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Virulence of nematodes against larvae of the south-American fruit fly in laboratory using soil from Porto Amazonas, Paraná, Brazil, as substrate

Virulência de nematoides contra larvas de mosca-das-frutas-sulamericana em laboratório, utilizando solo de Porto Amazonas, Paraná, Brasil, como substrato

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ABSTRACT

Anastrepha fraterculus *is an important orchard pest. Its management has been based in chemical sprays, but biological control is a growing demand. The objective of this work was to evaluate, under laboratory conditions, the virulence of eight nematode isolates to* ***A. fraterculus*** *in a soil collected in Porto Amazonas, Paraná, Brazil, and to estimate lethal doses (LD_{50} and LD_{90}) for the more virulent isolate.* ***Steinernema carpocapsae*** *CB 02, three* ***Heterorhabditis*** *sp., two* ***H. amazonensis*** *and two* ***Oscheius*** *sp. isolates were tested in laboratory against A. fraterculus third-instar larvae using as substrate a loam Cambisol collected in an apple orchard.* ***S. carpocapsae*** *CB 02 isolate caused the higher percent mortality of* ***A. fraterculus****.* ***Heterorhabditis*** *sp. isolates and LAMIP 9 (****Oscheius*** *sp.) isolates were intermediate, while LAMIP 92 (****Oscheius*** *sp.) didn't differ from the control.* ***S. carpocapsae*** *CB 02 is able to kill 50% and 90% of* ***A. fraterculus*** *population with 96.3 and 314.7 infective juveniles per larva, respectively, in that soil. As a conclusion, CB 02 is the most virulent to* ***A. fraterculus*** *when the substrate is Porto Amazonas' apple orchard soil and it is able to kill 50 and 90% larval population with 96.3 and 314.7 infective juveniles per larva, respectively.*

Key words: ***Steinernema carpocapsae****,* ***Heterorhabditis****,* ***Oscheius****,* ***Anastrepha fraterculus****.*

RESUMO

Anastrepha fraterculus *é uma importante praga em pomares. Seu manejo tem se baseado no uso de inseticidas químicos, porém, o controle biológico é uma demanda crescente. O objetivo do trabalho foi avaliar, em condições laboratoriais, a virulência de oito isolados de nematoides contra* ***A. fraterculus*** *em um solo coletado em Porto Amazonas, Paraná, Brasil, e estimar a dose letal do nematoide mais virulento.* ***Steinernema carpocapsae*** *CB 02, três isolados de* ***Heterorhabditis*** *sp., dois de* ***H. amazonensis*** *e dois de* ***Oscheius*** *sp. foram testados em laboratório contra larvas de terceiro instar de* ***A. fraterculus****, usando como substrato um Cambissolo franco coletado em um pomar de macieira.* ***S. carpocapsae*** *CB 02 provocou maior percentual de mortalidade de* ***A. fraterculus****. Isolados do gênero* ***Heterorhabditis*** *sp. e o LAMIP 9 (****Oscheius*** *sp.) foram intermediários, enquanto o LAMIP 92 (****Oscheius*** *sp.) não diferiu da testemunha.* ***S. carpocapsae*** *CB 02 é capaz de matar 50% e 90 % da população de larvas* ***A. fraterculus*** *com 96,3 e 314,7 juvenis infectivos por larva, respectivamente, naquele solo. Como conclusão, CB 02 é o mais virulento à* ***A. fraterculus*** *quando o substrato é solo de pomar de macieira de Porto Amazonas. Ele é capaz de controlar 50% e 90% da população de larvas com 96,3 e 314,7 juvenis infectivos por larva, respectivamente.*

Palavras-chave: ***Steinernema carpocapsae****,* ***Heterorhabditis****,* ***Oscheius****,* ***Anastrepha fraterculus****.*

INTRODUCTION

Apple [***Malus domestica*** (Borkhausen)] is one of the most important fruit crop in Southern Brazil. In Paraná State, Porto Amazonas and Lapa counties are responsible for about 30% of apple production (IBGE, 2015). The South-American fruit fly ***Anastrepha fraterculus*** (Wied.) (Diptera: ***Tephritidae***), is an apple and peach key pest in Southern Brazil and harms fruits by feeding and ovipositing on fruit (MONTEIRO & HICKEL, 2004). The extensive use of chemicals to control the pest has brought many environmental threats and human contamination. It has given rise to new researches on alternative control methods. Chemical-free or low-residue fruit are nowadays

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a demand. Biological control is an alternative measure, which includes the use of nematodes, since ***Anastrepha*** spp. have stages of larva and pupa in the soil (MONTEIRO & HICKEL, 2004).

