BIOLOGICAL CONTROL

Application Technology for the Entomopathogenic Nematodes *Heterorhabditis indica* and *Steinernema* sp. (Rhabditida: Heterorhabditidae and Steinernematidae) to Control *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) in Corn[1]

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Tecnologia de Aplicação para os Nematóides Entomopatogênicos *Heterorhabditis indica* e *Steinernema* sp. (Rhabditida: Heterorhabditidae e Steinernematidae) para Controle de *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) na Cultura do Milho

RESUMO - O efeito de diversas tecnologias de aplicação foi avaliado sobre a concentração, viabilidade e eficácia dos juvenis infectantes dos nematóides *Heterorhabditis indica* Poinar, Karunakar & David (IBCB-n5) e *Steinernema* sp. (IBCB-n6) no controle da lagarta-do-cartucho *Spodoptera frugiperda* Smith na cultura do milho. Para o controle da lagarta-do-cartucho no terceiro estádio em placas de Petri foram necessários 280 juvenis infectantes de *Steinernema* sp., enquanto que 400 juvenis infectantes de *H. indica* controlaram apenas 75% das lagartas. Podem-se pulverizar os entomopatógenos, sem que haja perda significativa na sua concentração e viabilidade, com equipamentos que forneçam carga elétrica à calda, ponta centrífuga e pontas hidráulicas. Entretanto, o emprego de pulverizadores com pontas que requerem elementos filtrantes com malha igual a 100 resultou em decréscimo na concentração de juvenis infectantes de *H. indica* e *Steinernema* sp., de 28% e 53%, respectivamente. Os tensoativos organosiliconado e etoxilados não afetaram a viabilidade dos juvenis infectantes de *Steinernema* sp. Nos experimentos de pulverização em plantas de milho (V6) com *Steinernema* sp., doses equivalentes a até 288 milhões de juvenis infectantes por hectare, diluídos em volume de calda de até 800 L ha^{-1} com 0,01 % do tensoativo etoxilado, ou nesse volume seguido de exposição a chuva artificial (lâmina de água de 6 mm), não foram suficientes para o controle de *S. frugiperda* em casa-de-vegetação.

PALAVRAS-CHAVE: Controle biológico, sistema de irrigação, lagarta-do-cartucho

ABSTRACT - The effects of different application technologies were evaluated on the concentration, viability, and efficiency of infective juveniles of the nematodes *Heterorhabditis indica* Poinar, Karunakar & David and *Steinernema* sp. (IBCB-n6) to control *Spodoptera frugiperda* Smith on corn plants. Two hundred and eighty infective juveniles of *Steinernema* sp. were required to kill 100% third-instar fall armyworms in petri dishes, as compared to 400 infective juveniles of the *H. indica* nematode to obtain 75% fall armyworm control. It is possible to spray entomopathogenic nematodes without significant loss in their concentration and viability, with equipment that produces electrical charges to the spraying mix, and with those using hydraulic and rotary nozzle tips. The concentrations of infective juveniles of *H. indica* and *Steinernema* sp. nematodes were reduced by 28% and 53%, respectively, when hydraulic spraying nozzles that require 100-mesh filtrating elements were used. Tensoactive agents of the organosilicone and ethoxylate groups did not affect the viability of infective juveniles of *Steinernema* sp. juveniles. Spraying corn plants (V6 growth stage) with up to 288 million infective juveniles of *Steinernema* sp. per hectare, diluted in the spraying mix up to 800 L ha^{-1}, with 0.01% ethoxylate tensoactive agent, or at the same volume followed by artificial rain (6 mm water depth) was not sufficient to control *S. frugiperda* in a controlled environment.

