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Repulsion of *Meloidogyne incognita* by Alginate Pellets Containing Hyphae of *Monacrosporium cionopagum, M. ellipsosporum,* or *Hirsutella rhossiliensis*[1]

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Abstract: The responses of second-stage juveniles (J2) of *Meloidogyne incognita* race 3 to calcium alginate pellets containing hyphae of the nematophagous fungi *Monacrosporium cionopagum, M. ellipsosporum,* and *Hirsutella rhossiliensis* were examined using cylinders (38-mm-diam., 40 or 72 mm long) of sand (94% <250-μm particle size). Sand was wetted with a synthetic soil solution (10% moisture, 0.06 bar water potential). A layer of 10 or 20 pellets was placed 4 or 20 mm from one end of the cylinder. After 3, 5, or 13 days, J2 were put on both ends, on one end, or in the center; J2 were extracted from 8-mm-thick sections 1 or 2 days later. All three fungal pellets were repellent; pellets without fungi were not. Aqueous extracts of all pellets and of sand in which fungal pellets had been incubated were repellent, but acetone extracts redissolved in water were not. Injection of CO_2 (20 μl/minute) into the pellet layer attracted J2 and increased fungal-induced mortality. In vials containing four randomly positioned pellets and 17 cm^3 of sand or loamy sand, the three fungi suppressed the invasion of cabbage roots by *M. javanica* J2. Counts of healthy and parasitized nematodes observed in roots or extracted from soil indicated that, in the vial assay, the failure of J2 to penetrate roots resulted primarily from parasitism rather than repulsion. Data were similar whether fungal inoculum consisted of pelletized hyphae or fungal-colonized *Steinernema glaseri.* Thus, the results indicate that nematode attractants and repellents can have major or negligible effects on the biological control efficacy of pelletized nematophagous fungi. Factors that might influence the importance of substances released by the pellets include the strength, geometry, and duration of gradients; pellet degradation by soil microflora; the nematode species involved; and attractants released by roots.

Key words: alginate, behavior, biological control, chemotaxis, *Hirsutella rhossiliensis, Meloidogyne incognita, Meloidogyne javanica Monacrosporium cionopagum, Monacrosporium ellipsosporum,* nematode, nematophagous fungi, *Steinernema glaseri.*

To achieve biological control of plant-parasitic nematodes, researchers have developed various formulations of control agents for addition to soil. For example, alginate pellets containing hyphae without nutrients have been studied for the endoparasitic fungus *Hirsutella rhossiliensis* and for various nematode-trapping fungi (14,15,22,31). These pellets can affect nematodes in several ways. Most obviously the fungi may produce structures that initiate infection when contacted by nematodes. Before contacting and infecting nematodes, the fungi also may attract or even repel nematodes.

Several studies have revealed that the mycelia or conidia of some fungi attract various mycophagous and bacteriophagous nematodes on agar plates (2,3,11,17, 19,23,24). Three obligately plant-parasitic tylenchids, *Ditylenchus dipsaci, Pratylenchus penetrans* and *P. fallax,* also were attracted (20). In theory, attraction would seem advantageous to the fungus because it increases the probability of fungus-nematode contact. The results of Jansson (18) support this notion. Among eight nematophagous fungi in his study, attraction on agar was positively correlated with predacity against the bacteriophagous nematode *Panagrellus redivivus* (18). Nematophagous fungi reported to attract nematodes include *Arthrobotrys dactyloides, A. entomopaga, A. musiformis, A. oligospora, A. superba, Cephalosporium balanoides, Dactylaria candida, D. gracilis, Harposporium anuillulae, Meria coniospora, Monacrosporium cion-*

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opagum, M. doedycoides, M. ellipsosporum, and *M. rutgeriensis. Arthrobotrys arthrobotryoides,* however, was repellent (19). To our knowledge, behavioral responses of nematodes to fungi have not been investigated in soil. Attraction of root-knot nematodes (*Meloidogyne* spp.) to fungi has not been studied in any matrix.

Our primary objective was to determine whether alginate pellets containing hyphae of *M. cionopagum, M. ellipsosporum,* or *Hirsutella rhossiliensis* attract or repel second-stage juveniles (J2) of *Meloidogyne incognita* in sand; ancillary experiments evaluated the possible contribution of chemotaxis in a standard efficacy assay currently in use. A second objective was to explore the potential of using a known nematode attractant, CO_2, to attract nematodes to pellets and thereby increase biological control.

Materials and Methods

Nematode and fungal preparations: The methods used for obtaining preparations of nematodes and fungi have been described in detail previously (13–16,30,34). Briefly, J2 of *M. incognita* race 3 were obtained from greenhouse tomato cultures at the USDA Southern Crops Research Laboratory, College Station, Texas; J2 of *M. javanica* were obtained from tomato hydroponic cultures at the Department of Nematology, University of California, Davis. At least 95% of the J2 used were spontaneously motile. Infective juveniles (J3) of *Steinernema glaseri* were obtained from *Galleria mellonella* caterpillars (34); fungal-colonized J3 were obtained by transferring healthy J3 to fungal cultures maintained on quarter-strength corn meal agar and individually collecting parasitized J3 with a needle after 24 hours (14).

Mycelia of *M. cionopagum* (ARSEF 3349), *M. ellipsosporum* (ARSEF 3348), and *H. rhossiliensis* (IMI 265748) for making alginate pellets were grown on potato-dextrose-broth in shake cultures for 4, 6, or 7 days, depending on the species and its growth rate (15). Pellets (ca. 1.5 mg) were made as described by Walker and Connick (33) and modified by Lackey et al. (22). This technique essentially consists of dripping a thoroughly blended 1:3 (g:ml) suspension of washed, fresh mycelial mass within 1% sodium alginate solution into a continually stirred 0.1 M $CaCl_2$ solution; the pellets that form in the $CaCl_2$ solution are removed, coated with sand to prevent fusing, and dried on a sheet of wax paper at room temperature. Pellets were prepared in four lots, stored at 4–5 °C, and packed in ice for shipment between California and Texas. Pellets in lot 1 were used in only one experiment and were asynchronously aged; the *M. cionopagum, H. rhossiliensis,* and *M. ellipsosporum* pellets were 0, 40, and 55 days old, respectively, on the day the experiment was started. Pellets from lots 2–4 were used in all other experiments and were stored for no longer than 4 weeks before experimental use.

Behavioral assay procedures: Nematode movement was studied in cylinders of moist sand inside 3.8-cm-i.d. acrylic tubes. Tubes were 20 cm long, and cylinders were either 4.0 cm or 7.2 cm long. Sand was a SiO_2 industrial abrasive (Black Magic NO. 5, TTEX, Houston, TX; white, no organic matter, pH 7.8, estimated conductivity at saturation 30 μmho/cm, specific density 2.5, bulk density 1.7, total porosity 30%, and a particle size distribution of 0.2% 0–75 μm, 23% 75–150 μm, 71% 150–250 μm, and 6% 250–425 μm). Sand was wetted to ca. 10% moisture during cylinder preparation with a synthetic soil solution (SSS) containing 3 mM NaCl, 0.5 mM KCl, 0.05 mM $CaCl_2$, and 0.05 mM $MgCl_2$ (pH 6.8).

