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SUPPRESSION OF *DIAPREPES ABBREVIATUS* IN POTTED CITRUS BY COMBINATIONS OF ENTOMOPATHOGENIC NEMATODES WITH DIFFERENT LIFESPANS

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ABSTRACT

El-Borai, F. E., J. D. Zellers, and L. W. Duncan. 2007. Suppression of *Diaprepes abbreviatus* in potted citrus by combinations of entomopathogenic nematodes with different lifespans. Nematropica 37:33-41.

Two experiments were conducted to test the hypothesis that augmenting entomopathogenic nematode (EPN) communities with short-lived EPN species can gradually reduce biological control of insect larvae by partially displacing longer-lived EPN species. Citrus seedlings growing in pasteurized soil mix were infested with *Steinernema riobrave* (Sr; short-lived) and *S. diaprepesi* (Sd; long-lived), either alone or in combination in both experiments, and with *Heterorhabditis zealandica* (Hz; short-lived) alone or combined with Sr or Sd in the second experiment. Larvae of the weevil *Diaprepes abbreviatus* were added to the pots periodically and plants were grown for up to 8.5 months in the first experiment and 13.6 months in the second. No interactions ($P \geq 0.05$) occurred between any treatment in either experiment. In the first experiment, the average weight of plants treated with Sd or Sd + Sr was greater than that of untreated controls ($P < 0.01$), but the main effect of Sr was not significant. The growth and survival of the citrus plants in the second experiment and the suppression of weevil larvae were greatest in the treatment combinations that contained Sd. Plant weights in all treatments containing Sd were greater than those of positive controls ($P \leq 0.01$) and did not differ from those of negative controls (no weevils). Treatments with Sr also produced plants that were heavier than the positive controls, but lighter than the negative controls ($P \leq 0.05$). Augmenting pots with Hz did not significantly affect plant weight or insect suppression. *Steinernema diaprepesi* was the only EPN species recovered by baiting soil with insect larvae at the end of either experiment. Neither experiment supported the hypothesis. Indeed, the long-lived *Sd* revealed a remarkable ability to invade adjacent pots where it became the dominant species.

Key words: Biological control, competition, competitive displacement, concomitant species interactions, longevity.

RESUMEN

El-Borai, F. E., J. D. Zellers, and L. W. Duncan. 2007. Supresión de *Diaprepes abbreviatus* en cítricos sembrados en macetas utilizando combinaciones de nematodos entomopatógenos con diferente duración de vida. Nematropica 37:33-41.

Se realizaron dos experimentos para probar la siguiente hipótesis: al aumentar las especies de corta duración de vida en las comunidades de nematodos entomopatógenos (EPN) se reduce gradualmente el control biológico de larvas de insectos debido al desplazamiento parcial de especies de larga duración de vida. Las plántulas de cítricos sembradas en suelo pasteurizado se infestaron con *Steinernema riobrave* (Sr; corta vida) y *S. diaprepesi* (Sd; larga vida), solos y combinados en ambos experimentos, y con *Heterorhabditis zealandica* (Hz; corta vida) solo o combinado con Sr ó Sd en el segundo experimento. Periódicamente, se adicionaron larvas de *Diaprepes abbreviatus* a las macetas y se mantuvieron las plantas durante 8.5 meses en el primer experimento y durante 13.5 meses en el segundo. No se observaron interacciones ($P \geq 0.05$) entre tratamientos en ninguno de los experimentos. En el primer experimento, el peso promedio de las plantas tratadas con Sd o con Sd + Sr fue mayor que el

de las no tratadas ($P < 0.01$), pero el efecto principal de Sr no fue significativo. El crecimiento y la supervivencia de las plantas en el segundo experimento, al igual que la supresión de las larvas fue mayor en los tratamientos con Sd. El peso de las plantas en todos los tratamientos que contenían Sd fue mayor que en los controles positivos ($P \leq 0.01$) y no se observó diferencia con los controles negativos (sin larvas del insecto). Los tratamientos con Sr también produjeron plantas más pesadas que los controles positivos, pero más livianas que los controles negativos ($P \leq 0.05$). Las infestaciones con Hz no afectaron el peso de las plantas o la supresión de los insectos. *Steinernema diaprepesi* fue la única epecie entomopatógena que pudo recuperarse utilizando larvas carnada al final de ambos experimentos. Ninguno de los experimentos probó la hipótesis. Ciertamente, el nematodo de larga vida Sd demostró gran habilidad para invadir macetas adyacentes, en donde se convirtió en la especie dominante.