KEY WORDS: Biological control, irrigation system, fall armyworm, entomopathogenic nematoda

The most usual form of pest control used by growers is chemical control; however, other forms, such as the application of biological products, have been developed and employed in integrated pest management (Alves 1986). Among the organisms studied to control populations of *Spodoptera frugiperda* Smith, entomopathogenic nematodes should be highlighted (Ferraz 1998, Grewal *et al*. 2001). When *S. frugiperda* larvae are lodged inside the corn whorl the disposition of the leaves prevents the direct contact with another one organism and reduces the larval control.

The quantity of infective juveniles (IJs) for application in the field varies according to the crop, target insect, formulation, and application technology, (e. g. up to 2.5 billion infective juveniles ha^{-1} (Nguyen & Smart 1996). Entomopathogenic nematodes can be applied with equipment developed for pesticides, including backpack, boom (with or without air assistance), aerial, and electrostatic sprayers (Georgis 1990). Poinar (1986) and Georgis (1990) stated that nematodes can be applied using tips with openings larger than 0.05 mm. Klein & Georgis (1994) did not detect any significant influence on the viability and concentration of EPNs when applied with different tip openings. The viability and concentration of the nematode *Steinernema feltiae* Filipjev also remained stable when sprayed with tips having 50 mesh, as reported by Nilson & Gripwall (1999). Garcia (2003) pointed out that infective juveniles could be prevented from passing through or even harmed depending on the filters in the sprayer's hydraulic system.

Pre- and post-application humidity is essential for nematode movement, persistence, and infection (Poinar 1986). Application volumes vary with soil type, compaction, structure, crop, target insect, target insect behavior, formulation, and plant architecture. Berg *et al*. (1987) suggested application volumes between 935 L ha^{-1} and 2,800 L ha^{-1} with entomopathogenic nematodes to pasture for controlling subterranean insect pests.

In various experiments conducted to test the compatibility between entomopathogenic nematodes and adjuvants, all authors concluded that tensoactive agents did not negatively influence effectiveness of the formers (Beattie *et al*. 1995, Schroeder & Sieblirth 1997, Schroer *et al*. 2005).

The methods employed to apply bioinsecticides through irrigation systems are: conventional sprinkling, center pivot irrigation, and sprinkler irrigation under the canopy. According to Valicente & Costa (1995), fall armyworm control with the virus *Baculovirus spodoptera* applied through pivot was effective and that in a constant water depth of 6 mm, mortality is crescent according to the *B. spodoptera* doses used.

The objective of this research was to evaluate the effect of different application technologies on IJs of the entomopathogenic nematodes *Heterorhabditis indica* Poinar, Karunakar & David strain IBCB-n5 (Rhabditida: Heterorhabditidae) and *Steinernema* sp. strain IBCB-n6 in corn, on fall armyworm (*S. frugiperda*) control effectiveness.

Material and Methods

Entomopathogenic nematode origin. *Steinernema* species used in the experiments is new to science and it is herein referred to as *Steinernema* sp., deposited in the entomopathogenic nematode collection of the Coleção Entomolopatológica "Oldemar Cardim de Abreu", Instituto Biológico, under accession number IBCB-n6, and was isolated from a soil sample collected in a native forest area in the city of Porto Murtinho, Mato Grosso do Sul. The nematode *H. indica* is deposited in the same entomopathogen collection, under accession number IBCB-n5, and was isolated from a soil sample collected in a citrus area in the city of Itapetininga, São Paulo state.

Infective juveniles measured in average 0.52 mm and 1.28 mm in length for *H. indica* and *Steinernema* sp., respectively. The nematodes were multiplied based on the method proposed by Bedding (1981), and the fall armyworm was reared as proposed by Batista Filho (1988).

