

Recycling Potential and Fitness of Steinernematid Nematodes Cultured in *Curculio caryae* and *Galleria mellonella*

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Abstract: The recycling potential of entomopathogenic nematodes in the pecan weevil, *Curculio caryae*, following inundative applications is an important factor in considering whether nematodes could be incorporated into a *C. caryae* management strategy. Our objective was to determine the recycling potential and fitness of *Steinernema carpocapsae* and *S. riobrave* cultured in *C. caryae*. To estimate fitness and quality, we reared nematodes in larvae of *C. caryae* and in the commonly used standard host, the greater wax moth, *Galleria mellonella*. Nematode lipid content, infectivity (power to invade), virulence (power to kill), and reproductive capacity (yield per insect) in *C. caryae* larvae were compared with *G. mellonella* data. Lipid content was higher in *S. carpocapsae* cultured in *C. caryae* than in *G. mellonella*, but *S. riobrave* lipid content was not affected by host source. Host source did not affect subsequent infectivity or virulence to *C. caryae* ($P > 0.05$) but did affect reproductive capacity ($P < 0.0001$). Both nematode species produced more progeny in *C. caryae* when they were first cultured in *G. mellonella* than when they were first passed through *C. caryae*. In terms of potential to recycle under field conditions, we predict that nematodes resulting from one round of recycling in *C. caryae* larvae would be equally capable of infecting and killing more weevils, but the potential to continue recycling in *C. caryae* would diminish over time due to reduced reproduction in that host.

Key words: *Curculio caryae*, entomopathogenic nematode, fitness, pecan, *Steinernema carpocapsae*, *Steinernema riobrave*.

The pecan weevil, *Curculio caryae*, is a key pest of pecan (Payne and Dutcher, 1985). Adults emerge from soil in late July-August and feed on and females oviposit in nuts (Harris, 1985). Larvae develop within the nut, and fourth instars drop to the ground where they burrow to a depth of 8 to 25 cm, form a soil cell, and overwinter. The following year, approximately 90% of the larvae pupate and spend the next 9 months in the soil cell as adults (Harris, 1985). The remaining 10% of the larval population spend an additional year in the soil as larvae and emerge as adults in the third year (Harris, 1985). Thus, *C. caryae*'s life cycle is usually 2 and sometimes 3 years (Harris, 1985). The bulk of *C. caryae* adults emerge from soil over a 4-to-6-week period usually beginning in mid-August (Harris, 1976); larvae emerge from nuts over several months thereafter (Boethel and Eikenbary, 1979; Harris and Ring, 1979).

Control recommendations for *C. caryae* currently consist of foliar applications of chemical insecticides (e.g., carbaryl) to kill the adults (Harris, 1999; Hudson et al., 2002). Due to environmental and regulatory concerns, research toward developing alternative control strategies is warranted. Entomopathogenic nematodes are one of the potential alternatives (Shapiro-Ilan, 2001a, 2001b, 2003).

Entomopathogenic nematodes are obligate parasites in the families Steinernematidae and Heterorhabditidae. Entomopathogenic nematodes kill insects with the

aid of a mutualistic bacterium, which is carried in their intestine (*Xenorhabdus* spp. and *Photorhabdus* spp. are associated with *Steinernema* spp. and *Heterorhabditis* spp., respectively) (Poinar, 1990). The nematodes complete two to three generations within the host, after which free-living infective juveniles emerge to seek new hosts (Poinar, 1990).

Entomopathogenic nematodes are pathogenic to both larval and adult *C. caryae*. Laboratory and field trials indicate that *Steinernema carpocapsae* and *Steinernema riobrave* are among the most virulent nematodes for *C. caryae* control (Shapiro-Ilan, 2001b; unpubl. data). Greenhouse experiments indicate that applications of *Heterorhabditis bacteriophora*, *S. carpocapsae*, or *S. feltiae* can result in moderate (ca. 35%) larval suppression (Nyczepir et al., 1992; Smith et al., 1993). Adult weevils are more susceptible than larval stages (Shapiro-Ilan, 2001b, 2003). In field trials using *S. carpocapsae*, up to 80% adult *C. caryae* suppression was achieved during the first week post-application, but treatment effects were no longer detectable 2 weeks after application (Shapiro-Ilan et al., unpubl. data). This short-term persistence is problematic because the bulk of *C. caryae* adults emerge from soil over a 4-to-6-week period (Harris, 1976), followed by several months of larval emergence from nuts. Thus, there is a need for extending the period that nematodes provide control, thereby decreasing the need for multiple nematode applications.

