Journal of Nematology 25(2):198–203. 1993.

"Pesta": New Granular Formulations for *Steinernema carpocapsae*

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Abstract: "Pesta," a new granular product for use with entrapped biocontrol agents, is based on a cohesive dough made of wheat flour, fillers, and other additives. Infective juveniles of the entomopathogen *Steinernema carpocapsae* strain All incorporated in Pesta granules emerged when the granules were softened by immersion in water. These granules may be useful for the biocontrol of insect pests in the soil. Storage temperature had the greatest effect on recovery of nematodes, followed by the moisture content of the granules. Recovery of nematodes was the same among the formulations tested and was unaffected by storage in nitrogen. Nematode recovery after storage at 21 C decreased to zero after 3–6 weeks. Storage of samples at 4 C and with a high moisture content (19.9–23.1%) greatly improved nematode viability.

Key words: Biological control, emergence, entomopathogenic nematode, formulation, nematode, Pesta, *Steinernema carpocapsae,* storage, wheat flour.

Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* are used successfully in agriculture for the biocontrol of numerous insect pests and are often as effective as chemical insecticides (7). The introduction of commercial nematode formulations has accelerated in recent years, but new formulation technology that produces stable, effective, and consistent products would expand markets even further (6,7). Spray application using a nematode suspension in water is straightforward, inexpensive, and often effective. However, capsules, bait pellets, and granular formulations that protect and (or) release nematodes in the soil are also desirable for insect control. Novel alginate capsules containing nematodes have been developed (10,11,14), as have wheat bran bait pellets (4) and alfalfa–wheat bait pellets (3).

A process for entrapping fungal weed pathogens (mycoherbicides) in a wheat gluten matrix has been developed recently, and the free-flowing granular products were also referred to as "Pesta" (5). It is possible that the Pesta-making process, which is simple to use and performed at ambient temperature, could be adapted for use with entomopathogenic nematodes. For this initial study, wheat flour, kaolin, and peat moss were selected as the formulation ingredients, rendering the granule matrix nontoxic and harmless toward the environment.

The principal objective of this study was the determination of *Steinernema carpocapsae* strain All viability after entrapment in Pesta and nematode capacity for emergence from wet granules. Additional objectives were the determination of the effects on product shelf life of formulation composition, moisture level, storage temperature, and storage atmosphere (air or nitrogen).

Materials and Methods

The nematode used in this study was *Steinernema carpocapsae* Strain #25 (All) from Biosys (Palo Alto, CA). Three aqueous suspensions containing between 744,000 and 770,000 live nematodes per ml were received on separate occasions and aerated at 5 C with an aquarium pump until used (randomly) in sample preparation within 3 days of receipt (with verification of viability immediately before formulation).

Preparation of Pesta granules: Semolina, a coarse durum wheat flour, was obtained from Tropical Nut and Fruit (Charlotte, NC). Kaolin, RC-32 type, was furnished by Thiele Kaolin Co. (Wren, GA). Sphagnum

Received for publication 24 June 1992.

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We thank Mrs. Kelley A. Williams, SRRC, and Ms. Antonette K. Malabey, PSI, for technical assistance, and Dr. Paul Pruitt, Biosys, for technical advice and nematodes.

peat moss (Premier Brands, New Rochelle, NY) was ground and sieved to pass an 80-mesh screen. Semolina was mixed thoroughly with kaolin and, for some samples, also with peat moss in the quantities shown in Table 1. Semolina content in the three Pesta formulations was held constant, and the amounts of kaolin (10–20%) and peat moss (0–10%) varied (Table 1). Twenty-five milliliters of nematode aqueous suspension was added to the flour mixture and kneaded by gloved hand to form a dough. Sometimes an additional 1–4 ml of tap water was necessary to produce a moist and cohesive, but not sticky, dough. Extra water was often needed when peat moss was included in the formulation. Water, not aqueous nematode suspension, was used for this purpose to maintain a more constant nematode concentration in the different samples. The dough was kneaded and passed through a pasta maker to produce a sheet that was 1.0–1.1 mm thick (5).

The dough sheet containing entrapped *S. carpocapsae* was placed to dry on a stainless steel wire mesh rack in a room conditioned at 21 C and 65% relative humidity. To obtain a low moisture content, samples were dried for 22 hours. For medium and high moisture contents, the freshly prepared dough sheets were covered with one or two paper towels, respectively, and dried for about 16 hours. Low moisture (13.3% water) samples were crisp; medium moisture (16.6–18.0% water) samples were slightly damp in the center and pliable with a slight crispness at the edge. For high moisture content (19.9–23.1%) samples, the whole dough sheet was slightly damp, pliable, and just dry enough for grinding. After drying, the dough sheets were ground in a Thomas-Wiley mill, intermediate model, equipped with a 10-mesh delivery tube (Thomas Scientific, Swedesboro, NJ). The grinds were sieved to pass 10-mesh (2.00 mm) screens and collected on 18-mesh (1.00 mm) screens. Samples were stored in air or nitrogen atmospheres in sealed glass or plastic vials at 4 C and 21 C. The nitrogen atmosphere was provided

TABLE 1. Data relative to the preparation of *Steinernema carpocapsae*/Pesta and properties of the granules.

