PAULO HENRIQUE DE SIQUEIRA SABINO

EFEITO DE INSETICIDAS UTILIZADOS NA CULTURA DO TOMATEIRO NA INFECTIVIDADE DE NEMATOIDES ENTOMOPATOGÊNICOS *Steinernema carpocapsae* e *Heterorhabditis amazonensis* (NEMATODA: RHABDITIDA)

Lavras - MG
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Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Entomologia, área de concentração Entomologia, para obtenção do título de mestre.

Orientador
Dr. Alcides Moino Junior

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Dr. Aldomario Santo Negrisoli Junior EMBRAPA

Dr. Luís Cláudio Paterno Silveira UFLA

Dr. Alcides Moino Junior
Orientador

LAVRAS – MG
2015

A Deus, princípio de toda sabedoria,
que está comigo em todos os momentos
dando-me forças para seguir firme na jornada da vida,

OFEREÇO

Aos meus pais, Roberto e Berenice
e a minha namorada Ticyane
DEDICO

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RESUMO GERAL

Os nematoides entomopatogênicos (NEP) são agentes de controle biológico de diferentes insetos-praga, podendo variar quanto ao seu grau de sucesso, em função das diferentes formas de ação, especificidade, capacidade de adaptação em novos ambientes, também podendo ocorrer sinergismo com outros agentes entomopatogênicos. Os NEP podem ser utilizados em aplicação conjunta com produtos fitossanitários, no chamado mistura de tanque, uma vez que os produtos não causam efeitos deletérios aos NEP. Dessa forma, o presente trabalho teve como objetivo relacionar a reserva de lipídios dos juvenis infectantes (JI), sua atividade metabólica e as bactérias mutualísticas com inseticidas que mantêm os JI viáveis, no entanto, reduzem sua infectividade a larvas *Galleria mellonella*. Foram utilizados os NEP *Heterorhabditis amazonensis* JPM4 e *Steinernema carpocapsae* ALL, que apresentam potencial no controle de *Tuta absoluta*. Foram utilizados oito inseticidas registrados para a cultura do tomateiro. A compatibilidade dos inseticidas com os NEP foi avaliada através da mortalidade e infectividade dos JI 48 horas após o contato com os inseticidas. Os inseticidas Vertimec® e Klorpan® reduziram a infectividade dos JI. Além disso, esses mesmos produtos reduziram o teor de lipídios e alteraram a atividade metabólica dos JI, no entanto não houve influência sobre as bactérias mutualísticas. A mudança na atividade metabólica e reserva de lipídios são fatores que influênciam na infectividade dos JI, no entanto outros estudos devem ser realizados para analisar quais outros fatores afetam a redução da infectividade.

Palavras-chave: Controle associado. Controle biológico. Seletividade.

GENERAL ABSTRACT

The entomopathogenic nematodes (EPNs) are biological control agents of different insect pests and may vary as to their degree of success, depending on the different action forms, specificity, adaptability to new environments, and can also occur synergism with other entomopathogenic agents. EPNs can be used in joint application with pesticides since that the products do not cause deleterious effects to EPNs. Thus, the present study aimed to correlate the reserve lipids of infective juveniles (IJs), their metabolic activity and mutualistic bacteria with insecticides that keeps viable IJs, however, reduces their infectivity to larvae *Galleria mellonella*. Two species of EPNs, *Heterorhabditis amazonensis* JPM4 and *Steinernema carpocapsae* ALL, which have potential to control *Tuta absoluta* were used. Eight insecticides registered for the tomato crop were used. The compatibility of insecticides with EPNs was assessed by mortality and infectivity of IJs 48 hours after contact with the insecticide. Vertimec® e Klorpan® reduce the infectivity of IJs. Moreover, these products reduced the lipid content and altered the metabolic activity of the IJs, however not influence the mutualistic bacteria. The change in metabolic activity and lipid reserves are factors that influence the infectivity of IJs, however other studies should be conducted to examine what other factors affect the reduction of infectivity.

Keywords: Associated control. Biological control. Selectivity

SUMÁRIO

1 INTRODUÇÃO GERAL

O controle biológico é um método que acompanha a evolução humana, tendo grande significado na agricultura mundial, visto que o seu emprego tem aumentado a cada ano e as exigências por produtos de qualidade e isentos de produtos fitossanitários estão constantemente pressionando os produtores a maiores investimentos na área. Além disso, a pesquisa tem auxiliado no desenvolvimento de técnicas e produtos que melhorem a eficiência dos agentes de controle de forma a minimizar seus custos.

Dentre os agentes de controle biológico os nematoides entomopatogênicos (NEP) estão entre os mais interessantes e utilizados em todo o mundo, principalmente devido a sua alta capacidade no controle de pragas, seletividade a inimigos naturais e controle associado com produtos químicos seletivos. A sua utilização tornou-se uma ferramenta importante no manejo integrado de pragas (MIP), sendo uma alternativa benéfica ao produtor. Apesar das várias vantagens que apresentam, pouco está sendo utilizado no campo. As causas prováveis desse pequeno interesse residem na falta de investimento das empresas em tecnologias que possam melhorar a eficiência desses agentes de controle biológico; ausência de pessoas capacitadas no seu manejo e pouco conhecimento dos produtores sobre os NEP.

No mercado altamente competitivo, a boa produtividade e alta qualidade das plantações dependem diretamente do manejo das pragas, e este, por sua vez depende de qual melhor método de controle. Sendo assim, é importante que se produza plantas de bom estado fitossanitário, através da utilização de agentes biológico, químicos ou com a associação entre ambos.

O uso de produtos fitossanitários, como fungicidas, inseticidas, nematicidas e acaricidas, entre outros, representa um dos principais métodos de controle de doenças e pragas agrícolas. A facilidade de aplicações, associadas à

possibilidade de resultados imediatos, tornaram o uso desses produtos muito difundido em diversas culturas. Entretanto, essa prática pode apresentar grandes problemas quando utilizados de forma indiscriminada, causando efeitos deletérios ao ambiente e aos animais, inclusive ao homem e aos inimigos naturais das pragas agrícolas.

Estudar o efeito de produtos fitossanitários sobre NEP torna-se altamente importante, uma vez que a partir dessas informações pode-se reduzir o efeito deletério dos produtos, buscando formulações que mantêm a infectividade dos nematoides e consequentemente melhorar a eficiência desse agente de controle biológico em condições de campo.

Os objetivos neste estudo foram

- Determinar a compatibilidade entre NEP e inseticidas utilizados na cultura do tomateiro.

- Medir o teor de lipídio dos NEP após o contato com inseticidas que reduziram sua infectividade.

- Estabelecer uma metodologia para medir a liberação de CO_2 a fim de quantificar sua atividade metabólica.

- Avaliar o efeito de inseticidas na liberação de CO_2 e sobre as bactérias mutualísticas dos NEP.

2 REFERENCIAL TEÓRICO

2.1 Nematoides entomopatogênicos na agricultura

Os NEP apresentam um futuro promissor na agricultura, devido às várias vantagens que apresentam: possuem ampla ação de controle perante as diversas pragas, resistem a vários produtos fitossanitários, não causam danos às plantas cultivadas e podem ser aplicados em pastagens, pois são nocivos a animais superiores (FERRAZ, 1998; KOPPENHOFER et al., 2000; KOPPENHOFER et al., 2002).

Atualmente, o principal método para redução de populações de pragas consiste no controle químico, que muitas vezes, pode levar a diversos e sérios problemas, tais como resíduo de produto nos alimentos, eliminação da população de inimigos naturais, contaminação humana e ambiental e conferindo resistência a populações de pragas (DIEZ–RODRIGUEZ; OMOTO, 2001). Uma ferramenta importante para redução dos problemas citados é o controle biológico, como os NEP que pertencem à ordem Rhabditida e possuem associação com bactérias mutualísticas, que quando introduzidas na hemocele do inseto pelos juvenis infectantes (JI) levam o hospedeiro a morte entre 24 e 48 horas (FERRAZ, 1998). Portanto, a utilização de NEP para o controle de pragas no Brasil é limitada. O nematoide *Beddingia siricidicola* (=*Deladenus siricidicola*) Bedding (Neotylenchidae) foi introduzido para o controle da vespa da madeira, *Sirex noctilio* Fabricius, 1793 no sul do Brasil (IEDE; PENTEADO; SCHAITZA, 1998). No entanto, pouco tem sido feito com os nematoides entomopatogênicos (Rhabditida: Steinernematidae e Heterorhabditidae), que possuem potencial para o controle biológico de insetos-praga (GREWAL; GEORGIS, 1998).

Existem várias pragas-alvo no Brasil que podem ser controladas com NEP. Há também oportunidades para encontrar novas espécies de nematoides adaptadas às condições ambientais que o inseto-praga habita.

2.2 Nematoides entomopatogênicos e produtos fitossanitários

Esses nematoides são aplicados em áreas que recebem diferentes insumos agrícolas como produtos fitossanitários, fertilizantes e corretivos de solo, sendo que alguns produtos podem reduzir a sobrevivência e infectividade desses nematoides (GREWAL; DE NARDO; AGUILLERA, 2001). Na maioria das vezes os testes de compatibilidade de produtos fitossanitários são realizados apenas com parasitoides e predadores, excluindo os entomopatógenos, desconsiderando assim sua importância no controle biológico aplicado e natural de insetos (ALVES; MOINO JÚNIOR; ALMEIDA, 1998). Assim, é importante a avaliação criteriosa de produtos fitossanitários e NEP, visando à inserção desses organismos em programas de MIP. O MIP encoraja a diminuição da utilização de produtos fitossanitários e fornece várias opções para se controlar a praga harmoniosamente, fazendo com que essa permaneça em baixos níveis populacionais e, assim, não causem danos econômicos à cultura (PAPACEK; SMITH, 1994).

A utilização de entomopatógenos para o controle de pragas exige diferentes estratégias no seu manuseio. Sendo assim, a estratégia mais prática é aquela que mantém o nível adequado de entomopatógenos no agroecossistema. Essa estratégia pode ser adotada com utilização racional de produtos fitossanitários e aplicação de produtos fitossanitários seletivos (ALVES; MOINO JÚNIOR; ALMEIDA, 1998).

A maioria dos estudos de compatibilidade de entomopatógenos com produtos fitossanitários tem sido realizada *in vitro* tendo a vantagem de expor ao máximo o microrganismo à ação do produto químico, fato que não ocorre em condições de campo, onde vários fatores servem de obstáculo a essa exposição, protegendo o entomopatógeno. Assim, constatada a inocuidade de um produto

fitossanitário em laboratório, não há dúvidas sobre a sua seletividade em condições de campo (ALVES; MOINO JÚNIOR; ALMEIDA, 1998).