Infective juvenile dose determination. The dose of IJs required to kill third-instar fall armyworm larva was defined in experiments conducted in completely randomized experimental designs, with five treatments: zero (control), 50, 100, 200, and 400 infective juveniles per insect, with four replicates. The nematode concentrations (percentages of viable infective juveniles per 1.0 ml) were determined under a stereoscopic microscope using Peters counting slide. The infective juveniles were released onto filter paper (49 mm diameter) placed in a Büchner funnel attached to a Kitasato flask (1.0 L) with a cork. The Kitasato flask was connected to a vacuum pump with a suction pressure of 42 kPa to extract the excess water, since the same mix dilution was used, varying the volume according to the dose applied in each treatment. After suction for 30 seconds, the filter paper was transferred to a petri dish with the same diameter and a 13 mm height. Each dish was added of 0.3 ml distilled water (pH 6.8) to facilitate nematode movement.

After different doses were applied, fall armyworm larva were transferred from glass vials to petri dishes where they were kept for 14h, without access to diet, in an incubator adjusted to 25 ± 2°C; RH 70 ± 10%; and 12h photophase. After that period, the larvae were transferred to sterilized glass vials containing fresh diet kept in the incubator.

Entomopathogenic nematode application. In order to evaluate the effect of electrostatic spraying on nematode viability, a 2.0 L container isolated from the environment was used and a 40 kV negative current of 1.0 μA was applied. Treatments consisted in supplying the current during zero, 10, 20, 30, 40, 50, and 60 min, with five replicates. The IJs viability without receiving current at the end of 60 min was considered a control. The nematode concentration in the suspension was 1.25 10^6 IJs L^{-1} to different strains.

The effect of different filter meshes located before the spray tip on the concentration of infective juveniles was measured by collecting the nematode solution after it passed through the hydraulic circuit containing filters with 25, 50, and 100 mesh, and comparing with the solution without passing through the hydraulic circuit of a PJH® manual backpack sprayer (control). A D8 tip was used, with a DC45 diffuser, with the largest mesh (25). Infective juvenile viability was assessed by collecting the solution that did not pass through the hydraulic circuit and collecting

the solution that had passed through the hydraulic circuit with rotary tip and AI 110015VS, AI 11003VS, D3, and D8 hydraulic nozzle tips, with five replicates. The counting of viable IJs in the solution (1.25 10^6 IJs L^{-1}) that did not go through the hydraulic circuit after 190 min was considered the control. Micro Plex® (for rotary tips) and PJH® sprayers (for other tips) were used. Viability was determined in a petri dish using a stereoscopic microscope 10 min after collection, according to a method adapted from Vainio (1992). A completely randomized experimental design was used.

***Steinernema* sp. spraying on corn plants.** We selected the corn cultivar BR201 susceptible to fall armyworm in order to define the dose required to control the larva inside the corn whorl (Viana & Potenza 2000). Seeding was made in 3 L pots, containing substrate (one third soil and equal parts of manure and sand) added 200 kg ha^{-1} of NPK fertilizer with 50-20-50 formula, corresponding to 100, 40 and 100 kg ha^{-1} of these elements. The pots were maintained in a controlled-environment greenhouse (25 ± 10°C; RH 70 ± 10%). Spraying took place in the Laboratório de Tecnologia de Aplicação de Defensivos Agrícolas (FCA – UNESP), when the corn plants were at growth stage V6 45 days after emergence (Gallo *et al*. 2002). The volume of the nematode suspension (800 L ha^{-1}) was defined when the product applied to the corn whorl started to run off (Garcia & Ramos 2004). The start to run off is identified by maximum capacity of spray retention of the central leaves or corn whorl in this growth stage. An AVI 11002 tip at 200 kPa was used in the sprays, with the boom traveling at 0.98 km h^{-1}. One third-instar larva *S. frugiperda* was transferred into the whorl of each corn plant, 60 min before spraying. The pots were lined up under the center of the jet provided by the tip installed on the boom. The distance between the tip and the top corn-plant height was 60 cm.

