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Isolation of entomopathogenic nematodes in an apple orchard in Southern Brazil and its virulence to *Anastrepha fraterculus* (Diptera: Tephritidae) larvae, under laboratory conditions

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(With 3 figures)

Abstract

Entomopathogenic nematodes (EPNs) are a promising alternative to integrated control in many fruit pests. Few studies were made on the relationship of *Anastrepha fraterculus* natural population with native EPNs population and other biotic and abiotic factors. The aim of this work was to verify the occurrence of endemic nematodes in an apple orchard, concerning environmental conditions and technical procedure, and access isolates virulence to *A. fraterculus* larvae. The experiment was conducted during a year taking monthly soil samples from an apple orchard, with and without fallen fruits just above the soil. Samples were baited with *Tenebrium molitor* and *A. fraterculus* larvae in laboratory. Canopy and fallen fruits were sampled to access the pest infestation. Seventy three EPN isolates were captured, in 23.2% soil samples, more with *T. molitor* than with *A. fraterculus* baits. From the 20 isolates tested against *A. fraterculus*, only five were pathogenic, and they were identified as *Oscheius* sp. The nematodes were captured during all seasons in a similar frequency. Soil and weather conditions, presence of fruit over the orchard soil, and *A. fraterculus* pupae in the fruits had no significant influence on the capture. As a conclusion, nematodes of the genera *Oscheius* are found in an apple orchard of Porto Amazonas constantly along the year, independently of fluctuations in *A. fraterculus* population, climate conditions and presence of fruit over the soil. Some of the isolates are pathogenic to *A. fraterculus.*

Keywords: South-American fruit fly, biological control, *Oscheius*, EPN, *Malus domestica*.

Isolamento de nematoides entomopatogênicos em pomar de macieira no sul do Brasil e virulência em larvas de *Anastrepha fraterculus* (Diptera: Tephritidae) em condições de laboratório

Resumo

Nematoides entomopatogênicos (NEPs) são alternativa promissora para o controle integrado de várias pragas de frutíferas. Foram feitos poucos estudos ecológicos sobre a relação de populações naturais de *Anastrepha fraterculus* com a de NEPs nativos e outros fatores bióticos e abióticos. O objetivo desse trabalho foi verificar a ocorrência de nematoides endêmicos de um pomar de macieira, considerando condições ambientais e procedimentos técnicos, e testar a virulência de isolados para larvas de *A. fraterculus*. O experimento foi conduzido durante um ano coletando mensalmente amostras de solo de um pomar de macieira com e sem frutos acima do solo. Foram feitas armadilhas com larvas de *Tenebrium molitor* e de *A. fraterculus* em laboratório. Frutos na copa e caídos no solo foram amostrados para quantificar a infestação da praga. Setenta e três isolados de NEPs foram capturados em 23,2% das amostras de solo, mais em armadilhas de *T. molitor* do que em *A. fraterculus.* Dos 20 isolados testados contra *A. fraterculus,* apenas cinco foram patogênicos, e eles foram identificados como *Oscheius* sp. Os nematoides foram capturados durante todas as estações e em frequência similar. Condições edáficas e climáticas, presença do fruto acima do solo amostrado e de pupas de *A. fraterculus* nos frutos não tiveram influência significativa na captura. Como conclusão, nematoides do gênero *Oscheius* ocorrem em pomar de macieira de Porto Amazonas de forma constante ao longo do ano, independentemente de flutuações na população de *A. fraterculus*, condições climáticas e presença de fruto sobre a superfície do solo. Alguns isolados são patogênicos para *A. fraterculus.*

Palavras-chave: moscas-da-fruta-sulamericana, controle biológico, *Oscheius*, NEP, *Malus domestica.*

1. Introduction

South-American fruit fly, *Anastrepha fraterculus* (Wied.) (Diptera: Tephritidae), has a wide distribution in America and can be found in almost all parts of South America. In southern Brazil, the species domains and can harm at least 67 hosts, including the exotic ones like apple (*Malus domestica* Borkh.) (Zucchi, 2000a). In laboratory condition, the adult can survive six months and the female is able to lay more than 400 eggs during lifetime (Salles, 2000). The larvae feed on fruit pulp and induce its earlier ripening and falling (Souza-Filho et al., 2009). On soil, late third-instar larva leaves the fruit and turns to pupa nearby into the soil. Last larval instar and pupal phase takes 15 days at 25 °C (Salles, 2000) and this is the suitable time for many soil biocontrolers to infect *A. fraterculus* (Rodrigues-Trentini, 1996).