Alguns produtos fitossanitários são altamente seletivos aos entomopatógenos, tendo esses que receber especial atenção em culturas nas quais o uso do controle químico seja indispensável, como frutíferas de clima temperado, citros, café, algodão, soja, etc, em que os problemas causados pelo ataque de pragas são considerados pontos-chave na condução das culturas (ALVES; MOINO JÚNIOR; ALMEIDA, 1998). De modo geral, as interações entre produtos fitossanitários e NEP podem ser positivas (ação sinérgica, aditiva) ou negativas (ação supressiva, antagonista), como descritas a seguir (NEGRISOLI JUNIOR, 2005).

2.3 Interações positivas

Alguns produtos fitossanitários são altamente seletivos aos entomopatógenos, contribuindo para sua ocorrência natural e o controle de uma determinada praga. Um estudo de compatibilidade com os NEP *Steinernema carpocapsae e Steinernema feltiae* foi realizado com 75 formulações comerciais de agrotóxicos. Os resultados indicaram que as formas JI de ambas as espécies toleram a maioria dos produtos testados, sendo que, entre os fungicidas, Dodine® foi o mais tóxico e entre os herbicidas, Alachor® e Paraquat®. Entre as formulações de inseticidas, acaricidas e nematicidas, os mais prejudiciais foram Parathion®, Aldicarb®, Methomyl®, Flubenzimina® e Fenamifós® (ROVESTI; DESEÖ, 1990).

Observando a ação de piretroides, Negrisoli Júnior, Garcia e Barbosa Negrisoli (2010) encontrou baixa mortalidade dos NEP (abaixo de 10%) quando expostos ao produto Decis®. A segurança de outros inseticidas à base de piretroides foi também comprovada por Rovesti et al. (1988) e Rovesti e Deseö

(1990), os quais verificaram alta viabilidade de *Heterorhabditis bacteriophora* e *S. carpocapsae*, após a exposição a esses produtos, o que amplia os resultados quanto à segurança deste produto aos NEP

2.4 Interações negativas

Efeito negativo também pode ocorrer entre produtos fitossanitários e NEP que pode estar relacionado tanto ao princípio ativo do agrotóxico como também aos materiais usados na formulação desses produtos. Sendo assim, alguns cuidados devem ser tomados ao misturar produtos químicos com agentes microbianos (ALVES; MOINO JÚNIOR; ALMEIDA, 1998).

Os grupos químicos mais tóxicos para *Steinernema* spp. e *Heterorhabditis* spp. são os organofosforados e carbamatos (HARA; KAYA, 1982; ROVESTI; DESEO, 1990; ROVESTI et al., 1988).

Hara e Kaya (1982) constataram que JI de *S. carpocapsae* foram afetados adversamente em seu comportamento e infectividade contra *Spodoptera exigua* (Lepidoptera: Noctuidae) por organofosforados (mevinfós, fenamifós e triclorfom) e por carbamatos (oxamil e methomil) em várias concentrações, havendo diminuição da virulência e movimentação dos JI.

Vários trabalhos de compatibilidade de produto fitossanitários com NEP mostram que os JI permanecem viáveis 48 horas após o contato com o produto, no entanto, reduzem a sua capacidade de infectividade, consequentemente sua capacidade no controle da praga. Além disso, a redução da infectividade pode ser ainda maior ao longo do tempo de contato entre produto e JI (ANDALÓ; MOINO JÚNIOR; SANTA-CECÍLIA, 2004). Negrisoli Júnior, Barbosa e Moino Júnior (2008), testando 18 produtos fitossanitários com os NEP *H. bacteriophora* e *S. carpocapsae* observaram que clorpirifós (Pyrinex®) manteve alta a viabilidade de *H. bacteriophora* (97.8%) e *S. carpocapsae* (97.8%), portanto reduziu a

capacidade de infectividade dos JI de *H. bacteriophora* (7.6%) e *S. carpocapsae* (13%). Os mesmos autores verificaram ainda que os produtos Cercobin® (tiofanato metílico) e Temik® (aldicarbe) são incompatíveis com *S. carpocapsae*, provocando redução de viabilidade e infectividade dessa espécie. Para *H. bacteriophora*, os produtos Actara® (tiametoxan), Cercobin® (tiofanato metílico), Temik® (aldicarbe) e Furadan® (carbofuran) foram considerados incompatíveis com o NEP.

Diversos podem ser os fatores relacionados à redução da infectividade dos JI como redução de lipídios, alteração na atividade metabólica, inibição das bactérias mutualístas, etc.

2.5 Lipídios: Energia corporal dos nematoides

Os JI não se alimentam e dependem do material de reserva para fornecimento de energia, o que demonstra que o nível de reserva corporal é um fator importante, afetando tanto sua viabilidade quanto sua infectividade (PATEL; STOLINSKI; WRIGHT, 1997; PATEL; WRIGHT, 1997; QIU; BEDDING, 1999). Os JI são adaptados a buscar o inseto hospedeiro e são capazes de sobreviver em sua ausência por semanas ou até mesmo por meses dependendo do seu teor de lipídio (HATAB; GAUGLER, 1999).

Os lipídios neutros são a principal fonte de energia corporal dos NEP e incluem triacilgliceróis, diacilgliceróis, ácidos graxos livres. O lipídio é uma importante energia de reserva aeróbica dos JI e varia entre 11% e 67% da matéria seca do corpo desses organismos (BARRETT; WRIGHT, 1998).

Patel, Stolinski e Wright (1997) encontraram uma variação de 74 a 77% do total de lipídios neutros de *S. carpocapsae*, *Steinernema riobraves*, *Steinernema feltiae* e *Steinernema glaseri.* Como os JI dependem de lipídios como fonte de energia enquanto procura por um hospedeiro (LEE; ATKINSON, 1977;

VAN GUNDY, 1985) com a redução de lipídios ocorre uma diminuição na capacidade de locomoção e infectividade no hospedeiro (LEWIS, 1995). Dessa forma, evitar condições de estresse que reduzam o lipídio, auxilia para manutenção da viabilidade e infectividade dos JI (WRIGHT; PERRY, 2002), como o uso de produtos fitossanitários que não afetam o teor de lipídios dos JI.

Lipídios neutros podem ser analisados usando cromatografia ou através da aplicação de métodos histológicos com base na coloração com *Oil red O* (CHRISTOPHERS et al., 1997). A análise cromatográfica é demorada e exige um grande número de namatoides, enquanto ensaio histológico é simples e pode ser aplicado a amostras individuais. A análise de imagem foi utilizada com sucesso para quantificar o grau de coloração com *Oil red O* e permitir o exame de muitas amostras em um curto período (QIU; BEDDING, 1999).

2.6 Atividade metabólica: liberação de CO_2

Os NEP são organismos aeróbicos, necessitando de oxigênio para sua sobrevivência (GLAZER, 2002). Os JI vivem livremente no solo, sendo que alguns tipos de solo são caracterizados por apresentarem baixa oxigenação, podendo reduzir sua sobrevivência (QIU; BEDDING, 1999). Baixas concentrações de oxigênio induzem o estágio de dormência (anaerobiose) em várias espécies de nematoides em estágio livre (WHARTON, 1986). A habilidade dos nematoides em sobreviver em condições aeróbicas e anaeróbicas é bastante variável entre as espécies e também entre diferentes estádios dentro da mesma espécie (FÖLL et al., 1999). Sendo que em condições aeróbicas e anaeróbicas o JI depende de carboidratos, lipídios e aminoácidos como fonte de energia (GLAZER, 2002). Células microbianas em condições aeróbicas, utilização de oxigênio para oxidar os materiais energéticos através de reações de catabolismo para obter energia para manutenção (MOREIRA; SIQUEIRA, 2006).

De uma maneira geral, no final do catabolismo de carboidratos, lipídios, aminoácido ocorre liberação de CO_2 pelos NEP (WRIGHT; PERRY, 2002).

Para medir a atividade metabólica de populações microbianas do solo, a quantidade de CO_2 liberado pela respiração dos microrganismos é um dos métodos mais tradicionais e utilizados (ZIBILSKE, 1994) e sua quantificação depende do estado fisiológico das células (MOREIRA; SIQUEIRA, 2006). A respiração reflete a atividade microbiológica dos organismos, sendo que ela pode ser medida pela quantificação de CO_2 liberado resultante da atividade dos microrganismos aeróbicos (RAMOS-RODRIGUES, 1999). Além dos nematoides, as bactérias simbiontes dos nematoides apresentam altas taxas de respiração (SMIGIELSKI; AKHURST; BOEMARE, 1994), podendo influenciar na liberação de CO_2.

2.7 Bactérias mutualísticas: *Xenorhabdus* e *Photorhabdus*

A grande importância atribuída às espécies de *Steinernema* e *Heterorhabditis* deve-se à associação mutualística com bactérias do gênero *Xenorhabdus* e *Photorhabdus*, respectivamente (BOEMARE, 2002). As bactérias do gênero *Xenorhabdus* ocorrem naturalmente em uma vesícula intestinal dos JI em *Steinernema* (AKHURST; BOEMARE, 1983), enquanto que *Photorhabdus* está localizada na parte anterior dos JI de *Heterorhabditis* (BOEMARE, 2002). O parasitismo inicia-se quando JI entra por aberturas nautrais (boca, ânus e espiráculo) ou diretamente no tegumento (principalmente *Heterorhabditis*) do inseto, liberando a bactéria através do ânus (BOEMARE, 2002). Na associação, os NEP contribuem oferecendo proteção à bactéria fora do corpo do inseto e atuando como transportadores das bactérias de um inseto morto para a hemocele de outro vivo. A contribuição da bactéria, por sua vez, está no fornecimento de nutrientes aos nematoides (BOEMARE, 2002).

Essas bactérias ocorrem em duas formas, referidas como primárias e secundárias. A forma primária suporta ou possibilita a formação de maior população de NEP, sendo capaz de produzir substâncias antibióticas. Podem ser isoladas do intestino de JI ou de insetos recém-parasitados, onde é a forma predominante (Fase I). Converte-se depois na forma secundária (Fase II), mais estável, menos rica em termos nutricionais e incapaz de produzir antibióticos, prevalecente em insetos parcialmente decompostos ou já mortos. As bactérias na hemocele do inseto se multiplicam rapidamente, levando à morte do hospedeiro, ficando tomado por uma verdadeira "sopa bacteriana", um meio rico em nutrientes constituídos pelas bactérias, a partir do qual os nematóides se alimentam (FERRAZ, 1998).

Segundo Smigielski, Akhurst e Boemare (1994) existe diferença na atividade respiratória entre as fases de ambos os gêneros de bactéria. Os mesmos autores observaram uma maior capacidade de absorção de nutrientes pela fase secundária em relação à fase primária, além disso, a atividade respiratória na fase secundaria foi de 15 a 100% maior em relação à fase primária, mostrando que as células bacterianas da fase primária são mais adaptadas às condições internas do nematoide e inseto, já as células bacterianas da fase secundária adaptadas a condições de solo.