Tubes were packed by filling them with dry sand vertically, then saturating the sand with SSS and removing excess water by applying a partial vacuum (80 cm H_2O) to a perforated suction plate taped on the bottom of the tube. The matric potential was estimated to be −0.06 bar based on a moisture retention curve obtained by the pressure plate extraction method (29). To position alginate pellets within sand cylinders, the dry sand was added in two parts, with 20 pellets and a thin, partial layer of

brightly colored sand in between to serve as a marker visible through the tube wall. After wetting, the tube was removed from the suction plate. The sand was pushed to one end and then to the other with a plunger, allowing excess sand to be cleanly cut away from both ends; this produced a sand cylinder of the desired length (4.0 or 7.2 cm long) and with the marker sand (and pellets) positioned exactly 20 mm from one end within 7.2-cm cylinders. The 7.2-cm control cylinders without pellets also had marker sand. The 4.0-cm cylinders had no marker sand and no pellets at this point. Next, the cylinder was carefully positioned relative to small holes drilled in the tube wall, which were used later to inject nematodes or CO_2. Where required, 10 pellets were buried 4 mm below the surface at one end of 4.0-cm sand cylinders by individually pressing them into the sand with a small rod. The ends of tubes were sealed.

Before adding nematodes, tubes were stored in the dark at room temperature (ca. 23 °C) for various intervals to allow fungi to grow. Then, 4,000–6,000 J2 of *M incognita* were syringe-injected into the center of 4.0-cm cylinders in 250 μl SSS, or were sprinkled in 7–8 drops onto the sand surface on one end. J2 were sprinkled onto both ends of all 7.2-cm cylinders (250 μl SSS/end and ca. 10,000 J2/cylinder).

In experiments testing responses to CO_2, hydrated CO_2 gas was injected continuously into the center of the marker sand at 15–20 μl/minute from the time of nematode introduction until the end of the experiment. When CO_2 injection was started (except where noted), caps on the ends of each acrylic tube were replaced with two-holed rubber stoppers, and the air space in each end of the tube was continually purged with a gentle flow (20 ml/minute) of hydrated air containing 1% CO_2 (30). This simulated the normally elevated CO_2 concentrations of soil but prevented CO_2 buildup and minimized evaporation on cylinder ends.

Experiments were ended 24 hours after adding nematodes to 4.0-cm cylinders and 42 hours after adding nematodes to 7.2-cm cylinders. The sand was extruded from each tube and sliced into 8-mm-thick sections, yielding five sections from 4.0-cm cylinders, with pellets (when included) in the first or last section, and yielding nine sections from 7.2-cm tubes, with pellets in the third or seventh section. As it was cut, each section (9 cm^3 in volume) was placed in a beaker with 20 ml water. Nematode numbers in each section were determined by capping and vigorously shaking the beaker, allowing the sand to settle for 45 seconds, and counting the nematodes in a 2-ml aliquot of water (ca. 60 J2 per aliquot, on average). In all but the first experiment, the nematodes that did and those that did not move within 3 seconds of observation were counted separately.

The vertical orientation of tubes during preparation resulted in a small moisture gradient (ca. 0.2%/cm). Therefore, in all experiments cylinders were assigned to treatments in random pairs and were used horizontally with the upward ends of the pair oriented oppositely relative to the placement of nematodes, CO_2, and (or) alginate pellets. Four replicate cylinders were used per treatment (except where noted). To facilitate comparisons within an experiment, the average numbers of moving and nonmoving nematodes counted in each section of each treatment were divided by the total number of nematodes extracted per cylinder in the control treatment. The total number of moving nematodes per cylinder for each treatment divided by the total number of moving nematodes per cylinder in the control was also calculated and is referred to as the survival index (SI). Data were subjected to one-way analyses of variance, and means were compared using protected least-significant differences.

The behavioral experiments with *M. incognita* included two experiments with pellets, four experiments with pellet extracts, and two experiments with CO_2 as a bait to attract nematodes to pellets. Four additional experiments with *M. javanica* examined nematode suppression by fungi

within vials. Some experiments were repeated; the original and repeated experiment are referred to as run 1 and run 2, respectively.

Behavioral assays with pellets: The first pellet experiment included 7.2-cm cylinders and five treatments: *H. rhossiliensis* pellets, *M. cionopagum* pellets, blank pellets (pellets without hyphae), marker sand only, and marker sand plus CO_2 injection. The *H. rhossiliensis* cylinders were prepared 14 days and the *M. cionopagum* cylinders 3 days before nematodes were introduced to the ends of all cylinders, based on known differences in the rates of growth of the fungi. The CO_2 treatment was included to verify that J2 were able to respond to attractants. The experiment was repeated without the CO_2 and *H. rhossiliensis* treatments.

The second pellet experiment included *M. cionopagum* pellets, 4.0-cm cylinders, and five treatments: (i) J2 injected in the center with pellets on one end; (ii) J2 injected in the center without pellets (control for treatment 1); (iii) J2 and pellets on the same end; (iv) J2 and pellets on opposite ends; and (v) J2 on one end without pellets (control for treatments 3 and 4). Pellets were buried 3 days before J2 introduction. The experiment was repeated with six rather than four replications for treatments 1 and 2.

Behavioral assays with extracts: These experiments examined four kinds of extracts from pellets containing fungi and two kinds of extracts from blank pellets. In each case, J2 were injected into the centers of 4.0-cm cylinders and a 250-μl aliquot of extract, distilled water, or SSS was applied simultaneously to the sand surface at one end. Acetone extracts were prepared by placing 720 mg of pellets in 15 ml cold acetone (4 °C) overnight, evaporating the acetone to dryness at room temperature, redissolving the residue in 15 ml water, ultrafiltering (0.2 μm), and freezing. Water extract was prepared identically, except that the water was ultrafiltered and frozen without evaporation. Freeze-dried water extract was simply water extract that was subsequently freeze dried and redissolved in an equivalent volume of distilled water. An SSS sand extract was obtained by incubating pellets in 11.0-cm petri dishes containing 88 g of sand moistened with 12 g SSS; after 7 days, the sand was repeatedly saturated with SSS and excess SSS removed several times on a Büchner funnel. The SSS extracted was filtered (Whatman No. 1) and frozen.

The first extract experiment included the acetone extract, water extract, and SSS sand extract from each fungal species plus a distilled water and an SSS control. The second experiment was done twice and included a distilled water control plus acetone extract, water extract, and freeze-dried water extract from *M. cionopagum* pellets. The third experiment included a distilled water control, water extract from blank pellets, and water extract from *M. cionopagum* pellets, in each case with and without an additional 250 μl of distilled water applied to the opposite end of the cylinder. The fourth experiment included water extracts of blank and *M. cionopagum* pellets that were prepared at six times the pellet:water ratio used previously, and then diluted six-fold with SSS to provide extracts in 84% SSS. These were compared with pure SSS.

