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Comparative dispersal and larvicidal activity of exotic and Azorean isolates of entomopathogenic nematodes against *Popillia japonica* (Coleoptera: Scarabaeidae)

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Abstract. The Japanese beetle, *Popillia japonica* Newman, is an introduced pest on Terceira, one of nine islands in the Azorean Archipelago. Research conducted on Terceira indicates that entomopathogenic nematodes in the families Steinernematidae and Heterorhabditidae provide good to excellent control of Japanese beetle larvae, but the species that have been evaluated are not native to the Azores. An efficacious species that is native to the archipelago might provide increased capabilities for persisting and recycling in Azorean soil and weather conditions. Surveys on the islands of Terceira and Santa Maria resulted in the isolation of two *Heterorhabditis* strains (São Mateus and Praia Formosa) with good larvicidal activity for *P. japonica.* Initial bioassays conducted with *Steinernema glaseri* (Steiner) originally from North Carolina against *P. japonica* third instar larvae and pupae produced LC_{50} values of 3.2×10^5 infective juveniles (IJs)/m^2 and 0.9×10^5 IJs/m^2, respectively. Comparative bioassays of the native isolates and *S. glaseri* against *P. japonica* revealed similar larvicidal activity. The LC_{50}s of the São Mateus and Praia Formosa isolates against third instar larvae were 3.64×10^5 and 4.44×10^5 IJs/m^2, respectively. The LC_{50} of *S. glaseri* ranged from 3.2 to 5.5×10^5 IJs/m^2. The higher larvicidal activity of the Azorean *Heterorhabditis* isolates for *P. japonica* indicates that native nematodes are as effective as *S. glaseri.* Heterorhabditid species also have demonstrated ability for persistence and apparent recycling under conditions where sustainable control of this introduced pest is needed. Studies comparing the dispersal behavior of the *Heterorhabditis bacteriophora* Poinar São Mateus isolate with that of *S. glaseri* and native and exotic strains of *Steinernema carpocapsae* (Weiser) revealed that the *H. bacteriophora* isolate demonstrated a greater propensity to disperse than other strains in the presence or absence of *P. japonica* larvae. In the presence of a host, a greater proportion of *H. bacteriophora* and *S. glaseri* dispersed than either of the two *S. carpocapsae* strains.

INTRODUCTION

The Japanese beetle, *Popillia japonica* Newman, is an introduced pest on Terceira Island (Azores, Portugal). Adults are present from June until October and are most abundant in July and August (Simões & Martins, 1985). The larvae and pupae inhabit the soil of pastures and lawns for the remainder of the year. Since its accidental introduction in the early 1970s into Terceira, it has spread to virtually every part of the island (Martins et al., 1988). Attempts to control the beetle with chemical insecticides have provided only temporary local suppression. Other sustainable methods for the long-term control of the beetle are needed. Biological control through the use of parasites and pathogens of *P. japonica* offers significant potential for sustainable control on an island habitat (Martins & Simões, 1988; Lacey et al., 1994). Entomopathogenic nematodes in the families Steinernematidae and Heterorhabditidae offer excellent potential for control of insects in soil habitats. (Klein et al., 2000). Research conducted on Terceira (Simões et al., 1993; Lacey et al., 1994) and in the USA (Klein & Georgis, 1992) indicates that entomopathogenic nematodes provide effective control of Japanese beetle larvae and, under certain conditions, persist and recycle in the host population. The nematode species that have been previously evaluated on Terceira were isolated from areas outside of the Azores. The most promising of these, *Steinernema glaseri* (Steiner), was originally isolated from Japanese beetle larvae in the United States (Glaser & Fox, 1930).

Surveys conducted around the world, including other island habitats, reveal a diverse fauna of entomopathogenic nematode species. Rather than introducing a non-native entomopathogenic nematode for Japanese beetle larval control, an efficacious species that is native to the Azorean Archipelago might provide increased capabilities for persisting and recycling under soil and climatic conditions in Terceira.