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ARTICLE 1 Compatibility of entomopathogenic nematodes (Nematoda: Rhabditida) with insecticides used in the tomato crop

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ABSTRACT: Entomopathogenic nematodes (EPNs) are agents that can be used for the biological control of pests associated with insecticides in a tank mix. Compatibility studies need to be conducted to analyze which products are compatible with nematodes. The aim of this work was to evaluate the compatibility between EPNs and the insecticides that are most used on the tomato crop, and to correlate the toxicological classification of the chemical products with two species of EPNs that have the potential to control tomato leaf miner, *Tuta absoluta*. Among the products tested, Certero® (triflumuron), Decis® (deltamethrin), Previcur® (dimethylamino-propyl), Ampligo® (lambdacyhalothrin + chlorantranilprole), Premio® (clorantranilprole), Engeo Pleno® (thiamethoxam + lambda-cyhalothrin) were compatible (IOBC class 1) with both nematode species.

Keywords: associated control, biological control, selectivity

INTRODUCTION

The tomato (*Lycopersicon esculentum* Mill) is considered one of the main agricultural crops worldwide, being currently distributed in all Brazilian regions, especially the Midwest and the South- East[1]. Among the principal insect pests of the tomato crop the tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), stands out. It is considered a key pest, causing damage to leaves, shoots and fruit, and may cause complete loss of the crop[2]. Chemical control is the most common way to reduce pest populations, but it can often lead to serious problems such as elimination of the population of natural enemies, human and environmental contamination and induced resistance in pest populations[3].

Biological control is an important tool in the reduction of the aforementioned problems. One biological control strategy is the use of entomopathogenic nematodes (EPNs), especially species from the genera

Steinernema and *Heterorhabditis,* due to the mutualistic association with bacteria from the genera *Xenorhabdus* and *Photorhabdus*, respectively, which cause rapid death to the insect. Parasitism starts when infective juveniles (IJs) enter through natural openings (mouth, anus and spiracle) or directly in the insect's tegument (especially *Heterorhabditis*), releasing the bacteria through the anus and causing host death[4].

Foliar application of nematodes presents great potential for controlling leaf miner larvae [5, 6]. The nematodes are applied in crops that receive varying agricultural inputs, such as fertilizers and chemical products applied on the leaves; some products may reduce the survival and infectivity of these nematodes[7]. In integrated pest control, selective insecticides are used together with biological control agents, and they may influence the activity of these organisms[8]. It has thus become very important to learn more about which insecticides help the nematodes in integrated control and, in consequence, reduce the establishment of populations with genes that confer resistance to a control agent[9]. Thus, it is vital to evaluate critically the compatibility of insecticides and entomopathogenic nematodes, aiming to introduce these organisms into integrated pest management (IPM).

The aim of this work was to evaluate the compatibility between insecticides that are most used on the tomato crop and correlate the toxicological classification of the products with two species of EPNs that have the potential to control tomato leaf miner.

MATERIALS AND METHODS

The nematodes used in the bioassay were *Steinernema carpocapsae* All (isolated in North Carolina, USA) and *Heterorhabditis amazonensis* JPM4 (isolated in Lavras, MG, Brazil, identified at the University of Florida)[10], which were maintained in an aqueous suspension (500 IJs / mL) at 16 ± 1 °C.

Production of *Galleria mellonella* (L) (Lepidoptera: Pyralidae) was carried out in accordance with the methodology adapted by Dutky et al.[11], using an artificial diet modified by Parra[12]. The entomopathogenic nematodes were multiplied on final-instar larvae of *G. mellonella*, in accordance with Kaya & Stock[13].

The nematodes obtained from *G. mellonella* larvae were kept in aqueous suspension at 16 ± 1 °C and stored for up to one week before being used in the experiment. The concentration of IJs in the final suspension was quantified with the use of polystyrene sheets containing 96 wells as used in serological tests, applying 0.1 mL of IJ suspension per well. At the end, the quantity of IJ per aliquot of 1 mL was obtained, and the mean value of three aliquot counts was taken.

Compatibility bioassay between entomopathogenic nematodes and insecticides

To determine the compatibility of the entomopathogenic nematodes with the insecticides used on tomato crops, the methodology modified by Negrisoli Jr et al.[14] was used, evaluating the viability and infectivity of the IJs after exposure to the products. The bioassay was carried out with eight insecticides normally used on the tomato crop (Table 1).

One liter of each formulated product was prepared, at double the recommended dose for application on one hectare. From this solution, one aliquot of 1 mL was taken and placed in each glass tube of each treatment, with each treatment being composed of five tubes (each tube as one repetition), to which 1 mL of suspension containing 2.500 IJs was added and shaken. The bioassay took place in a chamber climatized at 27 ± 1 °C, RH of 70 ± 10%. The viability of the nematodes was evaluated 48 hours after exposure to the products. For this, one aliquot of 0.1 mL (which corresponds to 125 IJs) was removed from the suspension and the IJs were observed under stereoscopic microscope to verify the effect caused by the tested products on nematode viability. Those that did not

respond to stimulation with scalpel were considered dead. Soon after evaluating viability, the infectivity test was carried out; for this 3 mL of distilled water was added to the glass tubes, which were then left to decant for 30 minutes in a chamber climatized at 27 ± 1 °C, RH of 70 ± 10%. The supernatant (about 3 mL) was discarded and washing was repeated three times. After the last wash, 0.2 mL (about 250 IJs) was removed from the bottom of each tube and pipetted on to five Petri dishes per treatment (9 cm in diameter), each containing one sheet of filter paper, previously moistened with 1.8 mL of distilled water.

Each dish received 10 final-instar *G. mellonella* larvae and was kept in a chamber climatized under the same conditions as the previous test, for three days. After this period, the dead larvae were transferred to Petri dishes (9 cm in diameter) containing filter paper, and maintained in a chamber climatized at 27 ± 1 °C, RH of 70 ± 10% for three more days. After this period they were observed under stereoscopic microscope and submitted to dissection to verify the presence of nematodes. The experimental design was completely random, and the mortality values of nematodes and larvae were submitted to analysis of variance.

The effect of the insecticides was analyzed for each nematode. The differences in the viability and infectivity of the EPN species were analyzed using Tukey test ($p < 0.05$), with the SISVAR program[15]. The effects of the treatments on EPN infectivity in *G. mellonella* were classified according to Peters & Poullot[16], based on the IOBC guide and the formula:

$E\% = 100 – (100 – \%\ \text{corrected mortality}) \times (100 – Red)$

In which Red = percentage reduction in infectivity in the treatment.

The corrected mortality was equal to zero for all treatments, and was thus not considered in the calculation of E%.

The percentage reduction in EPN infectivity was calculated by the formula:

$Red = (1 – It / Ic) \times 100$

It = mortality of *G. mellonella* in each treatment

Ic = mortality of *G. mellonella* in control treatment

Based on the value of E% the products were classified as: 1 – non-toxic (< 30%), 2 – slightly toxic (30 – 79%), 3 – moderately toxic (80 – 99%) and 4 – toxic (> 99%).

For the treatments that presented a percentage of dead larvae that was greater than in the control treatment, E% was considered equal to zero and the product was considered non-toxic.

RESULTS AND DISCUSSION

In relation to the viability of the IJs exposed to different insecticides, all provoked low mortality in the IJs from the two nematode species (Table 2). In relation to *S. carpocapsae* All, the control treatment was statistically equal to the products Vertimec®, Decis® and Klorpan®. The highest mortality registered for *H. amazonensis* JPM4 was with Previcur®, causing 24.6% of IJ mortality; this was the only product that differed statistically from the control treatment. Other products caused mortality lower than 20% on *H. amazonensis* JPM4 IJs.

S. carpocapsae All obtained higher mean infectivity in comparison to *H. amazonensis* JPM4 (Table 3). The products that most affected *H. amazonensis* JPM4 were Vertimec® and Klorpan®, provoking 30 and 26% mortality of *G. mellonella* larvae, respectively, being the only ones that differed statistically from the control treatment. The same happened with *S. carpocapsae* All, with Vertimec® and Klorpan® causing 20 and 50% of mortality of *G. mellonella* larvae, respectively.

In the present work, the two species of EPNs *S. carpocapsae* All and *H. amazonensis* JPM4 were not affected in relation to their viability when exposed to the insecticide Vertimec®, but a reduction in IJ infectivity took place. This result

may have been observed due to the nematicidal effect of this product, as abamectin can involve a toxic effect directly on the plant parasitic nematodes by damaging their sensorial organs, making it impossible to recognize the penetration site[17]. Work carried out by Head et al.[18] also demonstrated that Dynamec® caused low infectivity (0.1%) of IJs in *G. mellonella* larvae.

In the present work, the two species of EPNs presented insensitivity to the chitin-inhibiting insecticide Certero®. This result may be due to the absence of chitin in the cuticular structure of the nematodes, as their primary constitution is formed by collagens, cuticulins and other proteins[19]. In addition, the insecticide with action similar to that of Certero®, chitin-inhibitor diflubenzuron (unknown comercial product), did not provoke any inhibition in the reproduction and development of *S. carpocapsae* in *in vitro* tests carried out by Hara & Kaya[20]. Chitin-inhibiting insecticides had previously been observed not affecting the viability of *Heterorhabditis bacteriophora*[21], ratifying the results observed by Rovesti & Deseö[22] on *S. carpocapsae* and *Steinernema feltiae* and by De Nardo & Grewal[23] with the insecticide Adept IGR® on *S. feltiae*, also agreeing with the results obtained in the present work.

TABLE 1. Characteristics of the insecticides used in the bioassay.

Technical name	Commercial name	Formulation[a]	T.C[b]	Chemical group	Concentration/ ha[c]
Abamectin	Vertimec 18®	EC	III	Avermectin	1,2 l
Triflumuron	Certero®	SC	II	Benzoylurea	300 ml
Deltamethrin	Decis 25®	EC	III	Pyrethroid	400 ml
Dimethylamino-propyl	Previcur N®	CS	IV	Carbamate	1,5 l
Chlorpyrifos	Klorpan 480®	EC	I	Organophosphate	800 ml
Lambda-cyhalotrin + Chlorantranilprole	Ampligo®	SC	II	Pyrethroid + Anthranilamide	300 ml
Chlorantranilprole	Premio®	SC	III	Anthranilamide	200 ml
Thiamethoxan + Lambda-cyhalothrin	Engeo Pleno®	SC	III	Neonicotinoids + Pyrethroid	800 ml

[a]EC = Emulsionable concentrate; CS = concentrated suspension; SC = soluble concentrate
[b]T. C = Toxicological classification
[c]Highest recommended concentration

TABLE 2. Percentage of mortality (mean ± SE) of *Heterorhabditis amazonenis* JPM4 and *Steinernema carpocapsae* All exposure to the insecticides used on the tomato crop (27 ± 1º C, RU de 70 ± 10 %).

