ISELINO NOGUEIRA JARDIM

ESSENTIAL OILS AND ITS COMPONENTS IN THE CONTROL OF Meloidogyne incognita IN SOYBEANS AND TOMATOES

LAVRAS – MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Plantas Medicinais, Aromáticas e Condimentares, área de concentração em Biotatividade de Plantas Medicinais, para a obtenção do título de Doutor.

Prof. Dr. Paulo Estevão de Souza

Orientador

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Aos meus Pais; Jovino Marques Jardim e Maria de Nazaré Nogueira Jardim, responsáveis pela minha existência e perseverança!

Dedico

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RESUMO

Óleos essenciais derivados do metabolismo secundário de plantas podem ter atividades contra nematóides das galhas, *Melodogyne incognita*. Devido à necessidade de nematicidas mais eficientes e menos tóxicos para o homem e para o ambiente do que os disponíveis para o controle de *Meloidogyne incognita*, assim, neste trabalho objetivou-se estudar os óleos essenciais de *Cinnamomum cassia* e de *Allium sativum*, que foram descritos como ativos *in vitro* contra o nematóide Bursaphelenchus xylophilus. O óleo essencial de ambas as espécies foi obtido por hidrodestilação, dissolvidos em solução aquosa de Tween 80 a 0,01 g mL^{-1} e testado *in vitro* contra *M. incognita*. A 62 μg mL^{-1} as emulsões dos óleos foram mais ativas sobre ovos e juvenis de segundo estagio (J2) do que o Carbofuran a 173 μg mL^{-1}. De acordo com a análise de cromatografia gasosa-espectrometria de massas, o constituinte majoritário de *C. cassia* é o (E)-cinamaldeído (83,3%), enquanto para o óleo de alho foram trisulfeto de dialila (66.7%) e dissulfeto de dialila (21.3%). Esses constituintes explicaram a atividade nematicida in vitro do óleo essencial de cada espécie de planta. A emulsão do óleo de *C. cassia* (500 μg mL^{-1}), (E)-cinamaldeído (416 μg mL^{-1}) e alho (250 μg mL^{-1}) reduziram o numero de galhas e de ovos em plantas de soja e tomateiro, respectivamente, a valores estatisticamente iguais àquele obtido pelo Carbofuran (416 μg mL^{-1}). Vapores dos óleos essenciais e do (E)-cinamaldeído foram ativos tanto quanto o nematicida fumigante Basamid usado nos ensaios *in vitro* contra *M. incognita*. A infectividade e a reprodução de *M. incognita* em plantas de soja e de tomate cultivadas em substrato infestado artificialmente com ovos do nematóide e tratado com 0,2 mL (alho), 1,0 mL (E)-cinamaldeído e 0,25 g de Basamid foram estatisiticamente iguais entre si na redução da população do nematoide. Esses resultados inequivocamente provam a atividade nematicida dos óleos essenciais e da substância (E)-cinamaldeído contra *M. incognita*, portanto, tanto o óleo quanto o (E)-cinamaldeído são muito promissores ao desenvolvimento de novos nematicidas fumigantes para o controle de nematóides em plantas de soja e de tomate.

Palavras chave: Nematoide das galhas. *Allium sativum*. *Cinnamomum cassia*. Trisulfeto de dialila. Disulfeto de dialila. (E)-cinamaldeído. Biopesticida.

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ABSTRACT

Essential oils derived from secondary plant metabolism may have activities against root-knot nematodes, *Meloidogyne incognita*. Because nematicides that are more efficient and less toxic to humans and the environment than those available are desirable to control this pathogen, this work aimed at studying the essential oil of *Cinnamomum cassia* and *Allium sativum*, which were described as active in vitro against the nematode *Bursaphelenchus xylophilus*. The essential oils of both species were obtained by hydrodistillation, initially dissolved in aqueous solution of Tween 80 at 0.01 g mL^{-1} and tested *in vitro* against *M. incognita*. At 62 μg mL^{-1} the oil emulsions were more active on eggs and second stage juveniles (J2) than Carbofuran at 173 μg mL^{-1}. According to gas chromatography-mass spectrometry analysis, the major constituent of *C. cassia* is (E) -cinnamaldehyde (83.3%), while for garlic oil were diallyl trisulfide (66.7%) and diallyl disulfide (21.3%). These constituents explained the in *vitro* nematicidal activity of the essential oil of each plant species. The emulsion of the *C. cassia* (500 μg mL^{-1}), E)-cinnamaldehyde (416 μg mL^{-1}) and garlic (250 μg mL^{-1}) reduced the number of galls and eggs in roots soybean and tomatoes to values statistically equal to those obtained with Carbofuran at 415 μg mL^{-1}. Vapors from the essential oils and (E)-cinnamaldehyde were active as much as the basamid fumigant nematicide used in the *in vitro* assays against *M. incognita*. Infectivity and reproduction of *M. incognita* in soybean and tomato plants grown on substrate artificially infested with nematode eggs and treated with 0.2 mL (garlic), 1.0 mL (E)-cinnamaldehyde and 0.25 g of Basamid were statistically equal among themselves in reducing the nematode population. These results unequivocally proofs of the nematicidal activity of the essential oils and of the (E)-cinnamaldehyde substance against *M. incognita*, therefore, both oil (E)-cinnamaldehyde are very promising to the development of new fumigant nematicides for the control of nematodes in soybean and tomato plants.

Keywords: Root-knot nematodes. *Allium sativum*. *Cinnamomum cassia*. Diallyl trisulfide. Diallyl disulfide. (E)-cinnamaldehyde. Biopesticide.

SUMÁRIO

1 INTRODUÇÃO GERAL

De acordo com o relatório da ONU DESA, "World Population Prospects: The 2015 Revision", a população mundial deverá atingir 9,7 bilhões em 2050 (ORGANIZAÇÃO DAS NAÇÕES UNIDAS - ONU, 2015). Assim, é esperada uma demanda crescente por alimentos. Contudo, as estimativas atuais estão muito aquém do que é necessário (RAY et al., 2013). Portanto, produzir alimento que supra as necessidades desse quantitativo é um desafio sem precedentes na história da humanidade (ASH et al., 2010).

Diante de todos os fatores que reduzem a produção de alimentos, doenças causadas por vírus, bactérias, fungos e nematóides apresentam grande impacto sobre a agricultura. Uma vez que, são responsáveis por perdas significativas ou diminuem a qualidade e a segurança dos produtos agrícolas (MONTESINOS, 2007). Entretanto, conciliar a produtividade agrícola com outros componentes da sustentabilidade é um dos maiores desafios para a agricultura moderna (FOLEY et al., 2011). Uma questão central é a redução do uso de agrotóxicos por razões ambientais e de saúde (LECHENET et al., 2014). Contudo, o uso desses produtos continua sendo a principal estratégia de controle, por causa dos efeitos imediatos, tais como: a redução das perdas na produtividade e o aumento da oferta de alimentos (COOPER; DOBSON, 2007). Apesar da significativa contribuição desses produtos para a produção agrícola, alguns agrotóxicos, principalmente da classe dos organocarbamatos e organofosforados, tiveram seu uso proibido, em decorrência da alta toxicidade, atividade residual e grande poder bioacumulativo (PASSOS; REIS, 2013). Por conseqüência, algumas doenças de plantas de importância econômica têm sido gerenciadas com dificuldade, em razão da falta de compostos eficazes. Assim, é inteiramente desejável descobrir substitutos menos tóxicos e aceitáveis do ponto de vista ambiental para os agroquímicos comerciais.

Atualmente, há grande número de pesquisas buscando encontrar estratégias efetivas para o controle de fitopatógenos. Diversos trabalhos têm sido publicados, enfatizando a importância da obtenção de novos produtos com ação antimicrobiana, bactericida, fungicida e nematicida, mais efetivos e com menos toxicidade para humanos (ANDRÉS et al., 2012; BAKKALI et al., 2008; BURT, 2004; CASTRO et al., 2017). Nesse contexto, encontram-se os óleos essenciais e seus componentes individuais, como novas ferramentas para o tratamento de doenças de plantas (THOMIDIS; FILOTHEOU, 2016). Alguns deles constituem eficazes alternativas ou complementos aos compostos sintéticos da indústria química, sem demonstrar os mesmos efeitos secundários (GAHUKAR, 2012).

Considerando a alta demanda por alimentos mais saudáveis, o impacto que as doenças fitopatogênicas podem ocasionar na produção agrícola e também levando-se em consideração o menor risco ao meio ambiente e a saúde humana, no presente trabalho, objetivou-se selecionar e avaliar a ação de óleos essenciais e de seus componentes sobre *Meloidogyne incognita* (Kofold & White) Chitwood em plantas de soja e de tomateiro sob concentrações não tóxicas às plantas.

2 REFERENCIAL TEÓRICO

2.1 Os fitonematoides e sua importância econômica

Existem mais de 4100 espécies de nematóides fitoparasitas e, coletivamente, representam uma importante restrição a segurança alimentar (JONES et al., 2013). Nematoides fitoparasitas causam perdas agrícolas mundiais superiores a US $ 157 bilhões por ano, e os nematóides de galhas como *Meloidogyne incognita* podem infectar quase todas as plantas cultivadas (ABAD et al., 2008). No entanto, é provável que essa estimativa esteja aquém das verdadeiras perdas, uma vez que muitos produtores, particularmente os de países em desenvolvimento, possuem pouco conhecimento sobre nematóides fitoparasitas (JONES et al., 2013).

O gênero *Meloidogyne* spp. é composto por 98 espécies e parasitam quase todas as espécies de plantas vasculares (JONES et al., 2013). Moens, Perry e Starr (2009) designou como as "quatro principais espécies" *M. arenaria*, *M. incognita*, *M. javanica* e *M. hapla*. Dentre as espécies, *Meloidogyne incognita* pode ser considerado o patógeno de plantas que mais causa danos à agricultura. Tal afirmação se alicerça na distribuição global dessa espécie aliada à capacidade de parasitar a maioria das espécies vegetais cultiváveis (ABAD et al., 2008). Contudo, o controle desse patógeno é problemático, em razão do limitado número de nematicidas comerciais disponíveis, principalmente, após a retirada ou uso restrito de fumigantes eficazes do solo, especialmente o brometo de metila e nematicidas não fumigantes (OKA; SHUKER; TKACHI, 2009). Portanto, substitutos menos tóxicos e aceitáveis do ponto de vista ambiental para os nematicidas comerciais é bem-vindo, criando assim, uma oportunidade de mercado significativa para produtos alternativos e biológicos tais como nematicidas obtidos de produtos derivados de plantas, como os óleos essenciais (NTALLI; CABONI, 2012). Além disso, outros métodos de controle, por exemplo, o uso de variedades resistentes, rotação de culturas, controle biológico e solarização do solo, nem sempre são efetivos ou aplicáveis a todas as culturas ou regiões (OKA, 2014).

