Laboratory Studies of the Interactions of Environmental Conditions on the Susceptibility of Green June Beetle (Coleoptera: Scarabaeidae) Grubs to Entomopathogenic Nematodes[1]

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Abstract In the laboratory, four nematode species differed significantly in the percentage mortality that each caused in second-instar green June beetles (grubs), *Cotinis nitida* (L.). Water suspensions of 1,000 infective juveniles (IJ)/one grub/Petri dish each of *Heterorhabditis bacteriophora, Steinernema glaseri,* and *S. feltiae* caused 34, 22 and 18% grub mortality, respectively. These values were significantly ($P = 0.05$) greater than the grub mortality caused by either *S. carpocapsae* (12%) or the water check (0%) that were similar. Several factors affected third- and fourth-instar susceptibility in the laboratory when exposed to 10,000 *S. carpocapsae* or *H. bacteriophora* IJ in 75 g of soil (133 IJ/cm^3 soil)/plastic cup. Soil at 30% moisture by weight resulted in significantly more ($P = 0.006$) dead grubs (6.6%) than at 10% soil moisture (2.8%). Significantly more ($P = 0.03$) grubs died when at a grub density of 2 grubs/plastic cup (5.6%) than at 1 grub/plastic cup (3.8%). Significantly more ($P = 0.01$) grubs were killed by either *S. carpocapsae* and *H. bacteriophora* at a soil temperature of 25°C (6.5%) than at 12°C (2.8%). There was also a significant interaction of temperature and nematode species. *Heterorhabditis bacteriophora* killed significantly more ($P = 0.02$) grubs at 25°C (9.5%) than at 12°C (1.9%) and more than *S. carpocapsae* killed at either 12°C or 25°C (<3.9%). More virulent nematode strains or species than these four nematode species must be identified if green June beetles are to be controlled by nematodes.

Key Words *Cotinis nitida,* Scarabaeidae, nematode, entomopathogen, pest, biological control, *Steinernema glaseri, Steinernema feltiae, Steinernema carpocapsae, Heterorhabditis bacteriophora*

The green June beetle, *Cotinis nitida* (L.), is a pest in the southeastern United States (Ritcher 1945, Potter et al. 1991). Adult beetles emerge from pasture and turf in late June and July, mate, and oviposit as deep as 25 cm in soil beneath decomposed manure or haystacks. Larvae spend 10 months in the soil. During the day, larvae (grubs) inhabit deep vertical burrows (15 to 30 cm). Grubs feed at night at the soil surface on decaying organic matter such as green manure, and decaying straw. Adult green June beetles feed in aggregations and damage the fruit of apples, apricots, brambles, figs, grapes, peaches, and pears (Johnson et al. 1987, Domek and Johnson 1987, Tashiro 1987).

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Management practices vary for control for green June beetles. By the mid-1900's, fertilization practices in turf and row crops changed from the use of green manure to artificial fertilizers because of green June beetle grub infestations. Grubs burrowing near the surface often uproots and kills grass, young corn, oats, sorghum, and alfalfa in manured plantings (Davis and Luginbill 1921, Chittenden and Fink 1922, Miner 1951, Tashiro 1987, Potter et al. 1996). Insecticides are applied to ripe fruit to control green June beetle adults or to pastures to control the grubs. However, spraying ripe fruit results in a visible residue that is undesirable to consumers (Dominick 1950, Johnson and Mayes 1982). Chemical management of scarab grubs in turf is often ineffective because of the difficulty of moving insecticide residues through the thatch and into the root zone (Potter et al. 1996). Typically, green June beetle grubs are easily killed with carbaryl (D. T. J., unpubl. data). Broadcast applications of insecticide to pastures is not cost effective and some insecticides have either been banned or are not registered for use (Vittum 1985, Kard and Hain 1986). The public has a growing concern about pesticide safety, so crop managers search for an alternative to insecticides. Entomopathogenic nematodes have been used with some success in biological control of white grubs in turf and pastures (Forschler and Gardner 1991, Potter et al. 1996).