Heterorabditis amazonenses		*Steinernema carpocapsae*	
Engeo Pleno®	6,2[a] ± 0,19 a	Control	2,0 ± 0,20 a
Ampligo®	8,0 ± 0,12 a	Decis 25®	9,2 ± 0,44 ab
Premio®	8,4 ± 0,17 a	Klorpan 480®	12,8 ± 0,20 ab
Control	9,0 ± 0,20 a	Vertimec 18®	13,6 ± 0,17 ab
Certero®	9,2 ± 0,20 a	Engeo Pleno®	17,6 ± 0,36 b
Vertimec 18®	13,2 ± 0,19 ab	Certero®	18,2 ± 0,64 b
Klorpan 480®	16,2 ± 0,29 ab	Ampligo®	21,2 ± 0,35 b
Decis 25®	19,2 ± 0,25 ab	Premio®	21,6 ± 0,31 b
Previcur N®	24,6 ± 0,42 b	Previcur N®	22,8 ± 0,37 b
CV (%)	7,6		7,8

[a]Means followed by the same letter do not significantly differ according to Tukey's test at a 5% significance level.

TABLE 3. Mortality of *Galleria mellonella* larvae (mean ± SE) for *Heterorhabditis amazonenis* JPM4 and *Steinernema carpocapsae* All exposure to the insecticides used on the tomato crop (27 ± 1° C, RU de 70 ± 10 %).

Heterorabditis amazonenis				*Steinernema carpocapsae*			
	Mortality (%)[a]	E%[c]	C[d]		Mortality (%)[a]	E%[c]	C[d]
Klorpan 480®	26,0 ± 0,40 b	69,7	2	Vertimec 18®	20,0 ± 0,54 c	79,1	2
Vertimec 18®	30,0 ± 0,40 b	65,1	2	Klorpan 480®	50,0 ± 0,54 b	47,9	2
Decis 25®	70,0 ± 0,31 a	18,6	1	Previcur N®	80,0 ± 0,70 a	0	1
Ampligo®	76,0 ± 0,24 a	11,6	1	Certero®	82,0 ± 0,50 a	14,5	1
Certero®	76,0 ± 0,50 a	11,6	1	Engeo Pleno®	94,0 ± 0,40 a	2,0	1
Premio®	80,0 ± 0,70 a	6,9	1	Control	96,0 ± 0,40 a	-	
Previcur N®	82,0 ± 0,37 a	4,6	1	Decis 25®	96,0 ± 0,40 a	0	1
Control	86,0[b] ± 0,60 a	-		Ampligo®	100,0 ± 0,00 a	0	1
Engeo Pleno®	90,0 ± 0,44 a	0	1	Premio®	100,0 ± 0,00 a	0	1
CV (%)	13,8				14,1		

[a]Dead *Galleria mellonella* larvae.

[b]Means followed by the same letter do not significantly differ according to Tukey's test at a 5% significance level.

[c]Treatment effect: E % = 100 – (100 – % corrected mortality) x (100 – Red). % of corrected mortality was null in all treatments and therefore not considered for calculating E.

[d] IOBC toxicological classification of the insecticides: 1—harmless (< 30%), 2—slightly harmful (30 – 79%).

Studies carried out by other authors also show low IJ mortality for *S. carpocapsae* when exposed to chlorpyrifos[24, 25, 26]. One hypothesis that may explain this insensitivity in the EPNs involves the presence of butyrylcholinesterase in the synapse of parasitic nematodes, protecting the acetylcholinesterase, and thus acting as a frontline defense against such compounds[27]. Negrisoli Jr et al.[14] observed that Pyrinex® caused low mortality in *H. bacteriophora* (2.8%) and *S. carpocapsae* (2.2%), however, it caused a reduction in the infectivity of the IJs in *G. mellonella* larvae. The same result was found in the presente study, and this may be related to the reduction in lipids in the EPNs after contact with insecticides. The quantity of lipids present in the IJs directly influences host infectivity[28].

Observing the action of pyrethroids, Negrisoli Jr et al.[19] found low EPN mortality (below 10%) when exposed to the product Decis®. Similar results were found in this study, which broadens the results related to the safety of this product for EPNs. The safety of other insecticides based on pyrethroids was also proved by Rovesti et al.[21] and Rovesti & Deseö[22], who verified high viability of *H. bacteriophora* and *S. carpocapsae*, after exposure to these products.

In this study the EPNs presented viability and infectivity similar to the control treatment when exposed to the product Engeo Pleno®. Koppenhöfer et al.[29] evaluated the effect of Merit® and Meridian® combined with *H. bacteriophora* and *Steinernema glaseri*, obtaining synergic effects from both nematodes with each of the products on the same hosts. Actara® also maintained high viability in *Steinernema arenarium* (83%), *S. carpocapsae* (83.3%), *S. glaseri* (85.1%) and *H. bacteriophora* (83.4%) in a work carried out by Andaló et al.[30]. These authors also found high infectivity (over 80%) in these nematodes when exposed to Actara®. They found high infectivity (over 60%) in four species of nematodes tested when exposed to the insecticide Furadan®; the same was found with Previcur® for the two species of EPNs used in the present study.

The products that possess more than one active ingredient, such as Ampligo® and Engeo Pleno®, did not cause damage to the two species of nematodes, confirming the safety of these products in integrated control. Products with two active ingredients were also analyzed by Negrisoli Jr et al.[14], and the product Verdadero® reduced infectivity of *H. bacteriophora*, not causing an effect on *S. carpocapsae*.

The active ingredients of the chemical group anthranilamide did not present any difference in the viability and infectivity of the nematodes, but the active ingredients of the chemical group pyrethroid did present different effects on the viability of the two nematode species. This difference may be due to the formulation of the products, which may contain surfactants that are more or less toxic to the nematodes[31, 32].

There was a correlation in the toxicological classification of the products Decis®, Premio®, Engeo Pleno® (class 3) and Previcur® (class 4), causing a low effect on the viability and infectivity of the IJs, except for Vertimec® (class 3), which caused a loss in the infectivity of the IJs when compared to the control treatment. The same occurred with Klorpan®, reducing the infectivity of the IJs; however, correlating with its toxicological classification (class 2), the other products did not present a toxicological correlation with the nematodes, in agreement with the findings of Rovesti et al.[21].

Vertimec® and Klorpan® were considered slightly toxic (class 2 – slightly toxic) for the two species of EPNs, but the toxicity of a product *in vitro* does not always represent its toxicity in the field[8], since in the laboratory the contact is extreme and guaranteed for 48 hours. One way of using the incompatible nematodes and insecticides would be applying them at different moments after the period of persistence of the product, or vice versa[19]. Foliar applications have been severely limited due to environmental obstacles such as ultraviolet radiation, high temperatures and low humidity, reducing the survival and efficacy of the

nematodes[33]. Thus, the applications should be carried out at night or in the early morning when unfavorable environmental conditions can be avoided[34]. The other products used in the bioassay were considered compatible (class 1 – non-toxic) with the two species of nematodes.

In the present work, it was confirmed that the nematodes *Heterorhabditis amazonensis* isolate JPM4 and *Steinernema carpocapsae* All are considered compatible with most of the products tested for the tomato crop.

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ARTICLE 2 Effects of some insecticides on the neutral lipid percentage, survival and infectivity of *Steinernema carpocapsae* ALL and *Heterorhabditis amazonensis* JPM 4

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ABSTRACT: Lipids are an important energy source for entomopathogenic nematodes (EPNs) and directly influence their infectivity in the host. Some insecticides reduce the infectivity of infective juveniles (IJs) while keeping them viable after exposure. Thus, the objective of this study was to correlate the amounts of lipid reserves in the EPN *Heterorhabditis amazonensis* JPM 4 and *Steinernema carpocapsae* ALL with their survival and infectivity when exposed to insecticides that keep the nematodes viable but reduced their infective capacity against *Galleria mellonella*. Among the tested insecticides, Vertimec® and Klorpan® were incompatible (class 2) with the two EPN species because they reduced infectivity. The insecticides Vertimec® and Klorpan® maintained the viability of the IJs but reduced their infectivity and their lipid amounts after insecticide exposure.

Keywords: abamectin, chlorpyrifos, triflumuron, compatibility

INTRODUCTION

Entomopathogenic nematodes (EPNs) are utilized as biological control agents of various insect pests because of their different forms of action, specificity, and strong capacity to adapt to new environments; additionally, EPNs can occur in synergy with other entomopathogenic agents[1]. Among EPNs, the species in the genera *Steinernema* Travassos and *Heterorhabditis* Poinar are of great relevance due to their symbiotic association with bacteria of the genera *Xenorhabdus* Thomas & Poinar, and *Photorhabdus* Boemare, Louis & Kuhl, respectively, causing rapid insect death[2].

These nematodes are used in areas where different agricultural inputs are applied, such as chemicals, fertilizers and soil amendments[3]. However, this treatment can cause large problems if used indiscriminately. For instance, the treatment can cause detrimental effects to the environment and animals, including humans and natural enemies of the insect pests[4]. Various studies on the compatibility of insecticides with EPNs have been performed, and in some cases, the authors conclude that the agrochemical products keep the

nematodes viable but reduce their infectivity capacity[5, 6]. One fator related to the viability and infectivity of nematodes is the lipid amounts present in infective juveniles (IJs)[7]. The IJs are dependent on lipids as their energy source during their search for a host[8, 9], being able to survive and remain infective for weeks or even months until they find a susceptible host[10]. Thus, the controlled manipulation of lipid amounts in IJs has a considerable potential to increase EPN viability and infectivity[7].

Thus, the objective of this study was to correlate the amounts of lipid reserves in the EPNs *Heterorhabditis amazonensis* JPM 4 and *Steinernema carpocapsae* ALL with their survival and infectivity when exposed to insecticides that keep the nematodes viable but reduce their infective capacity against *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae.

MATERIALS AND METHODS

The nematodes used for this bioassay were *Steinernema carpocapsae* ALL (Weiser) Wouts, Mrácek, Gerdin & Bedding and *Heterorhabditis amazonensis* JPM 4, Andaló, Nguyen & Moino Jr., (obtained from soil samples from Lavras, Minas Gerais, Brazil).

The nematodes were reared in late instar larvae of the greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae)[11]. Rearing of *G. mellonella* was performed according to the method of Dutky[12] using a modified artificial diet[13].

The nematodes obtained from *G. mellonella* larvae were kept in aqueous suspension at 16 ± 1 °C and were stored for up to one week before the experiment.

Bioassay of compatibility between entomopathogenic nematodes and insecticides

To determine the compatibility of the EPNs with the insecticides, the methodology modified by Negrisoli Jr et al.[4] was used. For this bioassay, three insecticides registered for tomato crops were used: Vertimec 18® as

abamectin, toxicological classification (T.C.) III, 1.2 L/ha; Certero® as triflumuron, T.C. II, 300 mL/ha; and Klorpan 480® as chlorpyrifos, T.C. I, 800 mL/ha. One liter of each insecticide was prepared with twice the dosage recommended for application to one hectare: 400 L. From this solution, a 1-mL aliquot was transferred to each glass vial (one replicate), with a total of five vials per treatment. Then, 1 mL of the suspension containing 2,500 IJs was added to each vial, and they were manually agitated. The vials were kept in a climate-controlled chamber at 27 ± 1 °C.

