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Effect of cadaver coatings on emergence and infectivity of the entomopathogenic nematode *Heterorhabditis baujardi* LPP7 (Rhabditida: Heterorhabditidae) and the removal of cadavers by ants

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ABSTRACT

Entomopathogenic nematodes (EPNs) are used for the biological control of soil insect pests worldwide and are generally applied to the soil in aqueous suspension. An alternative application method that could be especially practical and effective under certain conditions is to apply the nematode-killed insect (referred to herein as infected insect cadavers) that are placed on or in the soil and from which the nematodes emerge to seek new hosts. However, physical damage to the insect cadavers during handling and application as well as the potential detrimental impact of various soil biotic and abiotic factors could reduce the efficacy of cadaver applications. Our objective was to test the effectiveness of various protective coverings applied to *Galleria mellonella* insect cadavers in terms of their potential impact on the emergence and virulence of infective juveniles of the EPN *Heterorhabditis baujardi* LPP7, and to evaluate whether these coverings influenced cadaver removal by ants (*Ectatomma* spp.). The protective covering treatments included a commercial calcareous powder, a commercial talc powder, and gelatin capsules. The number of emerging infective juveniles (IJs) from insect cadavers formulated with talc powder (9.722 ± 1.382) and gelatin capsules (7.892 ± 1.072) was similar to the control (6.346 ± 1.311), and indicated that these coverings do not interfere with IJ emergence. However, the powdered calcareous covering significantly reduced IJ emergence. High infectivity was observed for IJs that emerged from cadavers in all treatments. *Ectatomma* spp. ants removed all insect cadavers from the nest entrance to a distance of 20 cm, with the exception of insect cadavers formulated in gelatin capsules, which were not removed.

1. Introduction

Entomopathogenic nematodes (EPNs) belonging to the genera *Steinernema* and *Heterorhabditis* (Rhabditida: Steinernematidae and Heterorhabditidae) are used for the biological control of insect pests as part of integrated pest management (IPM) programs worldwide and have been successful against diverse pests that have part of their life cycle in the soil or cryptic environments (Klein, 1990). Entomopathogenic nematodes are frequently applied in aqueous suspension through irrigation systems or various spray equipment. However, recently, the application of EPNs formulated as insect cadavers is being considered as an effective alternative, at least under certain circumstances. In field experiments, Jansson et al. (1993) and Parkman et al. (1993) demonstrated that the application of insect cadavers was efficient and reliable for controlling the sweet potato weevil (*Cylas formicarius* Fabricius) and mole crickets (*Scapteriscus* spp.). Field experiments performed by Del Valle et al. (2008a,b) demonstrated the high migratory capability of infective juveniles (IJs) that emerged from insect cadavers infected with *Heterorhabditi baujardi* LPP7 Phan, Subbotin, Nguyen and Moens, as well as their pathogenicity to fourth-instar larvae of the guava weevil (*Conotrachelus psidii* Marshall). Studies performed with IJs that emerged from insect cadavers showed that these nematodes had higher migratory capacity, infectivity and persistence in soil compared to IJs that were applied in aqueous suspension (Shapiro and Glazer, 1996; Shapiro and Lewis, 1999).

For cadaver application, insect cadavers of *Galleria mellonela* (L.) (Lepidoptera: Pyralidae) infected with EPNs can be used to introduce IJs into the soil. Insect cadavers can also be formulated with protective coverings to improve handling and application viability and to avoid physical damage resulting from friction and rupture of the insect cadavers (Shapiro-Ilan et al., 2001). In addition, coverings might function to lower the stress generated by adverse environmental conditions (Hussaini et al., 2004). Calcareous coverings might change the environment around insect cadavers, prevent the multiplication of microorganisms, and could provide a beneficial osmotic effect (Andrén and Lagerlöf, 1983). Commercial talc powders contain sulfur, which could serve as a fungicidal function

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(Williams and Cooper, 2004). Gelatin capsules could effectively isolate insect cadavers from the soil.