CO_2 as a bait: These two experiments employed 7.3-cm (9-section) cylinders with CO_2 injected into the third or seventh section. The first experiment included two sets of nine treatments. The sets differed in the length of time between tube preparation and nematode addition, and in the inclusion or omission of end purge in cylinders receiving CO_2. Nematodes were added to both sets at the same time. In the first set, tubes were prepared 3 days before nematodes were added and end purge was omitted. However, all tubes were left loosely stoppered with moistened foam rubber plugs to allow gas exchange. In the second set, tubes were prepared 13 days before nematodes were added. The treatments for each set included cylinders with and without pellets of each of the three fungi, in each case both with and without

CO_2 injection (i.e., eight treatments), plus four extra tubes with blank pellets but no CO_2 injection as a ninth treatment.

In the second CO_2 experiment, *M. cionopagum* pellets were buried within 7.2-cm cylinders 5 days before J2 were added. Four treatments were included: pellets but no CO_2, CO_2 but no pellets, CO_2 plus pellets, and CO_2 plus pellets but with J2 extracted and counted using a different technique to increase the chances of extracting parasitized nematodes. With this technique, a 0.11-cm^3 core was removed from the center of each section and dispersed with water in a gridded 9.0-cm petri dish; all J2 found in the core were counted.

Suppression of nematodes in vials: These experiments utilized 25-cm^3 vials, each packed with 17 cm^3 of sand or loamy sand. The first experiment used a root invasion bioassay to evaluate the ability of the fungi to suppress nematodes in TTEX sand. Pellets (lot 1) containing hyphae of three fungi or blank pellets were added to TTEX sand (moistened to 10% water with SSS) or an unsterilized loamy sand (83% sand, 13% silt, 4% clay; 0.24% organic matter; pH 4.9; 8% moisture, ca. −0.1 bar matric potential) containing no endoparasitic nematodes (32). Four pellets were added per vial, with six replicate vials per treatment. After soil was added, vials were capped and kept at 20 °C in a moisture chamber. After 14 days, 100 ± 5 J2 (mean ± SE) of *M. javanica* in 0.5 ml of 4.5 mM KCl were added to the surface of the sand in each vial. Three days later, one germinated cabbage (*Brassica oleraceae* L. "Grand Slam") seedling was planted per vial, and vials were left uncapped in a moisture chamber under fluorescent light (20 °C). Five days later (22 days after adding soil), shoot and root lengths were determined and roots were stained (6). Nematodes in roots were counted.

In the second experiment, the root invasion bioassay was used to determine whether the efficacy of *M. cionopagum* and *H. rhossiliensis* pellets was lost after shipment from California to Texas and back. The assay was similar to that in the first experiment but only loamy sand was used, *M. ellipsosporum* was not included, and 103 ± 7 J2 were added to each of eight replicate vials.

The third experiment was designed to determine whether suppression of root invasion, as described in previous studies (14,15), was due to parasitism or repulsion. Pellets were mixed into soil, packed into vials, incubated, and inoculated with J2 of *M. javanica* as in the first experiment, except that each vial received 208 ± 5 J2. There were 16 replicate vials for each fungus and for the blank pellets. On day 17, eight vials per treatment were planted with cabbage, and these vials were incubated and data collected as in the first experiment. Also on day 21, nematodes were extracted from the soil of the remaining eight vials per treatment (vials without plants) via wet sieving (25-μm pore diam.) and centrifugal flotation (21). Parasitized and healthy nematodes were counted separately. The design of the experiment assumed that J2 parasitized by *H. rhossiliensis* would be extractable from soil and would be recognizably infected, whereas J2 that were parasitized by *M. cionopagum* or *M. ellipsosporum* would be trapped in mycelium and unextractable; the number parasitized, however, could be estimated based on the number extracted (18).

A final vial experiment was identical to the third experiment, except that fungal-colonized *S. glaseri* (20 cadavers/vial) rather than pellets were used as fungal inoculum. Moreover, each fungus was examined during a different time interval and with its own control (no *S. glaseri* added) to facilitate the labor-intensive preparation of fungal-colonized nematodes. In the *M. cionopagum, M. ellipsosporum,* and *H. rhossiliensis* assays, respectively, each vial received 216 ± 8, 187 ± 12, and 186 ± 12 J2.

Nematode counts from the vial assays were used to calculate the percentage suppression for each treatment, which was defined as $100 \cdot (1 - R_i/R_c)$, where R_i is the number of J2 within roots of treatment i and R_c is the number of J2 in roots of the control. In treatments without roots, sup-

pression was defined as $100 \cdot (1 - H_i/H_c)$, where H_i is the number of healthy J2 extracted from soil in treatment i and H_c is the healthy J2 extracted from soil in the control. Percentage suppression in vials without plants is, algebraically, the percentage complement of the SI parameter measured in the behavioral experiments.

RESULTS

Behavioral assays with pellets: In the first experiment, J2 readily penetrated the sand and dispersed more or less uniformly both in cylinders without pellets and in cylinders with blank (fungus-free) pellets (Fig. 1). Nematodes strongly accumulated in the section where CO_2 was injected; these nematodes were highly motile and appeared healthy. In cylinders containing *M. cionopagum* pellets, most J2 were extracted from sections near the ends, suggesting that they were repelled or poisoned by the pellets. The SI value for cylinders containing *M. cionopagum* pellets in run 2 (where moving as well as total nematodes were counted) was only 58%, indicating that many nematodes were dead, unextracted, or otherwise uncounted. Many dead, parasitized J2 were extracted from the pellet-containing section of *H. rhossiliensis* cylinders. The mean SI value for cylinders with blank alginate pellets (109%) was not different from 100% ($P = 0.05$), and there was no indication that blank pellets were repellent (Fig. 1).

Results obtained with 4.0-cm cylinders and *M. cionopagum* pellets in the second experiment (Fig. 2) confirmed the results obtained previously with 7.2-cm cylinders (Fig. 1). Whether J2 were placed at the center of cylinder, at the same end as the pellets, or at the end opposite the pellets, most moving nematodes were extracted 24 hours later from the half of the cylinder opposite the *M. cionopagum* pellets (Fig. 2). SI's indicated that 21–43% of the moving nematodes were missing, relative to control cylinders without pellets. It is possible that these nematodes were present within the sections containing pellets but were trapped by hyphae and unextractable.