Nematode survival was evaluated after 48 hours of exposure to the insecticides. A 0.1-mL alíquota (approximately 125 IJs) was observed under a stereoscopic microscope for assessing the effects of the insecticides. The nematodes that did not react to stimulation by a bamboo pick were considered dead. For the infectivity assay, IJs that survived in the presence of insecticide exposure were washed in 3 mL of distilled water. The solution was then allowed to decant for 30 minutes in the climatecontrolled chamber at 27 ± 1 °C. The supernatant (3 mL) was then discarded, and the washing process was repeated three times. After the last wash, a 0.2-mL aliquot (approximately 250 IJs) was removed from the bottom of each vial and pipetted into five Petri dishes (9 cm of diameter) per treatment. Each Petri dish contained a filter paper that had been previously soaked in 1.8 mL of distilled water. Tem *G. mellonella* larvae were placed on each Petri dish, and the dishes were then kept in a climate-controlled chamber for three days under the same conditions described above. After the three day period, the killed larvae were transferred to a dry chamber, mounted in Petri dishes (9 cm diameter) with filter paper, and kept in a climate-controlled chamber at 27 ± 1 °C at a relative humidity (RH) 70 ± 10% for three more days. After that period, the larvae were observed under a stereoscopic microscope and dissected to determine the presence of nematodes.

The experimental design used was a completely randomized one with four treatments, namely, a control (distilled water), Vertimec®, Certero® and Klorpan®, with five replicates per treatment; the experiment was conducted

two times. An analysis of variance was performed on the mortalities of nematodes and larvae. The differences in viability and infectivity for the EPN species were analyzed using Tukey's test ($p < 0.05$) in the SISVAR software program[14]. The effects of the treatments on the EPNs' infectivity in *G. mellonella* were classified according to Peters & Poullot[15] based on the guidelines of the International Organization for Biological Control (IOBC) and the following formula:

E% = 100 – (100 – % corrected mortality) × (100 – Red)

In which Red = percentage reduction in infectivity in the treatment.

The corrected mortality was equal to zero for all treatments, and was thus not considered in the calculation of E%.

The percentage reduction in EPN infectivity was calculated by the formula:

Red = (1 – It / Ic) × 100

It = mortality of *G. mellonella* in each treatment

Ic = mortality of *G. mellonella* in control treatment

Based on the value of E% the products were classified as: 1 – non-toxic (< 30%), 2 – slightly toxic (30 – 79%), 3 – moderately toxic (80 – 99%) and 4 – toxic (> 99%).

For the treatments that presented a percentage of dead larvae that was greater than in the control treatment, E% was considered equal to zero and the product was considered non-toxic.

Analysis of lipid percentage after insecticide exposure

To analyze the neutral lipid percentage, the IJs were exposed to the insecticides that maintained nematode viability but reduced their infectivity according to the results of the previous bioassay. The control group was exposed to distilled water. Following the period of exposure, 2 mL of IJ suspension (2,500 IJs/ml) was concentrated to a volume of 0.5 mL by

decantation. Using the suspension, the lipid amounts in the IJs' bodies were analyzed using the histological colorimetric method with "Oil Red O" dye[16].

A staining solution was prepared with 0.5 g of "Oil Red O" dye in 100 mL of 100% absolute alcohol. The solution was agitated for 15 minutes and then filtered with Whatman no. 1 filter paper and kept in the dark at 5 °C in a glass container. All glassware used to prepare the staining solution was previously cleaned and sterilized. Three milliliters of the "Oil Red O" staining solution was added to each IJ suspension concentrated to a volume of 0.5 mL, and the mixture was then heated in a water bath at 60 °C for 20 minutes. After cooling down to room temperature, the IJs, with the neutral lipids in their bodies stained red, were concentrated in 0.5 mL of staining solution by centrifugation at 2,000 rpm for 1 minute. Then, 3 mL of distilled water and pure glycerin (1:1) was added, and the solutions were stored at room temperature. The experimental design used was completely randomized with three treatments, namely, a control, Vertimec® and Klorpan®, with five replicates per treatment; the experiment was conducted two times. From each replicate, 2 IJs were randomly chosen for use, totaling 10 IJs per treatment.

Following the previous procedure, glass slides with IJs were obtained for each treatment and photographed using an Olympus Cx 31 trinocular microscope. Using the photographs, the total área of the IJs bodies was estimated, and the red stained area corresponded to the lipid area. For the área estimation, Image Tool Software version 3.0 for Windows was used. Thus, based on the red stained area, the percentage of neutral lipids in relation to the total IJ body area was calculated.

RESULTS AND DISCUSSION

The mortality of IJs from the two species of nematodes after exposure to insecticides was low (Table 1). The mortality rates of *S. carpocapsae* ALL IJs after exposure to the insecticides Vertimec® (13.6%), Certero® (18.2%), and Klorpan® (12.8%) were significantly different than the mortality rate in the control (2.0%) ($F = 6.94$, $df = 3$, $p < 0.05$, $CV = 6.59$) (Table 1). For *H.*

amazonensis JPM 4, the mortality rates caused by the treatments Vertimec® (13.2%), Certero® (9.2%), and Klorpan® (16.2%) did not differ from the mortality rate of the control (9.0%) (Table 1) ($F = 1.89$; $df = 3$; $p > 0.05$, CV = 6.38), and the treatments did not reduce the IJs' viability.

Table 1. Effect of phytosanitary products on the mortality percentages (mean ± SE) of *Heterorhabditis amazonensis* and *Steinernema carpocapsae* (27 ± 1°C, RH of 70 ± 10%).

Heterorabditis amazonenses		*Steinernema carpocapsae*	
Treatment	Mortality (%) [ns]	Treatment	Mortality (%)
Control	9,0 ± 0,20	Control	2,0[a] ± 0,20 c
Vertimec®	13,2 ± 0,19	Vertimec®	13,6 ± 0,17 ab
Certero®	9,2 ± 0,20	Certero®	18,2 ± 0,64 a
Klorpan®	16,2 ± 0,29	Klorpan®	12,8 ± 0,20 b

[a] Means followed by the same letter do not significantly differ according to Tukey's test at a 5% significance level.
[ns] not significant.

The nematode *S. carpocapsae* ALL had the highest mean percentage of infectivity against *G. mellonella* larvae in comparison to *H. amazonensis* JPM 4. Vertimec® and Klorpan® reduced the infectivity of *H. amazonensis* JPM 4, causing 30 and 26% mortality of *G. mellonella* larvae, respectively (Table 2). These values were significantly different from the control (86%) ($F = 36.06$, $df = 3$, $p < 0.05$, CV = 21.12). The insecticide Certero® did not reduce IJ infectivity, resulting in 76% mortality of *G. mellonella* larvae. Similar results were observed for *S. carpocapsae* ALL, with Vertimec® and Klorpan® significantly differing from the control (96%), causing 20 and 50% mortality of *G. mellonella* larvae ($F = 35.89$, $df = 3$, $p < 0.05$, CV = 20.45), respectively, and reducing the infectivity of this nematode. The Certero® treatment did not reduce IJ infectivity and resulted in 82% mortality of *G. mellonella* larvae (Table 2).

Table 2. Effect of phytosanitary products on the infectivity (mean ± SE) of *Heterorhabditis amazonensis* and *Steinernema carpocapsae* (27 ± 1°C, RH of 70 ± 10%) in *Galleria mellonella* caterpillars.

	Heterorabditis amazonenses			*Steinernema carpocapsae*		
Treatment	Infectivity (%)[a]	E%[c]	C[d]	Infectivity (%)	E%[c]	C[d]
Control	86,0 ± 0,60 a[b]	–		96,0 ± 0,40 a	–	
Vertimec®	30,0 ± 0,40 b	65,1	2	20,0 ± 0,54 c	79,1	2
Certero®	76,0 ± 0,50 a	11,6	1	82,0 ± 0,50 a	14,5	1
Klorpan®	26,0 ± 0,40 b	69,7	2	50,0 ± 0,54 b	47,9	2

[a]Dead *Galleria mellonella* caterpillars.
[b]Means followed by the same letter do not significantly differ according to Tukey's test at a 5% significance level.
[c]Treatment effect: E % = 100 – (100 – % corrected mortality) x (100 – Red). % of corrected mortality was null in all treatments and therefore not considered for calculating E.
[d] IOBC toxicological classification of the insecticides: 1—harmless (< 30%), 2—slightly harmful (30 – 79%)

The insecticides Vertimec® and Klorpan® were considered mildly toxic (class 2 – slightly harmful) for the two EPN species. There was a correlation in the toxicological classification of the product Klorpan® (class 2) and the reduction of IJ infectivity. There was no correlation in the toxicological classification of the product Vertimec® (class 3), which caused a loss in the IJ infectivity when compared to the control treatment. However, the toxicity of an *in vitro* insecticide may not always be related to its toxicity in the field[17] because in the laboratory, the contact is extreme and guaranteed for 48 hours. One possible way to use nematodes and incompatible insecticides on the same crop would be to time the application of the nematodes after the insecticide persistence period or vice-versa[18]. The insecticide Certero® was considered compatible (class 1 – harmless) with both nematode species.

These findings indicate that the viability and infectivity of the two studied species of EPNs, *S. carpocapsae* ALL and *H. amazonensis* JPM 4, were not affected by exposure to the chitin inhibitor insecticide Certero®. This result could be due to the absence of chitin in the nematodes' cuticle, which primarily consists of collagens, cuticulins and other proteins[18]. In addition, the insecticide with na action similar to that of Certero®, the chitin inhibitor diflubenzuron, did not provoke any inhibition in the reproduction and development of *S. carpocapsae* in *in vitro* tests[19]. Chitin inhibitor insecticides are known for not having an effect on the viability of *Heterorhabditis bacteriophora* Poinar[20], supporting the results of Rovesti & Deseö[21] for *S. carpocapsae* and *Steinernema feltiae* (Filipjev), Wouts, Mrácek, Gerdin & Bedding and those of De Nardo & Grewal[22] for *S. feltiae* using the Adept IGR® in addition to the results obtained in the present study.

Head[23] studied the compatibility of five insecticides with *S. feltiae* and found that Dynamec® caused low IJ infectivity (0.1%) in *G. mellonella* larvae. The same result was observed in the present study. Other studies have also shown low mortality of *S. carpocapsae* IJs when exposed to chlorpyrifos[24, 25, 26]. A possible explanation for the insensitivity of EPNs to this insecticide is

the presence of butyrylcholinesterase in the synapses of parasite nematodes, which protects the acetylcholinesterase from the insecticidal action, acting as a first line of defense[27]. Negrisoli Jr et al.[4] observed that Pyrinex® caused low mortality rates in *H. bacteriophora* (2.8%) and *S. carpocapsae* (2.2%) but reduced the IJs' infectivity in *G. mellonella* larvae, similar to the results of the present study.

