Journal of Nematology 19(3):287-291. 1987.
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Escape of *Steinernema feltiae* from Alginate Capsules Containing Tomato Seeds

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Abstract: The entomogenous nematode *Steinernema feltiae* was encapsulated in an alginate matrix containing a tomato seed. When these capsules were placed on 0.8% agar for 7 days, the seed germinated and ca. 20% of the nematodes escaped from the capsules, whereas only 0.1% escaped from capsules without seeds. When capsules containing nematodes and a seed were planted into sterilized or nonsterilized soil, nematodes escaped to infect *Galleria mellonella* larvae. When seed in capsules containing ca. 274 nematodes per capsule were planted in nonsterilized soil, *Galleria* mortality was 90% 1 week later. *Galleria* mortality declined to 27%, 23%, and 0% in weeks 2, 4, and 8 postplant, respectively. In sterilized soil, *Galleria* mortality was 96% and did not differ significantly from the nonsterilized soil in week 1, but was significantly higher in sterilized soil over nonsterilized soil for week 2 (81%) and week 4 (51%). When capsules containing nematodes only were used, *Galleria* mortality was 71% in sterilized soil 1 week after planting and 58%, 33%, and 12% in weeks 2, 4, and 8 postplant, respectively. In nonsterilized soil, *Galleria* mortality was 8%, 30%, 21%, and 28% after 1, 2, 4, and 8 weeks, respectively, using only encapsulated nematodes. When the number of nematodes per capsule was increased to ca. 817, *Galleria* mortality was 92% or higher in sterilized soil from week 1 to week 4.

Key words: biological control, calcium alginate, *Galleria mellonella, Neoaplectana,* sodium alginate, *Steinernema feltiae,* tomato.

Steinernematid nematodes show potential as biological control agents of insects in agroecosystems (8) because of their broad host range, safety to vertebrates, high efficacy, ability to seek out hosts, and ease of production and application (4). Although these nematodes can be applied using spray technology developed for chemical pesticides, this approach is not always appropriate. For example, applying nematodes directly to the soil surface may expose them to unfavorable conditions, including ultraviolet radiation and sunlight (5), high temperatures (7), and desiccation (1). Recently, Kaya and Nelsen (9) demonstrated that the entomogenous nematodes *Steinernema feltiae* (= *Neoaplectana carpocapsae*) and *Heterorhabditis heliothidis* can be encapsulated in an alginate matrix as a delivery system. One possible use of this system is to place nematodes and seeds within the same capsules; when they are planted, the nematodes can protect the roots of a young seedling from insect attack. Our objective was to demonstrate the feasibility of using *S. feltiae* encapsulated with tomato seeds for the protection of seedlings from soil insects.

MATERIALS AND METHODS

Encapsulation of nematodes and seeds: Nematodes were produced in *Galleria mellonella* larvae (2) and stored at 10 C until encapsulated as described by Kaya and Nelsen (9). A 2% sodium alginate solution (2 g alginate in 98 ml distilled water containing nematodes) was allowed to form droplets from a separatory funnel. A tomato (*Lycopersicon esculentum* cv. UC 82) seed was inserted manually into the sodium alginate droplet containing the nematodes before it went into the complexing solution (100 mM $CaCl_2$). Capsules were made with 1) nematodes plus seed, 2) nematodes alone, and 3) seed alone. Each capsule weighed 79 ± 7.6 (SD) mg.

The capsules were treated with a 0.5 M KNO_3 solution for 30 minutes as a seed arrestant and stored at 10 C for no longer than 3 weeks before use. The effect of the seed-arresting treatment on the nematodes was examined by dissecting capsules in distilled water 2 and 7 days after treatment, and 30 nematodes from each of three cap-

Received for publication 26 September 1986.
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sules with and without the KNO_3 treatment were examined for survival. In addition, three dissected capsules with nematodes were placed in a petri dish containing moist filter paper and their infectivity was assessed against five last-instar *Galleria* larvae. After 7 days, dead larvae were dissected and examined for nematode infection. There were three replicates.