Recycling of inundatively applied entomopathogenic nematodes can prolong pest suppression and reduce the need for subsequent applications (Klein and Georgis, 1992; Parkman et al., 1993; Shapiro-Ilan et al., 2002; Shields et al., 1999). Entomopathogenic nematodes have been reported to reproduce in *C. caryae* larvae (Nyczepir et al., 1992) and adults (Shapiro-Ilan, 2001b), and, due to factors such as stability and shading, orchard environments are among those most suitable to nematode recycling (Kaya, 1990). Therefore, research to determine the potential for entomopatho-

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genic nematodes to recycle in *C. caryae* and subsequent impact on population densities in pecan orchards is warranted.

Entomopathogenic nematode quality or fitness can be affected by nutritional factors associated with the host (Abu Hatab et al., 1998; Grewal et al., 1999). For example, Abu Hatab et al. (1998) reported higher total lipid content in *Steinernema glaseri* that were cultured in Japanese beetle, *Popillia japonica* compared with those cultured in greater wax moth larvae, *Galleria mellonella*; lipids are the primary energy source and food reserve for entomopathogenic nematodes and often are used as measures of quality. Abu Hatab and Gaugler (2001) demonstrated that in vitro media supplemented with insect lipids produced nematodes with higher reproductive capacities and faster development times compared with media based on other lipid sources. Thus, in estimating the potential of entomopathogenic nematodes to recycle in and impact subsequent *C. caryae* populations, it is important to not only determine the number of nematodes produced in the target host but also to assess the fitness and quality of the nematodes produced. Our objective was to determine the fitness of *S. carpocapsae* and *S. riobrave* cultured in *C. caryae* larvae vs. *G. mellonella*. To estimate fitness and quality of nematodes reared in the two hosts, we measured infectivity (power to invade), virulence (power to kill), and reproductive capacity (yield per insect) in *C. caryae* larvae. We also measured lipid content in the two hosts and in the nematodes reared in them. *Galleria mellonella* was chosen for comparison because it is the most common host used in laboratory and commercial in vivo culture (Shapiro-Ilan and Gaugler, 2002). Additionally, according to previous studies, the fitness of *S. carpocapsae* and *S. riobrave* reared on *G. mellonella* was found to be similar to the fitness of these species when produced commercially in liquid culture (Gaugler and Georgis, 1991; Shapiro and McCoy, 2000).

MATERIALS AND METHODS

Insect and nematode cultures: *Galleria mellonella* (last instar) were obtained from Webster's Waxie Ranch (Webster, WI). *Curculio caryae* cannot be continuously cultured in the laboratory; therefore, fourth instars were collected from infested nuts at the USDA-ARS Research Station, Byron, Georgia. The larvae were stored in autoclaved soil at 25 °C for 2 weeks (to remove diseased individuals), and remaining larvae were stored up to 5 months in sterile soil at 4 to 10 °C prior to experimentation (Shapiro-Ilan, 2001a). Average (\pm sd) weights of *C. caryae* and *G. mellonella* used were 109 \pm 21 and 215 \pm 38 mg per larva, respectively. Prior to this study, all nematodes, *S. carpocapsae* (All strain, originally obtained from K. Nguyen, University of Florida, Gainesville) and *S. riobrave* (original 355 strain, originally ob-

tained from Thermo Trilogy Inc., Columbia, MD) were routinely cultured in *G. mellonella* and stored at 13 °C according to standard laboratory procedures (Kaya and Stock, 1997).