A major determinant for selection of candidates for insect control is the ability of the nematode to find and penetrate its host as quickly as possible. The searching and dispersal behavior of entomopathogenic nematodes

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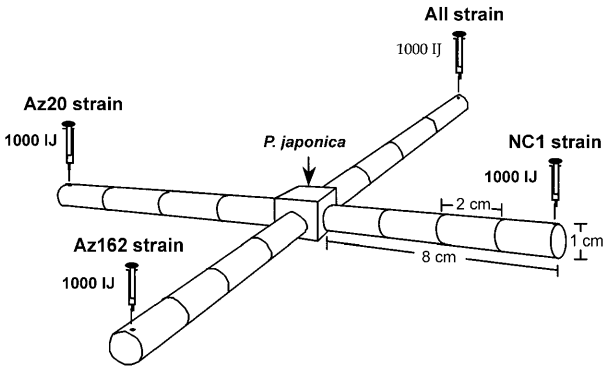

Fig. 1. Apparatus used to measure dispersal of infective juveniles of entomopathogenic nematodes in the presence and absence of a host insect. Az20= Azorean *Steinernema carpocapsae*; All=exotic *S. carpocapsae*; Az162=*Heterorhabditis bacteriophora*; NC1=*S. glaseri*.

has been investigated by numerous researchers for several nematode species. The propensity for nematodes to disperse is strongly correlated with the strategy for host finding (Kaya & Gaugler, 1993). The "ambushers" tend to wait for passing mobile host insects, while "cruisers" actively seek out hosts. A variety of environmental factors including the effect of temperature, soil type, depth and moisture, presence and type of vegetation, and proximity of host insects affect the distance and rate of vertical and horizontal movement of infective juveniles (Georgis & Poinar, 1983a, 1983b; Molyneux & Bedding, 1984; Choo & Kaya, 1991).

The objectives of our study were to determine the effectiveness of *S. glaseri* against third instars and pupae of *P. japonica,* to compare the efficacy of two native heterorhabditid isolates with *S. glaseri* against third instar *P. japonica*, and to study the comparative dispersal of endemic and exotic nematodes in the presence and absence of an insect host.

MATERIALS AND METHODS

Source of nematodes. Native *Heterorhabditis* sp. (Praia Formosa, Az 24 and São Mateus, Az 162) and *Steinernema carpocapsae* (Weiser) (Agualva, Az 20) were isolated from the islands of Santa Maria and Terceira using the *Galleria mellonella* (L.) bait method (Bedding & Akhurst, 1975; Kaya & Stock, 1997) during 1990 as part of a general survey of entomopathogenic nematodes in the Azorean Archipelago (Rosa et al., 2000). Koch's postulates showed that the IJs obtained using the baiting technique were infectious to *G. mellonella* larvae. A sample of adult nematodes from the São Mateus AZ162 isolate was identified by Dr. George Poinar, Oregon State University, Corvallis, Oregon as *Heterorhabditis bacteriophora* Poinar. The Praia Formosa Az 24 isolate was not identified to species. The NC1 isolate of *S. glaseri* originally isolated in North Carolina, USA and the All strain of *S. carpocapsae* originally isolated in Georgia, USA were obtained from cultures that had been maintained at the University of the Azores, Ponta Delgada.

Nematode culture. The methods of production of *S. glaseri* used in bioassays were published previously (Lacey et al., 1993). IJs were produced on a pork kidney medium as described by Bedding (1981). They were harvested 13–15 days after nematode inoculation and stored in distilled water at 8–10°C. IJs of the Azorean isolates of *Heterorhabditis* species were produced in *G. mellonella* larvae according to Dutky et al. (1964) and stored in distilled water at 8–10°C until used in bioassays. All IJs were used within 2 weeks after harvest. For dispersal studies, all IJs were produced in *G. mellonella* larvae as described above.