Palabras clave: control biológico, competencia, desplazamieto competitivo, interacción de especies concomitantes, longevidad.

INTRODUCTION

Diaprepes abbreviatus is a major weevil pest of many crops in the Caribbean Basin (Shapiro-Ilan *et al.*, 2006b). Plant damage is due primarily to feeding on roots by the subterranean larvae. When entomopathogenic nematodes (EPN) were used for augmentation biological control of *D. abbreviatus* in central Florida citrus orchards, we sometimes observed a period of elevated predation of sentinel insects by EPN followed by a short period of lower than normal predation (Duncan *et al.*, 2003a). An increase in the prevalence of nematophagous fungi and some microarthropods in response to EPN augmentation has been reported and may account for the reduced prevalence of EPN subsequent to some field applications (Jaffee and Strong, 2005; Duncan *et al.*, 2007; El-Borai *et al.*, 2007). Competition between augmented and endemic EPN was also proposed as a potential mechanism of reduced biological control, because endemic EPN are important natural biological control agents in central Florida and some endemic species are innately longer lived than the species used for augmentation (Duncan, unpublished; Koppenhöfer and Fuzy, 2006; Shapiro *et al.*, 2006a). EPN populations tend to be highly patchy and average densities are low compared to many nematode species in agricultural soils (Stuart and Gaugler, 1994). Partial displacement of endemic EPN by introduced species has been reported in this and other ecosystems (Millar and Barbercheck, 2001; Duncan *et al.*, 2003a). If short-lived species have patchier distributions than long-lived species, due to more frequent local extinctions when prey are unavailable, then partial competitive displacement of long-lived species could result in increased patchiness and a temporary reduction of biological control.

Here we report results from two greenhouse experiments that measured the efficacy against *D. abbreviatus* of long-lived (*Steinernema diaprepesi* Khuong and Duncan) and shorter-lived (*S. riobrave* Cabanillas Raulston and Poinar, *H. zealandica* Poinar) EPN species used alone and in combination. We tested the hypotheses that long-lived EPN will provide greater residual suppression of *D. abbreviatus* than will short-lived species and that combinations of species will provide intermediate residual control due to the partial competitive displacement of the long-lived EPN.

MATERIALS AND METHODS

Experiment 1

Five-month-old Swingle citrumelo (*Poncirus trifoliata* × *Citrus paradisi*) citrus seedlings were transplanted singly into plastic

pots (10 × 10 × 30 cm deep) filled to within 5 cm of the rim with an autoclaved soil mixture (50:50/v:v) of Candler fine sand (96:3:1; sand:silt:clay) and shredded Canadian sphagnum peat moss (Scotts, Inc., Sandusky, OH). The next day, 21 replications of the following treatments were established in a factorial design: *S. diaprepesi* (Sd), *S. riobrave* (Sr), Sd + Sr and untreated control (no EPN). Pots were arranged on benches in a completely randomized design, watered as needed, fertilized bi-weekly and treated with malathion and oil as needed to control whitefly, leafminer, and scale insects. Greenhouse temperature ranged between 13.9 and 30.5°C.