2.2 Óleos Esenciais

Entre os metabólitos secundários sintetizados pelas plantas, encontram-se os óleos essenciais. De forma geral, são misturas complexas de substâncias voláteis lipofílicas, geralmente odoríferas e líquidas, e evaporam a temperatura ambiente, o que os diferenciam dos óleos fixos. Podem ser sintetizados por todos os órgãos das plantas, como flores, folhas, caules, sementes, frutos, raízes, e são produzidos em tricomas glandulares e armazenados em

estruturas de secreção internas, como células idioblastos, cavidades e canais secretores (RAUT; KARUPPAYIL, 2014).

Na natureza, os óleos essenciais desempenham um papel importante na proteção das plantas como antibacterianos, antivirais, antifúngicos, inseticidas e também contra os herbívoros, reduzindo o seu desejo sobre essas plantas. Eles também podem atrair alguns insetos para favorecer a dispersão de pólens e sementes, ou repelir outros indesejáveis (BAKKALI et al., 2008).

Atualmente, são conhecidos vários métodos para a extração de óleos essenciais. Estes podem incluir o uso de dióxido de carbono líquido ou microondas, destilação de baixa ou alta pressão empregando água fervente ou vapor quente. Dependendo do método de extração, o perfil químico dos produtos de óleos essenciais pode diferir não só no número e no tipo de moléculas, mas também nas suas estruturas estereoquímicas, em resumo, o tipo de extração deve ser escolhido de acordo com o fim da utilização (BILIA et al., 2014). Além disso, o óleo essencial é um produto derivado do metabolismo secundário da planta, logo, a sua composição pode variar consideravelmente dependendo de vários fatores, como, tipo de extração, clima, composição do solo, órgão da planta, idade e fase de ciclo vegetativo (BAKKALI et al., 2008).

Os óleos essenciais têm sido amplamente utilizados por suas propriedades já observadas na natureza, isto é, pelas suas atividades antibacterianas, antifúngicas e inseticidas. Atualmente, são conhecidos cerca de 3000 óleos essenciais, dos quais 300 são comercialmente importantes, especialmente para as indústrias farmacêutica, agronômica, alimentar, sanitária, cosmética e perfumaria (RAUT; KARUPPAYIL, 2014).

2.3 Óleos essenciais com efeitos nematicidas

Em geral, os óleos essenciais têm sido estudados, principalmente, contra insetos e fungos, enquanto que à sua atividade nematicida ainda é pouco explorada por grupos de pesquisass ao redor do mundo (NTALLI; CABONI, 2012). As principais famílias produtoras de óleos essenciais, como Lamiaceae, Asteraceae, Myrtaceae, Rutaceae, Lauraceae e Poaceae têm sido amplamente estudadas *in vitro* para atividade nematicida (ANDRÉS et al., 2012).

A importância dos óleos essenciais para o controle de fitoparasitas, por exemplo, os nematóides residem no fato de que eles afetam simultaneamente vários alvos, consequentemente, a resistência ou adaptação desses organismos é diminuída, enquanto a toxicidade interespecífica de óleos e de seus compostos individuais são altamente incomuns (NTALLI; CABONI, 2012). Em geral, os óleos essenciais têm uma toxicidade baixa sobre

animais e seres humanos, e não são persistentes no ambiente, motivo pelo qual estão isentos dos requisitos de dados habituais para o registro nos EUA.

2.4 Sinergismo entre os componentes de óleos essenciais

Sabe-se que, em geral, os óleos essenciais são misturas complexas de numerosas moléculas, portanto, às suas propriedades biológicas devem ser o resultado de um sinergismo de todas as moléculas ou refletir apenas o efeito das principais moléculas presentes nos níveis mais altos de concentração de acordo com a análise cromatográfica gasosa (BAKKALI et al., 2008). Muitos estudos têm usado apenas os constituintes principais de certos óleos essências, em geral, eles têm refletido muito bem as características biofísicas e biológicas dos óleos essenciais dos quais foram isolados (IPEK et al., 2005). Além disso, a amplitude de seus efeitos tem sido dependente apenas de sua concentração quando testados isoladamente ou da sua quantidade existente no óleo essencial. Dessa forma, o efeito sinérgico de vários constituintes do óleo essencial, em comparação, ao efeito de um ou dois componentes do óleo essencial parece questionável (BAKKALI et al., 2008). Por outro lado, alguns constituintes inativos têm, por vezes, algum efeito sinérgico sobre os constituintes ativos e, embora não sejam ativos individualmente, a sua presença é necessária para atingir a toxicidade total (NTALLI; CABONI, 2012). De acordo com Ntalli e Caboni (2012), é muito importante compreender as interações de sinergia e antagonismo entre os constituintes individuais do óleo essencial, assim como, compreender os mecanismos biológicos subjacentes responsáveis pela atividade biológica.

3 CONCLUSÃO

Óleos essenciais bioativos podem ser desenvolvidos para uso direto como nematicidas, ou podem ser utilizados como compostos modelos para o desenvolvimento de derivados quimicamente sintetizados. Na excelente revisão de Andrés et al. (2012), encontra-se um número considerável de várias plantas produtoras de óleos essenciais com atividade nematicida sobre *Meloidogyne* spp. e *Bursaphelenchus xylophilus*. Outra excelente revisão foi realizada por Ntalli e Caboni (2012) que discutiram as principais substancias de origem vegetal que apresentavam atividade nematicida isoladamente ou combinadas, assim como, o seu modo de ação. Tomando como base essas duas revisões, podemos assumir que existe uma grande oportunidade para o desenvolvimento de nematicidas botânicos, baseado nos produtos derivados do metabolismo secundário de planta.

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SEGUNDA PARTE - ARTIGOS

ARTIGO 1 - (E)-CINNAMALDEHYDE FROM THE ESSENTIAL OIL OF *CINNAMOMUM CASSIA* CONTROLS *Meloidogyne incognita* IN SOYBEAN PLANTS

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ORIGINAL PAPER

(E)-cinnamaldehyde from the essential oil of *Cinnamomum cassia* controls *Meloidogyne incognita* in soybean plants

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Abstract Among the main problems faced by soybean producers is the nematode *Meloidogyne incognita*. Because nematicides that are more efficient and less toxic to humans and the environment than those available are desirable to control this pathogen, this work aimed at studying the essential oil of *Cinnamomum cassia*, which has been described as active in vitro against the nematode *Bursaphelenchus xylophilus*. At the concentration of 62 μg mL^{-1}, it performed better than the nematicide carbofuran at 173 μg mL^{-1} in an in vitro assay with *M. incognita* eggs and second-stage juveniles. The main components of this oil were identified by gas chromatography–mass spectrometry analysis and submitted to in vitro assays with the nematode, which showed (E)-cinnamaldehyde (83.3% of the oil) as responsible for the nematicidal activity. Emulsions of the oil (500 μg mL^{-1}) and this aldehyde (416 μg mL^{-1}) reduced the numbers of *M. incognita* galls and eggs in soybean plants to values statistically equal to those obtained with carbofuran (415 μg mL^{-1}). Vapors of the essential oil and (E)-cinnamaldehyde were also as active as the fumigant nematicide Basamid against *M. incognita* according to an in vitro assay. Cultivation of soybean plants in substrate inoculated with *M. incognita* eggs and treated with Basamid at 0.25 g (L of substrate)$^{-1}$ or (E)-cinnamaldehyde at 1.0 mL (L of substrate)$^{-1}$ caused a reduction in the nematode population to values statistically equal to each other. These results make (E)-cinnamaldehyde very promising for the development of new products to control *M. incognita* in soybean fields.

Keywords Biopesticide · *Trans*-cinnamaldehyde · *Cinnamomum* · Root-knot nematode · *Glycine max* · *Meloidogyne* spp.

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Key message

- New products to control the nematode *Meloidogyne incognita* in soybean fields are desired.
- To meet this demand, the essential oil of *Cinnamomum cassia* was studied.
- Emulsions of the essential oil and its main component, (E)-cinnamaldehyde, reduced the population of *M. incognita* in soybean plants to values statistically equal to those observed for the nematicide carbofuran.
- Vapor of (E)-cinnamaldehyde reduced the population of the nematode to values statistically equal to those obtained with the fumigant nematicide Basamid.

Introduction

Native to Southeast Asia, soybean [*Glycine max* (L.) Merr.] is one of the most cultivated oleaginous plants in the world (Subramanyam et al. 2012). The USA is the major

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producer of soybeans, followed by Brazil (USDA 2015), which produced about 96 million tons in the 2015/2016 harvest (CONAB 2016). Among the main problems faced by Brazilian soybean producers are diseases like those caused by root-knot nematodes (*Meloidogyne* spp.), which restrict the productivity of soybean (Soares et al. 2004). For example, *Meloidogyne incognita* (Kofoid and White) Chitwood causes losses of over 55% in soybean production in areas infested by this pathogen (Machado 2015). *M. incognita* is usually controlled in soybean plantations by commercial nematicidal substances, the use of which is generally more efficient than other methods to control plant-parasitic nematodes. However, these substances may persist in the environment and have side effects on humans and other non-target organisms (Sousa et al. 2015). Therefore, new nematicides that are more efficient and less toxic to humans and to the environment than the commercial products available are greatly welcome.

Plant metabolites are potentially useful to the development of new products able to circumvent the above-mentioned problems because some of them are known to be active against plant-parasitic nematodes (Douda et al. 2010). Some components of essential oils extracted from plants are among these metabolites (Ntalli and Caboni 2012; Regnault-Roger et al. 2012; Andrés et al. 2012), which can act alone or synergistically against nematodes. In addition to killing these organisms, these metabolites can attract or repel them, and stimulate or inhibit the eclosion of second-stage juveniles (J2) of these phytoparasites (Chitwood 2002; Faria et al. 2016).