Bioassays. Comparative bioassays of *S. glaseri* NC1 isolate were conducted against third instars and pupae of the Japanese beetle. Larvae and pupae of *P. japonica* were collected in the field and held overnight in soil to eliminate those that were damaged during collection. Larvae and pupae were then set up in soil in plastic containers. Bioassays were conducted in pasture soil that had been heat treated at 65°C for 24 h and stored at room temperature for at least 5 days before use. The soil was mixed with distilled water to bring the moisture content to 15%. Six hundred grams of soil were added to each 0.95 liter plastic container. The surface area of the soil was 122.7 cm^2 and soil depth was 5 cm. Ten *P. japonica* larvae were added to each container into separate depressions in the soil (2 cm deep, 1 cm diam.) and covered with soil. Ten *P. japonica* pupae per container were placed in other containers into separate depressions in the soil (1.5–2 cm deep) and covered with soil. Groups of three containers were treated with one of five concentrations of nematodes ranging from 10^5 to 10^6 IJs/m^2 (1,230–12,300 IJs per container). The IJs were applied in suspensions in 10 ml of water over the surface of the soil in each container. Ten ml of water was also applied to each of three control containers. The containers were covered with a perforated plastic lid and held at 24°C for 7 days, at which time larval and pupal mortality was assessed. The bioassays were repeated 4 times over a 5-week period.

Similar studies as described above were conducted with field-collected third instars of the Japanese beetle using *S. glaseri* and two Azorean isolates of the *Heterorhabditis* species. Groups of three containers were treated with one of five concentrations of nematodes ranging from 10^5 to 10^6 IJs/m^2 (1,230–12,300 IJs per container), and mortality was assessed 7 days later. Bioassays were repeated on six separate dates over an 18-week period. The relative infectivity ratios of *S. glaseri* and the *Heterorhabditis* species were calculated using LC_{50} values.

Dispersal assay. Comparative dispersal activity was assessed for native isolates of *S. carpocapsae* (Az 20) and *H. bacteriophora* (São Mateus, Az 162) and the exotic isolates of *S. glaseri* (NC1) and *S. carpocapsae* (All). Nematode dispersal was assessed in plastic tubes, 8 cm in length, each consisting of 4 sections, 2 cm length and 1 cm inner diameter, joined together with adhesive tape. Four such 8 cm tubes were connected to a central chamber (4 cm^3) (Fig. 1). The tubes were each filled with 10.5 g of washed and autoclaved sand that had been moistened with distilled water (10% w/w). The central chamber was filled with 5 g of sand. The distal ends of 4 different tubes were each inoculated with 1000 IJs of one of the 4 isolates of nematodes in 150 µl water. Assays were conducted with and without a third instar of *P. japonica* enclosed in the central chamber. After 24 h at 23°C, the plastic tubes were carefully separated and the number of nematodes in different sections was determined by washing the sand and counting all live nematodes using a dissecting microscope. The experiment was replicated 12 times with each of the 4 isolates, with and without *P. japonica*.

Statistical analysis. Mortality data from all assays were analyzed using probit analysis (LeOra Software, 1987). Students t-test and linear regression analyses were performed using SAS software (Ver. 6.12, 1996). Nematode migration data were subjected to analysis of variance (ANOVA) and Tukey's studentized range test at the P = 0.05 level (SAS, 1996). Percentage

TABLE 1. Mortality in third instar larvae and pupae of *Popillia japonica* exposed to five concentrations of *Steinernema glaseri* infective juveniles ranging from 10^5 to $10^6/m^2$ (1,230–12,300 infective juveniles per 122.7 cm^2).

	IJ conc. x $10^5/m^2$	
	Larvae	Pupae
LC_{50}	3.2	0.9
95% C.I.	(1.06–5.86)	(0.15–1.68)
R^2	0.99	0.89
Relative infectivity	1	3.56

4 replicated tests, conducted on separate dates; 30 third instar larvae/concentration and control/test. Control mortality (mean ± s.e.m.), was 11.5 ± 2.0% (larvae); 14.2 ± 2.1% (pupae). The relative infectivity values were calculated from the ratios of the LC_{50}s with the LC_{50} for larvae producing the baseline value of 1.0. The LC_{50} value for larvae divided by that for pupae produced the relative infectivity value for pupae.

values were normalized using arcsine transformation before analysis.

RESULTS

Bioassays. Pupal *P. japonica* had a considerably lower LC_{50} than larvae after exposure to *S. glaseri*, although there was slight overlap in the 95% C.I. (Table 1). Comparison of the mortality response of pupae and larvae to the discriminating concentration of 5 × 10^5 IJs/m^2 using Students t-test indicated a highly significant difference in mortality (85.9 ± 5.2% versus 55.9 ± 5.7%, respectively, (t = 3.92, df = 6, P = 0.008). There was a strong concentration-mortality response for both larvae and pupae of *P. japonica* exposed to increasing concentrations of *S. glaseri* IJs (Fig. 2).

