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The influence of mulch on the persistence of *Steinernema brazilense* (Nematoda: Steinernematidae) in sugarcane fields

Influencia del mantillo sobre la persistencia de *Steinernema brazilense* (Nematoda: Steinernematidae) en cultivo de caña de azúcar

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Abstract: Entomopathogenic nematodes (EPN´s) were tested for sugarcane soil dwelling pests, including the billbug, *Sphenophorus levis*. The successful application of EPN´s to protect sugarcane crops in Brazil requires the consideration of several aspects, including the widespread use of mechanical harvesting, which discards cane leaves in fields, resulting in a thick layer of mulch covering the ground. In this study, the influence of mulch on the persistence of *Steinernema brazilense* (strain IBCB n6) in sugarcane fields was examined. Nematode persistence in the soil was evaluated using *Galleria mellonella* larvae as an attractant. Results showed that *S. brazilense* can persists in sugarcane fields for at least 278 days after its application, and mulch does not affect its persistence, thus favoring its spread as compared to bare soil.

Key words: Entomopathogenic nematode. Biological control. Coleoptera.

Resumen: Los nematodos entomopatógenos (NEPs) son parásitos obligados de insectos, incluyendo al picudo de la caña *Sphenophorus levis*. Sin embargo, para la aplicación con éxito de NEPs en cultivos de caña en Brasil se debe tener en cuenta que la cosecha mecánica genera una capa de residuos de hojas (mantillo) en el suelo. En este estudio se evaluaron la influencia de la cobertura vegetal sobre la persistencia del nematodo *Steinernema brazilense* (cepa IBCB n6) en cultivo de caña, usando larvas de *Galleria mellonella*. *S. brazilense* puede persistir en el suelo de caña al menos durante 278 días después de su aplicación y el mantillo no afecta su persistencia, pero sí favorece su diseminación en comparación con el suelo desnudo.

Palabras clave: Nematodos entomopatógenos. Control biológico. Coleoptera.

Introduction

Sugarcane crop in Brazil occupies > 9 million hectares of land, just São Paulo state accounting for > 5 million hectares. Farmers expect that planted areas provide at least 5-6 ratton crops that mean the number of regrowth following a harvest. Meanwhile, several factors may affect the cane production in a way that the number of profiting ratton crops become much lower. Among these aspects, is notorious the importance of insect pests with emphasis on soil dwelling species that are the most diverse group, many of which are difficult to manage with chemical insecticides (Pinto *et al.* 2009). One promising alternative to this problem is to use entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis* (Nematoda: Steinernematidae, Heterorhabditidae) which are soil dwelling organisms that kill these pests and recycle on the cadavers (Leite *et al.* 2012).

EPNs are mutualistically associated with bacteria of the genera *Xenorhabdus* and *Photorhabdus*, which are voided from the nematode intestine into the hemolymph, propagate and kill the host insect by septicemia, usually within 48 h when infect susceptible hosts such as *Galleria mellonella* (Griffin *et al.* 2005). These nematodes possess a number of attractive qualities as biocontrol agents including a relative durable infective juvenile (IJ) stage, a broad host range, high virulence, great ability for host seeking, suitability to mass production and safety (Shapiro-Ilan and Gaugler 2002).

Several studies have tested EPNs against sugarcane pests, especially against the sugarcane billbug, *Sphenophorus levis* (Vaurie, 1978) (Coleoptera: Dryophthoridae), the larvae of which feed on sugarcane underground stems + rhizomes, damaging up to 80% of the stems and reducing cane productivity by 30%. Adults usually remain underground becoming also potential target for EPNs. Previous field trials applying *Steinernema brazilense* (strain IBCB n6) in the sugarcane rows (1×10^8 infective juveniles ha^{-1}), demonstrated that this nematode controls the billbug and enhance cane productivity (Leite *et al.* 2012).