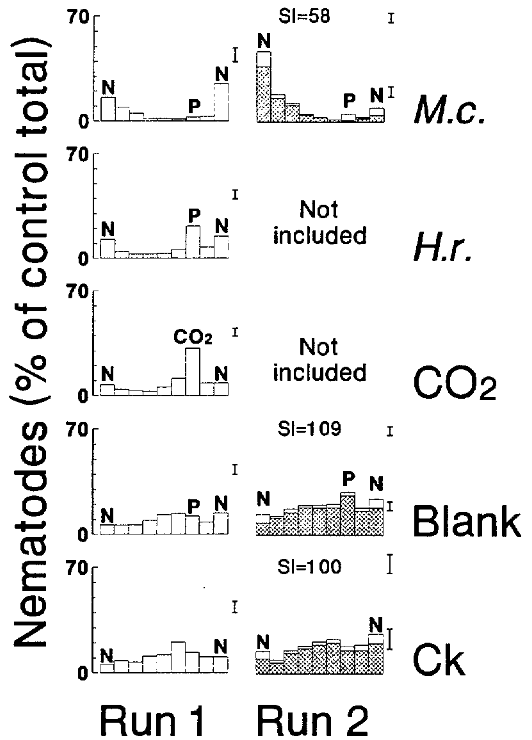

FIG. 1. First behavioral experiment with alginate pellets. Effects of pellets containing *Monacrosporium cionopagum* (*M.c.*), *M. ellipsosporum* (*M.e.*), *Hirsutella rhossiliensis* (*H.r.*), or no fungus (Blank) on the distribution of *Meloidogyne incognita* second-stage juveniles (J2) 42 hours after adding 10,000 J2 to cylinders of sand (7.2-cm-long, 3.8-cm-diam.) in which pellets had been buried. Hatched bars show moving and empty bars show total J2 extracted from nine 0.8-cm-thick sections. Lower and upper brackets are LSD's ($P = 0.05$) for moving and total nematodes. J2 and pellets were placed in sections denoted N and P, respectively. Cylinders denoted CO_2 received continuous CO_2 injection (20 µl/minute) into the section indicated. Cylinders denoted Ck received no pellets. SI denotes survival index (moving J2 divided by moving J2 for the no-pellet control). Only total J2 were counted in the first run of the experiment. Data are means of four replications.

Behavioral assays with extracts: All extract experiments (Figs. 3–5) indicated the presence of at least one repellent in *M. cionopagum* pellets, as well as in *M. ellipsosporum* and *H. rhossiliensis* pellets. SI values from extract experiments were 90–115% and not statistically different from 100% in most cases, indicating no measurable toxic effects. Water and acetone extracts had pH values of 5.5 and 6.3, respectively, with total salt concentrations of 25 and 0.3 mM, based on electrical conductivity.

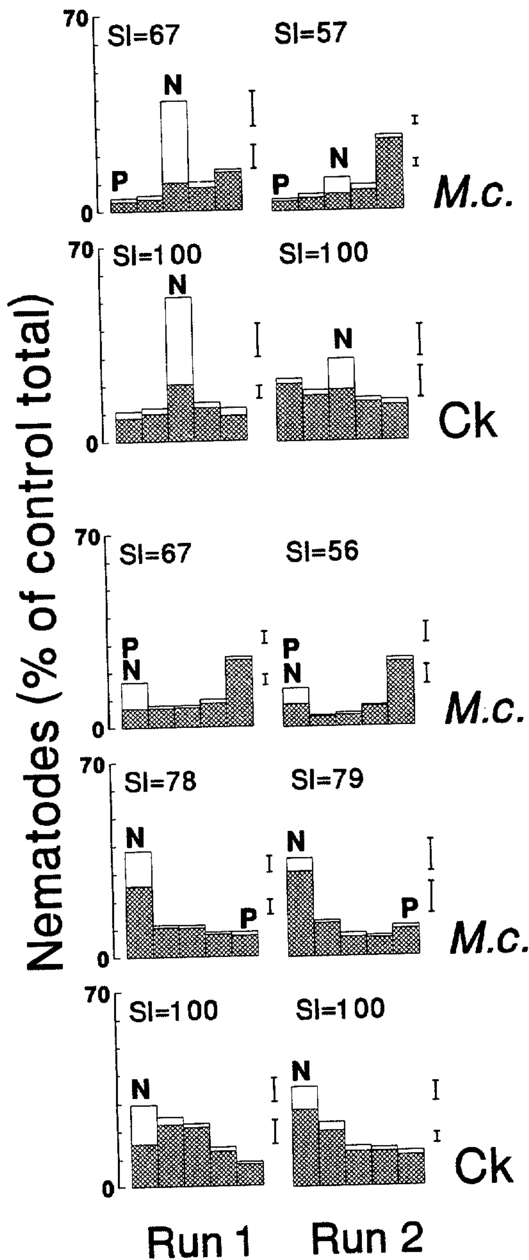

FIG. 2. Second behavioral experiment with alginate pellets. Effects of pellets containing *Monacrosporium cionopagum* (*M.c.*) on the distribution of *Meloidogyne incognita* second-stage juveniles (J2) 24 hours after adding 5,000 J2 to cylinders of sand(4.0-cm-long, 3.8-cm-diam.) in which pellets had been buried. Pellets (P) and nematodes (N) were placed where indicated. Control cylinders are denoted Ck and received no pellets. Hatched bars show moving J2, and empty bars show total J2 extracted from five 0.8-cm-thick sections. Lower and upper brackets are LSD's ($P = 0.05$) for moving and total nematodes. SI denotes survival index (moving J2 divided by moving J2 for the appropriate no-pellet control). Data in the upper two graphs of run 1 are means of six replications and others are means of four replications.

In the first extract experiment, the repellent(s) from all three kinds of fungal pellets was water-soluble and either unextractable with acetone or lost when the acetone was dried (Fig. 3). The SSS extracts obtained from 7-day-old sand cultures were less strongly repellent than the cold-water extracts, suggesting a time-dependent effect; however, these extracts also were more dilute than the aqueous extracts.

In the second experiment (Fig. 4), freeze-dried aqueous extract of *M. cionopagum* pellets appeared to retain the full activity of the aqueous extract, indicating that the repellent(s) was not volatile and thus probably not acetone extractable.

In the third and fourth extract experiments, aqueous extracts from the blank (fungus-free) pellets were weakly to moderately repellent, both when prepared with distilled water and when prepared with 84% SSS (Fig. 5). Sprinkling 250 μl of pure distilled water on the cylinder surface consistently attracted J2, while sprinkling 250 μl of SSS had no effect (Fig. 5). Extracts of *M. cionopagum* pellets were strongly repellent.

CO_2 as a bait: In the first CO_2 experiment (Fig. 6), J2 appeared to be repelled equally by pellets containing *M. cionopagum* whether J2 were introduced 3 days or 13 days after the pellets. A similar effect was noted for *M. ellipsosporum*. Thus, repellency persisted over a 10-day period, suggesting that a repellent substance was continually released or diffused very slowly. No repellency was evident in cylinders containing blank pellets (Fig. 6); in fact, there was a significant ($P = 0.05$) tendency for J2 to accumulate slightly in the pellet section. In tubes receiving CO_2 pronounced J2 accumulation occurred when pellets were absent, particularly in the 13-day cylinders, which received the end-purge treatment. These nematodes were motile and appeared healthy. A large number of nonmotile, apparently dead, and parasitized J2 accumulated in the pellet section of the *H. rhossiliensis* cylinders into which CO_2 was injected. A much

