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Hydraulic spray nozzles for entomopathogenic nematode application

Boquillas de pulverización hidráulicas para la aplicación de nematodos entomopatógenos

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Abstract: Biological control is a viable alternative to chemical control; however, some methods of releasing control agents still need to be improved in order to attract the attention of producers and to reduce costs. Therefore, this work was conducted to evaluate the effects of hydraulic spray nozzles on the viability and infectivity of entomopathogenic nematodes. The experiment was conducted using a completely randomized design with 37 treatments, of which there were three controls and 34 treatments using different spray nozzles with six repetitions. A spraying table was used for the applications. The working pressure was 400 kPa for all nozzles and the solution sprayed consisted of an aqueous suspension of the nematode *Heterorhabditis amazonensis* MC01 at a concentration of 400 infective juveniles (IJ) mL^{-1}. The viability of the IJ after being sprayed, as well as the infectivity of *Tenebrio molitor* larvae were verified. The results indicated that nematode viability was influenced by the nozzles and the living nematodes sprayed did not lose their capacity to infect the insect.

Key words: Application technology, biological control, hydraulic spray.

Resumen: El control biológico es una alternativa al control químico, sin embargo, algunos métodos para su aplicación aún requieren ser mejorados para que atraigan la atención de los usuarios y minimizar costos. En este sentido, este trabajo se llevó a cabo para evaluar el efecto de boquillas de pulverización hidráulica sobre la viabilidad y la infectividad de nematodos entomopatógenos. El experimento tuvo un diseño de bloques completamente al azar, con 37 tratamientos, tres controles y 34 boquillas de pulverización con seis repeticiones. Para las aplicaciones, se utilizó una mesa de pulverización con una presión de 400 kPa. Durante el experimento, se asperjó una suspensión acuosa del nematodo *Heterorhabditis amazonensis* MC01 a una concentración de 400 juveniles infectivos (JI) ml^{-1}. Se verificó la viabilidad de los JI después de haber sido pulverizado y su infectividad en larvas de *Tenebrio molitor*. Los resultados indicaron que la viabilidad de nematodos fue influenciada por las boquillas de pulverización hidráulica pero que aquellos viables, no perdieron su capacidad de infectar al insecto.

Palabras clave: Técnica de aplicación, control biológico, pulverización hidráulica.

Introduction

The entomopathogenic nematodes (EPNs) of the Heterorhabditidae and Steinernematidae families are increasingly studied with a view to controlling insect pests; these organisms present a suitable adaptability to environments and also the capacity to disseminate in search of their host (Grewal *et al*. 2001). EPNs can be applied by spraying, sprinkler or even drip irrigation systems (Wennemann *et al*. 2003; Garcia *et al*. 2008), in order to reach the insects located in the soil. This also allows the nematodes to remain in the soil over time (Navon *et al*. 2002). Moreover, these organisms are compatible with various pesticides; both biological and chemical control agents. In some cases, synergy is observed, enabling the use of these organisms in integrated pest management programs (Koppennhöfer and Kaya 1998).

The application of nematodes via hydraulic spraying has already been studied in works performed by Bellini and Dolinski (2012) and Leite *et al*. (2012); however, the interaction of nematodes with different hydraulic nozzles needs to be better understood, including the use of specific species of EPNs to control pests, since each species could react to the nozzles in a different way.

Bellini and Dolinski (2012) verified no significant interaction between *Heterohabditis baujardi* Phan, Subbotin, Nguyen and Moens, 2003 LPP7 strain and *Steinernema carpocapse* (Weiser, 1955) Wouts, Mracek, Gerdin & Bedding, 1982 All strain with the commercial adjuvants Gotafix® (Milenia) and Joint Oil® (Dow AgroSciences), when applying infective juveniles (IJs) in a sugarcane crop area. Leite *et al*. (2012) tested the nematodes *Heterorhabditis indica* Poinar, Karunakar and David, 1992 and *Steinernema brazilense* Nguyen, Ginarte, Leite, Santos and Harakava, 2010 with thiamethoxam (Actara® 250 WG) at doses of 500 and 1,000 g ha^{-1} of commercial product (c.p.), fipronil (Regent® 800 WG) 250 g ha^{-1} c.p., and no negative effect of these pesticides was observed on the nematodes' viability.