TABLE 2. Larvicidal activity of infective juveniles (IJ) of *Steinernema glaseri* and two Azorean isolates of *Heterorhabditis* sp. against third instar larvae of *Popillia japonica* (22–24°C).

	IJ conc. × $10^5/m^2$		
	Steinernema glaseri	*Heterorhabditis sp.*	
		São Mateus	Praia Formosa
LC_{50}	5.47	3.64	4.44
95% C.I.	(4.31–7.32)	(2.99–4.38)	(3.69–5.41)
R^2	0.76	0.96	0.86
Relative infectivity	1	1.45	1.16

6 replicated tests, conducted on separate dates; 30 third instar larvae/concentration and control/test; 5 test conc. ranging from 10^5 to 10^6 IJ/m^2 (1,230–12,300 per 122.7 cm^2). Control mortality, 5.0 ± 1.4%. The relative infectivity values were calculated from the ratios of the LC_{50}s with the LC_{50} for *S. glaseri* producing the baseline value of 1.0. The LC_{50} value for *S. glaseri* divided by that for the two heterorhabditids produced the relative infectivity values for each of those isolates.

The larvicidal activity of the two Azorean isolates of *Heterorhabditis* was comparable to or slightly better than that observed for *S. glaseri* (Table 2). Although the São Mateus Az 162 isolate of *H. bacteriophora* appears to be more virulent than *S. glaseri* based on the LC_{50} ratios, there was overlap in the 95% C.I. indicating the difference was not significant. Also the larval mortality data for

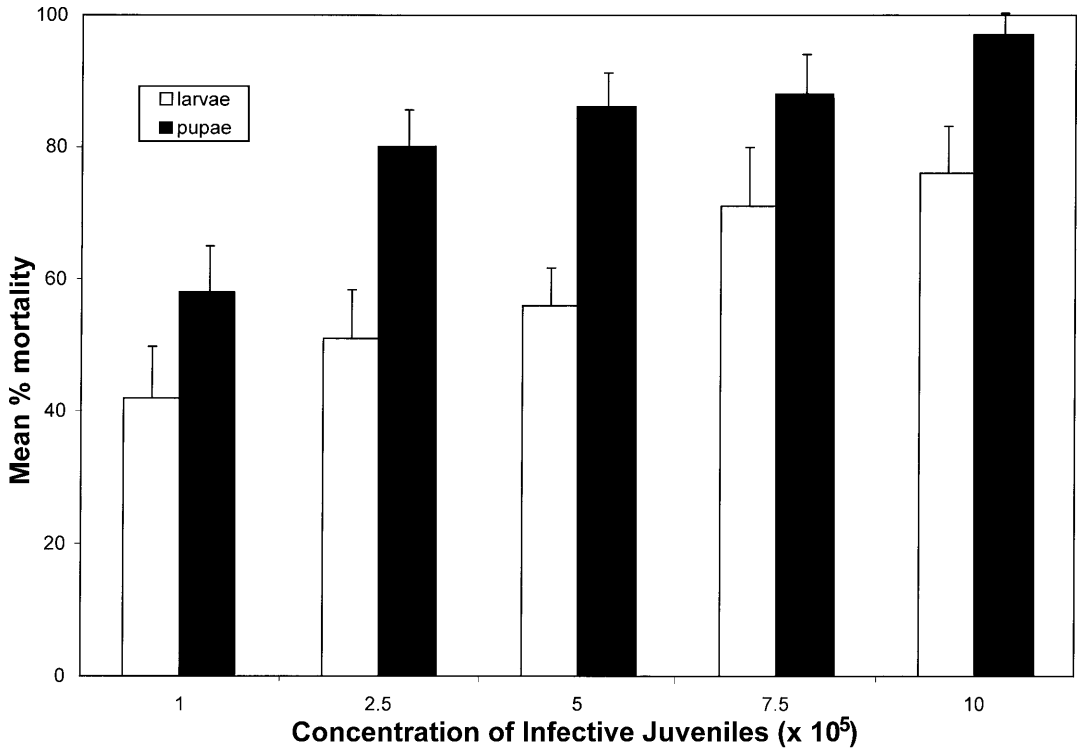

Fig. 2. The mortality response of larvae and pupae of *Popillia japonica* exposed to several concentrations of infective juveniles of *Steinernema glaseri*. Control mortalities in larvae and pupae were 11.5 ± 2.0% and 14.2 ± 2.1%, respectively.