The EPN were added to pots in the form of a single EPN-infected insect cadaver buried 3 cm deep (Perez *et al.*, 2004). To obtain infected cadavers, *D. abbreviatus* larvae (4-5-wk old, from a laboratory colony) were placed in individual Petri dishes (60-mm diam.) filled with autoclaved Candler fine sand (10% moisture) and 600 infective juveniles (IJ) of *S. diaprepesi* or *S. riobrave* (freshly emerged from insects in stock cultures) in 1 ml water were added to the dishes. The dishes were sealed with parafilm and stored at room temperature (23 ± 2°C). Cadavers were examined daily under a dissecting microscope for evidence of successful nematode reproduction. When nematode juveniles were clearly visible swarming beneath the insect cuticle, the cadaver was used immediately as inoculum. Care was taken to avoid damaging the cadaver by gently transferring the cadaver and surrounding sand from the incubation dishes to the experimental pots. Four weeks were required to infest all appropriate pots by this process. Ten cadavers containing each species were also incubated individually on moistened filter paper in Petri dishes until IJs emerged. Cadavers, paper and IJs were blended in 50 ml water and 5 ml of the IJ suspension were diluted 10-fold before counting a 5 ml aliquot to estimate the numbers of IJ of each species produced per cadaver.

Three weeks after EPN were added to a pot, three *D. abbreviatus* neonate larvae were buried 3 cm deep in the soil. Because the dates of adding EPN to pots varied, 4 weeks elapsed before all pots received live insect larvae. Insect larvae were added to controls on the same day they were first added to some of the EPN-treated pots. After the final pot received its initial treatment of insect larvae, an additional three larvae (4-5-wk old) were added to each pot at intervals of 2.5, 4, and 11 weeks. Approximately 6 weeks after the initial introduction of larvae, pots were sealed with plastic fly screen to prevent the escape of teneral adult weevils.

The experiment was terminated 46 weeks after seedlings were transplanted. Plant tops were weighed (fresh) and soil was sampled (2 cores, 2.5 cm diam. × 30 cm deep per pot), mixed and 113 cm^3 soil added to Petri dishes (60-mm diam.). To detect EPN in the soil, a single *D. abbreviatus* larva was added to each dish and after 7 days cadavers were placed in White traps (Glazer and Lewis, 2000) and monitored for EPN emergence.

Experiment 2

Seedlings were established as previously described and 18 replications of the following treatments were established: Sd, Sr, *Heterorhabditis zealandica* (Hz), Sd + Sr, Sd + Hz, Sr + Hz, positive control (insect larvae, but no EPN) and negative control (no insect larvae or EPN). Five thousand third-stage infective juveniles of EPN of the appropriate species in 5 ml water were pipetted onto the soil surface of each pot. All species of EPN were isolated from a citrus orchard with a history of augmentation biological control using Sr and maintained

in culture by periodically infecting *D. abbreviatus* larvae in moist (10%) autoclaved sand. Nematodes recovered from insect cadavers in White traps were stored in shallow water in transfer flasks (15°C) for between 1 and 5 days before use. At 0, 6, 16, 21, 37, and 51 weeks after EPN were introduced, two *D. abbreviatus* larvae (4-5-wk old) were introduced to pots of all treatments except the negative control. Plants were maintained in the same manner as described for the first experiment.

The experiment was terminated 2 months after the last addition of insect neonates and 13.6 months after the addition of EPN. Pots were watered a final time and the next day the roots were rinsed free of soil and the fresh weight of plant tops and roots was obtained. Insect adults, larvae, and pupae in pots were counted. To estimate the relative survival of EPN, the soil in each pot was mixed and 580 cm^3 soil and two *Galleria mellonella* (greater wax moth) larvae were added to plastic storage cartons maintained at 20-22°C. *Galleria mellonella* cadavers were recovered after 5 days, maintained in White traps, and emerging EPN were identified. Fresh larvae were added to replace each cadaver and the process was repeated twice, providing an opportunity for up to six larvae per pot to become infected.

Statistical Analyses

In both experiments, two-way analysis of variance was conducted for each possible pair of EPN species (General linear model; Minitab, Inc., Lancaster, PA). Mean separation was by Tukey's Honestly Significant Difference Test. The numbers of dead sentinel weevils in a pot at the end of experiment 2 were regressed (simple linear regression) against the number of adjacent pots that were initially infested with Sd.