Cinnamomum cassia (L.) J. Presl (Lauraceae Juss.), known as Chinese cassia or Chinese cinnamon, is a tree native to China and other countries of Southeast Asia, where it has been used as a spice or a medicine since ancient times (Geng et al. 2011). Nematicidal activity has been attributed to the essential oils obtained from barks of the trunk or brunches of this plant, since they were active in vitro against adults of the pine wood nematode [*Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle] (Kong et al. 2007). However, no report about the efficiency of these oils or their components is described in the literature for the control of plant-parasitic nematodes in soybean fields. As essential oils and their components apparently can decompose into non-toxic compounds and usually have few harmful effects on non-target organisms, an investigation into the essential oil of *C. cassia* and its main components was carried out to contribute to the development of a new fumigant nematicide to control *M. incognita* in fields for planting soybeans. To achieve such a goal, the objectives of the present work were to: (1) investigate the in vitro activity of the essential oil of *C. cassia* against *M. incognita*; (2) identify the main components of this essential oil through analysis by gas chromatography–mass spectrometry (GC–MS); (3) evaluate the in vitro activity against *M. incognita* by the main components of the essential oil obtained from *C. cassia*; (4) evaluate, under greenhouse conditions, the efficiency of the main component as a fumigant to prevent the development of *M. incognita* in soybean plants.

Materials and methods

M. incognita and chemicals

M. incognita was sampled from artificially infested tomato plants (*Solanum lycopersicum* L. cv. Santa Clara) grown under greenhouse conditions. Eggs were extracted from 60-day nematode-infested roots according to the method described by Hussey and Barker (1973). Eggs retained in a 500-mesh sieve (American Society for Testing and Materials, ASTM) were used in the experiments or transferred to a Baermann funnel (Whitehead and Heming 1965), and those second-stage juveniles (J2) of *M. incognita* that hatched after 24–48 h were collected to be used in the experiments.

The essential oil from barks of *C. cassia* was supplied by Empresa Ferquima Ind. Com. Ltda. (Vargem Grande Paulista, São Paulo, Brazil). It was obtained by hydrodistillation in January 2015 (lot 218) and is valid until January 2018. (E)-cinnamaldehyde (≥99%), *o*-methoxycinnamaldehyde (≥96%), benzaldehyde (≥99%) (Fig. 1) and carbofuran (98%) were supplied by Sigma-Aldrich Co. (Milan, Italy); while Basamid (98%) was purchased from BASF (Ludwigshafe, Germany).

In vitro motility and mortality of *M. incognita* J2 exposed to emulsions of the essential oil of *C. cassia*

According to the method described by Chen and Dickson (2000) and adapted by Amaral et al. (2003), an aqueous suspension (20 µL) containing approximately 20 *M. incognita* J2 and 100 µL of aqueous 0.01 g/mL Tween 80® solutions containing the essential oil of *C. cassia* at six different concentrations (1200, 600, 300, 150, 74.4 and 37.2 µg mL^{-1}) were put into 350-µL wells of a 96-well polypropylene plate. The final concentrations of the essential oil in the wells were 1000, 500, 250, 125, 62 and

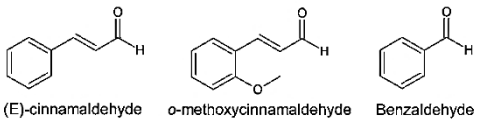

Fig. 1 Chemical structures of the main components of the essential oil obtained from *Cinnamomum cassia*

31 μg mL^{-1}. Water, Tween 80® at 0.01 g mL^{-1} and carbofuran (2,3-dihydro-2,2-dimethyl-1-benzofuran-7-yl *N*-methylcarbamate; final concentration 173 μg mL^{-1}) were used as controls. Six replicates were employed for each treatment. The plates were sealed with parafilm and kept at 26 °C for 48 h. Then, mobile and immobile nematodes were counted under a microscope, and one drop of a freshly prepared 1.0 mol L^{-1} NaOH solution was added to the content of each well and nematodes were counted again. J2 that changed their body shape within 3 min were considered to be alive, whereas the nematodes not responding to the addition of NaOH were considered dead.

In vitro hatching of *M. incognita* J2 from eggs exposed to emulstion of the essential oil of *C. cassia*

This experiment was set up similarly to that described above, but with 60 eggs of the nematode per well instead of 20 J2. The experiment time was also changed, as the evaluation was made seven days after the beginning of the experiment. This comprised counting intact eggs and hatched J2 (alive or dead).

GC–MS analysis of the essential oil of *C. cassia*

A gas chromatograph coupled to a mass spectrometer (model QP2010, Shimadzu, Japan), equipped with a RTX®-5MS capillary column (30 m × 0.25 mm ID × 0.25 μm film thickness; Restek), was employed in this work, which used helium at 1.0 mL min^{-1} as carrier gas. According to Adams (2007), the following conditions were adopted: (1) split/splitless injector temperature: 220 °C; (2) split ratio: 1:20; (3) initial temperature of the column: 60 °C; (4) elevation rate of the column temperature: 2 °C min^{-1} up to 200 °C and then 5 °C min^{-1}; (5) final temperature of the column: 250 °C; (6) temperature of the interface between the gas chromatograph and the mass spectrometer: 220 °C; (7) ionization of each molecule in the spectrometer: electron impact at 70 eV; (8) range of mass/charge (*m/z*) analyses in the mass spectrometer: 45–400; and (9) mass spectrum acquisition time: 0.5 s. The essential oil of *C. cassia* was dissolved in acetone to a concentration of 10 mg mL^{-1}, and 1 μL of this solution was injected in the gas chromatograph. A solution of homologous linear alkanes, containing C9–C20 carbon atoms, was used as an external standard. All mass spectra were compared to those in the NIST 05 Mass Spectral Library, 2005, and all peaks in the chromatogram with similarity index below 90% were considered unidentified. For each of the remaining peaks, the arithmetic index (AI) was calculated according to the following formulae: $AI = \{100P_z + 100[(RT - RTP_z)/(RTP_{z+1} - RTP_z)]\}$, where P_z = number of carbon atoms of the linear alkane with retention time immediately below that of the substance to be identified in the chromatogram; RT = retention time (min) of the substance to be identified in the chromatogram; RTP_z = retention time (min) of the linear alkane with number of carbon atoms equal to P_z; and RTP_{z+1} = retention time (min) of the linear alkane with number of carbon atoms equal to $P_z + 1$. Substances with calculated values of AI corresponding to an error ≥3% in relation to the AI described by Adams (2007) were considered not identified.

In vitro mortality of *M. incognita* J2 exposed to emulsions of the main components from the essential oil of *C. cassia*

This experiment was carried out as described above for the essential oil of *C. cassia*, but instead of this oil the following substances were evaluated: (E)-cinnamaldehyde (final concentrations: 833, 416, 208, 104, 52 and 26 μg mL^{-1}), *o*-methoxycinnamaldehyde (final concentrations: 71, 36 and 18 μg mL^{-1}) and benzaldehyde (final concentrations: 19, 10 and 5 μg mL^{-1}) (Fig. 1).

In vitro hatching of *M. incognita* J2 from eggs exposed to emulsions of (E)-cinnamaldehyde

This experiment was carried out as described above for the essential oil of *C. cassia*, with (E)-cinnamaldehyde (Fig. 1) at final concentrations of 833, 416, 208, 104, 52 and 26 μg mL^{-1}.

Effect of emulsions of the essential oil of *C. cassia* and (E)-cinnamaldehyde on the infectivity and reproduction of *M. incognita* in soybean plants

Seeds of soybean (*Glycine max* L. cv. BRS-284) susceptible to *M. incognita* were sown on a commercial substrate (Tropstrato®, Vida Verde Indústria e Comércio de Insumos Orgânicos Ltda., Mogi Mirim, São Paulo, Brazil) contained in 300-mL plastic pots. Twenty days later, plants were used in the experiment. An aqueous suspension (24 mL), containing about 3000 *M. incognita* J2 and emulsions (24 mL) of the essential oil of *C. cassia* (500, 250 and 125 μg mL^{-1}) or (E)-cinnamaldehyde (416, 208 and 104 μg mL^{-1}) (Fig. 1), in Tween 80 at 0.01 g mL^{-1}, was combined, resulting in eight different suspensions. Water, Tween 80® at 0.01 g mL^{-1} and carbofuran (415 μg mL^{-1}) were used as controls. A sample (8 mL, containing 500 J2) of each suspension was added to the substrate of each soybean plant through four equidistant holes (0.4 cm wide × 1.5 cm deep) around the stem. Plants were kept for 48 h in a room with no sun incidence and then moved to a greenhouse, where they were maintained for 30 days. After this period of time, roots

were removed, carefully washed, dried on paper towels and weighed. After counting galls, roots underwent eggs extraction according to the method described by Hussey and Barker (1973). Eggs retained in the 500-mesh (ASTM) sieve were suspended in 50 mL water and counted in a Peters chamber under a microscope. This experiment was carried out with six replicates for each treatment, under a random design.

In vitro mortality of *M. incognita* J2 exposed to vapors of the essential oil of *C. cassia* and (E)-cinnamaldehyde

Adapting the method described by Barros et al. (2014), sand (30 g) was sterilized by autoclaving at 120 °C for 30 min and poured into each Supelco™ SPME flask (28 mm wide × 80 cm deep, Sigma-Aldrich, Bellefonte, PA, USA). Two Eppendorf tubes (0.5 mL) were partially immersed in the sand of each flask, and 100 µL of the sample to be evaluated was poured into one of the tubes. The flask was immediately sealed with a screw cap internally coated with silicone and kept at 28 °C for 72 h. Employing a syringe with a needle to punch the silicon septa of the Supelco™ SPME flask, an aqueous suspension containing about 1000 *M. incognita* J2 was injected into each empty Eppendorf tube. After 48 h at 28 °C, the flasks were opened for the homogenization of the J2 suspension, from which 20 µL was withdrawn, diluted with 100 µL of water and submitted to mobile and immobile J2 count under a microscope. According to the method of Chen and Dickson (2000), adapted by Amaral et al. (2003), one drop of a freshly prepared 1.0 mol L^{-1} NaOH solution was added and immobile J2 under a microscope were considered dead. The products evaluated in this experiment were essential oils of *C. cassia* and (E)-cinnamaldehyde (Fig. 1). This experiment was carried out under a random design, with six replicates per treatment, employing the commercial fumigant nematicide Basamid® [(80 mg; BASF (Ludwigshafen, Germany)] and water as positive and negative controls, respectively. This experiment was carried out three times, resulting in similar values. Therefore, only one set of data is presented.