Insect cadavers in the soil are subject to the action of various arthropods and microorganisms (Kaya, 2002). Some ant species diminish the application efficiency of insect cadavers by feeding on them or removing them from the application site, especially when these are located close to nests (Kaya, 2002). Baur et al. (1998) showed that ants do not feed on cadavers infected with heterorhabditids, but that small fissures produced on the insect cuticle by the ants can result in the desiccation of the insect cadaver and lead to death of developing IJs and associated bacteria.

Ants of the genus *Ectatomma* (Hymenoptera: Formicidae: Ponerinae) are widely distributed in Brazil. The genus has 12 species of relatively large ants (approximately 8–11 mm length) that are endemic in neo-tropical regions and common in natural and disturbed habitats (Brown, 1958; Kugler and Brown, 1982; Levings and Franks, 1982). These ants have hypogeous and epigeous foraging and nesting habits (Fernández, 1991). Their nests are simple, they have low polymorphism between workers and queens, and they have relatively rudimental chemical communication (Wilson, 1971). These ants are considered polyphagous generalist predators of a variety of arthropods and annelids but seldom feed on honeydew exudates from Homoptera or flower nectar (Fernández, 1991; Del-Claro and Oliveira, 1999; Oliveira and Pie, 1998; Pie, 2004; Schmidt and Morais, 2007). Two species from this genus were observed removing insect cadavers located 5 cm under the soil surface in the field.

The objective of our study was to assess different protective coverings on *G. mellonella* cadavers infected with *H. baujardi* LPP7 for their potential impact on the infectivity and viability of emerging IJs, and on the behavioral response of two ant species of the genus *Ectatomma* toward insect cadavers located near their nest entrances.

2. Materials and methods

2.1. Entomopathogenic nematodes and insect cadavers

IJs of the EPN *H. baujardi* LPP7, isolated originally from the Amazon Forest in Rondonia State, Brazil, were used to infect last instar *G. mellonella* larvae. Larvae had an average weight of 300 mg and were infected in groups of five by the addition of 1000 IJs/ml in a petri dish (90-mm diameter) containing a filter paper disk (Whatman N° 1) on the dish base. Petri dishes were incubated in an incubation chamber at 25 °C and 80% humidity. After 4 days the cadavers were transferred to modified White traps (White, 1927). IJs were collected from traps during 6 consecutive days and stored in distilled water for a maximum period of 1 week at 16 °C before utilization in experiments. Insect cadavers for experiments were obtained from last instar larvae of *G. mellonella* that were infected with EPN as described above.

2.2. Cadaver coverings

The cadaver coverings used were: commercial calcitic calcareum (calcium carbonate 80%, Heringer Co., Paulínia, SP, Brazil), formulated as a powder and as an aqueous suspension (10%); talc (Granado®, Rio de Janeiro, RJ, Brazil) formulated as a powder and as an aqueous suspension (10%), and gelatin capsules. Chemical composition of the talc powder was of 42.3 g talc; 0.4 g orthoxibenzoic acid; 17.6 g sulfur; 3 g zinc oxide; 11.7 g starch and 1.5 g fragrance. Gelatin capsules (100% gelatin, size 000, Pfizer, São Paulo, SP, Brazil) had an average weight of 118 mg. Powdered formulated cadavers were obtained by carefully passing insect cadavers through 10 g of the covering product until a thin powder layer totally covered them. Aqueous formulated cadavers were obtained by immersion for 10 s in a 50 ml beaker containing a suspension of 10% of the covering product in sterilized water.

