

Parasitism of *Meloidogyne* eggs by a new fungal parasite

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Abstract: *Dactylella oviparasitica*, a fungus isolated from *Meloidogyne* egg masses, was shown to parasitize eggs on agar and in soil, when inoculated as either mycelium or conidia. The fungus also grew saprophytically on eggs killed with methyl bromide or heat. The amount of parasitism in the laboratory suggested that the fungus may be a useful biological control agent against *Meloidogyne*. *Key Words:* *Dactylella oviparasitica*, biological control.

During a study of nematodes in peach orchards in the San Joaquin Valley of California, Ferris *et al.* (2) had difficulty in finding peach orchards on Lovell rootstock that had high populations of root-knot nematodes (*Meloidogyne* spp.). They speculated that some of the orchards may have been situated in areas biologically unsuited to *Meloidogyne*. The possibility that the nematode was under natural biological control prompted a search of the area for antagonists of root-knot nematodes, which led to the discovery of a new fungal parasite of *Meloidogyne* eggs (5, 7). A description of the fungus, *Dactylella oviparasitica* Stirling and Mankau, and procedures for its isolation and culture have been published (7). Although there were indications that the fungus was actively parasitizing eggs, rather than living saprophytically in old egg masses and dead eggs (7), experiments described in this paper aim to resolve the question. Also explored is the potential of the fungus as a biological control agent against *Meloidogyne*.

MATERIALS AND METHODS

Parasitism of eggs on agar: Isolates C, K, and S of *D. oviparasitica* obtained from

Meloidogyne egg masses in three different peach orchards (7) were grown for 10 days in shake cultures of YPSS (yeast extract, 4 g; K₂HPO₄, 1 g; MgSO₄ · 7H₂O, 0.5 g; soluble starch, 20 g; distilled water, 1 liter). The mycelium was removed from the medium by filtration, washed with sterile water and macerated in sterile water with a Waring blender. A drop of macerated mycelium of each isolate was placed on the center of eight water agar (WA) plates 6 cm in diameter. Two days later, three young egg masses of *M. incognita* (Kofoid and White) Chitwood from greenhouse-grown tomatoes were added to each plate. Controls were egg masses on WA plates without the fungus.

The plates were maintained in the laboratory at about 24 C for an additional 10 days. The egg masses from six plates in each treatment were then removed, treated with 1% NaOCl for about 5 min to dissolve the gelatinous matrix, and macerated for 20 sec in water in the micro-attachment of a Sorvall Omnimixer. The maceration step separated the parasitized eggs, which were bound together by fungal mycelium.

Parasitized and unparasitized eggs were counted on a 1-ml Peters counting slide (Hawksley and Sons, Lancing, England) at a magnification of 100×. Hatched larvae were counted in an aliquot of the suspension obtained by macerating the agar in water with a blender. Egg masses on the remaining two plates were checked for parasitism and the fungus was reisolated by treating parasitized eggs briefly with 1%

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NaOCl, rinsing several times in sterile water, and placing groups of eggs on YPSS plates.

To check whether parasitism on agar was an artifact of the experimental conditions, a range of soil fungi (*Aspergillus* sp., *Fusarium solani*, *Monacrosporium ellipso-sporum*, *Mucor* sp., *Rhizoctonia solani*, *Trichoderma* sp., and *Dactylella oviparasitica*) were grown in liquid culture, macerated, and added to WA plates as described. *M. incognita* egg masses were added, and eggs were observed for parasitism over a period of 20 days.

Invasion of dead eggs on agar: WA plates containing *D. oviparasitica* (isolate K) were prepared as described previously. Egg masses collected from tomato plants were either treated with methyl bromide (32,000 μ l/liter) for 24 h, heated in water at 60 C for 5 min, or remained untreated. The egg masses were then added to plates containing *D. oviparasitica* or to plates without the fungus. Four egg masses were added to each plate, and treatments were replicated three times. Egg parasitism was assessed after 16 days at 24 C.

Parasitism of eggs in soil using mycelial inoculum: Mycelia of three isolates of *D. oviparasitica* from YPSS shake cultures were washed and macerated in sterile water. Suspensions of known quantities of moist mycelium were pipetted into an autoclaved, dry Hanford sandy loam soil, and the soil moisture content adjusted with sterile water to 7% (about 70% of field capacity). The dry-weight equivalent of the moist mycelium was obtained by oven-drying a sample of each isolate. Each fungal suspension was mixed thoroughly into the soil, and 10-g subsamples were placed into each of six glass vials. Small tomato root pieces bearing four *M. incognita* egg masses were added to the vials, which were lightly capped to allow gaseous exchange but reduce desiccation, and maintained at about 24 C for 20 days. Egg masses were then removed from each vial, the gelatinous matrix was partially dissolved in 1% NaOCl, and clumped eggs were examined microscopically for parasitism. Eggs were liberated from clumps by further treatment with 1% NaOCl followed by maceration, combined with those liberated by the first treatment, and parasitized and unparasitized eggs were