RESULTS

Experiment 1

The mean, standard error, and range of numbers of IJ of Sd that emerged from ten *D. abbreviatus* cadavers were 33,040 ± 6,544 (6,000-74,300), respectively, compared to 52,350 ± 9039 (20,400-107,700) IJ of Sr. Plant top weights in the first experiment were 70% greater on average in Sd treatments than in the untreated control (Sd main effect: DF = 1, 47; F = 7.67; P = 0.008) (Fig. 1). Addition of Sr to pots had no effect on plant top weight (F = 0.1; P = 0.906), and there was no interaction between the two EPN species (F = 0.33; P = 0.57). *Steinernema diaprepesi*, but not *S. riobrave*, was recovered from 9, 3, 11, and 3 pots from treatments Sd, Sr, Sd + Sr and untreated control, respectively.

Experiment 2

The results of the analyses of variance of plant top weights (not shown) were similar to those of root weights (Table 1). The main effect of Sd on root weight and num-

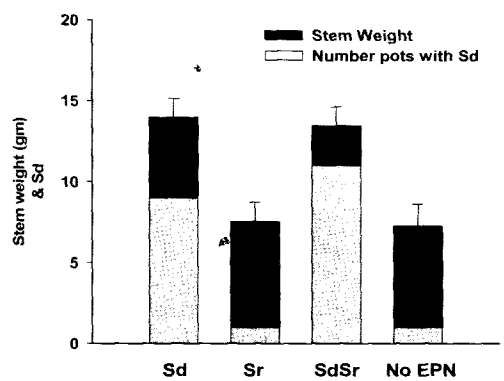

Fig. 1. Effects of *Steinernema diaprepesi* (Sd) or *S. riobrave* (Sr), alone or in combination, on citrus stem weight (black bars in background) and numbers of pots with *S. diaprepesi* (gray bars in foreground) recovered 46 weeks after treatment with nematodes. Error bars are mean standard errors of stem weight.

Table 1. Two-way analyses of variance (DF = 1,122) of the main effects and pairwise interactions of *Steinernema diaprepesi* (Sd), *S. riobrave* (Sr), and *Heterorhabditis zealandica* (Hz) on citrus root weight and numbers of *Diaprepes abbreviatus* recovered from pots 59 weeks after augmenting soil with entomopathogenic nematode (EPN) species alone or in pair-wise combinations and periodically adding weevil larvae.

	Root		Weevil	
EPN	F	P	F	P
Sd	35.56	0.001	25.71	0.001
Sr	14.50	0.001	1.88	0.173
Sd × Sr	0.46	0.500	1.52	0.220
Sd	25.34	0.001	24.87	0.001
Hz	0.02	0.895	0.02	0.893
Sd × Hz	0.37	0.544	0.07	0.788
Sr	4.76	0.031	0.14	0.713
Hz	0.27	0.604	0.97	0.327
Sr × Hz	3.57	0.061	0.54	0.462

bers of surviving weevils was highly significant when compared with either Sr or Hz. Significant main effects of Sr on plant growth contrasted with its lack of effect on the numbers of surviving weevils, suggesting that the influence of Sr on weevils waned over time compared to that of Sd. Hz did not affect plant weights or weevil survival. Interaction terms for each of the three possible pair-wise species comparisons were not significant.

Compared to the negative controls, *D. abbreviatus* alone reduced top and root weights by 97-98% (Fig. 2A-B). The effect of Sd on plant weight in the second experiment was similar to that in the first. The fresh weight means of roots treated with any combination of EPN containing Sd were between 29 to 45-fold greater than that of the positive control and did not differ from that of the negative control. In contrast to the first experiment, Sr afforded protection to the citrus plants. The treatments Sr and Sr + Hz produced heavier root systems and tops than those of the positive control, but significantly lighter than those of the negative control. *Heterorhabditis zealandica* had no affect on plant weights alone or in combination with Sd or Sr.

Numbers of weevils recovered from pots at the end of the experiment agreed with the effects of each species on plant growth (Fig. 2C). Fewer weevils remained in two treatments containing Sd than in the positive control or treatments without Sd. Numbers of weevils in treatments with Sr but no Sd did not differ from those for the positive control or the treatment with just Sd. *Heterorhabditis zealandica* had no effect on numbers of surviving weevils at the end of the trial.

Pots from all treatments, including many positive and some negative controls, were infested by Sd at the termination of the experiment (Fig. 2D). Neither Sr nor Hz were detected in any of the sentinel insect cadavers. In pots that were not treated with Sd, the rate of sentinel mortality was directly related to the number of adjacent Sd-treated pots (Fig. 3).