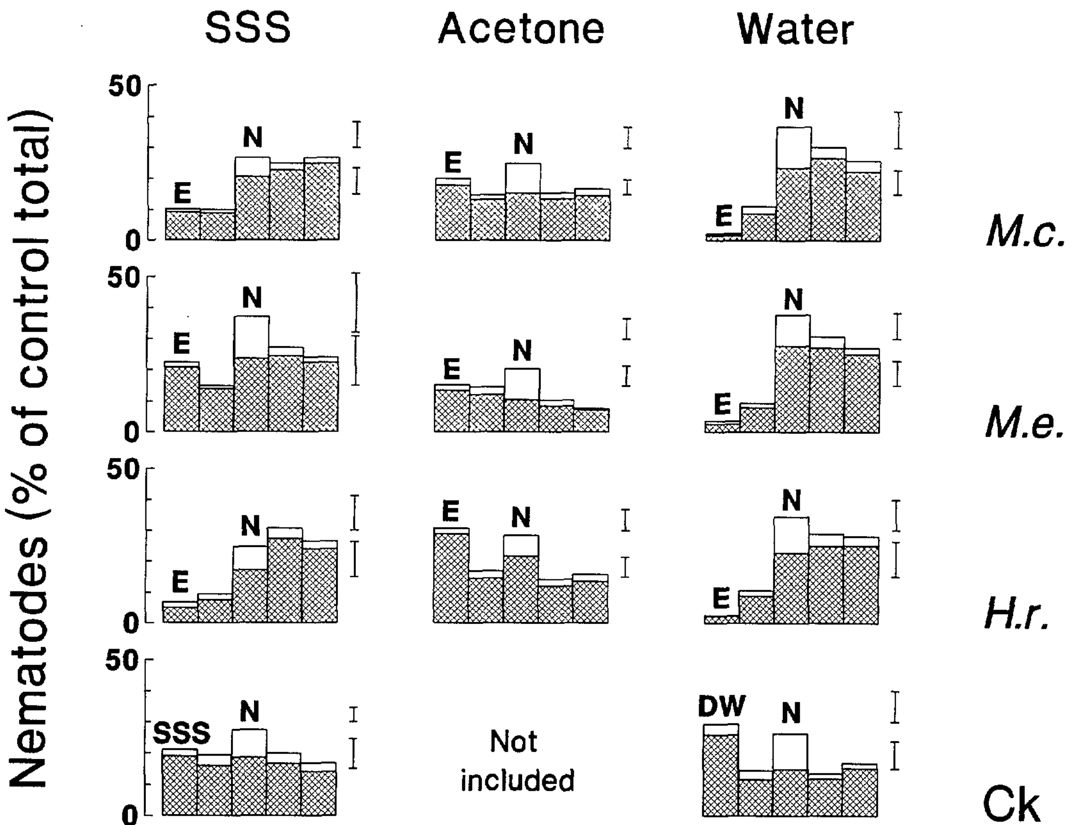

FIG. 3. First pellet extract experiment. Effects of extracts (SSS, Acetone, Water) of alginate pellets containing *Monacrosporium cionopagum* (*M.c.*), *M. ellipsosporum* (*M.e.*), or *Hirsutella rhossiliensis* (*H.r.*) on the distribution of *Meloidogyne incognita* second-stage juveniles (J2) 24 hours after adding 5,000 J2 and extracts to cylinders of sand (4.0-cm-long, 3.8-cm-diam.). In each cylinder, 250 μl of extract (E), nematode suspension (N), synthetic soil solution (SSS), or distilled water (DW) were placed where indicated. Hatched bars show moving J2, and empty bars show total J2 extracted from five 0.8-cm-thick sections. Lower and upper brackets are LSD's ($P = 0.05$) for moving and for total nematodes from four replications. The acetone extract was evaporated and redissolved in water before testing. Controls are denoted Ck and received pure SSS or DW. Applying acetone directly to the sand (center column, bottom row) was not tested.

weaker, but consistent tendency for J2 to accumulate similarly in the corresponding CO_2-treated *M. cionopagum* and *M. ellipsosporum* cylinders also occurred. About 15–50% of the nematodes in these cylinders were unextractable compared to the controls. Overall, about 50% more J2 were recovered from control tubes receiving CO_2 than from controls without CO_2.

In the second CO_2 experiment (Fig. 7), extraction of J2 from CO_2-treated *M. cionopagum* cylinders by the central core technique revealed a pronounced aggregation of J2 in the center of the pellet section. By comparison, the central cores of sections distant from the pellets had fewer J2 than would be expected based on counts obtained by the whole-section technique. Thus, J2 within the pellet-containing section were concentrated in the central core, where the tip of the CO_2-emitting needle was positioned, and were dispersed from the center in other sections; alternatively, they were absent from the center in other sections because they had moved to the CO_2 source. About 40% of the J2 in the central core of the *M. cionopagum* pellet section in tubes receiving CO_2 were nonmotile and appeared dead (Fig. 7). In other tubes, J2 were attracted to CO_2 in

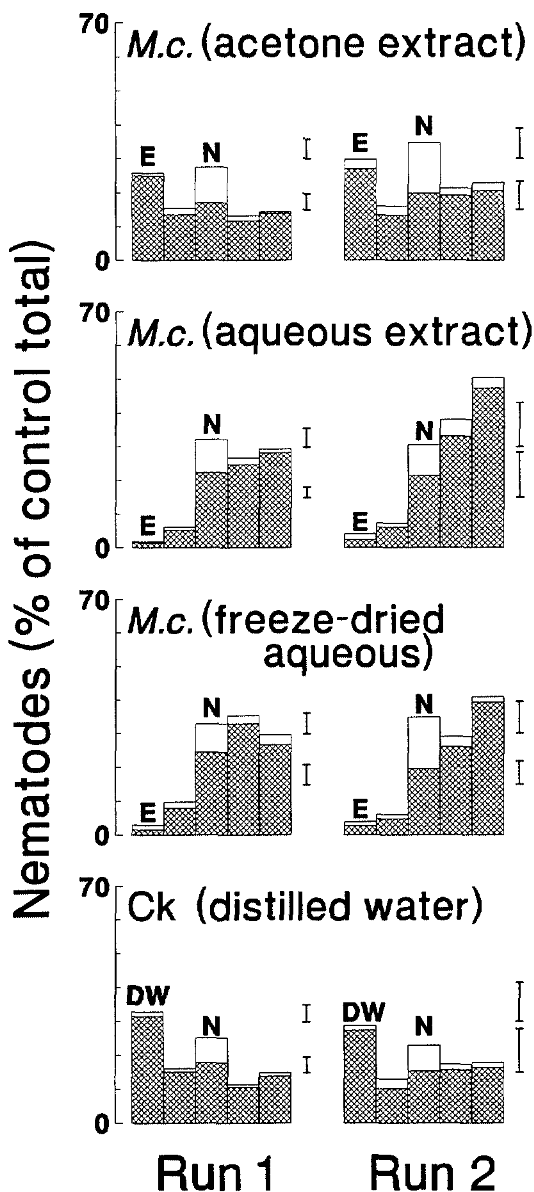

FIG. 4. Second pellet extract experiment. Effects of extracts of *Monacrosporium cionopagum* (*M.c.*) alginate pellets on the distribution of *Meloidogyne incognita* second-stage juveniles (J2) 24 hours after adding 5,000 J2 and extracts to cylinders of sand (4.0-cm-long, 3.8-cm-diam.). In each cylinder, 250 µl of extract (E), nematode suspension (N), or distilled water (DW) was placed where indicated. Hatched bars show moving J2, and empty bars show total J2 extracted from five 0.8-cm-thick sections. Lower and upper brackets are LSD's ($P = 0.05$) for moving and for total nematodes from four replications.

the absence of pellets, as seen in other experiments, and repelled by pellets in the absence of CO_2.