Only *Steinernema* sp. IJs were sprayed because they showed a better performance in previous tests. A completely randomized experimental design was adopted, with three controls (300 *Steinernema* sp. viable IJs in a petri dish containing one third-instar larva; lodged inside the corn whorl without application of nematode solution; and one third-instar larva lodged inside corn whorl with the application of 800 L ha^{-1} water). Other treatments were: 300, 400, 500, and 600 viable IJs in water per plant, with four replicates. Each replicate consisted of seven plants, infested with one larva per plant. Concentration and percentage of viable IJs per mL were determined, using Peters counting slide under a stereoscopic microscope. Ten min after spraying, the tip of the upper leaves was cut and the whorl leaves were wrapped with non-woven fabric (TNT) attached with a rubber band to prevent larvae from escaping.

Larval mortality percentages under the various doses of infective juveniles were evaluated 48h after treatment. For this evaluation the corn plants whorl were opened up. Surviving larvae were transferred to sterilized petri dishes containing parts of the central leaves of corn plants. Mortality percentage evaluations were performed again at 96h and 144h post-spraying.

Compatibility between *Steinernema* sp. and tensoactive agents. Tensoactive agents reduce the surface tension of water or aqueous solutions to form a liquid continuous film on leaf surface, thus aiding the displacement of entomopathogenic nematodes. Compatibility between *Steinernema* sp. and tensoactive agents was studied in a completely randomized experimental design, with four treatments and five replicates. The treatments consisted in adding to the spray suspension surfactant agents Break-Thru® (polyether-polymethylsiloxane-copolymer 750 g L^{-1} and polyether 750 g L^{-1}), Extravon® (alkyl-phenol-polyglycolether 250 g L^{-1}), Iharaguens-S® (polyoxyethylene alkylphenol ether 200 g L^{-1}), and a control (suspension without tensoactive agent). The tensoactive agent doses were 0.1% Break-Thru® and 0.01 % Extravon® or Iharaguens-S® relative to the solution (Andrei 1999). These tensoactive agents were selected because they have distinct compositions and belong to different chemical groups (Foy 1992). The test was set in petri dishes adding to spray suspension the surfactant agents only.

A concentration of 500 IJs per replicate was used, determined under a stereoscopic microscope using Peters counting slide. Distilled water at pH 6.8 was used. The petri dishes were placed in a controlled-environment room (24 ± 2°C and RH 65 ± 10 %) for 24h.

***Steinernema* sp. sprayed with tensoactive agents on corn plants.** The spray equipment with work-pressure and travel-speed control of the Laboratório de Tecnologia de Aplicação de Defensivos Agrícolas was used to apply the nematode suspension plus the surfactant on corn plants, according to the procedure previously described in this work. A completely randomized experimental design was adopted, with three controls (600 viable *Steinernema* sp. IJs in a petri dish containing third-instar larvae; one third-instar larvae lodged inside the whorl without application of nematode suspension; one third-instar larvae lodged inside the whorl with application of 800 L ha^{-1} water with 0.01% Iharaguens-S®), and suspension containing 0.01% Iharaguens-S® plus 600; 1,200; 2,400; and 4,800 IJs in water per plant, with four replicates. This tensoactive agent was chosen because it is registered as spray adjuvant at lowest doses (0.01%) to agricultural crops in Brazil (Andrei 1999).

Application of *Steinernema* sp. via the irrigation system in a controlled environment. We also studied the possibility to apply *Steinernema* sp. on corn plants via the irrigation system, using a water depth of 6.0 mm. The water depth was obtained with the same simulator used in the spray, with a TK-SS5 10 tip at 300 kPa and the boom traveling at 1.58 km h^{-1}. The experimental design was completely randomized, with seven treatments and four replicates. Each replicate consisted of seven plants infested with one larva per plant. The treatments consisted of three controls (600 viable *Steinernema* sp. IJs in a petri dish containing one third-instar *S. frugiperda* larva; one third-instar larvae lodged inside the whorl without nematode solution but with water depth; one third-instar larvae lodged in the whorl with the application of 800 L ha^{-1} water plus the water depth), and solution containing 600; 1,200; 2,400; or 4,800 viable *Steinernema* sp.