The actual number of nematodes per capsule was determined by dissolving six capsules in 9.5 ml of 0.5 M sodium citrate ($Na_3C_6H_5O_7 \cdot H_2O$) containing 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, MO). The capsules were stirred with a magnetic spinbar until dissolution (about 30 minutes), and the nematodes in 1 ml of suspension were counted using a Hawksley counting slide (Hawksley and Sons, Sussex, England). The number of nematodes averaged 274 ± 11 (SD) per capsule for the KNO_3 treatment, the escape from capsule experiment, and the first series of soil tests.

Escape of nematodes from capsules: A capsule with nematodes plus seed was placed in a test tube (150 × 20 mm) containing 10 ml of 0.8% agar. After 7 days, 2 ml of distilled water was added to the test tube and the contents emptied into a petri dish. The distilled water procedure was repeated and the number of nematodes counted. Nematodes in the test tube agar were also counted and added to the total. The height of the tomato plant from the surface of the agar was measured. Capsules with nematodes alone served as controls and were treated as described for capsules with nematodes plus seed. There were three trials with 12 to 20 capsules of each type in each trial.

Bioassay of escaped nematodes in petri dishes: Two capsules with nematodes plus seed, nematodes alone, or seed alone were placed in a petri dish containing moist filter paper and sealed with Parafilm. After 1 week, the capsules and germinated plants were removed and five *Galleria* pupae were placed in each petri dish to assess nematode infectivity. There were two trials with four petri dishes in each trial.

Bioassay of encapsulated nematodes in soil: Soil (61% sand, 20% silt, and 19% clay) from a former creek bed (Putah Creek) in Davis, California, was mixed uniformly in a cement mixer for 10 minutes. Half of the soil sample was steam sterilized at 121 C and 15 psi for 2 hours, stored in a garbage can, and used within 2 weeks. About 150 cm^3 sterilized or nonsterilized soil was placed in 6-oz (177.5-cm^3) styrofoam cups with four drainage holes in the bottom of each cup. The soil was watered, and the following day, two capsules with nematodes plus seed, nematodes alone, or seed alone were planted at a soil depth of ca. 2 cm. The cups were maintained in a greenhouse at 22 ± 3 C and watered daily. At 1, 2, and 4 weeks after planting in the first trial and 1, 2, 4, and 8 weeks in the second trial, six to nine cups were brought to the laboratory and the plants were cut at the soil surface. Three 9-mm-d holes, 3 cm deep, were bored into the soil around the cut plant with a number 4 cork borer. A last-stage *Galleria* larva, used to assess nematode presence by its infectivity, was placed in each hole. Moist sterilized sand was placed up to the rim of the cup, and the cups were covered with plastic petri dishes (100 × 15 mm), inverted to contain the insect hosts, and held at room temperature (25 ± 1 C); 1 week later the *Galleria* larvae were examined for nematode infection. Cups without tomato plants were similarly treated. In another series of tests, the same procedure was used except that only seed plus nematodes or seed alone was planted in nonsterilized soil. There were two trials with nine cups per trial; bioassays were done at 1, 2, 4, and 8 weeks after planting. Data were analyzed by chi-square tests.

Results

Treatment of the capsules with the seed-arresting chemical, KNO_3, had no significant effects on the nematodes as chemical-treated and untreated capsules contained 96.7% and 94.4% live nematodes, respectively. Bioassay of these nematodes against *Galleria* larvae resulted in 100% mortality,

TABLE 1. *Galleria mellonella* pupae (number) infected by nematodes from alginate capsules with and without tomato seeds.

Treatment	Pupae alive	Pupae dead	
		With nema	Without nema
Nema + seed	0	40†	0
Nema alone	38	1‡	1
Seed alone	40	0	0

† All pupae contained nematode adults and progeny.
‡ Pupa had nematode adult female only.

and nematode progeny were present in all larvae.

Capsules placed on 0.8% agar had 20% of the nematodes escaping from capsules with seed (n = 46) and 0.1% escaping from capsules without seed (n = 52) in 7 days. The mean number of nematodes escaping from the nematode plus seed and nematode alone capsules was 54.8 (± 18.3 [SD]) and 0.3 (± 0.7 [SD]), respectively. The average plant height at the end of the test was 64 mm (range 45–75 mm). In a few (n = 5) cases, the seed did not germinate from the capsules but 12–19 ($\bar{x}$ = 15) nematodes escaped. In the petri dish bioassay, all *Galleria* pupae placed in petri dishes containing capsules with nematodes plus seed were dead from nematode infection, while only one from the capsules with nematodes alone died from a nematode infection (Table 1).