Entomopathogenic nematodes (EPNs) are having promising results on biological control and are an alternative measure for many soil pests control (Stock, 2005). There are three different genus mostly known as EPNs: *Steinernema*, *Heterorhabiditis* and *Neosteinernema*. They are entomopathogenic bacteria vectors (Poinar-Junior, 1990). More recently the genus *Oscheius* was discovered as EPN (Torres-Barragan et al., 2011). There are few studies on demonstrating *Oscheius* contribution to biological control, since most species of this genus have facultative-parasite habit (Ye et al., 2011). *Oscheius* sp. have symbiotic relationship with entomopathogenic bacteria, caring them not inside its body like obligate EPNs do, but in its body surface. When bacteria are present, *Osheius* sp. are able to infect and kill insect species (Torres-Barragan et al., 2011), but they were not reported infecting *A. fraterculus* yet. In this work, for convenience, all isolated are called EPN, including *Oscheius*. EPNs have been studied as biocontrolers candidate of several fruit flies species (Lezama-Gutiérrez et al., 2006; Barbosa-Negrisoli et al., 2009; Rohde et al., 2012; Toledo et al., 2014). However, few studies at orchard condition were found in the literature and even less works reporting EPNs natural occurrence. In a Florida citrus orchard, the increase on EPN population was correlated only with soil temperature (Beavers et al., 1983). McGraw and Koppenhöfer (2009) found a positive correlation between the increase of native EPNs population and the host *Listronotus maculicollis* Kirby (Coleoptera: Curculionidae) in turfgrass.

The aim of this work was to verify the occurrence of isolate endemic entomopathogenic nematodes strains, characterize their seasonality and relation with biotic and abiotic factors in an apple orchard of Porto Amazonas, PR, Brazil, and select strains capable of infect and kill *A. fraterculus* in laboratory conditions.

2. Material and Methods

2.1. Orchard soil samples and EPN capture

An apple orchard (cultivar Eva), in Porto Amazonas, Paraná, Brazil (25°32'08"S 49°54'52"W, 865-m high, climate Cfb - Köppen) divided in six 100-m^2 sectors was sampled. Samples were taken away and under fallen apples, following Voss et al. (2009). From July 7 to September 19, 2013 (dates at Figure 1A), two sectors were sampled (total of 18 samples each date). From October 17, 2013, to August 8, 2014, all six sectors were sampled (total of 24 samples each date). In laboratory, 25±2 ºC and natural photophase and relative humidity, samples were baited with five late-instar *Tenebrium molitor* L. (Coleoptera: Tenebrionidae) in a 500-mL plastic pot (Voss et al., 2009), and alternative baits were made with *A. fraterculus* larvae in smaller pots (50-mL, because *A. fraterculus* is less mobile) sealed with parafilm. Dead insects with EPN symptoms were placed in White traps, kept at 24±1 ºC, dark and 70±10% relative humidity (Voss et al, 2009). The infective (dauer larvae) juveniles (IJ) were recovered every two days, purified and placed in tissue culture bottles. Isolates were tested for entomopathogenicity, and multiplied, in late-instar *Galleria mellonella* L. (Lepidoptera: Pyralidae) as Rio and Cameron (2000). The isolates were identified by a numeric code following the soil sample order. EPN stock samples were kept at 15 °C (Barbosa-Negrisoli et al., 2009).

2.2. Monitoring environmental condition and Anastrepha sp. population

Data on temperature in Lapa County (closer available data) and rainfall in Porto Amazonas were provided by Simepar (Sistema Meteorológico do Paraná, Brazil) (Figure 2). Physical and chemical soil characteristics were accessed for each sector by specialized laboratories. The soil is a Haplic Cambisol, loam in four orchard sectors (42.25% sand, 24.25% clay, 33.25% silt, 4.78% O.M., 450.9 mg/dm^3 K, 71.28 mg/dm^3 P, 2.92 cmol$_c$/dm^3 Mg, 13.13 cmol$_c$/dm^3 Ca, 6.38 pH) and sandy loam in the other two sectors (45.5% sand, 12.5% clay, 42% silt, 4.5% O.M., 435.6 mg/dm^3 K, 194.3 mg/dm^3 P, 2.6 cmol$_c$/dm^3 Mg, 16 cmol$_c$/dm^3 Ca, 6.6 pH).