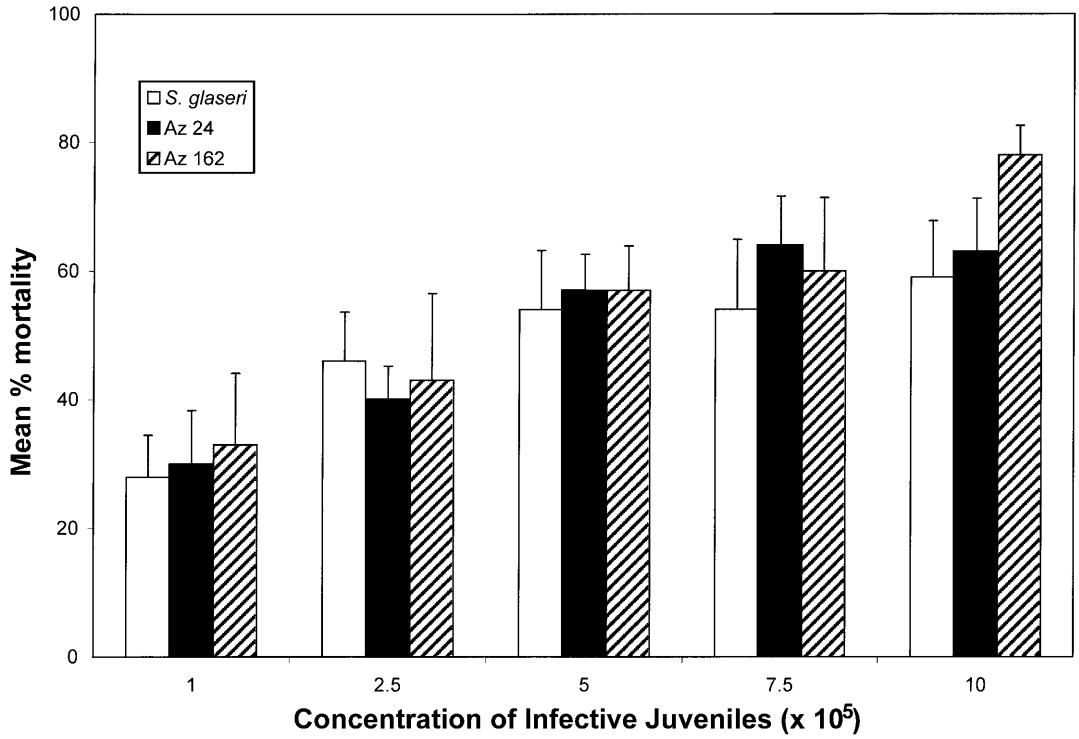

Fig. 3. The mortality response of *Popillia japonica* larvae exposed to several concentrations of infective juveniles of *Steinernema glaseri* and two Azorean *Heterorhabditis* isolates. Control mortality was 5.0 ± 1.4%.

S. glaseri in Table 1 were close to that observed for the *Heterorhabditis* isolates (Table 2). There was a strong concentration-mortality response of *P. japonica* larvae exposed to increasing concentrations of the two *Heterorhabditis* isolates (Fig. 3). In the bioassays of *S. glaseri* that were conducted concomitantly with the *Heterorhabditis* isolates, the concentration mortality response of *P. japonica* larvae was less pronounced than that observed for the *Heterorhabditis* isolates (Fig. 3) and the *S. glaseri* bioassays with larvae and pupae (Fig. 2).

