vulva rather than directly through the cuticle. Parasitism of females was infrequent when the nematode infected tomato but might have been more frequent under environmental conditions or on hosts less suited to egg production by the nematode.

There was no evidence to suggest that *D. oviparasitica* derived any nutritional benefit from the gelatinous matrix which surrounds *Meloidogyne* eggs since the fungus parasitized *Meloidogyne* eggs when the matrix was removed or when eggs were embedded in various agar media. This, and the ability of the fungus to parasitize eggs of all nematodes tested, suggested that *D. oviparasitica* was not a specific parasite of *Meloidogyne*. However, the aggregation of *Meloidogyne* eggs makes them more vulnerable to parasitism than the dispersed eggs of many other soil-inhabiting nematodes, since many eggs are destroyed once an egg mass is invaded.

The ability of *D. oviparasitica* to parasitize the eggs of microbivorous nematodes may be important in the ecology of the fungus. In areas where *Meloidogyne* eggs are an important food source for the fungus, it may survive periods when *Meloidogyne* eggs are absent by parasitizing the eggs of microbivorous nematodes, since they are usually abundant in soil. Eggs of microbivorous nematodes were extracted from field soil known to contain *D. oviparasitica*, but none of the few obtained were parasitized. Better techniques of extracting eggs are needed before the parasitism of nematode eggs occurring free in soil can be confirmed.

The cultivation of plants infected by *Meloidogyne* in soil in which *D. oviparasitica* was incorporated indicated that the fungus parasitized *Meloidogyne* eggs under relatively natural conditions. Although parasitism of eggs by *D. oviparasitica* suppressed the average number of galls on plants 50 days after inoculation, galling was retarded substantially only when more than half of the eggs were parasitized. Since a large number of viable eggs remained, such control was probably temporary. *Meloidogyne* populations are difficult to reduce when conditions are ideal for the nematode, since its potential for reproduction is so great that almost all viable eggs must be destroyed. Control might have been more prolonged if a host less suitable for reproduction of *M. incognita* had been used, or if environmental conditions had been different, since *D. oviparasitica* parasitizes greater numbers of eggs at temperatures below the optimum for the nematode (11). In the field, where conditions are not always ideal for the nematodes, and *Meloidogyne* populations need only be kept below economic thresholds, parasitism by *D. oviparasitica* may reduce *Meloidogyne* populations sufficiently to be of some economic benefit. Also, parasitism of the first eggs produced by females tends to extend the length of the life cycle of the nematode, and over long periods that might significantly affect its multiplication by reducing the number of generations possible each year.

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