IJs in water per plant. The spray method was described in the previous steps of this experiment. During conduction of the experiments, the climatic conditions were always maintained within the limits suggested by Poinar (1986).

Statistical analysis. In all steps of this study, sample size was defined by the stabilized mean and/or standard deviation of the data obtained in preliminary assays (Kranz 1988). The homocedasticity of variances was determined by Hartley's test, and data transformations were made as needed (Banzatto & Kronka 1995). F test and polynomial regression tests were used to determine possible differences between treatment variance estimates of larval mortality in relation to entomopathogenic nematode doses. We chose to adjust the variation of data by applying polynomial regression in order to derive the curve and define the optimal entomopathogen dose point (Banzatto & Kronka 1995). Nematode viability was analyzed via the F test to compare variance estimates; means comparisons were analyzed by the LSD test.

Results and Discussion

Data variability and sample size. The Hartley test indicated homocedasticity of variances; therefore, data transformations were not required in any of the statistical analyses performed in this research. When sample size was defined using the method proposed by Kranz (1988), we chose to use the smallest dose (50 IJs) per third-instar *S. frugiperda* larvae studied, because this dose showed the greatest data variation. The standard deviation for percentage of larvae killed by infective juveniles of *H. indica* and *Steinernema* sp. became stabilized at seven larvae per replicate. The viability rate of entomopathogenic nematodes was above 90% in all experiments.

Infective juvenile dose determination. Significant differences were observed between doses of entomopathogenic nematode infective juveniles. The mortality of fall armyworm larvae obtained with the application of 400 *H. indica* IJs was 75%. However, because significance was obtained with a first-degree regression, the point on the curve that is farthest from the horizontal axis was not determined, preventing a conclusion about which is the best ratio between larva mortality and doses under study. For the nematode *H. indica*, the significant regression was at a first-degree level (Fig. 1). With respect to *Steinernema* sp., the polynomial regression was significant for the quadratic equation (Fig. 2). The derivation of the curve of third-instar larvae mortality, with different doses of infective *Steinernema* sp. juveniles, determined that the best ratio was achieved with 280 nematodes. The results in this experiment give a partial idea of the EPN potential of the nematodes *H. indica* and *Steinernema* sp., as stated by Grewal *et al*. (2001).

Entomopathogenic nematode viability and application techniques. In the electrostatic spray, the F test did not indicate significant differences between treatments, dismissing data variation adjustments via the application of polynomial regression. The viability of *H. indica* IJs (overall mean 95%) and *Steinernema* sp. (overall mean 91%), was not affected by supplying of negative 40 kV (1.0 microampere) with time. Significant differences between treatments for concentrations of IJs of *H. indica* and *Steinernema* sp. were detected when they passed through the hydraulic circuit with different filter mesh sizes in the spray tips. For both nematodes, the concentration of infective juveniles significantly decreased when the 100 mesh was used (Table 1). These results corroborate the conclusions obtained by Nilson & Gripwall (1999), who did not detect significant differences in the viability and concentration of nematodes when sprayed with tips having 5.2 mm and 5.3 mm openings (mesh 50).

According to the data obtained in this experiment, the warning issued by Garcia (2003) on the special attention that should be paid to the filters in the sprayer's hydraulic