The successful application of EPNs to protect sugarcane crops in Brazil requires consideration of several aspects, including the widespread use of mechanical harvesting, which discards the cane leaves in the field, resulting in a thick lay of mulch covering the ground. In this study, the influence of mulch on the persistence of *S. brazilense* (strain IBCB n6) in a sugarcane field was examined.

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Methods

The nematodes were obtained by *in vitro* production using the sponge method (Bedding 1984) and were used soon after being collected, with the infective juveniles (IJs) showing more than 90% viability.

The study was done in a second ratton of a mechanically harvested sugarcane crop (variety SP 803280, planted in rows 1.5 m apart) located in the municipality of Santo Antonio de Posse in São Paulo state. Two identical experiments located 400 m apart were carried out, both starting on 10 December 2007, 91 days after the first harvest on 10 September 2007, when the plants were on average 40 cm height. For each experiment, four treatments were considered: (1) soil ground treated with nematodes and covered with mulch, (2) soil ground not treated with nematodes, but covered with mulch (control), (3) bare soil treated with nematodes, and (4) bare soil not treated with nematodes (control). Each treatment was replicated five times and each replicate consisted of a 10 x 7.5 m plot containing five rows 10 m long, randomly distributed in blocks. The plots were 5 m apart within blocks, and 4.5 m apart (three rows) between blocks. Both experiments were set up in an area covered with mulch, and plots without mulch (bare soil groups) were raked to remove any covering. The nematodes were applied just once, at a dose of 1 x 10^8 IJs ha^{-1}, on 24 December 2007, using a manually operated backpack sprayer fitted with a jet tip that delivered 3 L $plot^{-1}$ (400 L ha^{-1}) in a 15 cm wide strip along both sides of each row. For the mulch group, a 15 cm wide strip along both sides of each row was uncovered to allow the nematodes to be applied directly to the soil. After application, the sprayed area was covered with mulch again.

Nematode persistence in the soil was evaluated using *Galleria mellonella* (great waxmoth) larvae to bait the nematode. Evaluations were done 14 (10 December 2007) and 7 (17 December 2007) days before nematode application, and 3 (27 December 2007), 15 (8th January 2008), 32 (25 January 2008), 76 (9th March 2008), 105 (7th April 2008), 160 (1st June 2008), 225 (5th August 2008) and 278 (27 September 2008) days after nematode application. The last evaluation was done immediately after sugarcane harvesting. For each evaluation, three soil samples were randomly collected from each plot, from the 20 cm wide strip along each side of the three central rows. From each spot surveyed, 1 kg of soil was collected and placed in a plastic chamber (15 cm diameter x 10 cm high) and transported to the laboratory, were five artificially reared larvae of *G. mellonella* were buried in each chamber. Before burying, the larvae were held inside a 10 cm x 6 cm metal-screen cage (aperture: 1 mm), together with 35 g of soil, and then buried in the chambers at a depth of 5 cm. To improve conditions for the EPNs, ~100 mL of water was added to each chamber. The chambers were incubated for five days at 25 ºC on the dark. At the end of this period, the metal-screen cages were removed, opened and the insect mortality due to *Steinernema brazilense* was assessed. Larvae killed by ENPs had a flaccid body, with worms leaving the cadaver after being placed in a white trap. If the ENPs isolated belonged to the genus *Steinernema* they were cross-bred (Kaya and Stock 1997) to confirm whether they belonged to the same species as that used in the experiments (*S. brazilense*). However, if the EPNs belonged to the genus *Heterorhabditis*, they were promptly identified by the red color of the cadavers. Molecular analysis through sequencing of 16S ribosomal RNA gene was done to confirm species identity. These identifications were necessary since the experiments were conducted at field conditions, where some other EPN species may be inhabiting the soil and infect the *G. mellonella* larvae.

Both experiments were combined as single one to result 10 replications. The mortality of *G. mellonella* was analyzed by one-way analysis of variance (ANOVA) followed by the Tukey studentized range test for multiple comparisons. Mortality rates were arcsine $\sqrt{x/100}$ transformed prior to analysis and all means were transformed back to the original units for presentation. All statistical comparisons were done using SPSS version 10.0 software, with a p value < 0.05 indicating significance.