counted. Second-stage larvae were extracted by adding the soil to water, shaking the mixture vigorously, and allowing the suspension to settle briefly before decanting through 500- μ m and 25- μ m sieves. The material retained on the 25- μ m sieve was centrifuged in sugar solution (484 g sucrose/liter of water) at 1200 rpm (about 250 g) for 20 sec and the nematodes were collected from the supernatant on a 25- μ m sieve. Tests with soil inoculated with known numbers of *Meloidogyne* larvae showed that this method recovered $75 \pm 7\%$ of the larvae, and counts were adjusted for that extraction efficiency.

A tenfold-dilution series (75, 7.5, 0.75, 0.075 mg) of macerated mycelium of *D. oviparasitica* (isolate S) was added to six replicate vials containing 10 g of autoclaved dry Hanford sandy loam. The dry-weight equivalent of the moist mycelium was obtained as above. The initial soil moisture content was adjusted to 5%. Four days later, root pieces containing five *M. incognita* egg masses were added to each vial. After incubation at 24 C for 20 days, eggs and larvae were processed and counted as described previously.

Parasitism of eggs in soil using conidial inoculum: Isolate S of *D. oviparasitica* sporulates prolifically on some media, and the conidia germinate readily (7). Conidia of this isolate were obtained from heavily sporulating cultures on enriched YPSS (7) by washing the surface of the culture with sterile water. Conidia were counted in a hemocytometer, and a tenfold-dilution series was prepared so that 0.6-ml subsamples contained 10^6 , 10^5 , 10^4 , and 10^3 conidia. These subsamples were mixed with 10 g autoclaved, dry Hanford sandy loam soil to produce spore concentrations of 10^5 , 10^4 , 10^3 , and 10^2 conidia/g. Equal portions of each soil sample were placed in six vials; controls were prepared with water instead of the spore suspension. Five *M. incognita* egg masses were placed in each vial, and the number of eggs added was estimated by dispersing similar egg masses with 1% NaOCl. Vials were kept in the laboratory for 21 days at 24 C before egg parasitism was assessed.

RESULTS

Parasitism of eggs on agar: Although all

isolates of *D. oviparasitica* parasitized eggs, only isolates K and C caused a significant suppression in hatch (Table 1). The fungus was readily reisolated from egg masses containing parasitized eggs. *D. oviparasitica*

TABLE 1. Parasitism of *Meloidogyne incognita* eggs on agar by three isolates of *Dactylella oviparasitica* after 10 days.

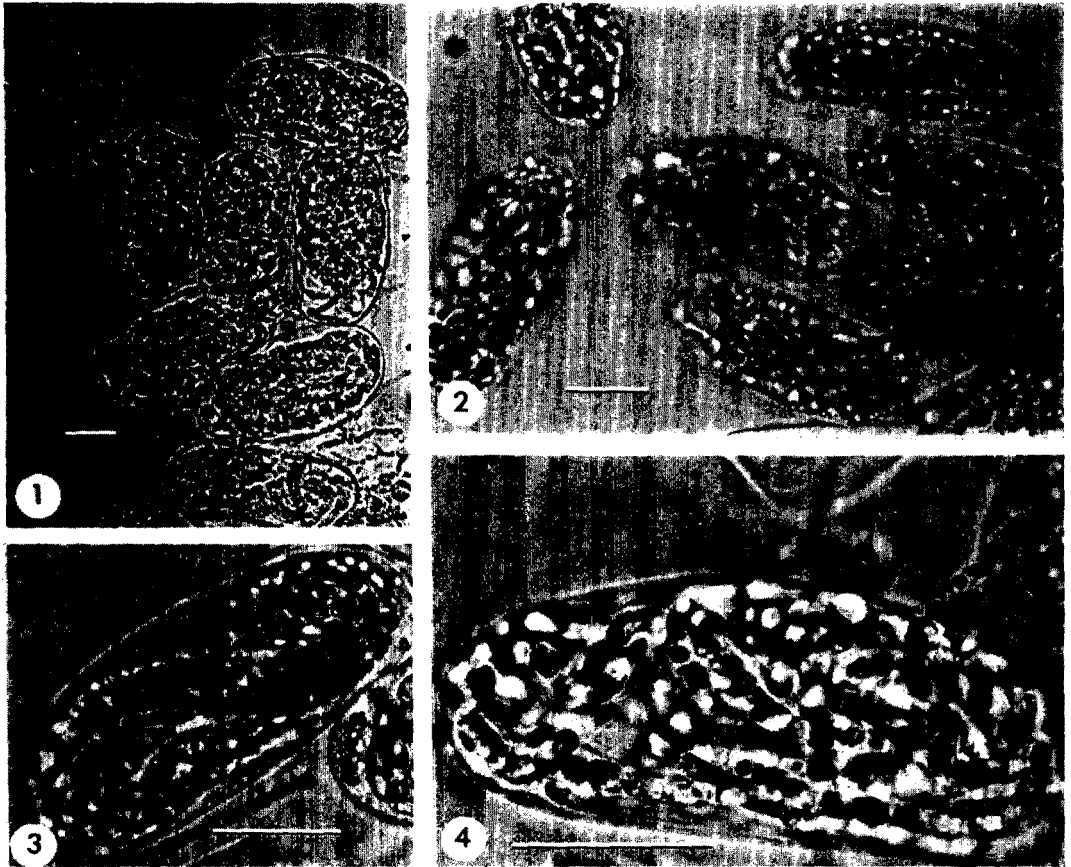
<i>D. oviparasitica</i> isolate	% eggs parasitized*	% hatch*
K	66.2a	25.9a
C	66.1a	20.6a
S	31.2b	43.9b
Control	0.0c	49.1b