Galleria mortality due to nematode infection occurred in sterilized and nonsterilized soil (Table 2). Highest *Galleria* mortality in the sterilized soil was observed during week 1 in the nematode plus seed and nematode alone treatments. Thereafter, mortality declined in both treatments until nematodes were detected at only low levels (12%) in the nematode alone treatment and were not detected in the nematode plus seed treatment after 8 weeks. In the nonsterilized soil, highest *Galleria* mortality was observed in week 1 in the nematode plus seed treatment; mortality declined thereafter, reaching 0% by week 8. The nematode alone treatment had low (8%) *Galleria* mortality in week 1, and in the ensuing weeks mortality ranged between 21 and 30%. *Galleria* mortality in the nematode alone treatment was significantly higher in nonsterilized than in sterilized soil (χ^2 = 30.13, P < 0.01) in week 1, but no significant difference was ob-

TABLE 2. *Galleria mellonella* larvae alive and dead after 8 weeks in sterile and nonsterile soil to which alginate capsules containing *Steinernema feltiae* with or without a tomato seed were added.

Treatment†	*Galleria* larvae in sterile soil			*Galleria* larvae in nonsterile soil		
		No. dead (%)			No. dead (%)	
	No. alive (%)	With nema	Without nema	No. alive (%)	With nema	Without nema
1 week						
Nema + seed	1 (3.3)	32 (96.3)	0 (0)	0 (0)	28 (90.3)	3 (9.7)
Nema alone	7 (20.0)	25 (71.4)	3 (8.6)	28 (77.8)	3 (8.3)	5 (13.9)
Seed alone	18 (100)	0 (0)	0 (0)	17 (100)	0 (0)	0 (0)
2 weeks						
Nema + seed	2 (5.6)	29 (80.5)	5 (13.9)	15 (50.0)	8 (26.7)	7 (23.3)
Nema alone	7 (21.2)	19 (57.6)	7 (21.2)	23 (69.7)	10 (30.3)	0 (0)
Seed alone	32 (86.1)	0 (0)	5 (13.9)	32 (100)	0 (0)	0 (0)
4 weeks						
Nema + seed	9 (18.4)	25 (51.0)	15 (30.6)	15 (34.9)	10 (23.2)	18 (41.9)
Nema alone	21 (38.9)	18 (33.3)	15 (27.8)	17 (40.5)	9 (21.4)	16 (38.1)
Seed alone	43 (93.5)	0 (0)	3 (6.5)	46 (95.8)	0 (0)	2 (4.2)
8 weeks						
Nema + seed	18 (100)	0 (0)	0 (0)	18 (100)	0 (0)	0 (0)
Nema alone	11 (64.7)	2 (11.8)	4 (23.5)	13 (72.2)	5 (27.8)	0 (0)
Seed alone	18 (100)	0 (0)	0 (0)	16 (88.9)	0 (0)	2 (11.1)

† Mean number of nematodes per capsule was 274 ± 11.

TABLE 3. *Galleria mellonella* larvae alive and dead after 8 weeks in nonsterile soil to which alginate capsules containing *Steinernema feltiae* with or without a tomato seed were added.

Treatment†	No. alive (%)	No. dead (%)	
		With nema	Without nema
1 week			
Nema + seed	0 (0)	48 (94.1)	3 (5.9)
Seed alone	50 (96.2)	0 (0)	2 (3.8)
2 weeks			
Nema + seed	0 (0)	52 (100)	0 (0)
Seed alone	53 (100)	9 (0)	0 (0)
4 weeks			
Nema + seed	1 (1.9)	49 (92.4)	3 (5.7)
Seed alone	50 (94.3)	0 (0)	3 (5.7)
8 weeks			
Nema + seed	29 (54.7)	10 (18.9)	14 (26.4)
Seed alone	51 (96.2)	0 (0)	2 (3.8)

† Mean number of nematodes per capsule was 817 ± 74.