In vitro hatching of *M. incognita* J2 from eggs exposed to vapors of the essential oil of *C. cassia* and (E)-cinnamaldehyde

This experiment was set up similarly to that described above, but with a suspension containing *M. incognita* eggs (3000 eggs mL^{-1}) instead of J2. The experiment time was also changed, as the evaluation was made seven days after the beginning of the experiment. This comprised counting intact eggs and hatched J2 (alive or dead). This experiment was carried out three times, resulting in similar values. Therefore, only one set of data is presented.

Effect of vapor of (E)-cinnamaldehyde on the infectivity and reproduction of *M. incognita* in soybean plants

M. incognita eggs (150,000) were added to 1 L of a commercial substrate (Tropstrato®, Vida Verde Indústria e Comércio de Insumos Orgânicos Ltda., Mogi Mirim, São Paulo, Brazil) contained in 2-L polyethylene terephthalate bottles. (E)-cinnamaldehyde (Fig. 1) was then splashed on the substrate to the following concentrations: 1.0, 0.5 and 0.2 mL (L of substrate)$^{-1}$. The commercial fumigant nematicide Basamid at 0.25 g (L of substrate)$^{-1}$ and water (1.0 mL) was used as positive and negative controls, respectively. All bottles were cap-closed, and the resulting mixtures were homogenized and remained standing at 28 °C for three days. The bottles were then opened, and after five days, the substrate inside was poured into cells (121.2 cm^3) of a 72-cell Styrofoam tray. Twenty-day-old soybean plants (*Glycine max* L. cv. BRS-284), susceptible to *M. incognita*, were transferred to the tray, which was kept in a greenhouse for 30 days. After this period of time, roots were carefully washed, dried with a paper towel and weighed. After counting galls, *M. incognita* eggs were extracted by the method described by Hussey and Barker (1973). Eggs retained in the 500-mesh (ASTM) sieve were suspended in 50 mL water and counted in a Peters chamber under a microscope. This experiment was carried out with six replicates for each treatment, under a random design. This experiment was carried out twice, resulting in similar values. Therefore, only one set of data is presented.

Statistical analyses

For the in vitro assays with *M. incognita* eggs and J2, values were transformed into a percentage and submitted to analysis of variance (ANOVA). Means were compared according to the Scott and Knott (1974) test ($P \leq 0.05$). The statistical calculation was carried out with the software SISVAR (Ferreira 2011). For the experiments with soybean plants, the statistical calculation was done without transformations of values into a percentage.

Results

In vitro activity against *M. incognita* by emulsions of the essential oil from *C. cassia*

When dissolved in an aqueous Tween 80® solution, the essential oil of *C. cassia* was very active against *M.*

incognita J2, as it caused 100% immobility and mortality of the nematode at a concentration of 62 μg mL^{-1}, while only 63% of the nematode individuals were dead after exposure to the commercial nematicide carbofuran at 173 μg mL^{-1} (Table 1). A similar result was observed when eggs of the nematode were exposed to the same emulsions, since the percentage of J2 hatching from eggs in contact with the essential oil at 62 μg mL^{-1} was about the same as observed for carbofuran at a concentration almost three times higher.

GC–MS analysis of the essential oil of *C. cassia*

The main components of the essential oil of *C. cassia* were (E)-cinnamaldehyde (83.3%), *o*-methoxycinnamaldehyde (7.1%), (E)-cinnamyl acetate (2.0%) and benzaldehyde (1.9%) (Fig. 1). These four compounds corresponded to about 94.3% of this essential oil constituents and presented a similarity index to their database spectra above 90% (Table 2). Furthermore, the arithmetic indexes calculated for them were very close (errors ≤ 0.63%) to those described in the literature for the same substances (Adams 2007).

In vitro activity against *M. incognita* by emulsions of the main components in the essential oil from *C. cassia*

(E)-cinnamaldehyde (Fig. 1), the main component of the essential oil (Table 2), was very active against *M. incognita*, since it caused 100% immobility and 97% mortality of J2 at 52 μg mL^{-1}, while carbofuran at 173 μg mL^{-1} took J2 mortality to only 63% (Table 1). Furthermore, despite the differences between the concentrations of these two emulsions, the percentages of J2 hatched from eggs exposed to them were statistically equal. It is also worth mentioning that (E)-cinnamaldehyde at concentrations corresponding to 83.3% of those employed for the essential oil, which is the same percentage of this substance in the essential oil according to the GC–MS analysis (Table 2), caused J2 mortality for about 90% of those observed for the essential oil. Regarding benzaldehyde and *o*-methoxycinnamaldehyde (Fig. 1), their activities against the nematode were very low at the concentrations studied (Table 1), which reflected their percentages in the essential oil according to the GC–MS analysis (Table 2).

Effect of emulsions of the essential oil of *C. cassia* and (E)-cinnamaldehyde on the infectivity and reproduction of *M. incognita* in soybean plants

Both the essential oil and its main component, (E)-cinnamaldehyde (Fig. 1), performed similarly to the commercial nematicide carbofuran, which reduced the number of galls and eggs of the nematode to values corresponding to about 14% and 7%, respectively, of those observed for water and Tween 80®, which were employed as controls (Table 3). In fact, (E)-cinnamaldehyde was somewhat more active than carbofuran, as with half of the concentration employed for this commercial nematicide afforded values statistically equal to those obtained with carbofuran.

Just as in the in vitro assay with emulsions of the essential oil and (E)-cinnamaldehyde (Table 1), when the concentration of this substance corresponded to 83.3% of the essential oil, values were very close to each other, tending to be statistically equal for all parameters (Table 3).

Table 1 Mobility and mortality of *Meloidogyne incognita* second-stage juveniles (J2) exposed to emulsions of the essential oil of *Cinnamomum cassia* and its main components, and J2 hatching from *M. incognita* eggs exposed to these emulsions

Treatments	Concentration (μg mL^{-1})	Immobile J2 (%)[a]	Dead J2 (%)[a]	Hatched J2 (%)[a]
Essential oil	250	100 f	100 f	5 c
Essential oil	125	100 f	100 f	8 c
Essential oil	62	100 f	100 f	13 b
Essential oil	31	60 d	45 d	–
(E)-cinnamaldehyde	208	100 f	100 f	6 c
(E)-cinnamaldehyde	104	100 f	100 f	10 b
(E)-cinnamaldehyde	52	100 f	97 f	11 b
(E)-cinnamaldehyde	26	54 c	39 c	–
o-Methoxycinnamaldehyde	18	21 b	7 b	–
Benzaldehyde	5	21 b	7 b	–
Carbofuran (control)	173	71 e	63 e	12 b
Water (control)	–	7 a	3 a	44 a

[a] Means followed by the same letter in each column do not differ statistically according to the Scott and Knott (1974) test ($P \leq 0.05$)

Table 2 Main components of the essential oil of *Cinnamomum cassia* according to analysis by gas chromatography–mass spectrometry

Number	Component	RT[a] (min)	AI[b]	SI[c] (%)	Area (%)
1	Benzaldehyde	6.673	958	99	1.9
2	Benzene acetaldehyde	9.894	1041	95	0.9
3	*o*-Anisaldehyde	20.685	1241	91	0.8
4	(E)-cinnamaldehyde	22.680	1273	97	83.3
5	Coumarin	32.304	1431	94	0.9
6	(E)-cinnamyl acetate	33.062	1443	95	2.0
7	(E)-cinnamic acid	33.306	1447	91	1.3
8	*o*-Methoxycinnamaldehyde	38.113	1528	98	7.1
Total					98.2

[a] RT = retention time

[b] AI = calculated arithmetic index

[c] SI = similarity index

In vitro activity against *M. incognita* by vapors of the essential oil of *C. cassia* and (E)-cinnamaldehyde

The essential oil, (E)-cinnamaldehyde (Fig. 1) and the commercial fumigant nematicide Basamid immobilized 100% of the nematode, but they were a little different in regard to the values of dead and hatched J2 (Table 4). Basamid was a little better when the parameter of dead J2 was taken into account, while the essential oil was a little better when the number of hatched J2 was considered.

Effect of vapor of (E)-cinnamaldehyde on the infectivity and reproduction of *M. incognita* in soybean plants

In all concentrations, used (E)-cinnamaldehyde (Fig. 1) reduced the population of *M. incognita* on soybean plants. At 1.0 mL (L of the substrate)$^{-1}$, both numbers of galls and eggs of the nematode on roots of the plants were statistically equal to those observed for the treatment corresponding to the commercial fumigant nematicide Basamid (Table 5).

Discussion

There is no doubt about the high direct activity of the essential oil of *C. cassia* against *M. incognita*. At lower concentrations than that used for carbofuran emulsions, such oil increased the in vitro J2 mortality to values above that observed for this commercial nematicide, while reduced the number of J2 hatched to less than that obtained with carbofuran (Table 1). This result is in complete agreement with the activity of such oil against the pine wood nematode *B. xylophilus* (Kong et al. 2007).