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Studies conducted by Schroer *et al*. (2005), indicated that it is possible to replace chemical control with biological control using *S. carpocapsae* and *Steinernema feltiae* (Filipjev, 1934) Wouts, Mracek, Gerdin and Bedding, 1982 to control the diamondback moth, *Plutella xylostella* (Linnaeus, 1758) (Lepidoptera: Plutellidae) in cabbage (*Brassica oleracea* var. *capitata*), achieving a satisfactory mortality index. These studies corroborate the possibility of using EPNs to control pests, even when associated with chemical pesticides, which makes it possible to apply different control agents in the same operation in the field, reducing costs.

Garcia *et al.* (2005) while conducted experiments in order to study the effect of working pressure on the viability of *Metarhizium anisopliae* (Metsch.) Sorokin, 1883, *Bacillus thuringiensis* var. *kurstaki* (Berliner, 115) and *Steinernema glaseri* (Steiner, 1929) Wouts, Mracek, Gerdin & Bedding, 1982, found that pressures up to 1,379 Mpa did not cause losses in the viability, keeping the nematodes alive and able to cause mortality in the pests.

Spray nozzles and sprayers are devices that can interfere in the application of EPNs, and there are no studies comparing different ways of spraying, such as aerial, hydraulic, hydropneumatic, pneumatic or electrostatic and their effects on the living organisms in the spray solution (Brusselman *et al.* 2012).

The effectiveness of the use of entomopathogenic organisms has been proved in several studies performed by Schroer *et al.* (2005), Leite *et al.* (2012) and Lanzoni *et al.* (2014). However, the interaction between the hydraulic nozzles routinely used for the application of pesticides and entomopathogenic nematodes is still little understood. Carrying out work that demonstrates results with different nozzles can assist the rural producer in selecting the best application method. It will also help the industry to develop nozzles that allow higher viability and for infectivity of the applied agents. Thus, the application of EPNs can be integrated with techniques already practiced on the farm making this procedure more feasible.

Highlighting the potential of EPNs to control insect pests, the aim of this study was to analyze the effect of the interaction between hydraulic spray nozzles and *Heterorhabditis amazonensis* Andaló, Nguyen and Moino Jr., 2006 strain MC01, a Brazilian native species, evaluating the indexes of viability and infectivity on *Tenebrio molitor* Linnaeus, 1758. (Coleoptera: Tenebrionidae) larvae.

Materials and methods

The experiment was conducted at the Entomology Laboratory and the Machinery and Mechanization Laboratory located at the Federal University of Uberlândia. The application was made in a controlled environment with mean temperature of 25 °C, mean relative humidity of 60 % and no wind, using a uniform distribution table with three nozzle supports spaced 0.5 meters apart.

Heterorhabditis amazonensis MC01 is an EPN isolated from the soil by bait insect technique using *T. molitor*. They were identified by morphological and molecular techniques. These species have a mean of 589 µm in length and 23 µm in diameter (Andaló *et al*. 2006).

The population was maintained in Erlenmeyer flasks in acclimated chambers at 16 ± 1 °C, in an aqueous suspension containing 500 to 1,000 IJs mL^{-1}. This nematode strain was chosen because of its adaptability to the local climate, since it is a native population with potential use to control pests. Nematodes were reared on *Galleria mellonella* larvae (Linnaeus, 1758) (Lepidoptera: Pyralidae) grown according to the methodology described by Dutky *et al.* (1964), using an artificial diet modified by Parra (1998). The nematode culture and maintenance were done as described by Molina and López (2001). The nematode suspension was diluted and quantified using a stereomicroscope to adequate the concentration for the experiment. The nematodes used emerged from day 2 to day 5 and were stored for up to 3 days until used in the experiments.