Results and discussion

No *Steinernema* were found in the two surveys done before application, indicating that this genus did not occurred naturally in both experimental areas (Table 1). In contrast, indigenous *Heterorhabditis* sp. was detected at a low density and accounted for <16% of the mortality of *G. mellonella* larvae. Three days post application (PA), *S. brazilense* accounted for 49.3% of the mortality in *G. mellonella* larvae in both nematode treated groups (with and without mulch), indicating that *S. brazilense* IBCB n6 was introduced successfully in the sugarcane field. Several factors may have contributed to this introduction, including the high rainfall that helped to maintain soil moisture and the soil type, which was slightly sandy (clay = 23.8%, silt = 22.8%, total sand = 53.4%) favoring the action of the nematodes. In agreement, Susurluk (2009) reported that colonization and persistence of the nematodes *S. feltiae* and *H. bacteriophora* in different field crops and rotation regimes were correlated with the weekly precipitation following nematode release.

After introduction, there was a decreasing in *G. mellonella* infection rates that was not significantly affected by the presence or absence of mulch. In our study, the soil samples were rehydrated for baiting with *G. mellonella*, implying that the decrease in the mortality of the greater waxmoth was directly related to the decrease in the nematode population. Bednarek and Gaugler (1997) found that increased organic matter (organic manure) appeared to stimulate nematode establishment and recycling. The progressive decrease in the percentages of *G. mellonella* infected by *S. brazilense,* with no significant difference ($F_{39\,360} = 29.145$; $P = 0.124$) up to the 32th day PA in the nematode-mulch group, and up to 76th day in the nematode-soil, suggests that this nematode persisted for about one to two months before losing its effectiveness as an agent for controlling sugarcane pests. However, despite this decrease, *S. brazilense* remained the main nematode responsible for *G. mellonella* mortality, except prior to application, when the indigenous *Heterorhabditis* sp. was the only nematode present. According to Kung *et al.* (1991), *S. carpocapsae* and *S. glaseri* survived for 32 days at low soil moistures of 2 and 4%, when soil samples were kept at RH of 100%.

By 76 days PA, the nematode *S. brazilense* was also found in the control plots (confirmed by cross-breeding identification), which were located 4.5 - 5 m from the neighboring plots where the nematodes were sprayed. This finding indicates that the nematode spread throughout the experimental area, migrating at least 5 m in 76 days, i.e., at

Table 1. Mortality (%) of *Galleria mellonella* larvae (± standard error) caused by the nematodes *Steinernema brazilense* IBCB n6 (S) and *Heterorhabditis* sp. (indigenous) (H) in the *S. brazilense*-treated groups, with mulch (Nema-mulch) and without mulch (Nema-soil), and in the respective controls groups, with mulch (Control-mulch) and without mulch (Control-soil).