The insecticide Certero® did not affect EPN infectivity and therefore was not used for the lipid colorimetric assay. The neutral lipid percentages in the IJs were reduced in both species after insecticide exposure (Figure 1). For *H. amazonensis* JPM 4, the lipid percentages were 72.6% after exposure to Klorpan® and 63% after exposure to Vertimec®. The lipid percentage after exposure to Vertimec® was significantly different from that of the control (80.48%) ($F = 5.47$, $df = 2$, $p < 0.05$; $CV = 16.34$). Regarding *S. carpocapsae* ALL, the lipid percentages were 41.82% after exposure to Klorpan® and 45.14% after exposure to Vertimec®, both of which were significantly different from the control (74.7%) ($F = 37.86$, $df = 2$, $p < 0.05$, $CV = 17.25$).

The nematode *S. carpocapsae* has the capacity to survive for long periods of time in water by remaining quiescent in a J-shaped posture, thus saving energy[28]. Because of this behavior, it can be inferred that IJ locomotion may spend more energy from its reserves and may consequently lead to a decrease in infectivity[29]. In addition, the IJ's size and its initial lipid amounts appear to be related to their survival and infectivity[30]. Furthermore, the *Steinernema* isolates can survive longer periods than *Heterorhabditis,* as the lipid content is depleted more rapidly in the latter[31, 32]

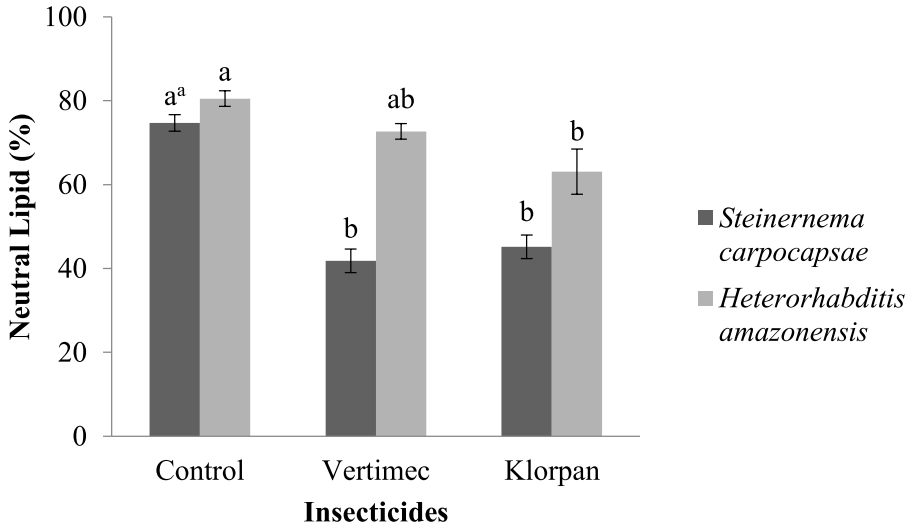

Figure 1. Effect of the phytosanitary products on the neutral lipid percentage in infective juveniles after exposure. [a]Means followed by the same letter do not significantly differ according to Tukey's test at a 5% significance level.

According to Menti et al.[33], the lipid contents of the three EPNs *S. feltiae, Heterorhabditis megidis* GR and *H. megidis* UK 211 Menti, Wright & Perry were [38] and 45%, respectively. These results are similar to the results of Fitters et al.[31]. Moreover, EPNs reared in *G. mellonella* (*in vivo*) exhibit higher initial lipid contents than those reared *in vitro*[7]. Andaló et al.[29] observed neutral lipid mean values of 97.4% for *Steinernema riobrave* Cabanillas, Poinar & Raulston, 90.3% for *S. carpocapsae* and 90.7% for *Heterorhabditis* sp. JPM 4. The same authors observed that *Heterorhabditis* isolates were less tolerant than *Steinernema* isolates at both low (8 °C) and high temperatures (24 and 28 °C).

According to Wright et al.[34], the infectivity of *S. carpocapsae* IJs declines rapidly after 90 days of storage. In *Heterorhabditis*, there is also a direct correlation between neutral lipids and infectivity[35]. Wright et al.[34] observed high levels of infectivity after 180 days of storage in water for *S. feltiae*, whereas for *S. carpocapsae,* the infectivity was stable up to 60 days.

However, after this period, there was a decline in infectivity, and only a few individuals were infective after 120 days[34].

All the above observations clearly demonstrate that the quality and quantity of IJ lipids are particularly important for the use of EPNs as pest control agents, as these factors critically influence nematode viability and infectivity. The possibility of using EPNs in integrated pest management programs (IPMs) depends on careful study of the selection of adapted species or populations to provide information on their physiological and biochemical attributes, and so to allow their efficiency to be increased for commercial use[36].

Based on these results, it is possible to observe that the use of some insecticides in association with EPNs reduces the amounts of lipids they store. Lipids are an essential resource for IJs when searching for hosts. The formulation of insecticides compatible with EPNs that are capable of maintaining adequate amounts of lipids is of critical importance. Other factors may also be involved in the reduction of IJ infectivity in the present work, such as alteration of metabolic activity, inhibition of symbiotic bactéria and damage in sensorial organs; these should be studied further. In the present work, the nematodes *H. amazonensis* JPM 4 and *S. carpocapsae* ALL were considered incompatible with the insecticides Vertimec® and Klorpan®. These two products reduce the infective capacity of IJs and their lipid amounts.

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ARTICLE 3 A method for measuring the concentration of CO_2 released by entomopathogenic nematodes

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Abstract: This study aimed to standardize a method to measure the concentration of CO_2 released by infective juveniles (IJs) to assess their metabolic activity. The nematodes used in this experiment were *Heterorhabditis amazonensis* JPM4 and *Steinernema carpocapsae* All. A gas chromatography method was used for the CO_2 analysis. There was a linear increase in the CO_2 concentration associated with increased numbers of IJs. Additionally, the CO_2 concentration obtained for *S. carpocapsae* was higher than that for *H. amazonensis*. The standardized method was adequate to measure the concentration of CO_2 released by the IJs.

Key Words*:* metabolism, standardization, Steinernematidae, Heterorhabditidae

Introduction

Entomopathogenic nematodes (EPNs) are biological control agents of various insect pests because of the distinct forms of action, specificities and abilities to adapt to new environments of these types of nematodes, which can act synergistically with other entomopathogenic agents (Ferraz, 1998). The cycle of parasitism begins with third-stage infective juveniles (IJs), which carry symbiotic bacteria internally. The IJs penetrate the host through natural openings such as the mouth, anus or spiracles or even through the cuticle, as exemplified by some species of the genus *Heterorhabditis*. Once inside the host, the IJs release their internal bacteria (Poinar, 1990). These bacteria multiply rapidly, causing septicemia and death of the host within 24 to 48 h (Grewal, 2001).

Nematodes are aerobic organisms that can enhance their survival under conditions of low oxygen availability by inducing a state of dormancy (anaerobiosis) (Glazer, 2002). The ability of nematodes to survive in aerobic and anaerobic conditions is highly variable among species and among different life stages within a species (Föll *et al*. 1999).

The amount of CO_2 released by the respiration of microorganisms is one of the most traditional and commonly used methods to measure the

metabolic activity of soil microbial populations (Moreira and Siqueira, 2006). Respiration reflects the microbiological activity of organisms and can be measured by quantifying the released CO_2, which results from the activity of aerobic microorganisms (Ramos-Rodrigues *et al*. 1999). Therefore, both EPNs and mutualistic bacteria have high respiration rates (Smigielski, 1994).

In this context, the present study aimed to standardize a method to measure the concentration of CO_2 released by the IJs of two species of nematodes to assess their metabolic activity.

Materials and Methods

Multiplication of EPNs. The nematodes used were *Steinernema carpocapsae* All (isolated from a soil sample in North Carolina, USA) and *Heterorhabditis amazonensis* JPM 4 (isolated from a soil sample in Lavras, Minas Gerais, Brazil), which were maintained in aqueous suspension (500 IJs/ml) at 16±1°C at the Laboratory of Insect Pathology, Federal University of Lavras, Minas Gerais, Brazil. The rearing of the *Galleria mellonella* specimens followed the methodology adapted by Dutky *et al*. (1964) using an artificial diet modified by Parra (1998).

The EPNs were multiplied in the last-instar specimens of *G. mellonella* following the methodology of Kaya and Stock (1997) and maintained in an aqueous suspension at 16±1ºC up to 1 week before using the EPNs in the experiment. Various concentrations of the IJs were prepared in 96-well polystyrene plates used in serological tests. To this end, 0,1 ml of the IJ suspension per well was added to a total of ten wells, thus obtaining the desired amount of IJs in 1 ml of suspension.

Bioassay to measure the release of CO_2. The bioassay was performed with five replicates of the following six treatments: control (distilled water), 500, 750, 1,000, 1,250 and 1,500 IJs/ml. One milliliter of nematode suspension and 1 ml of distilled water were added to each 9-ml tube vacuum used for blood collection. Prior to the CO_2 analyses, the IJs were stored in the tubes for 48 h in a climate-controlled chamber at 27±1°C and a relative humidity (RH) of

70±10%. The bioassay was conducted under aerobic conditions because the lids of the tubes were opened to inoculate the IJs.

The CO_2 analysis was performed in the Center for Analysis and Chemical Prospecting (Central de Análises e Prospecção Química - CAPQ), Department of Chemistry, UFLA. For the CO_2 analysis, the gas chromatography was performed using a GC-2010 apparatus with a thermal conductivity detector (TCD) at 250ºC with a +50 mV polarity, an injector temperature of 220ºC and He carrier gas at a linear velocity of 50 cm/s. The initial temperature of the column (Rt-QPLOT - 30 m x 0.32 mm ID x 10 μm) was 50ºC for a period of 3.5 min, followed by a heating ramp of 50ºC/min up to 150ºC and maintenance at that temperature for 2 min. The injection was performed in a 1:20 split mode. The total analysis time was 7.5 min. The quantification was performed via external standardization. The data were subjected to an analysis of variance and a regression analysis using the SISVAR software (Ferreira, 2011).

Results and Discussion

According to the results, there was a linear increase in the CO_2 concentration with increasing concentrations of the IJ suspensions for the two species of nematodes (Fig. 1). It was also observed that the IJs of *S. carpocapsae* resulted in a higher CO_2 concentration (1.62×10^{-4} mol/L) compared with the IJs of *H. amazonensis* (1.34×10^{-4} mol/L) (Fig. 2).