There are already some studies on the main entomopathogenic nematode (EPN) genera ***Steinernema*** and ***Heterorhabditis*** as fruit flies biocontrolers. LEZAMA-GUTIÉRREZ et al. (2006) and TOLEDO et al. (2014) studied EPNs against ***A. ludens*** Loew; TOLEDO et al. (2005), in ***A. obliqua*** (Macquart); and TOLEDO et al. (2006) in ***A. serpentina*** (Wied). BARBOSA-NEGRISOLI et al. (2009) and RODRIGUES-TRENTINI (1996) tested the virulence of some EPNs against ***A. fraterculus,*** using sterile substrates. ***Oscheius*** genus contain species recently found to be facultative entomopathogenic nematodes (FEPNs): ***O. carolinensis*** (TORRES-BARRAGAN et al., 2011), ***O. chongmingensis*** (LIU et al., 2012) and ***O. gingeri*** (PERVEZ et al., 2014), but they were not reported infecting ***A. fraterculus*** yet.

Few studies about EPNs and FEPNs against ***A. fraterculus*** were made under soil influence. Soil characteristics as organic matter content, moisture, texture and biotic antagonists can affect nematode action (KAYA, 1990; LEZAMA-GUTIÉRREZ et al., 2006). Therefore, trials in sterile substrate like sand or Petri dishes with paper can estimate the potential efficiency of EPNs but trials in not sterilized soil reflects more closely the answer at field conditions for that specific soil (VOSS et al., 2009).

Thus, the objective of this work was to evaluate the virulence of nematode isolates of the genera ***Steinernema, Heterorhabditis*** and ***Oscheius*** to ***A. fraterculus*** larvae and estimate lethal doses for the more efficient nematode, having as substrate a loam Haplic Cambisol from an apple orchard of Porto Amazonas (Paraná State, Brazil), in laboratory condition.

MATERIAL AND METHODS

A. fraterculus utilized in the trial were obtained from laboratory rearing from the CENA (Centro de Energia Nuclear na Agricultura), São Paulo - Brazil, started eight years before in Piracicaba, SP. The insects were reared according to SALLES (1992).

Five ***Heterorhabditis*** sp. isolates were obtained from UENP (Universidade Estadual do Norte do Paraná) and from IB (Instituto Biológico), and also one ***Steinernema carpocapsae*** from IB (Table 1). Two ***Oscheius*** sp. strains were isolated in Porto Amazonas (-25º32'07'', -49º54'41''), Paraná State, BR, by the authors, from a loam soil of an apple orchard, and identified by genetic markers at Florida University (unpublished results). After the initial purification, all the nematodes were inoculated on late-instar ***Galleria mellonella*** L. (Lepidoptera: ***Pyralidae***) larvae and maintained in culture tissue bottles, with sponge, at 15°C (VOSS et al., 2009).

Eight nematode isolates (Table 1) were experimented against ***A. fraterculus*** late third-instar larvae in a completely random design with ten repetitions (two sets of five repetitions at different times). Each plot consisted of 12 ***A. fraterculus*** larvae disposed in a 50-mL plastic container with 14.4g sieved (2mm) loam Haplic Cambisol from a Porto Amazonas orchard (22% clay, 41% silt; 37% sand, pH 5.9, 255mg dm^{-3} P, 462mg dm^{-3} K, 3.8% organic matter, 22% gravimetric moisture), equivalent to 13,04cm^2 of soil surface. The plastic cup was covered with parafilm to avoid loss of soil moisture. The soil was collected as VOSS et al.

Table 1 - Specie, source and origin of the entomopathogenic nematode isolates used in the experiment against ***A. fraterculus*** larvae.

Identification code	Specie	Source	Origin
Alho	***Heterorhabditis*** sp.	UENP[1]	Minas Gerais, BR
CB 02	***Steinernema carpocapsae***	Instituto Biológico[2]	Florida, USA
CB 24	***H. amazonensis***	Instituto Biológico[2]	São Paulo, BR
RSC 05	***H. amazonensis***	UENP[1]	Amazonas, BR
JPM4	***Heterorhabditis*** sp.	UENP[1]	Minas Gerais, BR
NEPET 11	***Heterorhabditis*** sp.	UENP[1]	Rio Grande do Sul, BR
LAMIP 9	***Oscheius*** sp.	UFPR[3]	Paraná, BR
LAMIP 92	***Oscheius*** sp.	UFPR[3]	Paraná, BR

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[3]Universidade Federal do Paraná - Laboratório de Manejo Integrado de Pragas (LAMIP).