2.3. Emergence and infectivity of infective juveniles

Plastic cups (200 ml) were filled with 120 g of soil from a commercial guava orchard (58% sand, 23% clay and 19% silt; pH 4.6 and 2.4% organic matter). Insect cadavers with different coverings were placed individually 2 cm under the soil surface. These cadavers were formulated as previously described at 4 and 8 days after infection. Plastic cups were watered at 2 day intervals to maintain the contents as close as possible to field capacity. Twenty days after the experiment began, the extraction of IJs was performed according to the method of Jenkins (1964). Living IJs were differentiated from other juvenile nematode species based on their characteristic locomotion, size and morphology, and counted under a dissecting microscope on a Peters counting slide.

The experiment was performed as a completely randomized design with four repetition in time and 25 replicates for each treatment. Analysis of variance was performed on the data and differences between treatment means were assessed by Tukey's test at 5% probability using the software SAEG (SAEG, 1990).

Infectivity of IJs that emerged from the formulated insect cadavers was also evaluated. Plastic cups (200 ml) were filled with 120 g of soil (as previously described) and one insect cadaver of each formulated treatment 8 days after infection. Twelve days later, IJ infectivity was assessed. Each 120 g of soil was placed in a 1.5 l plastic bucket together with 1.5 kg of soil that previously tested negative for EPNs plus five last instar larvae of *G. mellonella*. Buckets were inverted and kept at laboratory temperature (25 ± 3 °C). The number of dead *G. mellonella* larvae with signs of nematode infection and nematodes present inside the larvae was recorded after 10 days.

The experimental design was completely randomized with 10 replicates for each treatment. The infectivity test was conducted in triplicate. The results were subjected to analysis of variance and treatment means compared by Tukey's test and a significant difference of $P < 0.05$ probability using the software SAEG (SAEG, 1990).

2.4. Observations of Ectatomma spp. behavioral response toward insect cadavers

Observations on two species of *Ectatomma* spp. ants were conducted in a guava orchard in the experimental area at the Universidade Estadual do Norte Fluminense Darcy Ribeiro campus, in Campos dos Goytacazes, Rio de Janeiro State, Brazil. Three nests of each ant species were randomly selected in the experimental area. Insect cadavers infected 8 days previously were formulated with different treatments as previously described. One insect cadaver of each treatment was placed randomly on the soil surface at a 5 cm distance from the nest entrance. The insect cadaver was considered 'removed' whenever ants displaced the cadaver at least 20 cm from the nest entrance. Observations were performed starting at 1700 h on each of five evaluation days. Time spent by ants removing insect cadavers was also recorded.

The experiment was a completely randomized factorial design with three replicates for each treatment. The entire experiment was done four times. Data were analyzed using analysis of variance and means were compared using Tukey's HDS test at $P < 0.05$ probability using the software SAEG (SAEG, 1990).

3. Results and discussion

There were no significant differences among the formulations tested concerning the number of IJs that emerged when cadavers were formulated 4 days after infection ($F = 0.68$; d$f = 5, 144$; $P = 0.6414$). However, insect cadavers formulated 8 days after infection showed significant differences among treatments in IJ emergence ($F = 2.68$; d$f = 5, 144$; $P = 0.0239$; Table 1). This difference suggests that the covering effect becomes evident as cadavers begin to lose hardness due to the process of cadaver degradation. Emergence of IJs observed from insect cadavers formulated with talc powder (9.722 ± 1.382) showed that this covering did not interfere with IJ emergence in the soil and might be especially favorable for emergence, perhaps by preserving cadaver integrity.

Our results differed from those of Shapiro-Ilan et al. (2001) for clay, gluten, lignin and starch coverings on *G. mellonella* larvae infected with *H. bacteriophora* Poinar strain Hb. These authors found greater IJ survival and tolerance to desiccation for insect cadavers formulated 4 days after infection compared with those formulated after 8 days. These differences might be related to differences in the beginning of IJ emergence from insect cadavers infected by these EPN species. Cadavers infected with *H. bacteriophora* have levels of physical damage above 80% 9 days after infection (Shapiro-Ilan et al., 2001), whereas insect cadavers infected with *H. baujardi* LPP7 still have physical integrity 8 days after infection. This allows the use of covering formulations at later periods closer to the time of IJ emergence, which begins12 days after infection (Dolinski et al., 2007).