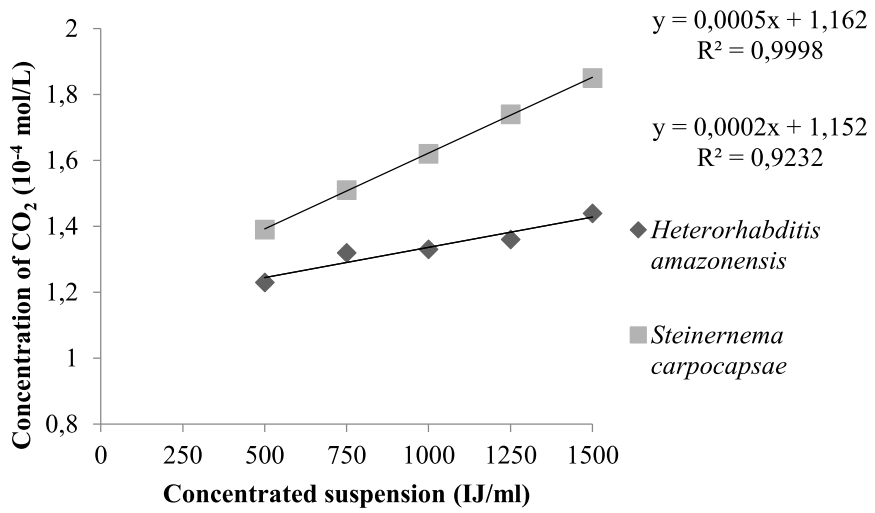

Figure 1. CO_2 concentration in the different suspensions of the infective juveniles (IJs) of *Heterorhabditis amazonensis* and *Steinernema carpocapsae*. Control = 1,049

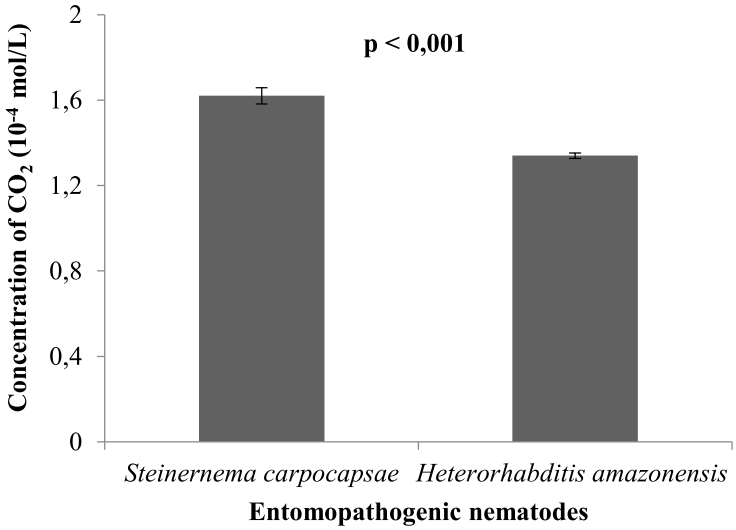

Figure 2. CO_2 concentration released by the infective juveniles (IJs) of *Heterorhabditis amazonensis* and *Steinernema carpocapsae*. [a]The means followed by the same letter do not differ according to the Tukey test at a 5% probability.

The release of CO_2 is related to the metabolic activity of organisms; it was found that it is possible to quantify this process via gas chromatography.

The proposed method allows the concentration of CO_2 released by IJs to be measured in a practical way, which enables the standardization of this method, thus assisting in various studies and enabling a better understanding of the metabolic activities of EPNs.

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ARTICLE 4 Effects of insecticides on CO_2 release and the mutualistic bacteria of entomopathogenic nematodes

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ABSTRACT - Entomopathogenic nematodes (EPNs) may be used in combination with insecticides in tank mixes for the control of various insect pests to reduce application costs and to achieve higher insect pest mortality rates. However, compatibility studies must be performed to analyze the insecticide effects on the viability and the infectivity of infective juveniles (IJs) when performing a combined application. Thus, the present study aimed to evaluate the CO_2 release by IJs and the viability of mutualistic bacteria of EPNs when exposed to contact with synthetic insecticides. Two species of EPNs, *Heterorhabditis amazonensis* JPM4 and *Steinernema carpocapsae* ALL, were included. The applied insecticides were Vertimec® (abamectin) and Klorpan® (chlorpyrifos). Gas chromatography was employed for CO_2 analysis. The bacteria were isolated in sterile Petri dishes, and the colony development was evaluated. The Vertimec® product triggered a greater CO_2 release by the nematodes, associated with changes in their metabolic activity, than the Klorpan® product. The Vertimec® and Klorpan® products did not inhibit the growth of the bacterial colonies. The change in metabolic activity is likely related to the reduction in infectivity, although other factors may be involved and should be studied.

KEYWORDS: Compatibility, infectivity, abamectin, chlorpyrifos.

Introduction

Entomopathogenic nematodes (EPNs) (Rhabditida: Heterorhabditidae, Steinernematidae) are key control agents of insect pests because of their association with mutualistic *Xenorhabdus* and *Photorhabdus* bacteria, which are released into the hemocoel of insects by infective juveniles (IJs) of the genera *Steinernema* and *Heterorhabditis*, respectively, causing septicemia and rapid death of the host within 24 to 48 h (Adams & Nguyen 2002). The use of EPNs within integrated pest management (IPM) has been increasingly studied, and the introduction of nematodes in agricultural systems involves their application combined with various other agricultural inputs, including chemical products, fertilizers, and acidity correctors.

However, those products may reduce the viability and infectivity of EPNs (Grewal *et al* 2001).

Selecting products that do not alter the viability of EPNs and their ability to infect the host is of the utmost importance for supporting their use in IPM. Several studies of insecticide compatibility with EPNs have already been conducted. However, while several products do not affect nematode viability, there is a reduction in infectivity to the host in certain cases (Koppenhöfer & Grewal 2005), and various factors may be related to this reduction in infectivity, including changes in metabolic activity and/or the inhibition of mutualistic bacteria.

The measurement of CO_2 release is one of the most frequently employed methods to quantify the metabolic activity of populations of soil organisms (Moreira & Siqueira 2006), and metabolic changes may reduce pathogen infectivity, given the greater energy expenditure (Wright & Perry 2002). Another relevant factor is the activity of mutualistic bacteria associated with EPNs (Boemare 2002), which may be inhibited following contact with specific products, preventing their development and action on the insect and, therefore, the death of the host.

Thus, an understanding of the physiological and biochemical aspects of the effects of synthetic insecticides on EPNs and their mutualistic bacteria is an important condition for the combined use of these organisms within pest control programs. Thus, the present study aimed to evaluate the CO_2 release by IJs and the activity of mutualistic bacteria of EPNs when exposed to contact with synthetic insecticides to assess factors associated with reductions in EPN infectivity to insect pests.

Materials and Methods

The nematodes used for the bioassay were *Steinernema carpocapsae* ALL (Weiser) Wouts, Mrácek, Gerdin, and Bedding and *Heterorhabditis amazonensis* JPM4, Andaló, Nguyen & Moino Jr, which have potential for the control of *Tuta absoluta* (Meyrick; Lepidoptera: Gelechiidae) in tomato

crops. They were prepared from stocks maintained as aqueous suspensions (500 IJs mL^{-1}) at 16 °C ± 1 °C.

The EPNs were multiplied in the last larval instar of *Galleria mellonella* L. (Lepidoptera: Pyralidae), according to Kaya & Stock (1997). The *G. mellonella* larvae used in the assays were generated according to the rearing method adapted by Dutky *et al* (1964) using the artificial diet modified by Parra (1998).

The insecticides were selected from the data reported in the study by Sabino et al. (2014a), wherein the said insecticides had no effect on the viability of IJs but reduced their ability to infect *G. mellonella* larvae.

Effects of insecticides on CO_2 release by entomopathogenic nematodes. The method suggested by Sabino (unpublished data) was used to analyze the CO_2 release by the nematodes to evaluate the effect of insecticides on their metabolic activity. The synthetic insecticides evaluated were Vertimec 18 CE™ active ingredient abamectin, toxicological classification (TC) III, 1.2 L 400 L^{-1} water application dose and Klorpan 480 CE™ active ingredient chlorpyrifos, TC I, 800 mL 400 L^{-1} water application dose. The dose of each product evaluated is the recommended dose for the control of *T. absoluta* in a tomato crop.

The method suggested by Negrisoli Jr. *et al* (2008) was employed to expose the IJs to the insecticides. One liter of each pesticide broth was prepared at double the recommended dose for use in one hectare. A 1-mL aliquot was collected from that solution and added to a volume of 9 mL in a Vacuette®-type plastic tube (16 x 100 mm). Following that procedure, 1 mL of suspension containing 1.500 IJs was added to each tube. The following treatments were established for each of the two nematode species: control (distilled water + IJ suspension), IJ suspension + Vertimec®, and IJ suspension + Klorpan®, performing five replicates per treatment and considering each tube a replicate.

The tubes were maintained in a temperature-controlled chamber at 27 ± 1 °C for 48 h after starting the experiment. The gas chromatography analysis

was then performed using a GC 2010 gas chromatograph with a thermal conductivity detector (TCD) at 250 °C with a polarity of +50 mV, a 220 °C injector temperature, and He as the loading gas at a linear speed of 50 cm s^{-1}. The initial column (RT-QPLOT - 30 m x 0.32 mm DI x 10 µm) temperature was 50 ºC, which was maintained for 3.5 min and then increased at 50 °C min^{-1} to 150 °C, remaining at 150 °C for 2 min. The injection was conducted in the 1:20 split mode. The total analysis time was 7.50 min. The quantification was performed by external standardization. The bioassay was conducted under aerobic conditions because oxygen entered the tubes when they were opened to add nematodes and insecticides. This has no effect on the results because the amount is negligible for the analyses.

Effects of insecticides on *Xenorhabdus* sp. and *Photorhabdus* sp. bacterial activities. Aqueous suspensions of 1000 IJs mL^{-1} of the nematodes *S. carpocapsae* and *H. amazonensis* were prepared to evaluate the effects of the insecticides on the bacteria. A 1-mL aliquot was collected from each of those suspensions and added to a flat-bottom glass test tube with a diameter of 2 cm and a height of 8 cm. Subsequently, 1 mL of an insecticide, Vertimec® or Klorpan®, prepared at the same ratio as in the previous assay, or distilled water (for the control treatment) was added to each tube, performing five replicates per treatment. Thus, the treatments were the following: control (distilled water + IJ suspension), Vertimec® + IJ suspension, and Klorpan® + IJ suspension. The tubes were kept in a temperature-controlled chamber at 27 ± 1 °C for 48 h for the subsequent evaluation of bacterial activity.

The method modified by Voss *et al* (2009) for isolating mutualistic bacteria was followed; a 50-µL aliquot was collected from the mixture of nematodes and insecticide and mixed with 50 µL of 2% sodium hypochlorite for 5 minutes in a 9-cm-diameter sterile Petri dish. That process was conducted in a laminar flow hood to promote external asepsis of IJs. The mixture was re-suspended using a micropipette, removing 50 µL and transferring it to the same volume of sterile deionized water. Mixing of the suspension was performed by pipetting up and down with the micropipette

and distributing the entire volume (100 µL) onto the surface of the nutrient agar (NA) culture medium (Kaya & Stock 1997) in a sterile glass Petri dish (9 cm in diameter) before spreading the suspension using a Drigalski spatula.