		Evaluation dates and days before and after nematode application								
	Treatment	**10/12/07 -14**	**17/12/07 - 7**	**27/12/07 3**	**08/01/08 15**	**25/01/08 32**	**09/03/08 76**	**07/04/08 105**	**01/06/08 160**	**05/08/08 225**	**27/09/08 278**
S	Control-mulch	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	34.0 ± 8.6 fgh	6.7 ± 4.2 abc	0.0 a	0.0 a	9.3 ± 1.6 bcde
	Nema-mulch	0.0 a	0.0 a	49.3 ± 1.6 h	45.3 ± 4.4 gh	40.0 ± 3.0 gh	21.3 ± 3.9 defg	14.7 ± 3.9 cdef	1.3 ± 1.3 ab	2.7 ± 2.7 ab	9.3 ± 4.4 abcd
	Control-soil	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	4.7 ± 2.5 abc	5.3 ± 2.5 abc	1.3 ± 1.3 ab	0.0 a	4.0 ± 2.7 abc
	Nema-soil	0.0 a	0.0 a	49.3 ± 4.5 h	40.0 ± 5.6 gh	36.0 ± 3.4 fgh	28.0 ± 6.5 efgh	8.0 ± 2.5 abcd	2.7 ± 2.7 ab	1.3 ± 1.3 ab	8.0 ± 3.3 abcd
H	Control-mulch	13.3 ± 3.0	8.0 ± 4.9	18.7 ± 6.8	4.9 ± 4.9	13.3 ± 3.7	4.0 ± 2.7	0.0	0.0	0.0	10.7 ± 9.1
	Nema-mulch	15.9 ± 6.9	5.3 ± 3.9	9.3 ± 4.5	3.3 ± 3.3	8.0 ± 6.5	2.7 ± 2.7	6.7 ± 6.7	0.0	0.0	5.3 ± 2.5
	Control-soil	12.0 ± 3.9	2.7 ± 2.7	13.3 ± 2.1	4.9 ± 4.9	14.7 ± 3.9	0.0	13.3 ± 6.3	0.0	0.0	5.3 ± 5.3
	Nema-soil	13.3 ± 2.1	2.7 ± 1.6	9.3 ± 3.4	2.1 ± 2.1	6.7 ± 2.1	0.0	0.0	0.0	0.0	9.3 ± 3.4

Means followed by the same letter in each row and column for *S. brazilense* (S) did not differ significantly by the Tukey test ($P < 0.05$). The data were arcsine $\sqrt{x/100}$ transformed prior to analysis.

least 1 m every 15 days. According to Weischer and Brown (2001), some species can move 3-6 m in approximately two months, or 1.5-2.0 m per month, which is similar to the rate observed in the current study. Passive dispersion by rain, wind, soil, humans or insects may occur over kilometers whereas active dispersal generally occurs over only a few centimeters (Smart and Nguyen 1994).

The high percentage of *G. mellonella* infected by *S. brazilense* in control-mulch group, not significantly different ($F_{39\ 360} = 29.145$; $P = 0.124$) from the nematode-treated groups at 3th day PA (49.3%), but significantly greater ($F_{39\ 360} = 29.149$; $P < 0.001$) compared to the control-soil group at 76th day PA (4,7%), probably reflected the greater suitability of mulch plots to enhance the nematode movement as well as to retain rainwater containing the nematodes from the treated plots, in addition to keeping the soil moist for a longer time. This result is in agreement to the study by Hsiao and All (1998), who showed that mulch can enhance the movement of some entomopathogenic nematodes in agricultural systems, with *S. carpocapsae* moving 3.5 cm/day on bare soil and 7.5 cm in rye mulch-covered soil.

Beyond 76 days PA, the percentages of *G. mellonella* infected by *S. brazilense* decreased in almost all treatments and remained low from 160 to 225 days PA, when the larva mortality was < 3.0%. From June to August (160-225 days PA), rainfall was low and may have accelerate a decreasing in the nematode population by drying the soil. This time has been a drought season for this region in Brazil. The level of moisture is one of the most important factors in the soil environment to influence the survival, virulence and persistence of nematodes (Klein 1990).

Beyond 225 days PA, the nematode population started to increase again, resulting in 9.3% mortality of *G. mellonella* larvae in both control-mulch and nematode-mulch groups by 278 days PA (last evaluation). The increase in the nematode population coincided with the beginning of the rainy season. Our results show that *S. brazilense* survived the unfavorable conditions associated with winter dry season, persisting in the soil by surviving as IJs or by recycling in the insect cadaver, for at least 278 days since its application.

S. brazilense can persist in the sugarcane field for at least 278 days after its application on the soil, and the mulch does not affect its persistence, favoring its spread compared to bare soil. The percentage of *G. mellonella* infected by the nematode decreased as the time approached to the draught season, dropping significantly after one - two month from the nematode application.

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