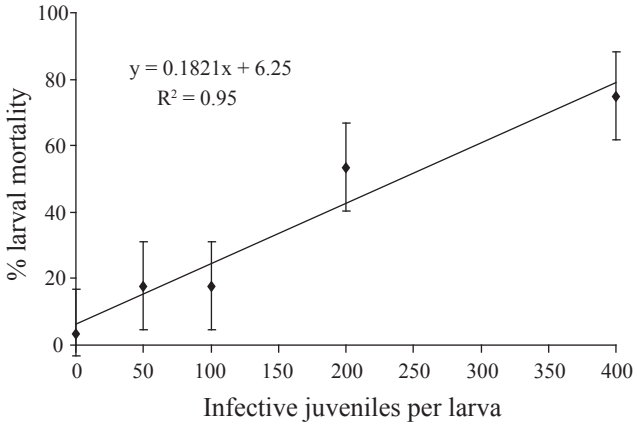

Fig 1. Mean percentage mortality of third-instar fall armyworm larvae (*S. frugiperda*), 48h after applying different doses of infective *H. indica* juveniles in a controlled environment (25 ± 2°C; RH 70 ± 10 %; and 12h photophase).

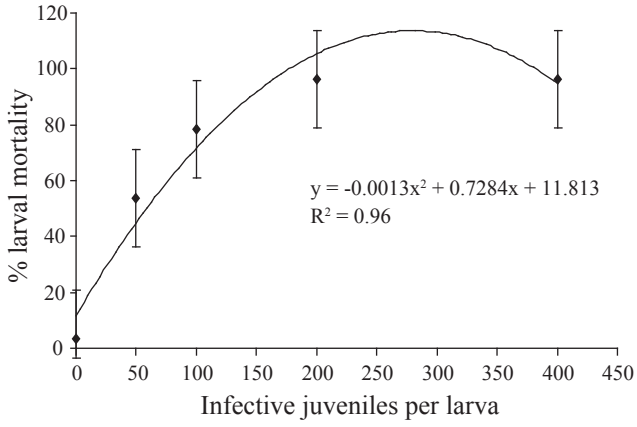

Fig 2. Mean percentage mortality of third-instar fall armyworm larvae (*S. frugiperda*), 48h after applying different doses of infective *Steinernema* sp. juveniles in a controlled environment (25 ± 2°C; RH 70 ± 10 %; and 12h photophase).

Table 1. Concentration of infective juveniles of *H. indica* and *Steinernema* sp. after passing through the hydraulic circuit of a PJH® sprayer (200 kPa, D8 tip and DC45 diffuser), with nozzle filters of different meshes.

Treatments	Concentration of *H. indica* (infective juveniles ml^{-1})	Concentration of *Steinernema* sp. (infective juveniles ml^{-1})
Control [1]	419 ± 14 a	1,464 ± 26 a
25 mesh nozzle filter	414 ± 13 a	1,457 ± 23 a
50 mesh nozzle filter	411 ± 15 a	1,447 ± 24 a
100 mesh nozzle filter	303 ± 10 b	690 ± 20 b
Overall mean	387 ± 13	1,265 ± 23
C.V. (%)	3.4	1.8

[1]The concentration of juveniles present in the mix that did not pass through the sprayer's hydraulic circuit was considered as control.
Means followed by the same letter in the columns do not differ significantly from each other by the LSD test (P > 0.05).

system is relevant, since they can act as barriers against infective juveniles. On the other hand, the results obtained do not confirm the conclusions of Poinar (1986) and Georgis (1990), as the nematodes can be applied with 0.05 mm openings tips (meshes lowest at 200). In addition, the data obtained did not confirm the significant influence on the viability and concentration of the entomopathogens, when *H. bacteriophora* and *Steinernema* sp. are sprayed with XR8001 tips (100 mesh filter), as identified by Klein & Georgis (1994).

Significant differences between treatments for the viability of *H. indica* and *Steinernema* sp. infective juveniles when passing through the hydraulic circuit with different spray tips were observed. For both nematodes, infective juvenile viability significantly decreased when the tip AI 110015-VS was used (Table 2). The other treatments were not different from one another (P > 0.05). Again, the significant differences identified between treatment means for the concentration and viability variables were due to the filter element, with a mesh count of 100 wires per inch.