The plates were incubated in a temperature-controlled chamber at 23 ± 1 °C and evaluated after 48 and 72 h. The bacterial growth was analyzed after that period by counting the number of colonies per plate.

Statistical analysis. The data recorded for the difference in CO_2 released by both nematode species and the colony numbers per plate were submitted to analysis of variance (ANOVA) and Tukey's test for comparison of means ($p < 0.05$). The effect of the interaction between time and insecticide product was analyzed separately for each bacterial species.

Results

Effects of insecticides on CO_2 release by entomopathogenic nematodes. The results demonstrate that Vertimec® caused an increase in the concentration of CO_2 released by the IJs of *S. carpocapsae* ALL and *H. amazonensis* JPM 4 when in contact with the insecticide Klorpan® from 28.86 x 10^{-4} mol L^{-1} to 59.08 x 10^{-4} mol L^{-1} and from 13.3 x 10^{-4} mol L^{-1} to 21.47 x 10^{-4} mol L^{-1}, respectively, compared to the control treatment (Fig 1).

The insecticide Klorpan® reduced the concentration of CO_2 released by the IJs of *S. carpocapsae* ALL (28.86 x 10^{-4} mol L^{-1}) compared to the control treatment (38.72 x 10^{-4} mol L^{-1}). Conversely, the concentration of CO_2 released by *H. amazonensis* JPM 4exposed to Klorpan® was the same as that recorded for the control treatment (13.3 x 10^{-4} mol L^{-1}; Fig 1).

The values recorded for the CO_2 concentrations both in the treatment with Klorpan® and in the control were lower than the values found in the treatment with Vertimec® for both nematode species tested (Fig 1).

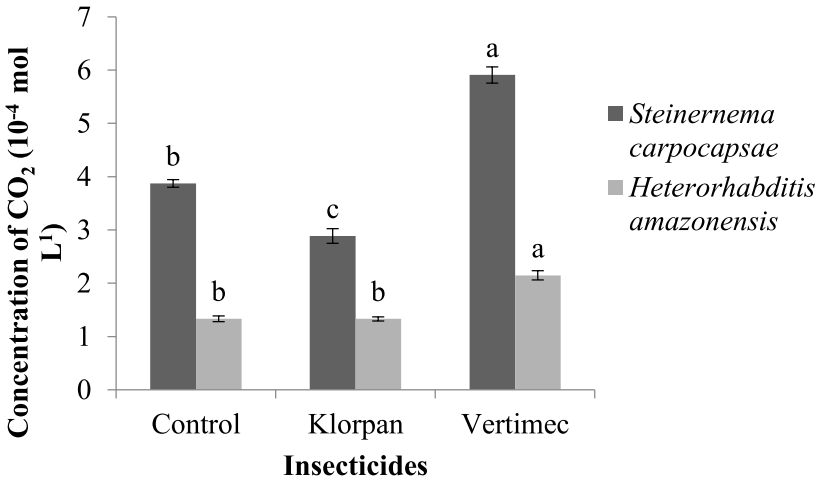

Fig 1. CO_2 release by infective juveniles of *Steinernema carpocapsae* and *Heterorhabditis amazonensis* following exposure to the insecticides Vertimec® and Klorpan®. Means followed by the same letter are not significantly different from each other according to Tukey's test at the 5% probability level.

Effects of insecticides on *Xenorhabdus* sp. and *Photorhabdus* sp. bacterial activities. There were no significant differences among the control, Vertimec®, and Klorpan® treatments ($F = 0.39$; $df = 2$; $p > 0.05$) in the mean colony numbers of the *Xenorhabdus* sp. bacteria after 48 and 72 h of exposure to the insecticides (Table 1). However, the *Photorhabdus* sp. bacterial colony number of the control treatment differed from the Vertimec® and Klorpan® treatments at both 48 h and 72 h of growth ($F = 12.94$; $df = 2$; $p < 0.05$). Although the products reduced the number of *Photorhabdus* sp. bacteria colonies, they did not cause their complete death (Table 1). There was a significant difference between the two sampling times for both *Xenorhabdus* sp. ($F = 37.08$; $df = 1$; $p < 0.05$) and *Photorhabdus* sp ($F = 14.47$; $df = 1$; $p < 0.05$), with greater colony numbers at 72 h than at 48 h, indicating that the products fail to prevent the development of bacteria over time (Table 1).

Table 1. Mean colony numbers of *Xenorhabdus* sp. and *Photorhabdus* sp. (mean ± SE) after exposure to the insecticides Vertimec® and Klorpan® at two different times, 48 and 72 hours (27 ± 1 °C, 70 ± 10% relative humidity, RH).

Treatment	*Xenorhabdus*		*Photorhabdus*	
	Time (h)			
	48	72	48	72
Control	3.4[a] ± 0.2 B a	6.8 ± 0.2 A a	4.4 ± 0.2 B a	6.6 ± 0.37 A a
Vertimec	3.6 ± 0.31 B a	5.8 ± 0.37 A a	3.4 ± 0.31 B b	4.8 ± 0.4 A b
Klorpan	4.0 ± 0.58 B a	6.2 ± 0.37 A a	3.2 ± 0.58 B b	4.8 ± 0.58 A b

[a]Means followed by the same uppercase letter within a row and lowercase letter within a column are not different from each other according to Tukey's tests at 5% significance.

Discussion

Several studies of compatibility between nematodes and insecticides have already been conducted (Hara & Kaya 1982, Rovesti *et al* 1988, Rovesti & Deseo 1990, Negrisoli *et al* 2010), although little has been published regarding the effects of insecticides on reducing the infectivity of nematodes, even without affecting their viability.

The metabolic activity of microorganisms may be measured by the amount of CO_2 released by respiration according to Moreira & Siqueira (2006), thus enabling the observation that insecticides altered the metabolic activity of nematodes in the present study because insecticides caused changes in the CO_2 released by nematodes.

Studies conducted by other authors have reported low mortality of IJs of *S. carpocapsae* when exposed to chlorpyrifos, the active ingredient of Klorpan® (Zimmerman & Cranshaw 1990, Alumai & Grewal 2004, Gutierrez *et al* 2008). A hypothesis that may explain the insensitivity of EPNs to this product suggests that the presence of butyrylcholinesterase found in the nerve synapses of parasitic nematodes protects the acetylcholinesterase from the pesticide action, thus acting as a front line of defense against such compounds (Selkirk *et al* 2001). The IJs of *S. carpocapsae* exposed to the Klorpan® product remained alive but exhibited a reduction in the release of CO_2 compared to the control treatment in the present study because the nematode *S. carpocapsae* has the ability to adapt the shape of its "J-shaped" growth curve, thus remaining in quiescence (Grewal 2000) and reducing its metabolic activity.

The nematodes evaluated in this bioassay experienced maximum exposure to the insecticides, which does not occur under field conditions (Alves *et al* 1998). Furthermore, the insecticides were prepared at double the dose recommended for application to one ha. According to Ishibashi & Takii (1993), IJs may maintain "J-shaped" growth when exposed to certain insecticides at high concentrations (from 800 to 400 *µg/ml)*, thereby reducing their metabolic activity. This shape was observed for the IJs in the present study following their exposure to insecticides.

The increases in CO_2 release by the nematodes caused by Vertimec® may be associated with the mechanism described by Sabino *et al* (2014b), who reported reductions in the lipid levels for the same nematodes because nematodes usually consume oxygen to metabolize lipids and release CO_2 at the end of the process, according to Wright & Perry (2002). Thus, this process may explain the correlation between the lipid percentage and CO_2 release.

Lipid levels are another factor related to the reduction in nematode infectivity, as in the study conducted by Sabino *et al* (2014b), wherein Vertimec® and Klorpan® reduced the lipid levels of IJs, which are essential for detecting their hosts. A similar result was observed by Andaló *et al* (2009), who reported that the herbicides Ranger® and Topeze® reduced the lipid levels of *H. amazonensis*.

Bacterial populations present in soils experience the effects of insecticides when the insecticides are applied to soils to control insect pests, and chlorpyrifos and abamectin have the capacity to be persistent in soils and possibly act on microorganisms (Santos & Monteiro 1994, Gebremariam *et al* 2012, Milhome *et al* 2009).

The insecticides Vertimec® and Klorpan® have high octanol/water partition coefficients (log K_{ow}), ranging from 4 to 5 (Gebremariam *et al* 2012, Milhome *et al* 2009). The low toxicity of these products to the bacteria may be related to their high lipophilicity, which may reduce their ability to penetrate inside the nematodes because of the presence of lipids in their body,

without directly affecting the bacteria. Another factor that may complicate the penetration of the insecticides inside the nematodes is the retention of the cuticle from the J2 stage, prior to JI, with the nematode containing both cuticles. Furthermore, the cuticles of nematodes contain a small amount of lipids, which further inhibits the penetration of the insecticides (Fetterer & Rhoads 1993).

Infective juveniles of *Steinernema* carry the bacteria *Xenorhabdus* in a specialized intestinal vesicle, whereas the bacteria are dispersed in the midgut region and are encased in tissues of different structures in nematodes of the *Heterorhabditis* genus (Forst & Clarke 2002). In the present study, there was a significant difference in the growth of the *Photorhabdus* bacteria between the control and the Vertimec® and Klorpan® insecticide treatments, with a small delay in colony development, which was not observed for the *Xenorhabdus* bacteria. This difference in effect may result from the locations of the bacteria within the nematodes. The delay in bacterial colony development may enable the immune system of the insect to act more effectively because it will have more time to produce substances to fight the nematodes and bacteria by encapsulation and melanization (Dowds & Peters 2002, Manachini *et al* 2013). The study conducted by Madhaiyan *et al* (2006) demonstrated that the insecticide endosulfan (at its commercial dose) promoted an initial delay in the growth of *Gluconacetobacter diazotrophicus;* however, after 70 h, the growth had already returned to a level similar to the control treatment.

The results demonstrate that certain insecticides alter the metabolic activity of EPNs. The insecticide Vertimec® caused a greater release of CO_2 by both nematode species than the insecticide Klorpan®, and the Vertimec® and Klorpan® insecticides delayed the bacterial growth of *Xenorhabdus* sp and *Photorhabdus* sp, despite failing to cause their death. Studies that examine the physiological and biochemical traits of EPNs must be conducted to support their use in IPM programs. Other approaches should be performed under field

conditions to evaluate the application of nematodes combined with the application of insecticides.

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CONCLUSÕES GERAIS

A maioria dos inseticidas testados são compatíveis aos NEP. Apenas Klorpan® e Vertimec® foram considerados incompatíveis com as duas espécies de nematoides estudada. Esses dois inseticidas mantém os nematoides viáveis, no entanto reduz sua infectividade a larvas de *G. mellonella*. A redução da infectividade pode estar relacionada à redução do teor de lipídios e alteração na atividade metabólica. No entanto, diversos são os fatores que podem estar relacionados a redução da infectividade como danifcação nos orgão sensoriais e esses devem ser análisados.