The relationship of the proportion of EPN-positive samples with clay, sand and organic matter soil content, precipitation cumulated and mean air temperature two until six days before sampling, was analyzed through Spearman correlation.

Anastrepha fraterculus population fluctuation was determined by monitoring fruit fly apple infestation. Fruit samples were collected from July 11, 2013 to July 03, 2014. Samples from each sector were composed by twenty fruits (from four plants of a row) every week during harvest season and six fruit (from three plants) biweekly after and before harvest. Fallen fruit were sampled the same way when available. In laboratory, fruit were laid on vermiculite according to Nascimento et al. (2000). Pupae were placed in Petri dishes with filter paper and moist with distilled water until fly emergency. Two days after emergency, flies were registered and stored in ethanol 70% to identification (Nascimento et al., 2000; Zucchi, 2000b; Marsaro Júnior et al., 2013).

The relationship of the proportion of EPN-positive samples with *A. fraterculus* pupae was analyzed through Spearman correlation. The proportions of EPN-positive

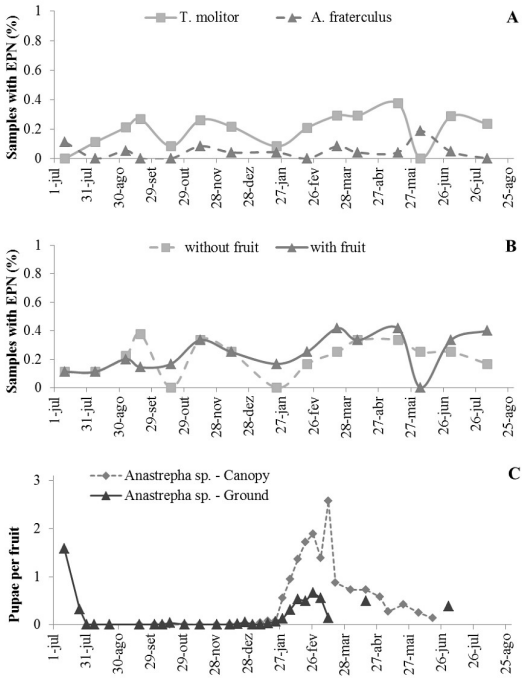

Figure 1. Relative frequency of EPNs - positive soil samples when the bait was *Tenebrio molitor* or *Anastrepha fraterculus* larvae (A), and when samples were collected without or with an apple over the soil (B). In chart (C) the number of *Anastrepha* sp. pupae obtained from apples collected in the canopies or orchard ground. Time is about one 'Eva' apple production cycle in Porto Amazonas, PR.

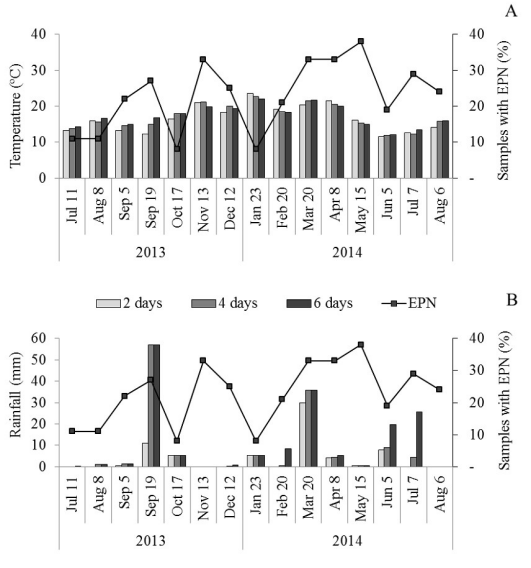

Figure 2. Mean air temperature measured in Lapa- PR (A) and rainfall cumulated in Porto Amazonas- PR (B), 2, 4 and 6 days before soil sampling (sampling day included) from Jul 11, 2013 to Aug 6, 2014, and percentage of samples with entomopathogenic nematode (EPN) captured (right axis) in soil from an apple orchard in Porto Amazonas. Weather data provided by Simepar.

samples in the subsets with and without fruit upon the sample site were compared by chi-square tests. The software BIOESTAT 5.0 was used to both tests (Ayres et al., 2007). Additionally, data on EPN capture were split by season and the proportion on EPN-positive samples was compared through a chi-square test (Campbell et al., 1995).