Subsequently, for the purposes of this study, nematodes (stored less than 2 weeks) were passed once in either *G. mellonella* or *C. caryae*. Approximately 600,000 (for *C. caryae* infection) or 81,000 (for *G. mellonella* infection) infective juveniles (IJ) of either nematode species were applied to 2 liters of soil in plastic tubs (ca. 29 cm \times 18 cm \times 10 cm deep) containing 300 to 500 insects (depending on how many were in the storage bin); a higher inoculation rate was necessary for *C. caryae* to ensure a sufficient infection level for IJ production. Infected insects were placed on White traps, and IJ were collected and stored (for up to 3 weeks) in refrigeration according to Kaya and Stock (1997).

Virulence experiment: Virulence was evaluated based on procedures described by Shapiro-Ilan (2001a). Experimental units were plastic cups (3-cm i.d. on bottom tapering to 4 cm on top, and 3.5 cm deep) (Bioserv Inc., Frenchtown, NJ) filled with oven-dried soil from the USDA-ARS pecan orchard (Byron, GA) and containing one *C. caryae* larva each. The soil was a loamy sand with the percentage sand:silt:clay = 84:10:6, pH = 6.1, and organic matter = 2.8% by weight. Approximately 500 IJ (ca. 40 IJ/cm²) were pipeted onto the soil surface of each cup in 0.5 ml of water so that the final moisture was standardized at field capacity (14%). Treatments included each of the nematode species cultured in *C. caryae* or *G. mellonella* and a nontreated control. *Curculio caryae* mortality was determined after 7 and 14 days of incubation at 25 °C. Analysis of treatment effects on virulence, however, was based on cumulative mortality (at 14 days); 7-day mortality was assessed only to ensure that dead larvae were collected for reproduction experiments before nematode progeny began to emerge (see below). The experiment, arranged in a completely randomized design, contained four replicates of 10 insects (cups) per treatment and was repeated once (the data from the two trials were pooled).

Reproduction experiment: Insect cadavers showing signs of nematode infection (Kaya and Stock, 1997) were collected at 7 and 14 days from virulence experiments and placed on modified White traps (at 25 °C) to estimate reproduction; the number of IJ in the trap was estimated using dilution counts after emergence had appeared to cease (a minimum of 30 days post-infection). The number of replicates (infected weevils) obtained for reproduction from the virulence experiment was low. Our goal was to obtain at least 10 replicates/treatment, but this was not achieved. Therefore, additional insects were infected to bolster the number of replicates in the reproduction experiment. Approximately 10,000 IJ of either species were pipeted onto soil (ca. 4 mm deep and at 14% moisture) in petri dishes

(90 mm) containing 20 *C. caryae* larvae. Weevil mortality was checked 5, 7, and 10 days after inoculation. Dead insects exhibiting signs of nematode infection were placed on White traps along those from the virulence experiment, and reproduction was assessed as described above (data were combined; no effect of infection method on reproduction was detected, $P = 0.75$). In total there were 14 replicates (infected insects) per treatment: 4 from the first inoculation method and 10 from the second.

Infectivity experiment: Experimental units were soil cups as described above for the virulence experiment, but filled with only 10 g of soil. For each of the four nematode treatments 1,000 IJ were applied to the soil cups in 0.5 ml tap water. After 3 days insects were removed and the number of IJ that had invaded was estimated using the pepsin digestion method (Mauléon et al., 1993). There were 10 replicates per treatment, and the experiment was repeated once (the data from the two trials were pooled).

Lipid analysis: Total percentage lipids in insects (*C. caryae* and *G. mellonella* larvae) and nematodes (ca. 2.5 g wet weight of *S. carpocapsae* or *S. riobrave* IJ from each of the insects) were extracted with chloroform/methanol (2:1, v/v) 3 times and washed with 1% NaCl (saturated with chloroform). Dry weight of nonextractable materials was determined by placing the combined material in an oven overnight at 105 °C (Abu Hatab and Gaugler, 2001; Folch et al., 1957). Sterols were obtained using 0.25 g total lipids through extraction with ether, elutriation in an aluminum oxide column, and evaporation in a rotary evaporator. Sterols were dissolved in hexane and weight was determined after drying under a stream of N₂ gas (Abu Hatab and Gaugler, 2001; Goodnight and Kircher, 1971).