Calcareum may have negatively influenced the survivorship of IJs due to detrimental effects related to osmotic potential (Andrén and Lagerlöf, 1983). Calcareum formulations applied 8 days after infection negatively affected IJs, perhaps as a consequence of the shorter period between formulation and the beginning of IJ emergence. Lower calcareum concentration around cadavers formulated with the aqueous suspension caused an added increment of emerged IJs. Apparently, the osmotic potential is reduced by dilution and time. Therefore, in field applications the physical–chemical characteristics of the soil must be considered because these might influence the covering used on cadavers (Barbercheck, 1992).

Gelatin capsules protected cadavers without any effect on IJs' emergence, which was not significantly different from the control treatment.

High infectivity of emerged IJs to *G. mellonella* larvae was observed for the different coverings. There were no significant differences in the number of infections produced by the coverings tested ($F = 1.38$; d$f = 5, 18$; $P = 0.2768$) (Fig. 1). These data suggest that the covering treatments might affect the number of IJs emerging, but that there is no effect on pathogenicity.

High numbers of insect cadaver removals were observed when comparing the behavior of *Ectatomma* spp. ants with respect to cadavers with different coverings (Fig. 2). However, there was no significant difference among the treatments, except for the gelatin capsules, which were apparently ignored by *Ectatomma* spp. ants and not removed at all ($F = 5.50$; d$f = 5, 54$; $P = 0.0004$). The average number of insect cadavers removed by *Ectatomma* sp. 1 ranged from 73% to 80%, while those removed by *Ectatomma* sp. 2 ranged from 40% to 67% (Fig. 2).

These results show that both ant species are responsive to the insect cadavers and readily remove them to an average distance of more than 20 cm from the nest entrances. *Ectatomma* sp. 1 showed behavior that is more aggressive in the presence of the insect cadavers and removed them faster and in greater quantities than *Ectatomma* sp. 2. EPN-infected hosts produce volatile compounds and secretions that are released into the environment and might mediate this kind of ant behavior (Grewal et al., 1997; Glazer, 1997).

We considered "removed" as all insect cadavers transported to a distance of at least 20 cm, but a large number of cadavers were transported to a distance of at least 100 cm from the nest, and some were removed more than 400 cm. Some control insect cadavers were transported into the nests, especially by *Ectatomma* sp. 1. This behavior might be a consequence of the polyphagous habit of the species (Schmidt and Morais, 2007). Field experiments by Baur et al. (1998) showed that Linepithema humile (Mayr) ants feed on cadavers infected by steinernematids, but rarely feed on cadavers infected by heterorhabditids. These results are consistent with our present work, and support the conclusion that Heterorhabditis-infected cadavers are not attractive to ants for feeding. It has been suggested that the Photorhabdus bacteria within the cadaver might be responsible for this apparent ant repellency (Zhou et al., 2002).

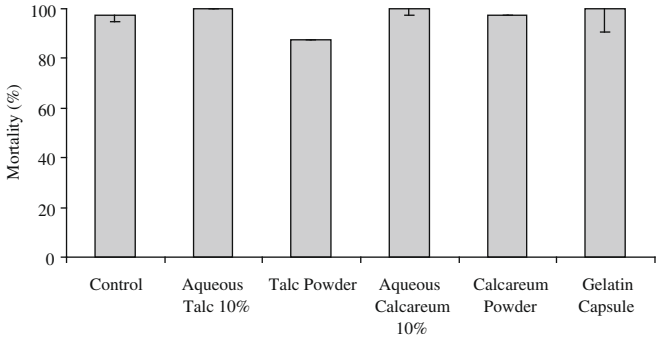

Fig. 1. Average mortality of last instar *Galleria mellonella* larvae used as trap-insects in the soil with previous additions of insect cadavers infected with *Heterorhabditis baujardi* LPP7 and formulated with different coverings 8 days after infection ($n = 30$). Bars are standard errors and values are not statistically different based on Tukey's HSD means separation test at $P < 0.05$.