2.3. Virulence to A. fraterculus

An *A. fraterculus* population from Cena (Centro de Energia Nuclear na Agricultura, Piracicaba – Brazil), was reared following Walder et al. (2014) to obtain late third-instar larvae. They were used in an experiment completely random designed, with five repetitions composed by a nine-centimeter Petri dishes (Rohde et al., 2012) with 12 larvae, in a dark climatic chamber (24±2 °C, U.R. 70±10%). Twenty nematode isolates from Porto Amazonas apple orchard, pathogenic to *G. mellonella,* were tested split in three trials, each one including a negative control (distilled water) and a positive control with *Steinernema carpocapsae* CB 02 (provided by Instituto Biológico de Campinas), multiplied as in Voss et al. (2009). The IJ concentration was determined by counting 20-microliter drops with five replicates, using an optical microscope (Voss et al., 2009). Each larva (except in water control) was treated with 100 IJ (Barbosa-Negrisoli et al., 2009) suspended in 0.6 mL distilled water. The number of flies emerged was registered twenty five days after inoculation. Dead insects were dissected under amplification for confirmation of infection by nematode (Barbosa-Negrisoli et al., 2009). The control efficiency was calculated according to Abbott (1925). Data were square-root transformed to reach normality and submitted to analysis of variance and Tukey test using R 3.02 (R Development Core Team, 2008).

2.4. Isolates identification

Five isolates were selected based on its virulence to *A. fraterculus* and sent to identification by Dr. Tesfamariam Mengistu at Florida University. DNA was extracted from five first generation female nematodes from each isolate placed in 1.5-mL Eppendorf tubes each containing 20 µl 0.25 M NaOH and incubated at 25 °C overnight (Floyd et al., 2002). Thereafter, samples were incubated at 99 °C for tree minutes and allowed to cool to room temperature, and then 4 µl 0.25 M HCl, 10 µl 0.5 M Tris-HCl, (pH 8.0), and 5 µl 2% Triton X-100 were added to each tube. Samples were vortexed, spun down, incubated at 99 ºC for tree minutes, cooled to room temperature, and stored at −20 °C until further use. ITS-rRNA gene was amplified using the ITS primers TW81 (5'-GTTTCCGTAGGTGAACCTGC-3') and AB28 (5'-ATATGCTTAAGTTCAGCGGGT-3') (Subbotin et al., 2001) by PCR in an Applied Biosystems 2720 Thermocycler (MJ Research, Waltham, MA, USA). The PCR conditions were: denaturation at 94 °C for five minutes; followed by 35 cycles of 94 ºC for one minute, 52 ºC for one minute, and 72 ºC for one minute; and a final extension at 72 ºC for 10 minutes. Eight microliters of the amplification product was electrophoresed on a 1.8% agarose gel and stained with ethidium bromide.

The sizes of amplified products were determined by comparison with a 1 kb molecular weight ladder (Invitrogen). For direct sequencing, PCR products were purified with the QIA quick PCR purification kit (Qiagen) and sequenced. DNA sequences were compared to sequences from the GenBank National Centre for Biotechnology Information (NCBI) using BLAST search with standard algorithm parameters (NCBI, 2015).

3. Results

3.1. EPN capture

Seventy eight pathogenic isolates were captured from a total of 315 soil samples. Seventy tree samples were positive for EPN (23.2%), because some samples were positive in both the trap types. The baits with *T. molitor* captured more EPNs than the ones with *A. fraterculus* (Chi-square test, p< 0.05) (Figure 1A). EPNs were captured in 63% *T. molitor* baits against 15% with *A. fraterculus*.