Suppression of nematodes in vials: Cabbage seedling growth in the TTEX sand was comparable to growth in the loamy sand (Table 1). Almost identical numbers of J2 invaded roots in the two sets of vials containing blank pellets. Suppression of root invasion by all three fungi was greater in the TTEX sand than in the loamy sand.

In the second vial experiment, addition of 103 ± 7 (mean ± SE) *M. javanica* J2/vial resulted in 31 ± 2 J2/root in vials with blank pellets, 4 ± 1 J2/root in vials with *H. rhossiliensis* pellets, and 1 ± 1 with *M. cionopagum* pellets. Thus, pellets remained effective after shipment from California to Texas and back.

In the third experiment, suppression was substantial, and the ranking of suppression among the three fungi (*M. cionopagum* > *M. ellipsosporum* > *H. rhossiliensis*) was the same, whether based on J2 in roots or J2 extracted from soil (Table 2). As in the cylinder experiments, the total numbers of J2 extracted from soil in control vials or vials inoculated with *H. rhossiliensis* were similar, and a large percentage was parasitized by the fungus. Also as in the cylinder experiments, a large proportion of the J2 in vials inoculated with *M. cionopagum* and *M. ellipsosporum* was unextractable from soil. Washing of bottoms of lids and other vial surfaces did not yield any nematodes; nematodes probably were not extracted because they were trapped and held in place by *M. cionopagum* and *M. ellipsosporum* in the vials. Data in the fourth experiment (Table 2) were similar to those in the third, suggesting that processes resulting in suppression were similar whether pelletized hyphae or colonized nematodes were used as fungal inoculum.

Discussion

Pelletized hyphae influenced the behavior of *M. incognita* J2, but not as expected. Based on in vitro experiments in which a

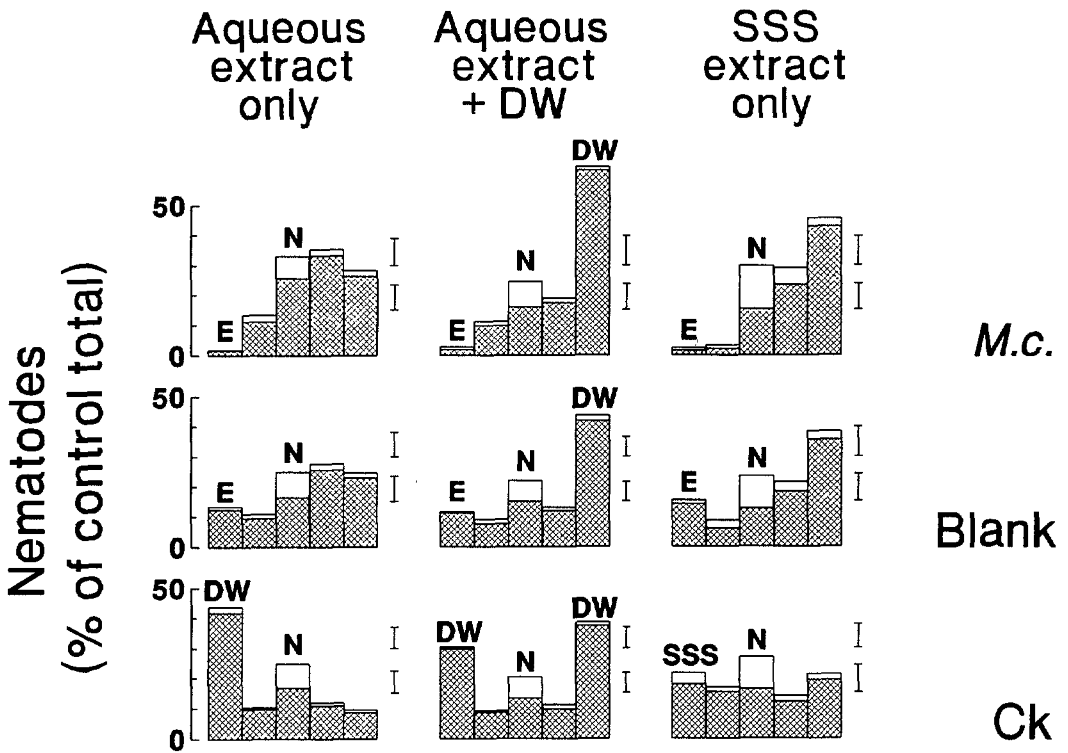

FIG. 5. Third and fourth pellet extract experiments. Distribution of *Meloidogyne incognita* second-stage juveniles (J2) 24 hours after adding 5,000 J2 and extracts to cylinders of sand (4.0-cm-long, 3.8-cm-diam.), showing comparative effects of aqueous and of synthetic soil solution (SSS) extracts from fungus-free alginate pellets (Blank) and from alginate pellets containing *Monacrosporium cionopagum* (*M.c.*). Aqueous extracts were tested in the third and SSS extracts in the fourth experiment. In each cylinder, 250 µl of extract (E), nematode suspension (N), synthetic soil solution (SSS) or distilled water (DW) was placed where indicated. Hatched bars show moving J2, and empty bars show total J2 extracted from five 0.8-cm-thick sections. Lower and upper brackets are LSD's (P = 0.05) for moving and for total nematodes from four replications. Controls are denoted Ck.

variety of nematophagous fungi had been observed to attract nematodes on agar (2–4,11,17–20,24), pellets were expected to attract root-knot nematodes or else have no effect on their movement if behaviorally effective gradients were not established. In our behavioral assays in sand, however, pellets containing *M. cionopagum* and *M. ellipsosporum* consistently repelled J2 of *M. incognita.* Pellets containing *H. rhossiliensis* also were repellent, but less so than those of the other two fungi. For biological control, the repellent could be counteracted by an attractant such as CO_2. The addition of CO_2 near the pellets substantially increased fungal parasitism, especially by *H. rhossiliensis.* Thus, the results demonstrate that pellet efficacy can be increased by manipulating nematode behavior.

