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Original Article

Virulence of entomopathogenic nematodes and their symbiotic bacteria, under laboratory conditions, aiming controlling *Saccharicoccus sacchari* (Cockerell, 1895) (Hemiptera: Pseudococcidae) on sugarcane

Virulência de nematoides entomopatogênicos e suas bactérias simbióticas, sob condições de laboratório, visando controlar *Saccharicoccus sacchari* (Cockerell, 1895) (Hemiptera: Pseudococcidae) na cana-de-açúcar

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Abstract

Sugarcane crops *Saccharum* spp. (Poales: Poaceae) produces different derivatives to the world: sugar, ethanol and bioenergy. Despite the application of pesticides, insect pests still cause economic losses, among these the pink sugarcane mealybug *Saccharicoccus sacchari* (Cockerell, 1895) (Hemiptera: Pseudococcidae) causing direct and indirect damage to the plant. This study assess the virulence of three entomopathogenic nematodes (EPNs) species and their symbiont bacteria against the pink sugarcane mealybug, under laboratory conditions. Fourteen treatments represented by control (distilled water), *Heterorhabditis bacteriophora* Poinar, 1976 (HB EN01) (Rhabditida: Heterorhabditidae), *Steinernema rarum* (Doucet, 1986) (PAM25) and *Steinernema carpocapsae* Weiser, 1955 (All) (Rhabditida: Steinermatidae) at concentrations of 25, 50, 75 and 100 infective juveniles (IJs)/insect, and the standard chemical product, thiamethoxam, were assayed. In a second experiment, the bacteria *Photorhabdus luminescens* (Thomas and Poinar, 1979), *Xenorhabdus szentirmaii* Lengyel, 2005 and *Xenorhabdus nematophila* (Poinar and Thomas, 1965) (Enterobacterales: Morganellaceae) at 3.0 x 10^9 cells/ml were assessed for each treatment. Ten replications were stablished, each one counting ten females/mealybugs inside a 10 cm Petri dish, amounting 100 individuals/treatment. All treatments were kept under stable conditions (25±1 ºC, H 70±10%, in the dark). All nematodes species infected *S. sacchari*. *Steinerma rarum* (PAM25) provided the highest mortality against the pink sugarcane mealybug (79.25%), followed by *H. bacteriophora* (HB EN01) (58.25%) and *S. carpocapsae* (All) (42.50%) (P<0.001). The mortality rate caused by *X. szentirmaii, P. luminescens* and *X. nematophila* were 40, 45 and 20%, respectively. *Steinerma rarum* (PAM25) has conditions to be a potential agent to be incorporate into the integrated pest management in sugarcane.

Keywords: *Heterorhabditis*, *Photorahbdus*, pink sugarcane mealybug, *Saccharum* spp., *Steinernerma*, *Xenorhabdus*.

Resumo

A cultura da cana-de-açúcar *Saccharum* spp. (Poales: Poaceae) produz diferentes derivados para o mundo: açúcar, etanol e bioenergia. Apesar da aplicação de pesticidas, os insetos-praga ainda causam prejuízos econômicos, dentre eles a cochonilha rosada da cana-de-açúcar *Saccharicoccus sacchari* (Cockerell, 1895) (Hemiptera: Pseudococcidae) causando danos diretos e indiretos à planta. Este estudo avaliou a virulência de três espécies de nematoides entomopatogênicos (NEPs) e suas bactérias simbiontes contra a cochonilha rosada da cana-de-açúcar, em condições de laboratório. Quatorze tratamentos representados pelo controle (água destilada), *Heterorhabditis bacteriophora* Poinar, 1976 (HB EN01) (Rhabditida: Heterorhabditidae), *Steinernema rarum* (Doucet, 1986) (PAM25) e *Steinernema carpocapsae* Weiser, 1955 (All) (Rhabditida: Steinermatidae) nas concentrações de 25, 50, 75 e 100 juvenis infectantes (JIs)/inseto, e o produto químico padrão, tiametoxam, foram testados. Em um segundo experimento, a bactéria *Photorhabdus luminescens* (Thomas e Poinar, 1979), *Xenorhabdus szentirmaii* Lengyel, 2005 e *Xenorhabdus nematophila* (Poinar e Thomas, 1965) (Enterobacterales: Morganellaceae) em 3,0 x 10^9 células/ml foram avaliadas para cada tratamento. Dez repetições foram estabelecidas, cada uma contendo dez fêmeas/cochonilhas dentro de uma placa de Petri de 10 cm, totalizando 100 indivíduos/tratamento. Todos os tratamentos foram mantidos em condições estáveis (25±1 ºC, U 70±10%, no escuro). Todas as espécies de nematoides infectaram