Statistical analysis: Treatment effects in virulence, reproduction, and infectivity experiments were detected through analysis of variance. If the *F* test was significant ($\alpha = 0.05$), treatment differences were further elucidated through the LSD test using SAS software (SAS Institute, Cary, NC). Prior to analysis, percentage data (mortality) were arcsine of square root transformed, and count data (reproduction and infectivity) were square-root transformed (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

The host that nematodes were cultured in (*C. caryae* vs. *G. mellonella*) did not affect subsequent virulence or infectivity to *C. caryae* larvae. All of the nematode treatments caused greater larval mortality in *C. caryae* than was observed in the control, but no differences were detected among these treatments (Fig. 1) ($F = 5.24$; $df = 4, 70$; $P = 0.0009$). Further, no differences in number of nematodes infecting *C. caryae* were detected among *S. riobrave* and *S. carpocapsae* cultured in *C. caryae* or *G. mellonella* (Fig. 2) ($F = 1.4$; $df = 3, 76$; $P = 2.50$). The

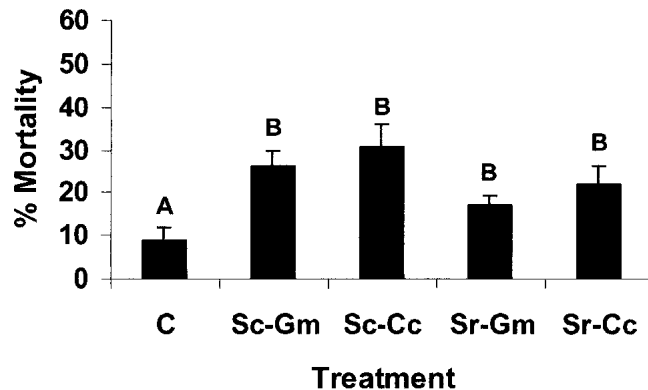


FIG. 1. Mortality of *Curculio caryae* larvae following exposure to nematodes: Sc = *Steinernema carpocapsae* (Sc); Sr = *S. riobrave*. Prior to exposure the nematodes were cultured either in *Galleria mellonella* (Gm) or *C. caryae* (Cc). C = Control. Bars with the same letter above are not significantly different ($P \leq 0.05$, LSD test).

relatively low levels of nematode-induced mortality observed in this study are similar to levels observed in previous laboratory experiments using the same application rate (Shapiro-Ilan, 2001a).

In contrast to virulence and infectivity, host source had a substantial effect on the nematode's subsequent reproductive capacity. For both *S. carpocapsae* and *S. riobrave*, more progeny were produced in *C. caryae* larvae from nematodes previously cultured in *G. mellonella* than nematodes cultured in *C. caryae* (Fig. 3) ($F = 23.04$; $df = 3, 72$; $P < 0.0001$). *Steinernema riobrave* cultured in *G. mellonella* had the highest reproductive capacity, followed by *S. riobrave* cultured in *C. caryae* and *S. carpocapsae* cultured in *G. mellonella* (which were not different from each other). The lowest reproduction was observed in *S. carpocapsae* cultured in *C. caryae* (Fig. 3).

The cause(s) for differential reproductive potential is not clear. Genetic processes such as selection can cause changes in nematode's beneficial traits (Gaugler et al., 1989; Stuart and Gaugler, 1996), but selection can essentially be ruled out as a factor, which explains our results because it is unlikely a single passage in different