(2009) and the samples were tested for EPNs and FEPNs absence by exposing ***Tenebrio molitor*** L. (Coleoptera: ***Tenebrionidae***) larvae during 15 days (VOSS et al., 2009). After ***A. fraterculus*** larvae had reached late third instar, they were removed from the semiliquid diet and mixed to an EPN-free soil for at least 30 minutes before use them in the bioassay, to avoid carrying excess of moisture and diet residues to the experimental plots. Then, 12 larvae were placed on the plots to penetrate the soil by themselves. Those that didn't penetrate the soil after 30 minutes were replaced (VOSS et al., 2009). EPN infective juveniles (IJ) were obtained from inoculated ***G. mellonella*** larvae and had less than 20 days since its production. The IJ concentration was determined in 20-microliter EPNs suspension drop with 5 replicates, with micropipette, using a stereomicroscope, as described by VOSS et al. (2009). After soil larvae penetration, 1,200 IJ (100IJ larva^{-1}) were distributed on the soil surface suspended in 1.2mL distillated water, after homogenized, using a micropipette. The control plots received only distilled water in same volume. Plots were incubated in a controlled chamber at 25±2°C, RH 70±10 % (BARBOSA-NEGRISOLI, 2009).

Seven days later, the soil was sieved and each larva or pupa (since some larvae turned to pupae) was placed in a 1.1-cm² well of a tissue culture plate (Kasvi®, model K12-024), with two layers of filter paper. They were observed every two days to recover moisture of the filter paper. Twenty days after inoculation individuals not giving rise to an adult were dissected and observed under stereomicroscope, to verify the presence/absence of EPNs. To access virulence, only insect individuals infected with nematode were counted for the mortality data (LEZAMA-GUTIÉRREZ et al., 2006). The virulence was calculated by the equation: $V = (Y - X)/(1-X)*100$, where V is the virulence, *X* is the mean mortality in the control and *Y* is the mortality observed in each plot (ABBOTT, 1925). Data on mortality of ***A. fraterculus*** were transformed [$(y+1)^{0.5}$] to normalize and submitted to analysis of variance and a Tukey tests using R 3.0.2 (R DEVELOPMENT CORE TEAM, 2008).

Lethal doses (LD_{50} and LD_{90}) were determined for the more virulent isolate, using the previous methodology for nematode inoculum, insect larvae preparation, EPN-free soil, arena dimensions, incubation time, mortality determination and experimental design. The treatments were 0 (control), 75, 150, 225, 300, 375IJ larva^{-1}. Lethal concentrations were estimated submitting the mean corrected mortality for each dose to a Probit regression using PoloPlus 1.0. (ROBERTSON et al., 1980).

RESULTS AND DISCUSSION

Nematodes virulence against ***A. fraterculus***

All isolates of nematodes tested were able to infect ***A. fraterculus***. Infection rates ranged from 28 to 84.2% among the treated larvae, significantly higher than the control ($F=19.26$; $P<0.05$). Irrespective to the treatment, most of the infected insects (at least 79%) reached pupal phase during the first week after inoculation (Figure 1A). The methodology couldn't define the time of infection: pupal or larval stage. The higher amount of pupae indicates infected insects died slowly and could reach pupal phase before, probably because ***A. fraterculus*** larva had active defenses against nematode by retarding its development on hemolymph (RODRIGUES-TRENTINI, 1996). However, the insect died anyways.

The virulence of the treatments against ***A. fraterculus*** in the loam soil is showed in figure 1B. CB 02 had the highest virulence (90.5%). CB 24 and JPM4 reached 47.4 and 45.3% mortality of ***A. fraterculus***, respectively. Moreover, Alho, LAMIP 9, RSC 05 and NEPET 11 formed another group with lower virulence, but still different