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S. sacchari. *Steinerma rarum* (PAM25) proporcionou a maior mortalidade contra a cochonilha rosada da cana-de-açúcar (79,25%), seguida por *H. bacteriophora* (HB EN01) (58,25%) e *S. carpocapsae* (All) (42,50%) (P<0,001). As taxas de mortalidade causada por *X. szentirmaii, P. luminescens* e *X. nematophila* foram de 40, 45 e 20%, respectivamente. *Steinerma rarum* (PAM25) tem condições de ser um agente potencial a ser incorporado ao manejo integrado de pragas da cana-de-açúcar.

Palavras-chave: *Heterorhabditis*, *Photorahbdus*, cochonilha rosada da cana-de-açúcar, *Saccharum* spp., *Steinernerma*, *Xenorhabdus*.

1. Introduction

Sugarcane crop *Saccharum* spp. (Poales: Poaceae) is important to the world as producing ethanol, sugar and bioenergy, standing out Brazil the world's largest producer and exporter of sugarcane derivatives (Embrapa, 2020).

In this production, many insect pests provoke economic losses, and among them, scale insects (Hemiptera: Coccoidea) cause direct and indirect damage that affect the phenology of the plant (Novoa et al., 2015; Jayanthi et al., 2016; Mohamed et al., 2009; Monteiro et al., 2021b).

There are 18 species of scale insects associated to sugarcane in Brazil (Monteiro et al., 2019). The most important one is the pink sugarcane mealybug *Saccharicoccus sacchari* (Cockerell, 1895) (Hemiptera: Pseudococcidae) attacking roots and from basal to apical nodes (Tohamy et al., 2008; Monteiro et al., 2021b).

Saccharicoccus sacchari, of unknown origin (Zhang et al., 2018) is associated to *Saccharum* spp., being reported in 79 countries around the world (García Morales et al., 2016) and widely spread throughout the state of São Paulo (Monteiro et al., 2021a). In India and Cuba, the insect caused growth delay and plant death of sugarcane shoots due to the intense sap suction (Novoa et al., 2015; Nrip and Gaikwad, 2017). In Egypt, severe infestations of *S. sacchari* caused reduction in the weight and diameter of stems (Jayanthi et al., 2016), as well as reductions of 13 to 21% of sugar production (Mohamed et al., 2009). Sucking habit of *S. sacchari* facilitates the entry of phytopathogenic microorganisms, being also a vector of the Sugarcane Bacilliform Virus (ScBv) (Autrey et al., 1995).

This insect infests newly-planted sugarcane rhizomes, up to 30 cm of depth, and as the plant grows, the species forms colonies under the leaf sheaths, on the nodes of the plants (Tohamy et al., 2008; Monteiro et al., 2021a). This cryptic habit makes difficult to control the pseudococcid, including its dispersal between plants by ants, the movements of the air provided by jet-transported equipments and infested stems left after harvest, favouring reinfestation of the pink sugarcane mealybug to the next crop cycle (Rajendra, 1974; Tohamy et al., 2008).