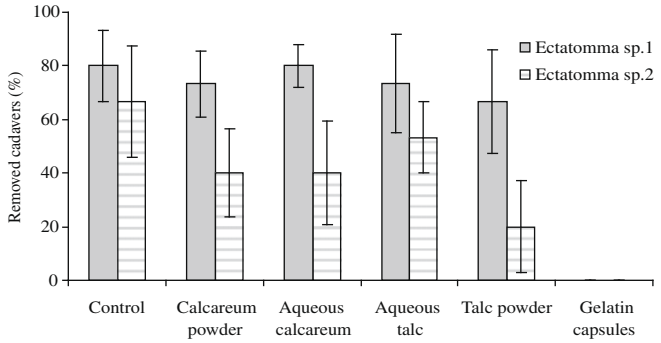

Fig. 2. Average number of insect cadavers formulated with different coverings that were removed by *Ectatomma* spp. ants from the nest vicinity. Cadavers in gelatin capsules were not removed at all. Bars are standard errors, and values are not statistically different based on Tukey's HSD means separation test at $P < 0.05$.

Table 1
Mean number of *Heterorhabditis baujardi* LPP7 infective juveniles that emerged from insect cadavers formulated with different coverings at 4 and 8 days after infection (mean value ± standard error). Values within columns followed by same letter are not statistically different based on Tukey's HSD means separation test at $P < 0.05$.

Covering treatment	4 days		8 days	
Calcareum powder	6.956 ± 1.029	A	4.154 ± 942	a
Aqueous talc 10%	7.100 ± 932	A	6.038 ± 986	ab
Aqueous calcareum 10%	5.359 ± 923	A	6.071 ± 1.189	ab
Control	6.397 ± 964	A	6.346 ± 1.311	ab
Gelatin capsule	7.815 ± 1.189	A	7.892 ± 1.072	ab
Talc powder	6.755 ± 906	A	9.722 ± 1.382	b

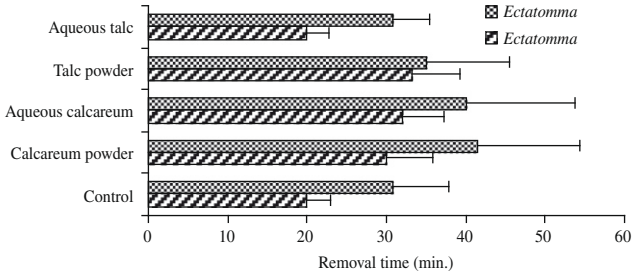

Fig. 3. Time taken by *Ectatomma* spp. to remove formulated insect cadavers from the nest vicinity. Bars are standard errors, and values are not statistically different based on Tukey's HSD means separation test at $P < 0.05$.

Regarding the time taken for cadaver removal, control cadavers (without covering) were removed most rapidly compared to the other treatments (Fig. 3). This shows that the different coverings tested here might effectively reduce ant responses to cadavers and offer some protection for cadavers against ants of this genus.

Our results indicate that gelatin capsules might be an especially practical formulation for insect cadavers containing EPN for use in biological control because they are easily manipulated and applied, effectively prevent damage to the cadaver, and allow for the emergence of a high proportion of highly infective IJs. In general, the behavior of *Ectatomma* spp. ants, especially *Ectatomma* sp. 1, might be problematic for the use of insect cadavers as an EPN application method. We suggest that the application of insect cadavers be avoided in areas with a high population density of these ants or that cadavers used in such areas be formulated in gelatin capsules. However, additional technology to simplify and improve cadaver formulations still needs to be developed.

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