***Steinernema* sp. spraying on corn plants.** The F test and the polynomial regression test could not be applied due to the lack of variance among the results obtained in the treatments. The control with infective juveniles in the petri dish resulted in 100% larval mortality after 144h. Control ineffectiveness may have resulted from dose, poor distribution, and lack of humidity for the nematode to act upon the larva, as discussed by Poinar (1986), Berg *et al*. (1987), and Nguyen & Smart (1996). However, the application of spray volumes higher than 800 L ha^{-1} with boom sprayers is well above the volumes used in chemical control and may render the biological control under study unfeasible. The dose issue might be resolved by using quantities higher than 600 infective juveniles per plant and different application technologies in future experiments. With regard to distribution and humidity, it can be speculated

Table 2. Viability of infective *H. indica* and *Steinernema* sp. juveniles after passing through the hydraulic circuit with different spray tips.

Treatments				*H. indica* (%)	*Steinernema* sp. (%)
Tips	Filter mesh	Sprayer	Pressure (kPa)		
Suspension not passing through the sprayer	-	-	-	94 ± 0.9 a	81 ± 0.5 a
AI110015VS	100 (nozzle)	PJH®	200	90 ± 1.1 b	75 ± 1.0 b
AI11003VS	50 (nozzle)	PJH®	200	93 ± 0.9 a	79 ± 2.6 a
D3 - DC25	25 (nozzle)	PJH®	200	92 ± 1.1 a	79 ± 1.9 a
D8 - DC45	25 (nozzle)	PJH®	200	93 ± 0.8 a	80 ± 2.7 a
Rotary	25 (tank)	Micro Plex®	-	93 ± 0.8 a	82 ± 1.5 a
Control [1]	-	-	-	93 ± 1.1 a	81 ± 1.9 a
Mean				93 ± 1.0	79 ± 1.9
C.V. (%)				1.1	2.4

[1]The viability recount of infective juveniles in the mix that did not pass through the sprayer's hydraulic circuit at the end of the 190 minutes spent in the assay, in order to identify a potential viability decrease with time, was considered as control.
Means followed by the same letter in the column do not differ significantly from each other by the LSD test (P > 0.05).

that because the larva is located, in general, in the center of the corn-plant whorl, the availability of sufficient humidity for the infection to occur would be more important than the distribution over all central leaves. Because high solution volumes are only economically feasible via the irrigation system, the properties imparted by tensoactive agents to the solution could be utilized.

Compatibility between *Steinernema* sp. and tensoactive agents. The viability of *Steinernema* sp. infective juveniles was not affected when the tensoactives Break-Thru® at 0.1%, and Extravon® and Iharaguens-S® at 0.01% were added to the suspension ($P > 0.05$). The results obtained corroborate the data presented by Beattie *et al*. (1995), Schroeder & Sieblirth (1997), and Schroer *et al*. (2005). All authors concluded that tensoactive agents did not negatively influence effectiveness of entomopathogenic nematodes (*Steinernema riobravis* and *Steinernema carpocapsae*).

***Steinernema* sp. spraying with tensoactive agents on corn plants.** The percent larval mortality in the treatment without tensoactive agent (control) using infective juveniles in petri dishes was 95% after 144h. The spray volume of 800 L ha^{-1} with the added tensoactive properties of Iharaguens-S® at 0.01% was not sufficient to provide the conditions required for the entomopathogen to infect the larvae.

Application of *Steinernema* sp. via the irrigation system in a controlled environment. No larval mortality was observed when the entomopathogen was applied via the irrigation system on corn plants in a controlled environment with the adoption of a water depth of 6 mm. Even when an application volume 17 times greater than that required to cause larval death in petri dishes, the conditions required to control *S. frugiperda* larvae in corn using *Steinernema* sp. infective juveniles were not met. Because of leaf architecture and insertion angle into the corn plant stem, such lack of control effectiveness could be related to the lack of a minimum water depth necessary for the nematode to move up to the host. Additional studies on the application technology of entomopathogenic nematodes for fall armyworm control are needed.

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