The green June beetle grub has several natural enemies, but none have been tested for grub control (Chittenden and Fink 1922, Davis and Luginbill 1921). Dutky (1941) reported that milky disease, caused by *Bacillus popilliae* Dutky, would not infect green June beetle grubs. Recent unpublished studies showed that green June beetle grubs were not infected by *B. popilliae, B. thuringiensis* Berliner var. *tenebrionis,* or *Beauveria bassiana* (Balsamo) Vuillemin (D. C. S. and D. T. J., unpubl. data). Townsend et al. (1994) killed >45% of third- and fourth-instar green June beetle grubs with a peroral injection of 1,000 infective juvenile (IJ)/grub of each of three steinernematid nematodes, e.g., *Steinernema carpocapsae* (Weiser) (All strain), *S. glaseri* (Steiner), *S. feltiae* (Filipjev) (NC strain), and one heterorhabditid nematode *H. bacteriophora* (=*heliothidis*) Poinar (HP88 strain).

Tests of nematodes against other scarabaeid grubs have yielded varying results. It was reported that nematode species differed in their activity against white grubs because of their host-finding behavior (Poinar 1990, Campbell and Gaugler 1993). Also, several environmental factors (e.g., temperature, soil moisture, inoculum rate, host defense, and host density) affect the susceptibility of white grubs to nematodes (Greany et al. 1977, Gray and Johnson 1983, Zervos et al. 1991, Kaya et al. 1993, Selvan et al. 1994, Wang et al. 1994, 1995, Koppenhofer and Kaya 1995).

Two laboratory studies were conducted: to determine the susceptibility of green June beetle grubs to water suspensions of each of four entomopathogenic nematode species (*S. carpocapsae, S. glaseri, S. feltiae,* and *H. bacteriophora*); and to identify the effects of nematode species, grub density, soil moisture, soil temperature, and their interactions on the susceptibility of green June beetle grubs to *H. bacteriophora* and to *S. carpocapsae.*

Materials and Methods

Insect rearing. Adult beetles were collected from infested grape clusters from several vineyards in White Co., AR, during June and July of 1991 and 1992. Adult beetles were allowed to oviposit in 19-liter glass aquaria that contained heat-sterilized soil (3 h at 93°C) at 16 to 18 cm depth. These aquaria were held in an outdoor

insectary at the Arkansas Agricultural Research and Extension Center in Fayetteville, AR. After oviposition, beetle eggs were sifted from the soil, and placed in aquaria containing sifted sterile soil, and held in the laboratory at 20 to 28°C with a 14L:10D photoperiod. The soil was analyzed and described as a silty loam (23.4% sand, 63.1% silt, 13.5% clay) by the University of Arkansas Soil Characterization Laboratory in Fayetteville. Soil was sifted periodically to remove second and third instars. Perforated plastic boxes (40 × 60 × 18 cm deep) were lined with fiberglass window screen and filled with sterilized soil to a depth of 14 to 16 cm. The screen prevented grubs from crawling out of the perforations and allowed for ventilation. A maximum density of 50 grubs/box, which equated to 1.16 grubs/1,000 cm^3 of soil, insured that few grubs wounded others and minimized disease. The soil surface was watered daily to keep the soil moist. Approximately 200 ml of ground alfalfa meal was added weekly to the soil surface as a food source. These boxes were placed in an insectary, and the temperature was maintained above 4°C using a thermostatically-regulated heating system during the winter months.

Nematode cultures. Stock cultures of *S. carpocapsae* and *H. bacteriophora* were obtained from biosys (Palo Alto, CA). Stock cultures of *S. glaseri* and *S. feltiae* were provided by G. Thurston at the University of California-Davis. These nematode strains were the same as used by Townsend et al. (1994).

Nematodes were cultured using large *Galleria mellonella* (L.) larvae as hosts as described as Woodring and Kaya (1988). Each nematode species was propagated by inoculating lots of 10 large wax moth larvae in a Petri dish with a 1-ml suspension containing 200 nematodes (20 nematodes/larva). White traps, as described in Woodring and Kaya (1988) and Townsend et al. (1994), were used to collect the nematode progeny for use in experiments. *Heterorhabditis bacteriophora* were stored in tissue culture flasks at room temperature, while the *Steinernema* spp. were held at 4°C. Estimates of the number of IJ/ml in each stored nematode suspension were determined by the serial dilution method of Woodring and Kaya (1988).

