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Biological Control 30 (2004) 439–445
Biological Control
www.elsevier.com/locate/ybcon

Virulence of new and mixed strains of the entomopathogenic nematode *Steinernema riobrave* to larvae of the citrus root weevil *Diaprepes abbreviatus*

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Received 24 August 2003; accepted 20 November 2003

Abstract

Steinernema riobrave ranks as the most efficacious entomopathogenic nematode for inundative biological control (biopesticide) applications against larvae of the root weevil *Diaprepes abbreviatus* in Florida citrus groves and has been commercially available for several years. However, only one strain is known, and others could be more effective. Therefore, we used two soil sampling techniques (cores and shovel/bucket samples) and collected soil samples at 10 sites near the type locality of Weslaco, Texas. Soil samples were baited with either *Galleria mellonella* or *D. abbreviatus* larvae and produced 10 new isolates of *S. riobrave* from five sites, and an undescribed species of entomopathogenic nematode in the genus *Heterorhabditis* from one site. All new isolates were from *G. mellonella* larvae, and eight of the *S. riobrave* isolates were from soil cores. In virulence assays comparing the new *S. riobrave* strains, the commercial strain, and a mixed strain (formed by pooling the new strains), average larval mortality for five strains (including the mixed strain) was significantly greater than for the commercial strain. Larval mortality for the 12 strains ranged from 57.7 to 84.1% with the commercial strain and the mixed strain being the extremes, and the mixed strain killing 45.7% more than the commercial strain. Among the 10 new strains, larval mortality ranged from 65.8 to 79.3%, with two strains from the same site being the extremes and the only strains significantly different from one another. Thus, these strains have potential for improved biological control of *D. abbreviatus* and further comparisons are warranted.

Keywords: *Steinernema riobrave*; *Diaprepes abbreviatus*; Biological control; Entomopathogenic nematode; Diaprepes root weevil; Citrus; Virulence; Florida

1. Introduction

The root weevil, *Diaprepes abbreviatus* (L.), originally from the Caribbean, is a major introduced pest of citrus, ornamentals, and other crops in Florida (McCoy, 1999; Simpson et al., 1996). In citrus, larval feeding damages roots, reduces yield and kills trees by girdling or by facilitating infection by plant pathogens such as *Phytophthora* spp. (Graham et al., 2003; McCoy, 1999). *D. abbreviatus* infestations can lead to rapid tree decline and devastate entire groves within a few years of detection. Adults are long lived and feed on foliage, especially new growth. Mating occurs in the canopy, and eggs are laid in masses between leaves glued together by the female during oviposition. The larvae hatch, escape from the sealed leaf envelope, drop to the soil and burrow down to the roots where they begin feeding. Larvae move to larger roots as they grow, and pupate after 9–11 instars (McCoy, 1999; Quintela et al., 1998).

The application of entomopathogenic nematodes for inundative biological control (i.e., as biopesticides) is one of the few tactics available to control *D. abbreviatus* larvae in Florida citrus groves (McCoy et al., 2003). Nematode

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doi:10.1016/j.biocontrol.2003.11.004

products for weevil control have been commercially successful in Florida, but the confidence of growers and researchers in this approach remains weak because laboratory, greenhouse, and field trials often produce variable results and sometimes fail (McCoy et al., 2000; Shapiro-Ilan et al., 2002). Numerous factors could be responsible for these problems but differences in soil type and texture are considered of primary importance (Duncan et al., 2001; McCoy et al., 2002). In general, nematode virulence to a specific target insect under a particular set of environmental conditions determines the rate at which nematodes should be applied in inundative biological control and is of fundamental importance in determining application costs and the economic viability of this control tactic. However, research indicates that application rates currently recommended by nematode suppliers might often be insufficient to provide high levels of efficacy, especially in finer textured soils (Duncan et al., 2001; McCoy et al., 2002; Shapiro et al., 2000). Thus, research is necessary to establish appropriate rates for various combinations of nematodes and environmental conditions, and it might be especially important to find new and more efficacious nematode species or strains or to develop other improvements to maintain costs and efficacy at reasonable levels.