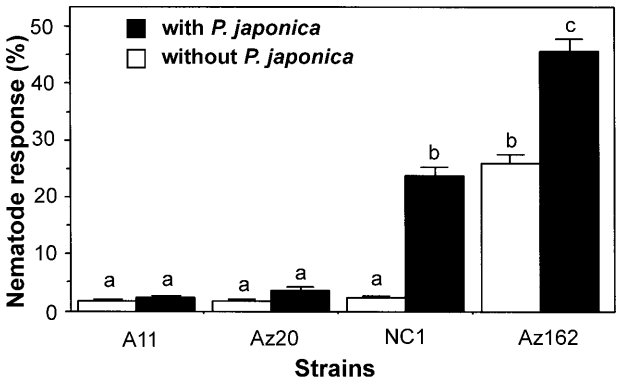

Fig. 4. Influence of insect presence on the dispersal of infective juveniles of four nematode strains 2 cm or farther in horizontal sand columns, 24 hours after adding 1000 IJ to the distal end of each column. Bars with the same letter are not significantly different from one another at the P = 0.05 level (Tukey's multiple range test). (F = 194.8; df = 88; P < 0.01). Az20= Azorean *Steinernema carpocapsae*; All=exotic *S. carpocapsae*; Az162=*Heterorhabditis bacteriophora*; NC1=*S. glaseri*.

Dispersal studies. The distance covered by the four strains was significantly different among nematode species (F = 169.58; df = 44; P = 0.0001). The percentage of the two *S. carpocapsae* strains dispersing beyond 2 cm within 24 h was minimal and not significantly different from one another (Fig. 4) in the presence or absence of a host insect. Less than 1% of IJs of the two *S. carpocapsae* strains dispersed 8 cm in the presence or absence of a host (Table 3). A significantly higher proportion of the native *H. bacteriophora* and NC1 *S. glaseri* dispersed farther than 2 cm in the presence of a host insect compared to the two *S. carpocapsae* strains (F = 211.70; df = 44; P = 0.0001). The percentage of IJs dispersing more than 2 cm was significantly higher in *H. bacteriophora* than in *S. glaseri* in the presence and absence of a host insect (Fig. 4). In the absence of a host, the percentage of response in *S. glaseri* was not significantly different from that of the two *S. carpocapsae* strains. The percentage of IJs dispersing 8 cm was significantly higher for both *H. bacteriophora* and *S. glaseri* with a host insect present than in the absence of a host (Table 3). The mean (± s.e.m.) overall recovery rate of NC1, All, Az 20, and Az 162 IJs was 95.7 ± 1.0%, 94.6 ± 1.7%, 94.9 ± 1.2%, and 84.7 ± 1.5%, respectively. Mortality of the *P. japonica* larvae used in the dispersal studies was not monitored due to the brevity of the experiments.

DISCUSSION

The larvicidal activity of the Azorean *Heterorhabditis* isolates against *P. japonica* demonstrates that suitable biological control agents are available within the archipelago. The heterorhabditids are as effective as *S. glaseri*;

TABLE 3. The percentage of infective juveniles dispersing in horizontal sand columns in 24 h period in the presence and absence of an insect host.

	Mean percentage of nematodes ± SEM			
	Assays with *P. japonica*			
Distance (cm)	*S. carpocapsae* (Az20 strain)	*S. carpocapsae* (All strain)	*S. glaseri* (NCl strain)	*H. bacteriophora* (Az162 strain)
0–2	96.5 ± 0.5 Ac	97.7 ± 0.4 Ac	76.6 ± 1.8 Ab	54.4 ± 1.9 Aa
2–4	2.6 ± 0.4 Bc	1.3 ± 0.2 Bc	16.7 ± 1.5 Bb	32.7 ± 1.7 Ba
4–6	0.6 ± 0.1 Bb	0.5 ± 0.1 Bb	3.6 ± 0.6 Cb	10.8 ± 1.6 Ca
6–8	0.3 ± 0.1 Bb	0.6 ± 0.2 Bb	3.1 ± 0.6 Ca	2.2 ± 0.2 Da
	Assays without *P. japonica*			
0–2	98.1 ± 0.3 Ab	98.2 ± 0.3 Ab	97.5 ± 0.4 Ab	74.3 ± 1.7 Aa
2–4	1.5 ± 0.3 Bb	1.1 ± 0.2 Bb	1.7 ± 0.3 Bb	22.3 ± 1.5 Ba
4–6	0.3 ± 0.1 Bb	0.4 ± 0.1 Bb	0.6 ± 0.1 Bb	2.9 ± 0.3 Ca
6–8	0.1 ± 0.03 Bb	0.2 ± 0.1 Bb	0.2 ± 0.1 Bb	0.7 ± 0.3 Ca