Petri dish bioassay. Assays were conducted with wax moth larvae to confirm the infectivity of all four nematode species. Ten wax moth larvae were placed in a 10 × 1.5 cm Petri dish lined with moistened 9-cm Whatman #1 filter paper and no soil. Wax moth larvae were inoculated with a 1-ml suspension of 200 nematodes from the same batch as used in the green June beetle tests. Concurrent with the wax moth assay, we ran each of five replications using green June beetle grubs. Each replicate consisted of ten second-instar green June beetle grubs placed individually in each of ten 10 × 1.5 cm glass Petri dishes lined with 9.0 cm Whatman #1 filter paper but no soil. The four treatments included *S. carpocapsae, S. feltiae, S. glaseri,* and *H. bacteriophora.* A single dose of 1,000 IJ/grub of one of the four species of nematodes was added in 1 ml of deionized water to each Petri dish. Grubs were held for 3 d at 26-30°C with a 14L:10D photoperiod. Mortality was assessed daily for both wax moth larvae and green June beetle grubs. A larva or grub was noted as dead only if the legs did not move after being probed with an insect pin.

Soil environmental effects. In the laboratory, we evaluated the effect of grub density, soil environment (e.g., moisture and temperature), and the their interactions on the susceptibility of third- and fourth-instar green June beetle grubs to *S. carpocapsae* and *H. bacteriophora.* Approximately a 4-cm depth of soil (75 g or 100 ml of soil) was placed in 148 ml polystyrene cups (Northwest Arkansas Paper Co., Springdale, AR). The cups were covered by plastic lids pierced with small air holes to maintain moisture in the cups during the assay period. For 2 wk prior to the addition

of the nematodes, a density of 1 or 2 grubs/plastic cup (equates to 10 or 20 grubs/1,000 cm^3 of soil) were held in temperature chambers set at either 12°C or 25°C with a 14L:10D photoperiod. A 1-ml deionized water suspension containing 10,000 IJ (24 h old) was added to the soil in each plastic cup. Additional water was added to the soil in each cup to total either 7.5 or 22.5 ml/cup, equating to 10% or 30% soil moisture by weight, respectively. No more water was added to the cups during the assay period.

Either 1 or 2 grubs/plastic cup of soil were used to assess the effect of green June beetle grub density on the susceptibility of the grubs to entomopathogenic nematodes. Ten plastic cups were used per treatment replicate (5 replications/treatment). Treatment cups were placed in the appropriate temperature chamber and held for 2 wk, then grub mortality was assessed as above. Grubs were fed 5 g of ground alfalfa meal weekly. No moisture was added to the treated plastic cups during the experimental period. Dead green June beetle grubs were removed and placed in White traps.

Data analysis. Data in the Petri dish liquid suspension bioassay were evaluated by analysis of variance with raw or arcsin √(proportion dead) transformed means separated using least-significant difference (LSD, $P = 0.05$) (SAS Institute 1996). Transformed means produced similar results, so raw data were presented in Table 1 and in the text. Data from the soil study were evaluated as a 2^4 factorial design in a completely randomized experimental design with five replicates. The main factors analyzed included temperature, soil moisture, grub density and nematode species and all possible interactions. When appropriate, PROC GLM with LSMEANS (LSD, $P = 0.05$) as used to determine the statistical differences among arcsin √(proportion dead) transformed treatment means (SAS Institute 1996). The actual mean percent mortality values are presented in the text and in Table 3 along with the transformed letters of significance.

Results and Discussion

All four entompathogenic nematode species cultured for use in these tests were viable and infective. Simultaneous inoculations of wax moth larvae in Petri dish bioassays resulted in 100% mortality within 48 h.

In the laboratory, the susceptibility of second-instar green June beetle grubs varied ($P = 0.002$) among four entomogenous nematode species but the overall mortality was low (<35%). Grubs in Petri dishes exposed to a liquid suspension of each of four nematode species for a 72 h period experienced the following mortality: *H. bacteriophora* killed 34%, *S. glaseri* (22%), *S.* feltiae (18%), or *S. carpocapsae* (12%). Only *H. bacteriophora* caused significantly more ($F = 3.05$; df = 4, 19; $P < 0.05$) grub mortality than did *S. carpocapsae,* the latter equaled the water check (Table 1).