DISCUSSION

The long-lived Sd was a more effective biological control agent than shorter lived steinernematid and heterorhabditid species under the conditions of these experiments. In contrast to our expectation, competition with Sr and Hz was not shown to mitigate the effectiveness of Sd. It is possible that multiple augmentations of the shorter lived species would have reduced the numbers of Sd and eventually all EPN, because we did not recover any Sr or Hz at the end of either experiment. Our use of pasteurized soil also favored the survival of larger numbers of Sd than occurs naturally (Ishibashi and Kondo, 1986). Therefore, in the concomitant species treatments, even if all weevils initially added to some pots were killed by Sr or Hz, in the absence of natural enemies, the inoculum potential of Sd may have remained unaffected for weevils that were subsequently introduced.

The propensity of Sd, and perhaps the other EPN species, to migrate between experimental units was striking. The plants were watered carefully, and it is unlikely that the nematodes were moved though splashing water. Rather, they probably migrated between drainage holes in the bottoms of pots when pot and bench surfaces were wet following irrigation. The highly significant relationship between proximity of invaded pots to pots treated with Sd argues against the possibility that pots were inadvertently inoculated with Sd. Although by the end of the experiment, Sd was almost as prevalent in the positive control pots as in Sd treated pots, migration, and establishment of Sd did not occur soon enough to protect the positive control plants from near total destruction. Similarly, the protection of plants by treatments Sr and Sr+Hz demonstrates that Sr controlled weevils early in the experiment even though the nematode was undetected after 13 months. Nevertheless, the eventual widespread contamination of all treatments by Sd may have mitigated the

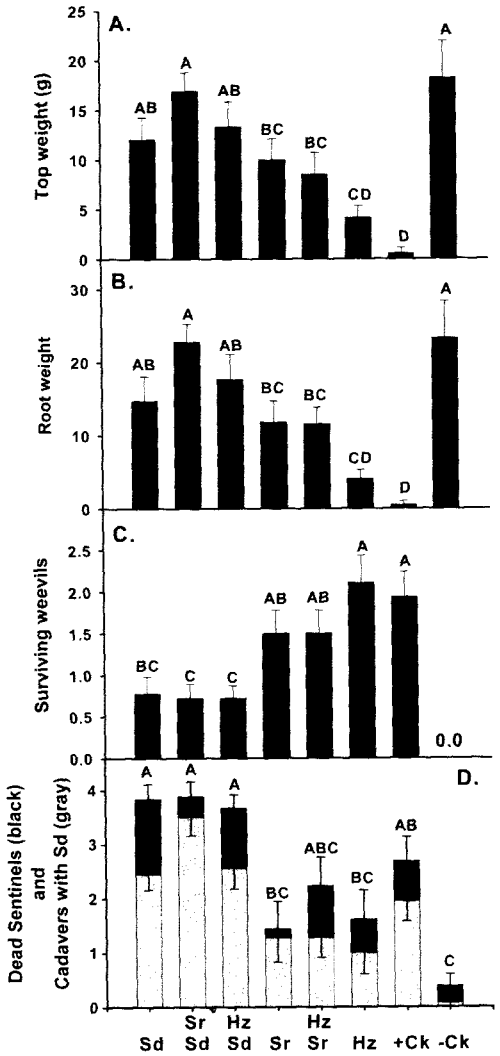

Fig. 2. Effects of *Steinernema diaprepesi* (Sd), *S. riobrave* (Sr), and *Heterorhabditis zealandica* (Hz), alone or in pair-wise combinations, on citrus top weight (A), root weight (B), numbers of *Diaprepes abbreviatus* recovered from pots 59 weeks after treatment with EPN (C), and numbers of dead *Galleria mellonella* sentinels (D; black bars in background) and *G. mellonella* containing *S. diaprepesi* (D; gray bars in foreground). Error bars are mean standard errors.