served in the nematode plus seed treatment between sterilized and nonsterilized soil (Table 2). Significantly higher *Galleria* mortality was observed in the nematode plus seed treatment than in the nematode alone treatment both in sterilized soil ($\chi^2 = 5.35$, $P < 0.05$) and in nonsterilized soil ($\chi^2 = 48.13$, $P < 0.01$). In week 2, significantly higher *Galleria* mortality occurred in the nematode plus seed treatment ($\chi^2 = 21.02$, $P < 0.01$) and in the nematode alone treatment ($\chi^2 = 10.62$, $P < 0.01$) in the sterilized soil than in the nonsterilized soil. *Galleria* mortality was significantly higher in the nematode plus seed treatment than in the nematode alone treatment in the sterilized soil ($\chi^2 = 4.42$, $P < 0.05$), but there was no significant difference in mortality between the same treatments in the nonsterilized soil. Similar trends were observed in week 4, but by week 8 *Galleria* mortality was higher in the nonsterilized than sterilized soil in the nematode alone treatment.

Capsules each containing 817 ± 74 (SD) nematodes showed greater activity over time than capsules containing 274 ± 11 (SD) nematodes (Table 3). Nematode activity was very high (> 92%) and chi-square analysis showed no significant difference in *Galleria* mortality 1–4 weeks after planting, but after 8 weeks mortality was significantly lower than after 1–4 weeks ($\chi^2 = 52.41$, $P < 0.01$).

DISCUSSION

The concept of using alginate capsules as a delivery system for biological control agents against pest organisms has been attempted with pathogens of weeds (11) and antagonists of plant pathogens (3,10). In those studies conidia, ascospores, chlamydospores, and mycelial fragments were encapsulated in the alginate with a bulking agent, such as kaolin or bran, and dried. Generally, the fungi remained viable in these dehydrated capsules and showed potential usefulness as biological control agents of plant pathogens and weeds.

S. feltiae has been successfully encapsulated in alginate (9). Lepidopterous insects fed upon these capsules releasing the nematodes which infected the insects, but the system worked effectively only if adequate moisture was present to prevent desiccation and mortality of the nematodes within the capsules. Thus, the application of these capsules to a soil ecosystem where adequate moisture prevails is reasonable. However, if anhydrobiotic nematodes can be used in these capsules, the application could be expanded into the development of dried bait systems.

Kaya and Nelsen (9) suggested that encapsulation of entomogenous nematodes with seed has potential to protect roots from insect attack. By placing a tomato seed into the alginate matrix containing nematodes, we demonstrated that nematodes escaped from the capsules within 7 days when placed in a tube containing agar. When the seed germinated, the nematodes had an opening to escape from the capsule. Similarly, in soil the nematodes escaped from the capsule and infected the insect host upon seed germination. When more nematodes were placed in the capsules, higher *Galleria* mortality was observed for longer periods in nonsterilized soil (Tables 2, 3). Our bioassay was conducted near the soil surface, however, and we do not know the

nematode dispersion through the root zone at greater depths.

Our data showed that the nematodes were more effective in sterilized than in nonsterilized soil, particularly in weeks 2 and 4 after planting (Table 2). Ishibashi and Kondo (6) observed that nematode survival and mortality of insects were enhanced in sterilized soil. They suggest that the absence of microflora in the sterilized soil creates a favorable environment for nematode survival, and our data support their observations.

The higher *Galleria* mortality for nematodes alone the first week in sterilized soil compared with nonsterilized soil (Table 2) is in direct contrast to data obtained in the laboratory test tube tests where few nematodes escaped from capsules containing no seed. Because the sterilized soil had little or no microflora or microfauna, biological capsule breakdown was more apt to occur in the nonsterilized soil. Since greater insect mortality was observed in the sterilized soil, we suggest that the freed nematodes were in a more favorable environment for survival and infection of the host insect.

The concept of incorporating a biological control agent with seed in alginate capsules is feasible. Although seed arrestants are available to prevent or slow seed germination, problems of long-term seed storage in a moist capsule that must be overcome include growth of contaminating micro-organisms and seed viability. An alternative to this approach is to place a capsule containing the nematodes adjacent to the seed to protect the root system from insect attack. The advantage of such a system would be the slow release of nematodes and protection of nematodes from micro-organisms in the soil. Placement of nonencapsulated nematodes directly with the seed would expose them to antagonists resulting in a loss of nematodes and reducing their effectiveness as biological control agents.

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