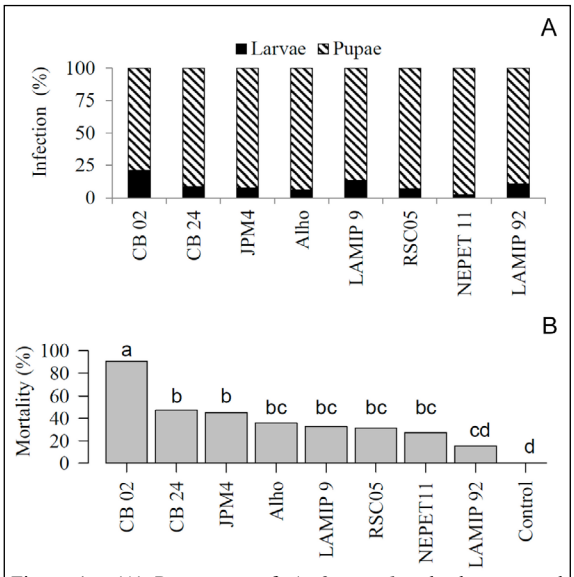

Figure 1 - (A) Percentage of ***A. fraterculus*** deaths occurred at larval or pupal phase following infection by entomopathogenic nematode isolates. (B) Mortality caused by entomopathogenic nematodes isolates (100IJ.larva^{-1}) on ***A. fraterculus***. In B, statistical analysis was performed in transformed data [$y'=(y+1)^{0.5}$], and columns with the same letter do not differ (Tukey test, $\alpha=0.05$). All data were obtained in a loam soil from an apple orchard of Porto Amazonas, PR, under laboratory condition (25±2ºC, RH 70±10%).

from the control (Figure 1B). However, LAMIP 92 not surpassed the control.

S. carpocapsae CB 02 was highly virulent, killing 90.5% of the larvae treated. As a comparison, using an unspecified isolate of ***S. carpocapsae***, RODRIGUES-TRENTINI (1996) observed insect mortality lower than 31% using clay loam soil (100IJ larva^{-1}). Five times more IJ larva^{-1} were necessary to cause 91.7% mortality. Besides the possible inherent difference in virulence among the two isolates, the higher clay content of the soil can have influenced negatively the infection by the nematodes, especially because clay reduces soil pore size and aeration which hampers host finding by the EPN (KAYA, 1990). The present experiment was performed in a loam soil, with less clay, and probably enhanced CB 02 action. A similar but sandier soil (sandy loam) was found to be more appropriated than sand, clay loam, and clay soils to ***S. carpocapsae*** survival and pathogenicity (KUNG et al., 1990). Beyond the differences in soil texture, RODRIGUES-TRENTINI (1996) used a bigger soil volume per larva, compared to this work, what caused dilution of the pathogen in the soil. In Petri dishes (best substrate for EPNs), 316IJ larva^{-1} were needed to kill 90% of ***A. fraterculus*** larvae, what suggests CB 02 is more virulent.

BARBOSA-NEGRISOLI et al. (2009) tested other EPN species, ***H. bacteriophora*** and ***S. riobrave*** (100IJ larva^{-1}), against ***A. fraterculus*** and found 55 and 58% of larvae mortality, respectively, using sterile sand as substrate. These results are better than the virulence of the most part of the EPNs tested in this work. Sand is better than fine-textured soils to general EPNs action (KAYA, 2009), what helps explain the difference. However, the ***S. carpocapsae*** CB 02 was still more virulent (Figure 1B) even in loam soil.

Heterorhabditis sp. isolates performed worse than ***S. carpocapsae*** in the trials now presented. ***Heterorhabditis*** differs from ***Steinernema*** by its searching behavior that helps in the pray finding (KAYA, 1990). ***S. carpocapsae*** is ambusher, whilst most of the ***Heterorhabditis*** species are cruisers. However, in the present work, this characteristic could have been not important. The passive dispersal of the nematodes by the water through the soil pore space and the small soil depth could have helped ***S. carpocapsae*** to spread in the soil. The temperature is another possible explanation, since ***Steinernema*** genus was observed to be more efficient at 25°C (temperature of this experiment) while ***Heterorhabditis*** showed higher efficiency at 30°C (ROHDE et al., 2010). Furthermore, Steinernematids seem to be more adaptable to different textures of soil than ***Heterorhabditis*** sp. (KAYA, 1990).