EPN-positive samples without fruit over the soil were 21% of the total against 25.7% with some fruit (Figure 1B). The difference was not significant (Chi-square test, p= 0.39). Furthermore, there was no significant correlation between the number of *A. fraterculus* pupae in the fruits of the sector and the proportion of positive samples (r= 0.52; p= 0.09) (Figure 1C). Ninety one percent of the pupae emerged from fruits gave rise to *Anastrepha* sp. and, among them, all females were *A. fraterculus*. In the same way, the proportion of EPN-positive samples among the year seasons was not significantly different (Chi-square test, p= 0.36), besides the differences in air temperature which fell during winter to 16-12 ºC (Figure 2A).

The correlation between cumulated rainfall two to six days before sampling and the proportion of EPN-positive samples was not significant (r= -0.01; p= 0.96). Some rain happened in the six days preceding soil collection in almost all evaluations (Figure 2B). No rain occurred before the sampling in November 2013 and August 2014 and EPNs were captured as well.

There was no correlation between sand or clay content and proportion of EPN positive samples (r= 0.26, p= 0. 62 and r= -0.8, p= 0.10, respectively). In the same way, no correlation was observed between soil organic matter content and the proportion of EPN positive sample (r= -0.39, p= 0.27).

3.2. Virulence against A. fraterculus and isolates identification

Among 20 isolates tested, native from Porto Amazonas apple orchard soil, five caused significantly higher mortality of *A. fraterculus* larvae than the control without nematode: 158, 222, 288, 304 and 319 (Figure 3). Isolates 304 and 319 scored virulence of 73 and 71%, respectively (Figure 3B). For the other tree pathogenic isolates, *A. fraterculus* mortality ranged from 33 to 54%, also differing from the control (Figure 3A and 3C). Among the five pathogenic isolates, two (158 and 288) had significantly worse results on attacking *A. fraterculus* larvae than *S. carpocapsae* CB 02, which killed all the larvae exposed.

DNA amplification for the five isolates pathogenic to *A. fraterculus* with the primers TW81 and AB28 produced

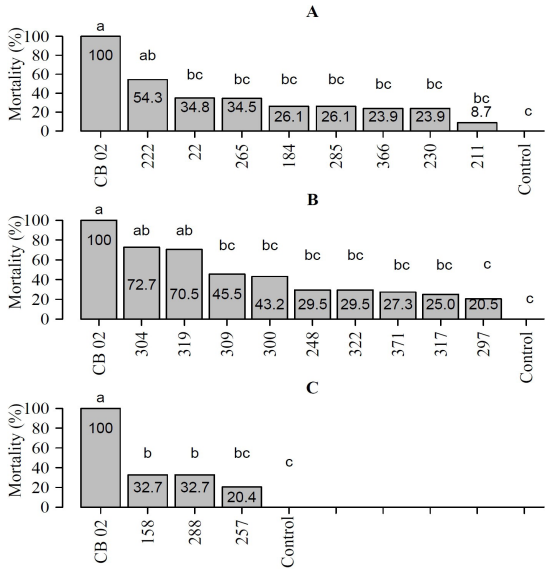

Figure 3. Mortality of *Anastrepha fraterculus* larvae following inoculation of different nematode strains isolated from soil in Porto Amazonas, PR, and *Steinernema carpocapsae* CB 02, in three trials performed in Petri dishes (100 IJ.larva^{-1}) (A, B and C). Mortality was transformed by square root for statistical analysis, resulting in coefficient of variation 9.58, 8.75 and 6.26 for A, B and C, respectively; columns with the same letter above are not different (Tukey test, α=0.05).

sequences ranging from 802 to 820 base pars. When compared to the gene bank, the isolate 222 showed 88% identity with *Oscheius* sp. isolate TEL-2014 (accession number KM492926.1) and 85% with *Oscheius* sp. isolate MCB (accession number KF684370.1) (Table 1). All the other isolates showed higher identity (98%) with *Oscheius* sp. isolate MCB (accession number KF684370.1), with null e-values (Table 1).