Based on laboratory, greenhouse, and field studies, *Steinernema riobrave* Cabanillas, Poinar, and Raulston ranks as the most effective entomopathogenic nematode yet tested for inundative biological control of *D. abbreviatus* larvae (Shapiro and McCoy, 2000a,b; Shapiro-Ilan et al., 2002). This nematode is the basis for the commercial product BioVector 355 (Certis Corporation, Columbia, MD), which has been used in Florida citrus groves for several years. However, only a single strain of this species is known, and others might be more effective. Characteristics important for biological control that are known to differ among strains within species of entomopathogenic nematodes include virulence (Morris and Converse, 1991; Shapiro and McCoy, 2000a,b), reproductive potential (Somasekhar et al., 2002), and environmental tolerance (Glazer, 2002; Shapiro et al., 1996; Somasekhar et al., 2002). Therefore, in an effort to find new strains of *S. riobrave*, we took soil samples at 10 sites in the vicinity of the type locality near Weslaco, Texas (Cabanillas et al., 1994). Here, we report the results of our field sampling, and subsequent virulence assays with the new strains against *D. abbreviatus* larvae.

2. Materials and methods

2.1. Isolating new strains

Soil samples were collected at 10 sites in the lower Rio Grande Valley near Weslaco, Texas (sites #1–6), and Reynosa, Mexico (sites #7–10), April 17–19, 2001. The collection sites were in agricultural habitats and included citrus groves (#1,2, and 4), a pecan orchard (#3), a corn field (#5), a cucumber field (#6), sorghum fields (#7–9), and beside an irrigation canal (#10). Site #1 is the only site in Texas where *D. abbreviatus* has been reported (French and Skaria, 2000). Two soil sampling methods were used. The first method followed the procedures of Stuart and Gaugler (1994) and involved taking a series of 20 soil cores (2.5-cm dia. $\times$ 21-cm depth) per site (except site #3 where only 10 cores were taken) and placing the soil from each core into a separate plastic bag (total $n = 190$ samples). Soil cores were generally taken at ca. 4-m intervals along linear transects at each site. However, at site #3, each sample was taken near the trunk of a different pecan tree. The second sampling method involved collecting three 2 L buckets of soil per site (see Shapiro-Ilan et al., 2002) with the exception that two series of bucket samples were taken at site #5; one in an area using conventional tilling and one in a no-till area (total $n = 33$ samples). In taking bucket samples, three locations approximately 20–30 m apart were selected at each site for each sample. At each location, five full shovels of soil were taken and mixed thoroughly in a plastic bag. Then 2 L of the mixed soil were taken as the sample. Soil samples were kept on ice in coolers for transport to the laboratory.

In the laboratory, each core sample was baited with a single wax moth larva *Galleria mellonella* (L.) (Webster's Waxie Ranch, Webster, WI). Bucket samples were subdivided into two equal portions, one of which was baited with five wax moth larvae and the other with five *D. abbreviatus* larvae (obtained from the Florida Department of Agriculture, Division of Plant Industry, Gainesville, FL). We used two insects for isolations because *G. mellonella* is highly susceptible to nematode infection and its use should maximize the number of isolates obtained, whereas, some nematode species are very host specific (e.g., *Steinernema scapterisci* Nguyen and Smart, 1990) and using *D. abbreviatus* as bait might enable us to isolate an especially virulent host-specific nematode that might not respond to *G. mellonella*. Soil samples were checked weekly for dead larvae, which were removed and replaced with live larvae for additional rounds of baiting. Dead larvae that exhibited signs of infection with entomopathogenic nematodes were placed in modified White traps (Hara et al., 1991). Nematodes that emerged from any individual bait larva were considered a separate isolate.