As there is no efficacious product against this pest, the producers are dependent by natural enemies (Cruz et al., 2019). Entomopathogenic nematodes (EPNs) (Steinernematidae and Heterorhabditidae) are interesting microbial control agents due to their low impact on the environment and selective to natural enemies (Brida et al., 2017; San-Blas et al., 2019). The families Heterorhabditidae and Steinernematidae are associated respectively to bacteria of the genus *Photorhabdus* spp. and *Xenorhabdus* spp. (Almenara et al., 2012) and both of them can be artificially cultivated (Kim et al., 2005). The bacteria is inside the infective juvenile (IJ) digestive system killing the host by septicemia (Poinar Junior and Grewal, 2012). The high susceptibility of the pseudococcids *Dysmicoccus brevipes* (Cockerell, 1893) (Hemiptera: Pseudococcidae) (Zart et al., 2021) and *Dysmicoccus texensis* (Tinsley, 1900) (Hemiptera: Pseudococcidae) (Alves et al., 2009) to EPNs suggests the pink sugarcane mealybug could be a potential target.

This study aimed to assess the virulence of three species of EPNs and their respective symbiotic bacteria against *S. sacchari* under laboratory conditions.

2. Material and Methods

2.1. Biological materials acquisition

The EPNs: *Heterorhabditis bacteriophora* Poinar, 1976 (HB EN01) (Rhabditida: Heterorhabditidae), *Steinernema rarum* (Doucet, 1986) (PAM25) and *Steinernema carpocapsae* Weiser, 1955 (All) (Rhabditida: Steinermatidae), and their respective bacteria *Photorhabdus luminescens* (Thomas and Poinar, 1979), *Xenorhabdus szentirmaii* Lengyel, 2005 and *Xenorhabdus nematophila* (Poinar and Thomas, 1965) (Enterobacterales: Morganellaceae), were provided by the Laboratory of Biological Control of the Instituto Biológico, Campinas, São Paulo, Brazil.

Adult females of *S. sacchari* were collected in sugarcane field in the municipality of Jaboticabal, São Paulo, Brazil, 21°13'22" S, -48°16'81" W, altitude of 605 m, and stored in conical tubes of 50 mL.

2.2. Virulence of entomopathogenic nematodes

The pink sugarcane mealybug were prepared in permanent slides using the technique described by Willink (1996) to identify the species. The identification took place under an optical microscope through morphological characteristics, using the work of Williams and Willink (1992).

Fourteen treatment tested *H. bacteriophora*, *S. rarum* and *S. capocapsae* at four concentration, tiamethoxam and distilled water (control). For each treatment, eight replications were established, each replication consinting of a Petri dish (10 cm diameter) contaning a filter paper covering the botton and ten adult females of *S. sacchari*. For each nematode strain, four doses were tested, 25, 50, 75 and 100 IJs/insect, 2 mL of IJ suspension were applicated over the filter paper. Thiamethoxam (3-(2-chloro-1.3-thiazol-5-ylmethyl)-5-methyl-1.3.5-oxadiazinan-4-ylidene(nitro)amine), was tested at the pattern dose of 1.2 g L^{-1} adding same volume per dish, following the recommendation of 1200 g/ha for the

control of the coffee root mealybug *D. texensis* (Agrofit, 2021). Once this pest is from the same family of the pink sugarcane mealybug.

Mealybug mortality was assessed seven days, to obtain the maximum potential for nematode mortality. After the application of the suspensions, mortality caused by nematodes was confirmed by observing the symptoms in a Zeiss Stemi 508 (Alves et al., 2009) and observing them inside and on the dead *S. sacchari* body.