Pressure was monitored by a Wika brand manometer, CL.A model, with full scale between zero and 2,000 kPa. The working pressure was 400 kPa for all nozzles, which is the pressure range recommended by the manufacturer. The spray was activated by a centrifugal pump with a flow rate of 50 L min^{-1}, KSB brand, Hidroblc P 1000 model.

The experiment was conducted in a randomized complete design with 37 treatments, being 34 models of hydraulic nozzles with different spectrum of droplets e three controls; for each nozzle model were used six repetitions (six different nozzles of the same models), following Lanzoni *et al.* (2014) methodology, and three control tests. The applications were performed using a 50 mesh filter. The nozzles were used with line filters according to the manufacturer's recommendations, although Garcia *et al.* (2005) considered that the filters could interfere in the results, so they removed them.

The control group consisted of three evaluations. The first evaluation was the viability of IJ collected directly in the spray tank after five minutes of agitation; the second was also obtained from the suspension tank six hours after spraying; and the third control was obtained nine hours after spraying, at the end of the evaluations. The controls were used to determine the effect of the hydraulic system on viability and infectivity of EPNs.

A volume of 50 L of the nematode *H. amazonensis* MC01 suspension was put in a tank at a concentration of 400 IJ mL^{-1}, following Brusselman *et al.* (2012). The water used in the experiment came from a well, with a pH varying between 6.0 and 6.5; without treatment, aiming to expose the nematodes to conditions close to those in the field. Therefore, the water quality was similar to that used by the producer to perform spraying in agriculture, in contrast to the study by Garcia *et al.* (2005), which used deionized and sterile water to prevent the growth of contaminants.

After the beginning of the application and the stabilization of the flow in the nozzle, 30 seconds after the pump was activated, a sample of at least 2 mL was collected from the sprayed suspension to perform the viability and infectivity tests, of which 1 mL was used for each test. The viability test was conducted to verify the survival of IJ after being sprayed, while the infectivity test aimed to verify if the juveniles are still able to infect the insect.

To perform the viability test, 0.1 mL of the nematode suspension was added to each well of an ELISA plate (commonly used for serological tests, containing 96 wells) up to a total of 10 wells and the data of each nozzle were obtained by the average means of these readings. The counting was done using a stereoscopic microscope (Nikon model). The number of live and dead nematodes in each well was counted, obtaining the percentage of live IJ from a total

of one milliliter of suspension, using a methodology similar to that of Lanzoni *et al.* (2014).

To evaluate the infectivity test, 1 mL of the sprayed IJ suspension was collected. The suspension was applied to a Petri dish (9 cm diameter) containing two sheets of filter paper and four larvae of *T. molitor* in fourth or fifth instars (*T. molitor* was chosen because is considerate a sentinel host to isolate EPNs and it is an insect with high mortality response (Yan *et al*. 2016), so it was used as a differential and additional test host, since *H. amazonensis* was isolated in *G. mellonella* larvae), resulting in 24 larvae per treatment (four larvae per repetition). The dishes were placed in B.O.D. (Biochemical Oxigen Demand) at 26 ± 1 °C and a photoperiod of 12 h. The verification of the nematode infectivity on the larvae was performed after five days, confirming mortality through the symptoms of the *Heterorhabditis* genus, with a methodology adapted from Noosidum *et al.* (2016).

The data were submitted to analysis of variance; if data were considerate significant for the F test, the Scott-Knott test was applied at 5 % probability to compare means. The data showed normal distribution by the Shapiro-Wilk test and homogeneity of variances by the Levene test, so transforming data was not necessary.

Results and discussion

Weather conditions inside the laboratory were favorable to conduct the experiment without interfering in the treatments. According to Noosidum *et al.* (2016), the survival of the nematodes is mainly dependent on the method of application, weather conditions and the habits of the nematode and of the host insect. Due to this importance, the weather factor was monitored and observed to be constant while the experiment was conducted.