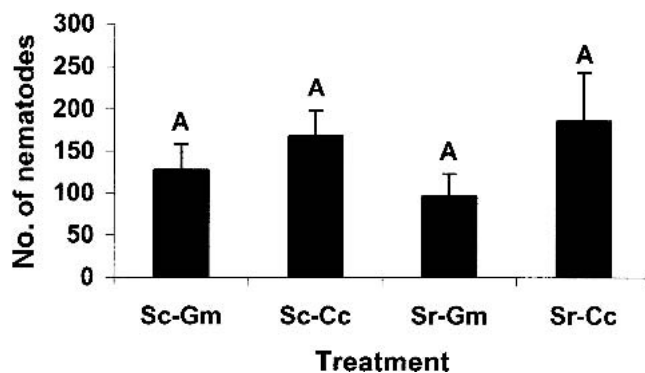


FIG. 2. Number of nematodes invading *Curculio caryae* larvae. Sc = *Steinernema carpocapsae*; Sr = *S. riobrave*. Prior to exposure to *C. caryae*, the nematodes were cultured either in *Galleria mellonella* (Gm) or *C. caryae* (Cc). Bars with the same letter above are not significantly different ($P \leq 0.05$, LSD test).

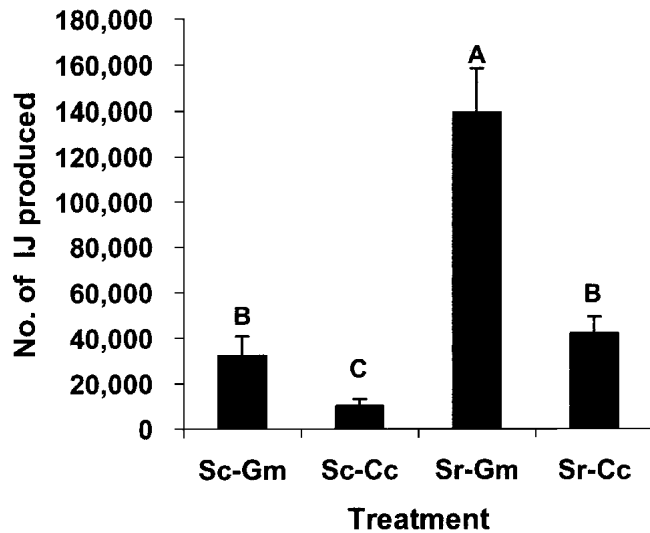


FIG. 3. Number of nematodes exiting *Curculio caryae* infected larvae. Sc = *Steinernema carpocapsae*; Sr = *S. riobrave*. Prior to exposure to *C. caryae*, the nematodes were cultured either in *Galleria mellonella* (Gm) or *C. caryae* (Cc). Bars with the same letter above are not significantly different ($P \leq 0.05$, LSD test).

hosts would be sufficient to cause genetic selection. Moreover, one would expect to have observed the opposite trend, i.e., passage in *C. caryae* would lead to greater reproduction in *C. caryae*. Nutritional differences between *C. caryae* and *G. mellonella* are a more likely explanation for the observed differences in nematode reproductive potential. The nutritional makeup of artificial media can affect nematode fitness characters such as yield or efficacy (Abu Hatab and Gaugler, 2001; Gaugler and Georgis, 1991; Han et al., 1992). Further, Grewal et al. (1999) reported reduced virulence in *S. scapterisci* when the nematode was cultured in a non-preferred host (the house cricket, *Acheta domesticus*). Similarly, poor nutritional compatibility in *C. caryae* larvae may have contributed to subsequent reduced reproductive success in *S. carpocapsae* and *S. riobrave*.

Insect hosts also can carry certain plant-derived toxins that are detrimental to entomopathogenic nematodes. For example, cucurbitacins (secondary compounds produced by many plants in the Cucurbitaceae), which can be carried in insect hosts, reduce entomopathogenic nematode reproduction, infectivity, and bacterial growth (Barbercheck and Wang, 1996; Barbercheck et al., 1995). Additionally, Kunkel et al. (2004) reported that alkaloids derived from endophytic fungi (and potentially sequestered by insect hosts) were detrimental to *S. carpocapsae* and their bacterial symbionts. Conceivably, *C. caryae* could sequester secondary plant compounds that contributed to the reduced nematode reproduction observed in our experiments. Pecan produces various defensive compounds such as juglone (Borajani et al., 1985; Gueldner et al., 1994).