In previous studies (14–16,22), suppression of root invasion by root-knot and cyst nematodes (*Heterodera* spp.) was assumed to be caused by fungal parasitism, not by chemical repellents. Perhaps the importance of parasitism had been overestimated and repulsion had prevented nematodes from penetrating roots. In the case of *H. rhossiliensis,* however, parasitism had been directly quantified (22), and parasitism was the primary factor preventing J2 from invading roots; this was confirmed in the present study. In contrast, parasitism by the two trapping fungi cannot be measured directly because suitable methods to extract trapped nematodes from soil are lacking (15); however, had parasitism been unimportant in the vial assays, a relatively large proportion of nematodes would have

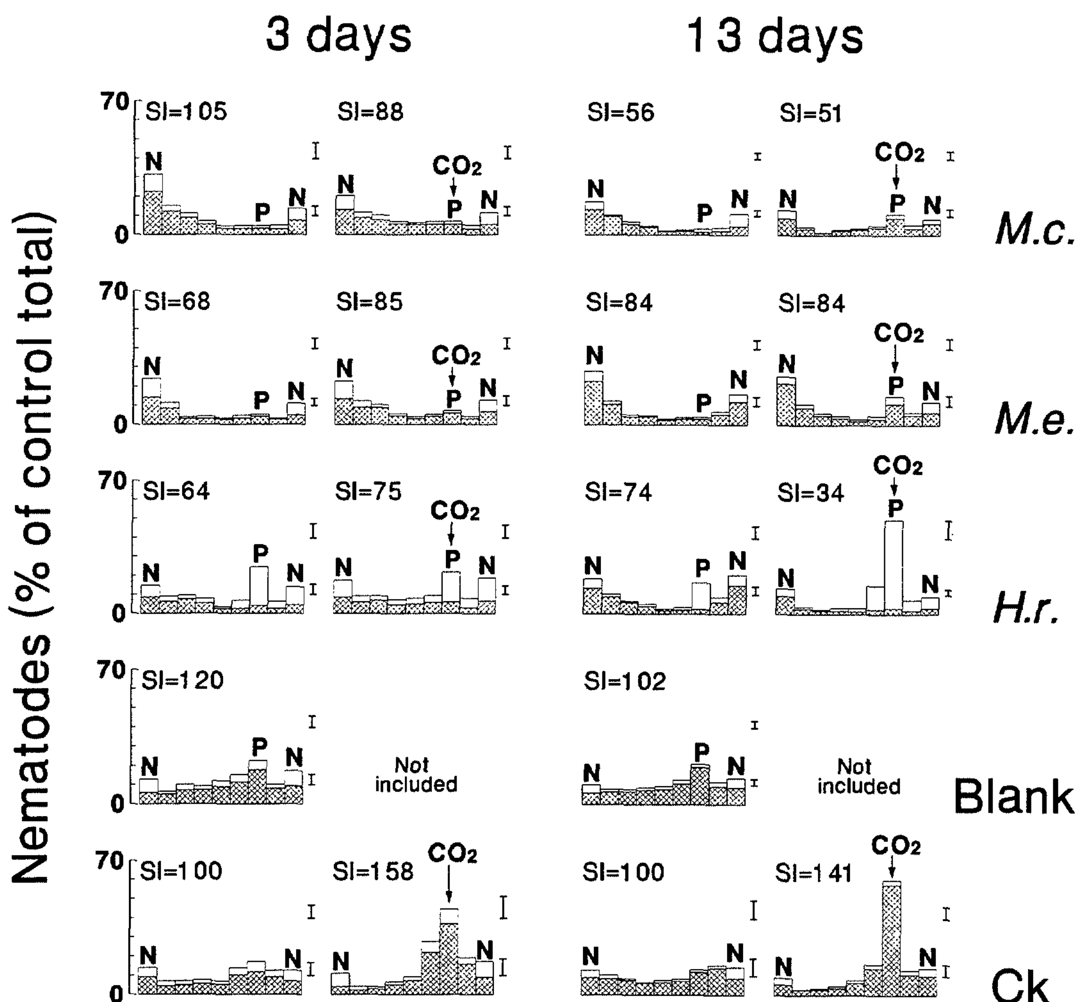

FIG. 6. First CO_2 bait experiment. Effects of CO_2 and alginate pellets containing *Monacrosporium cionopagum* (*M.c.*), *M. ellipsosporum* (*M.e.*), *Hirsutella rhossiliensis* (*H.r.*) or no fungus (Blank) on the distribution of *Meloidogyne incognita* second-stage juveniles (J2) 42 hours after introducing 10,000 J2 to cylinders of sand (7.2-cm-long, 3.8-cm-diam.) in which pellets had been buried 3 and 13 days previously. Hatched bars show moving J2, and empty bars show total J2 extracted from nine 0.8-cm-thick sections. Lower and upper brackets are LSD's ($P = 0.05$) for moving and for total nematodes from four replications. J2 and pellets were placed in sections denoted N and P, respectively. Where indicated, CO_2 was injected (20 µl/minute) continuously into the third section from the right for the last 42 hours. SI denotes survival index (moving J2 divided by moving J2 for the no-CO_2, no-pellet control). Controls for the 3- and 13-day sets are the first and third graphs in the bottom row, denoted Ck.

been extracted from soil. This was not the case, and we cautiously conclude that suppression of J2 root invasion by *M. cionopagum* and *M. ellipsosporum* in vials was caused primarily by parasitism.

The fungi appeared to repel nematodes in the behavioral assay cylinders but not in the vials. Unknown behavioral differences between the two nematode species tested (*M. incognita* in cylinders vs. *M. javanica* in vials) would seem an unlikely explanation, in view of their ecological, developmental, and morphological similarities. Perhaps in vials where cabbage seedlings were planted, attractants from roots were stronger than repellents from fungi. Also, concentration gradients may have been weaker in vials than in behavioral assay cylinders, because pellets in vials were not concentrated in layers as in cylinders, and

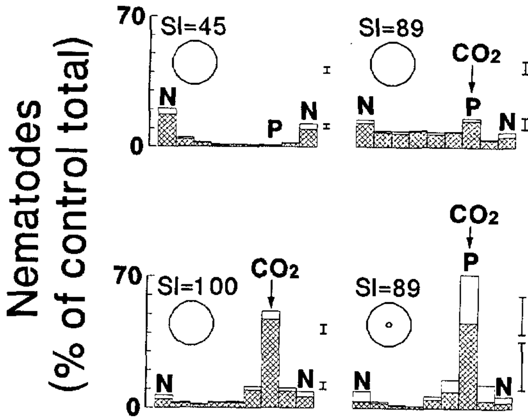

FIG. 7. Second CO_2 bait experiment. Effects of CO_2 and alginate pellets of *Monacrosporium cionopagum* on the distribution of *Meloidogyne incognita* second-stage juveniles (J2) 42 hours after introducing 10,000 J2 to cylinders of sand (7.2-cm-long, 3.8-cm-diam.) in which pellets had been buried for 5 days. Hatched bars show moving J2, and empty bars show total J2 extracted from nine 0.8-cm-thick sections. Data in the lower right-hand graph were obtained by dispersing and counting all J2 in a 0.11-cm^3 central core; data in other graphs were obtained by counting nematodes extracted by flotation from the entire 9.1-cm^3 section. Lower and upper brackets are LSD's ($P = 0.05$) for moving and for total nematodes from four replications. J2 and pellets were placed in sections denoted N and P, respectively. Where indicated, CO_2 was injected (20 µl/minute) continuously into the third section from the right for the last 42 hours. SI denotes survival index (moving J2 divided by moving J2 for the no-pellet control).

because microorganisms present in the loamy sand, but not in the pure sand, could have degraded the repellent. Use of the unsterilized loamy sand in the behavioral assay cylinders would permit the latter possibility to be tested. Alternatively, repellency could have occurred in vials without reducing fungal parasitism because nematodes were in the vials for a longer period (5 days) than in cylinders (24–42 hours) and because nematodes were introduced into vials closer to the pellets than in the cylinders, in most cases.