Given the potential detected by in vitro tests for the utilization of essential oil of *C. cassia* in developing new nematicides, it was analyzed by GC–MS, which revealed (E)-cinnamaldehyde (Fig. 1) as the main constituent, accounting for approximately 83.3% of the oil (Table 2). This result is in total agreement with previous work carried out by other research groups, according to which the percentage of this aldehyde in such essential oil can vary in the 42–92% range (Kocevski et al. 2013; Kim et al. 2016; Khaled et al. 2015; Geng et al. 2011; Chou et al. 2013; Giordani et al. 2006). This wide range can be easily explained by factors such as the age of the plant, humidity

Table 3 Effect of emulsions of (E)-cinnamaldehyde and essential oil of *Cinnamomum cassia* on the numbers of *Meloidogyne incognita* galls and eggs on the roots of soybean plants, and on the mass of their roots

Treatments	Concentration (μg mL ^{1})	Galls[a]	Eggs[a]	Root mass (g)[a]
(E)-cinnamaldehyde	416	0 a	7 a	8.35 a
(E)-cinnamaldehyde	208	2 a	80 a	8.02 a
(E)-cinnamaldehyde	104	7 b	203 b	8.15 a
Essential oil	500	3 a	107 a	8.38 a
Essential oil	250	4 a	178 b	8.23 a
Essential oil	125	6 b	299 b	8.23 a
Carbofuran (control)	415	3 a	51 a	7.95 a
Tween 80® (control)		22 c	674 c	8.10 a
Water (control)		22 c	759 c	8.10 a

[a] Means followed by the same letter in each column do not differ statistically according to the Scott and Knott (1974) test ($P \leq 0.05$)

Table 4 Mobility and mortality of *Meloidogyne incognita* second-stage juveniles (J2) exposed to vapors of the essential oil of *Cinnamomum cassia* and (E)-cinnamaldehyde, and J2 hatched from the nematode eggs exposed to the same vapors

Treatment	Immobile J2 (%)[a]	Dead J2 (%)[a]	Hatched J2 (%)[a]
Essential oil	100 a	80 b	8 a
(E)-cinnamaldehyde	100 a	84 c	13 b
Basamid (control)	100 a	92 d	12 b
Water (control)	3 b	0 a	41 c

[a] Means followed by the same letter in each column do not differ statistically according to the Scott and Knott (1974) test ($P \leq 0.05$)

Table 5 Effect of (E)-cinnamaldehyde vapor on the numbers of *Meloidogyne incognita* galls and eggs on the roots of soybean plants, and on the mass of their roots

Treatments	Amount per liter of substrate	Galls[a]	Eggs[a]	Root mass (g)[a]
(E)-cinnamaldehyde	1.0 mL	19 a	603 a	2.18 b
(E)-cinnamaldehyde	0.5 mL	27 b	1335 b	2.26 b
(E)-cinnamaldehyde	0.2 mL	31 b	1746 b	2.26 b
Basamid (control)	0.25 g	18 a	322 a	1.94 a
Water (control)	–	53 c	2291 c	2.23 b

[a] Means followed by the same letter in each column do not differ statistically according to the Scott and Knott (1974) test ($P \leq 0.05$)

and method to prepare the essential oil (Lahlou 2004). The other constituents, in much lower concentrations according to the present work (Table 2), are also found in this essential oil according to these authors.

The assay with emulsions of benzaldehyde, *o*-methoxycinnamaldehyde and (E)-cinnamaldehyde (Fig. 1), at concentrations proportional to their amounts in the essential oil, revealed the latter substance as responsible for the nematicidal activity of such oil (Table 1). This result is consistent with the previously described in vitro activity of this substance against *B. xylophilus* (Kong et al. 2007) and *M. incognita* (Caboni et al. 2013). Apparently, it acts against the nematode inhibiting its V-ATPase enzyme, which is a vacuolar-type proton *trans*-locating ATPase that pumps protons across membranes, energized by ATP hydrolysis. This enzyme may be involved in nematode nutrition, osmoregulation, cuticle synthesis, neurobiology and reproduction (Caboni et al. 2013).

As the results so far discussed made clear the in vitro activity against *M. incongita* by the essential oil of *C. cassia* and its main component, (E)-cinnamaldehyde (Fig. 1), emulsions of both materials underwent an assay with soybean plants inoculated with *M. incognita* J2, which corroborated the potential of (E)-cinnamaldehyde to the development of new nematicides (Table 1). In almost all concentrations of this aldehyde, the numbers of galls and eggs in soybean plants were statistically equal to those observed in plants treated with the essential oil of *C. cassia* at concentrations equal to concentrations of (E)-cinnamaldehyde × 1.2. Thus, this substance really is responsible for the activity of the oil against the nematode, which is in accordance with its in vitro and in vivo activity against *Meloidogyne javanica* (Treub) Chitwood described in the literature (Oka 2001). Furthermore, in the present work this aldehyde was as efficient as carbofuran, which is a commercial nematicide used to reduce the populations of several plant-pathogenic nematodes, including *Meloidogyne* spp., in different cultures (Adegbite and Agbaje 2007; Khan et al. 2012; Jada et al. 2011). This result also seems in line with the work by Ntalli et al. (2016), who observed the nematicidal activities against *M. incognita*, *M. javanica* and *Meloidogyne arenaria* Chitwood, by (E,E)-deca-2,4-dienal and (E)-dec-2-enal, which are also aldehydes. It is worth mentioning that although some plant metabolites that are potentially useful for the control of plant pathogens can present phytotoxic effects (Roh et al. 2011), both essential oil and (E)-cinnamaldehyde have not affected root development (Table 3), suggesting that these materials are not phytotoxic. Actually, according to Oka (2001), this aldehyde can increase the shoot weight of tomato plants.

Despite the excellent results obtained with emulsions of (E)-cinnamaldehyde (Fig. 1) and the essential oil in the assay with soybean plants inoculated with *M. incognita* J2, it seemed very important to take into account the volatility and high sensitivity of (E)-cinnamaldehyde to oxidizing agents (López-Serna et al. 2016), which can cause its persistence in soil to be very low when compared to the commercial non-fumigant nematicides. Although these features sound undesirable for this class of nematicide, they are excellent for the development of a product characteristic of the fumigant class, which can be used to reduce the nematode population in the field before planting.

Therefore, the in vitro effect of vapors from (E)-cinnamaldehyde and essential oil of *C. cassia* on motility, mortality and hatching of *M. incognita* J2 was also studied. Values obtained for these vapors were very close to each other (Table 4), corroborating once again this aldehyde as the component accounting for the nematicidal activity of the essential oil. Furthermore, they were also close to values obtained with the commercial nematicide Basamid, which suggests that (E)-cinnamaldehyde has great potential for the development of new fumigant nematicides.

To assess the efficiency of (E)-cinnamaldehyde (Fig. 1) vapor against the nematode under conditions closer to those observed in the field, an experiment was carried out using substrate inoculated with *M. incognita* eggs, which correspond to most of the nematode population in fields (Evans and Perry 2009). The in vitro results were confirmed, as this aldehyde reduced both numbers of galls and eggs of the nematode to values close to those observed for the commercial nematicide Basamid (Table 5). Furthermore, these results are in accordance with the activity of this aldehyde against *M. incongita* in tomato plants, which was detected under conditions different from those employed in the present work (Ishibashi and Kubo 1987).

In view of the above-mentioned results, there is no doubt about the potential of (E)-cinnamaldehyde (Fig. 1) for the development of new products to control *M. incognita* in soybean fields. Furthermore, both this aldehyde and the product of its oxidation, (E)-cinnamic acid, present low toxicity to non-target organisms (Bickers et al. 2005), which is very important for the development of environmentally friendly nematicides.

Author contribution statement

INJ conceived, designed the research, conducted the experiments and wrote the manuscript. GHS carried out the GC–MS analysis and interpreted the results. VPC supervised the experiments with the nematode. PES conceived and designed the research. DFO supervised all the work and reviewed the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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ARTIGO 2 - GARLIC ESSENTIAL OIL EMULSION AND VAPOR REDUCE THE POPULATION OF *MELOIDOGYNE INCOGNITA* IN TOMATO PLANTS

Artigo formatado de acordo com o periódico CROP PROTECTION.

Garlic essential oil emulsion and vapor reduce the population of *Meloidogyne incognita* in tomato plants

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ABSTRACT

As the nematode *Meloidogyne incognita* causes large losses to the production of tomato, new methods/products to control this parasite are desirable. Garlic essential oil and its main components are potentially usefull to satisfy this desire, because theirs nematicidal activity against *M. incognita* is unequivolly proven by this study. In the present work garlic essential oil, obtained by hydrodistillation, was initially dissolved in water and assayed *in vitro* with *M. incognita*. At 62 μg mL^{-1} the oil was more active against *M. incognita* eggs and second stage

juveniles (J2) than de commercial nematicide Carbofuran at 173 μg mL^{-1}. The main components of the oil, according to gas chromatography-mass spectrometry analysis, are diallyl trisulfide (66.7%) and diallyl disulfide (21.3%), which explained the *in vitro* nematicidal activity of the oil. A solution of the oil at 250 μg mL^{-1} reduced the numbers of galls and eggs of *M. incognita* in tomato roots to values statistically equal to those obtained with Carbofuran at 415 μg mL^{-1}. Vapor of the oil also was as active *in vitro* against *M. incognita* eggs and J2 as the commercial fumigant nematicide Basamid®. The infectivity and reproduction of *M. incognita* in tomato plants cultivated in substrate inoculated with eggs of the nematode and treated with 0.2 mL of oil were statistically equal to those observed when the oil was replaced by 0.25 g of Basamid®. These findings are unequivolly proofs of the activity against *M. incognita* by garlic essential oil and its components, which are very promising for the development of a new fumigant nematicide to control the nematode in tomato plants.

Keywords: Allium sativum, Biopesticide, Root-knot nematode, Diallyl trisulfide, Diallyl disulfide, Organosulfur compounds

1. Introduction

Tomato (*Solanum lycopersicum* L.) is one the most cultivated vegetables in the world. In 2014, among the 170 million tons of tomatoes produced worldwide, about 4 million were due to Brazil (FAO, 2016). The yield could be higher if problems like those caused by root-knot nematodes (*Meloidogyne* spp.) could be avoided (Jones et al., 2013). In addition to directly affecting tomato plant, these parasites make the plant more susceptible to other diseases (Zhou et al., 2016). Apparently, the most destructive root-knot nematode species for

tomato plants is *Meloidogyne incognita* (Kofoid & White) Chitwood, which also affects several other crops of global economic importance (Sikora and Fernandez, 2005).

Currently, application of chemical nematicides is generally the method most used by farmers to control plant parasitic nematodes (Wesemael et al., 2011). Although the nematicides show good overall efficacy on plant-parasitic nematodes, they may have several undesirable effects on non-target organisms, in addition to risks for human health and negative environmental impact (Wesemael et al., 2011).