In both tests and treatments the average of infective juveniles in 1 mL aliquot after the spraying was variable, however similar to the number in the original suspension (400 JI mL^{-1}), since it was not observed parts of infective juveniles that suffered damaged.

The hydraulic application affected the viability and infectivity of nematodes on *T. molitor* larvae, indicating that mortality may occur after passing through the hydraulic system. However, the living (viable) nematodes maintained their ability to infect and cause insect death (Table 1).

The infective juvenile's viability before the experiment was 99 % and the infectivity to *T. molitor* larvae was 100 %. The passage of the nematodes through the centrifugal pump in the first five minutes demonstrated that the nematode's lifetime in the solution was affected, as observed in control treatments for tests 1, 2 and 3, since different viability occurred over time. However, after nine hours of stirring, the viability rate remained high even for test 3. The nematode infective capacity was not influenced by the stirring time, indicating that even after an extended period of stirring the viable nematodes remained infective (Table 2).

The nematodes that went through the spray pump and the ones that returned to the tank, although they differed from each other, did not suffer drastic reductions in their viability. Viability was 98.6, 95.7 and 92.7 % in the treatments with tests 1, 2 and 3, respectively. Despite the statistical difference between the control treatments, it is undeniable

Table 1. Effect of hydraulic application with different nozzles on the viability and infectivity of *Heterorhabditis amazonensis* MC01 infective juveniles.

Variation sources	Viability	Infectivity
Nozzles	47.3*	767.4*
Error	0.5	1,355.8
CV (%)	1.63	24.8

* Significant at 5 % probability level by F test.

Table 2. Effect of hydraulic nozzles on the viability of the nematode *Heterorhabditis amazonensis* MC01 and its infectivity on *Tenebrio molitor* larvae.

Nozzle	Viability (%)	Infectivity (%)
GAT 110 02	99.2 ± 0.15 a	91.6 ± 2.15 a
GRD 120 02	98.7 ± 0.24 a	58.3 ± 6.27 b
Test 1	98.6 ± 0.10 a	95.8 ± 1.70 a
GAT 110 03	98.3 ± 0.19 a	91.6 ± 2.15 a
GAT 110 025	98.0 ± 0.20 a	100.0 ± 0.0 a
GRD 120 03	98.0 ± 0.10 a	79.1 ± 3.14 b
GAT 110 04	97.7 ± 0.14 a	91.6 ± 2.15 a
ADGA 120 03	97.7 ± 0.25 a	79.1 ± 1.70 b
ST-IA 140 04	97.5 ± 0.18 a	91.6 ± 2.15 a
ADGA 120 04	97.5 ± 0.26 a	87.5 ± 2.28 a
GRD 120 04	96.9 ± 0.10 a	87.5 ± 2.28 a
AVI 110 10	96.4 ± 0.15 b	75.0 ± 3.72 b
JAP 110 04	96.3 ± 0.11 b	70.8 ± 4.87 b
ATR 80 04	96.0 ± 0.14 b	79.1 ± 4.87 b
AD-IA 110 03	95.9 ± 0.10 b	100.0 ± 0.0 a
Test 2	95.7 ± 0.23 b	87.5 ± 3.49 a
JHC 80 04	95.7 ± 5.05 b	58.3 ± 0.1 b
JSF 110 04	95.7 ± 0.18 b	66.6 ± 3.40 b
JSF 110 03	95.6 ± 0.26 b	87.5 ± 3.49 a
CV-IA 100 04	95.4 ± 0.10 b	70.8 ± 4.10 b
JAP 110 05	95.0 ± 0.14 b	66.6 ± 4.30 b
ADI 110 03	94.8 ± 0.32 b	75.0 ± 6.45 b
JHC 80 03	94.8 ± 0.20 b	87.5 ± 2.28 a
ST-IA 140 03	94.5 ± 0.15 b	79.1 ± 1.70 b
CV-IA 100 03	94.2 ± 0.19 c	100.0 ± 0.0 a
CV-IA 110 02	93.7 ± 0.30 c	79.1 ± 3.14 b
AXI 110 06	93.5 ± 0.26 c	75.0 ± 3.73 b
AD-IA 110 02	93.5 ± 0.21 c	95.8 ± 1.70 a
JAP 110 03	93.4 ± 0.34 c	66.6 ± 4.30 b
AXI 110 03	93.3 ± 0.26 c	79.1 ± 4.88 b
AXI 110 04	93.3 ± 0.43 c	62.5 ± 4.38 b
Test 3	92.7 ± 0.22 c	83.3 ± 5.04 a
GAT 110 05	91.7 ± 0.13 d	79.1 ± 1.70 b
ST-IA 140 02	91.2 ± 0.42 d	83.3 ± 2.15 a
GRD 120 05	91.2 ± 0.31 d	83.3 ± 4.30 a
JHC 80 06	89.6 ± 0.34 e	87.5 ± 2.29 a
JSF 110 08	85.9 ± 0.75 f	75.0 ± 2.63 b