Means in the same row followed by the same lowercase letter are not significantly different from one another at the 0.05 level (Tukey's multiple range test). Means in the column in the same treatment group (presence or absence of *P. japonica*) followed by the same uppercase letter are not significantly different from one another at the 0.05 level (Tukey's multiple range test).

moreover, they have the ability to persist and recycle as indicated by their natural occurrence in the Azores (Rosa et al., 2000). The two isolates used in our study were collected within 200 m of the sea. São Mateus is a port village and the Praia Formosa site is a sandy beach with clumps of grasses. However, most pastures that are positive for *P. japonica* larvae occur above 150 m elevation. Rosa et al. (2000) found that the majority of sites (70%) positive for *Heterorhabditis* species were below 150 m elevation in soil ranging from pH of 5.6 to 6.3 and more often in association with native vegetation than introduced vegetation. In contrast, the *Steinernema* species were more prevalent above 300 m and not found in association with native vegetation. Thus, further field trials with promising native isolates of *Heterorhabditis* and *Steinernema* species should be conducted where *P. japonica* larvae are causing serious problems to determine environmental conditions that might influence the efficacy and persistence of these native species.

The susceptibility of insect pupae to entomopathogenic nematodes is variable ranging from highly susceptible to resistant to infection (Kaya & Hara, 1980; Henneberry et al., 1995). The high susceptibility of pupal *P. japonica* might be misleading because in nature, prior to pupation, mature third instar larvae make an earthen cell in which to pupate (Vittum et al., 1999). Although *S. glaseri* IJs readily infected the pupae, their entrance into the pupal cells under natural conditions could be restricted by the barrier of the earthen cell surrounding the pupa.

The dispersal behavior of the two *S. carpocapsae* strains, *H. bacteriophora* and *S. glaseri* was consistent with the host seeking behavior previously observed for each of these species. *S. carpocapsae* did not disperse very well in sand in the presence or absence of a host and confirmed the earlier dispersal studies conducted with this species (Kaya, 1990). In contrast, *S. glaseri* and *H. bacteriophora* demonstrated a high degree of dispersal behavior. Our results with *S. glaseri* were similar to observations made by Lewis et al. (1992) who documented that this species responds strongly to host chemo-attractants, but in the absence of a host insect, its dispersal response was minimal. On the other hand, over 25% of the Azorean *H. bacteriophora* IJs dispersed beyond 2 cm within a 24 h period in the absence of host cues. With a host present, a significantly greater proportion of IJs (45%) dispersed beyond 2 cm. Overall, these data show that the heterorhabditids are more active than *S. glaseri.*

The behavior of the nematodes is critical to effective control of Japanese beetle larvae. Georgis & Gaugler (1991) evaluated 380 field treatments of entomopathogenic nematodes against this insect and concluded that *S. carpocapsae* was ill-adapted to infect Japanese beetle larvae under a wide range of conditions. One of the main factors is that *S. carpocapsae* is an ambusher strategist and is ineffective against an insect pest that occurs deep in the soil profile (Kaya et al., 1993). *H. bacteriophora* was effective against Japanese beetle larvae, but only when the hatch depth and soil moisture, temperature and type were favorable (Georgis & Gaugler, 1991). Another critical factor is that *H. bacteriophora* is a "cruiser" strategist and is found throughout the soil profile (Campbell et al., 1996; Gaugler et al., 1997) and is well adapted to find, infect and kill Japanese beetle larvae (Wang et al., 1995) . Our studies indicate that the two Azorean heterorhabditid isolates are excellent biological control agent candidates for the Japanese beetle.

Native nematodes offer alternatives to the introduction of exotic species such as *S. glaseri* in island habitats where increasing public concern and/or legislation may restrict or prohibit such introductions. The documented and potential negative impact of introduced biological control agents into island habitats has been reviewed by Howarth (1991), Follett & Duan (1999) and others. In addition to untoward permanent effects of introduced spe-

cies on nontarget insects, there is also the potential for antagonistic effects on endemic species of entomopathogenic nematodes (Barbercheck & Millar, 1999). Accordingly, wherever feasible, the use of native entomopathogenic nematodes against soil insect pests should be explored before introducing exotic ones.

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