2.3. Virulence of the symbiotic bacteria

The bacteria *P. luminescens, X. szentirmaii* and *X. nematophila* were grown in Luria–Bertani (LB) medium, consisting of 0.1% tryptone, 0.05% yeast extract, 0.05% NaCl, 0.1% bacterial agar and 97% distilled water at pH 7.0 for three days at 28 °C. The bacteria were tested at the dose 3 x 10^9 cells/mL by adding 2 mL of the culture inside a Petri dish, over a filter paper circle. The bacteria were compared to the chemical (thiamethoxam) at the dose of 1.2 g L^{-1}, and with distilled water (control). Each treatment contained ten replications and each replication consisted of ten pink sugarcane mealybug, female/Petri dish, on the filter paper.

2.4. Data analysis

All experiments were conducted in a completely randomized design. The data were submitted to the tests of normality of residues by Shapiro-Wilk (Royston, 1995) and homogeneity of variances by Levene (Gastwirth et al., 2009) at 5% probability. Once the normality and homogeneity were verified, the data were submitted to analysis of variance and the means compared by the Tukey test (5%) using the Agroestat Online software (Maldonado, 2020).

3. Results

All nematodes were pathogenic to *S. sacchari*. *Steinernema rarum* (PAM25) provided the highest mortality to the pseudococcid (79.25%), followed by *H. bacteriophora* (HB EN01) (58.25%) and *S. carpocapsae* (All) (42.50%) (P<0.001). Thiamethoxam, caused 100% mortality (Figure 1A).

Although the overall efficiency of *S. rarum* (PAM25) was higher than another isolates, there was no difference (P=0.206) among tested concentrations, as well as no difference in the mortality of *S. sacchari* in *H. bacteriophora* (HB EN01) concentrations (P=0.338). However, concentration of 25 IJs of *S. carpocapsae* (All) provided the lowest mortality rate, killing only 21% of mealybugs (P<0.01).

Different mortalities were observed in the application of the isolated bacteria (P<0.001) (Figure 1C). There was no difference between the bacteria *X. szentirmaii* and *P. luminescens*, which caused 40 and 45% mortality, respectively. The lowest mortality (20%) was observed with the use of *X. nematophila*, but it was still higher than that obtained in the control (10%). Thiamethoxam caused the greatest mortality (100%).

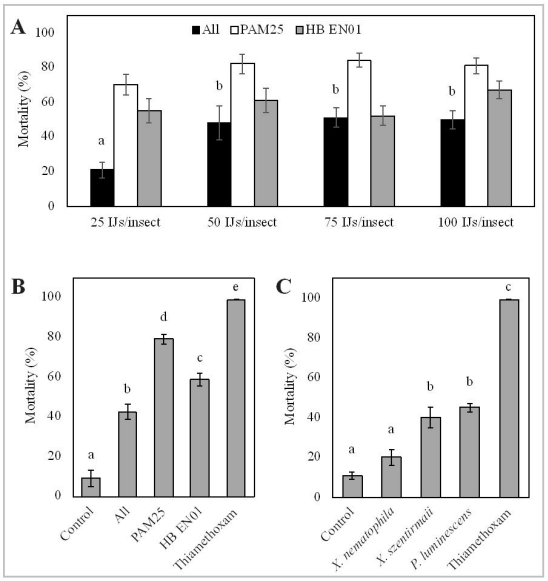

Figure 1. Effect of different strains of entomopathogenic nematodes (EPNs) and their respective symbiotic bacteria, on the control of *S. sacchari*. (A) Different concentrations of *S. rarum* (PAM 25), *H. bacteriophora* (HB EN01), *S. carpocapsae* (All) on the mortality of *S. sacchari*; (B) Main effects of different EPNs on the mortality of *S. sacchari*; (C) Effect of *X. szentirmaii*, *P. luminescens* and *X. nematophila* at concentration of 3 x 10^9 cells/ml on the mortality of *S. sacchari*. For each group, bars with different letters are significantly different (P<0.05); unidentified bars do not differ (P>0.05).