4. Discussion

Tenebrio molitor baits captured significantly more EPN than *A. fraterculus* ones. This difference is probably due to the bigger soil volume in *T. molitor* baits. Besides, *T. molitor* moves inside the soil, which made the infection more likely. The proportion of EPN-positive samples obtained was considered low, when compared with other works using *G. mellonella* as bait: samples with nematodes reached 50% (Rio and Cameron, 2000); Campbell et al. (1995) found nematode-positive soil samples between 25 and 38%. Besides the possible difference in EPN density in the soils, for most EPNs *G. mellonella* is better than *T. molitor* because of its higher susceptibility and body size (Boff et al., 2000), what can explain at least partially the lower frequency observed here.

The presence of a fallen fruit over the soil is expected to increase arthropods abundance nearby, because of fruit fly larvae and pupae came from the fruit (Salles, 2000) and other insect species that feeds on the decomposing fruit are stimulated. These arthropods are possible hosts for EPNs.

Table 1. Scores of identity (%) and e-values (e) output in the comparisons of DNA sequences of eight accessions available at NCBI nucleotide data bank (NCBI, 2015) with sequences found in five nematode isolates from Porto Amazonas, PR.

Accession[1]	Porto Amazonas' isolates									
	158		222		288		304		319	
	%	e	%	e	%	e	%	e	%	e
KF684370.1	98	0.0	85	0.0	98	0.0	98	0.0	98	0.0
KM492926.1	84	0.0	88	0.0	84	0.0	84	0.0	84	0.0
KF500235.1	85	$1e^{-100}$	89	$3e^{-92}$	85	$1e^{-100}$	85	$1e^{-100}$	85	$1e^{-100}$
EF503690.1	85	$2e^{-99}$	89	$3e^{-92}$	85	$2e^{-99}$	85	$2e^{-99}$	85	$2e^{-99}$
EU273598.1	85	$7e^{-98}$	89	$1e^{-90}$	85	$7e^{-98}$	85	$7e^{-98}$	85	$8e^{-98}$
JQ002565.1	85	$3e^{-96}$	89	$1e^{-90}$	85	$3e^{-96}$	85	$3e^{-96}$	85	$4e^{-96}$
FJ547241.1	89	$4e^{-66}$	90	$1e^{-70}$	89	$4e^{-66}$	89	$4e^{-66}$	89	$4e^{-66}$
AM398825.1	81	$8e^{-53}$	87	$8e^{-53}$	81	$8e^{-53}$	81	$8e^{-53}$	81	$8e^{-53}$

[1]KF684370.1| *Oscheius* sp. MCB; KM492926.1| *Oscheius* sp. TEL-2014; KF500235.1| *Heterorhabditidoides chongmingensis* isolate FUMN101; EF503690.1| *Heterorhabditidoides chongmingensis*; EU273598.1| *Rhabditis* sp. Tumian-2007; JQ002565.1| *Heterorhabditidoides* sp. RG081015 ; FJ547241.1| *Oscheius carolinensis*; AM398825.1| *Pellioditis mediterranea*.

So, it was expected an increase in EPN capture when soil samples is taken underneath a fallen apple, but it was not observed here (Figure 1B), despite fruit infestation by *A. fraterculus* (Figure 1C). This trial didn't regard the total host range in the apple orchard, so the nematodes could have other suitable host or food source, even because *A. fraterculus* is not present during the entire year. Furthermore, a great number of arthropods live in orchards and are possible nematode hosts during all seasons, regardless fallen fruit (Glazer et al., 1996).

EPN-positive samples were not different across year seasons. Temperatures between 16-10 °C as observed in Porto Amazonas during winter usually reduces EPN efficacy (Klein, 1990; Mejia-Torres and Sáenz, 2013). However, winter temperatures seemed to be high enough to assure nematode capture during the entire year, even because samples were kept at 25 ºC in the laboratory, what favored the infection. The homogeneous occurrence of EPNs along the year corroborates Campbell et al. (1995) findings on *Heterorhabditis* sp. constancy during all seasons, and Puza and Mracek (2005) on *Steinernema affine* (Gerdin and Beddind). The capture of EPNs over different seasons, and recoveries in the same area during and after winter, indicates its population is well established.