Plants are promising sources of substances to circumvent the above-mentioned problems, as some of their metabolites present potential to be used directly as pesticides or as starting models for the synthesis of improved chemical structures (Ntalli and Caboni, 2012). Among these metabolites are the compounds found in essential oils, which are volatile secondary metabolites produced by plants. Many of them can be used as natural antimicrobials (Júnior et al., 2014), and some of them are reported to be active against nematodes (Isman, 2000; Barbosa et al., 2010; Faria et al., 2013; Andrés et al., 2012).

Among the essential oil producing plants is garlic (*Allium sativum* L.), which has been used for culinary and medicinal purposes since antiquity (Martins et al., 2016). In part, this is due to the antioxidant, antibacterial and antifungal activities of metabolites produced by this plant (Block, 2010). Some of these metabolites have also been reported to be active against insects (Chaubey, 2016; Zhao et al., 2013) and even the pine wood nematode *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle (Park et al., 2005). The cold pressing oil from garlic was also reported to be active against *M. incognita* (Cetintas and Yarba, 2010). The essential oil of garlic is rich is organosulfur compounds such as ajoene, diallyl disulfide (DADS), diallyl trisulfide (DATS), allyl methyl trisulfide and diallyl sulfide (Block, 2010; Corzo-Martinez and Villamiel, 2007). Apparently, the sulfur compounds are responsible for the nematicidal activity of garlic essential oil (Park et al., 2005).

Although no error was observed in the work done with *B. xylophilus* (Park et al., 2005), only *in vitro* tests were performed by the authors to evaluate the activity of garlic essential oil and its components against this nematode. Therefore, the action of garlic essential oil under *in vivo* conditions is unknown. Regarding the work done with *M. incognita* (Cetintas and Yarba, 2010), only second stage juveniles (J2) of the nematode were assayed, but it is well known that the majority of the nematode population in the field is in the form of eggs (Evans and Perry, 2009). Furthermore, the oil studied by Cetintas and Yarba (2010) was obtained by cold pressing, which affords oils containing several non-volatile compounds (Ferhat et al., 2007). As the oil used by the authors was not analysed, their components effectively active against *M. incognita* were not identified. For example, such active components could be fatty acids that according to Zhang et al. (2012) can be active against nematodes. Or could be alicin, a non-volatile substance that inhibits the hatching of *M.incognita* J2 at 0.5 μL mL^{-1} (Gupta and Sharma, 1993).

In addition to the above-mentioned issues, it is important to mention that in both works with *B. xylophilus* (Park et al., 2005) and *M. incognita* (Cetintas and Yarba, 2010) no nematicidal substance was used as reference. It is therefore difficult to assess whether the products obtained from garlic are in fact potentially useful for the development of new nematicides. It is also important to take into account the volatile properties of the essential oils in general, as well as the propensity to oxidation by the sulfur components produced by garlic (Yang et al., 2001). These characteristics probably make the persistence of the essential oil of garlic in the soil very low in relation to non-fumigant nematicides. It is likely that, under field conditions after planting tomato, several *M. incognita* individuals will not come across with the essential oil due to its low persistence, resulting in inefficient control of the nematode. Consequently, it makes more sense to use this oil as a fumigant nematicide to reduce the population of *M. incognita* in the soil before planting.

In view of the above-mentioned issues, the present study was designed to: 1) Evaluate the *in vitro* activity against J2 and eggs of *M. incognita* by aqueous solutions of garlic essential oil; 2) Identify the components of garlic essential oil through gas chromatography-mass spectrometry analyses; 3) Evaluate solutions of the main components of garlic essential oil through *in vitro* assays with *M. incognita* J2 and eggs; 4) Investigate the *in vitro* activity against *M. incognita* J2 and eggs by vapor of garlic essential oil; 5) Evaluate the effect by vapor of garlic essential oil on the infectivity and reproduction of *M. incognita* on tomato plants.

2. Materials and methods

2.1. Meloidogyne incognita inoculum

M. incognita was multiplied in tomato plants (*Solanum lycopersicum* L. 'Santa Clara'), kept in a greenhouse for 50 days after inoculation nematodes eggs were then extracted from the roots according to the Hussey and Barker (1973) technique and placed on an adapted Baermann funnel at 28 ºC, for hatching J2 during the first 24 h were discarded, while those hatched between 24-48 h were imediately used in the bioassays.

2.2. Garlic essential oil and chemicals

Bulbs of garlic *in natura* (1000 g) were purchased in the local market (Lavras - MG, Brazil), ground in a blender with distilled water and submitted to hydrodistillation for 60 min in a Clevenger apparatus type. The obtained essential oil was separated from water and treated with anhydrous sodium sulphate to yield 7 mL of a pale yellow liquid that was stored in a freezer at -20 ºC. Diallyl disulfide (DADS, 80 %), diallyl trisulfide (DATS, 98 %) and Carbofuran (2,3-dihydro-2,2-dimethyl-1-benzofuran-7-yl N-methylcarbamate, 98%) were

125 purchased from Sigma-Adrich Co. (Milan, Italia), while Basamid® (98%) was purchased from
126 BASF (Ludwigshafe, Germany).
127
128 *2.3. In vitro effect on mobility and mortality of M. incognita J2 by solutions of garlic essential*
129 *oil*
130 Garlic essential oil was dissolved in an aqueous 0.01 g mL^{-1} Tween 80® solution to
131 10.000 µg mL^{-1}. The resulting emulsion was then diluted with 0.01 g mL^{-1} Tween 80® to five
132 different concentrations (2500, 1250, 625, 312 and 156 µg mL^{-1}). Each of the resulting
133 solution (100 µL) and an aqueous suspension (400 µL) containing aproximately 100 *M.*
134 *incongnita* J2 were placed into Eppendorf tubes (0.5 mL). The final concentrations of the oil
135 were 500, 250, 125, 62 and 31 µg mL^{-1}. Water, 0.01 g mL^{-1} Tween 80® and Carbofuran at 865
136 µg mL^{-1} (final concentration: 173 µg mL^{-1}) were used as controls. This experiment was
137 carried out under a randomized design, with six repetitions for each treatment. After 48 h at
138 28 ºC, the Eppendorf tubes were agitated, opened and pipetted 200 µL of their contents to the
139 350 µL wells of a 96-well polypropylene plate. The number of mobile and imobile J2, under a
140 microscope, was estimated. Mortality was avaliated according to the method described by
141 Chen and Dickson (2000), adapted by Amaral et al. (2003). For that one drop of freshly
142 prepared 1.0 mol L^{-1} NaOH was added to each well and nematodes were counted again. J2
143 that moved within 3 min were considered to alive, whereas the nematodes not responding to
144 addition of NaOH were considered dead.
145
146 *2.4. In vitro effect on hatching of M. incognita J2 by solutions of garlic essential oil*
147 A similar procedure to the described above (see 2.3) was employed. However 240 *M.*
148 *incognita* eggs per Eppendorf tube was used instead of 100 J2 used in above assay. Also the

149 contact time of the eggs to the solutions was prolonged to seven days differing from previous
150 assay. Hatching and unhatched eggs were counted under the microscope.
151
152 *2.5. GC-MS analysis of the essential oil of garlic*
153 A gas chromatograph coupled to a mass spectrometer (model QP2010, Shimadzu,
154 Japan), equipped with a RTX®-5MS capilar column (30m x 0.25 mm ID x 0.25 µm film
155 thickness; Restek), was employed in this work, which used helium at 1.0 mL min^{-1} as mobile
156 phase. According to Adams (2007) the following conditions were adopted: 1) split/splitless
157 injector temperature: 220 ºC; 2) split ratio: 1:20; 3) initial temperature of the column: 60 ºC;
158 4) elevation rate of the column temperature: 2 ºC min^{-1} up to 200 ºC and then 5 ºC/min; 5)
159 final temperature of the column: 250 ºC; 6) temperature of the interface between the gas
160 chromatograph and the mass spectrometer: 220 ºC; 7) ionization of each molecule in the
161 spectrometer: electron impact at 70 eV; 8) range of mass/charge (*m/z*) analyses in the mass
162 spectrometer: 45-400; 9) mass spectrum acquisition time: 0.5 s. The essential oil of garlic was
163 dissolved in acetone to a concentration of 10 mg mL^{-1} and 1 µL of this solution was injected
164 in the gas chromatograph. A solution of homologous linear alkanes, containing C9-C20
165 carbon atoms, was used as external standard. All mass spectra were compared to those in the
166 NIST 05 Mass Spectral Library, 2005, and all peaks in the chromatogram with similarity
167 index below 90% were considered unidentified. For each of the remaining peaks the
168 arithmetic index (AI) was calculated according to the following formulae: $AI = \{100X_z +$
169 $100[(RT - RTX_z)/(RTX_{z+1} - RTX_z)]\}$, where X_z = number of carbon atoms of the linear
170 alkane with retention time immediately below that of the substance to be identified in the
171 chromatogram; RT = retention time (min) of the substance to be identified in the
172 chromatogram; RTX_z = retention time (min) of the linear alkane with number of carbon atoms
173 equal to X_z; RTX_{z+1} = retention time (min) of the linear alkane with number of carbon atoms