Curculio caryae larvae contained slightly higher total percentage lipids compared with *G. mellonella* larvae (Table 1). The higher lipid content might have been

TABLE 1. Percentage total lipid and sterol content.

Treatment	% Total lipids	% Sterols
Sc from Gm	48.80	7.55
Sc from Cc	84.40	6.60
Sr from Gm	55.66	8.50
Sr from Cc	56.73	5.10
Gm (insect)	71.30	10.37
Cc (insect)	80.47	8.96

Percentage of nematode dry weight. Nematodes *Steinernema carpocapsae* (Sc) or *S. riobrave* (Sr) were cultured in either *Galleria mellonella* (Gm) or *Curculio caryae* (Cc).

expected because *C. caryae* larvae have a higher caloric content than various other arthropods (Dutcher, 1983). One may also expect that lipid content in the nematodes would reflect that of its host, e.g., as observed by Abu Hatab et al. (1998) when comparing lipid content of *S. glaseri* cultured in *P. japonica* vs. *G. mellonella*. Indeed, in our study *S. carpocapsae* cultured in *C. caryae* contained substantially more total lipids than *S. carpocapsae* cultured in *G. mellonella* (although sterol content did not follow that trend) (Table 1). *Steinernema riobrave* did not, however, follow this trend; percentage lipid content was similar for the nematodes cultured on the two hosts (Table 1).

We did not observe any advantage in nematodes with higher lipid content. In fact, the *S. carpocapsae* with higher lipid content (from *C. caryae*) exhibited reduced reproduction. Similarly, Barbercheck et al. (2002) also observed an inverse relationship between lipid content and reproduction. However, lipid contents in *S. riobrave* did not support this trend in reproduction. Persistence of entomopathogenic nematodes is associated with lipid content (Gaugler and Georgis, 1991; Grewal and Georgis, 1999) and may be the trait most likely affected. Future studies are needed to determine if nematodes (e.g., *S. carpocapsae*) reproduced in *C. caryae* persist longer than nematodes cultured in other hosts.

Based on the number of IJ produced per *C. caryae* larva, we can speculate on the potential for recycling under field conditions. The question is whether the number of infected larvae resulting from an entomopathogenic nematode application would produce sufficient IJ to provide additional pest suppression. In general, a minimum of 2.5×10^9 IJ/ha are required to provide pest suppression under field conditions (Shapiro-Ilan et al., 2002), but a pecan orchard would not require complete coverage because the weevils occur only under the tree canopy. Thus, we might speculate that approximately one-fourth of the standard rate, i.e., 625 million IJ, would be a minimum requirement for significant *C. caryae* suppression. Maximum average reproduction in *C. caryae* larvae was approximately 32,000 IJ for *S. carpocapsae* and 140,000 IJ for *S. riobrave* (Fig. 3). If we consider that the economic threshold of adult weevils is approximately 500 to 3500 per ha depending on quality and value of the nuts, and that each female

produces approximately 75 larvae each (Harris et al., 1981), then we might expect a range of up to 18,750 to 131,250 larvae/ha in managed orchards. If (based on our lab study) we can expect that at least 20% of the larvae would become infected following nematode application, then either of the nematode species could conceivably infect enough weevils to produce moderate additions in pest suppression resulting from recycling (e.g., 131,250 larvae \times 0.20 infected \times 32,000 IJ per larva). However, the number of IJ actually produced per infected larva under field conditions could be hampered by various abiotic (such as temperature and humidity) (Brown and Gaugler, 1997) and biotic factors (such as microbial antagonists) (Kaya, 2002). The potential for nematode recycling and resulting *C. caryae* suppression would have to be verified through field studies.

In summary, we observed no effects of host source on virulence or infectivity, but nematodes cultured in *G. mellonella* subsequently reproduced more in *C. caryae* than nematodes reared in *C. caryae*. Thus, we can expect that nematodes resulting from one round of recycling in *C. caryae* larvae would be equally capable of infecting and killing more weevils but the potential to continue recycling in *C. caryae* would diminish over time due to reduced reproduction in that host.

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