Soil moisture is one of the most critical factors on nematodes movement and survival (Kaya, 1990), as observed in a *Citrus* sp. grove where higher humidity close to the roots was responsible by higher number of EPN-positive soil samples (Glazer et al., 1996). However, in the present work there was no correlation between rainfall and the proportion of samples with EPN captured, probably because sample soil humidity was corrected in laboratory (Voss et al., 2009), so this influence could have been diminished. It's prudent to have in mind that between-work differences could be related to the species considered. However, results for *Oscheius* sp. are still few or inexistent.

Different textures of soil didn't affect the capture of nematodes. It was expected that sandy soil could have more EPNs captured, especially because of the pore sizes that enhance nematode efficiency (Kaya, 1990). Hoy et al. (2008) observed that *Rhabditis* sp. bacteria feeders were found in most vegetable cultivated soil, beside the differences in sand content from 29.7 to 49.1%. In the same way, organic matter was not an important factor in EPN capture. Organic matter richer soils have more microorganism activity, which means more food sources for facultative EPNs like *Oscheius*. Bacterial activity in the soil correlated positively with number of the facultative-EPN *Rhabditis* sp. collected (Hoy et al., 2008). The soil collected in this trial was rich in organic matter (3.8 - 5%) what can be enough to ensure good microorganism activity.

Five isolates from this trial were found to be close related to two accessions of *Oscheius* sp. (MCB and TEL-2014). Besides other accessions were found to be more identical to isolate 222, e-values for TEL-2014 and MCB pairing were lower (0.0), because the query cover was higher. The smaller the e-value, the higher the chance of matches not to be random, but due to relativeness. The accession MCB was already supported as entomopathogenic (Serepa and Gray, 2014). The isolate *Oscheius* sp. TEL-2014 was collected in a loam sandy soil on *G. mellonella* baits in South Africa (NCBI, 2015).

In South America, some *Oscheius* species [*Oscheius colombiana* and *Rhabditis* (*Oscheius) pheropsophi* n. sp.] were already found associated with insect in Colombia and southeast Brazil, respectively (Smart and Nguyen, 1994; Stock et al., 2005). However, it is possible that the captured isolates are new species since there is no report of this genus in south Brazil. The identification of these new facultative insect-parasitic nematodes is important since they can be natural pest enemies, and also can help on equilibrating soil insect populations. Further studies are necessary on the species-level identification, morphometric characteristics, associated bacteria identification and the relationship between them, involving biotic and abiotic factors.

It was observed differences in virulence to *A. fraterculus* between 304 and 297 as well as 319 and 297 (Figure 3B). Assuming the isolates are the same species (if not, it would explain the differences), the difference in virulence can be explained by the kind and amount of bacteria each

isolate carries. In *Oscheius* sp., the association between nematode and bacteria is symbiotic, not mutualistic as in *Heterorhabiditis* and *Steinernema* genera. Although the associated bacterium was not studied in this work, it is known that *Oscheius*-associated bacterium doesn't live inside its body, but on its surface (Torres-Barragan et al., 2011). Four different bacteria species were associated with *Rhabditis* (*Oscheius*) *blumi* Sabhaus. Only two were pathogenic to *G. mellonella* and with different virulence (Park et al., 2011). So, it is likely to be similar in the isolates captured here. Still, *Oscheius* sp. isolates which were less virulent to *A. fraterculus* can have other suitable food source at field since they have facultative-parasitic behavior.

It was expected that EPNs captured in the orchard would be more virulent to the same orchard pest *A. fraterculus* than *S. carpocapsae* CB 02, which was cllected in Florida (USA). However, it didn't happen. Similar observation was already reported in other insects (Grewal et al., 2002). Rodrigues-Trentini (1996) also found high virulence of *S. carpocapsae* to *A. fraterculus* larvae. *Steinernema carpocapsae* is an obligate parasite, with a wide range of hosts, and needs to be highly effective to survive, while *Oscheius* sp., as explained above, can feed in sources other than live insects.

As a conclusion, nematodes of the genera *Oscheius* are found in an apple orchard of Porto Amazonas. Some of them are pathogenic to *Anastrepha fraterculus*. Facultative EPNs population is constant along the year in the orchard, independently of fluctuations in *A. fraterculus* population, air temperature, pluvial precipitation and presence of fruit over the soil surface.

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