* Means followed by the same letter in the column do not differ by the Scott-Knott test at $P < 0.05$. M ± SE (M).

that the percentage of viability is still high, and therefore the application of these organisms can be performed at field level using a hydraulic spray system and enabling other application operations usually adopted for plant protection products. Shapiro-Ilan *et al.* (2016), evaluated *S. carpocapsae* in peach production systems to control *Synanthedon pictipes* (Grote and Robinson, 1868) (Lepidoptera: Sesiidae) testing simple jet nozzles. They verified high viability of the nematodes even when applied with the insecticide. The biggest difference in viability was 13.3 %, observed between nozzles GAT 110-02 and JSF 110-08. Should be considerate that *S. carpocapsae* has a smaller length than *H. amazonensis* MC01, with an average of 558 µm; but it is slightly wide, with an average of 25 µm (Nguyen and Smart Jr. 1992).

The time that the nematode remained in the tank was not a limiting factor for its application via hydraulic spraying, according to the results observed in test treatments 1, 2, and 3. This corroborates the results of Lanzoni *et al.* (2014). Noosidum *et al.* (2016) observed high infectivity of *S. carpocapsae* and *Steinernema* sp. even after 72 hours, when controlling *P. xylostella* larvae, applied in combination with foliar desiccant.

The results obtained for control tests 1, 2 and 3 also indicated that the agitation of the nematode suspension in the tank does not affect infectivity. Hydraulic agitation exerted by the return to the spray tank is fundamental for the distribution of nematodes on the target, corroborating Moreira *et al.* (2013) and Beck *et al.* (2015). In cases where the nematode suspension agitation is insufficient, sedimentation of nematodes can occur, reducing the quality of the application.

The results obtained with the GAT nozzle series indicated that the differences in flow rates are not limiting for the use of nozzles to apply EPNs. Build differences could be the main factors that interfere in the interaction between hydraulic nozzles and nematodes, as the suspension's movement inside the nozzles of greater flow could lead to higher friction between the nematodes, resulting in lower infectivity. However, additional tests should be conducted in order to better understand this dynamic, which would allow greater survival in lower flow nozzles. Besides, tests could be performed throughout all the working pressure of each selected nozzle in order to map its effect on the viability of nematodes.

The GRD and ADGA nozzle series behaved similarly to the GAT series as regards the viability; however, infectivity was higher in lower flow nozzles. These results demonstrate that there are differences caused by movement of the nematode suspension according to the flow and the type of nozzle. Even so, it is also possible to affirm that the hydraulic application is a viable alternative to release entomopathogenic organisms, which was also verified by Garcia *et al.* (2008), considering that nematodes were still able to cause mortality of *T. molitor* larvae.