In the laboratory, several factor affected green June beetle grub mortality when exposed to 10,000 *S. carpocapsae* or *H. bacteriophora* IJ in 75 g of soil (133 IJ/cm^3 soil)/plastic cup (Table 2). Soil at 30% moisture by weight killed significantly more ($P = 0.006$) grubs (6.6%) than at 10% soil moisture (2.8%). Significantly more ($P = 0.03$) grubs died at a grub density of 2 grubs/plastic cup of soil (5.6%) than at 1 grub/plastic cup of soil (3.8%). Significantly more ($P = 0.01$) third- and fourth-instar green June beetles were killed by either *S. carpocapsae* and *H. bacteriophora* at a soil temperature of 25°C (6.5%) than at 12°C (2.8%) (Table 3). There was also a significant interaction of temperature × nematode species ($P = 0.02$). *H. bacteriophora* killed

Table 1. Mean percentage mortality of second-instar green June beetles exposed to a liquid suspension of each of four species of entomopathogenic nematodes in the laboratory

Nematode	Mean (±SEM) % mortality
Heterorhabditis bacteriophora	34 ± 5.1a
Steinernema glaseri	22 ± 6.6ab
Steinernema feltiae	18 ± 6.6ab
Steinernema carpocapsae	12 ± 5.8bc
Control	0c

Means followed by the same letter were not significantly different (LSD = 0.05, df = 4, 20).

Table 2. Factors that significantly effected green June beetle grub mortality relative to two species of entomopathogenic nematodes in soil in the laboratory

Source	MS*	F	Pr > F
Soil moisture by weight (df = 1)	570	7.93	0.006
Density of grubs (df = 1)	374	5.19	0.03
Temperature of soil (T) (df = 1)	478	6.65	0.01
T × Nematode species (df = 2)	307.4	4.27	0.02

* MSE (df = 74) was the total of MS values for all non-significant factors.

significantly more grubs at 25°C (9.5%) than at 12°C (1.9%) and more than *S. carpocapsae* killed at either 12°C or 25°C (all <3.9%) (Table 3).

The lower grub mortality caused by *S. carpocapsae* compared to the other three species may be attributed to species differences in host finding behavior (Table 1). Yeh and Alm (1992) applied 969 IJ/grub of *H. bacteriophora, S. glaseri, S. feltiae,* and *S. carpocapsae* (All strain) to soil, and caused 86.7, 85.6, 32.8, and 28.9% mortality of Japanese beetle grubs, respectively. Poinar (1990) and Campbell and Gaugler (1993) noted that *S. carpocapsae* IJ lays in wait for a host; whereas, the larger nematode species, *H. bacteriophora, S. glaseri,* and *S. feltiae,* each actively search the substrate for hosts.

Green June beetle grub defense mechanisms appear to play a role in minimizing their susceptibility to or survival of these four nematode species. This was noted by differences in efficacy due to the method of grub exposure to nematodes. Peroral injections of 1000 IJ/third- and fourth-instar green June beetles caused the greatest grub mortality, e.g., *S. carpocapsae* (65%), *S. glaseri* (65%), *H. bacteriophora* (63%), and *S. feltiae* (45%) (Townsend et al. 1994). In our study, a liquid suspension of any one of these four entomopathogenic nematodes killed less than 35% of second-instar green June beetles (Table 1). In the best natural soil environment, only 15% of third- and fourth-instar grubs died when held at a density of two grubs/plastic cup and

Table 3. Factors affecting the percent mortality of third- and fourth instar green June beetles in soil in the laboratory

Source	Mean % mortality	
Main effects*		
Soil temperature	12°C	25°C
	2.8b	6.5a
Soil moisture	10% moisture	30% moisture
	2.8b	6.6a
Grub density	1 grub/cup	2 grubs/cup
	3.8b	5.6a
Interactions**	12°F	25°F
Steinernema carpocapsae	3.8b	3.5b
Heterorhabditis bacteriophora	1.9b	9.5a

Percent mortality corrected by Abbott's (1925) formula.