Nematode strains were maintained on *G. mellonella* larvae with 18 larvae being infected per strain during each round of infection. The large number of cadavers per strain is important to maintain a sufficiently large breeding population at each cycle of infection to prevent deterioration of strains due to inbreeding depression, genetic drift, or founder effects (Hopper et al., 1994; Roush, 1990; Stuart and Gaugler, 1996). All nematodes

that emerged over a 3-week period for each strain were collected and pooled prior to experimentation or the next cycle of infection. This protocol provides a broad sampling of nematodes of each strain for experimental purposes and is necessary to eliminate possible experimental bias because nematodes emerging earlier can differ dramatically from those emerging later with respect to various characteristics that can influence virulence (Lewis and Gaugler, 1994; Stuart et al., 1996).

2.2. Virulence comparisons

Tests comparing the virulence of the new strains of *S. riobrave*, the commercial strain (BioVector 355), and a mixed strain (formed by pooling infective juveniles of the 10 new strains) against *D. abbreviatus* were conducted based on procedures described by Shapiro and McCoy (2000a). Our version of the commercial strain was obtained from a sample of commercial product at about the same time the new strains were isolated. The mixed strain was formed by mixing a large number of infective juveniles from each of the new strains shortly after they were isolated. Thereafter, the commercial strain and the mixed strain were reared in parallel with the new strains. Experiments were conducted in 25-dram snap-cap plastic vials (3.5-cm i.d., 8.5-cm deep) filled with oven-dried Candler sand (estimated soil characteristics are sand:silt:clay = 96.6:2.0:1.4%, pH 6.3, organic matter = 0.3%) at 8% moisture by weight and containing one *D. abbreviatus* larva each. The rate of application was 200 infective juveniles per container, which were pipetted onto the soil surface of each vial. Each replicate included an untreated control (only water added). Individual vials were randomly assigned to three or four open plastic boxes and placed in incubators at 24 °C and 70% RH. Sand columns were opened and larval mortality was recorded 10 days post-inoculation. The larvae were held for an extra two days in closed plastic containers and rechecked to confirm mortality and nematode infection.

The seven replicates reported here represent five successive rearings of the nematodes and were conducted from 29 April 02 to 18 Feburary 03. Replicates 2–3 and 6–7 were conducted with nematodes from the same rearings but were otherwise independent. The replicates varied with respect to several characteristics (Table 1), which included the total number of sand columns in each replicate (ca. 20–30 vials per treatment per replicate), and the total rearing and storage period for the nematodes prior to each replicate (i.e., from *Galleria* infection, through the rearing and harvest period, and until the inoculation of the sand columns) (Table 1). After rearing, the nematodes were stored in distilled water at room temperature. The *D. abbreviatus* larvae used in the study were reared on artificial diet by the Florida Department of Agriculture, Division of Plant Industry, and represent age cohorts between 35 and 60 days of age at the time of the experiments. Based on a random sample of 30 individuals per replicate, average weight, and instar of the *D. abbreviatus* larvae used in the replicates varied and there were significant differences among replicates (Table 1). Instars were determined by measuring head capsule width (Quintela et al., 1998). The percent mortality for each treatment was arcsine transformed and compared among treatments and replicates by two-way ANOVA and LSD tests (PROC ANOVA, SAS Institute Inc., 1990). Possible relationships among conditions and results of different replicates were assessed using correlation analysis (PROC CORR, SAS Institute Inc., 1990)

3. Results

3.1. Isolating new strains

Seven of the 190 core samples (3.7%) and three of the 33 bucket samples (9.1%) proved positive for entomopathogenic nematodes. This included a total of 10 isolates of *S. riobrave* from five sites. Eight of the

Table 1
Details of the seven replicates of the virulence study with the total number of sand columns in each replicate, the rearing and storage period for the nematodes prior to experimentation, instar and weight for the *Diaprepes abbreviatus* larvae, and percent mortality for the 12 strains (experimental) and the controls