Moreira *et al.* (2013) showed that hydraulic nozzles allow the maintenance of viability and infectivity of entomopathogenic nematodes. In the present study it was found that there was a variation between the models of nozzles tested, but most maintained high levels of viability and infectivity of IJ. The ST-IA nozzle series maintained the highest viability for nozzles with a higher flow, but for infectivity the results were inferior. The JAP nozzle series provided higher viability in the models of higher flow rates, while infectivity did not vary with flow rate. The AD-IA nozzle series provided the greatest viability for higher flow models, and resulted in the highest infectivity.

The JSF series with higher flow showed the lowest observed viability, while the lower flow produced the highest infectivity observed. For the CV-IA series the observed viability was highest at maximum flow; however, the infectivity was highest with the nozzle at flow 03, which was intermediary among the analyzed flows. The nozzles of the AXI series, regardless of flow, showed similar viability and infectivity of the IJ. The AVI, ATR and ADI series resulted in intermediate viability and infectivity in relation to the other nozzles used in the experiment.

In this way, the results obtained in the present study demonstrated that the impact or contact of nematodes inside the nozzles can affect their viability and infectivity; however, how this happens needs to be better studied. Under field conditions, the effect of the hydraulic system on the nematodes can be expanded, considering the difficulties that the nematode would have in finding the insects in the soil and also as a function of the increased number of filters present in sprays. This was discussed by Shapiro-Ilan *et al.* (2006), and despite these challenges, their work considered that EPNs can be used in all types of applications, even aerial.

Garcia *et al.* (2005) tested the influence of pressurization on *S. glaseri*, verifying that pressure applied up to 200 psi can be used without loss in nematode viability and infectivity. Nilson and Gripwall (1999) found no significant difference in the viability of nematode *H. indica* when applied through nozzles with different apertures (5 x 2 mm and 5 x 3 mm). The results obtained with these nematodes are similar even they are species with different sizes, which could interfere in the response over pressurization and nozzles. *Steinernema glaseri* is in a group of large nematodes, average length of 1130 µm and width of 43 µm (Nguyen and Smart Jr. 1995), while *H. indica* is smaller, averages of 528 µm in length and 20 µm in diameter, even less than *H. amazonensis* (Andaló *et al*. 2006)

Considering the results of viability and infectivity in general, it is possible to notice that even if one nozzle provides lower viability of IJs compared to the others, as it was observed for JHC 80 06, the nematodes remain infective, since they are found in sufficient amount to cause the death of *T. molitor* larvae in laboratory conditions. Further evidence of interaction between the hydraulic nozzles and the nematodes is observed by the results with nozzle GRD 120 02, which presented one of the highest percentages of viability; however, the infectivity of nematodes after passing through the nozzle was 58.3 %.

Considering the results, parameters as the concentration of IJs suspension can be adjusted when applied in the field, as well as the possible mixture of EPNs and others entomopathogens, such as fungi, bacteria and viruses, for which the application systems could be a limiting factor for their efficiency. Thus, IJs could be used as a vector to these entomopathogens and aid them to reach different niches; and the mixture of these organisms with chemicals should also be highlighted, which can generate reduction of active ingredients.

The results in the present work provide evidence that the time and frequency of the nematode passage through the hydraulic system is the main factor in the reduction of viability and infectivity. It is also evident that the hydraulic nozzle influences the effectiveness of the nematode. However, in

most cases, infectivity was maintained even when a reduction in viability was observed. In this way, it can be concluded that the application of the nematode *H. amazonensis* MC01 via hydraulic spraying can be an alternative for pest control in the field, allowing different cultural practices to be brought together in a single operation.

Conclusions

The hydraulic spray system interferes in the nematodes' viability; however, this parameter still remains at high levels. This is also the case with infectivity, since the nematodes that stayed alive remained infectious. The nematode viability is also influenced by most hydraulic spray nozzles, but the living nematodes did not lose their ability to infect *T. molitor*. Therefore, the controlled spraying of these organisms through the selection of hydraulic nozzles that have better interaction with EPNs could allow for more effective control of insect pests, and this can be an operationally advantage for the producer.

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