The repellent(s) detected may alter biological control in the field or may be of value as a nematode management tool independent of the fungi, and their structure and activity should be characterized. One repellent appears to be highly polar or ionic. Many such substances could be

TABLE 1. Cabbage seedling growth, and suppression of root invasion by *Meloidogyne javanica* second-stage juveniles (J2) within 25-ml vials containing alginate pellets of nematophagous fungi (*Monacrosporium cionopagum, M. ellipsosporum,* or *Hirsutella rhossiliensis*) and 17 cm^3 of an unsterilized loamy sand soil or an inert industrial abrasive (TTEX sand).

Fungus[b]	Shoot length (cm)		Root length (cm)		Total J2 per root		Suppression[a] (%)	
	Loamy sand	TTEX sand	Loamy sand	TTEX sand	Loamy sand	TTEX sand	Loamy sand	TTEX sand
M. cionopagum	2.2 (0.1)[c]	2.1 (0.1)	4.1 (0.3)	4.0 (0.3)	11 (1)	1 (1)	72	97
M. ellipsosporum	1.8 (0.5)	1.8 (0.1)	1.4 (0.2)	2.8 (0.2)	35 (4)	24 (3)	12	38
H. rhossiliensis	2.1 (0.2)	1.7 (0.1)	3.0 (0.2)	3.1 (0.2)	24 (3)	9 (2)	41	77
Blank pellets	1.9 (0.1)	1.4 (0.2)	1.4 (0.2)	1.6 (0.4)	40 (2)	39 (8)	—[d]	—

[a] Suppression is defined as $100 \cdot (1-R_t/R_c)$ where R_t = J2 in roots of treatment i and R_c = J2 in roots of the blank pellet control; the number of J2 added to each vial was 100 ± 5 (mean ± SE)]
[b] Each vial had four pellets containing the fungus indicated; blank pellets contained no fungus.
[c] Values in parentheses are standard errors based on six replications.
[d] Not calculated; 0% by definition.

TABLE 2. Cabbage seedling root growth, suppression of root invasion by *Meloidogyne javanica* second-stage juveniles (J2), and suppression of J2 populations in the soil, when nematophagous fungi (*Monacrosporium cionopagum, M. ellipsosporum,* or *Hirsutella rhossiliensis*) were added to each assay vial as four calcium alginate pellets, or as 20 colonized J3 of *Steinerenema glaseri.*

	Vials with cabbage seedlings			Vials without cabbage seedlings			
				M. javanica extracted from soil			
Fungus	Root length (cm)	Total J2 per root	Suppression (%)	Healthy	Parasitized	Total	Suppression[a] (%)
Vials with calcium alginate pellets							
M. cionopagum	5.0 (0.2)[b]	4 (1)	93 (1)	19 (2)	2 (1)	20 (3)	79 (3)
M. ellipsosporum	3.5 (0.3)	17 (3)	74 (5)	33 (4)	8 (1)	42 (3)	62 (4)
H. rhossiliensis	3.2 (0.4)	26 (6)	59 (10)	44 (5)	60 (4)	104 (5)	51 (5)
Blank pellets	1.6 (0.2)	63 (7)	—[c]	88 (4)	0 (0)	88 (4)	—
Vials with colonized *Steinernema glaseri*							
M. cionopagum	4.3 (0.2)	20 (2)	73 (2)	26 (1)	7 (1)	33 (2)	65 (2)
Control[d]	1.4 (0.1)	73 (7)	—	76 (4)	0 (0)	76 (4)	—
M. ellipsosporum	5.1 (0.2)	4 (1)	94 (2)	29 (3)	7 (1)	36 (3)	66 (3)
Control	1.3 (0.2)	66 (7)	—	85 (5)	0 (0)	85 (5)	—
H. rhossiliensis	2.8 (0.4)	27 (4)	43 (8)	26 (2)	56 (4)	82 (3)	63 (3)
Control	1.5 (0.3)	48 (6)	—	69 (7)	0 (0)	69 (7)	—

[a] Suppression in vials containing cabbage seedlings is defined as $100 \cdot (1-R_i/R_c)$ where R_i = J2 in roots of treatment i and R_c = J2 in roots of the blank pellet control. Suppression in vials without cabbage seedlings is defined as $100 \cdot (1-H_i/H_c)$ where H_i = healthy J2 extracted from soil in treatment i and H_c = healthy J2 extracted from soil in the fungus-free (no *S. glaseri*) control.

[b] Values in parentheses are standard errors based on eight replications.

[c] Not calculated; 0% by definition.

[d] Control vials received no *S. glaseri*.

present, including Na^+, Ca^{++}, and Cl^- from the pellet preparation process as well as ammonium salts or other trace contaminants from the fungal nutrient broth. Various inorganic salts repel *M. incognita* (7,8) as well as other *Meloidogyne* species (25–28), and ammonia was implicated as the toxin used by *Arthrobotrys* species to kill nematodes (2). The consistent observation that aqueous extract from blank pellets is moderately repellent during the first 24 hours following application to sand, while intact blank pellets have no repellent activity 3 to 13 days after application, suggests that gradients of the repellent from blank pellets dissipate within 3 days and that some other substance, perhaps released metabolically, is subsequently responsible for repellency of pellets containing fungi. Extracts of the fungal pellets could contain both substances. Pellets were extracted with 21 μl water/mg pellet and weighed about 1.5 mg. Thus, the 250 μl of extract applied to each 4.0-cm cylinder was roughly equivalent to eight pellets; 10 pellets were applied to the 4.0-cm cylinders in the pellet experiments.

Surprisingly, there was no evidence of attractants, even though *M. cionopagum, M. ellipsosporum,* and several other nematophagous fungi are known to attract ecologically diverse nematodes on agar (17,24). Perhaps a substance that attracts *M. incognita* is in fact released by these fungi but is not active in the presence of repellents that also are released.

Results of the CO_2 bait experiments indicate that appropriate attractants could be used to overcome repellent effects and may increase parasitism. From a practical standpoint, though, combining a suitable CO_2 source with nematophagous fungi within pellets may be difficult. An inorganic reaction that releases CO_2 would need to progress slowly to achieve behaviorally effective release rates (30), and a microbiological source might compete with the pelletized fungus. Nematode pheromones (1,12) or other attractants (4,5,9,10) might be substituted. Recently, tannins have been identified as potent attractants for *M. incognita* (pers. comm., T. E. Hewlett, University of Florida). It might even be advantageous to incorporate repellents into the pellets intentionally. In crops that are particularly sensitive to nematode infection during the seedling stage, such pellets could be strategically placed where the taproot is expected to grow. Nematodes already in this zone would in theory be killed by the fungi, and nematodes outside the zone would be repelled and thus prevented from reaching the root.

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