Replicate No.	No. of columns	Rearing + storage (days)	Instar range	Larval instar[a] (mean ± SE)	Larval weight (g)[a] (mean ± SE)	% Mortality experimental[a] (mean ± SE)	% Mortality control
1	292	n.d.[b]	6–9	7.6 ± 0.2 a	nd[b]	60.0 ± 3.4 a	5.0
2	275	23	6–9	7.4 ± 0.1 ab	0.81 ± 0.03 a	62.9 ± 3.9 a	4.5
3	351	32	6–8	7.3 ± 0.1 ab	0.74 ± 0.02 b	67.6 ± 4.0 a	18.5
4	404	51	5–9	7.1 ± 0.2 b	0.24 ± 0.02 c	69.6 ± 4.6 a	0.0
5	301	27	6–8	7.2 ± 0.1 b	0.25 ± 0.03 c	87.0 ± 2.9 b	4.2
6	330	34	5–8	6.3 ± 0.2 c	0.13 ± 0.02 d	90.3 ± 2.8 b	0.0
7	296	40	5–8	7.0 ± 0.2 b	0.20 ± 0.02 c	65.1 ± 4.9 a	8.7

[a] Common letters following values indicate no significant difference within columns based on ANOVA and LSD tests, $P = 0.05$.
[b] n.d., no data.

S. riobrave isolates were obtained from seven core samples and two were from two bucket samples. All isolates were from wax moth larvae. Five of the soil core isolates came from site #3 (pecan grove), and two of these were obtained from a single soil core. In addition, a single soil core from each of three Mexican sites (#7, 8, 9; sorghum fields) produced a *S. riobrave* isolate. One bucket sample from site #3 produced a *S. riobrave* isolate, and an isolate was obtained from a bucket sample from site #5 (no-till corn). A bucket sample from one of the Mexican sites (#10, irrigation canal) produced a series of heterorhabditid isolates ($n = 6$), which have been determined to be a new species that is in the process of being described and characterized (Khuong B. Nguyen, David I. Shapiro-Ilan, Byron J. Adams, Robin J. Stuart, Clay W. McCoy, and Rosalind R. James, in preparation). The new *S. riobrave* isolates are designated by site and core numbers for those derived from soil core samples (i.e., 3–2, 3–3, 3–7, 3–8a, 3–8b, 7–12, 8–14, and 9–5), or by site number only for bucket samples (i.e., 3 and 5).

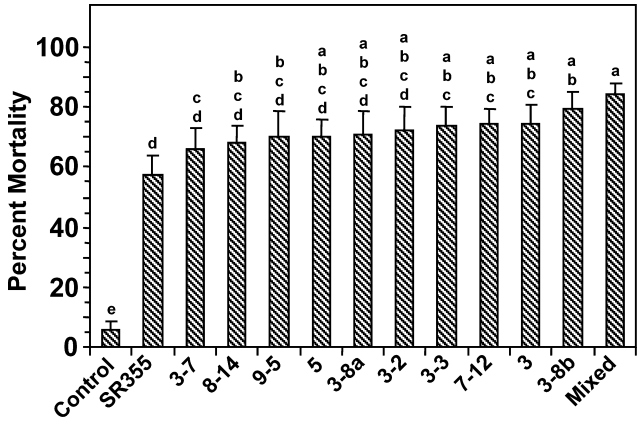

Fig. 1. Results of seven replicates of virulence testing in which 10 new *Steinernema riobrave* strains were compared to the commercial strain (SR355) and a mixed strain formed by combining all 10 new strains (Mixed). Common letters above bars indicate no significant difference based on ANOVA and LSD tests (SAS Institute Inc., 1990).

3.2. Virulence comparisons

Significant differences in percent mortality were observed among strains ($F = 14.41, df = 12, 72, \ P = 0.0001$) (Fig. 1). For the 12 strains being compared, the commercial strain and the mixed strain formed opposite extremes of the virulence continuum, and four strains in addition to the mixed strain produced mortality levels significantly greater than the commercial strain. The commercial strain produced an average mortality level of 57.7% ($SE = \pm 5.8$) and the mixed strain 84.1% (± 4.1). Thus, the mixed strain killed 45.7% more larvae than the commercial strain. Mortality levels for the 10 new strains ranged from 65.8 (± 7.2) to 79.3%(± 5.6), with two strains from the same site (3–7 and 3–8b) representing the extremes and being significantly different. Significant differences in percent mortality also occurred among replicates ($F = 9.91, df = 6, 72, P = 0.0001$) with mortality levels in replicates 5 and 6 being significantly greater than in the remaining replicates (Table 1). For the six replicates with complete data (Table 1), a correlation analysis of the relationship between the length of the nematode rearing and storage period, mean larval instar, mean larval weight, percent mortality pooled for all strains, and percent mortality in the controls, revealed no significant associations ($P > 0.05$, PROC CORR, SAS Institute Inc., 1990) (Table 2).