Sample composition†	Formulation					Dough pH	H_2O (%)	Water activity (a_w)	Dried product wt., g		No. active nematodes/g
	Moisture type	Semolina (g)	Kaolin (g)	Peat moss (g)	Nematode‡ suspension (ml)				Gross	10–18 mesh	
80S/20K	Medium	32	8	0	25	5.9	16.6	0.89	44.7	26.9	416,000
80S/20K	High	32	8	0	25	5.9	19.9	0.94	46.4	26.4	415,000
80S/15K/5P	Low	32	6	2	25	5.2	13.3	0.71	42.1	23.1	449,000
80S/15K/5P	Medium	32	6	2	25	5.2	17.4	0.88	44.1	22.2	431,000
80S/10K/10P	Medium	32	4	4	25	4.7	18.0	0.89	44.2	21.9	428,000
80S/10K/10P	High	32	4	4	25	4.7	23.1	0.95	47.5	25.7	405,000

Each value is the average of at least two determinations.
† Numbers refer to percentage by weight of dry ingredients; S = semolina, K = kaolin, and P = peat moss.
‡ An additional 1 to 4 ml of tap water was added to get a dough that was moist and cohesive, but not sticky.

by introducing a stream of the gas for about 30 seconds through a glass pipet that was pushed through the Pesta granules to the bottom of the storage vial. It is possible that trace levels of oxygen remained in the vial.

pH, water content, and water activity: The pH of a freshly prepared dough sheet was measured with a flat tip combination electrode (Fisher Scientific, Pittsburgh, PA). Water content of granules was determined by Karl Fischer titration (AquaStar V1B titrator and Model EV-6 Solid Evaporator, EM Science, Cherry Hill, NJ), using 0.20-g samples and 135 C chamber temperature. Water activity (a_w) of granules (1.5-g samples) was measured with a CX-1 instrument (Decagon Devices, Pullman, WA). All pH, water content, and a_w measurements were duplicated and averaged for each sample.

Emergence and counting of nematodes: A 0.200 ± 0.002 g aliquot of each sample was weighed in a disposable weighing boat (8-cm square at top). Freshly drawn tap water (10 ml) was added and the sample was left undisturbed for 20–24 hours at 21 C. Then the water with immersed sample was swirled, four bursts of air from a disposable pipet were injected to aerate the nematodes and aid mixing, and the liquid was decanted quickly into a 10-ml graduated cylinder. Four tap water rinses were used to transfer the nematodes into the graduated cylinder. If the granules had mostly disintegrated after soaking, all the material in the weighing boat was rinsed into the cylinder. The volume was brought to 10 ml again (about 4–6 ml had evaporated during the soak period) with tap water, and the cylinder was stoppered and inverted several times to mix the contents. An aliquot was quickly withdrawn from the center of the cylinder, diluted if necessary, and placed in a 1-ml eelworm counting slide (Hawksley & Sons, Lancing, West Sussex, England). The number of active nematodes (unprovoked movement) that emerged from the water-softened or partially disintegrated granules was counted and expressed as the number per gram of sample. The number of live nematodes was probably higher because *S. carpocapsae* infective juveniles can remain motionless even though alive. A second aliquot was withdrawn and counted and the results were averaged. Nematode emergence was determined for the freshly prepared Pesta and at 1 week, 3 weeks, and 3-week intervals for as long as active nematodes were present up to 36 weeks.

Assay for infectivity after storage: A standardized nematode infectivity test that regularly provides 90–100% wax moth larval mortality in 72 hours was used (I. Popiel and P. Pruitt, Biosys, pers. comm.). This method utilized a nematode suspension of 100–120 third-stage infective juveniles in 2 ml of water applied to two filter papers in a petri dish (100 × 15 mm) into which 10 wax moth larvae were added. The resultant 90–100% wax moth larval mortality in 72 hours was used as an indicator that nematode infectivity was retained after being stored in Pesta granules.