*Percentages of eggs added originally. Means are from six replicates with three egg masses (average of 1,740 eggs) per replicate. In each column, means followed by the same letter are not significantly different ($P = 0.05$) by Duncan's multiple-range test.

also utilized dead eggs as a substrate, since it invaded 99% of the eggs treated with methyl bromide or heat. In untreated egg masses, second-stage larvae hatched from some of the eggs, so that significantly less (57%) of the eggs originally added were parasitized. Eggs parasitized by *D. oviparasitica* on agar (Figs. 2-4) appeared similar to those collected from field soil (Fig. 1).

Of the fungi tested, only *D. oviparasitica* was able to parasitize eggs. *Monacrosporium ellipsosporum* grew into the egg mass and trapped second-stage larvae which had hatched from eggs, but unhatched eggs were never parasitized. Eggs appeared to develop and hatch normally in the presence of all other fungi.

Parasitism of eggs in soil: Incorporation of macerated mycelium of three isolates of *D. oviparasitica* into autoclaved soil at concentrations of about 1 mg dry mycelium/g dry soil resulted in parasitism of 38% to



FIGS. 1-4. *Dactylella oviparasitica* 1) Parasitized *Meloidogyne* sp. eggs collected in the field. 2-4) Eggs of *M. incognita* parasitized by *D. oviparasitica* (isolate S) in the laboratory. Egg in Fig. 3 contains parasitized larva. Bars represent 25 μ m.

71% of the eggs, and a lower hatch than in controls (Table 2). When the concentration of mycelial inoculum of isolate S was varied from 0.8 to 0.0008 mg dry mycelium/g dry soil, the degree of parasitism increased with the amount of inoculum (Table 3). Parasitized eggs were not observed at the lowest inoculum level.

When conidia were added to soil at inoculum densities of 10^5 , 10^4 , 10^3 and 10^2 conidia/g, the average number of parasitized eggs was 368, 400, 363, and 140 respectively. The last figure was signif-

icantly different ($P = 0.05$) from the others. No more than 14% of the eggs added to the soil were parasitized, regardless of spore density. Only 7% of the eggs were parasitized in a similar experiment with inoculum levels of 5,000 conidia/g, suggesting that parasitism is relatively low when conidia are used for inoculum under these experimental conditions.

DISCUSSION

These experiments confirm suggestions (7) that *D. oviparasitica* is an active parasite of *Meloidogyne* eggs. Eggs were parasitized on agar and in autoclaved soil infested with either mycelium or conidia. Eggs parasitized by *D. oviparasitica* were similar in appearance to those observed in the field, and the fungus was reisolated from infected eggs. Since *D. oviparasitica* appeared incapable of parasitizing second-stage larvae, its efficacy as a biocontrol agent depends on its ability to parasitize eggs before they hatch. Eggs of *Meloidogyne* develop and hatch in about 20 days at 23-24 C (1, 8) and our experiments showed that the fungus was active enough to parasitize eggs extensively within that period.

Dactylella oviparasitica grows saprophytically in eggs killed with methyl bromide or heat. However, the greater proportion of infected eggs in treated compared with healthy egg masses probably did not reflect a preference of the fungus for dead eggs, but rather that none of the dead eggs hatched and so all remained available for infection.