4. Discussion

Similar results were obtained by EL Roby (2018), with *Steinernema* spp. and *Heterorhabditis* spp. against *S. sacchari*, with mortality up to 48.7 and 70.8%, respectively. In general, the increase in the concentration of nematodes did not improve the control. This suggests that the control efficiency is more linked to the strain quality than the concentration of EPNs. Although *S. carpocapsae* (All) isolate was less efficient at the concentration of 25 IJs, the highest concentration also showed low mortality (<51%) (Figure 1B). These results show that virulence depends on the nematode species, highlighting the importance to screem the best nematode for each targert.

Other studies with *Steinernema* spp. and *Heterorhabditis* spp. also proved their virulence against other groups of scale insects, such as *D. texensis*, with up to 70% mortality (Alves et al., 2009). Laboratory tests with four nematodes species demonstrated that *S. carpocapsae* strain caused 78% mortality of scale insects with 25 IJs/insect (Andaló et al., 2004).

Evaluating the virulence of EPNs to *D. texensis*, Alves et al. (2009) verified that *Heterorhabditis* sp. (CCA), *H. bacteriophora* (HB EN01), *Heterorhabditis* sp. (JPM3 1) and *Heterorhabditis* sp. (JPM3) provided high virulence, in the concentration of 100 IJs/insect, reaching mortality of 100, 94, 93.6 and 80.9% respectively. The isolates (CCA) and (JPM3) also were efficient controlling the pseudococcids

sheltered in crypts, which caused mortality of 84 and 93% of the insects respectively.

The mortality obtained with the use of bacteria can be related to the secondary metabolites produced that came into contact through the natural openings such as mouthpieces, spirals, circles and anus of the mealybug. There are a large number of genes in the entomopathogenic bacteria responsible for the production of toxins and secondary metabolites. These molecules guarantee the insect's pathogenic process and recognition by the nematode, maintaining symbiosis (Clarke, 2008).

Photorhabdus luminescens synthetizes anthraquinone secondary metabolites and antibiotics of the stilbene class, which are pathogenic toxins (Brachmann et al., 2007; Duchaud et al., 2003; Joyce et al., 2008). The bacterium *X. nematophila* produces antibiotics of different classes, xenocoumarin and xenorhabdin. Antibiotic biosynthesis prevents other species of bacteria from infecting the corpse colonized by EPNs (Bradley et al., 1999). The first characterized toxins of *Photorhabdus* spp. (Tc) and (Mcf) are examples of proteins involved on controlling the immune response of the infected insect, inducing apoptosis in the cells of the immune system. These bacteria produce compounds that inhibit the phospholipase A2 enzyme of the infected insect. This enzyme is involved in the biosynthesis of eicosanoids that regulates the cellular immune response (Kim et al., 2005; Stanley, 2006).

The *X. nematophila* bacteria has genes encoding a toxin complex in its genome (Almenara et al., 2012). Among them, Brown et al. (2004) identified a 42 kDa protein, toxic to insects, which, when expressed in a recombinant system, this toxin (Tc) caused deaths to larvae of *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae) and *Helicoverpa armigera* (Hubner, 1809) (Lepidoptera: Noctuidae) in doses of 30 to 40 ng/g. Other example of secreted toxin is the protein Xpt, also with insecticidal activity (Sergeant et al., 2006). These proteins kill insects when injected artificially, released during infection with EPNs or administered orally (Herbert and Goodrich-Blair, 2007).

However, the mortality below 50% caused by the bacteria might be related to the hydrophobic waxes on the scale insect body, which hinder the action of contact of the bacteria culture and its metabolites to the body of the mealybug, and also by the pseudococcid sucking behaviours, requiring the ingestion of bacteria or toxic compounds to reach the target site to confer insecticidal action.

Steinernema rarum (PAM25), as evidenced under laboratory conditions, has the potential as biological control agent to be incorporate into the integrated pest management program in sugarcane against the pink sugarcane mealybug. However, further studies are needed to reveal the field performance of EPN isolates tested.

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