Statistical analysis: Four experimental factors were considered across time: formulation, moisture level, storage temperature, and storage atmosphere. Polynomial (i.e., linear, quadratic, or cubic) regression models were fitted across time to the square root of observed nematode count triplicates for each of the 24 treatment combinations: four temperature by atmosphere treatments for each of the six samples listed in Table 1 (15). Two analyses of variance were conducted using time as a fifth factor. The first analysis crossed all effects with the exception of moisture level, which was nested within formulation to allow examination of specific differences among levels of moisture for a given formulation. The second analysis nested formulation within moisture level to examine differences among formulations at a given level of moisture and crossed all other effects. An analysis of covariance (15) was conducted to compare overall nematode counts and rates of release for specific samples as suggested by the analyses of variance results. The curves representing the number of live nematodes re-

leased across time were not forced through the actual number released at time zero. Thus, the method of least squares could be used to fit the data.

Results

Formulations that contained more than 10% peat produced doughs with cohesiveness insufficient for easy processing. Doughs from each of the formulations were dried to two different moisture levels. Low moisture was the designation given to Pesta with a water content of 13.3% and a water activity of 0.71. Medium moisture was 16.6–18.0% water and a_w = 0.88–0.89. High moisture included the range of 19.9–23.1% water and a_w = 0.94–0.95. The pH of fresh dough without peat moss was 5.9; addition of peat acidified the dough (pH 4.7 with 10% peat). The product weight of 10–18 mesh granules averaged 54 ± 6% of the dried dough sheet weight before grinding. The calculated number of active nematodes in each sample averaged 424,000 per gram. For each formulation, the sample with higher water content had fewer nematodes because of dilution by the extra water. No allowance for this difference in nematode content was made in subsequent data analysis.

Effect of moisture and formulation at 21 C: Survival of nematodes entrapped in Pesta granules stored in air at 21 C was relatively poor for all formulations and moisture levels. A rapid loss in nematode viability was observed (Fig. 1) and, by 6 weeks, few live nematodes remained. The low moisture sample had the poorest survival rate. At a given moisture level, there were no significant (α = 0.05) differences in survival rate due to formulation composition. Fungal growth of an undetermined nature frequently was observed on the granules, especially those with high moisture content, and the infested granules became matted together and were no longer free-flowing.

Effect of moisture and formulation at 4 C: Survival rates of nematodes were high for the two formulations of high moisture content (19.0–23.1%) that were stored in air at

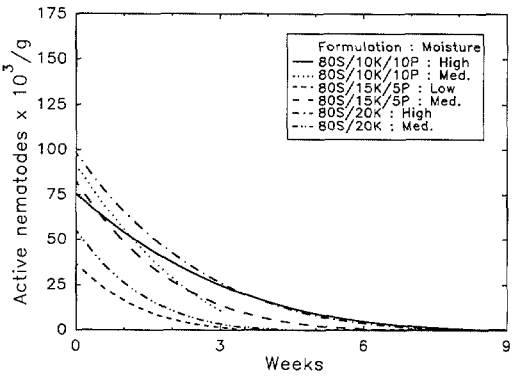

FIG. 1. Effect of moisture and formulation on nematode emergence in water after storage of *S. carpocapsae*–Pesta granules at 21 C in air. Fitted regression curves explain between R^2 = 83% and R^2 = 98% of the total variability observed in the data. Formulation key: numbers refer to percentage by weight of dry ingredients; S = semolina, K = kaolin, and P = peat moss.

4 C (Fig. 2). More than 60,000 active nematodes per gram were released from these samples after 18 weeks. The three replicates of sample composition 80S/20K at high moisture were tested at 36 weeks. The average number of active nematodes released per gram of Pesta was 15,500, and each replicate caused 100% mortality of wax moth larvae (with typical infection by the bacterial associate).

Samples with a medium moisture level

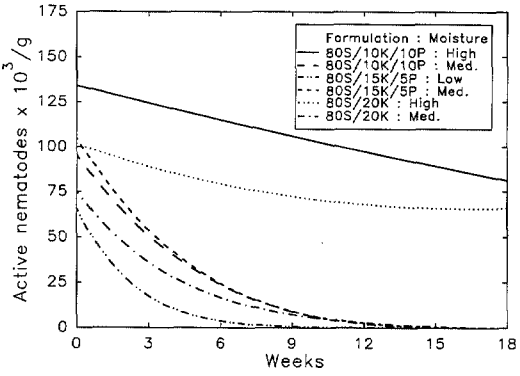

FIG. 2. Effect of moisture and formulation on nematode emergence in water after storage of *S. carpocapsae*–Pesta granules at 4 C in air. Fitted regression curves explain between R^2 = 59% and R^2 = 97% of the total variability observed in the data. Formulation key: numbers refer to percentage by weight of dry ingredients; S = semolina, K = kaolin, and P = peat moss.

were not as stable as those with high moisture, and the decline in numbers of emerging nematodes was more rapid over the first 6 weeks. There were few viable nematodes observed after 6 weeks. Survival of the nematodes was poorest in the low moisture sample. For each formulation composition, the higher moisture level allowed significantly ($P < 0.0082$) better nematode emergence than the lower level. At comparable moisture levels, there were no significant differences ($\alpha = 0.05$) due to formulation composition. The gluten matrix of these formulations continued to entrap a significant number of nematodes, even after a 24-hour immersion in water (Table 1, Figs. 1–3). When granules that had been rinsed of emerged nematodes after the standard 24-hour test were resubmerged in water for an additional 24 hours, a further recovery of 8–29% of the number initially counted often was observed (data not shown).