174 equal to X_z + 1. Substances with calculated values of AI corresponding to an error ≥ 3 % in
175 relation to the AI described by Adams (2007) were considered not identified.
176
177 *2.6. In vitro effect of the main components of the essential oil of garlic on hatching, mobility*
178 *and mortality of M. incognita J2*
179 The experiments were carried out as described above for the essential oil of garlic, but
180 instead of it the following substances were tested in different concentrations: DATS (final
181 concentrations: 335, 168, 84, 42 and 21 µg mL^{-1}) and DADS (final concentrations: 106, 53,
182 27, 14 and 7 µg mL^{-1}), by evaluating J2 mobility, mortality and hatching.
183
184 *2.7. Infectivity and reproduction of M. incognita in tomato plants after exposition to aqueous*
185 *solution of garlic essential oil*
186 Seeds of tomato plant (*Solanum lycopersicum* L. 'Santa Clara') susceptible to *M.*
187 *incognita* were sown on a commercial substrate (Tropstrato ®, Vida Verde Indústria e
188 Comércio de Insumos Orgânicos Ltda., Mogi Mirim, São Paulo, Brazil) contained in 121 mL
189 wells of a 72-well Styrofoam tray. An aqueous suspension (24 mL) containing approximately
190 3,000 *M. incognita* J2 and aqueous solutions (24 mL) of garlic essential oil (250, 125 and 62
191 µg mL^{-1}) in 0.01 g mL^{-1} Tween 80® were combined. Water, Tween 80® at 0.01 g mL^{-1} and
192 Carbofuran (415 µg mL^{-1}) were used as controls. Aliquots (8 mL, approximately 500 J2) of
193 each resulting suspension was added to the substrate of each 20 days plant through four
194 equidistant holes (0.4 cm wide x 1.5 cm deep) around the stem. This experiment was carried
195 out under a completely randomized design, with six repetitions for each treatment. The tray
196 was kept in a dark room, at 28 ^{o}C, for 48 h, and then it was transfered to a greenhouse, where
197 it was mantained for 30 days. After the period, roots were carefully removed, washed with
198 water, dried on paper towels and weighed. After gall counting, roots underwent eggs

199 extraction in accordance to the Hussey and Barker (1973) technique. Eggs retained in a 500
200 mesh (ASTM) sieve were suspended in 50 mL water and counted in a Peters chamber under a
201 microscope. This experiment was carried out twice, resulting in similar values. Therefore,
202 only one set of data is presented.
203
204 *2.8. In vitro immobility and mortality of M. incognita J2 by vapor from garlic essential oil*
205 Adapting the method described by Barros et al. (2014), sand (30 g) was sterilezed by
206 autoclaving at 120 ºC for 30 minutes and poured into each Supelco™ SPME flask (28 mm
207 wide x 80 cm deep, Sigma-Aldrich, Bellefonte, PA, USA). Two Eppendorf tubes (0.5 mL)
208 were partially immersed in the sand of each flask and 100 µL of garlic essential oil was
209 poured into one of the tubes. The flask was immediately sealed with a screw cap internally
210 coated with silicone and kept at 28 ºC for 72 h. Employing a syringe with a needle to punch
211 the silicon septa of the Supelco™ SPME flask, 100 µL of an aqueous suspension (1,000 J2
212 mL^{-1}) was injected into each empty Eppendorf tube. The commercial fumigant nematicide
213 Basamid® [80 mg; BASF (Ludwigshafen, Germany)] and water (100 µL) were used as
214 positive and negative controls, respectively. This experiment was carried out under a
215 completely randomized design, with six replicates for each treatment. After 48 h of the J2
216 inserted at 28 ^{o}C, the flasks were opened for the homogenization of the J2 suspension, from
217 which aliquots (20 µL) were transferred to 350 µL wells of a 96-well polypropylene plate.
218 These aliquots were diluted with 100 µL water before counting mobile and immobile J2 under
219 a microscope. Employing the method of Chen and Dickson (2000), adapted by Amaral et al.
220 (2003), the mortality was evaluated.
221
222 *2.9. The in vitro hatching of M. incognita J2 by vapor of garlic essential oil*

This experiment followed similar procedure as described anteriorly. But instead of J2, *M. incongita* suspension of 3,000 eggs mL^{-1} was used and exposed to the vapor by seven days instead of three days as described previously. After the period, non-hatched eggs and hatched J2 (alive or dead) were estimated. This experiment was carried out three times, resulting in similar values. Therefore, only one set of data is presented.

2.10. Effect of vapor of garlic essential oil on the infectivity and reproduction of M. incognita on tomato plants

M. incognita eggs (150,000) were added to 1 L of a commercial substrate (Tropstrato®, Vida Verde Indústria e Comércio de Insumos Orgânicos Ltda., Mogi Mirim, São Paulo, Brazil) contained in 2 L polyethylene terephthalate bottles. Essential oil of garlic was then splashed on the substrate surface to the following concentrations: 0.5, 0.2 and 0.1 mL (L of substrate)$^{-1}$. The commercial fumigant nematicide Basamid® at 0.25 g (L of substrate)$^{-1}$ and water (1.0 mL) were used as positive and negative controls, respectively. This experiment was carried out with six replicates for each treatment, under a randomized design. All bottles were cap closed and the resulting mixtures were homogenized and remained standing at 28 ºC for three days. The bottles were then opened and five days later, the substrate inside them was poured into cells (121.2 mL) of a 72-cell Styrofoam tray. Twenty-day old tomato plants (*Solanum lycopersicum* L. 'Santa Clara') were transferred to the tray, which was kept in a greenhouse for 30 days. After this period of time, roots were carefully washed, dried with a paper towel and weighed. After counting galls, *M. incognita* eggs were extracted through the method described by Hussey and Barker (1973). The eggs obtained were counted in a Peters chamber under a microscope. This experiment was carried out twice, resulting in similar values. Therefore, only one set of data is presented.

248 *2.11. Data analysis*

249 Values from the *in vitro* tests were converted into percentage to be submitted to
250 analysis of variance (ANOVA). Means were compared according to the Scott and Knott
251 (1974) test ($P \leq 0.05$). For the *in vivo* assays values underwent the same analysis without
252 convertion into percentage. The software SISVAR (Ferreira 2011) was used to carry out the
253 statistical calculations.

254

255 **3. Results and discussion**

256

257 *3.1 In vitro effect on mobility, mortality and hatching of M. incognita J2 by solutions of garlic*
258 *essential oil and its main components*

259 Solutions of garlic essential oil were very active against *M. incognita*. For example, at
260 62 µg mL^{-1} the oil caused more deaths to J2 than the commercial nematicide Carbofuran at
261 173 µg mL^{-1}, while at 125 µg mL^{-1} reduced J2 hatching to a value statistically equal to that
262 obtained with the nematicide (Table 1). Although a quantitative comparison can not be done
263 to the results previously obtained in studies with *B. xylophilus* (Park et al., 2005) and *M.*
264 *incognita* (Cetintas and Yarba, 2010), because those authors did not use a nematicide as
265 control, the present results seem to be qualitatively in line with those described by these
266 authors.

267

268 **Table 1**

269 Effects of the essential oil of garlic and its main components, diallyl disulfide (DADS) and
270 diallyl trisulfide (DATS), on mobility, mortality and hatching of *Meloidogyne incognita*
271 second-stage juveniles (J2).

Treatment	Concentration ($\mu g\ mL^{-1}$)	Imobile J2 (%)[a]	Dead J2 (%)[a]	Hatched J2 (%)[a]
Oil	500	100.0 f	100.0 h	5.8 c
DATS+DADS	335+106	100.0 f	100.0 h	0.0 a
DATS	335	100.0 f	100.0 h	0.0 a
DADS	106	81.7 e	49.0 e	0.0 a
Oil	250	100.0 f	100.0 h	9.8 d
DATS+DADS	168+53	100.0 f	100.0 h	0.0 a
DATS	168	100.0 f	100.0 h	0.0 a
DADS	53	29.3 c	12.6 b	1.3 b
Oil	125	100.0 f	100.0 h	11.0 d
DATS+DADS	84+27	100.0 f	100.0 h	0.0 a
DATS	84	100.0 f	100.0 h	0.7 a
DADS	27	24.6 b	9.5 b	2.7 b
Oil	62	85.5 e	82.3 g	16.1 e
DATS+DADS	42+14	100.0 f	100.0 h	0.0 a
DATS	42	82.2 e	44.4 e	1.3 b
DADS	14	18.5 b	5.2 a	5.7 c
Oil	31	67.7 d	34.9 d	21.1 f
DATS+DADS	21+7	92.9 f	75.9 g	2.0 b
DATS	21	40.0 c	16.9 c	5.2 c

DADS	7	4.9 a	2.5 a	10.0 d
Oil	16	34.7 c	17.4 c	--
DATS+DADS	10+4	61.8 d	38.5 d	4.8 c
DATS	10	21.3 b	10.0 b	--
DADS	4	1.8 a	0.90 a	--
Water (control)	--	1.0 a	1.0 a	51.6 g
Tween 80® (control)	--	1.0 a	1.0 a	51.8 g
Carbofuran (control)	173	71 d	63 f	11.8 d

[a] Means followed by the same letter in each column do not differ statistically according to the Scott and Knott (1974) test ($P \leq 0.05$).

3.2. GC-MS analysis of the essential oil of garlic

Seven compounds were identified in the garlic essential oil. They represent 93.8% of the total. The most abundant are DATS (66.7%) and DADS (21.3%) (Table 2). Although this result is in agreement with previous reports (Martinez-Velazquez et al. 2011, Kocic-Tanackov et al. 2012, Zhao et al. 2013, Foe et al. 2016, El-Sayed et al. 2017), it is worth mentioning the large variation described in the literature for the composition of garlic essential oil. For example, in a sample from Egypt, diallyl sulfide (21.5%), allyl methyl disulfide (3.3%), DADS (27.4%), allyl methyl trisulfide (8.0%), DATS (26.3%), allyl methyl tetrasulfide (3.4%) and diallyl tetrasulfide (9.93%), were identified (Nashwa, 2015), while in a sample from Tunisia the identified substances were DATS (30,38%) and DADS (49,1%) (Chekki et al., 2014). In part, this may be due to factors such as climatic condition, harvesting period and distillation technique (Lahlou, 2004; Block, 2010).

289 **Table 2**

290 Main components of the essential oil of garlic according to analysis by gas chromatography-
291 mass spectrometry.

Number	Component	RT[a] (min)	AI[b]	SI[c] (%)	Area[d] (%)
1	Diallyl sulfide	4.034	874	93	0.4
2	Allyl methyl disulfide	5.362	916	82	0.4
3	Diallyl disulfide (DADS)	11.572	1078	85	21.3
5	3-Vinyl-1,2-dithiacyclohex-5-ene	18.758	1209	87	0.3
6	Diallyl trisulfide (DATS)	24.100	1298	96	66.7
7	Diallyl tetrasulfide	38.379	1533	91	4.7
Total					93.8

292 [a] RT = retention time in the chromatogram. [b] AI = calculated arithmetic index. [c] SI =
293 similarity index between the mass spectrum obtained and that in the NIST 05 Mass Spectral
294 Library. [d] Relative area of the peak in the chromatogram.