* Main effects, mean values in the same row followed by the same letter are not significantly different (LSD = 0.05, LSMEANS ± 0.85 SEM).

** Interactions, any paired combination of the four mean values followed by the same letter are not significantly different (LSD = 0.05, LSMEANS ± 1.2 SEM).

exposed to *H. bacteriophora* in soil held at 25°C and 30% soil moisture (Table 3). Japanese beetle grubs were reported to use grooming and encapsulation to inhibit the penetration and survival of *S. glaseri* and *H. bacteriophora* (Wang et al. 1995). Wang et al. (1995) increased the number of penetrating nematodes of *H. bacteriophora* from 0.1 to 1.2% and *S. glaseri* from 1 to 53% by restraining Japanese beetle grubs. Japanese beetle grubs strongly encapsulated or melanized *H. bacteriophora* and *S. carpocapsae,* but not *S. glaseri,* when injected into the hemocoel. The survival rate of *H. bacteriophora* injected into the midgut and hemocoel fluid of Japanese beetle grubs was less than that of *S. glaseri,* but Japanese beetle grubs died after 8 and 24 h exposures to *H. bacteriophora* and *S. glaseri,* respectively. In our studies, we have not determined how the green June beetle grub gut or hemocoel interacts with each of these four nematode species.

Steinhaus (1958) stated that crowding appeared to change the metabolic activity of an insect, which could make the insect more susceptible to infectious agents. In Table 2, significantly more green June beetle grubs were killed by either *H. bacteriophora* or *S. carpocapsae* at a grub density of 2/plastic cup (equates to 20 grubs/1,000 cm^3 of soil) than at 1/plastic cup of soil (Table 2). Crowded grubs tend to be more active and inflict biting wounds on each other at densities in excess of 1 larva/1,000 cm^3 of soil in the rearing box (D. T. J., unpubl. data). This increased grub

activity and wounding could result in increased encounters with nematodes and nematode invasion.

Environmental conditions are important factors in both the success of the nematode infection and increased susceptibility of the host insect to nematodes or other pathogens. As in our study, changes in environmental factors may cause a significant change in the susceptibility of an insect to a pathogen or natural enemy. Other studies have shown the importance of the effect of environment on the success of a pathogen to invade and colonize a host (Donegan and Lighthart 1989, Greany et al. 1977, Lighthart et al. 1988, Steinhaus 1960). Gray and Johnson (1983) noted a reduction in the survival rate of *S. carpocapsae* in soil with less than 20% moisture. This may explain the lower grub mortality by nematodes in our study in soil held at 10% versus 30% moisture (Table 3). Applying this to the field, Selvan et al. (1994) and Yeh and Alm (1995) successfully killed Japanese beetle grubs with a daytime application of *S. glaseri* to soil, followed by either an immediate 1.32 cm water rinse of the treated grass surface or daily applications of 3.2 mm of water for 14 d, respectively. However, Gaugler and Kaya (1990) indicated that limited oxygen supplies and higher nematode metabolic activity occurred in saturated soil, which may result in lower levels of survival and pathogenicity for the nematode.

Temperature also significantly affected the green June beetle grub mortality as did temperature × nematode species interaction (Table 2). *Heterorhabditis bacteriophora* killed significantly more grubs at 25°C (9.5%) than at 12°C (1.9%); whereas *S. carpocapsae* killed similar numbers of grubs at both soil temperatures (Table 3). Kaya et al. (1993) reported that at 22°C, both *S. carpocapsae* and *H. bacteriophora* killed their respective target species; whereas, at 16°C, only *S. carpocapsae* was effective. Other reports indicate that heterorhabditid species were tropical in origin and performed better at higher temperatures than did the temperature steinernematids (Kung et al. 1991, Molyneux 1986, Woodring and Kaya 1988).

Our results indicated that second- through fourth-instar green June beetles were not easily killed by these four entomopathogenic nematodes. Thus, we must identify nematode species or strains with higher infectivity if we are to achieve practical control of green June beetles.

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