Effect of storage atmosphere: In general (comparing levels of atmosphere using averages of levels of the other three factors), there was no significant difference ($\alpha = 0.05$) between storage of samples in air or in nitrogen. This is shown in Fig. 3, which is a plot of data from the 80S/10K/10P formulation at high and medium moisture levels stored at 4 C under air or nitrogen.

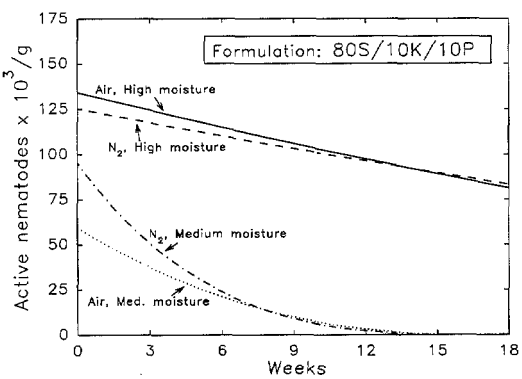

FIG. 3. Effect of moisture and atmosphere on nematode emergence in water of *S. carpocapsae*–Pesta granules made with 80% semolina, 10% kaolin, and 10% peat moss and stored at 4 C in air or in nitrogen. Fitted regression curves explain between $R^2 = 27\%$ and $R^2 = 96\%$ of the total variability observed in the data.

DISCUSSION

Pesta dough, like pasta, gets its desirable cohesive properties from wheat gluten in the wheat flour. A sufficient quantity of semolina, usually about 70–80% by weight, is needed in the formulation to make a satisfactory dough. Preliminary testing indicated that high protein bread flour performed similarly to semolina. Fillers and adjuvants can affect dough properties, but the Pesta process is amenable to the incorporation of a wide variety of solid and liquid additives. For proper consistency, the dough usually requires a flour:water ratio of 1:0.6–0.8, depending on the effect of additives. We have not evaluated the Pesta granules as a bait, but the wheat flour composition and the compatibility with substances like wheat bran, sugars, vegetable oils, etc., suggest that a bait could be formulated readily.

An improved method of granulating the Pesta dough is desirable. Grinding dried dough in a Wiley mill produces a substantial amount of fine particles that release fewer nematodes than larger granules. Undoubtedly, the grinding that produced the small granules decreased nematode survival.

Kaolin is an inexpensive filler, but other clays may be more compatible with nematodes (1,16). Peat moss holds water effectively, and granules made with 10% peat are softer and disintegrate faster in water than those made without peat. The number of nematodes that can be incorporated in Pesta is governed by the concentration of nematodes in the aqueous suspension and by the amount of this suspension that can be added to the flour and other ingredients to make a satisfactory dough.

Generally, Pesta formulation differences were not significant for the three that were tested. Analysis of the nematode release data clearly showed that storage temperature had the most profound effect, followed by moisture content. Shelf life at 21 C was 6 weeks or less, regardless of moisture content. Storage of high moisture content granules at 4 C maintained a high

level of viable nematodes over the 18-week test period. The 80S/20K sample was still effective after 36 weeks at 4 C. The benefits of refrigerated storage and high moisture levels to nematode survival are well known (6,9,11,13).

Oxygen requirements of entomopathogenic nematodes in storage can vary (2,8, 12,17). We found no significant differences in shelf life for Pesta granules stored in air or nitrogen. Storage in air is simpler and cheaper, but storage in nitrogen may sometimes be advantageous to control the growth of microbial contaminants. The formulation work described was not performed under sterile conditions, and viable fungi and bacteria are present and can grow under favorable conditions. This was evident particularly in samples with high moisture content. Storage in nitrogen seemed to reduce the growth of these microorganisms, but a suitable antimicrobial agent is needed to preserve the products in storage.

Pesta products are worth further development and investigation in greenhouse and small-scale field tests. Free-flowing granules that are easy to apply to soil would be a commercially attractive means of applying entomopathogenic nematodes. A sustained release of nematodes from Pesta may occur in soil and make application timing a little less critical for effective insect control. The temperature and rate of drying of the dough with entrapped nematodes should be optimized to prolong shelf life. Further improvements in recovery of nematodes from granules and in prolonging shelf life at ambient temperature are needed and may result from incorporation of, as yet, untested adjuvants. The goal of further formulation development is a commercially acceptable biocontrol product for the home gardener, turf specialist, and farmer.

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