295

296 3.3 *In vitro effect of the main components of the essential oil of garlic on hatching, mobility*
297 *and mortality of M. incognita J2*

298 As DADS and DATS were the main components of the garlic essential oil (Table 2),
299 only these substances were assayed. Their concentrations in the assays were proportional to
300 their amounts in the oil. Therefore, DADS and DATS activities against *M. incognita* that were
301 smaller than or equal to those observed for the oil were expected. However, the oposite was
302 observed. For example, at 106 µg mL^{-1} DADS reduced J2 hatching to a value statistically
303 lower than that observed for the essential oil at 500 µg mL^{-1}. A similar behavior was observed
304 for DADS at 53, 27, 14 and 7 µg mL^{-1}. DATS also always reduced J2 hatching to values
305 statistically lower than those observed for the oil at proportional concentrations (Table 1).

These results suggest that other components in the oil may interfere in the action of DADS and DATS against *M. incognita*.

When isolated, both DADS and DATS could not afford values of imobile or dead J2 greater than those observed for the oil at proportional concentrations. However, when combined these substances were more active than the essential oil. For example, DADS+DATS at 14+42 µg mL^{-1} imobilized and killed 100% of J2, while the oil at 62 µg mL^{-1} increased J2 imobility and death to 85.5% and 82.3 %, respectively (Table 1). Again, these results suggest interference in the action of DADS and DATS against *M. incognita* by other components of the oil. This is normal behavior for some samples of natural origin. For example, it is possible to mention the work by Jiang et al. (2009), who observed the sinergistic effect of some components of the essential oils of *Litsea pungens* Hemsl. and L. *cubeba* (Lour.) Pers. (Lauraceae), on the active compounds. Analgously, Miresmailli et al. (2006) reported a sinergistic effect between inactive and acitive components of the essential oil of *Rosmarinus officinalis* L.

Both DADS and DATS were more active against *M. incongita* eggs than the commercial nematicide Carbofuran at 173 µg mL^{-1}. For example, at 14 and 42 µg mL^{-1}, respectively, they reduced J2 hatching to 5.8% and 1.3%, respectively, while the value observed for Carbofuran corresponded to 11.8% (Table 1). Consequently, these substances are very promising for the development of new nematicides, especially when taken into account that the majority population of *M. incognita* in the field is in the form of eggs (Evans e Perry, 2009).

DADS seems to be as active against J2 as Carbofuran, while DATS certainly is more active than this nematicide. For example, at 84 µg mL^{-1} DATS killed 100% J2, while Carbofuran at 173 µg mL^{-1} killed only 63% and DADS at 106 µg mL^{-1} killed 49.0%. However, the combination of DADS and DATS is more efficient than that commercial

nematicide. For example, DADS+DATS at 7+21 µg mL^{-1} killed 76.0% J2. Thus, this combination is also very promising for the development of a new nematicide. Furthermore, this result suggests a sinergistic effect between DADS and DATS. Although there is no report of such behavior in assays carried out with nematodes, synergistic effect by components of garlic essential oil has already been observed (Amagase et al., 2001). For example, the combination of allyl alcohol with DATS and DADS increases the antifungal activity against *Candida utilis* (Henneberg) Lodder & Kreger-van Rij (Chung et al., 2007).

Anyway, the results obtained so far suggest that DADS and DATS account for the nematicidal activity observed for garlic essential oil in the present work, which is in accordance with the work by Park et al. (2005), who demonstrated the *in vitro* activity of these substances against the pine wood nematode *B. xylophilus*. It is also worth mentioning the work by Anastasiadis et al. (2011), who demonstrated the nematostatic and nematicidal activity of DADS at 2 µL mL^{-1} against *Meloidogyne javanica* (Treub) Chitwood.

3.4. Infectivity and reproduction of M. incognita in tomato plants after exposition to aqueous solution of garlic essential oil

Although garlic essential oil reduced the number of eggs and of galls when used at 63 and 125 µg mL^{-1}, only at 250 µg mL^{-1} it afforded values statistically equal to those obtained for Carbofuran at 415 µg mL^{-1}. As root mass was not affected by the oil, apparently it presents no phytotoxic effect whithin the concentration range studied (Table 3). This result seems to be in line with the work by Anastasiadis et al. (2011), who reported no phytotoxic activity of DADS against tomato plants. Thus, the components of this oil present great potential for de development of new nematicides.

Table 3

Numbers of galls (NG) and eggs (NE) and root mass (RM) of tomato plants inoculated with *Meloidogyne incognita* second stage juveniles (J2) and treated with solutions of garlic essential oil.

Treatment	Concentration (µg mL^{-1})	NG[a]	NE[a]	RM (g)[a]
Oil	250	6 a	31 a	1.7 a
Oil	125	26 b	111 b	1.6 a
Oil	63	46 c	193 b	1.7 a
Carbofuran (control)	415	3 a	15 a	1.6 a
Tween 80® (control)	--	47 c	471 c	1.8 a
Water (control)	--	55 c	483 c	1.7 a

[a] Means followed by the same letter in each column do not differ statistically according to the Scott and Knott (1974) test ($P \leq 0.05$).

This result also seems to be in accordance with the previously described assay with garlic oil obtained by cold pressing (Cetintas and Yarba, 2010), which reduced the number of galls of *M. incognita* in tomato roots to aproximately 13% of that observed for the untreated plants, and no phytotoxic effect was observed by the authors. However, the number of eggs was reduced to aproximately 67% by the cold pressing oil, while in the present work this parameter was reduced by garlic essential oil (250 µg/mL) to aproximately 7% of that obtained for untreated plants (Table 3). Consequently, it is unclear whether the effect on the nematode by garlic oils obtained by cold pressing and by steam distillation are exactly the same.

373 *3.5. In vitro by vapor from garlic essential oil immobilityon, mortality and hatching of M.*
374 *incognita J2*

375 Despite the excellent results obtained with solutions of garlic essential oil (Table 3), it
376 seemed important to take into account the volatility of this oil and the sensitivities of its
377 components to oxidizing agents (Yang et al., 2001), which can cause its persistence in soil to
378 be low. These characteristics are not appropriate for a non-volatile nematicide, but are
379 excellent for a fumigant, which can reduce the nematode population in the field before
380 planting. Therefore, the *in vitro* effect of vapors from garlic oil on *M. incognita* J2 was also
381 studied, resulting in values that were statistically equal to those obtained for the commercial
382 fumigant nematicide Basamid® (Table 4). This result shows important, mainly because the
383 vapor of the essential oil could affect nematode eggs, which corresponde to the major part of
384 the nematode population under field conditions (Evans and Perry, 2009). Essential oil vapor
385 of other plants and ethanol vapor have shown effect on reducing hatching, and increasing
386 immobility and mortality of nematodes (Silva et al., 2017).

387

388 **Table 4**

389 Effect of vapor from garlic essential oil on motility, mortality and hatching of *Meloidogyne*
390 *incognita* second stage juveniles.

Treatment	Amount	Imobile (%)[a]	Dead (%)[a]	Hatched (%)[a]
Oil	100 µL	100.0 b	91.5 b	12.7 b
Water (control)	--	1.0 a	0.4 a	47.6 a
Basamid® (control)	80 mg	100.0 b	91.5 b	11.5 b

391 [a] Means followed by the same letter in each column do not differ according to the Scott and
392 Knott (1974) test ($P \leq 0.05$).

393

394 *3.6. Effect of vapor of garlic essential oil on the infectivity and reproduction of M. incognita*
395 *in tomato plants*

396 To further evaluate the potential of garlic essential oil as a fumigant nematicide, it was
397 used in an *in vivo* assay with eggs of the nematode. At 0.2 mL (L of substrate)$^{-1}$ this oil
398 reduced the numbers of galls and eggs of *M. incognita* to values that were statistically equal to
399 those obtained with the commercial nematicide Basamid® at 0.25 g (L of substrate)$^{-1}$. In
400 addition, the oil had the advantage of having no apparent phytotoxic effect, since root weights
401 of plants treated with it were statistically the same as those of the water-treated plants, while
402 root weights of plants treated with Basamid® were smaller (Table 5). These results
403 corroborate the potencial of the garlic essencial oil to be used as a fumigant nematicide.
404 Fumigation obtained by incorporation of Brassicas plants has been proved effective against
405 plant-parasitic nematodes (Riga, 2011).

406

407 **Table 5**

408 Numbers of galls (NG) and eggs (NE) and root mass (RM) of tomato plants cultivated in
409 substrate inoculated with *Meloidogyne incognita* eggs and treated with garlic essential oil.

Treatment	NG[a]	NE[a]	RM (g)[a]
Oil at 0.5 mL (L of substrate)$^{-1}$	10 a	12 a	4.8 b
Oil at 0.2 mL (L of substrate)$^{-1}$	43 a	104 a	4.1 b
Oil at 0.1 mL (L of substrate)$^{-1}$	812 c	2214 b	4.8 b
Water (control)	822 c	2592 b	4.8 b
Basamid® at 0.25 g (L of substrate)$^{-1}$ (control)	8 a	10 a	3.6 a

410 [a] Means followed by the same letter in each column do not differ according to the Scott and
411 Knott (1974) test ($P \leq 0.05$).

412

4. Conclusions

When dissolved in water, the essential oil obtained from bulbs of garlic was more active *in vitro* against *M. incognita* J2 and eggs than the commercial nematicide Carbofuran. This property can be attributed to DADS and DATS, which when combined reveal a synergistic effect that makes such substances much more active than Carbofuran. Apparently, other substances in the oil interfere with the activity o DADS and DATS, which in concentrations proportional to those in the oil are more active than the oil. When in solution the oil also reduced the infectivity and reproduction of *M. incognita* in tomato plants. The vapor of garlic essential oil was also active against *M. incognita in vitro* and reduced the infectivity and reproduction of the nematode to levels statistically similar to those observed for the commercial fumigant nematicide Basamid®, when substrate inoculated with *M. incognita* eggs was treated with the oil and used to cultivate tomato plants. This result suggests that garlic essential oil or its components can be used as fumigant to reduce the population of the nematode before planting tomato.

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