The ***Oscheius*** sp. isolate LAMIP 9 was able to infect and kill ***A. fraterculus*** larvae (Figure 1). This pathogenic relationship has not been previously reported. Both ***Oscheius*** sp. were able to develop and kill ***G. mellonella***. Some ***Oscheius*** sp. are known to be facultative parasites like: ***O. carolinensis*** (TORRES-BARRAGAN et al., 2011); ***O. chongmingensis*** (LIU et al., 2012); and ***O. gingeri*** (PERVEZ et al., 2013). Difference between LAMIP 9 and LAMIP 92 virulence, considering they are the same species, can be explained by the amount and kind of bacteria they are associated. An unique ***Oscheius*** species can carry more than one bacteria outside its body (TORRES-BARRAGAN et al., 2011), with different virulence.

LAMIP isolates performed as well or worse than other-origin ones. In agreement with this result, GREWAL et al. (2002) found that it was common the EPNs from an insect species to be less virulent than isolates from other insect and/or geographical origin. Specificity seems to be a more important factor in EPN virulence than adaptation to the environment. It's possible that LAMIP isolates facultative parasitic behavior allows them to survive on other suitable food sources than ***A. fraterculus***, since this insect is not present in the orchards during the entire year.

Lethal doses of CB 02

S. carpocapsae CB 02 lethal doses are presented in figure 2. Its virulence rose as the dose of

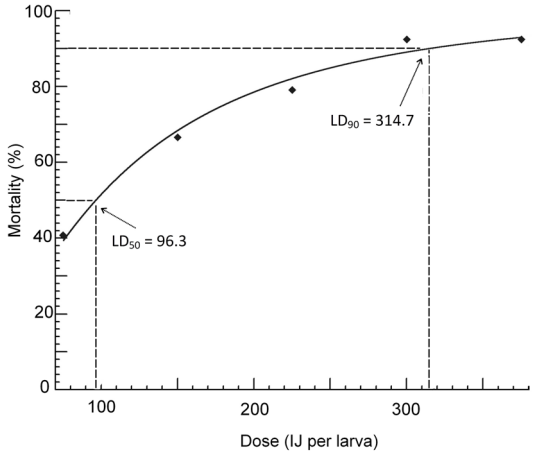

Figure 2 - Probit regression (dark line) for observed mortality [corrected according ABBOTT (1925)] of the fruit fly ***A. fraterculus*** following increasing doses of ***S. carpocapsae*** CB 02, in a loam soil of an apple orchard of Porto Amazonas (PR, Brazil) as substrate, at laboratory condition. LD50 and LD90 are indicated by dash lines.

IJ increased until 300IJ larva^{-1}, and reached 92.2%. The lethal doses, LD_{50} and LD_{90}, of ***S. carpocapsae*** CB 02 on ***A. fraterculus*** larvae were estimated to be 96.3IJ larva^{-1} (confidence interval: 80.6 to 110.2IJ larva^{-1}) and 314.7IJ larva^{-1} (confidence interval: 272.7 to 380.9IJ larva^{-1}), respectively (χ^2=2.47, heterogeneity=0.82).

RODRIGUES-TRENTINI (1996) estimated the confidence interval of LD_{50} for ***S. carpocapsae*** against ***A. fraterculus***, in a loam clay soil, to be 261.5 to 429.0IJ cm^{-2}, similar to LD_{90} of the ***S. carpocapsae*** CB 02. Besides differences of inherent virulence among the isolates tested, the lower LD_{50} estimated in this work for ***S. carpocapsae*** CB 02 can be due to the substrate used. Comparing to other studies (RODRIGUES-TRENTINI, 1996; BARBOSA-NEGRISOLLI et al., 2009), the relative low LD of ***S. carpocapsae*** CB 02 in the loam soil is a suitable characteristic for its possible use as a biocontrolers in the orchard.

Insect mortality usually falls down after EPN concentration over an optimal point because of IJ competition (ROHDE et al., 2012). However, this work did not report decrease in control efficiency of ***S. carpocapsae*** CB 02 against ***A. fraterculus*** larvae, corroborating the observation by RODRIGUES-TRENTINI (1996).

CONCLUSION

All ***Heterorhabditis*** sp. isolates, ***S. carpocapsae*** CB 02 and ***Oscheius*** sp. LAMIP 9 are virulent to ***A. fraterculus*** larvae when the substrate is Porto Amazonas' apple orchard loam soil. ***Oscheius*** sp. LAMIP 9 and LAMIP 92 are less effective than EPNs from other origins tested in this trial. CB 02 is the most efficient on ***A. fraterculus*** control, and is able to kill 50% and 90% of ***A. fraterculus*** population with 96.3 and 314.7 infective juveniles per larva, respectively, in that soil.

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