4. Discussion

Our results demonstrate that there are significant differences in virulence among *S. riobrave* strains, and that some of the new strains are significantly more virulent to *D. abbreviatus* larvae than the commercial strain. In our study, the mixed strain formed by pooling all 10 new strains killed 45.7% more *D. abbreviatus* larvae than the commercial strain and ranked highest in virulence whereas, the commercial strain ranked lowest.

Table 2
Correlation matrix examining potential relationships among some of the factors that differed among replicates and the results that were obtained (see Table 1)

		Nematode rearing and storage	Host instar	Host weight	Percent experimental mortality
Host instar	r	−0.2470			
	P	0.6370			
Host weight	r	−0.5421	0.6967		
	P	0.2665	0.1241		
Percent experimental mortality	r	−0.1419	−0.6684	−0.5998	
	P	0.7886	0.1467	0.2082	
Percent control mortality	r	−0.2358	0.4679	0.5696	−0.4445
	P	0.6529	0.3494	0.2380	0.3771

r, Pearson correlation coefficient; *P*, probablity value.

If these differences in virulence are similar under field conditions then the rate of nematode application (and the associated cost) could be reduced considerably while maintaining the same level of efficacy. Future research will compare various characteristics of these strains under the range of conditions common in Florida citrus.

The 10 new strains showed relatively little difference in virulence and caused from 65.8 to 79.3% mortality to *D. abbreviatus* larvae. Nonetheless, larval mortality for the two most extreme strains was significantly different, even though these strains were collected at the same site (site #3, pecan orchard). This reinforces the view that local populations of entomopathogenic nematodes can be genetically heterogeneous and variable for important biological traits (Blouin et al., 1999; Hominick et al., 1999; Somasekhar et al., 2002).

It is noteworthy that the mixed strain ranked as the most virulent in our study. Although, the genetic diversity of the new strains are unknown, they are unlikely to be homozygous (e.g., Shapiro et al., 1997), and the superior performance of the mixed strain cannot easily be attributed to hybrid vigor or heterosis because all strains were maintained as large breeding populations, and our replicates represent successive rearings, each of which probably involved multiple cycles of reproduction (Stuart and Gaugler, 1996). Rather, the enhanced genetic diversity of the mixed strain was probably advantageous by providing a greater array of adaptations for locating and penetrating target insects and overcoming their immune responses (see Wang et al., 1995). The strong performance of the mixed strain suggests that genetic variability is an important asset and could be useful for producing and maintaining high levels of virulence in strains for inundative biological control. Genetic variability might also be beneficial when nematode products are intended for use against multiple target species, or for inoculative biological control where colonization and establishment is desired (Hopper et al., 1994; Roush, 1990).

The poor performance of the commercial strain of *S. riobrave* in our study could be due to various factors. When a population is isolated and reared in the laboratory for multiple generations or mass-produced for commercial purposes, certain processes can cause apparent deterioration of traits important for fitness. These processes can include environmental factors such as nutritional deficiencies and disease, or involve genetic changes associated with genetic bottlenecks, genetic drift, inbreeding, and laboratory adaptation (Hopper et al., 1994; Roush, 1990; Stuart and Gaugler, 1994; Wang and Grewal, 2002). Most of these genetic changes occur when the number of breeding individuals in the population becomes relatively small either temporarily or over more prolonged periods, and lead to detectable strain deterioration under laboratory conditions that can also apply to the field. However, laboratory adaptation can occur in large populations and involves enhanced laboratory fitness that is only indirectly associated with reduced fitness in the field (Hopper et al., 1994; Roush, 1990). Commercial producers of entomopathogenic nematodes should anticipate strain deterioration and maintain programs to isolate, test, and store new strains for future incorporation into nematode products.