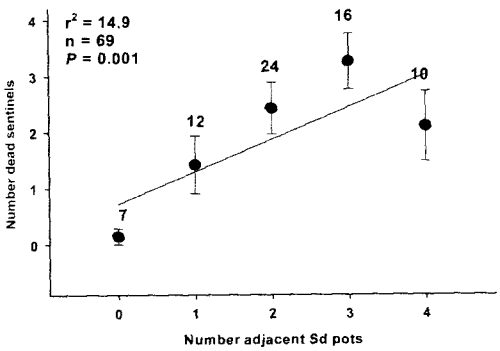

Fig. 3. The incidence of sentinel insect mortality in pots not inoculated with *Steinernema diaprepesi* in relation to the numbers of immediately adjacent pots (in 4 directions) that were treated with *S. diaprepesi*. Error bars are mean standard errors. Numbers above data points are numbers of replicates in a category.

differences between EPN treatments. Less likely is the possibility that Sr and Hz were able to migrate in sufficient numbers to effect the outcome in Sd-treated pots, because the effects of the Sd treatment did not differ from those of Sd+Sr or Sd+Hz, despite the initially large numbers of the concomitant species.

The design of this study favored EPN with greater longevity by providing prey at intervals as long as 11 and 14 weeks in the first and second experiments, respectively. Infective juveniles of Sd and Sr remained infective and viable for more than 2 years and less than 3 months, respectively, in the absence of hosts in autoclaved moistened sand in the laboratory (Duncan, unpublished). Longevity of Sr in water was reported to be 5 months (Grewal, 2000) and persistence of Hz in sandy loam soil at water potential between -10 and -100 kPa was negligible after 56 days, although persistence increased at lower moisture levels (Koppenhöfer and Fuzy, 2006). Shapiro-Ilan *et al.* (2006a) concluded that Sd and *S. carpocapsae* had the highest survival capacity among 29 strains of 11 EPN species, whereas survival of Sr was intermediate in sandy loam soil at field capacity. Thus, although Sr can be highly effective for augmentation biological control of *D. abbreviatus* (Schroeder, 1994; Duncan *et al.*, 1996; Bullock *et al.*, 1999), it is not surprising that Sd survived longer and provided greater plant protection when prey availability was restricted to long intervals. These data demonstrate the potential importance of IJ longevity in selecting EPN species for augmentation and emphasize the need to better understand relationships between the spatial and temporal patterns of insects and EPN in the soil (Fenton *et al.*, 2000, 2001).

In addition to the interaction between innate longevity of IJ EPN and food availability, predation by EPN antagonists is a key force in organizing patterns of EPN (Ishibashi and Kondo, 1986; Jaffee and Strong, 2005). Compared to heterorhabditids, steinernematid EPN contain the longest lived species (Strong, 2002); however, the greater tendency of infective steinernematids to loose the protective sheath (second-stage cuticle) makes them more susceptible than heterorhabditids to some forms of predation (Timper and Kaya, 1989, 1992). If the high mortality rates generally reported for augmented EPN (Strong, 2002) are due primarily to predation, it is difficult to predict whether innate longevity can affect EPN survival in the field enough to significantly modulate efficacy. Nevertheless, Sd exhibits several k-selected strategies and thus the relative susceptibility to antagonists of these large, long-lived EPN compared to smaller species merits additional study. Indeed, several traits found to be related positively to IJ longevity within populations of *H. bacteriophora* include tolerance of heat stress, ultraviolet radiation, and hypoxia, but not desiccation (Grewal *et al.*, 2002). Larger EPN were more fit than small species when competing with other EPN or free living

bactivorous nematodes for resources in the insect cadaver (Koppenhöfer *et al.*, 1995; Duncan *et al.*, 2003b). Matching the life strategies (k- or r-selected) of EPN with insects that are either continuously or occasionally present in soil, may be as important as matching EPN search strategies (ambusher vs. cruiser) with insect pests based on their relative motility (Kaya *et al.*, 1993). Whereas pests such as pink bollworm that merely overwinter in soil can be controlled with a single annual application of the more r-selected *S. riobrave* (Gouge *et al.*, 1997), highly persistent EPN may provide better control of larvae such as *Diaprepes abbreviatus* that are recruited into the rhizosphere throughout the year.

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