Although controls were used in all experiments to check for possible egg parasites in greenhouse cultures of *M. incognita*, none were observed, probably because the egg masses used were always removed from roots inoculated 30-40 days previously with second-stage larvae. *M. incognita* eggs containing the fungus *Cylindrocarpon* sp. have been observed occasionally in cultures in which the nematode population had passed through several generations on the same plant, suggesting that egg masses from greenhouse cultures should always be checked for fungal parasites and saprophytes.

Interpretation of the experimental results was complicated because egg masses

TABLE 2. Parasitism of *Meloidogyne incognita* eggs in autoclaved soil infested with one of three isolates of *Dactylella oviparasitica*.*

<i>D. oviparasitica</i> isolate	No. of infected egg masses [†]	% eggs parasitized [‡]	% hatch [‡]
C	22	70.8a	6.4a
K	12	37.5b	12.9a
S	21	49.3b	16.5a
Control	0	0c	49.2b

*The amounts of inoculum used for isolates C, K, and S were respectively 1.02, 0.80, and 0.91 mg dry mycelium per g dry soil.

†Percentages of eggs added originally. Means are from six replicates with four egg masses (average of 4,800 eggs) per replicate. In each column, means followed by the same letter are not significantly different ($P = 0.05$) by Duncan's multiple-range test.

TABLE 3. Parasitism of *Meloidogyne incognita* eggs in autoclaved soil infested with different quantities of *Dactylella oviparasitica* (isolate S) mycelium.

Inoculum level (mg/g) ^x	% eggs parasitized [†]	% hatch [‡]
0.8	59.6a	36.4b
0.08	42.8b	47.5ab
0.008	7.8c	47.1ab
0.0008	0c	55.7a
Control	0c	65.6a

^xInoculum added in mg dry mycelium per g dry soil.

[†]Percentages of eggs added originally. Means are from six replicates with five egg masses (average of 4,420 eggs) per replicate. In each column, numbers followed by the same letter are not significantly different ($P = 0.05$) by Duncan's multiple-range test.

were used which contained various numbers of eggs in different stages of development, and between 33 and 50% of the eggs contained second-stage larvae. Many of these eggs hatched almost immediately, and were essentially unavailable for parasitism, but they contributed to the pool of hatched larvae observed at the end of the experiments. Thus, moderate and heavy amounts of parasitism may lead to little measurable reduction in hatch. Also, the exposure of *D. oviparasitica* to fully developed egg masses differs from the real situation, in which the fungus may contact eggs as soon as they are exposed to soil. Early invasion of egg masses may result in parasitism of an increased proportion of the eggs, but that hypothesis will need further testing.

Despite those limitations, the techniques used demonstrate the parasitic nature of the fungus, and should be useful in further studies of its biology and ecology. Mycelium was a better source of inoculum than conidia since it resulted in higher levels of parasitism and was easier to produce in culture. The knowledge that *D. oviparasitica* will parasitize egg masses following the addition of relatively small amounts of mycelium should provide a basis for the study of the infection of egg masses on plants.

During the search for useful biological control agents against *Meloidogyne*, egg parasites have received little attention. There has been more interest in antagonists which parasitize or prey on second-stage larvae in soil. Since second-stage larvae remain in soil for only a short time before entering roots, and only a small proportion need to invade the roots to maintain nematode populations, rapid and specific predation or parasitism is necessary for effective control. Organisms such as nematode-trapping fungi, predacious microarthropods and predacious nematodes undoubtedly consume some nematodes in soil, but our knowledge of their biology and ecology (6) suggests a limited capacity to reduce populations of *Meloidogyne* below economically damaging levels.

Organisms which prevent or limit reproduction by attacking females or eggs are more likely to be useful biological control agents. Because parasitic larvae and adults are protected by the root, potential para-

sites and predators must be highly specialized and able to enter the root with the second-stage larvae or attack the female after she breaks the root cortex at maturity. *Bacillus penetrans* Mankau, a prokaryotic endoparasite which infects the nematode from spores attached to the second-stage larvae, is the only organism known to parasitize parasitic larvae and adults of *Meloidogyne* (3, 4).

Of all the stages in the life cycle of *Meloidogyne*, the eggs may be the most vulnerable to parasitism and predation. They are clumped together in a gelatinous matrix on the outside of the root, and even under conditions optimum for the development and hatch of eggs, individual eggs are present in the egg mass for at least 9 days (1, 8). In the field the period is generally much longer, and in overwintering eggs it may last for 3 or 4 months. Consequently, it is not surprising that some soil organisms can use *Meloidogyne* eggs as a food source. *Dactylella oviparasitica* is apparently the first such organism to be studied. The amount of parasitism observed in the laboratory, and the occurrence of the fungus in the field in areas where *Meloidogyne* populations are low, suggests that *D. oviparasitica* can contribute to the biological control of root-knot nematodes.

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