Numerous factors might have been responsible for the variability in mortality among replicates that we observed in our study. Nematode age can influence infection and mortality rates (e.g., Perez et al., 2003), and *Diaprepes* larvae become less susceptible to entomopathogenic nematodes as they age (Shapiro et al., 1999). Although, we found no significant correlations between these factors and mortality levels in the present study, relatively small sample sizes or additional factors might have obscured these relationships.

All new isolates in our study were derived from *G. mellonella* larvae and none from *D. abbreviatus* larvae, even though the bucket samples were split into two portions, and baited equally with the two insects. Evidently, the high susceptibility of *G. mellonella* to entomopathogenic nematodes enabled extractions of the nematodes from these relatively small soil samples whereas the inherent resistance of *D. abbreviatus* larvae to nematode infection effectively prevented extractions with this bait insect. Thus, although it would be beneficial to isolate nematodes from soil using the target insect of interest, and such isolations might reveal host specialists (e.g., Nguyen and Smart, 1990), this approach might not be practical for small soil samples. Nonetheless, it might work well for bait insects placed in the field where nematodes can be extracted from larger areas, provided multiple visits by researchers to field sites over a period of several days are possible.

In our study, the core sampling technique and the bucket sampling technique produced similar levels of success in obtaining new isolates. The 190 core samples (ca. 19.6 L of soil) produced eight isolates from four of the 10 sites that were sampled, whereas, the 33 bucket samples (ca. 66 L of soil) produced eight isolates (including the six heterorhabditids) from three of the 10 sites. It is noteworthy that three of the four sites found positive by the core method were negative by the bucket method, and two of the three sites found positive by the bucket method were negative by the core method. Only one site was found positive using both methods (site #3). We attribute this result to the relative rarity and patchiness of entomopathogenic nematode populations (Campbell et al., 1996; Stuart and Gaugler, 1994) and conclude that both the number and volume of soil samples can be relevant in assessing the occurrence, abundance, and distribution of entomopathogenic nematodes within and among sites.

Although, the two soil sampling methods in our study produced similar results, they differ in practicality, efficiency, and usefulness. Based on our sampling protocols for an individual site, core samples comprise about a third of the volume of bucket samples and, therefore, are easier to carry, pack, and transport. Moreover, since multiple species can be found at the same site (Stuart and Gaugler, 1994) and conspecific isolates from the same site can exhibit significant differences in important biological characteristics (Blouin et al., 1999; Hominick et al., 1999; Somasekhar et al., 2002), the larger number of independent samples obtained by the core method can be important for assessing issues such as abundance, distribution, and diversity (e.g., Stuart and Gaugler, 1994). Soil cores can usually be obtained quickly and efficiently provided the soil is sufficiently moist but are extremely difficult to take in heavy, dry, and/or rocky soils; and the time needed to label large numbers of individual samples can slow the sampling process. In contrast, shovel and bucket samples can be obtained under almost any field conditions; and the mixing and sub-sampling of bucket samples in the field can reduce the volume of soil being transported while assuring that a sufficiently large number of areas at a site are sampled. Although, the mixing of samples results in the loss of information regarding distribution and might effectively pool nematodes with different characteristics from different locations within sites, these issues are not important for all studies. Thus, we consider both sampling methods to be useful and that the optimal sampling method depends on the goals, logistics, and field conditions for particular studies.

Acknowledgments

We thank Victor French and Orlando Zamora for assistance in the field, Angel Hoyte and Ian Jackson for technical support, Suzanne Fraser for providing *Diaprepes* larvae, and Larry Duncan, Herb Nigg, and two anonymous reviewers for comments on the manuscript. This research was supported by the Florida Agricultural Experiment Station and a grant from the Florida Citrus Production Research Advisory Council, and approved for publication as Journal Series No. R-09715.

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